CBP is required for environmental enrichment-induced neurogenesis and cognitive enhancement

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The epigenetic changes of the chromatin represent an attractive molecular substrate for adaptation to the environment. We examined here the role of CREB-binding protein (CBP), a histone acetyltransferase involved in mental retardation, in the genesis and maintenance of long-lasting systemic and behavioural adaptations to environmental enrichment (EE). Morphological and behavioural analyses demonstrated that EE ameliorates deficits associated to CBP deficiency. However, CBP-deficient mice also showed a strong defect in environment-induced neurogenesis and impaired EE-mediated enhancement of spatial navigation and pattern separation ability. These defects correlated with an attenuation of the transcriptional programme induced in response to EE and with deficits in histone acetylation at the promoters of EE-regulated, neurogenesis-related genes. Additional experiments in CBP restricted and inducible knockout mice indicated that environment-induced adult neurogenesis is extrinsically regulated by CBP function in mature granule cells. Overall, our experiments demonstrate that the environment alters gene expression by impinging on activities involved in modifying the epigenome and identify CBP-dependent transcriptional neuroadaptation as an important mediator of EE-induced benefits, a finding with important implications for mental retardation therapeutics.

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Introduction

Animals have developed a complex nervous system for adapting their conduct to the ever-changing environmental conditions. This flexibility at the behavioural level depends on the ability of neuronal circuits to evolve based on previous experiences through cellular mechanisms, such as functional and structural plasticity. These processes rely on the activation of specific and complex transcriptional programmes. A novel idea that has come under discussion in recent years is that these gene programmes are also subjected to activity-driven modulation through epigenetic modification of the chromatin of neural cells (Borrelli *et al*, 2008; Zocchi and Sassone-Corsi, 2010). Importantly, the malfunction of these processes can contribute to the molecular aetiology of cognitive disorders (Graff and Mansuy, 2009). A good example of epigenetic disorder is the Rubinstein–Taybi syndrome (RSTS), a complex autosomal-dominant disease characterized by cognitive impairments and skeletal abnormalities (Rubinstein and Taybi, 1963; Wiley *et al*, 2003) associated to mutations in the gene encoding the CREB-binding protein (CBP) (Petrij *et al*, 1995). CBP is a transcriptional co-activator with lysine acetyltransferase (KAT) activity and thereby it has the ability to leave epigenetic marks on the chromatin (Chan and La Thangue, 2001). The recent characterization of several mouse models for RSTS has demonstrated a direct role of the KAT activity of CBP in RSTS pathology and highlighted the importance of histone acetylation in neuronal plasticity and memory in the normal brain (Bourtchouladze *et al*, 2003; Alarcon *et al*, 2004; Korzus *et al*, 2004; Wood *et al*, 2005, 2006; Chen *et al*, 2010; Viosca *et al*, 2010; Barrett *et al*, 2011; Valor *et al*, 2011).

Environmental enrichment (EE) has been found to be beneficial in a number of cognitive disorders (Nithianantharajah and Hannan, 2006), including different forms of mental retardation. Exposing laboratory rodents to continuous or repeated sessions of EE increase dendritic branching and spine number in hippocampal neurons, promotes neurogenesis and the integration of newborn neurons in functional circuits and improves learning and memory (van Praag *et al*, 2000; Nithianantharajah and Hannan, 2006). These events likely require the activation of complex gene networks, but the nature and sequence of the genetic programme underlying these experience-driven changes in the structure and function of neuronal circuits remains elusive. Interestingly, EE has been shown to promote hippocampal histone acetylation (Fischer *et al*, 2007), a process that is impaired in RSTS mice (Alarcon *et al*, 2004).

We explore here the benefits of EE in a mouse model of RSTS mice. Our results reveal that EE promoted synaptic growth and alleviated some behavioural and cognitive deficits associated to RSTS. However, CBP-deficient mice showed a strong defect in environment-induced neurogenesis that correlated with attenuation of the transcriptional

programme associated with this process and with impaired EE-mediated enhancement of spatial navigation and pattern separation. Our results support a specific role for CBP in environment-induced neurogenesis and identify CBP-dependent transcription and histone acetylation as important mediators of environment-induced benefits.

Results

EE ameliorates some behavioural deficits in *cbp*^{+/-} mice, but does not cause an improvement of spatial memory and discrimination

EE is known to trigger major structural and functional changes in the hippocampus (Nithianantharajah and Hannan, 2006). To examine the efficacy of behavioural therapy in RSTS, we compared cohorts of *cbp*^{+/-} and control littermate mice housed either in standard cages (SC) or in a large EE in a number of paradigms.

We first examined whether EE promoted synaptic growth in this mouse models of RSTS by assessing dendritic spine density and morphology in CA1 pyramidal neurons of Thy1-EGFP-M/*cbp*^{+/-} double mutants in different housing conditions. These animals exhibit an *in vivo* Golgi-like staining in the CA1 area in which few neurons are intensively labelled with EGFP expression (Feng *et al*, 2000). The examination of the number of dendritic spines in the *stratum radiatum* demonstrated that EE triggered similar structural changes in hippocampal pyramidal neurons of *cbp*^{+/-} mice and control littermates (Figure 1A and B; spine density two-way ANOVA: $F_{(1,24)housing} = 5.32$, $P = 0.03$). Neither EE nor CBP deficiency altered the morphology of dendritic spines and hippocampal dendritic organization (Supplementary Figure S1).

EE is also known to have important consequences in the animals' behaviour (Nithianantharajah and Hannan, 2006). We observed that *cbp*^{+/-} and control littermates behaved similarly in the open field regardless of their housing condition (Supplementary Figure S2A, B, and that both genotype and housing had a mild impact in the elevated plus maze task (Supplementary Figure S2C, D). EE, however, caused a remarkable improvement of the animals' performance in an accelerated RotaRod task in both genotypes (Figure 1C; latency to fall two-way ANOVA repeated measures: $F_{(1,36)genotype} = 5.55$, $P = 0.02$; $F_{(1,36)housing} = 5.21$, $P = 0.03$), reversing previously described deficit of *cbp*^{+/-} mice (Alarcon *et al*, 2004). The *cbp*^{+/-}-EE group stayed in the RotaRod as long as the wild type (WT)-SC group, but did not reach the performance level of the WT-EE group. Importantly, the reversal of behavioural deficits was not restricted to locomotor impairments. *Cbp*^{+/-} mice, like other CBP-deficient strains (Barco, 2007), are impaired in diverse memory tasks, including contextual fear conditioning (Alarcon *et al*, 2004). We subjected cohorts of *cbp*^{+/-} and control littermates housed either in SC or in EE to contextual fear conditioning and observed a similar effect of EE (Figure 1D; freezing time two-way ANOVA: $F_{(1,35)genotype} = 13.52$, $P = 0.001$; $F_{(1,35)housing} = 10.72$, #: $P = 0.002$). The *cbp*^{+/-}-EE group showed similar memory as compared with the WT-SC group (*t*-tests; *cbp*^{+/-}-SC versus WT-SC: $t_{(18)} = 3.76$, $P = 0.001$; *cbp*^{+/-}-EE versus WT-SC: $t_{(17)} = 0.24$, $P = 0.81$), but lower than the WT-EE group (*t*-test; WT-EE versus *cbp*^{+/-}-EE: $t_{(17)} = 2.14$, $P = 0.05$).

Figure 1 EE-mediated structural, behavioural and cognitive benefits. (A) Representative confocal images showing spines protruding from dendritic segments of hippocampal CA1 pyramidal neurons from *cbp*^{+/-} mice and control littermates in SC or EE conditions. Scale bar: 5 μm. (B) Mice exposed to EE, independently of the genotype, show increased density of dendritic spines compared with animals kept in SC. Two-way ANOVA, #: significant housing effect; $n = 6-8$ mice per group. These EE-dependent changes did not affect the morphology of dendritic spines (Supplementary Figure S1A-C) or the general cytoarchitecture of the hippocampus (Supplementary Figure S1D and E). (C) *Cbp*^{+/-} mice show deficits in RotaRod performance and the exposure to EE causes a recovery in mutant mice and an improvement of the performance of WT mice. Two-way ANOVA, #: significant housing effect, \$: significant genotype effect; $n = 10$ mice per group. (D) *Cbp*^{+/-} mice show an impairment in contextual fear conditioning. EE rescues this deficit and improves the contextual memory of WT mice. Two-way ANOVA, #: significant housing effect, \$: significant genotype effect. *t*-Tests compared with WT-SC group: * $P < 0.05$; ** $P < 0.005$; $n = 9-10$ mice per group.

We also examined whether EE has a beneficial impact in the navigation skills of *cbp*^{+/-} mice. In agreement with the reversal of motor deficits in the RotaRod task, the *cbp*^{+/-}-EE group increased their swimming speed in the Morris water maze (MWM) task (Supplementary Figure S2E), reversing the previously reported locomotor deficit (Alarcon *et al*, 2004). As previously reported (Alarcon *et al*, 2004), *cbp*^{+/-} and control littermates maintained in SC performed equally well the hidden platform task in the MWM ($F_{(1,16)genotype} = 2.44$, $P = 0.14$). As expected, EE improved the performance of WT mice both during training ($F_{(1,17)housing} = 6.97$, $P = 0.02$; Figure 2A, upper panel) and in the two probe trials (Figure 2B, upper panel). In contrast, *cbp*^{+/-} mice showed the same performance regardless of the housing condition ($F_{(1,14)housing} = 0.07$, $P = 0.79$; Figure 2A and B, lower panels). Interestingly, the large number of annulus crossings in the WT-EE group indicates that these animals show better spatial discrimination ability than the other three groups (Figure 2B).

To complete this comprehensive behavioural analysis, we assessed working memory and pattern separation ability in a water radial maze (WRM) task in which the mice were tested for the ability to select, from a choice of two arms, the arm harbouring the escaping platform (Figure 2C). We performed

Figure 2 *Cbp*^{+/-} mice show impaired EE-enhanced spatial navigation and pattern recognition ability. (A) The two-way ANOVA analysis of path lengths in the water maze task revealed a significant housing effect ($F_{(1,31)}^{\text{housing}} = 4.81$, $P = 0.04$), no genotype effect ($F_{(1,31)}^{\text{genotype}} = 0.37$, $P = 0.55$), and indicated a possible genotype $\times$ housing interaction ($F_{(1,31)}^{\text{genotype} \times \text{housing}} = 3.53$, $P = 0.07$). More precisely, EE improved the performance in WT mice (upper panel, two-way ANOVA, #: significant housing effect), but not in *cbp*^{+/-} mice (lower panel, NS: non-significant); $n = 8$ –10 mice per group. (B) EE housed wt mice showed more annulus crossings in the first and the second probe trials (upper panels: P1, $P = 0.06$ no significant difference; P2, $*P = 0.03$), whereas *cbp*^{+/-} mice did not exhibit any housing effect (lower panels: P1, $P = 0.90$; P2, $P = 0.70$). (C) (Upper panels) Schematic representation of the WRM tests used to measure pattern separation. Mice were tested for their pattern separation ability by comparing their performance in two types of tests: low separation tests (LOW) in which the target arm (T, where the platform is located) and the choice arm (C) were contiguous, and high separation tests (HIGH) in which the target and the choice arms were separated by a closed arm. Graph: In the third day of training, the mice were subjected to two symmetrical low and high separation tasks and the average performance was calculated. WT mice housed in an enriched environment (WT-EE) performed well both kind of tests (percentage of correct HIGH: $t_{(7)} = 2.65$, $P = 0.03$; percentage of correct LOW: $t_{(7)} = 3.42$, $P = 0.01$), whereas WT housed in standard cages (WT-SC) were only successful in the high separation tests (percentage of correct HIGH: WT-SC, $t_{(9)} = 3.00$, $P = 0.02$). In contrast, *cbp*^{+/-} mutants failed in both the HIGH and the LOW tests. $*P < 0.05$ *t*-tests versus 50 (chance); $n = 8$ –10 mice per group.

four probe trials (T1–T4) and tested whether mice could differentiate between locations that were presented closely in space (LOW: T2 and T4) versus those that were more

highly separated (HIGH: T1 and T3). *Cbp*^{+/-} mice were impaired in the spatial discrimination task (two-way repeated measures ANOVA: $F_{(1,30)}^{\text{genotype}} = 5.55$, $P = 0.02$). In addition, only WT-EE mice performed equally well at low and high separations, whereas *cbp*^{+/-} mice (regardless of the housing condition) and WT-SC mice could not discriminate between the choice and the target arms when presented in close spatial proximity (Figure 2C). These results indicate that EE enhanced pattern separation ability in WT mice, an skill that is likely to be in higher demand when the animal lives in an enriched, changing environment.

Overall, our comprehensive behavioural analysis demonstrated that EE has a beneficial effect in various motor and cognitive abilities in *cbp*^{+/-} mice and unveiled new deficits associated to impaired CBP function, namely reduced EE-enhanced spatial navigation and pattern separation ability.

CBP is specifically required for environment-induced neurogenesis

According to current theoretical models describing the role of the DG in the processing and storage of spatial information, newborn neurons in the subgranular zone (SGZ) are hypothesized to facilitate pattern separation and spatial memory resolution (Deng *et al*, 2010; Aimone *et al*, 2011; Sahay *et al*, 2011b). Therefore, our results in the MWM and WRM may suggest a specific defect in environment-induced neurogenesis. To assess this hypothesis, we examined adult neurogenesis in *cbp*^{+/-} mice by BrdU uptake.

Adult neurogenesis is a complex and dynamic phenomenon that takes several weeks. Neural progenitors go through different stages before becoming mature granule cells integrated in functional circuits (Kronenberg *et al*, 2003; Zhao *et al*, 2008). Five weeks after BrdU administration, most BrdU⁺ cells (LRC, label-retaining cells) were double labelled with the marker for mature neurons NeuN (Figure 3A). In this condition, the quantification of LRC in the two neurogenic regions in the adult mouse brain, the SGZ and the SVZ (subventricular zone), demonstrated that basal adult neurogenesis is not affected in *cbp*^{+/-} mice (Figure 3B and C; Supplementary Figure S3). We next examined EE-induced neurogenesis and found that EE increased >10-fold the number of newborn neurons in the SGZ of WT mice, whereas *cbp*^{+/-} mice only showed a modest increase (Figure 3B; $F_{(1,13)}^{\text{housing}} = 40.05$, $P < 0.001$; $F_{(1,13)}^{\text{genotype}} = 14.57$, $P < 0.01$; $F_{(1,13)}^{\text{genotype} \times \text{housing}} = 15.91$, $P < 0.01$). Our experiments therefore revealed a good correlation between EE-enhanced neurogenesis and spatial navigation and pattern separation abilities, supporting a specific role for newborn neurons in these skills.

To define more precisely the impairment associated to CBP deficiency, we examined the anatomy and cellular composition of the hippocampus of mutant mice and control littermates by magnetic resonance imaging (MRI; Supplementary Figure S4) and histological analyses (Supplementary Figure S5). *Cbp*^{+/-} mice had normal hippocampal structure, cell density and hippocampus/brain ratio despite of their slightly smaller body size, facial dysmorphia and skull abnormalities (Viosca *et al*, 2010). We also examined neuronal differentiation in the SGZ using different markers. The number of quiescent type-1 progenitors (radial nestin⁺ cells) did not show significant difference between genotypes or housing conditions (Supplementary Figure S6A). In contrast, the

Figure 3 Impaired induced neurogenesis in the SGZ of *cbp*^{+/-} mice. **(A)** Animals received two daily injections of BrdU (100 mg/kg) for 5 consecutive days starting at day 11 of EE and were perfused (P) 35 days later. The right image shows cells in the SGZ immunolabelled with antibodies against BrdU (red) and NeuN (green) and nuclei counterstained with DAPI (blue). Five weeks after the last administration of BrdU, the vast majority of surviving cells were NeuN⁺. **(B)** CBP-deficient mice show a severe impairment in EE-induced neurogenesis in the SGZ. The right panels show representative images of BrdU (brown nuclei) immunostaining showing newborn neurons in the SGZ of WT and *cbp*^{+/-} mice in SC and EE. Two-way ANOVA, #: significant housing effect, §: significant genotype effect, &: significant genotype $\times$ housing interaction; $n = 3$ –4 mice per group. Scale bars: 100 μm . **(C)** KA-induced neurogenesis is also impaired in *cbp*^{+/-} mice. Six days after a single administration of KA (20 mg/kg) (KA) or vehicle (Veh), WT and *cbp*^{+/-} mice received two daily BrdU injections for 5 consecutive days. Five weeks later, the mice were perfused (P) and adult newborn cells were stained for BrdU. The right panels show representative images of LRC immunolabelling in the SGZ of WT and *cbp*^{+/-} mice treated with KA or vehicle. Two-way ANOVA, #: significant housing effect, §: significant genotype effect; $n = 4$ –5 per group. Scale bars: 100 μm .

examination of maturing neurons with doublecortin (dcx) immunolabelling revealed no difference between genotypes in the basal condition and a reduction in *cbp*^{+/-} mice housed in an EE (Supplementary Figure S6B; $F_{(1,22)\text{housing}} = 15.03$, $P < 0.01$; $F_{(1,22)\text{genotype}} = 4.23$, $P = 0.05$; $F_{(1,22)\text{genotype} \times \text{housing}} = 4.39$, $P < 0.05$). This result indicates that EE-induced neurogenesis is already impaired at early differentiation stages. In line with this finding, the determination of immature neurons by LRC count at earlier times after BrdU administration revealed no difference between genotypes in the basal condition (Supplementary Figure S6C).

We examined next whether CBP deficiency also interfered with activity-dependent regulation of neurogenesis in a paradigm unrelated with EE: the cellular response to a single injection of the pro-epileptic drug kainic acid (KA), a manipulation which is known to trigger long-term structural and functional changes in the hippocampus (Parent *et al*, 1997). As expected, KA-injected WT mice displayed more BrdU/NeuN-positive cells than those injected with vehicle, whereas there was no significant increase in neurogenesis in the case of *cbp*^{+/-} mice (Figure 3C;

$F_{(1,19)\text{treatment}} = 6.97$, $P < 0.05$; $F_{(1,19)\text{genotype}} = 5.65$, $P < 0.05$; $F_{(1,19)\text{genotype} \times \text{treatment}} = 3.48$, $P = 0.08$).

CBP's paralog p300 has been also associated to RSTS; however, the cognitive deficits associated to p300 deficiency in both humans and mice are more modest than for CBP (Zimmermann *et al*, 2007; Viosca *et al*, 2010). To evaluate the specificity of the role of CBP in environment-induced neurogenesis, we performed equivalent BrdU uptake experiments in *p300*^{+/-} mice. This mutant strain showed normal adult neurogenesis in the SGZ both in standard conditions and after EE (Figure 4; $F_{(1,18)\text{housing}} = 31.17$, $P < 0.001$; $F_{(1,18)\text{genotype}} = 0.31$, $P = 0.59$).

Together, these results indicate that CBP plays a specific and important role in the adaptive response to tonic changes in the activity of hippocampal circuits triggered by experience.

Impaired transcriptional neuroadaptation and histone acetylation in the hippocampus of *cbp*^{+/-} mice

To identify the genes downstream of CBP involved in these deficits, we determined the gene expression profile of CBP-deficient mice and WT littermates housed in SC or after 2

Figure 4 Environment-induced neurogenesis is not altered in p300^{+/-} mice. p300-deficient mice show normal basal and EE-induced neurogenesis in the SGZ. The right panels present representative images of BrdU (brown nuclei) immunostainings. Two-way ANOVA, #: significant housing effect; $n = 3$ –6 per group. Scale bar: 100 μ m.

weeks of EE using microarrays. This analysis revealed that CBP deficiency had a very modest effect on basal gene expression, whereas EE triggered changes in >150 genes (Figure 5A).

None of the very few transcript clusters (TCs) significantly altered in the hippocampus of *cbp*^{+/-} mice at the basal stage showed a fold change (FC) larger than 1.2 (Supplementary Table SI). Interestingly, although the function of most of these low confidence candidate genes is poorly understood, some of them have been previously associated with mental retardation (e.g. *asl*, *spred2*, *srgap3*) and the development of the nervous system (e.g. *rtn4rl1*, *ffx1*, *crim1*) and may, therefore, contribute to RSTS neurological traits. As a validation of the microarray experiment, analysis of the individual probe sets comprised into CBP's TC confirmed a 50% reduction in the signal corresponding to exon 2, as it would be expected considering the gene targeting strategy used to generate these mice (Supplementary Figure S7A). Quantitative RT-PCR (qRT-PCR) assays using independent samples reproduced this result and confirmed that the reduction of CBP levels does not cause a compensatory upregulation of the paralog gene *Ep300* (Supplementary Figure S7B).

Regarding the transcriptional response to EE, our microarray analysis provided a comprehensive list of EE-induced genes in the mouse hippocampus (Supplementary Table SII). Interestingly, the *in silico* prediction tool PSCAN (Zambelli *et al*, 2009), which searches for TF consensus binding sequences in promoter regions, revealed a significant enrichment for CREB-binding sites, among other CBP-interacting transcription factors (Kasper *et al*, 2006), within the promoter region of these genes (Supplementary Table SIII). Gene Ontology (GO) analysis identified *Neurogenesis and neuron differentiation*, *Ion transport and homeostasis* and *Synaptic transmission* as the main biological functions affected by this condition (Figure 5E; Supplementary Figure S7C; Supplementary Table SIV).

The transcriptional programme induced by EE was clearly attenuated in *cbp*^{+/-} mice. Both gene upregulations and downregulations were affected (Figure 5A–D). Although all the GO functional groups listed above showed a significant interaction with genotype (Supplementary Figure S7D), *Neurogenesis and neuron differentiation* showed the most pronounced effect, manifested both in the number of entities affected and the level of statistical significance. The list of EE-regulated related to *Neurogenesis and neuron differentiation* (Supplementary Table SV) included 13 genes showing a

significant genotype effect and 16 genes showing significant genotype $\times$ housing interaction. Figure 5F presents several examples of genes directly related to neurogenesis whose expression was enhanced in WT mice housed in an EE, but not in *cbp*^{+/-} mice. Interestingly, PSCAN analysis revealed that the promoters of these genes were enriched in transcription factor binding site motifs recognized by some relevant CBP partners, such as NF- κ B, p53 and NRSF (Supplementary Table SVI). Independent qRT-PCR assays for some of these genes, such as the pro-differentiative cytokine *lif*, the pro-neurogenic transcription factor *neurog1* and the microtubule-associate protein *dcx*, confirmed the microarray data (Figure 5G).

Given the cellular heterogeneity of the hippocampus, our gene profiling experiment cannot discriminate between transcriptional changes in specific cellular types and changes in the cellular composition of the tissue. Interestingly, the list of neurogenesis-related genes showing a significant condition per genotype interaction (Supplementary Table SV) included both genes that likely play a cell-autonomous effect (i.e. *nes*, *gli2*) and genes encoding for proteins that may have a paracrine function in neurogenesis (i.e. *lif*), suggesting that transcriptional changes in both mature and newborn neurons can contribute to the altered gene profile. Importantly, in agreement with our observations regarding structural changes in CA1 neurons, the microarray data and independent qRT-PCR assays confirmed that CBP deficiency does not cause a general impairment in EE-regulated gene expression since we observed similar upregulation of a number of genes related to synaptogenesis, such as *bdnf*, *nptx2* and *vgf*, in both genotypes (Supplementary Figure 7E and F).

We next examined the acetylation state of mature neurons and neuroprogenitor cells in the DG of *cbp*^{+/-} mice. Previous analyses in these mice had demonstrated a reduction in hippocampal histone H2B acetylation. This modification has been associated to active transcription (Karlic *et al*, 2010) and appears to be one of the main reaction catalysed by the KAT activity of CBP *in vivo* (Alarcon *et al*, 2004; Valor *et al*, 2011). In agreement with this observation, we found that both, the proliferating cells in the SGZ (Figure 6A and B) and the surrounding mature granule cells (Figure 6C; Supplementary Figure S8A), have lower level of acetylated histone H2B (AcH2B) than the corresponding cells in WT mice. We, however, did not observe any effect of long-term exposure to EE in the bulk acetylation level of different histones and specific lysine residues (Supplementary Figure S8B and C).

Figure 5 Impaired neuroadaptive transcriptional response to EE in the hippocampus of *cbp*^{+/-} mice. **(A)** The hierarchical cluster of the 159 TCs differentially regulated in response to EE (corrected *P*-value < 0.05, FC > 1.3) reveals an attenuated transcriptional response in *cbp*^{+/-} mice. **(B)** Number of TCs upregulated (white) and downregulated (black) in response to EE in WT and *cbp*^{+/-} mice (referred to the respective SC groups) with FC > 1.3. **(C)** Venn diagram showing the number of EE-regulated TCs. **(D)** Scatter plot comparing, in WT and *cbp*^{+/-} mice, the FC of the 84 TCs differentially upregulated in response to EE. The dotted line indicates the threshold for FC. Most dots are located in the upper left quadrant, indicating that the changes are larger in WT animals. *r*₍₈₂₎ = 0.31, *P* < 0.05. **(E)** Pie diagram showing the number of unique entities associated to a GO term in each of the major functional categories identified in the analysis of gene sets differentially expressed in EE mice. **(F)** Bar graph showing the expression level of specific neurogenesis-related genes whose induction by EE is impaired in *cbp*^{+/-} mice (expression values extracted from microarray data). Two-way ANOVA, \$: significant genotype effect, &: significant genotype × housing interaction (non-corrected *P*-values). All these genes show a significant housing effect. Some interesting genes showing borderline significance are also presented. **(G)** qRT-PCR validation of EE-mediated hippocampal induction for the neurogenesis-related genes *lif*, *neurog1* and *dcx*. Two-way ANOVA, #: significant housing effect, \$: significant genotype effect, &: significant genotype × housing interaction; *n* = 3 per group.

Next, we explored through chromatin immunoprecipitation (ChIP) assays whether the reduced bulk histone H2B acetylation affected the acetylation state of the promoters of some of the neurogenesis-related genes differentially induced by EE in *cbp*^{+/-} mice and control littermates. Two-way ANOVA of ChIP data revealed significant effects of both genotype and housing condition. Whereas CBP deficiency caused a significant reduction in the acetylation state of histone H2B, EE caused a significant increase. We also found that CBP deficiency prevented the increase of AchH2B induced by EE (Figure 7A). Interestingly, ChIP assays using antibodies against AchH3 showed that CBP deficiency also caused deficits in the acetylation of histone

H3 at the promoters of the genes *dcx* and *nes* (Figure 7B) despite of the absence of significant deficits at bulk acetylation level (Supplementary Figure S8B and C). These local histone acetylation deficits were not restricted to the neurogenesis-related genes differentially induced in *cbp*^{+/-} mice, NPY which is not affected at the transcription level by genotype also showed reduced levels of histone H2B acetylation at its promoters (Figure 7A and B).

Environment-induced neurogenesis is extrinsically regulated by CBP function in granule cells

To further clarify the role of CBP-dependent mechanisms in gene expression in environment-induced neurogenesis, we

examined the consequences in adult neurogenesis of restricted ablation of CBP in mature neuron using CaMKIIa-creERT2/CBP^{f/f} mice. This regulatable and restricted CBP knockout strain shows a complete ablation of CBP activity in mature granule neurons of the dentate gyrus few days after tamoxifen administration together with a dramatic reduction in the acetylation level of neuronal histones (Figure 8A; Supplementary Figure S9), whereas neuroprogenitor cells and immature neurons show normal levels of CBP and histone acetylation (Figure 8A and B). CaMKIIa-creERT2/CBP^{f/f} and control littermates in SC and upon EE exposure received BrdU injections 12 weeks after tamoxifen administration. The quantification of the number of LRC in these four experimental groups showed that the specific loss of CBP in mature granule cells is sufficient to cause impaired environment-induced neurogenesis in the SGZ (Figure 8C; $F_{(1,17)\text{housing}} = 62.77$, $P < 0.001$; $F_{(1,17)\text{genotype}} = 10.91$, $P < 0.01$; $F_{(1,17)\text{genotype} \times \text{housing}} = 8.03$, $P < 0.05$). This result discards that the reported adult neurogenesis deficit were a consequence of developmental defects, and indicates that

activity-dependent extrinsic regulation of hippocampal neurogenesis requires proper levels of CBP in mature granule neurons of the dentate gyrus.

Discussion

Our experiments in a mouse model for RSTS mental retardation demonstrate that EE ameliorates some of the deficits associated to RSTS and unveil new impairments caused by CBP deficiency. *Cbp*^{+/-} mice have a strong defect in environment-induced neurogenesis that correlates with the attenuated induction of EE-regulated genes, with impaired EE-dependent histone acetylation at specific promoters and with impaired modulation of pattern separation and spatial navigation abilities. Additional experiments with conditional knockout mice indicate that environment-induced neurogenesis is extrinsically regulated by CBP function in granule cells. Moreover, our rigorous microarray analysis contributes to elucidate the still poorly defined gene programme induced in the hippocampus in response to EE. This information will represent a useful resource for future studies since we identified dozens of new genes involved in the neuronal response to this condition. Although CBP deficiency had a minor impact in basal hippocampal gene expression, our screen also identified possible CBP downstream genes that may be relevant for RSTS pathology and should be further explored.

Reversal of behavioural deficits in *cbp*^{+/-} mice: implications in RSTS therapeutics

Our interdisciplinary analyses demonstrate that long-term exposure to EE had a beneficial effect in various motor and cognitive abilities of *cbp*^{+/-} mice and promoted synaptic growth and induced a number of plasticity-related genes in the hippocampus regardless of the genotype. Our data, therefore, suggest that behavioural therapy may be, as for other cognitive disorders (Nithianantharajah and Hannan, 2006), beneficial for RSTS children. However, EE also unveiled new deficits associated to CBP hemideficiency. The result of two independent water maze experiments, the classical hidden platform task and a radial maze task adapted to examine pattern separation ability, showed that *cbp*^{+/-} mice, contrary to their control littermates, do not become more precise in the localization of the escape platform as result of the EE. There

Figure 6 Reduced histone acetylation in the dentate gyrus of *cbp*^{+/-} mice. (A) Representative confocal images of the DG labelled with BrdU (red) and acetylated histone H2B (green). Scale bar: 25 μm. (B) Quantification of fluorescence intensity in individual BrdU⁺ cells demonstrates that the acetylation of histone H2B is reduced in the proliferating neuroprogenitors of *cbp*^{+/-} mice. * $P < 0.05$ (unpaired two-tailed *t*-test), $n = 12$ –16 cells per group. (C) Quantification of fluorescence intensity in individual granule cells (BrdU⁻ cells adjacent to the BrdU⁺ cells shown in B) demonstrates a general reduction of histone H2B acetylation in the DG of *cbp*^{+/-} mice. * $P < 0.05$ (unpaired two-tailed *t*-test), $n = 46$ –50 cells per group.

Figure 7 Reduced histone acetylation at the promoters of neurogenesis-related genes. (A) ChIP assays using an antibody against ACh2B. (B) ChIP assays using an antibody against ACh3. Two-way ANOVA, #: significant housing effect, \$: significant genotype effect; $n = 3$ mice per sample, three samples per condition.

Figure 8 The environment-induced neurogenesis defect is not developmental and depends on intact levels of CBP in mature granule cells. (A) In forebrain-restricted inducible CBP knockout mice, tamoxifen injection causes the elimination of CBP immunoreactivity (red) in granule cells and severe hypoacetylation (green). The few remaining CBP⁺ cells in the inner blade of the DG show normal levels of AcH2B (arrow heads) and are NeuN⁺ (see Supplementary Figure S9), suggesting that only progenitors and newborn neurons still express CBP. Nuclei were stained with DAPI (blue). Scale bar: 50 μ m. (B) Immunohistochemistry for CBP (red) and DCX (green) in coronal sections of CaMKIIa-creERT2/CBP^{f/f} and WT/CBP^{f/f} mice treated with tamoxifen demonstrate that most of the remaining CBP⁺ nuclei in the SGZ of CaMKIIa-creERT2/CBP^{f/f} mice belong to Dcx⁺ cells (solid arrowheads). The empty arrowheads denote cells in the SGZ of the DG that were CBP⁺ and Dcx⁺. Nuclei were stained with DAPI (blue). Right, magnification of the dotted squares in the left images show CBP⁺ and DCX⁺ cells in the SGZ of the DG. Scale bar: 15 μ m. (C) Tamoxifen was administered to 2-month-old mice and 12 weeks later, mice were housed in EE or maintained in SC. Animals received two daily injections of BrdU (100 mg/kg) for 5 consecutive days starting at day 11 of EE. Five weeks after the last administration of BrdU, the animals were perfused (P) for immunohistochemistry. CaMKIIa-creERT2/CBP^{f/f} mice show a severe impairment in EE-induced neurogenesis in the SGZ. The right panels show representative images of BrdU (brown nuclei) immunostaining showing newborn neurons in the SGZ of CaMKIIa-creERT2/CBP^{f/f} mice and control littermates housed in either SC or EE. Two-way ANOVA, #: significant housing effect, \$: significant genotype effect, &: significant genotype $\times$ housing interaction; $n = 4-5$ per group. Scale bar: 100 μ m.

is a great deal of discussion about the role of hippocampal newborn neurons in learning and memory (Deng *et al*, 2010; Aimone *et al*, 2011; Ming and Song, 2011; Sahay *et al*, 2011b) and the extent to which increased neurogenesis is relevant for cognitive improvement in hippocampal-dependent tasks (Meshi *et al*, 2006; Jaholkowski *et al*, 2009). Our behavioural experiments provide additional correlative evidence supporting the recently presented hypothesis that newborn neurons in the DG play a specific role in spatial memory resolution and pattern separation (Clelland *et al*, 2009; Creer *et al*, 2010; Sahay *et al*, 2011a). In addition, these results indicate that there are two components in the neurological deficits observed in RSTS mice, one of them susceptible of recovery through EE (e.g. locomotor and fear conditioning impairments) and another resistant to this condition and related to the production of newborn neurons in the SGZ. Overall, these findings suggest a role of defective neurogenesis in RSTS cognitive impairments and predict that the efficacy of environmental therapies might be more limited than in other mental impairment disorders (Jirtle and Skinner, 2007). Our study might also have important implications for other neurological diseases since a similar specific defect in enrichment-mediated hippocampal neurogenesis has been

reported for mice expressing Presenilin 1 (PS1) variants linked to early-onset familial Alzheimer's disease (FAD) (Choi *et al*, 2008) and for PS1-deficient mice (Feng *et al*, 2001). Since PS1 deficiency causes a reduction of the expression of CBP (Saura *et al*, 2004), our finding provides an attractive molecular explanation for FAD-linked defects in neurogenesis.

Role of CBP in environment-induced neurogenesis

Although CBP has been recently shown to regulate embryonic neural differentiation (Wang *et al*, 2010), our experiments indicate that a normal level of CBP is neglectable for steady-state neurogenesis in the adult hippocampus, but becomes again necessary for modulating the rate of neurogenesis in response to environmental challenges, such as EE and induced seizures, suggesting a general role for CBP in the adaptation of hippocampal circuits to external stimuli. CBP does so likely in coordination with other epigenetic factors like Gadd45b, a protein previously involved in DNA repair, whose loss also causes a deficit in environment-induced neurogenesis (Ma *et al*, 2009a). CBP plays a dual role in transcriptional regulation: it acts as transcriptional co-activator for a large number of transcription factors and as

epigenetic enzyme with intrinsic KAT activity (Chan and La Thangue, 2001). It is likely that both functions will contribute to the neurogenesis and transcriptional defects observed in CBP-deficient mice.

The most studied partner of CBP is CREB, an activity-regulated transcription factor that plays important roles in cognition (Benito and Barco, 2010) and adult neurogenesis (Merz *et al*, 2011). CREB regulates different phenomena during neurogenesis, from proliferation and survival of neuroprogenitors to the maturation and integration of newborn neurons in neuronal circuits (Zhu *et al*, 2004; Jagasia *et al*, 2009; Merz *et al*, 2011). Other transcription factors involved in adult neurogenesis also use CBP as co-activator (Medrano and Scrable, 2005; Denis-Donini *et al*, 2008). These include NF- κ B and p53 that are, in addition, direct substrates of CBP's KAT activity (Ito *et al*, 2001; Nadiminty *et al*, 2006). Interestingly, we observed a strong and significant enrichment for the binding motifs of some of these transcription factors in the promoters of neurogenesis-related genes regulated by EE and showing a significant genotype effect or genotype $\times$ housing interaction.

Histone acetylation also plays an important role in neurogenesis (Lee and Lee, 2010). *In vitro* experiments using HDAC inhibitors (HDACi), with few exceptions, have consistently shown that the treatment with HDACi reduces neural cell proliferation and promotes neuronal differentiation (Hao *et al*, 2004; Hsieh *et al*, 2004; Yu *et al*, 2009; Umka *et al*, 2010). However, the results of *in vivo* experiments are more difficult to interpret (Jessberger *et al*, 2007; Kim *et al*, 2009), probably because the reduction of proliferation by HDACi may result in a net reduction of neurogenesis in spite of the potential activity of these compounds to promote neuronal differentiation. Interestingly, the deficiency in HDAC2 results in specific and cell-autonomous defects in neural differentiation during adult neurogenesis (Jawerka *et al*, 2010). Therefore, the balance between histone acetylation/deacetylation seems to be critical for the correct activation and/or inactivation of neurogenic programmes.

CBP and neuroadaptation to environmental changes

It has been proposed that epigenetic mechanisms, such as DNA methylation or histone modification, serve as key conduits for the extrinsic regulation of adult neurogenesis by a wide variety of stimuli, including the environment and internal physiological states (Ma *et al*, 2009b, 2010). Since the recruitment of CBP to specific promoters is regulated by neuronal activity and depends on the sort of stimuli (Hardingham *et al*, 1999), this protein is in a privileged position to link neuronal circuit activity to epigenetic modification of the chromatin, leading to persistent or permanent changes in neuronal circuits through changes in gene expression.

We and others have proposed that the reduction of CBP and the subsequent hypoacetylation of histones may interfere with the transcriptional response driven by activity, thus contributing to the cognitive deficits observed in mouse models for RSTS (Barco, 2007). Acetylation marks in the chromatin are considered a feature of active transcription (Kouzarides, 2007). In particular, the presence of acetylated H2B has been associated to highly transcribed genes and with the maintenance of transcriptional competence at specific *loci* (Myers *et al*, 2003; Karlic *et al*, 2010). Recent results indicate

that this may be a consequence rather than a cause of the high transcriptional activity (Kasper *et al*, 2010; Valor *et al*, 2011). Histone acetylation, or at least some specific histone acetylation marks, may be associated to the ability to respond to certain stimuli rather than be an exact readout of the transcriptional activity of the loci. This would explain why the histone acetylation defects shown in Figures 6 and 7 do not have a direct correlate in basal hippocampal gene expression and basal adult neurogenesis. Given the role of CBP in the setting of epigenetic marks, it is maybe not surprising that the transcriptional consequences of its deficiency became more evident in the context of the establishment of a new long-term transcriptional stage, such as the response to EE. In agreement with this view, our microarray experiment indicates that most of the transcriptional programme induced by EE was affected by CBP deficiency.

The study of gene–environment interactions has experimented important progress in the last years. The epigenetic modification of the genome provides mechanisms that allow the stable propagation of gene activity states from one generation of cells to the next (Jaenisch and Bird, 2003). Referring to neurons, the same epigenetic events can underlie the long-term maintenance, maybe for the whole life of the individual, of new gene activity states, providing a plausible link between experience and long-lasting alterations in gene expression in the brain (Fischer *et al*, 2007). In fact, recent studies have shown that some of the benefits of EE can be transmitted to the offspring, which necessarily involves the participation of epigenetic mechanisms (Arai *et al*, 2009). Our study demonstrates that the environment can alter gene expression and its functional outputs by impinging on activities involved in modifying the epigenome, highlighting the importance of neural histone acetylation in gene–environment interaction and adult neurogenesis (Hsieh and Eisch, 2010). It also identifies CBP-dependent transcription and histone acetylation as important mediators of environment-induced benefits.

Materials and methods

Maintenance, treatment and housing of mice

The generation of *cbp*^{+/-} (Tanaka *et al*, 1997), *p300*^{+/-} (Yao *et al*, 1998), *CBP*^{f/f} (Zhang *et al*, 2004), *CaMKII α -creERT2* (Erdmann *et al*, 2007) and *Thy1-EGFP* (line M) (Feng *et al*, 2000) mice have been previously described. The experiments with *cbp*^{+/-} mice were performed on a DBA and C57BL/6J mixed background, since these mutants are not viable in a pure C57BL/6J background (Alarcon *et al*, 2004). The genetic background of all other mice was C57BL/6J. Experiments were performed in 2- to 7-month-old animals and in all cases the mice used as control were littermates of the mutant mice. For CBP ablation experiments, tamoxifen (T5648; Sigma-Aldrich) was administered to ~2-month-old *CaMKII α -creERT2* / *CBP*^{f/f} mice using a gastric probe for 5 consecutive days (total consumption = 20 mg per animal); control animals were *CaMKII α -creERT2* / *CBP*^{f/f} treated with the same volume of the vehicle (corn oil C8267; Sigma-Aldrich) and non-cre recombinase expressing *CBP*^{f/f} treated with tamoxifen. To observe nuclear translocation of cre recombinase in a control experiment, tamoxifen was administered once more the day before sacrifice. Mice were maintained according to animal care standards established by the European Union and all the protocols were approved by the Institutional Animal Care and Use Committee. The mice were kept on a 12-h light/dark cycle and food and water were provided *ad libitum*. Standard housing consisted of 30 $\times$ 15 $\times$ 11 cm³ clear cages occupied by up to five mice. The EE boxes were large white plexiglass boxes (> 1 m²) and were occupied by a maximum of 20 mice. We used natural materials, plastic tubing, running wheels and toys to create

an EE whose configuration was modified every 48 h shortly before the start of the dark cycle. The samples from EE animals used in the western blot, immunohistochemistry, qRT-PCR and microarray experiments were obtained at least 16 h after the last change to prevent any interference between the response to novelty and to EE. Intraperitoneal (i.p.) KA injection (20 mg/kg in saline solution) caused overt seizures 5–10 min later, which lasted for ~2–3 h before they spontaneously stopped. All mice displayed status epilepticus that started with jerking of the forelimbs, often followed by seizures and involuntary falling. One death occurred among 12 animals both in the WT and in the *cbp*^{+/-} group. For BrdU uptake experiments, the animals received i.p. injections of BrdU (100 mg/kg in saline solution) at the times indicated in the legends of Figures 3, 4 and 8; Supplementary Figures S3 and S9.

Antibodies

In this study, the following primary antibodies were used: α -ACh2A, α -ACh2B, α -ACh3 and α -ACh4 (Sanchis-Segura *et al*, 2009); α -H2B (07-371), α -ACh2B-K5 (07-382), α -ACh2B-K12 (07-336), α -ACh2B-K15 (07-343), α -ACh2B-K20 (07-347), α -H3 (05-499), α -pH3-S10 (06-570), α -ACh3-K14 (06-911), α -ACh4-K8 (07-328) and α -NeuN (MAB377) from Millipore (Billerica, MA, USA); α -ACh2A-K5 (5276) from Cell Signaling (Beverly, MA, USA); α -H3 (ab1791), α -BrdU (ab6326), α -dcx (ab18723), α -nestin (ab11306) and α -ACh3-K14 (ab52946) from Abcam (Cambridge, UK); α - β -actin (F5441), α -GFAP (G9269), and α -MAP2 (M4403) from Sigma-Aldrich Química SA (Madrid, Spain); α -GFP (A-11122) from Invitrogen (Carlsbad, CA, USA); α -CBP C1 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); α -Cre recombinase (Kellendonk *et al*, 1999). See Supplementary data for secondary antibodies details.

Quantitative western blotting

Western blot analyses were carried out as previously described (Sanchis-Segura *et al*, 2009). Equal amount of protein extracted from isolated hippocampi was loaded in each lane, and the intensity of the protein bands was measured using FUJIFILM LAS-100 equipment (Fujiphoto Film Co.) and quantified using Quantity One 4.6 software (Bio-Rad, Inc.).

Immunohistochemistry and structural analyses

Mice were anaesthetized with a ketamine/xylazine mixture immediately and perfused with paraformaldehyde (4% in 0.1 M phosphate buffer); brains were postfixed overnight. Immunostaining was performed on 50 μ m free-floating sections. For BrdU staining, sections were incubated in HCl 2 N for 30 min at 37°C, rinsed in 0.1 M borate buffer, pH 8.5, and thoroughly washed in Tris-buffered saline, pH 7.4. For diaminobenzidine (Sigma) immunostaining, sections were pretreated with 0.6% H₂O₂ to block endogenous peroxidase reaction. For immunofluorescence, sections were counterstained with DAPI (Molecular Probes). For the structural analysis of dendritic spines, the transgenic line Thy1-EGFP (Feng *et al*, 2000) was crossed with *cbp*^{+/-} strain to generate Thy1-EGFP/*cbp*^{+/-} double mutants. Two-way ANOVA and two-tailed unpaired *t*-test were used to analyse histological data. The experimenters were blind to the genotypes and housing of the mice for all quantifications. See Supplementary data for additional details on the analysis of dendritic spines and description of MRI methods.

Microarray analysis

Total RNA extracted from the hippocampi of three to four age, sex- and genotype-matched mice were used to produce one pooled sample. We analysed 16 samples (8 per genotype): 5 control (SC) and 3 EE. RNA samples were hybridized to GeneChip[®] Mouse Gene 1.0 ST Arrays according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). The microarray data were then analysed using GeneSpring GX 11 (Agilent Technologies, Inc., Santa Clara, CA). RMA (Robust Multichip Average) algorithm was used for data normalization. Principal component analysis revealed clustering of samples according to the batch of replicates; therefore, normalization was conducted using the median of the corresponding control samples values as reference. TCs were then filtered on signal intensity by establishing a lower cutoff at the 20th percentile (20th–100th percentile). Genotype and EE differentially regulated TC sets were obtained using two-way ANOVA. TCs differentially regulated by 'genotype' were also identified using unpaired *t*-test since no TC passed the thresholds defined in the two-way ANOVA analysis. In significance analysis, *P*-values were obtained by

asymptotic computation and corrected for multiple testing with Benjamini–Hochberg FDR method. Hierarchical clustering was also performed using GeneSpring software. GO enrichment analysis for differentially regulated genes upon EE was performed using the web-based gene set analysis toolkit (WebGestalt) (Zhang *et al*, 2005). PSCAN Versión 1.1.1 was used for transcription factor binding site discovery (Zambelli *et al*, 2009). See Supplementary data for additional detail. Microarray data are accessible through the Gene Expression Omnibus (GEO) database (GEO Series accession number GSE30880).

Quantitative RT-PCR and ChIP assays

Whole hippocampi were dissected and treated with RNAlater solution (Qiagen). DG subregions were microdissected from two 500- μ m slices obtained from dorsal hippocampus with a tissue chopper (Stoelting). Total RNA was extracted using RNeasy kit (Qiagen). Reverse transcription was performed using RevertAid First-Strand cDNA synthesis kit (Fermentas). qRT-PCR was carried out using SYBR GreenER mix (Invitrogen, Carlsbad, CA) and primers specific for the genes of interest. ChIP experiments were performed according to the protocol in the Millipore ChIP kit (Millipore) with minor modification based on Wells and Farnham (2002). See Supplementary data for addition details. Primer sequences used in ChIP and qRT-PCR assays are listed in Supplementary Table SVII.

Behaviour

For all behavioural tasks, we used adult female mutant and control littermates to prevent the fights observed between males housed in an EE. The experimenters were blind to genotypes. The result of the PCR-based genotyping was provided as a factor for statistical analysis of the behavioural data once the battery of tasks was concluded. The open field, elevated plus maze RotaRod, MWM and fear conditioning tasks were performed as previously described (Viosca *et al*, 2010). The training protocol in the MWM consisted of three trials per day with a 45-min inter-trial interval. If the mice did not find the platform after 120 s, they were gently guided to it. Memory retention trials of 60 s were performed at the beginning of day 5 (P1) and 24 h after concluding the training on day 8 (P2). The number of annulus crossings was calculated considering an area double than the platform. For the pattern separation experiment, we adapted the protocol described into a WRM (Clelland *et al*, 2009). A six-arm WRM was positioned in the centre of a pool (1.7 m of diameter) filled with opaque water. A platform of 10 cm diameter was placed 1 cm below the water level at the end of one selected arm. We positioned external cues in the walls to facilitate spatial navigation. The animals were trained per 3 consecutive days changing the platform position each day to ensure that mice use a hippocampus dependent, allocentric strategy to solve the task; the platform position and the target arm were changed and balanced between trials. The animals were exposed each day to 14 training trials and four probe trials. In each trial, mice were gently positioned in the centre of the maze facing to a closed arm and allowed to search for the platform for a maximum of 120 s. In the probe trials, the animals had to choose between two arms open (the target and the choice arms) at variable distance (HIGH: high spatial separation with one closed arm in between; or LOW: low spatial separation, in which the arms were contiguous). A trial was considered successful when the mouse entered into the target arm without making any error. See Supplementary data for addition details.

Statistical methods

Precise description of the statistical methods used in each experiment is presented in the text. In the graphs, error bars represent s.e.m.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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