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HP1-β is required for development of the cerebral neocortex and neuromuscular junctions


1Division of Immunogenetics, Department of Immunology and Cell Biology, Research Center Borstel, D-23845 Borstel, Germany
2Division of Genetics and Genomics, Institute of Molecular Medicine, University of Edinburgh, Edinburgh EH2 2HL, Scotland, UK
3Department of Developmental Biology, Uppsala University, Uppsala, Sweden
4Institute of Medical Research, University of London, London NW7 1AA, England, UK
5School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, England, UK
6Department of Anatomy and Cell Biology, University of Ulm, D-89069 Ulm, Germany
7Clinic of Obstetrics and Gynecology, University Hospital Schleswig-Holstein, D-24105 Kiel, Germany
8Department of Radiation Oncology, Washington University School of Medicine, St. Louis, MO 63108

HP1 proteins are thought to be modulators of chromatin organization in all mammals, yet their exact physiological function remains unknown. In a first attempt to elucidate the function of these proteins in vivo, we disrupted the murine Cbx1 gene, which encodes the HP1-β isotype, and show that the Cbx1-/-null mutation leads to perinatal lethality. The newborn mice succumbed to acute respiratory failure, whose likely cause is the defective development of neuromuscular junctions within the endplate of the diaphragm. We also observe aberrant cerebral cortex development in Cbx1-/- mutant brains, which have reduced proliferation of neuronal precursors, widespread cell death, and edema. In vitro cultures of neurospheres from Cbx1-/- mutant brains reveal a dramatic genomic instability. Our results demonstrate that HP1 proteins are not functionally redundant and that they are likely to regulate lineage-specific changes in heterochromatin organization.

Introduction

In mammals, there are three homologues of Drosophila melanogaster HP1, termed HP1-α, HP1-β, and HP1-γ (Jones et al., 2000). They share a high degree of sequence similarity and localize, to a lesser or greater extent, to constitutive heterochromatin: HP1-α and HP1-β are usually found enriched at sites of constitutive heterochromatin, whereas HP1-γ has a more uniform distribution (Dialynas et al., 2007). All three proteins are comprised of an N-terminal chromodomain (CD), an intervening "hinge" region, and a C-terminal chromoshadow domain (CSD) that dimerizes to form a hydrophobic pocket that can accommodate a pentapeptide sequence, PxVxL, found in several HP1-interacting partners (Thiru et al., 2004). Of the three proteins, HP1-β is the most studied.

HP1-β localizes to constitutive heterochromatin through a variety of interactions with chromatin. One involves a dynamic interaction (association constant in the micromolar range) of the HP1-β CD with Me(3)K9H3 that results from the enzymatic activities of KMT1A/B (Rea et al., 2000; Cheutin et al., 2003; Festenstein et al., 2003). In cells taken from double-null KMT1A/B mutant mice, which are viable albeit runted (Peters et al., 2001), the enrichment of both Me(3)K9H3 and Me(3)K20H4 at centromeric heterochromatin is lost, and HP1 proteins appear homogeneously distributed throughout both the eu- and heterochromatin (Kourmouli et al., 2004, 2005; Schotta et al., 2004). Structural...
analysis of the HP1-β CD–Me(3)K9H3 interaction reveals that the histone tail inserts as a β strand, completing the β-sandwich architecture of the HP1-β CD (Nielsen et al., 2001). The binding of HP1-β to Me(3)K9H3 is part of an epigenetic pathway in mammals: HP1-β bound to Me(3)K9H3 acts as an adapter to recruit KMT5B/C methyltransferases that trimethylate lysine 20 on histone H4 (Kourmouli et al., 2004, 2005; Schotta et al., 2004). The pathway from Me(3)K9H3 to Me(3)K20H4 via HP1 is thought to be important for the assembly of HP1-containing constitutive heterochromatin (Kourmouli et al., 2004, 2005; Schotta et al., 2004). A second mode of interaction is the binding of the HP1-β CD with the histone fold domain of histone H3 (Nielsen et al., 2001). This binding is of high affinity (in the nanomolar range; resistant to 0.6 M of salt) and is thought to represent the immobile HP1-β fraction (~5%) in heterochromatin observed in FRAP experiments (Nielsen et al., 2001; Schmiedeberg et al., 2004; Dialynas et al., 2006). An interaction of HP1-β with methylated K26 histone H1.4 has also been shown (Daujat et al., 2005), although its significance is not known.

As part of constitutive heterochromatin, HP1 homologues are found at both the centromeres and telomeres of nearly all eukaryotic chromosomes, and the proper maintenance of these chromosomal regions is critical for genome integrity (Fanti and Pimpinelli, 2008). Mammalian HP1 proteins found at pericentric heterochromatin have been implicated in recruiting both the cohesin complex and kinetochore proteins that are necessary for chromosome segregation at mitosis and the avoidance of an-cohesin complex and kinetochore proteins that are necessary for eukaryotic chromosomes, and the proper maintenance of these (Daujat et al., 2005), although its significance is not known.

In this study, we show that the mammalian Cbx1 gene, which encodes HP1-β, is essential for viability; thus, the HP1 isoforms are not functionally redundant. The loss of HP1-β protein leads to defective neuromuscular and cerebral cortex development. The defect in the latter is likely to be the result of a dramatic increase in genomic instability.

Results and discussion

We disrupted the Cbx1 gene using standard techniques (Fig. 1, A–C). Crosses between Cbx1+/− heterozygotes, which were indistinguishable from wild-type littermates, revealed that embryonic day 19 (E19) Cbx1−/− embryos were significantly smaller than their Cbx1+/− and wild-type littermates (Fig. 1 C). The Cbx1−/− homozygotes died at, or a few hours after, birth. HP1-β was not detected in Cbx1−/− nuclear extracts using specific antibodies to either the N or C terminus of HP1-β (see Fig. 3 I and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200804041/DC1). For initial characterization, we tested whether the Cbx1 mutation was a modifier of position-effect variegation (PEV) by introducing the mutation into mice heterozygous for the hCD2-I.3B variegating transgene located within centromeric constitutive heterochromatin (Festenstein et al., 1999). As shown in Fig. 1 D, there is a highly significant difference between hCD2 expression in the Cbx1−/− animals and the parental genotypes (P < 0.001). The difference between Cbx1−/− and Cbx1+/− is not significant. These data confirm that HP1-β can regulate heterochromatin-mediated gene silencing in vivo but that the sensitivity of hCD2 expression to Cbx1 gene dosage is coarse because only when both copies of Cbx1 are mutated is there a significant reduction in hCD2 repression (Fig. 1 D).

The coarse sensitivity of hCD2 variegation to Cbx1 gene dosage contrasts with the situation in Drosophila, in which loss of a single copy of the HP1 gene by introduction of the Su(var)205 mutation into flies that variegate for white significantly reduces repression (Eissenberg et al., 1990). This apparent species-specific difference in the sensitivity of PEV to gene dosage might be explained by the so-called mass action model of PEV (Locke et al., 1988). In this model, changes in the dosage of genes whose products are incorporated as tetramers (or higher order oligomers) have a much greater effect on heterochromatin formation (and thus repression) than genes whose products are incorporated monomers or dimers into heterochromatin. Given the coarse sensitivity of hCD2 variegation to Cbx1 gene dosage (Fig. 1 D), we suggest that HP1-β is incorporated into the mammalian heterochromatin in a dimeric form. This would be consistent with structural evidence, which shows that HP1-β forms stable dimers through CSD–CSD interactions (Thiri et al., 2004), and with a kinetic model based on FRAP analysis, which indicates that swi6p (the fission yeast HP1) is incorporated into yeast heterochromatin through one-to-one homotypic interactions (Cheutin et al., 2004). The fine sensitivity of Drosophila white variegation to HP1 gene dosage (Eissenberg et al., 1990) might reflect incorporation of the Drosophila HP1 protein into heterochromatin as higher order oligomers. Thus, although certain structural components of heterochromatin (proteins or RNAi) may be conserved across species, their stoichiometry during heterochromatin formation might be species dependent.

Cbx1−/− neonates exhibited no gross morphological abnormalities in the major organs. However, we observed that the lung alveoli remained collapsed after birth, indicating that perinatal death was a result of respiratory failure (Fig. 2 A). This suggested a defect in neuromuscular function. Whole mount staining of E19 diaphragms (Fig. 2 B) using antibodies to neurofilament (NF) to stain axons and α-bungarotoxin to stain postsynaptic acetylcholine receptors (AChRs) revealed that axonal growth is unaffected in Cbx1−/− animals, although there is sometimes a reduction in the amount of branching (Fig. 2 B, bottom). However, in Cbx1−/− diaphragms, we observed a significant reduction in the number of AChR clusters per micrometer of nerve (P < 0.004; Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200804041/DC1), indicating that the likely proximate cause of death is the inability of the diaphragm to respond to the activating signals from the intramuscular nerve. The staining of thigh muscle revealed a similar decrease in AChR clustering (P < 0.001; Table S1), indicating that the Cbx1−/− mutation has a widespread effect on neuromuscular development in mutant embryos.

The defect in neuromuscular development prompted us to examine other neuronal structures, such as the developing cerebral cortex in wild-type and Cbx1−/− embryos. The staining of developing neocortices at days E17 (Fig. 3, A, B, E, and F) and E19 (Fig. 3, C, D, G, and H) with a marker of postmitotic neurons,
neuronal nuclei (NeuN), showed clear differences between wild-type and Cbx1−/− neocortices. Specifically, we observed that subplate (SP) neurons of the E17 wild-type neocortex were stained with NeuN and formed a distinct boundary between the cortical plate (CP) and the intermediate zone, but in E17 Cbx1−/− brains, SP neurons were very weakly stained (Fig. 3, compare E with F).

Figure 1. Cbx1 gene function is essential, and its product, HP1-β, is a modifier of PEV. (A) The relevant regions in the wild-type Cbx1 locus (top; see Materials and Methods), the TK-Neo targeting vector (middle), and the targeted gene (bottom). Coding regions are depicted by closed boxes. Noncoding regions are denoted by striped boxes. The DNA probe used to screen for targeting events is shown as a shaded rectangle. (B) Southern blot authentication of germline transmission of the Cbx1 mutation. BamHI digest of genomic DNA produces an 11.6-kb fragment for the wild-type (wt) allele and a diagnostic 5.3-kb fragment for the targeted allele. (C) Images of wild-type (left) and Cbx1−/− (right) neonates. (D) Scatter plots showing the results of flow cytometry analysis of the proportion of transgenic embryonic DP (CD4+CD8+) thymocytes that express hCD2 taken from the three Cbx1 genotypes. Each point represents the result from a single embryo. The mean expression of hCD2 in embryonic thymocytes from Cbx1−/− animals is higher than that for Cbx1+/+ animals, but not significantly. The mean expression of hCD2 in thymocytes taken from Cbx1+/− animals is significantly different from expression in thymocytes taken from the parental genotypes (P < 0.001). Black lines represent the means.
At E19, the prominent NeuN staining of CP neurons in wild-type brains is absent in CP neurons of Cbx1−/− E19 brains (Fig. 3, compare G with H). The amount and heterochromatic localization of Me(3)K9H3 and Me(3)K20H4 were unchanged in Cbx1−/− brains (Fig. 3, I–K). Similarly, the amount and distribution of HP1-α and HP1-γ were also unchanged in Cbx1−/− brains (Fig. 3 I and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200804041/DC1).

The loss of NeuN staining in postmitotic neurons is thought to be a marker of cell death, where the loss of NeuN staining is
the result of a loss in antigenicity rather than absolute protein levels (Kuan et al., 2004; Unal-Cevik et al., 2004; Collombet et al., 2006). Therefore, we explored the possibility that the loss of NeuN staining in the Cbx1−/− brains might be caused by cell death; Western blot analysis confirmed that NeuN protein levels in Cbx1−/− E19 neocortices were the same as in the wild type (Fig. 3 J). We undertook an examination of the integrity of cortical lamination using Nissl staining of semithin sections of neocortex. 

Figure 3. Immunohistochemical and Western blot analysis of Cbx1−/− brains. (A–D) Hematoxylin and eosin–stained sagittal sections of E17 (A and B) and E19 (C and D) neocortices correspond to the antibody-stained cortices in E–H. Genotypes of the embryos are marked above the photographs. (E–H) α-NeuN–stained E17 and E19 neocortices. The staining of the wild-type E17 cortex with the α-NeuN antibody (E) detects a layer of SP cells that separates the CP from the intermediate zone (IZ). These cells are very weakly stained in the Cbx1−/− neocortex (F). Similarly, CP cells are strongly stained with the α-NeuN antibody in the E19 neocortex (G), but such staining of CP cells is reduced to background levels in the E19 Cbx1−/− neocortex (H). MZ, marginal zone. (I) HP1-β protein is not detected in extracts from Cbx1−/− brains using an antibody to the C terminus of HP1-β, an N-terminal antibody also fails to detect HP1-β in the same way (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200804041/DC1). The levels of HP1-α, HP1-γ, Me(3)K9H3, Me(3)K20H4, and NeuN are not significantly changed in Cbx1−/− compared with wild-type brain extracts. The bottom panel is the actin-loading control. Identical results were obtained using whole embryo extracts (not depicted). (J) Me(3)K9H3 heterochromatic distribution is not affected by the Cbx1−/− mutation. Cbx1−/− and Cbx1+/+ CP neurons show identical Me(3)K9H3 staining patterns. (K) Me(3)K20H4 heterochromatic distribution is not affected by the Cbx1−/− mutation. Cbx1−/− and Cbx1+/+ CP neurons show identical Me(3)K20H4 staining patterns. Bars: (A–H) 100 μm; (J and K) 10 μm.
CP cells. In the E19 the E19 wild-type neocortex (A) shows the typical ordered arrangement of reduced proliferation of neuronal precursors. (A and B) Nissl staining of the CP layers. (C – E) In the E19 wild-type neocortex is severely disturbed with patches of edema (C and D, arrows) and clusters of dying or dead cells (Fig. 4 E, arrows). These data indicate a strong effect of the Cbx1<sup>−/−</sup> mutation on both cortical lamination and integrity and also show that Cbx1<sup>−/−</sup> E19 embryos are haploinsufficient, as they exhibit a partial disruption of laminar organization in the developing neocortex, although Cbx1<sup>−/−</sup> adults are viable and fertile.

It is known that cortical neurons are generated from the proliferating neuronal stem cell precursors located in the ventricular zone (VZ), which lines the lateral cerebral ventricle (Rakic, 1988). Postmitotic neurons migrate away from the VZ and form the distinct cortical layers in an inside-first, outside-last pattern (Desai and McConnell, 2000). We examined cell proliferation in the VZ by staining E17 and E19 wild-type and Cbx1<sup>−/−</sup> brains. On E17, the layer of pKi-67-positive proliferating neural progenitors is thicker in the wild-type VZ compared with the Cbx1<sup>−/−</sup> VZ.

As shown (Fig. 4 A), control CPs from E19 wild-type neocortices had a normal columnar organization, which was partially disrupted in E19 Cbx1<sup>−/−</sup> brains (Fig. 4 B). In stark contrast, we observed gross changes in the columnar organization in Cbx1<sup>−/−</sup> neocortices (Fig. 4, C–E) that were accompanied by edematous areas (Fig. 4, C and D, arrows) and clusters of dying or dead cells (Fig. 4 E, arrows). These data indicate a strong effect of the Cbx1<sup>−/−</sup> mutation on both cortical lamination and integrity and also show that Cbx1<sup>−/−</sup> E19 embryos are haploinsufficient, as they exhibit a partial disruption of laminar organization in the developing neocortex, although Cbx1<sup>−/−</sup> adults are viable and fertile.

Figure 4. Cbx1<sup>−/−</sup> mutants exhibit defective cerebral corticogenesis and reduced proliferation of neuronal precursors. (A and B) Nissl staining of the E19 wild-type neocortex [A] shows the typical ordered arrangement of CP cells. In the E19 Cbx1<sup>−/−</sup> neocortex (B), the ordered arrangement of CP cells is disturbed, and the boundaries at the top and bottom edges of the CP layer are blurred. (C–E) In the E19 Cbx1<sup>−/−</sup> neocortex, the lamination of the neocortex is severely disturbed with patches of edema (C and D, arrows) and clusters of dying cells (E, arrows) scattered through the cortical layers. (F) Proliferating neural progenitors are depleted in the VZ of Cbx1<sup>−/−</sup> brains. (C) Proliferating neural progenitors have been observed in in vitro Cbx5 (encoding HP1-α) siRNA experiments, in which knockdown of Cbx5 gene expression results in derepression of E2F-responsive genes and
the initiation of abnormal cell cycles that can lead to cell death (Panteleeva et al., 2007). It is unlikely that changes in Cbx5 gene expression contribute to the Cbx1−/− phenotype reported here (Figs. 2–5) because (a) HP1-α protein levels and distribution are not significantly changed in Cbx1−/− brain extracts (Fig. 3 I, Fig. S2, and not depicted), and (b) Cbx5−/− null mutants are viable and fertile and exhibit no overt neuronal phenotype (not depicted). Rather than the derepression of cell cycle–promoting genes, we suggest that the reduced proliferation and cell death phenotype in Cbx1−/− cortices is caused by a severe genomic instability that is the result of an improper constitutive heterochromatin assembly/organization in Cbx1−/− cortical neurons. The observed chromosomal aberrations (Fig. 5, A–E) are all consistent with this view. PCD has been observed in the swi6p (fission yeast HP1 homologue) mutant (Nonaka et al., 2001); polyplody can result from defective kinetochore assembly (Storchová et al., 2006), which requires a platform of HP1-containing heterochromatin (Zhang et al., 2007); undercondensation of centromeric heterochromatin can lead to the exclusion of chromosomes into micronuclei (Guttenbach and Schmid, 1994); and diplochromosomes can result from inefficient deconcatenation of centromeric DNA at the end of mitosis, leading to an extra round of DNA replication without chromatid separation (Sumner, 1998).

It is a matter of speculation as to how the loss of HP1-β could affect the heterochromatin assembly/organization in Cbx1−/− cortical neurons. First, it is possible that the loss of HP1-β could result in the misregulation of a critical HP1-β–regulated gene that is required for heterochromatin formation/assembly in neurons; HP1 proteins are known to regulate transcription both positively and negatively (Fanti and Pimpinelli, 2008). Second, it is possible that the defect is structural. According to a current model, the HP1-β–Me(3)K9H3 interaction is weakened by S10-H3 phosphorylation at metaphase, leading to a release of the initiation of abnormal cell cycles that can lead to cell death (Panteleeva et al., 2007). It is unlikely that changes in Cbx5 gene expression contribute to the Cbx1−/− phenotype reported here (Figs. 2–5) because (a) HP1-α protein levels and distribution are not significantly changed in Cbx1−/− brain extracts (Fig. 3 I, Fig. S2, and not depicted), and (b) Cbx5−/− null mutants are viable and fertile and exhibit no overt neuronal phenotype (not depicted). Rather than the derepression of cell cycle–promoting genes, we suggest that the reduced proliferation and cell death phenotype in Cbx1−/− cortices is caused by a severe genomic instability that is the result of an improper constitutive heterochromatin assembly/organization in Cbx1−/− cortical neurons. The observed chromosomal aberrations (Fig. 5, A–E) are all consistent with this view. PCD has been observed in the swi6p (fission yeast HP1 homologue) mutant (Nonaka et al., 2001); polyplody can result from defective kinetochore assembly (Storchová et al., 2006), which requires a platform of HP1-containing heterochromatin (Zhang et al., 2007); undercondensation of centromeric heterochromatin can lead to the exclusion of chromosomes into micronuclei (Guttenbach and Schmid, 1994); and diplochromosomes can result from inefficient deconcatenation of centromeric DNA at the end of mitosis, leading to an extra round of DNA replication without chromatid separation (Sumner, 1998).

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of much of the Me(3)K9H3-bound HP1-β into the nucleoplasm (Fischle et al., 2005). However, it is possible that an immobile fraction of HP1-β bound to the H3 histone fold (Dialynas et al., 2006) still remains associated with the constitutive heterochromatin, and it is the loss of this immobile fraction that leads to the dramatic genomic instability seen in Cbx1−/− cortical neurons. We favor the latter because the Cbx1−/− phenotype is more severe than the viable double-null KMT1A/B compound mutation in which the dynamic histone tail–HP1 interactions within constitutive heterochromatin are disrupted (Peters et al., 2001; Kourmouli et al., 2004). This possibility is further supported by the observation that the overall Me(3)K9H3 and Me(3)K20H4 levels and distribution are unchanged in Cbx1−/− neurons (Fig. 3, I–K), although we cannot exclude the possibility that critical sites might be affected. Future work will be directed toward exploring the role of HP1-β in regulating the heterochromatin structure in developing/migrating neurons during cerebral neurocortex development.

Materials and methods

Targeted disruption of the Cbx1 gene and generation of Cbx1−/− mice

The targeting vector was constructed from the XhoI–HindIII genomic fragment of Cbx1 (for a schematic diagram see Fig. 1 A; http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG000000018666). For ease of construction, the TkNeo gene was inserted into a unique SmaI site found in exon 4 of the Cbx1 gene (Fig. 1 A). Exon 4 gives rise to boxes 320–413 of the Cbx1 mRNA [A of AUG given as 1] encoding amino acids 108–137 of HP1-β, which lies adjacent to the C terminus of the CD (Ball et al., 1997). The targeting of Cbx1 with the construct was detected using a 0.3-kb probe from the HP1-β cDNA (Fig. 1 A, shaded boxes). After the digestion of genomic DNA by BamHI, this probe produces a fragment of 11.6 kb for the wild-type allele and 5.3 kb for the targeted allele (because of the introduction of a BamHI site in the Neo gene). Blastocyst injections for the production of germline chimeras and Southern blotting were performed according to standard protocols (Hogan et al., 1994).

Determination of the effect of Cbx1 gene dosage on hCD2 variation.

The hCD2-1.3B transgene contains the hCD2 promoter driving an hCD2 minigene and 1.3 kb 3′ flanking sequences with a partial locus control region (LCR); i.e., the LCR includes hCD2 DHS1 and DHS2 but lacks DHS3, a site known to be essential for position-independent LCR function and avoidance of PEV. When integrated into pericentric heterochromatin, the transgene variegates (Festenstein et al., 1999). To avoid background effects, heterozygous Cbx1+/− mice were backcrossed onto the CBA mouse strain background [more than nine backcrosses]. Because homozygous Cbx1−/− mice die around birth, we set up timed matings to obtain homozygous embryos with the hCD2 transgene at days 17–19 of embryonic development. Accordingly, we set up the following crosses: mice homozygous for Cbx1−/− and hemizygous for hCD2 (Cbx1−/− and hCD2+) were mated with mice heterozygous for Cbx1 (Cbx1+/−). Day 17–19 embryos resulting from this cross were genotyped for the Cbx1 mutation by Southern blot analysis (Fig. 1 B), whereas the presence of the hCD2-1.3B transgene was determined by measuring hCD2 expression using FACS.

We measured hCD2 expression in CD4+/CD8+ double-positive (DP) embryonic thymocytes. Single-cell suspensions were prepared from embryonic thymus and stained for FACS analysis. 106 embryonic thymocytes were stained with the following antibody combinations: (a) FITC-conjugated anti-hCD2, allophycocyanin-conjugated anti-mCD4 (BD), and peridinin-chlorrophyl protein complex–conjugated anti-mCD8 (BD) or (b) FITC-conjugated anti-hCD2 (BD), phycoerythrin-conjugated anti-mCD4 (BD), and tricolor-conjugated anti-mCD8 (BD). Three-color FACS analysis was performed on a laser instrument (FACS Calibur; BD) and analyzed using CellQuest software (BD). Live cells were then gated in a ferric-sorbitol-citrate/SSC dot blot, and this gate was used to display CD4 and CD8 expression in a dot blot. Gates for the different populations (double-negative, CD4 positive, CD4/CD8 DP, and CD8 positive) were set, and hCD2 expression in each population was displayed as a histogram. To obtain the percentage of hCD2-positive cells, marker regions were set. The percentage of hCD2 expression in CD4+/CD8− DP cells from each sample was compiled in a table and displayed as a graph sorted in accordance with the genotype of the sample (Fig. 1 D).

Histology

Whole embryos or brains embedded in paraffin blocks were sectioned to 7 μm and mounted on SuperFrost Plus slides (Menzel-Gläser). Hematoxylin and eosin staining were performed according to standard procedure.

Whole mount immunohistochemistry analyses

E19 dissected thymus and diaphragms were fixed in 2% PFA at 4°C overnight, rinsed briefly with PBS, and incubated in 0.1 M glycine/PBS for 1 h followed by permeabilization in 0.5% Triton X-100/PBS. Tissues were blocked in 10% normal goat serum for 2 h and incubated with primary rabbit antibodies against the light chain of NF (1:200; Millipore) in antibody dilute (Dako) overnight at 4°C. After extensive washing, the tissues were incubated with TRITC-conjugated swine anti-rabbit IgG (1:200; Dako) plus 5 ng/μl AlexaFluor-488–conjugated bungarotoxin (Invitrogen) overnight at 4°C. Tissues were then washed four times for 30 min each with 0.5% Triton X-100/PBS, postfixd in 1% PFA, and mounted in mounting medium (Vector Laboratories).

Immunostaining of cryosections of optimal cutting temperature–embbeded brains

Optimal cutting temperature–embbeded brains were sectioned at 14–16 μm. The brains were then fixed in 4% PFA for 30 min and blocked in 10% normal goat serum. The primary antibodies used were rat α–HP1-β (MAC 353; 1:50; recognizes the C terminus of HP1-β; Wreggelt et al., 1994) and mouse α-neuN (1:200; Millipore); FITC, fluorescein, or TRITC-conjugated secondary antibodies (Dako) were used to visualize the primary antibody staining. For detection of Me(3)K9H3 and Me(3)K20H4, we used the protocol described in Scholzen et al. (2002). Rabbit antibodies (Me(3)K9H3 and Me(3)K20H4) were detected with an AlexaFluor-488–coupled goat anti-rabbit secondary antibody (Invitrogen). The rat α–HP1-β (Wreggelt et al., 1994) antibody was detected with an AlexaFluor-546–coupled goat anti-rat secondary antibody (Invitrogen).

Western blotting of mouse brains

Murine embryonic fibroblast (MEF) cell extracts were prepared according to Cowell et al. (2002). E19 wildtype and Cbx1+/− brains were lysed in Laemmli buffer. The extracts were analyzed in 12.5 or 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with the antibodies according to standard procedures (Cowell et al., 2002). The antibody to the N terminus of HP1-β was used at a concentration of 1:100 and was a gift from E. Chan (University of Florida Health Science Center, Gainesville, FL).

Nissl staining of E19 brains

Embryos were perfused via the left ventricle of the heart with 1–2 ml of a 9% NaCl solution containing 0.5% heparin and 0.0266% NaNO2. Subsequently, 20 ml of a 0.1-M Na-acacodylate buffer, pH 7.3, 4% PFA, 2.5% glutaraldehyde, and 1% saccharose were perfused. Heads were postfixed in the same fixative for 3 h at room temperature, and brains were removed, stored overnight in freshly prepared fixative at 4°C, and finally transferred to 0.1-M Na-acacodylate buffer containing 15% saccharose for 2 h. Pieces of occipital cortices of both sides were postfixed in 2% OsO4, dehydrated, and embedded in epoxy resin. The staining of semithin sections was performed according to Richardson (1966). Before perfusion, the yolk sac was removed for DNA extraction and genotyping.

Neurosphere culture and generation of chromosome spreads from neurospheres

Neurosphere cultures from E19 embryonic brains and the production of chromosomes from the neurospheres was performed according to Frappart et al. (2005).

Chromosome experiments and detection of telomeres

Detection of telomeres on metaphase chromosomes was obtained by FISH by using a Cy3-labeled telomere sequence–specific peptide nucleic acid (PNA) probe (Sharma et al., 2003). Giemsa-stained chromosomes of metaphase spreads were analyzed for chromosome aberrations (Fig. 5 F). Cbx1−/− and wildtype MEFS were generated according to the 3T3 protocol of Kamijo et al. (1997). Unfixed metaphase chromosomes from the MEFS were stained with anti–HP1-β and α–HP1-α antibodies according to Wreggelt et al. (1994).
Statistical analysis
Embryo weights. Data were collected at two sites, A and B. There were seven litters in mouse house A and four litters in mouse house B. Four embryos were removed from the analysis, three because of uncertainty about the genotyping and one because it had no tail. The sizes of the litters used in the statistical analysis varied between 2 and 10. A linear mixed model incorporating effects for site, genotype, and litter was fitted to the data. Site and genotype were fitted as fixed effects. Litter was fitted as a random effect. Neither the interaction between site and genotype nor the effect of site was found to be statistically significant. The effect of genotype was highly significant (P < 0.001). The embryo weights for +/+ and +/− are not significantly different from each other and are so similar that they can be combined for comparison with the mean weight for −/−. The weights for −/− are significantly lower than those for the other two genotypes (using a t-test for the difference between two means; P < 0.01). On average, −/− embryos are significantly lower than those for the other two genotypes (using a combined t-test for the mean weight for both mutant and wild-type (diaphragm and thigh) muscles is given in Table S1). The range of the percentage of CD2 cells per embryo is from six litters. The range of the percentage of CD2 cells per embryo is 8.5–72.7% across all three genotypes. The means and standard deviations for the genotypes (ignoring litter effects) are as follows: +/+: mean = 24.3%, SD = 9.6; +/−: mean = 32.1%, SD = 11.3; and −/−: mean = 50.2%, SD = 14.1. Litter was fitted as a random effect and genotype as a fixed effect in a restricted maximum likelihood model fitted using the Genstat statistical package (Lawes Agricultural Trust). This showed that there is a highly significant difference between −/− and the other two genotypes (P < 0.001). The difference between +/+ and +/− is not significant.

Neurospores. There is a borderline difference between genotypes when all three experiments are fitted separately (P = 0.10 on 2 and 46 degrees of freedom). There is no interaction between experiments; i.e., even though the experiments differ in the mean numbers of neurospores, these differences are consistent across genotypes. Most of the difference between genotypes clearly lies between −/− and the other two (+/+ and +/−). The total variation resulting from genotype is reduced only very slightly by combining +/+ and +/− and comparing them with −/− (variation explained falls from 4.3 on 2 df to 4.1 on 1 df). In this case, the difference between +/+ and +/− is weakly significant (F = 4.63 on 1 df and 47 df; P < 0.04); the trend is that fewer neurospores are generated from Cbx1−/− brains.

Chromosomal aberrations in neurosphere cells. For PCD, the raw data per 100 cells are for +/+; 10 for +/−; and 59 for −/−. There is a significant difference between −/− and +/+ and +/− combined (P = 0.001; χ² test = 90.1 on 1 df). There is a borderline difference between +/+ and +/− because of the low number of incidences (P = 0.10; χ² test = 2.8 on 1 df). For ploidy, the raw data per 100 cells are for 0 for +/+; 4 for +/−; and 38 for −/−. There is a significant difference between −/− and +/+ and +/− combined (P = 0.001; χ² test = 70.4 on 1 df). There is a borderline difference between +/+ and +/− because of the low number of incidences (P = 0.053; χ² test = 4.1 on 1 df). For micronuclei, the raw data per 100 cells are for 0 for +/+, 5 for +/−; and 63 for −/−. There is a significant difference between −/− and +/+ and +/− combined (P = 0.001; χ² test = 37 on 1 df). There is a borderline difference between +/+ and +/− because of the low number of incidences (P = 0.037; χ² test = 2.75 on 1 df). AChRs/micrometer of nerve in mutant (Cbx1−/−) and wild-type (Cbx1+/+) diaphragms and thigh muscle. The number of AChRs/micrometer of nerve for both mutant and wild-type (diaphragm and thigh) muscles is given in Table S1. Using a simple t-test, there are significantly fewer AChRs/micrometer in mutant muscles compared with wild-type muscles (P < 0.004 for mutant diaphragm vs. wild-type diaphragm; P < 0.001 for mutant thigh muscle vs. wild-type thigh muscle).

Image acquisition and manipulation
Make and model of microscope. For Fig. 2 A, we used a Leica MZ16 microscope. For Figs. 2 B, 3 (A–H), and 4 (A–F), we used a Leica DMRXE microscope. For Fig. 3 (J and K), we used a confocal scanning microscope (TCS-SP; Leica). For Fig. 5, we used a motorized microscope (Axioplan 2ie; Carl Zeiss, Inc.). (TCS-SP; Leica). For Fig. 3 (J and K), we used a confocal scanning microscope (TCS-SP; Leica). For Fig. 5, we used an in situ imaging system FISH imaging software (MetaSystems).

Subsequent software used for image processing. Quantitation of AChRs was performed using ImageJ version 1.41i (National Institutes of Health). The confocal z series of diaphragms and thigh muscles stained for both NF and bungarotoxin were taken on a confocal laser microscope (SPS, Leica) using a 20× objective. The number of ACHR clusters was fitted in 3D z stacks using an ImageJ plug-in 3D Object Counter (Cordellies and Jackson, 2007) after background subtraction. The lengths of the NF-positive nerves on maximum intensity-projected images were calculated by tracing each individual nerve using the ImageJ plug-in Neuron J (Meijering et al., 2004).

For Figs. 2 (A and B), 3 (A–H), and 4 (A–F), the pictures were assembled using Photoshop CS2 version 9.0 (Adobe Systems, Inc.) for Macintosh. For Fig. 3, I, pictures of specific bands of the indicated antibodies have been assembled. For Fig. 3 (J and K), pictures were processed and assembled with ImageJ and Photoshop 6.0. For Fig. 5, composite images were composed using Photoshop 7.0.1, and the input level was adjusted to match the black background.

Online supplemental material
Figs. S1 shows the lack of N- and Terminal regions of HPI-β in Cbx1−/− brains extract. Fig. S2 shows the immunolocalization of HPI-α and HPI-γ proteins in Cbx1−/− and Cbx1+/+ E19 cortical sections. Table S1 tabulates the number of ACHR clusters/micrometer of nerve in diaphragms and thigh muscle taken from E19 Cbx1−/− and Cbx1+/+ brains. Online supplemental material is available at www.jcb.org/cgi/content/full/jcb.200804041/DC1.

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For Fig. 3 (J and K), samples were mounted in Dabco solution (0.25% 1, 4-diazabicyclo[2.2.2]octane [Sigma-Aldrich], 15 mM NaCl, 0.8 mM NaH2PO4, and 0.2 mM KH2PO4 in 90% [vol/vol] glycerin, pH 8.6).

Fluorochromes. In Figs. 2 B, 3 (E–H), and 4 F, the fluorochromes used were either FITC- or TRITC-conjugated antibodies or AlexaFluor-488-conjugated bungarotoxin. In Fig. 3 (J and K), AlexaFluor-488 (displayed in green) or AlexaFluor-546 (displayed in red) was used. For Fig. 5, the FNA probe was labeled with Cy3, and the chromosomes were counterstained with DAPI.

Camera make and model. For Fig. 2 A, a Progres C14 camera (Jenoptik) was used. For Figs. 2 B, 3 (A–H), and 4 A–F, we used OpenLab 3.0.9 software (PerkinElmer). The acquisition software used for Fig. 3 (J and K) was Leica TCSNT software. For Fig. 5, we used in situ imaging system FISH imaging software (MetaSystems).

For Figs. 2 A, B, 3 (A–H), and 4 A–F, we used OpenLab 3.0.9 software (PerkinElmer). The acquisition software used for Fig. 3 (J and K) was Leica TCSNT software. For Fig. 5, we used in situ imaging system FISH imaging software (MetaSystems).

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