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Demethylation-Induced Developmental Pleiotropy in *Arabidopsis*

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The function of DNA methylation in higher plants was investigated by expression of a complementary DNA encoding a cytosine methyltransferase (MET1) from *Arabidopsis thaliana* as an antisense RNA in transgenic plants. This expression resulted in a 34 to 71 percent reduction in total genomic cytosine methylation. Loss of methylation was observed in both repetitive DNA and single-copy gene sequences. Developmental effects included altered heterochrony, changes in meristem identity and organ number, and female sterility. Cytosine demethylation prolonged both vegetative and reproductive phases of development. These findings implicate DNA methylation in establishing or maintaining epigenetic developmental states in the meristem.

Plant genomes contain relatively large amounts of the modified nucleotide 5-methylcytosine (5mC) (*1*). Despite evidence implicating cytosine methylation in plant epigenetic phenomena, such as repeat-induced gene silencing (RIGS), cosuppression, and inactivation of transposable elements (*2*), the role of cytosine methylation in plant developmental processes is not clear. In *Arabidopsis*, *ddm* (*decrease in DNA methylation*) mutants have been isolated with reduced levels of cytosine methylation in repetitive DNA sequences, although these mutations do not result in any detectable change in DNA methyltransferase enzymatic activity (*3*, *4*). After several generations of self-pollination, *ddm* mutants exhibit a slight delay (1.7 days) in flowering, altered leaf shape, and an increase in cauline leaf number (*4*).

To address the role of DNA methylation in plant development, we used an antisense strategy to interfere with *MET1*, a DNA methyltransferase (MTase) gene of *Arabidopsis*, previously cloned by homology to the mouse gene (Fig. 1) (*5*, *6*). The *MET1* gene represents one member of a small gene family in *Arabidopsis* (*5*) that maps to position 68.9 on chromosome 5, nonallelic to the *ddm1* locus (*7*). The *MET1* gene is expressed in seedling, vegetative, and floral tissues; in the inflorescence, expression is seen at highest levels in meristematic cells by in situ RNA hybridization (*8*). To inhibit expression of the *MET1* gene, we introduced an antisense construct (Fig. 1), consisting of a 4.3-kb *MET1* cDNA in the antisense orientation under the control of a constitutive viral promoter (CaMV 35S) (*9*), into *Arabidopsis* strain Columbia (*10*) by *Agrobacterium*-mediated transformation (*11*). In one experiment, a total of nine primary transformants (T0 generation) were recovered; progeny tests indicated that six lines that were further characterized contained single-locus transferred DNA (T-DNA) insertions (*12*).

Methylation patterns in repetitive DNA sequences were examined by Southern (DNA) hybridization (*13*). Genomic DNAs were digested with the isochizomers Hpa II or Msp I (Fig. 2, upper panels) and probed with a centromeric repeat or a 5S ribosomal DNA sequence; both repeats are methylated in wild-type genomic DNA (*3*). Hpa II digestion is inhibited if either cytosine in the CCGG target site is methylated; Msp I can cleave $C^{5m}CGG$ but not $^{5m}CCGG$ (*14*). With both probes, Hpa II digestion revealed a high extent of demethylation in three of six antisense lines (Tr244, 246, and 248: designated "strong") and in the *ddm1* mutant. Three antisense lines (Tr242, 243, and 245: designated "weak") showed near wild-type levels of methylation. Msp I digestion was more complete in strong antisense lines than in wild-type or weak antisense lines. These results indicate that strong antisense lines contain substantial demethylation of these repeated sequences at both $C^{5m}CGG$ and $^{5m}CCGG$ sites (*15*).

DNA methylation was examined at four single-copy gene sequences (*16*) (Fig. 2, lower panels). Substantial demethylation of all four genes was seen only in the strong antisense lines; the *ddm1* mutant showed little

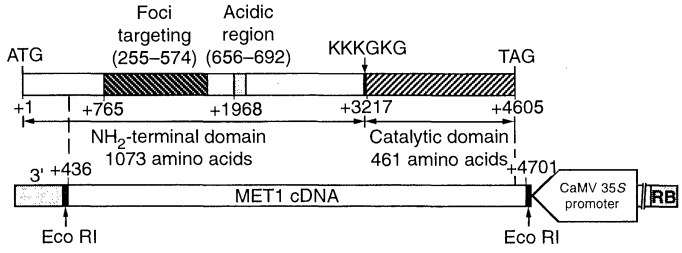

Fig. 1. The predicted *MET1* gene product and antisense construct. The predicted gene product of the *MET1* locus is a 1534–amino acid protein with a high degree of homology to the mouse MTase, particularly in the catalytic and NH_2-terminal foci targeting domains (*5, 30*). The *MET1* antisense construct is shown below. A 4.3-kb *MET1* cDNA spanning the positions indicated was inserted in the reverse orientation with respect to the CaMV 35S promoter in the pMON530 T-DNA vector (*9*).

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or no demethylation relative to wild-type DNA, consistent with published reports (*3*). Total genomic levels of 5mC were also measured in these lines by high-performance liquid chromatography (HPLC) (*17*) (Table 1). Cytosine methylation levels in wild type (6.4% of total genomic cytosine) and the decrease seen in the *ddm1* mutant (75%) agree with previously published estimates (*3*, *18*). Total genomic 5mC content in strong lines Tr246 and Tr248 was reduced 71% relative to wild-type levels; the weak line Tr245 showed a 34% reduction. The decrease in 5mC levels in the strong lines by a factor of 3.5 is comparable to reductions seen in the *ddm1* mutant and the MTase knockout mouse (*19*). Unlike the pattern of demethylation of the *ddm1* mutant, however, *MET1* antisense expression resulted in substantial demethylation of both repetitive DNA and single-copy gene sequences.

Normal patterns of development were perturbed in strong antisense lines. Under long day conditions (*12*), Tr244, Tr246, and Tr248 plants initiated 30 to 35 vegetative nodes with delayed abaxial trichome production and flowered after 45 to 48 days (Table 2 and Fig. 3, A to D); these phenotypes were fully penetrant in all T-DNA–containing progeny of the strong antisense lines. After a transition from vegetative to reproductive development, wild-type plants initiated a primary inflorescence axis with two to five secondary inflorescence branches subtended by cauline leaves, followed by an abrupt transition to the production of solitary floral meristems (Table

Table 1. Cytosine methylation levels in *Arabidopsis* wild-type (WT), *ddm1* mutant, and three antisense lines. We determined 5mC content by reversed-phase HPLC using the method of Gehrke *et al.* (*17*). All values represent the averages of two to four individual replicates and were calculated by integration of peak areas with Dynamax HPLC Method Manager version 1.2 (Rainin). Percentages of total 5mC content [5mC/(5mC + C)] are normalized for absorbance differences between cytosine and 5mC.

Line	Total 5mC (%)	% WT levels	% Decrease
Wild type	6.38 ± 0.69	100	0
Tr245	4.24 ± 0.59	66.5	33.5
Tr246	1.84 ± 0.27	28.9	71.1
Tr248	1.83 ± 0.16	28.7	71.3
ddm1	1.60 ± 0.04	25.0	75.0

Table 2. Characterization of vegetative and inflorescence traits of wild-type (WT) and *MET1* antisense lines. Quantitative aspects of vegetative and inflorescence traits are given for wild-type Columbia and six antisense lines. Flowering time is measured in days after germination (*28*); leaf number refers to the number of vegetative leaves initiated before emergence of the primary inflorescence; and secondary branches refers to the number of branches initiated on the primary inflorescence axis. Values are calculated from a minimum of four individual plants from each line (*29*).

Line	Flowering time	Leaf number	Secondary branches
WT	26.2 ± 1.7	9.2 ± 1.3	4.3 ± 0.5
Tr242	24.5 ± 2.1	10.0 ± 1.4	4.5 ± 1.1
Tr243	27.8 ± 0.4	11.0 ± 0.7	4.3 ± 0.4
Tr244	47.7 ± 4.0	34.0 ± 5.7	20.3 ± 4.9
Tr245	27.7 ± 7.5	11.5 ± 1.5	4.3 ± 0.8
Tr246	45.9 ± 3.6	32.5 ± 3.2	20.7 ± 1.4
Tr248	46.3 ± 4.7	32.9 ± 2.7	20.5 ± 2.0

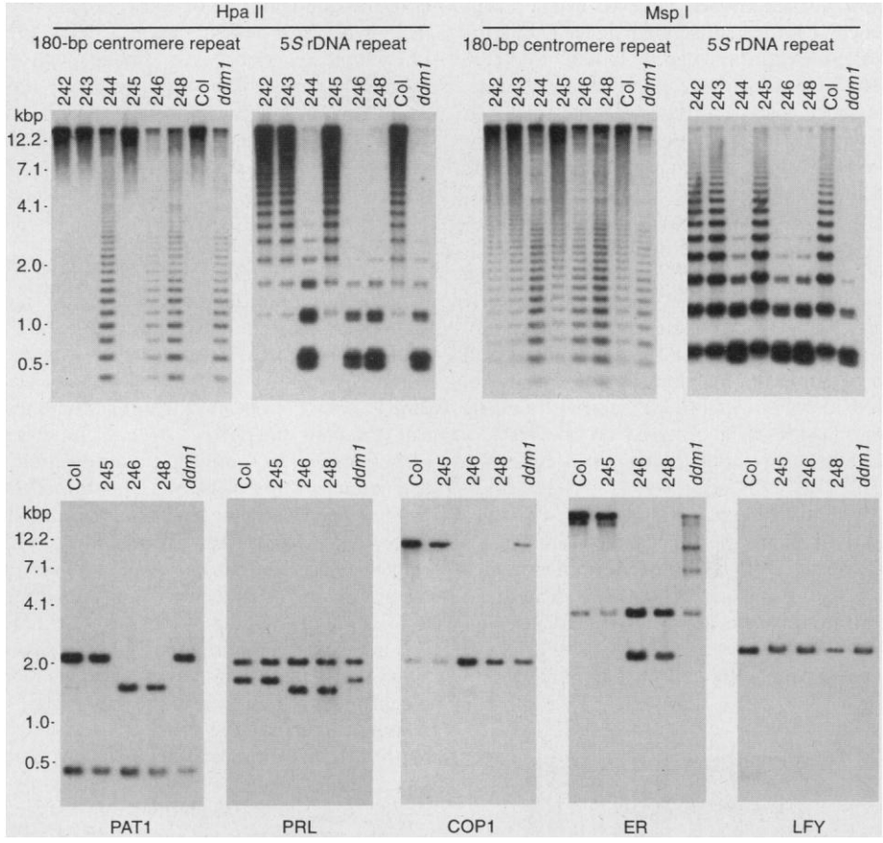

Fig. 2. Southern analysis of repetitive and single-copy DNA methylation patterns. Total genomic DNA (3 μg per lane) from antisense lines, wild type, and the *ddm1* mutant were digested with Hpa II or Msp I, subjected to electrophoresis in 0.8% agarose, and transferred to Zeta-Probe membranes (Bio-Rad) and hybridized as described (*13*). (**Upper panels**) Filters were probed with a 180-bp centromere repeat (*31*) and 5*S* rDNA (*32*). (**Lower panels**) Filters containing Hpa II–digested genomic DNA were probed with four single-copy gene probes—PHOSPHORIBOSYLANTHRANILATE TRANSFERASE 1 (PAT1), PROLIFERA (PRL), CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), and ERECTA (ER) (*15*)—shown by differential Hpa II–Msp I digestion of wild-type DNA to contain $C^{5m}CGG$ methylation (*27*). The panel on the right was hybridized to a control gene, LEAFY (LFY) (*33*), shown not to be methylated in wild-type DNA.

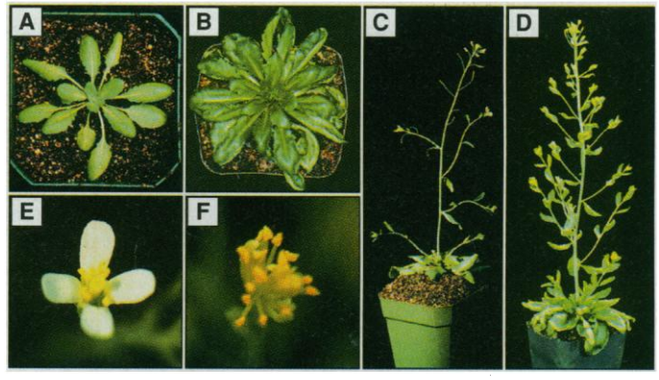

Fig. 3. Phenotypes associated with wild-type and strong antisense lines of *Arabidopsis*. Wild-type (*34*) (**A** and **C**) and strong antisense line Tr248 (**B** and **D**) plants shown at the onset of inflorescence emergence (A and B) and during early reproductive growth (C and D). Floral morphology of wild-type (**E**) and Tr248 (**F**) flowers initiated at the apical region of secondary inflorescences is shown. The flower from line Tr248 contained 17 stamens.

2 and Fig. 3C) (*20*). In strong antisense lines, the primary inflorescence shoot produced an average of 20 secondary branches (Table 2 and Fig. 3D) before the production of flowers. The basal-most branches in strong antisense lines often assumed characteristics of vegetative rosettes, including enhanced spiral phyllotaxy of 10 to 20 vegetative-like leaves and shortened internodes, followed by the emergence of an inflorescence bolt recapitulating the primary inflorescence. Unlike wild-type plants, occasional secondary branches were produced apically to flowers in the transition zone. Early-initiating flowers from strong antisense lines were normal in appearance and male-fertile, but were often female-sterile. Apical flowers initiated on secondary and tertiary inflorescences showed gross abnormalities, including a threefold increase in stamen number (Fig. 3F) and sterile, incompletely fused carpels lacking stigmas. Despite the severe disruptions in floral morphology seen in these late-initiating flowers, no obvious defects in pollen viability or paternal transgene transmission were observed on the basis of segregation ratios of transgenes in outcrosses (*21*).

One trivial explanation for the *MET1* antisense pleiotropy is that it represents an indirect effect of the transgene rather than a direct consequence of genomic demethylation; however, several lines of evidence support a direct role for DNA methylation in development. Data collected and analyzed in additional experiments revealed an identical pleiotropy in 12 independent transgenic lines. In analyses of outcross progeny from strong antisense lines, the severe phenotype cosegregated with the presence of the transgene, and a slightly attenuated pleiotropy was seen in progeny that had lost the transgene but retained a demethylated genome (Fig. 4). The latter finding is not unexpected, because the rate of genomic remethylation is slow (*3*). Phenotypic revertants seen among outcross progeny had reestablished near wild-type levels of genomic methylation (Fig. 4, lanes 5 and 11). In sum, it appears that demethylation is sufficient to maintain developmental pleiotropy in the absence of the transgene, whereas genomic remethylation is required to restore a wild-type phenotype.

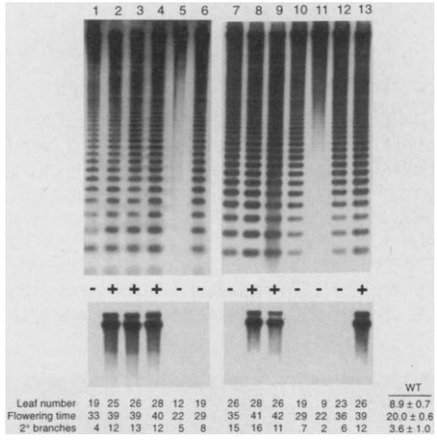

Fig. 4. Southern and phenotypic analyses of Tr246 outcross progeny. Genomic DNAs (4 μg per lane) from outcross progeny of strong antisense line Tr246 to wild-type *Arabidopsis* (*34*) were digested with Hpa II (upper panels) or Eco RI (lower panels). Southern analyses were done as described in Fig. 2. Filters were probed with the 180-bp centromere repeat (*31*) (upper panels) or the CaMV 35*S* promoter fragment from pMON530 (lower panels). Symbols indicate the presence (+) or absence (−) of the antisense transgene. Phenotypic data for each individual plant are shown below each lane. Plants were grown under continuous light at 21°C.

The demethylation phenotype produced by antisense inhibition of *Arabidopsis* MTase differs markedly from the phenotype produced by the treatment of *Arabidopsis* seeds with the nonmethylatable cytosine analog 5-azacytidine (5-azaC), but bears some similarity to the *ddm1* mutant phenotype. 5-AzaC produces early flowering in some late-flowering strains and mutants of *Arabidopsis* (*22*), but this effect has not been correlated with a quantitative decrease in cytosine methylation. More recent evidence suggests that the primary effect of 5-azaC treatment is due to toxicity resulting from covalent trapping of DNA MTase (*23*) or 5-azaC incorporation into RNA. The overall level of demethylation in the *ddm1* mutant is equivalent to that seen in the strong *MET1* antisense lines, yet the phenotype exhibited by the *MET1* antisense lines is much more severe and pleiotropic. This discrepancy may be due to substantial demethylation observed at single-copy gene sequences in *MET1* antisense lines not seen in the *ddm1* mutant.

On the basis of these studies, we suggest that DNA methylation is an essential component in the process of phase transitions and meristem determinacy. Methylation may serve as a primary signal to restrict meristem determinacy, or it may represent a secondary process required to maintain an epigenetic state once established. Phase transitions involve an interplay of both cell autonomous and diffusible signals (*24*). Evidence that methylation may represent an autonomous component in this process comes from the observation that phase transitions are often irregular in strong antisense plants—the location of branches apical to flowers in the inflorescence transition zone may represent an autonomous switching of individual cells in the meristem resulting in developmental mosaicism. Methylation effects have also been shown to be progressive in the plant meristem (*25*), and it is intriguing to speculate that a methylation gradient might be established during meristem growth to serve as an autonomous signal that directs meristem determinacy. In this light, the strong antisense phenotype could be explained by delayed establishment of this hypothetical gradient. Such a model predicts that meristem potential becomes progressively more epigenetically restrictive and that repression cascades will be an underlying theme in plant determinacy, a process implicit in the pathway controlling inflorescence and floral development in *Arabidopsis* (*26*).

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6. The *MET1* cDNA (B6-2) used in these experiments was cloned independently; it lacked 436 base pairs (bp) downstream of the deduced translation start site and differed from the published sequence by a thymine-to-cytosine substitution at position +2981.
7. The *MET1* gene was mapped with an Eco RI polymorphism between *Arabidopsis* ecotypes WS and W100 with RI lines (Dupont) and is nonallelic to the DDM1 locus (*4*).
8. H. Rodriguez-Rodriguez, M. Ronemus, A. Calderon-Urrea, S. L. Dellaporta, unpublished data.
9. *MET1* cDNA B6-2 was cloned into the Eco RI site of T-DNA vector pMON530 (Monsanto) in the antisense orientation to the CaMV 35*S* promoter through use of Eco RI sites present in cDNA linkers. The pMON530 T-DNA confers resistance to kanamycin (50 μg/ml) on transgenic plants.
10. In all transformations and analyses, an *Arabidopsis* strain Columbia line homozygous for a mutation at the *gl1* locus was used as a marker (but otherwise wild type), except as noted.
11. Transformation was by the protocol of N. Bechtold, J. Ellis, and G. Pelletier [*C. R. Acad. Sci. Paris* **316**, 1194 (1993)], with modifications by A. Bent.
12. Six single-locus lines were identified by kanamycin segregation. Southern blot analyses revealed that all six lines contained independent insertions consisting of tandem repeats of the T-DNA. The plants used in this study represent the kanamycin-resistant progeny of T1 outcrosses from single-locus lines Tr242, 243, 244, 245, 246, and 248. Before germination, all seeds were plated on MS media (Gibco) with (all transgenic lines) or without (controls) kanamycin (50 μg/ml, Sigma) and incubated for 3 days at 4°C in the dark. Growth conditions were 16 hours light, 8 hours dark at 21°C, except as noted. Seedlings were transplanted into soil 8 days after germination.
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17. HPLC conditions were as described [C. W. Gehrke *et al.*, *J. Chromatogr.* **301**, 199 (1984)], with the following exceptions: genomic DNAs were isolated from plants immediately after the onset of flowering, treated with 20 μg of ribonuclease A (Sigma) for 30 min at 37°C, followed by passage through a Sepharose CL-6B (Pharmacia) spin column. Nucleosides were resolved on a Varian Vista 5500 Liquid Chromatograph with a Rainin Dynamax 5-μm Spherical Microsorb C18 column (100 Å pore size, 4.6 mm inner diameter by 15 cm length) with a 20-min isocratic gradient of 2.5% methanol, 50 mM KH_2PO_4 (pH 4.0), followed by a 10-min linear gradient to 8.0% methanol, 50 mM KH_2PO_4 (pH 4.0).
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28. Flowering time refers to the number of days elapsed from seed germination until emergence of an inflorescence bolt 0.5 to 1.0 cm in height.
29. Pool sizes for each line were as follows: wild-type, Tr242, Tr245: $n = 6$; Tr243 and Tr244, $n = 4$; Tr246, $n = 11$; and Tr248, $n = 15$.
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Reduction of Morphine Abstinence in Mice with a Mutation in the Gene Encoding CREB

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Chronic morphine administration induces an up-regulation of several components of the cyclic adenosine 5′-monophosphate (cAMP) signal transduction cascade. The behavioral and biochemical consequences of opiate withdrawal were investigated in mice with a genetic disruption of the α and Δ isoforms of the cAMP-responsive element-binding protein (CREB). In CREBαΔ mutant mice the main symptoms of morphine withdrawal were strongly attenuated. No change in opioid binding sites or in morphine-induced analgesia was observed in these mutant mice, and the increase of adenylyl cyclase activity and immediate early gene expression after morphine withdrawal was normal. Thus, CREB-dependent gene transcription is a factor in the onset of behavioral manifestations of opiate dependence.

Adaptations in the cAMP signal transduction pathway underlie the mechanisms of opiate tolerance and dependence, and up-regulation of these components plays an important role in the onset of the withdrawal syndrome (*1–3*). In particular, the activation of the transcription factor CREB is implicated in naloxone-precipitated withdrawal syndrome (*4*). Here, the behavioral manifestations of abstinence were investigated during naloxone-precipitated morphine withdrawal in mice with a targeted mutation of the gene encoding CREB (*5*). This mutation causes a hypomorphic allele of the CREB gene such that two of the three known transcriptionally active isoforms are disrupted, CREB α and Δ (*6*). A minor isoform of the CREB gene, CREBβ, is up-regulated in these mutant mice. However, given that the transcriptional activity of CREBβ is lower than that of CREBΔ and that the overall level of CREB protein, based on protein immunoblot and immunohistochemical analysis, is reduced (*6*), we estimate that the homozygous mutants have about 10 to 20% of residual CREB activity. In vivo studies have shown that this reduction in CREB activity leads to impairment of memory consolidation in these mice (*7*).

Opiate dependence was induced by repeated morphine injection, and the behavior of the mice was observed in transparent round plastic boxes with white floors before and after naloxone administration. The mutant mice treated with saline appeared healthy and their spontaneous behavior nearly normal, except for the presence of a lower number of rears (14.54 ± 4.68, mean ± SEM) compared with control wild-type animals [44.61 ± 7.43, $t(1,22) = 3.27$, $P < 0.005$, two-tailed Student's t test]. To further characterize the behavior of mutant mice, we also evaluated their responses in the open-field test (*8*). No significant difference was observed between wild-type and mutant mice for any of the behavioral parameters quantified in this test (only a tendency of mutant mice to cross more squares was observed: 317.9 ± 30.8 squares compared with 259.9 ± 10.7 in wild type, mean ± SEM). This result indicates that mutant mice elicit a normal behavioral response to a stressful situation. Analgesia was measured after the first morphine administration [20 mg per kilogram of body weight, administered intraperitoneally (i.p.)] with the hot plate test in which the animal is placed on a hot plate and the elapsed time for several behavioral responses is recorded. The hot plate was set to shut off after 90 s (*9*). There was no difference between the mutant and the wild-type mice in the latency of either morphine-increased licks (wild type = 64.9% analgesia, mutant = 54.4% analgesia) or jumps (wild type = 95.1% analgesia, mutant = 100% analgesia) (*10*). Morphine treatment induced classical responses in both mutant and wild-type mice, such as the Straub reflex (reflex posture of the tail exhibited by rodents after opiate administration) and increased horizontal locomotor activity. Thus, the acute analgesic responses and the changes in locomotor activity induced by opiates were not affected in CREBαΔ mutant mice.

In chronically treated wild-type mice, naloxone administration [1 mg/kg, administered subcutaneously (s.c.)] precipitated the standard behavioral signs of withdrawal in morphine-treated animals and did not trigger behavioral changes in saline-injected control groups (Fig. 1). In contrast, a dramatic decrease in all nine classical signs of withdrawal was observed in the mutant mice. Sniffing and ptosis (drooping of the upper eyelid) were almost completely absent in the CREBαΔ mutant mice. All seven other parameters an-

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