The cockayne syndrome B protein is essential for neuronal differentiation and neuritogenesis

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Cockayne syndrome (CS) is a progressive developmental and neurodegenerative disorder resulting in premature death at childhood and cells derived from CS patients display DNA repair and transcriptional defects. CS is caused by mutations in csa and csb genes, and patients with csb mutation are more prevalent. A hallmark feature of CSB patients is neurodegeneration but the precise molecular cause for this defect remains enigmatic. Further, it is not clear whether the neurodegenerative condition is due to loss of CSB-mediated functions in adult neurogenesis. In this study, we examined the role of CSB in neurogenesis by using the human neural progenitor cells that have self-renewal and differentiation capabilities. In this model system, stable CSB knockdown dramatically reduced the differentiation potential of human neural progenitor cells revealing a key role for CSB in neurogenesis. Neurite outgrowth, a characteristic feature of differentiated neurons, was also greatly abolished in CSB-suppressed cells. In corroboration with this, expression of MAP2 (microtubule-associated protein 2), a crucial player in neuritogenesis, was also impaired in CSB-suppressed cells. Consistent with reduced MAP2 expression in CSB-depleted neural cells, tandem affinity purification and chromatin immunoprecipitation studies revealed a potential role for CSB in the assembly of transcription complex on MAP2 promoter. Altogether, our data led us to conclude that CSB has a crucial role in coordinated regulation of transcription and chromatin remodeling activities that are required during neurogenesis.

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Abbreviations: DAPI, 4,6-Diamidino-2-phenylindole nuclear staining; UV, ultraviolet; EGF, Epidermal growth factor; FGF, Fibroblast growth factor; HRP, horseradish peroxidase

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A recent article has described that an early and limited window of postnatal neurogenesis that occurs in the subventricular zone (SVZ) is associated with a major migratory pathway that targets these new neurons not only to the olfactory bulb but also to the human prefrontal cortex. In support, postnatal neurogenesis was reported during the functional development of cerebellum until 18 months. Our principal hypothesis is that CS patients are not able to support brain plasticity and repair events occurring at perinatal and postnatal stages resulting in severe neurodegeneration. Therefore, a systematic evaluation of the role of CSB in neurogenesis is mandatory for exploring the molecular cause for neurodegeneration in CS patients. In this study, we used human immortalized (ReNcell VM) neural progenitor cells with efficient self-renewal and multilineage differentiation capabilities to gain insights into the role of CSB in neurogenesis. Our study provides evidence that CSB deficiency affects neuronal differentiation capabilities of human neural progenitor cells suggestive of a crucial role for CSB in adult neurogenesis.

Results

CSB suppression dramatically affects neuronal differentiation. To induce neuronal differentiation, cells were seeded on poly-ornithine and Laminin-coated slides/dishes and cultured without epidermal growth factor (EGF) for an additional 3 days. Growth medium without EGF and fibroblast growth factor (FGF) was then replaced every 3 days to promote the generation of neurons, oligodendrocytes, and astrocytes. Differentiation of ReNcell VM cells into neurons, oligodendrocytes and astrocytes was achieved at days 10, 17 and 24, respectively. Technical procedure followed for neuronal differentiation is shown in Figure 1a and in accordance with differentiation protocol, typical neurite outgrowth and establishment of neuronal polarity were visualized by Tuj1 staining at day 10 (Figure 1b). Successful accomplishment of differentiation of the neural progenitor cells into neurons was indicated by the strong upregulation of one of the neuronal markers, microtubule-associated protein 2 (MAP2) and downregulation of the cell proliferation marker Ki67 (Figure 1c).

To evaluate the role of CSB in human neurogenesis, we employed a lentiviral-based strategy. Control (Scrambled non-specific) and CSB-specific shRNA vectors were used to create a CSB-proficient cell line (sh-K) and a CSB-suppressed cell line (sh-CSB), respectively (Figure 1d). Stably transduced cells were selected by puromycin (2 µg/ml) resistance. Both RT-PCR and western blot analyses of puromycin resistant ReNcell VM cells showed a good suppression of CSB expression (more than 80%) in sh-CSB cell line relative to scrambled vector-transfected cells (Figure 1e). A hallmark feature of CSB-deficient human and hamster cells is their increased sensitivity to UV-C radiation. To determine whether sh-K and sh-CSB lines were sensitive to UV-C radiation, cells were exposed to 10 J/m² of UV-C radiation. As expected, sh-CSB neural progenitor cells showed increased apoptotic death relative to sh-K cells (Figure 1f). This observation clearly demonstrates that the CSB knockdown neural progenitor cells exhibit a typical UV sensitive phenotype, a characteristic feature of CSB deficiency.

Figure 1 (a) Schematic diagram showing the procedural steps involved in the differentiation of human neural progenitor cells (RenCell VM) into neurons and glial cells. RenCell VM cell line is endowed with efficient self-renewal and multilineage differentiation capabilities. (b) Immunofluorescence staining for neuronal marker Tuj1 in sh-K cells at days 3 and 10 of differentiation. (c) Quantitative analysis of neuronal MAP2 and stem Ki67 markers expression at different times of differentiation (mean ± S.D. of three independent experiments). (d) Schematic diagram illustrating the technical procedure followed for the generation of stably transfected cell lines with empty vector (sh-K) and CSB shRNA vector (sh-CSB) used in this study. (e) Analysis of CSB mRNA expression by RT-PCR in normal and CSB-suppressed cells (left panel) and western blotting analysis of CSB protein expression (right panel). (f) Histogram illustrates the percentage of apoptotic cells in mock and UV-treated cells (mean ± S.D. of three independent experiments).
Therefore, we used this cell model system to evaluate the role of CSB in neurogenesis. More specifically, we wanted to determine whether or not csb gene is crucial for viability and differentiation capabilities of NSC. Upon addition of neuronal differentiation medium, sh-K ReNcell VM cells showed prominent neuronal differentiation starting at day 9–12, with a marked neurite extension (Figures 2a–c and g–i). In contrast, neuronal differentiation was greatly diminished in sh-CSB neural progenitor cells (Figures 2d–f and l–n). Neurite outgrowth, a characteristic feature of differentiating neurons, was greatly abolished in CSB-suppressed cells.

Further, immunostaining intensity of fluorescein conjugated neuronal cytoplasmic marker B-tubulin III (Tuj1) was reduced in sh-CSB cells with an absolute lack of neuronal polarity.

Neuritogenesis and growth cone pathfinding are affected in CSB-suppressed cells. As shown in sh-K cells (Figures 3a–d), shortly after the initiation of differentiation process (6–8 days) and preceding the initial neurite outgrowth, ruffling on the cell surface, which is primarily due to disruption of the subcortical actin network (F-actin), was observed. During neuritogenesis, the lamellipodium...
becomes segmented at one or more sites followed by extension from the soma leading to the formation of growth cone (visualized by combined staining of actin and drebrin) concurrent with advancement of microtubules (MT) into the initiation site and formation of an ordered microtubule array (white arrow). The newly formed protrusion then elongates (red arrow) and MT become tightly packed into parallel arrays inside the nascent neurite shaft (yellow arrow). Actin–microtubule interactions were observed in lamellipodia at all stages of neurite outgrowth. All of these processes of neuritogenesis were detected in CSB-proficient cells (Figures 3a–d).

In contrast, upon CSB suppression, the subcortical actin network was not destabilized and a fairly uniform lamellipodium (F-actin) surrounding cell soma was observed with drebrin extensions (Figures 3e–h, white arrow). Further, MT were not found to interact with either actin or drebrin and the cells showed an increased cytoplasmic volume. These morphological features seemed to suggest the lack of cell polarity and motility in CSB-suppressed neural cells (Figures 3e–h, red arrow).

Although no quantitative analysis was done, it was observed that the nuclei of CSB-suppressed cells appeared to have kidney-shaped morphology and were slightly bigger than the nuclei of CSB-proficient (sh-K) cells. It is not entirely clear whether CSB suppression alters the morphology of nuclei through modulation of DNA metabolic activities. Interestingly, morphology of the two cell lines differed noticeably when sh-K and sh-CSB cells were grown for longer times in neuronal differentiation medium (Figure 4). In sh-K cells (a–d), the neuronal differentiation appeared to be completed after 15–18 days after the addition of differentiation medium. A complex network of neurites, visualized by Tuj1 immunostaining, showed the connective network between adjacent neurons; actin staining was found confined to the soma as well as the drebrin indicating the completeness of neuritogenesis process. In contrast, neuritogenesis was greatly reduced in sh-CSB cells (e–h) as judged by the lack of connecting network of neuritic growth between adjacent cells. Strikingly, CSB-suppressed cells displayed lack of polarization and abortive growth cone-like structures visualized by actin and drebrin staining. Further, tubulin, which is not part of this external structure, appeared to be dispersed in the cytoplasm.

**MAP2 expression is reduced in CSB-suppressed cells.** Studies on the dynamics of cytoskeletal components during neuritogenesis have implicated actin, tubulin and MAPs in sprouting. Actin, in association with surface adhesion molecules, has an important role in the pathfinding of the processes. MT enter the growth cone to initiate neurite extension and this process appears to be facilitated by MAPs, in particular MAP2. Cunningham et al. demonstrated that MAP2c initiates neurite outgrowth through its interaction with the subcortical actin network. It has been suggested that the interaction of MAP2c with the actin-rich subcortical network might be responsible for the switch from an actin-based lamellar structure to a microtubule-based neuritic structure during neuritogenesis. Observation of highly reduced neuritogenesis in sh-CSB cells led us to hypothesize that MAP2c expression may be deregulated by lack or reduced CSB expression.

A time course analysis of MAP2c protein accumulation during differentiation revealed a clear difference between CSB-proficient (sh-K) and CSB-suppressed (sh-CSB) cells. Western blotting performed at different times during differentiation (Figures 5a and b) showed upregulation of MAP2c protein in normal cells with a peak of activation at day 12. In contrast, MAP2 induction was respectively five and ninefold less in sh-CSB cells, at days 12 and 15. Immunofluorescence studies were also performed at days 12 and 24 to analyze the expression of MAP2 in parallel with GFAP (Glial Fibrillary Acidic Protein) a glial marker expressed in neural progenitor cells. Representative pictures shown in Figure 5c showed that expression of MAP2 peaked at day 12 in the majority of cells and then declined at day 24 with a gradual increase in GFAP-positive cells, reflecting the gradual time-dependent progression from neurons to glial cell populations. In contrast

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**Figure 4** Immunofluorescence staining for Tuj1, Drebrin (an F-actin associated protein) and actin in sh-K (a–d) and sh-CSB (e–h) cells after growth in differentiation medium at 15–18 days. Cells were counterstained for DNA using 4',6-diamidino-2-phenylindole nuclear staining.
to CSB-proficient cells, sh-CSB cells displayed a much lower content of MAP2, defects in neuritic outgrowth and neuronal polarity were also apparent (Figures 5d and e). Furthermore, expression of both neuronal and glial markers (MAP2 and GFAP) were simultaneously detected in sh-CSB cells indicating that the differentiation programs are somewhat distorted and the transition stage between neurons and glial cells less defined or the cells were not entirely committed to differentiate. However, GFAP expression (assayed by both western blot and immunofluorescence techniques) was found to be grossly similar between sh-K and sh-CSB cells at the earlier step of the differentiation, while significantly higher in sh-CSB at the later times of the differentiation (Figures 5d and e), demonstrating that the GFAP expression was not drastically altered by CSB deficiency during the early phase of differentiation.

To determine whether the reduced expression of MAP2 observed in CSB-suppressed cells was a consequence of a reduction of its transcriptional level, MAP2 mRNA expression was monitored by quantitative RT-PCR (Figure 5f). MAP2 mRNA level increased in CSB-proficient cells during differentiation and MAP2 induction began at day 3 (1.6-fold induction) and reached its maximum at day 12 (4.5-fold induction). In contrast, MAP2 expression was consistently reduced by ~50% in CSB-suppressed cells (2.3-fold induction, at day 12). CSB-dependent induction of MAP2 during the differentiation process led us to investigate whether or not CSB expression is also changing during the differentiation process (Figure 5g). Intriguingly, CSB mRNA was also induced during differentiation in normal cells starting to be noticeable at day 6 and reaching its peak at day 12, exhibiting kinetics of induction similar to MAP2. A similar trend for CSB was also observed in CSB-suppressed cells albeit much reduced due to suppression of CSB by shRNA. These findings clearly indicate that optimal csb gene dosage is required for efficient neurogenesis.

CSB interacts with nBAF complex. Although CSB has been shown to interact with some of the functional units of basal transcription factor TFIIH and RNA polymerase elongation complexes, identification of additional proteins and protein complexes interacting with CSB would help to unravel some of the yet unidentified functions of CSB. With this objective, tandem affinity purification (TAP) tag technology coupled with mass spectrometry was employed to identify the proteins associated to CSB. TAP technique is often used to isolate proteins, which can be identified by subsequent mass spectrometry analysis. In our approach, a TAP tag was cloned in frame with CSB cDNA (Figure 6a) and the construct was subsequently transfected into suitable host cells (CSIAN cell line, derived from CS patient without a functional CSB protein). CSIAN cells were...
transfected with either pZome-1-N (mock), or pZome-1-N-TAP-CSB for stable expression of CSB–TAP-tagged protein. For stable transfection, CSB-deficient cells were chosen because this allows testing the functionality of the CSB–TAP protein. CSIAN cells stably expressing TAP-CSB or TAP alone were selected with puromycin (2 μg/ml) for 3 weeks. The selected clones were amplified and screened for the expression of TAP-tagged CSB protein. To avoid possible artifacts due to CSB–TAP overexpression, we chose a stably transfected clonal cell line, whose CSB expression level was grossly similar to the endogenous CSB level observed in CSB-proficient MRC5 cells (data not shown). Cell survival after UV exposure showed that the CSB–TAP fusion protein was indeed functional and it protected CSIAN cells from UV induced cell death relative to vector alone-transfected cells. Further, cellular resistance to UV in CSB–TAP fusion protein expressing CSIAN cells was almost identical to CSB-proficient wild-type MRC5 cells (data not shown). Proteins associated with CSB–TAP were isolated through two sequential affinity purification steps, as described in Materials and Methods. Finally, the isolated proteins were size fractionated on SDS polyacrylamide gels (Figure 6b) and analyzed by mass spectrometry essentially as described before. Using this approach, we found that CSB co-purified with components of a chromatin remodeling complex (npBAF complex), which is specific for neuronal progenitor cells (Figure 6c). The BAF complex consists of different subunits (BAF170, BAF53A and BAF47) all of which are known to participate in transcriptional activation through an ATP-dependent chromatin remodeling process. Additional proteins that act as transcriptional repressors such as HDAC1 (histone deacetylase 1), and GATA2A and GATA2B (GATA zinc finger domain containing 2A or 2B) were also found to co-purify with CSB. Some of these interactions were confirmed by protein–protein interaction studies using recombinant tagged-proteins (Figure 6d).

CSB coordinates transcription and chromatin remodeling activities on gene promoters including MAP2. Impaired neuritogenesis coupled with the reduced expression of MAP2 at mRNA and protein levels observed in CSB-suppressed cells prompted us to investigate the possibility that CSB had a role in the transcriptional regulation of MAP2 either alone or in combination with other transcription and chromatin remodeling proteins. Regulation of chromatin structure is emerging as an important level of transcriptional control during neural development. SWI/SNF complex is a well-studied chromatin remodeler that uses the energy derived from ATP hydrolysis to regulate nucleosome mobility and chromatin accessibility. One such SWI/SNF-like chromatin-remodeling enzymatic complex is Brg/Brm-associated factor (nBAF) that specifically regulates dendrite growth and axon myelination. The fact that CSB belongs to the SWI/SNF family makes it imperative to determine the interaction of CSB with other chromatin remodeling factors that are specifically involved in neurogenesis.
Co-purification of CSB–TAP with components of npBAF and nBAF complexes observed in vitro indicates the possibility that CSB may protect the functional integrity of different brain cell types (neural stem/progenitor cells, neurons and glial cells) in vivo through successful coordination of transcription and chromatin remodeling activities during neurogenesis and neuritogenesis. To further explore this exciting possibility, chromatin immunoprecipitation (ChIP) assays were performed to study the assembly of the transcriptional machinery and the chromatin remodeling factors during neuronal differentiation. We initially focused on MAP2 because of CSB suppression reduced its expression both at mRNA and protein levels. To investigate whether reduced MAP2 expression was due to defect(s) in the assembly of transcriptional apparatus, kinetics of the occupancy of RNA polymerase II and its associated factors to map2 promoter were examined by ChIP assay at different days after the initiation of differentiation process. Antibodies directed against various components of the transcription machinery were used to precipitate the genomic DNA fragments that were further analyzed by quantitative PCR (qPCR). ChIP experiments showed a significant increase in both RNA pol II and TFIIB occupancy on the map2 promoter in sh-K cells, most likely due to transcriptional activation triggered by neuronal differentiation process (Figures 7b and c). Conversely, we found that both RNA pol II and TFIIB were not recruited to the same extent on the map2 promoter in sh-CSB cells. To gain further insights into the chromatin modifications imposed by gene activation, extent of nucleosome histone acetylation around the map2 promoter region was investigated. In particular, we focused on H3K9 acetylation, an epigenetic marker specific for transcriptionally active chromatin (Figure 7d). Strikingly, H3K9 acetylation was highly reduced at the map2 promoter in sh-CSB cells. The reduced level of H3K9 acetylation observed in sh-CSB cells seems to correlate with the impaired recruitment of the p300 histone acetyltransferase at the map2 promoter (Figure 7e). p300 functions as an essential cofactor for adult neurogenesis. Further, ChIP assay revealed the presence of CSB at the map2 promoter site and the recruitment of CSB was further enriched at the promoter site during/after differentiation (Figure 7f). As expected, recruitment and retention of CSB was reduced in sh-CSB cells. It should be noticed that in sh-K cells, during differentiation, BAF complex was detected at the map2 promoter with progressive enrichments of its Brg1 and BAF170 subunits (Figures 7g and h). This indicates that the positioning of BAF chromatin remodeling complex at the map2 promoter is required for its activation. Collectively, these results suggest that the reduced MAP2 expression

Figure 7 Interaction of CSB protein with transcription and chromatin remodeling factors detected by ChIP. (a) Real-time PCR was performed using gene promoter-specific primers to examine the relative enrichment for the promoter region of map2 sites immediately upstream of the transcription initiation site. Soluble chromatin was prepared from sh-K and sh-CSB cells and subjected to ChIP assay using antibodies against RNA pol II (b), TFIIB (c), K9-Acetylated histone H3 (d), p300 (e), CSB (f), Brg1 (g), BAF170 (h), CREST (i), HDAC3 (j) and HDAC1 (k). ***P<0.001, **P<0.01, *P<0.05, (unpaired two-tailed Student’s t-test)
observed in CSB knockdown cells is most probably due to impaired recruitment of BAF complex on map2 promoter and that CSB is necessary for efficient assembly of transcriptional complex on this promoter. In addition, we found that the activation of map2 promoter that occurred during neural differentiation correlated well with the loss of HDAC3 in sh-K cells but not in sh-CSB cells (Figure 7j). However, there was no change in the retention of HDAC1 at the map2 promoter during neural differentiation either in sh-K or sh-CSB cells (Figure 7k). Thus, it appears that the increased binding of CSB to map2 promoter paralleled the recruitment of BAF, thereby implying a role for CSB in BAF recruitment. This is partly supported by the observation of CSB interacting to some of the subunits of the BAF complex. BAF53b, that seems essential for the recruitment of BAF complex at the gene promoter sites, does not have a DNA binding domain. Therefore, a likely possibility is that CSB can mediate the binding of BAF53b to the promoter sites. In support, interaction of CSB not only with Brg1 and BAf170 but also with Baf53 (a and b) was demonstrated. Alternatively, histone acetylation changes imposed by CSB via its interaction with HAT p300 would also facilitate the recruitment of essential factors to BAF. Both hypotheses, although not mutually exclusive, confer an important role to CSB in MAP2 gene activation during neuronal differentiation. Along this line of evidence, it has been shown that nBAF complex together with CREST has a role in regulating the activity of genes essential for dendrite growth.46

Our finding of reduced expression of MAP2 in CSB-suppressed cells prompted us to investigate the expression of yet another neuronal marker NEUROD1 in sh-K and sh-CSB cell lines. NEUROD1 mRNA expression was monitored by quantitative RT-PCR (Figure 8a). NEUROD1 mRNA level increased in CSB-proficient cells during differentiation and reached its maximum at day 15 (6.2-fold induction). In contrast, NEUROD1 expression was consistently reduced by ~50% in CSB-suppressed cells (2.9-fold induction, at day 15). As observed for MAP2 promoter, also NEUROD1 promoter exhibited impaired recruitment of RNA polymerase II in CSB-suppressed cells (Figure 8c). ChIP assay also revealed the presence of CSB at the neurod1 promoter site in CSB-proficient cells and the recruitment of CSB was further enriched at the promoter site during/after differentiation (Figure 8d). As expected, recruitment and retention of CSB was reduced in sh-CSB cells due to overall reduced CSB expression in these cells.

**Discussion**

In this report, we demonstrate that CSB suppression affects the neuronal differentiation capability of human neural progenitor cells. Most strikingly, CSB suppression led to a highly reduced expression of MAP2 accompanied by impaired cell polarization and neuritogenesis. Collectively, these findings appear to be relevant to explain the molecular basis of at least some of the neurological symptoms reported in CS patients. Although earlier studies have addressed this aspect in double XP/CS knockout mouse model system,48–50 our study is the first to our knowledge to explore the role of CSB in neurogenesis using a human model system.

Brain has long been considered to have ceased the development beyond fetal and early postnatal stages but recent studies have demonstrated that neurons are continually generated from adult neural stem/progenitor cells in two well-defined neurogenic regions: SVZ of the lateral ventricles and subgranular zone of the dentate gyrus and for a limited period, within the white matter tracts. Moreover, NSC have been isolated in the external granular layer of the cerebellum in rodents and hence their presence in cerebellum cannot be completely excluded in human brain.51 Development of the brain structure and complexity occurring during early infancy is critical for the postnatal neurogenic activity.52,53 Homeostasis of the brain tissue requires the replenishment of neurons as well as protection of brain cell types, including neural stem/progenitor cells, against endogenous and exogenous DNA damage. Therefore, impairment of either of these processes, neurogenesis and DNA repair, is likely to result in neurodegeneration. In this study, we demonstrate

![Figure 8](image_url)

**Figure 8** NEUROD1 (a) mRNA expression analysis in sh-K and sh-CSB cells during the entire time course of neural differentiation (mean ± S.D. of three independent experiments). (b–d) ChIP analysis of RNA polymerase II and CSB recruitment on NEUROD1 promoter region. Real-time PCR was performed using gene promoter-specific primers to examine the relative enrichment of RNA polymerase II and CSB protein at the promoter region of NEUROD1 gene (b). Soluble chromatin was prepared from sh-K and sh-CSB cells and subjected to ChIP assay using antibodies against RNA pol II (c) and CSB (d).
that the efficient differentiation of neural progenitor cells into neurons requires a functional csb gene product. Impaired neuronal differentiation coupled with reduced expression of neuronal-specific marker MAP2 in CSB-suppressed cells strongly indicate that csb gene is pivotal for the neuronal differentiation process. Thus, the positive correlation observed between CSB deficiency and defective neurogenesis in the present study suggests that the neurological symptoms observed in CS patients are indeed primarily due to CSB deficiency but not to secondary effects. It is reasonable to assume that a defect in the postnatal development of prefrontal cortex due to lack of postnatal neurogenesis may result in features such as microcephaly that characterize CS. Likewise, a deficiency in neurogenesis in the subcortical white matter could also account for the dysmyelination/demyelination features displayed by CS. Along these lines, defective neurogenesis particularly in the cerebellum could account for the cerebellar degenerative features displayed by CS patients. Alternately, an additive effect of defective neurogenesis superimposed by TCR defect can also contribute to other neurological aspects including cataract and cerebellar atrophy in CS patients.

In contrast to our findings, a recently published article reported that the neural precursor functions are not affected in CSB-deficient mice. It was previously established that the neurological symptoms in CSB-deficient mice are not as severe as in humans and that CSB mouse model system does not recapitulate the human CSB phenotype. Severe neurological symptoms in human CSB patients can only be induced in mice after functional inactivation of csb in combination with xpc (xeroderma pigmentosum complementation group C) gene. In this context, it is known that XP mutations in human confer neurologic defects per se, which however differ from that associated with CS. Although XP is a primarily neurodegenerative disease, owing to the degeneration of neurons, CS appears to be more of a neurodevelopmental disease because of the neurologic features such as early microcephaly, cerebellar hypoplasia and hypomyelination.

The onset of neurological defects observed after birth in CS patients raises an intriguing question: why is the CSB protein essential for adult neurogenesis but not during embryonic brain development? Strikingly, patients either with the classical form (CS type 1) or congenital form (CS type II) display normal intrauterine somatic and brain growth with developmental crisis after birth: immediately after birth in type II, in the first or second year of life in type I. This led us to hypothesize that the csb gene is dispensable during the fetal stage but not during postnatal development. Manifestation of developmental defects especially of neurological symptoms after birth of CS patients led us to postulate that csb gene may perform essential functions during the extrauterine life when the child is no longer protected by the controlled environment in mother’s womb in terms of angiogenic factors and gaseous oxygen. Oxygen homeostasis could very well represent one of the Achilles’ heel of CS patients during extrauterine life, which is amply supported by an abnormal transcriptional response of CSB-deficient cells to hypoxia involving HIF-1 (hypoxia inducible factor-1)-mediated signaling pathway. HIF-1 is implicated in the regulation of a vast array of genes that control multiple cellular functions such as angiogenesis, glucose metabolism and cell survival. This finding might have great implications for neurodegeneration in CS because the oxygen tension in brain is lower than in other organs/tissues and therefore optimal activation of HIF-1 pathway is crucial for the functional integrity of brain cell types including neural stem/progenitor cells. Another likely possibility is that hypoxia in certain metabolically active regions of brain may stimulate ROS production in a mitochondrial dependent fashion and increased ROS production in turn can lead to DNA damage accumulation resulting either in senescence or cell death. In corroboration, we observed an increased fraction of senescent cells with exacerbation of defects in neurogenesis when CSB-suppressed neural progenitor cells are grown under hypoxic conditions (Supplementary data 1).

Observations of localization of CSB protein in mitochondria and its role in the repair of mitochondrial oxidative DNA damage further add a new dimension to the multitude of complex cellular functions of CSB in maintaining the functional integrity of mitochondria. A recent paper has demonstrated that CSB prevents the accumulation of damaged mitochondria by promoting mitochondrial autophagy. CSB also promotes the transcriptional elongation in mitochondria through communications with mitochondrial proteins. As brain cells are metabolically highly active, protection of mitochondrial DNA from excessive generation of ROS and RNS is crucial for the integrity of brain cells. Therefore, loss of CSB is expected to lead to deleterious effects in terms of neuronal loss owing to enhanced DNA damage accumulation in addition to the impact on neurogenesis. Collectively, our findings suggest a multifaceted role for CSB not only in neurogenesis but also in protecting the functional integrity of neural progenitor cells under low oxygen tension through transcriptional activation of factors involved in hypoxia signaling pathway.

Given that MAP2 is a neuronal marker, reduced amount of MAP2 observed in CSB-suppressed neural progenitor cells may be viewed per se as a simple consequence of a reduced number of neurons. Interestingly, demonstration of transcriptional regulation of MAP2 by CSB indicates that the reduced MAP2 expression is primarily due to CSB deficiency and not due to reduced neuronal differentiation. It is currently unclear whether optimal MAP2 expression is in fact the driving force for neuronal differentiation besides its role in neuritic outgrowth. Although we cannot exclude this, a likely possibility is that CSB may also participate in the transcriptional activation of other genes essential for neurogenesis. This possibility is greatly strengthened by our finding of the reduced expression of NEUROD1 in CSB knockdown cells and that neurod1 promoter is also bound by CSB. Therefore, it is highly likely that CSB upregulates those genes that are essential for neuronal differentiation through chromatin opening/remodeling activities as described in the case of map2. Although additional experiments are clearly warranted for unraveling the functional complexity of CSB in neurogenesis, this study in our opinion has paved the way for future mechanistic studies.

Materials and methods
Cell culture, silencing and differentiation. ReNCell VM cells were grown as an adherent monolayer on poly-ornithine(0.002%) laminin (2 μg/ml)-coated tissue culture flasks in the presence of 20 ng/ml of human recombinant

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EGF and bFGF2 in DMEM:F12 medium with nutrients optimized for neural progenitor cell growth. Cells in exponential growth phase were transfected with lentiviral particles (1 × 10^5 infectious units of virus) containing either three target-specific constructs that encode 19 nt (plus hairpin) shRNA designed to knockdown CSB gene expression (Santa Cruz Biotechnology, sc.37794-V) or shRNA non-targeting control (Santa Cruz Biotechnology, Dallas, TX, USA; sc.108080). Puromycin selection (2 µg/ml) is performed to achieve stable gene silencing. Withdrawal of the growth factors from the maintenance medium, as depicted in Figure 1A, resulted in spontaneous differentiation of these progenitor cells. Immunofluorescence labeling of differentiating neural progenitor cells were purchased from Santa Cruz Biotechnology. Antibodies against Flag (F3165), MCM2 (20429-1-AP, Millipore, Billerica, MA, USA), polyclonal anti-MAP2 (AB15452, Millipore, Billerica, MA, USA), polyclonal anti-GAP (AB18-0063, Invitrogen, Carlsbad, CA, USA), polyclonal anti-drebrin A/E (AB10140, Millipore), followed by incubation with fluorescein-conjugated secondary antibodies. Actin was stained using Rhodamine-phalloidin (R415, Molecular Probes, Carlsbad, CA, USA) staining. Slides were analyzed with a confocal microscope system (Zeiss LSM 710, Oberkochen, Germany).

Construction of tagged vector, transfection and selection. The mammalian CSB expression plasmid used in the TAP procedure (pZome-1-N-TAP-CSB) has been generated by inserting the full-length coding region of human CSB cDNA into the BamHI site of pZome-1-N (Euroscarf, Heidelberg, Germany). In this construct, the TAP tag consists of the protein A (Prot. A) and the calmodulin-binding peptide affinity sequences that are separated by the recognition sequence for tobacco etch virus (TEV) protease, permitting proteolytic elution of the fusion protein from the IgG affinity resin. CSIAN (CSB deficient), normally grown in DMEM/F10 medium containing 10% serum and antibiotics, have been transfected with either pZome-1-N (mock), or pZome-1-N-TAP-CSB using JetPEI (Polyplus) DNA transfection reagent. CSIAN cells stably expressing TAP-CSB or TAP alone were selected with puromycin (0.3 µg/ml) for 3 weeks.

Western blot analysis. Cells were lysed for 10 min on ice in RIPA buffer. The whole cell extracts were centrifuged at 13 000 × g for 5 min and the supernatant was recovered. Protein concentration was determined by Bradford protein assay kit (BioRad, Hercules, CA, USA). Proteins (50 µg) were separated on polyacrylamide gradient gel (4–20%) electrophoresis and blotted onto PVDF membrane (Amersham, Pittsburgh, PA, USA) following the standard procedures. The membrane was incubated with TBST (20 mM Tris–HCl, pH 7.4, 137 mM NaCl; 0.2% Tween 20) buffer containing 5% non-fat dried milk for 60 min at RT and subsequently incubated with primary and horseradish peroxidase-conjugated secondary antibodies (Vector, Burlingame, CA, USA). The signal was detected using the enhanced chemiluminescence method following the manufacturer’s instructions (Amersham). Antibodies against actin (sc-47778) and CSB (sc-25370) were purchased from Santa Cruz Biotechnology. Antibodies against Flag (F3165) and Myc (C3956) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Retrotranscription and real-time qPCR. RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). cDNA synthesis was performed using the First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). Real-time qPCR was carried out with SYBR green master mixture (AB15452, Millipore, Billerica, MA, USA) using the amplification of the CSB gene expression (Santa Cruz Biotechnology, sc.37794-V) or shRNA non-targeting control (Santa Cruz Biotechnology, Dallas, TX, USA; sc.108080). Puromycin selection (2 µg/ml) is performed to achieve stable gene silencing. Withdrawal of the growth factors from the maintenance medium, as depicted in Figure 1A, resulted in spontaneous differentiation of these progenitor cells. ChIP. Cells were cross-linked with 1% formaldehyde solution for 10 min at RT. Cross-linking was stopped by addition of glycin to 125 mM final concentration. Samples were sonicated to generate DNA fragments below 500 bp. For immunoprecipitations, protein extract (1 mg) was precleared for 2h with 50 ml of a 50% slurry of 50-50 protein A/G-Sepharose before adding the indicated antibodies. Then, 2 mg of p53 antibody was added to the reactions and incubated over night at 4 °C in the presence of 50 ml of protein A/G beads. After serial washings, the immunocomplexes were eluted twice for 10 min at 65 °C and cross-linking was reversed by adding 200 mM NaCl and incubating 5 h at 65 °C. Further proteinase-K digestion was performed for 2 h at 42 °C. DNA was purified by using Qiaquick PCR purification Kit. Immunoprecipitated DNA was quantified by real-time qPCR. Primers sequences are available upon request.

Conflict of Interest

The authors declare no conflict of interest.

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