DIPARTIMENTO DI SCIENZE ECOLOGICHE E BIOLOGICHE

Corso di Dottorato di ricerca in Ecologia e Gestione Sostenibile delle Risorse Ambientali - XXIX ciclo

The DNA repair proteins CSA and CSB play a key role in the regulation of cellular division and differentiation

s.s.d. BIO/18

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A.A. 2016/17

A Te che sei l'orso e la grotta,

La mia forza e la mia debolezza,

Il mio tormento e la mia delizia.

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Introduction

In the environment many substances have a genotoxic effect as they are able to interact with genetic material either directly or through metabolic activation. This interaction can lead to the modification of genetic material through structural alteration of the DNA molecule and can be expressed as adducts, chemical alteration of nitrogen bases, cross−links and break of the single and double strand. Generally, these types of alterations can be repaired by various cellular repair systems. However, the lesions that are not repaired correctly can lead to abnormalities of the genetic material such as chromosomal aberrations, gene mutations and cancer.

The aim of the present PhD thesis was to contribute to the knowledge of the systems used to safeguard the genome by genotoxic damages arising also from everyday exposure to environmental agents, as ultraviolet (UV) light, which has strong genotoxic effects to produce DNA damage, and to environmental pollution, in particular from heavy metals, benzene, polyaromatic hydrocarbons, dioxins and ultrafine particulate, that determine a state of instability of our genome and set the stage to mutations that give rise to tumors. Especially my aim was to study / understand the pleiotropic role of two DNA repair proteins involved in protection processes described above, *Cockayne syndrome group A* and *B* proteins (*CSA* and *CSB*).

The 396-amino-acid (44-kDa) CSA protein belongs to the family of WD-40 repeat proteins, which are typically involved in coordinating interactions among multi-protein complexes. Along this line, CSA showed to be a component of *ubiquitin E3 ligase* complex composed of *Cul4*, *RBX1* and *DDB1*. The CSB protein of 1493 amino acids (168 kDa) is a member of the *SWI2/SNF2* family of chromatin remodelers and harbors conserved motifs for ATP binding and hydrolysis (Fig. 1).

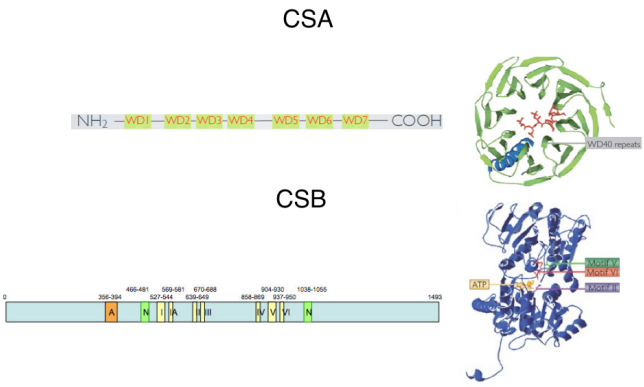

Figure 1: CSA and CSB proteins domains and folding.

Both CSA and CSB have pleiotropic functions: these proteins play a role in the transcription coupled repair pathway of nucleotide excision repair, which rapidly corrects certain transcription-blocking lesions located on the transcribed strand of active genes. In addition, CSB plays a role during basal and activated transcription by stimulating all three classes of nuclear RNA polymerases. CSA and CSB were demonstrated to be key regulators of *p53*, by stimulating its ubiquitination and degradation.

Finally, CSB functions as an anti-apoptotic factor over-expressed in a variety of cancer cells and tissues, so it represents a strategic target for anticancer therapy: the inhibition or down regulation of CSB in cancer cells makes these cells hypersensitive to a variety of commonly used cancer chemotherapeutic agents.

Mutations in either of the two genes originate *Cockayne syndrome* (*CS*), an autosomal recessive disorder that affects the development, the growth and the maintenance of a wide range of tissues and organs (Fig. 2).

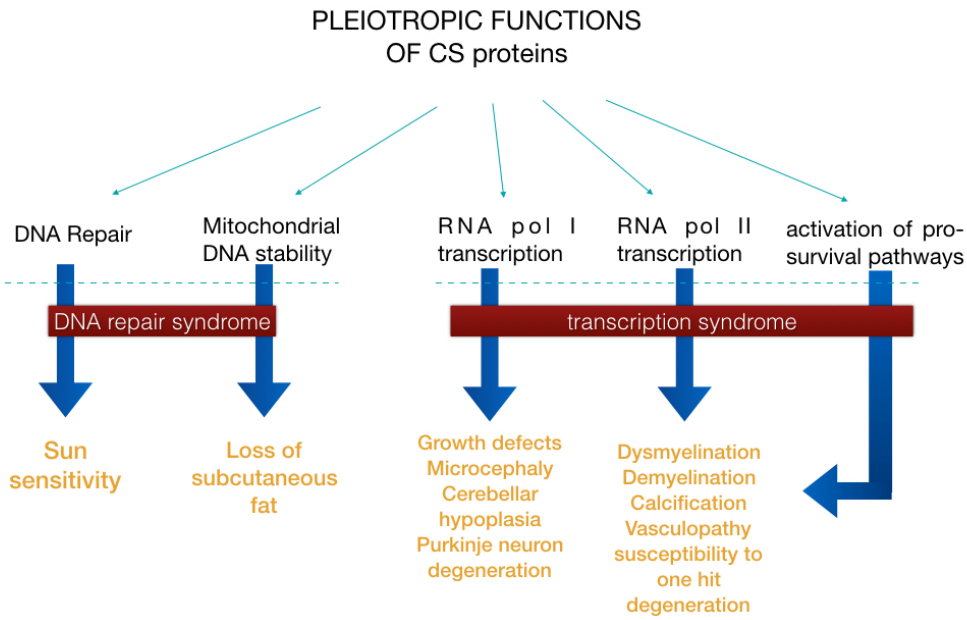

Figure 2: Pleiotropic functions of CS proteins can explain the wide range of symptoms of Cockayne syndrome patients. Cockayne syndrome can be classified both as a DNA repair syndrome, which leads to sun sensitivity and loss of subcutaneous fat, and as a transcription related syndrome. However, this is not enough to explain all the characteristics seen in CS patients.

Aims

CSA and CSB are proving more and more to be key proteins at the crossroad in the management and response to DNA damage. Firstly, they have a fundamental role in DNA repair; both CSA and CSB, in fact, are involved in a subpathway of *Nucleotide Excision Repair* known as *Transcription-Coupled Repair* (*TCR*), which removes transcription blocking DNA lesions, located on the transcribed strand of active genes; inefficiency or lack of TCR triggers an apoptotic signal, which depends on the functional status of CSB. This protein, in fact, also plays a critical role in cell robustness negatively modulating p53 activity after cellular stress, including DNA damage and hypoxia and counteracting p53-independent apoptosis. Also, CSB mediates the transcriptional programs following exposure to cellular stressors such as UV, oxidative damage, inflammation and hypoxia.

Evidently, it seems to be that the repair is tuned with the cell-cycle progression and / or induction of death. During my PhD activity I have studied an unexpected new role for CSA and CSB proteins. They not only participate in DNA repair and / or adjust the trigger of apoptosis but also regulate cell fate decision by taking part in the last step of mitosis, cytokinesis, or cell differentiation. So, my aim was to better understand the key role of these proteins, that seem to be the judges of cell life or death in response to genotoxic agents and of cell differentiation. These studies have given rise to the drafting of two scientific papers below reported.

In the first work, I've investigated about abnormalities in the regulation of *RNA pol I* and *II* transcription, that might provide plausible explanations for many of the somatic features, including neurological symptoms associated with CS. Observation of neurological symptoms detected either at birth or during early childhood raises the possibility that CSB may have a crucial role in the transcriptional programs that govern the plasticity and the maintenance of the central nervous system during (perinatal and postnatal)

pediatric life. The neurogenesis process, initiated by neural stem cells (NSC), continues during adult life involving the generation of new neurons and other cell types thereby maintaining the turnover of neural cells and cognitive plasticity. Further, it is well established that generation and migration of neurons is crucial not only for postnatal brain enlargement but also for establishing normal synaptic connection and functionality in the brain.

Our principal hypothesis was 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 was mandatory for exploring the molecular cause for neurodegeneration in CS patients. For this aim, 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, in order to verify if CSB deficiency might affect neuronal differentiation capabilities of human neural progenitor cells, suggestive of a crucial role for CSB in adult neurogenesis.

Furthermore, although many of the CSA and CSB protein functions, including DNA repair, are explained by their nuclear localization, we observed that CSA and CSB localization was not restricted to the nucleus. In my last work, using confocal microscopy, I observed that CSA and CSB localize within the intercellular bridges at the *midbody*, a transient structure that connects the two daughter cells at the end of cytokinesis, and that loss of function or down-regulation of CS proteins results in abscission impairment and cytokinesis failure, giving rise to mitotic abnormalities and formation of both multinucleated cells and multipolar mitotic spindles.

This finding was suggestive of a new and previously unpredicted role for both CSA and CSB in the abscission control.

OPEN

Citation: Cell Death and Disease (2014) 5, e1268; doi:10.1038/cddis.2014.228

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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.
Cell Death and Disease (2014) **5,** e1268; doi:10.1038/cddis.2014.228; published online 29 May 2014
Subject Category: Neuroscience

Cockayne syndrome (CS) is a rare human autosomal recessive disorder. CS patients are characterized by severe photosensivity, growth retardation, cachectic dwarfism, features of premature aging and progressive neurological abnormalities of the central nervous system including microcephaly, cerebellar atrophy and demyelinating peripheral neuropathy.[1–3]

CS patients have been assigned to two complementation groups (CSA and CSB), whose corresponding genes (*csa* and *csb*) have been cloned.[4–6] CSB protein has critical roles in a subpathway of nucleotide excision repair known as transcription-coupled repair (TCR).[7–10] TCR removes transcription-blocking DNA lesions, located on the transcribed strands of active genes and inefficient or lack of TCR triggers an apoptotic signal, which depends more on the functional status of CSB.[11,12] Besides its involvement in TCR of ultraviolet (UV) damage, CSB has also been implicated in base excision repair and in tumorigenesis.[13,14]

Although a defect in TCR pathway could potentially account for the enhanced photosensitivity of CS patients, other pathological features including neurodegeneration may not be solely explained by TCR defect.[15] This notion is somewhat strengthened by the demonstrated involvement of CSB in basal transcription mediated by RNA polymerases I and II.[16–22] More recently, we and others have demonstrated that CSB mediates the transcriptional programs following exposure to cellular stressors such as UV, oxidative damage, inflammation and hypoxia.[23–27] Therefore, abnormalities in the regulation of RNA pol I and II transcription might provide plausible explanations for many of the somatic features, including aspects of neurological symptoms associated with CS. Observation of neurological symptoms detected either at birth or during early childhood raises the possibility that CSB may have a crucial role in the transcriptional programs that govern the plasticity and the maintenance of the central nervous system during (perinatal and postnatal) pediatric life. The neurogenesis process, initiated by neural stem cells (NSC), continues during adult life involving the generation of new neurons and other cell types thereby maintaining the turnover of neural cells and cognitive plasticity.[28,29] Further, it is well established that generation and migration of neurons is crucial not only for postnatal brain enlargement but also for establishing normal synaptic connection and functionality in the brain.[30]

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Keywords: Cockayne syndrome; neurodegeneration; neurogenesis; neuritogenesis; chromatin remodeling complexes; transcription complex assembly and progeroid syndromes
Abbreviations: DAPI, 4,6-Diamidino-2-phenylindole nuclear staining; UV, ultraviolet; EGF, Epidermal growth factor; FGF, Fibroblast growth factor; HRP, horseradish peroxidase
Received 24.2.14; revised 28.3.14; accepted 14.4.14; Edited by A Verkhratsky

A recent article has described that an early and limited window of postnatal neurogenesis that occurs in the subventricolar 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.[31] In support, postnatal neurogenesis was reported during the functional development of cerebellum until 18 months.[32,33] 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-L-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.[11] To determine whether sh-K and sh-CSB lines were sensitive to UV-C radiation, cells were exposed to 10 J/m^2 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.

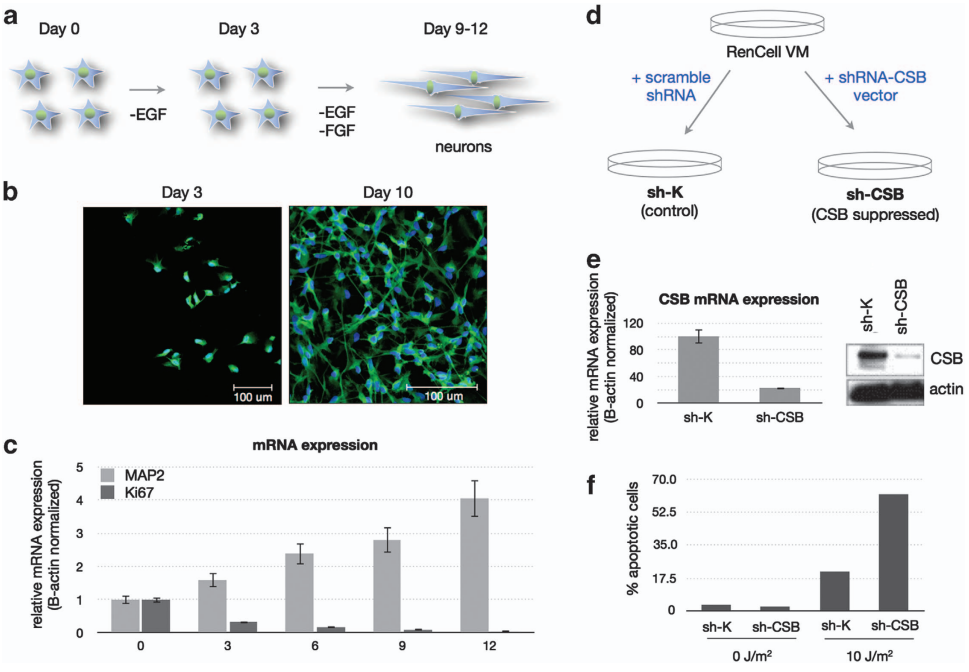


Figure 1 (**a**) Schematic diagram showing the procedural steps involved in the differentiation of human neural progenitor cells (RenCell VM) into neurons and glial cells. RenCell VM cell line is endowed with efficient self-renewal and multilineage differentiation capabilities. (**b**) Immunofluorescence 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

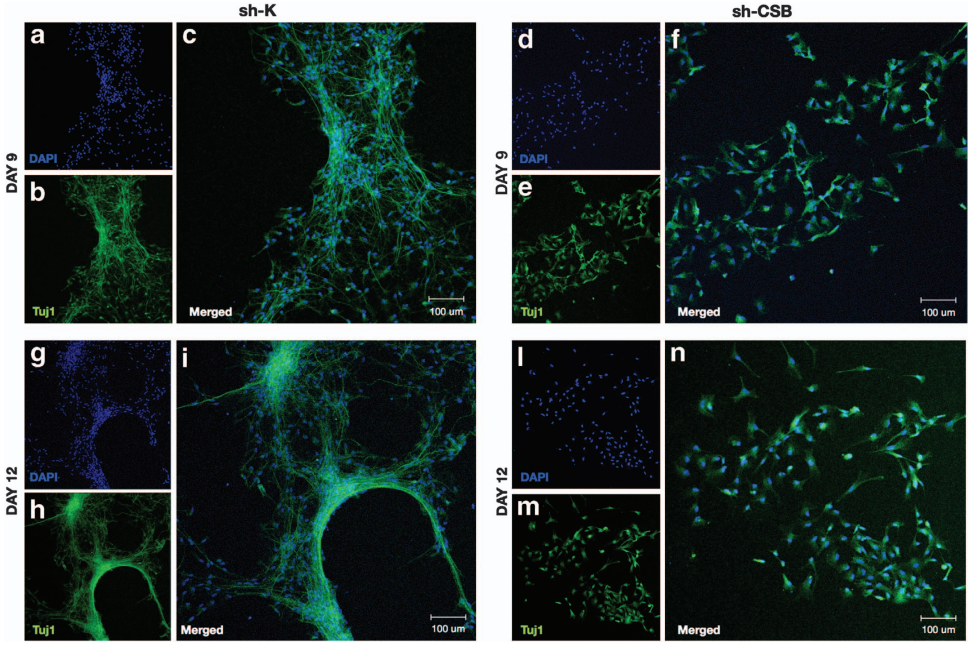


Figure 2 Immunofluorescence staining for neuronal marker Tuj1 in sh-K (**a–c** and **g–i**) and sh-CSB (**d–f** and **l–n**) cells after differentiation at days 9 and 12. Cells were counterstained for DNA using 4',6-diamidino-2-phenylindole

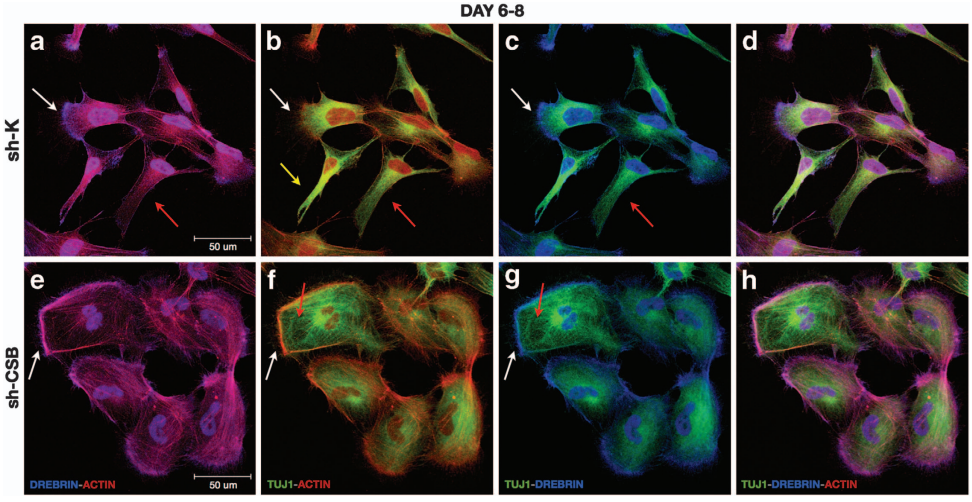


Figure 3 Immunofluorescence staining for Tuj1, Drebrin and actin in sh-K (**a–d**) and sh-CSB (**e–h**) cells after differentiation at days 6–8. In **a–d**, arrow indicates three different stages of neuritic outgrowth: growth cone formation (white arrow), elongation of the new formed growth cone (red arrow) and microtubules packaging inside the nascent neurite (yellow arrow). In **e–h**, white arrow indicates no destabilization of subcortical actin network and red arrow indicates absent advancement of microtubules within the lamollipodium, both as occuring in sh-CSB cells

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.*[34] 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

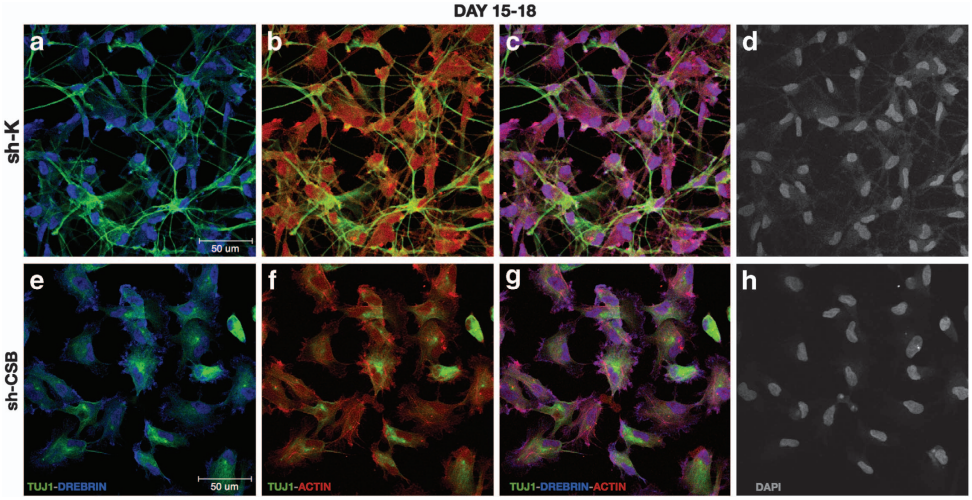


Figure 4 Immunofluorescence staining for Tuj1, Drebrin (an F-actin associated protein) and actin in sh-K (**a**–**d**) and sh-CSB (**e**–**h**) cells after growth in differentiation medium at 15–18 days. Cells were counterstained for DNA using 4′,6-diamidino-2-phenylindole nuclear staining

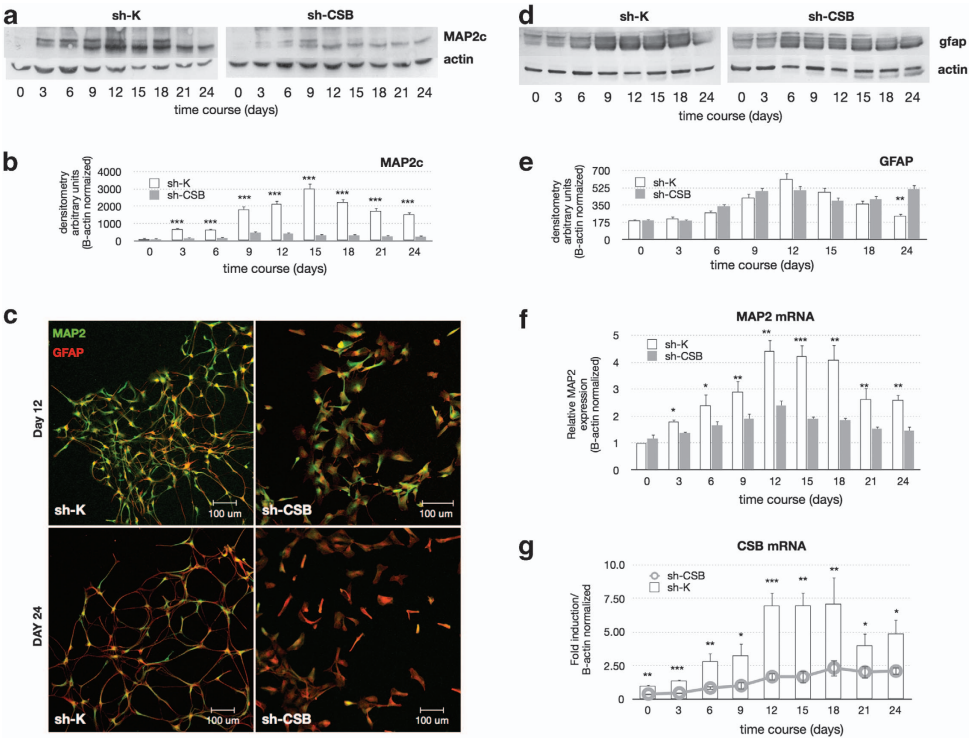
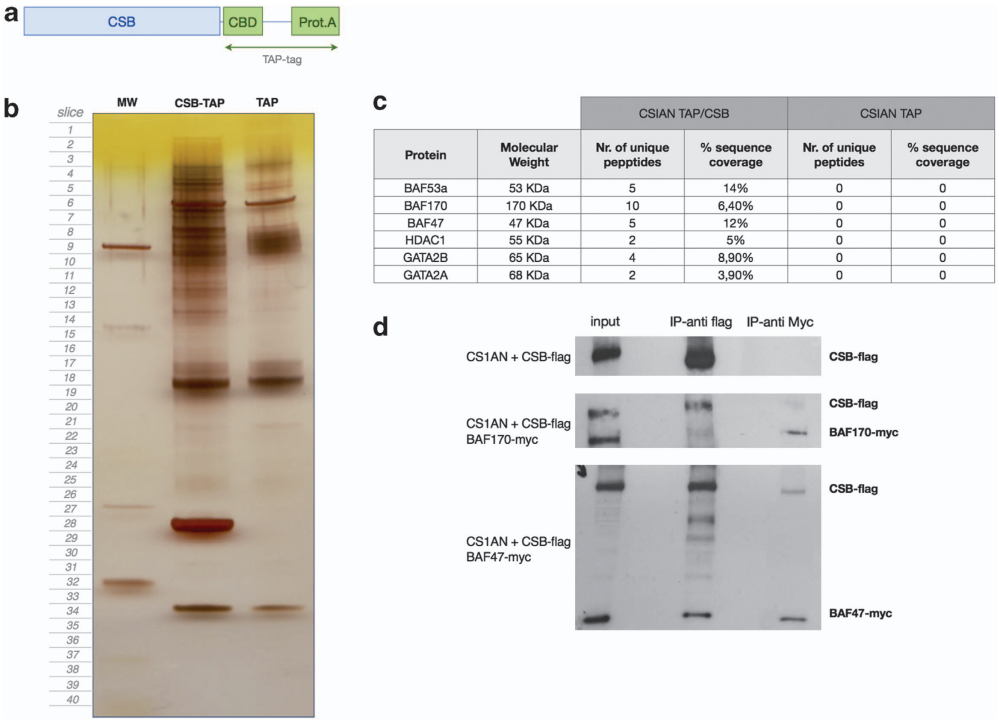


Figure 5 (**a**) Western blot analysis of MAP2 and β-actin expression detected in the whole cell extracts (WCE) of sh-K and sh-CSB cells during the entire time course of neural differentiation. WCE were collected at the indicated times. (**b**) Histogram showing quantification of MAP2 protein expression (mean ± S.D. of three independent experiments) during neural differentiation using Image J software (NIH). Data have been normalized by β-actin. (**c**) Immunofluorescence staining for MAP2 and GFAP in sh-K and sh-CSB cells at days 12 and 24 of differentiation. (**d**) Western blot analysis of GFAP and β-actin proteins detected in the WCE of sh-K and sh-CSB cells during the entire time course of neural differentiation. Protein extracts were collected at the indicated times. (**e**) Histogram shows the quantification of GFAP (mean ± S.D. of three independent experiments) during neural differentiation using Image J software (NIH). Data have been normalized by β-actin. MAP2 (**f**) and CSB (**g**) 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). ***$P<0.001$, **$P<0.01$, *$P<0.05$, (unpaired two-tailed Student's *t*-test)

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,[35] 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.[36–38] 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



Protein	Molecular Weight	CSIAN TAP/CSB		CSIAN TAP	
		Nr. of unique pepptides	% sequence coverage	Nr. of unique peptides	% sequence coverage
BAF53a	53 KDa	5	14%	0	0
BAF170	170 KDa	10	6,40%	0	0
BAF47	47 KDa	5	12%	0	0
HDAC1	55 KDa	2	5%	0	0
GATA2B	65 KDa	4	8,90%	0	0
GATA2A	68 KDa	2	3,90%	0	0

Figure 6 CSB co-precipitates with BAF complex. (**a**) TAP tag is fused with CSB cDNA and then cloned into a mammalian expression vector to express CSB–TAP-tagged protein. (**b**) Proteins associated either with CSB–TAP and or TAP alone were isolated by tandem affinity purification, separated on a 4–12% Bis-Tris gel and visualized by silver staining. (**c**) Table summarizes the names and the biological processes of selected proteins co-purifying with CSB–TAP fusion protein, but were absent in the purification from control cells expressing TAP tag alone. (**d**) WCE prepared from CSIAN cells with stable expression of CSB-flag and transient expression of BAF170-Myc or BAF47-Myc were immunoprecipitated (IP) using either flag or Myc specific antibodies. The input, the flow through (Ft) and the IP fractions were analyzed by western blotting using antibodies indicated on the right

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.[39,40] 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.[41] 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.[42–44]

SWI/SNF complex is a well-studied chromatin remodeler that uses the energy derived from ATP hydrolysis to regulate nucleosome mobility and chromatin accessibility.[45] One such SWI/SNF-like chromatin-remodeling enzymatic complex is Brg/Brm-associated factor (nBAF) that specifically regulates dendrite growth and axon myelination.[46] 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 acetyltrasferase at the *map2* promoter (Figure 7e). p300 functions as an essential cofactor for adult neurogenesis.[47] 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

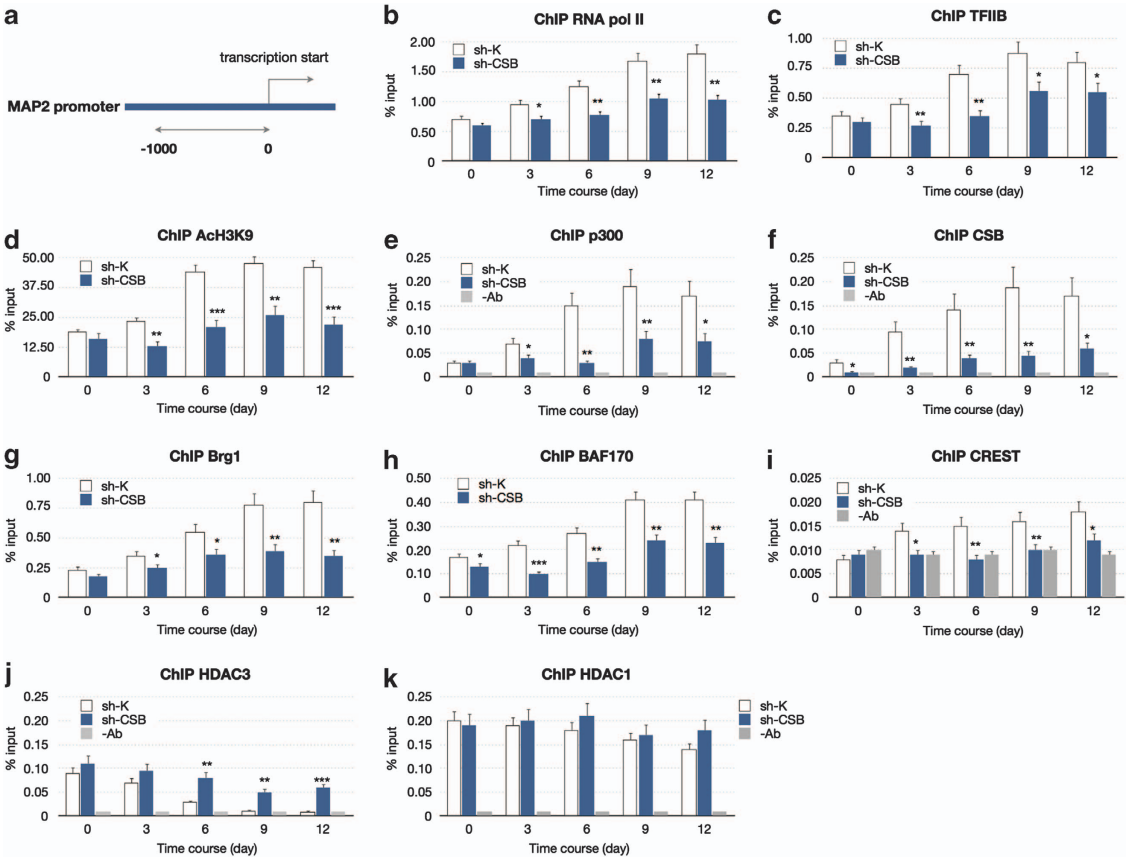


Figure 7 Interaction of CSB protein with transcription and chromatin remodeling factors detected by ChIP. (**a**) Real-time PCR was performed using gene promoter-specific primers to examine the relative enrichment for the promoter region of *map2* sites immediately upstream of the transcription initiation 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

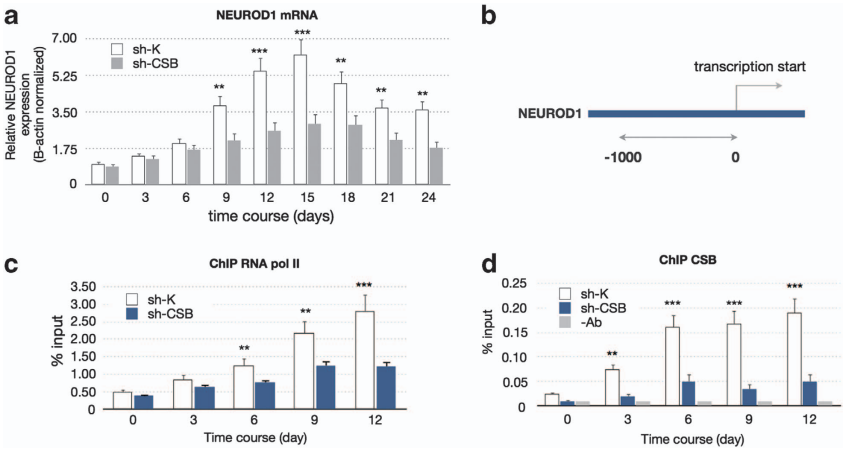


Figure 8 NEUROD1 (**a**) mRNA expression analysis in sh-K and sh-CSB cells during the entire time course of neural differentiation (mean ± S.D. of three independent experiments). (**b–d**) ChIP analysis of RNA polymerase II and CSB recruitment on NEUROD1 promoter region. Real-time PCR was performed using 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.[50] 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.[48] 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[54,55] 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.[24] 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[56] 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.[57] A recent paper has demonstrated that CSB prevents the accumulation of damaged mitochondria by promoting mitochondrial autophagy.[58] CSB also promotes the transcriptional elongation in mitochondria through communications with mitochondrial proteins.[59] 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

EGF and bFGF2 in DMEM:F12 medium with nutrients optimized for neural progenitor cell growth. Cells in exponential growth phase, were transduced with lentiviral particles (1×105 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). Puromicin 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 the spontaneous differentiation of these progenitor cells. Immunofluorescence labeling of differentiating neural progenitor cells ReNCell VM cells grown on Laminin-coated glass coverslips were induced to differentiate, fixed in paraformaldehyde and incubated with monoclonal antibodies against class III beta-tubulin (MMS-435P, Covance, Princeton, NJ, USA), polyclonal anti-MAP2 (AB15452, Millipore, Billerica, MA, USA), polyclonal anti-GFAP (AB18-0063, Invitrogen, Carlsbad, CA, USA), polyclonal anti-drebrin A/E (AB10140, Millipore), followed by incubation with fluorochrome-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 technique (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 $13\,000 \times g$ 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, Duren, 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 (Promega, Fitchburg, WI, USA) using Mx3005P Real-Time PCR system (Agilent, Santa Clara, CA, USA). Results were normalized to β-actin. Primers sequences are available upon request.

TAP. The cells were scraped from plates in ice-cold PBS and pelleted by centrifugation at $2000 \times g$ for 10 min at 4 °C. After removal of excess PBS, the cell pellet (30 ml) was resuspended in 60 ml of ice-cold IPP150 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, complete protease inhibitors, 1 mM PMSF). The cells were homogenized with 40 strokes in a Dounce homogenizer with a tight-fitting pestle and incubated on ice for 5 min. Insoluble material was removed by centrifugation at $16\,000 \times g$ for 20 min at 4 °C. The cell extracts were incubated with 500 µl of IgG sepharose beads for 2 h at 4 °C on a rotating wheel. The IgG beads were washed twice with 60 ml of ice-cold IPP150 lysis buffer and 30 ml of TEV cleavage buffer (10 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT). The washed IgG beads were resuspended in 2 ml of ice-cold TEV cleavage buffer supplemented with 40 μl of AcTEV protease (400 U) and complete protease inhibitors and incubated at 16 °C for 2 h on a rotating wheel. The TEV eluate was adjusted with CaCl2 to 3 mM final concentration, mixed with 6 ml of calmodulin-binding buffer 1 (10 mM β-mercaptoethanol, 10 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM imidazole, 1 mM Mg-Acetate, 2 mM CaCl2) and 150 μl calmodulin beads and incubated for 2 h at 4 °C on a rotating wheel. The calmodulin beads were washed with 30 ml of ice-cold calmodulin-binding buffer 1 and with 20 ml of calmodulin-binding buffer 2 (1 mM β-mercaptoethanol, 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM Mg-Acetate, 2 mM $CaCl_2$). The bound proteins were eluted from beads by boiling in the LDS sample buffer, separated on a 4–12% Bis-Tris gel and visualized by silver staining.

ChIP. Cells were cross-linked with a 1% formaldehyde solution for 10 min at RT. Cross-linking was stopped by addition of glycine to 125 mM final concentration. Samples were sonicated to generate DNA fragments below 500 bp. For immunoprecipitations, protein extract (1 mg) was precleared for 2 h 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 adjusting to 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 Qiagen (Valencia, CA, USA) columns (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.

Acknowledgements. This work has been supported by Telethon (GGP11176) and Italian Association for Cancer Research (AIRC-IG2012-13074).

1. Weidenheim KM, Dickson DW, Rapin I. Neuropathology of Cockayne syndrome: evidence for impaired development, premature aging, and neurodegeneration. *Mech Ageing Dev* 2009; **130**: 619–636.
2. Laugel V, Dalloz C, Durand M, Sauvanaud F, Kristensen U, Vincent MC *et al.* Mutation update for the CSB/ERCC6 and CSA/ERCC8 genes involved in Cockayne syndrome. *Hum Mutat* 2010; **31**: 113–126.
3. Natale V. A comprehensive description of the severity groups in Cockayne syndrome. *Am J Med Genet* 2011; **155A**: 1081–1095.
4. Henning KA, Li L, Iyer N, McDaniel LD, Reagan MS, Legerski R *et al.* The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. *Cell* 1995; **82**: 555–564.
5. Troelstra C, van Gool A, de Wit J, Vermeulen W, Bootsma D, Hoeijmakers JH. ERCC6, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. *Cell* 1992; **71**: 939–953.
6. Lehmann AR. Three complementation groups in Cockayne syndrome. *Mutat Res* 1982; **106**: 347–356.
7. Venema J, Mullenders LH, Natarajan AT, van Zeeland AA, Mayne LV. The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. *Proc Natl Acad Sci USA* 1990; **87**: 4707–4711.
8. Licht CL, Stevnsner T, Bohr VA. Cockayne syndrome group B cellular and biochemical functions. *Am J Hum Genet* 2003; **73**: 1217–1239.
9. Laine JP, Egly JM. When transcription and repair meet: a complex system. *Trends Genet* 2006; **22**: 430–436.
10. Sarasin A, Stary A. New insights for understanding the transcription-coupled repair pathway. *DNA Repair (Amst)* 2007; **6**: 265–269.
11. Mayne LV, Lehmann AR. Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum. *Cancer Res* 1982; **42**: 1473–1478.
12. Balajee AS, Proietti-De-Santis L, Brosh RM, Selzer R, Bohr VA. Role of the ATPase domain of the Cockayne syndrome group B protein in UV induced apoptosis. *Oncogene* 2000; **19**: 477–489.
13. Aamann MD, Muftuoglu M, Bohr VA, Stevnsner T. Multiple interaction partners for Cockayne syndrome proteins: implications for genome and transcriptome maintenance. *Mech Ageing Dev* 2013; **134**: 212–224.
14. Caputo M, Frontini M, Velez-Cruz R, Nicolai S, Prantera G, Proietti-De-Santis L. The CSB repair factor is overexpressed in cancer cells, increased apoptosi resistence, and promotes tumor growth. *DNA Repair* 2013; **12**: 293–299.

15. Brooks PJ. Blinded by the UV light: how the focus on transcription-coupled NER has distracted from understanding the mechanisms of Cockayne syndrome neurologic disease. *DNA Repair* 2013; **12**: 656–671.
16. Balajee AS, May A, Dianov GL, Friedberg EC, Bohr VA. Reduced RNA polymerase II transcription in intact and permeabilized Cockayne syndrome group B cells. *Proc Natl Acad Sci USA* 1997; **94**: 4306–4311.
17. Selby CP, Sancar A. Cockayne syndrome group B protein enhances elongation by RNA polymerase II. *Proc Natl Acad Sci USA* 1997; **94**: 11205–11209.
18. Tantin D, Kansal A, Carey M. Recruitment of the putative transcription-repair coupling factor CSB/ERCC6 to RNA polymerase II elongation complexes. *Mol Cell Biol* 1997; **17**: 6803–6814.
19. van Gool AJ, van der Horst GT, Citterio E, Hoeijmakers JH. Cockayne syndrome: defective repair of transcription? *EMBO J* 1997; **16**: 4155–4162.
20. Bradsher J, Auriol J, Proietti de Santis L, Iben S, Vonesch JL, Grummt I *et al.* CSB is a component of RNA pol I transcription. *Mol Cell* 2002; **10**: 819–829.
21. Yuan X, Feng W, Imhof A, Grummt I, Zhou Y. Activation of RNA polymerase I transcription by cockayne syndrome group B protein and histone methyltransferase G9a. *Mol Cell* 2007; **27**: 585–595.
22. Brooks PJ, Cheng TF, Cooper L. Do all of the neurologic diseases in patients with DNA repair gene mutations result from the accumulation of DNA damage? *DNA Repair* 2008; **7**: 834–848.
23. Proietti-De-Santis L, Drane P, Egly JM. Cockayne syndrome B protein regulates the transcriptional program after UV irradiation. *EMBO J* 2006; **25**: 1915–1923.
24. Filippi S, Latini P, Frontini M, Palitti F, Egly JM, Proietti-De-Santis L. CSB protein is (a direct target of HIF-1 and) a critical mediator of the hypoxic response. *EMBO J* 2008; **27**: 2545–2556.
25. Frontini M, Proietti-De-Santis L. Cockayne syndrome B protein (CSB): linking p53, HIF-1 and p300 to robustness, lifespan, cancer and cell fate decisions. *Cell Cycle* 2009; **8**: 693–696.
26. Newman JC, Bailey AD, Fan HY, Pavelitz T, Weiner AM. An abundant evolutionarily conserved CSB-PiggyBac fusion protein expressed in Cockayne syndrome. *PLoS Genet* 2008; **4**: e1000031.
27. Velez-Cruz R, Egly JM. Cockayne syndrome group B (CSB) protein: at the crossroads of transcriptional networks. *Mech Ageing Dev* 2013; **134**: 234–242.
28. Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C *et al.* Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science* 2007; **315**: 1243–1249.
29. Sanai N, Berger MS, Garcia-Verdugo JM, Alvarez-Buylla A. Comment on 'Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension'. *Science* 2007; **318**: 393.
30. Tau GZ, Peterson BS. Normal development of brain circuits. *Neuropsychopharmacology* 2010; **35**: 147–168.
31. Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai HH, Wong M *et al.* Corridors of migrating neurons in the human brain and their decline during infancy. *Nature* 2011; **478**: 382–386.
32. Bonfanti L, Peretto P. Adult neurogenesis in mammals—a theme with many variations. *Eur J Neurosci* 2011; **34**: 930–950.
33. Spreen O, Risser AH, Edgell D. *Developmental Neuropsychology*. Oxford University press: New York, 1995.
34. Cunningham CC, Leclerc N, Flanagan LA, Lu M, Janmey PA, Losik KS. Microtubule-associated protein 2c reorganizes both microtubules and microfilaments into distinct cytological structures in an actin-binding protein-280-deficient melanoma cell line. *J Cell Biol* 1997; **136**: 845–857.
35. Tantin D. RNA polymerase II elongation complexes containing the Cockayne syndrome group B protein interact with a molecular complex containing the transcription factor IIH components xeroderma pigmentosum B and p62. *J Biol Chem* 1998; **273**: 27794–27799.
36. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Séraphin B. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 1999; **17**: 1030–1032.
37. Rumpf C, Cipak L, Dudas A, Benko Z, Pozgajova M, Riedel CG *et al.* Casein kinase 1 is required for efficient removal of Rec8 during meiosis I. *Cell Cycle* 2010; **9**: 2657–2662.
38. Rumpf C, Cipak L, Schleiffer A, Pidoux A, Mechtler K, Tolić-Nørrelykke IM *et al.* Laser microsurgery provides evidence for merotelic kinetochore attachments in fission yeast cells lacking Pcs1 or Clr4. *Cell Cycle* 2010; **9**: 3997–4004.
39. Cipak L, Spirek M, Novatchkova M, Chen Z, Rumpf C, Lugmayr W *et al.* An improved strategy for tandem affinity purification-tagging of *Schizosaccharomyces pombe* genes. *Proteomics* 2009; **9**: 4825–4828.
40. Gregan J, Riedel CG, Petronczki M, Cipak L, Rumpf C, Poser I *et al.* Tandem affinity purification of functional TAP-tagged proteins from human cells. *Nat Protoc* 2007; **2**: 1145–1151.
41. Ho L, Crabtree GR. Chromatin remodelling during development. *Nature* 2010; **463**: 474–484.
42. Hong EJ, West AE, Greenberg ME. Transcriptional control of cognitive development. *Curr Opin Neurobiol* 2005; **15**: 21–28.
43. Hsieh J, Gage FH. Chromatin remodeling in neural development and plasticity. *Curr Opin Cell Biol* 2005; **17**: 664–671.
44. Goldberg AD, Allis CD, Bernstein E. Epigenetics: a landscape takes shape. *Cell* 2007; **128**: 635–638.
45. Wang W, Xue Y, Zhou S, Kuo A, Cairns BR, Crabtree GR. Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev* 1996; **10**: 2117–2130.
46. Wu JI, Lessard J, Olave IA, Qiu Z, Ghosh A, Graef IA *et al.* Regulation of dendritic development by neuron-specific chromatin remodeling complexes. *Neuron* 2007; **56**: 94–108.
47. Chatterjee S, Mizar P, Cassel R, Neidl R, Selvi BR, Mohankrishna DV *et al.* A novel activator of CBP/p300 acetyltransferases promotes neurogenesis and extends memory duration in adult mice. *J Neurosci* 2013; **33**: 10698–10712.
48. Laposa RR, Huang EJ, Cleaver JE. Increased apoptosis, p53 up-regulation, and cerebellar neuronal degeneration in repair-deficient Cockayne syndrome mice. *Proc Natl Acad Sci USA* 2007; **104**: 1389–1394.
49. Brace LE, Vose SC, Vargas DF, Zhao S, Wang XP, Mitchell JR. Lifespan extension by dietary intervention in a mouse model of Cockayne syndrome uncouples early postnatal development from segmental progeria. *Aging Cell* 2013; **12**: 1144–1147.
50. Sacco R, Tamblyn L, Rajakulendran N, Bralha FN, Tropepe V, Laposa RR. Cockayne syndrome b maintains neural precursor function. *DNA Repair (Amst)* 2013; **12**: 110–120.
51. Walton RM. Postnatal neurogenesis: of mice, men, and macaques. *Vet Pathol* 2012; **49**: 155–165.
52. Yang Z, Ming GL, Song H. Postnatal neurogenesis in the human forebrain: from two migratory streams to dribbles. *Cell Stem Cell* 2011; **9**: 385–386.
53. Feliciano DM, Bordey A. Newborn cortical neurons: only for neonates? *Trends Neurosci* 2013; **36**: 51–61.
54. Robbins JH, Brumback RA, Mendiones M, Barrett SF, Carl JR, Cho S *et al.* Neurological disease in xeroderma pigmentosum. Documentation of a late onset type of the juvenile onset form. *Brain* 1991; **114**: 1335–1361.
55. Przedborski S, Vila M, Jackson-Lewis V. Neurodegeneration: what is it and where are we? *J Clin Invest* 2003; **111**: 3–10.
56. Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp Physiol* 2006; **91**: 807–819.
57. Aamann MD, Sorensen MM, Hvitby C, Berquist BR, Muftuoglu M, Tian J *et al.* Cockayne syndrome group B protein promotes mitochondrial DNA stability by supporting the DNA repair association with the mitochondrial membrane. *FASEB J* 2010; **24**: 2334–2346.
58. Scheibye-Knudsen M, Ramamoorthy M, Sykora P, Maynard S, Lin PC, Minor RK *et al.* Cockayne syndrome group B protein prevents the accumulation of damaged mitochondria by promoting mitochondrial autophagy. *J Exp Med* 2012; **209**: 855–869.
59. Berquist BR, Canugovi C, Sykora P, Wilson DM, Bohr VA. Human Cockayne syndrome B protein reciprocally communicates with mitochondrial proteins and promotes transcriptional elongation. *Nucleic Acids Res* 2012; **40**: 8392–8405.

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)

CSA and CSB proteins localize to the midbody during cytokinesis and regulate abscission through PRC1 degradation

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ABSTRACT

Biochemical studies have indicated that CSA and CSB proteins are involved in DNA repair and transcription activities. Here, we report that CSA and CSB proteins, so far primarily considered nuclear proteins, localize to the midbody, a transient structure that connects the two daughter cells at the end of cytokinesis. Loss of function or down-regulation of CS proteins results in abscission impairment and cytokinesis failure, resulting in mitotic abnormalities that lead to formation of multinucleated cells and multipolar mitotic spindles. Our findings further demonstrate that CSA and CSB participate in the ubiquitylation and degradation of protein regulator of cytokinesis 1 (PRC1) which plays a fundamental role in the regulation of abscission. Altogether these findings reveal novel and unpredicted roles of CS proteins in the context of mitosis and open a new scenario in the understanding of Cockayne syndrome features beside those resulting in defect in transcription and DNA repair.

INTRODUCTION

CSA protein (44-kDa) belongs to the family of WD-40 repeat proteins, which are typically involved in coordinating interactions among multiprotein complexes [Zhang and Zhang, 2015]. Along this line, CSA showed to be a component of ubiquitin E3 ligase complex composed of Cul4, RBX1 and DDB1 [Lee and Zhou, 2007]. CSB protein (168 kDa) is a member of the SWI2/SNF2 family of chromatin remodelers and harbors conserved motifs for ATP binding and hydrolysis [Selby and Sancar, 1997; Tantin et al., 1997]. Both CSA and CSB proteins play a role in the transcription coupled repair pathway of nucleotide excision repair, which rapidly corrects certain transcription-blocking lesions located on the transcribed strand of active genes [Litch et al., 2003; Lainé and Egly, 2006]. In addition, CSB plays a role during basal and activated transcription by stimulating all three classes of nuclear RNA polymerases [Selby and Sancar, 1997; Balajee et al., 1997; Tantin et al., 1997; Bradsher et al., 2002; Yuan et al, 2007]. Finally, CSA and CSB have been demonstrated to be key regulators of p53, by stimulating its ubiquitination and degradation [Latini et al., 2011; Frontini and Proietti-De-Santis, 2012]. Both, CSA and CSB, are at the origin, when mutated, of Cockayne syndrome [Henning et al., 1995; Troesltra et al., 1992], a human autosomal recessive disorder that affects the development, the growth and the maintenance of a wide range of tissues and organs [Weidenheim et al., 2009; Natale, 2011]. Overexpression of CSB is common in many types of human cancer (Caputo et al., 2013).

Cytokinesis is the process during cell division in which the cytoplasm of a single mother cell is divided into two daughter cells. Cytokinesis process begins in the early stages of anaphase after the chromosomes have segregated. This requires the assembly of an actomyosin contractile ring, which shrinks the plasma membrane, between the newly formed nuclei, and compacts midzone microtubules to form a transient structure, termed midbody, which is located in the center of the intercellular bridge that connects the two daughter cells at the end of cytokinesis [Mierzwa and Gerlich, 2014]. The resolution of this intercellular bridge, through the severing of the microtubules sited at the midbody, a process known as abscission, is the last step of cytokinesis that separates the two daughter cells (Normand and King, 2010). Despite the understanding of the midbody assembly and regulation is still limited, we know that its principal function is to localize the site of abscission and to serve as a sort of scaffold for the recruitment of the huge number of assembly and disassembly factors as well as negative and positive regulators that have been demonstrated to be necessary for the successful completion of cytokinesis (Hu et al, 2012). Here, we show that CSA

and CSB proteins localize to the midbody and participate in the triggering of the abscission, through the ubiquitination and degradation of Protein Regulator of cytokinesis 1 (PRC1). Accordingly with this emerging finding, we demonstrated that loss of function of CS proteins results in abscission impairment and cytokinesis failure, resulting in mitotic abnormalities that lead to formation of multinucleated cells and multipolar mitotic spindles.

RESULTS

CSA and CSB proteins localize to the midbody

Using a monoclonal CSB antibody and confocal microscopy, we showed that during cytokinesis in Hela cells, CSB localized with the midbody within the intercellular bridges (Fig.1A panels a-d). This observation was confirmed during cytokinesis of both neuroblastoma SKNBE-2c cells (Fig.1A e-h) and breast cancer MCF7 (Fig.S1-A). Next, we found that CSA, the binding partner of CSB (Laine and Egly, Tantin), also localized to the midbody in both HeLa and SKNBE-2c cell lines (Fig 1B a-h) as well as in MCF7 cells (Fig.S1-B). To exclude non-specific, off-target effects of the antibodies raised against CS proteins, we transiently expressed wt flag-tagged CSB and CSA proteins in CS patients' fibroblasts. Antibody against the flag tag confirmed that both CSB and CSA proteins localizes to the midbody (Fig.S1-C). Next, we purified midbody (MID) fraction from telophase-enriched proliferating cells. Western blot analysis showed that MID fraction contained CSA and CSB proteins, in addition to structural components of the midbody (REF) such as α-tubulin, γ-tubulin and chromosomal passenger protein Aurora B (Fig. 1C lane 3). Actin and the transcription factor Sp1, used as markers of cytoplasmic and nuclear contaminations, respectively, were not found in MID, while they were present in either asynchronous cells (A-TCE) or in telophase enriched cells (T-TCE) (Fig. 1C, lanes 1 and 2).

Dynamic localization of CSA and CSB proteins during the cell cycle

We next investigated the localization of both CSA and CSB proteins during the cell cycle progression as schematized (Fig.2A). Both CSB and CSA appeared to be nuclear during interphase, according to their well-established role in DNA-metabolic processes; during metaphase, instead, following the disassembly of the nuclear envelop, the two proteins occupied distinct territories from the chromatin (Fig.2B and 2C, panels a). Aurora B localized on the inner centromere of the chromosomes during metaphase (Figs. 2B and 2C, panels a), then translocated to the central spindle

(also called midzone) in early anaphase (Figs. 2B and 2C, panels b) and finally accumulates in the midbody from telophase to cytokinesis (Figs. 2B and 2C, panels c-n).

In late anaphase, during cleavage furrow contraction, CSB and CSA localized to the external boundary of the spindle midzone, which was intensely stained by Aurora B (Figs. 2B and 2C, panels c-e). We noted that the localization with the spindle midzone is more pronounced for CSB as compared to CSA (Figs. 2B and 2C, panels e). During telophase CSB and CSA accumulated at the midbody, where CSB has a characteristic ring-like arrangement surrounding the midbody in a region called "bulge zone**"**, while CSA localized more precisely in the so-called "dark-zone", a narrow region sited in the center of the midbody (Figs. 2B and 2C, panels f-n). However, using a different antibody and a deconvolution software, we were able to visualize CSA also in the surrounding bulge zone (Fig. S2).

CSB silencing resulted in multipolar mitotic spindles and multinucleated cells.

To further investigate the role of CS proteins during the last steps of cytokinesis, we knocked-down CSB in SKNBE-2c cells (SKNBE-2c/sh-CSB), using a lentiviral-based strategy, (Fig. 3A).

Spindle pole staining by γ-tubulin antibody showed that CSB suppression resulted in the appearance of multipolar mitotic cells with supernumerary poles scattered among dispersed chromatin (Fig. 3B). In SKNBE-2c/sh-CSB cells, we observed up to 85% of multipolar cells as well as a significant accumulation of multinucleated cells characterized by the presence of differently sized nuclei with abnormal and irregular shape, as compared to SKNBE-2c/sh-K control cells (Fig. 3C and D). Furthermore a deeper analysis showed a high percentage (around 70%) of dividing cells interconnected by dramatically elongated intercellular bridges (long IBs) (Fig. 3E,F). Interestingly, treatment of control SKNBE-2c/sh-K cells by the cytokinesis inhibitor, cytokalasin-B, resulted in the formation of both multinucleated and multipolar mitotic cells similar to the phenotype previously observed in CSB-silenced cells (Fig. 3G and 3C).

To shed light on the origin of multinucleated cells, we followed the progression of the cell cycle of EGFP-tubulin transfected, asynchronous SKNBE-2c cells by time-lapse imaging. We showed that CSB-silenced cells actually executed cell division, leading to the formation of the daughter cells, that however failed to fully separate (Fig. 4B and Movies S1 and S2). As observable in the movies, the SKNBE-2c/sh-CSB cells remained connected, engaged in a sort of *tug of war*, and further fused back giving rise to bi- or multinucleated, cells. More in detail, in a time range of 4-6 hrs, 53±11,5 % (n = 50) of the dividing SKNBE-2c/sh-CSB cells were still connected by intercellular bridges and/ or failed abscission, with the daughter cells collapsing back into binucleated/multinucleated cells.

On the contrary, after an average of 2.5 hrs from the formation of the intercellular bridges, 94±3 % (n = 50) of the SKNBE-2c/sh-K control cells divided into two daughters cells. (Fig.4 upper panels).

Altogether our data strongly indicates a crucial role of CSA and CSB proteins in the cytokinesis step of the cell cycle division.

Delocalization of PRC1 protein in CS deficient cells

We further investigated the role of CSB and CSA in CS patients-derived, immortalized fibroblasts, CSIAN and CS3BE, respectively deficient for CSB and CSA. These cells exhibited the typical mitotic defects already observed in CSB silenced cells (Figs. 5A,C). Notably, the high percentage of binucleate and multipolar cells, and of cells still connected by long intercellular bridges significantly ($P \leq 0.05$) decreased upon expression of CSB and CSA wt proteins (Figs. 5B and D).

In all the rescued cells, we observed the progression of the intercellular bridge toward the establishment of the two secondary ingression-constriction zones (Fig. 6B, panels e-h and m-p), schematized in Fig.6A. In CS deficient cells, instead, the elongated IBs lacked secondary ingression zones (Fig.6B, panels a-d and i-l). These observations prompted to investigate the behavior of key proteins involved in cytokinesis: the 55 KDa centrosomal protein (CEP55), polo-like kinase 1 (Plk1), protein regulator of cytokinesis (PRC1), kinase-like protein KIF23 (MKLP1) and Apoptosis-Linked Gene 2-Interacting Protein X (ALIX).

Confocal microscopy showed that the localization of CEP55, ALIX and MKLP1 was not CSA and CSB dependent (Fig. S3). Indeed, CEP55, ALIX and MKLP1 (correctly) localized in the dark zone of the midbody in both the Long or the normal IBs (with clearly apparent secondary ingression zones.)

In contrast, in the absence of (functional) CS proteins, PRC1 became dramatically mislocalized. Indeed, in CS mutant cells, PRC1 occupied, in addition of the dark zone, also one of the two arms that form the intercellular bridge (Fig, 6B, panels a-d and i-l).

Interestingly, the localization of PLK1, the anaphase-specific binding partner of PRC1 (Neef et al., 2007), was not modified in the absence of CS proteins (Fig. 6C).

CSA and CSB proteins are involved in the ubiquitin-proteasome degradation of PRC1

The above data suggests a strong relationship between CS protein absence, PRC1 mislocalization and ineffective abscission. Since the separation the two daughter cells was associated with a very active ubiquitin-proteasome degradation process concentrated at midbody (Pines, 2006; Grenfell et al., 1994; Wojcik et al., 1995), we investigated the influence of CS proteins on the presence (positioning, recruitment) of their ubiquitin ligase proteins partners, at midbody. In CSA rescued cells, CUL4, a partner of CSA within the ubiquitin ligase complex (Groisman et al., 2003), localized at the midbody (Fig. 7A, panels d-f). Instead, in the absence of CSA (CS3BE cells) CUL4 was no longer visible (Fig. 7A, panel a-c). Interestingly, MDM2 ubiquitin ligase, previously found to be associated with CSA ubiquitin ligase complex (Latini et al., 2013; Epanchintsev et al., submitted) also localized at the midbody in CSA rescued cells (Fig. 7B, panels d-f) while was absent in CSA mutant cells (Fig. 7B, panels a-c). In CSB deficient cells (CSIAN), in which CSA is functional, CUL4 as well as MDM2 pattern was unaffected (Fig. 7C and D, panels a-c).

Having ascertained mislocalization of PRC1 in absence of functional CS proteins, we wondered whether the recruitment of ubiquitin ligases at the midbody was required for the ubiquitination of PRC1. Thus, we immunoprecipitated ubiquitinated proteins using antibody against ubiquitin and then immunodetected PRC1 using a specific antibody. In CSA-rescued CS3BE extracts we visualized a ladder of three PRC1 bands, ranging from 130 to 160 KDa (Fig.7E, line 3). The three PRC1 ubiquitinated bands are also apparent in the CSA-rescued CS3BE input fraction when the film was overexposed (Fig.7E, lane 1), while short exposure time revealed the expected size (73 KDa) of PRC1 protein when devoid of any post-translational modification (lane 5). In contrast, in CS3BE protein extracts, PCR1 ubiquitination was not found (lanes 2 and 4).

To follow the fate of PRC1 during the progression of the cytokinesis, midbody extracts were collected at different times after the release from a metaphase block. In CSA and CSB rescued cells, PRC1 degradation clearly occurs starting from 150 minutes after the block release (Figs.7F and G, lanes 5-8). In contrast, in CSA and CSB mutant cells PRC1 degradation does not occurs throughout the entire time course analyzed (either at 180 minutes) after the block release (Figs.7F and G, lanes 1-4). Interestingly the diminution of PRC1 signal in rescued cells is preceded (at 120 minutes) by a peak of CSA and MDM2 engagement at the midbody (Fig.7F, lanes 5-8). In line with immunofluorescence observations (Fig.7B), in the absence of CSA, the MDM2 peak was not instead visible (Fig.7F, line 1-4).

Further immunofluorescence experiments showed that CSB is essential for the recruitment of the non-ATPase regulatory subunit 1 (PSMD1) of the 26S proteasome complex at the midbody (compare Fig. 7I, panels a-c and d-f). Whereas, the PSMD1 recruitment at the midbody did not depend on CSA (compare Fig. 7H, panel a-c and d-f).

DISCUSSION

With the present study we provided molecular and cellular evidences on an unpredicted role of CSA and CSB proteins in the cytokinesis execution. First, we showed the dynamic localization of both proteins during the different phase of the cell cycle. In addition to previous observations on the nuclear and mitochondrial localization of CS proteins (Kamenish et al., 2010, Aamann et al., 2010), our results clearly indicated, for the first time, that they translocated to the central spindle during anaphase and finally accumulated at the midbody during late telophase. Then, our results clearly showed that CSA and CSB peculiar mitotic localization was not casual but reflected their true role in cell division. Finally, we presented sound evidences that CSA and CSB play a key role in the last step of the cytokinesis, the abscission, partecipating in the ubiquitylation and degradation of the midbody protein, PRC1.

From fluorescence confocal analysis and from the immunodetection of CS proteins in midbody extracts, we gained definitive evidence that in late mitotic cells, CSA and CSB localized with the midbody. The use of Cockayne Syndrome mutant cells, naturally lacking either of CS proteins, and of CSB-interfered wildtype cells, allowed us to demonstrate that CS proteins deprivation dramatically hampered the regular cytokinesis progression. The occurrence of both multipolar mitoses and multinucleated cells in CSA- and CSB-deprived cells raised the question of whether the former were the cause of the latter, or viceversa. Using time-lapse live imaging, we could conclude that binucleated cells were first generated by the abscission failure. This conclusion was strongly supported by the similarity between the CSA- and CSB-deficiency phenotypes and that induced by the cytokinesis inhibitor cytokalasin-B.

How CSA and CSB worked to regulate cytokinesis execution became clear by investigating the effects of CS proteins withdrawal on the behavior of a panoply of proteins playing crucial roles in

the last steps of cell division. Of these, only PRC1 appeared delocalized in CS mutant cells with respect to rescued ones. PRC1 is a microtubule (MT) bundling protein essential for the architecture of the central spindle and the formation of the midbody (Mollinari et al., 2002). Actually, both its suppression and its overexpression dramatically affected the success of the cytokinesis. Indeed, it has been suggested that PRC1 suppression disturbs the early event of cell division and cytokinesis because of the not proper bundling of microtubules. In contrast, its overexpression would presumably affect the severing of microtubules which is required for the final step of the abscission. It is therefore evident the need for a tight and fine tuning of PRC1 amount during the different steps of cell division. Previous studies revealed a role of ubiquitylation and proteasomal activity in regulating cytokinesis (Pines and Lindon, 2005). Along this line, ubiquitin-activating enzymes and the proteasome have been found concentrated on midbodies (Grenfell et al., 1994 and Wojcik et al., 1995), and both proteolytic and non-proteolytic functions of ubiquitin seem to play a role. Our results strongly support this model. Indeed, in normal cells, at the final step of cytokinesis, PRC1 was rapidly removed from the midbody, via ubiquitylation and degradation (Fig. 7E-G). In contrast, in CSA and CSB deficient cells, PRC1 remained associated to the midbody and accumulated all along one arm of the intercellular bridges (Figs. 7F-G, and Fig. 6B, panels a-d and i-l). The normal disappearance of PRC1 from the midbody followed the engagement of the ubiquitin ligase complex, thus implying that the ubiquitiylation of PRC1 is required for its release from the midbody. The engagement of CSA and CSB proteins at the midbody and the “stalling” of PRC1 at the midbody, when either CSA or CSB were not functional, compellingly indicated that the CS proteins were involved in the removal of PRC1 from the midbody via ubiquitylation and proteasomal degradation.

Our results indicated the pleiotropic role of CS proteins in cell metabolism. We can ask why these two DNA repair proteins are also implicated in the modulation of PRC1, the gatekeeper that regulates the final step of cell division, the abscission. Interestingly, we previously demonstrated that CSA and CSB proteins also stimulates the ubiquitylation/degradation of both p53 (Latini et al., 2012) and ATF3 proteins (Epanchintsev et al., submitted). These results led us to postulate that a crosstalk there exists between caretakers (CS proteins) and gatekeepers (PRC1, p53 and ATF3) that may represent a cellular integrated surveillance strategy coupling cell growth regulation and DNA integrity signaling to protect from DNA damage accumulation. The sequestering of CS proteins to the DNA damage sites, delaying PRC1 degradation would block cell division and proliferation, and slowing down p53 and ATF3 degradation would target the cell to p53-mediated apoptosis.

The newly identified role of CS proteins may have implications for the phenotype exhibited by the patients. We showed that both CS-A and CS-B patients' derived (immortalized) fibroblasts displayed both centrosome amplification and multipolar mitotic spindles as a consequence of the cytokinesis failure-induced tetraploidization . Interestingly, it was recently demonstrated that cytokinesis failure-induced tetraploidy triggers age-related processes in subcutaneous tissues, such as the composition of cutaneous progenitor cells, adipocytes and fibroblasts, which also result in premature subcutaneous fat loss (Tanaka et al., 2015). This could underline a possible etiologic role in the dramatic cachexia displayed by CS patients.

MATERIALS AND METHODS

Cells, Culture Conditions and Antibodies

Hela cells were grown in DMEM medium supplemented with 10% Fetal Bovine Serum and 2mM L-Glutamine. SK-N-BE (2c) cells were grown in a MEM/DMEM F12 medium supplemented with 10% Fetal Bovine Serum and 2mM L-Glutamine. CS1AN-CSB-Ty1-TET and CS3BE-CSA-Ty1-TET were grown in DMEM/F10 medium supplemented with 10% Fetal Bovine Serum and 2mM L-Glutamine.This lines are inducible for the expression of functional proteins by adding Tetracycline. Cells in exponential growth phase were transduced with lentiviral shRNA particle (1 x 105 infectious units of virus - Santa Cruz Biotechnology) expressing sh-RNA targeting CSB or sh-RNA non-targeting control. Puromicin selection (2ug/ml) was performed to achieve stable gene silencing. The following antibodies were employed: anti-Aurora B (6/AIM-1) from BD Biosciences, anti-alpha-tubulin (T9026), anti-gamma-tubulin (T3559) and anti-FLAG M2 from SIGMA, anti-Ty1 from Diagenode, anti-CSB [N2C1] and anti-ERCC8 [N2C2] from GeneTex; anti-CSB (H300), anti-PLK1 (H-152), anti-Sp1 (PEP2), anti-beta actin (C4), anti-MDM2 (HDM2-323), anti-Cul4 (H-66), MKLP1 (H110), anti-PLK1 (F-8), anti-PRC1 (H-70), anti-Alix (3A9) and anti-Cep55 Ab (B-8) from Santa Cruz biotechnology.

Midbody Isolation and Extraction

Cells were enriched in telophase by treatment with nocodazole (100 ng/ml for 3 hr) followed by mitotic shake-off, nocodazole wash-out, and incubation of the collected cells for about 80 min to reach telophase stage. For time-course experiments, cells where collected also after 120, 150 and

180 min. Midbodies were isolated as described by Kuriyama (Kuriyama et al., 1984) and extracted using an extraction buffer (50 mM Tris-HCl [pH 7.4], 600 mM NaCl, 0.1% SDS, 0.5% NP40, 1 mM DTT, 5 mM EDTA) supplemented with protease- and phosphatase-inhibitor mix (Roche). TCEs from nonsynchronized interphase and telophase-enriched cells were obtained in the same manner and analyzed by WB together with midbody extracts.

Western Blot

For WB, cells were lysed for 10 min on ice in RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, 1% Triton) supplemented with protease-inhibitor mix (Roche). The whole cell extracts were centrifuged at 13000 g for 5 min and the supernatant was recovered. Protein concentration was determined by Qubit Fluorometer (ThermoFisher scientific). Proteins (70 ug) were separated on polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Bio-Rad, Hercules, CA, 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 40 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).

Immunofluorescence Studies

For immunofluorescence experiments, cells were seeded onto 35 mm glass bottom dishes (Ibidi). Cells were fixed in ice-cold methanol (gamma-tubulin, alix, PRC1 and MKLP1) or 2% formaldehyde, washed three times in phosphate buffered saline (PBS), permeabilized in 0.25% Triton X- 100 in PBS for 10 min., and then blocked in 3% bovine serum albumin (BSA) in PBS for 30 min before the required primary Abs were applied.

Live Cell Imaging

Cells were seeded in 35 mm glass bottom dishes (Ibidi), transfected whit CellLight BacMan 2.0 GFP-tubulin (ThermoFisher scientific) and incubated for 16 hours at 37°C for fluorescent protein expression; then cells were observed under a a confocal microscope system (Zeiss LSM 710). During the whole observation, cells were kept in a microscope stage incubator at 37°C and 5% CO2.

Acknowledgements

This work was supported by grants from AIRC (IG13074) and Telethon (GGP11176). Confocal analysis was performed at the Centro Grandi Apparecchiature (CGA) of the University of Tuscia.

LEGENDS

Figure 1: **CSA and CSB localize at the midbody.** (A) Confocal micrographs of HeLa and SKNBE-2c cells, immunostained for α-tubulin (green) and CSB (red) and stained for DNA with DAPI (blue). CSB localizes to the midbody during cytokinesis in 92.6 ± 2.4% s.d. of cells (n = 500) (B) Confocal micrographs of HeLa and SKNBE-2c, immunostained for α-tubulin (green), and CSA (red) and stained for DNA with DAPI (blue). CSA localizes to the midbody during cytokinesis in 78.6 ± 1.8% s.d. of cells (n = 500). (C) Western blotting showing enrichment of CSA and CSB in the midbody protein fraction of SKNBE-2c cells. MID: midbody associated protein fraction; A-TCE: total cellular extracts from asynchronous cells; T-TCE: total cellular extract from telophase enriched cells .

Figure 2: Dynamic localization of CSA and CSB during the cell cycle. (A) Schematic representation showing the distribution of Aurora B, CSA and CSB through the different stages of the cell cycle, including interphase, mitosis and cytokinesis. Confocal micrographs showing staining of (B) DNA, Aurora B and CSB or (C) DNA, aurora B and CSA in SKNBE-2c cells, during different cell division stages.

Figure 3: **CSB suppression causes multipolar mitosis and multinucleation.** (A) Western blotting demonstrating the significant decrease of CSB protein after siRNA CSB knockdown. *CSB-PGDB3 is a conserved fusion protein in which the first 5 exons of CSB are alternatively spliced to the PGBD3 transposase (Newman et al., 2008). (B) Confocal micrographs of SKNBE-2c cells stained for centrosomes (anti-γ-tubulin, green) and DNA (DAPI, blue) with (right) or without (left) siRNA CSB depletion. White arrows indicate normal methaphases, while pink arrows indicate multipolar metaphases. (C) Confocal micrographs of SKNBE-2c cells immunostained for α-tubulin (green) and DNA (DAPI, blue) after treatment with either non-specific siRNA (left) or CSB siRNA (right). (D) Graph showing the relative percentages of canonical and aberrant (multipolar) metaphases after treatment of SKNBE-2c with either non-specific siRNA (sh-K) or CSB siRNA. (E) Confocal micrographs of SKNBE-2c cells immunostained for aurora B (green) and stained for DNA (DAPI, blue). Left image depicts a normal intercellular bridge while in the right panel a long intercellular bridge is shown. (F) Graph showing the relative percentages of normal and long IBs either before or after CSB knockdown. (G) Confocal micrographs of SKNBE-2c/sh-K cells immunostained for α-tubulin (green), γ-tubulin (red) and stained for DNA (DAPI, blue) after treatment with cytokalasin B.

Figure 4: SKNBE-2c cells were transiently transfected with a plasmid encoding EGFP/α-tubulin and recorded by time-lapse phase contrast microscopy. Images at the indicated time-points (h:min) are displayed. Abscission occurred between 2:40 and 2:50 in normal cells (SKNBE-2c/sh-K) while did not occur in CSB suppressed cells (SKNBE-2c/sh-CSB) where a thin midbody (arrows) still persisted at 4:15.

Figure 5: (A) Confocal micrographs of CSIAN (CSB deficient) cells immunostained for α-tubulin (green), γ-tubulin (red) and stained for DNA (DAPI, blue), showing typical multipolar mitoses. (B) graphs showing percentages of binucleated cells, multipolar mitoses and long IBs, either before or after the expression of wildtype CSB protein. (C) Confocal micrographs of CS3BE (CSA deficient) cells immunostained for α-tubulin (green), γ-tubulin (red) and stained for DNA (DAPI, blue), showing typical multipolar mitoses. (D) Graphs showing percentages of binucleated cells, multipolar mitoses and long IBs, either before or after expression of wildtype CSA protein.

Figure 6: (A) schematic design depicting the comparison between normal late intercellular bridge (Late IB) and abnormal long intercellular bridge (Long IB). (B) Confocal micrographs of CS3BE (CSA deficient) and CSIAN (CSB deficient) cells showing delocalization of PRC1 (red) in the absence of either of CS proteins. (C) Confocal micrographs of CS3BE (CSA deficient) and CSIAN (CSB deficient) cells showing that PLK1 pattern (red) is not affected by CS proteins deprivation. Cells are also immunostained for Aurora B (green) and stained for DNA (DAPI, blue).

Figure 7: Confocal micrographs showing the localization (in red) of Cul4 (A and C), Mdm2 (B and D) and PSMD1 (H and I) at the midbody, in CS3BE (CSA deficient) and CSIAN (CSB deficient) cells, before and after stimulation of wildtype CSA or CSB proteins, respectively. Cells are also immunostained for Aurora B (green) and stained for DNA (DAPI, blue). (E) PRC1 immunoblotting from CS3BE (CSA deficient) cell total protein extracts (lane 1,2 and 5) or anti-ubiquitin immunoprecipitated fraction (lane 3 and 4), before and after expression of wildtype CSA. Time-course analysis of midbody associated proteins of CS3BE (F) and CSIAN (G) cells without or after expression of wildtype CSA or CSB proteins, respectively. MID extracts were collected at different time (1h and 20', 2h, 2h and 30' and 3h) after the release from the metaphase

Supplementary figures

Fig. S1: Confocal micrographs of MCF7 stained for α-tubulin and CSB (A) or CSB (B). CSIAN and CS3BE cells transfected with either CSB-flag (C, panels a-c) and CSA-flag (C, panels d-f) respectively, were stained with anti-flag ab. DNA was stained with DAPI (blue).

Fig. S2: Confocal micrographs of SKNBE-2c cells stained for DAPI (DNA), aurora B and CSA (panels a-b) and only CSA (panels c-d). In panels c-d deconvolution software **have been** used for restoring the 3-D information.

Fig. S3: Confocal micrographs of CS3BE and CSIAN cells stained for CEP55 (A), MKLP1 (B) and ALIX (C) either at long IBs or at late IBs before and after expression of wtCSA or wtCSB proteins. Cells are also stained for Aurora B and DAPI.

Figure 1

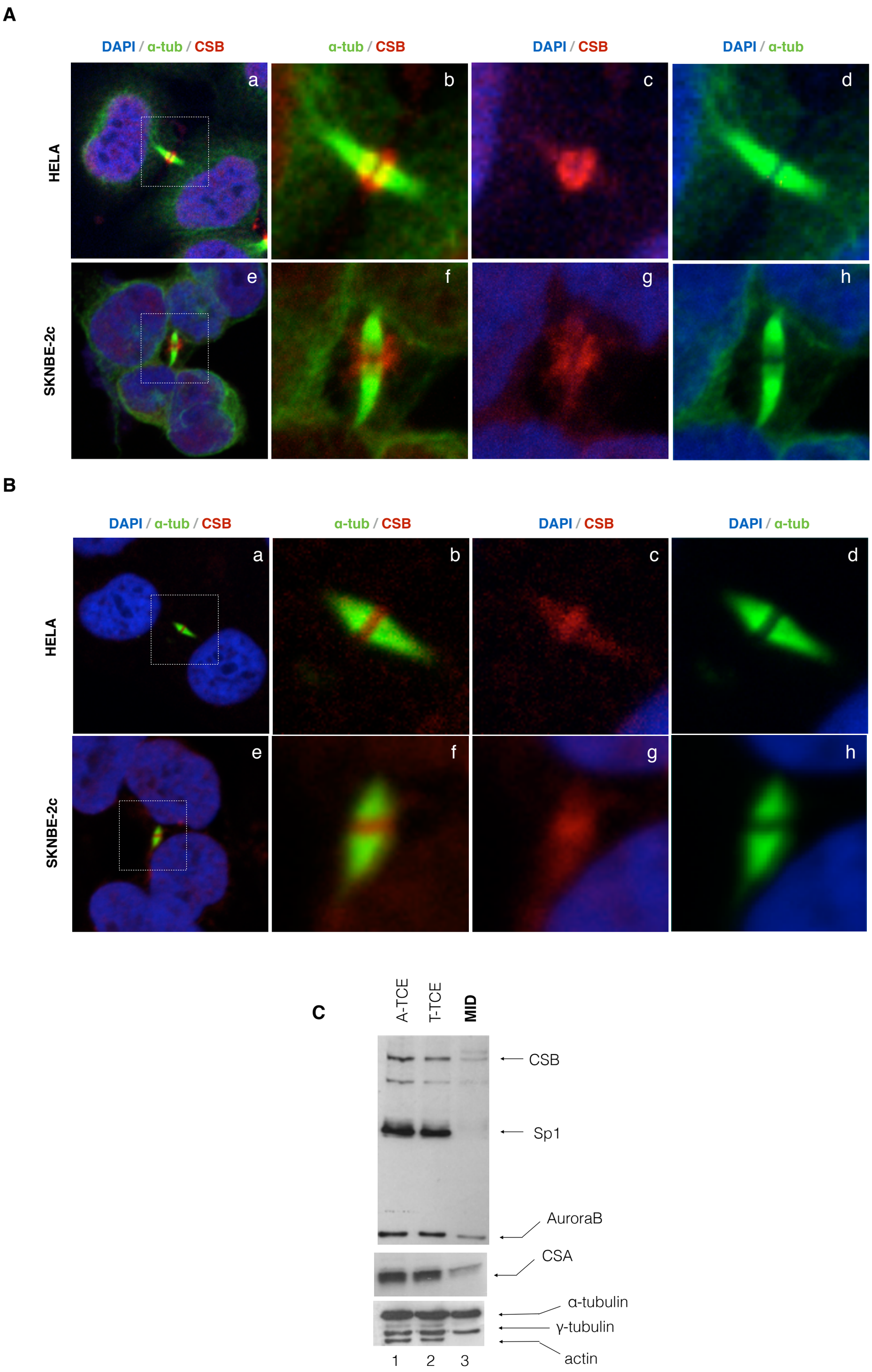

A
DAPI / α-tub / CSB
α-tub / CSB
DAPI / CSB
DAPI / α-tub
HELA
SKNBE-2c
a
b
c
d
e
f
g
h
B
DAPI / α-tub / CSB
α-tub / CSB
DAPI / CSB
DAPI / α-tub
HELA
SKNBE-2c
a
b
c
d
e
f
g
h
C
A-TCE
T-TCE
MID
CSB
Sp1
AuroraB
CSA
α-tubulin
γ-tubulin
actin
1
2
3

Figure 2

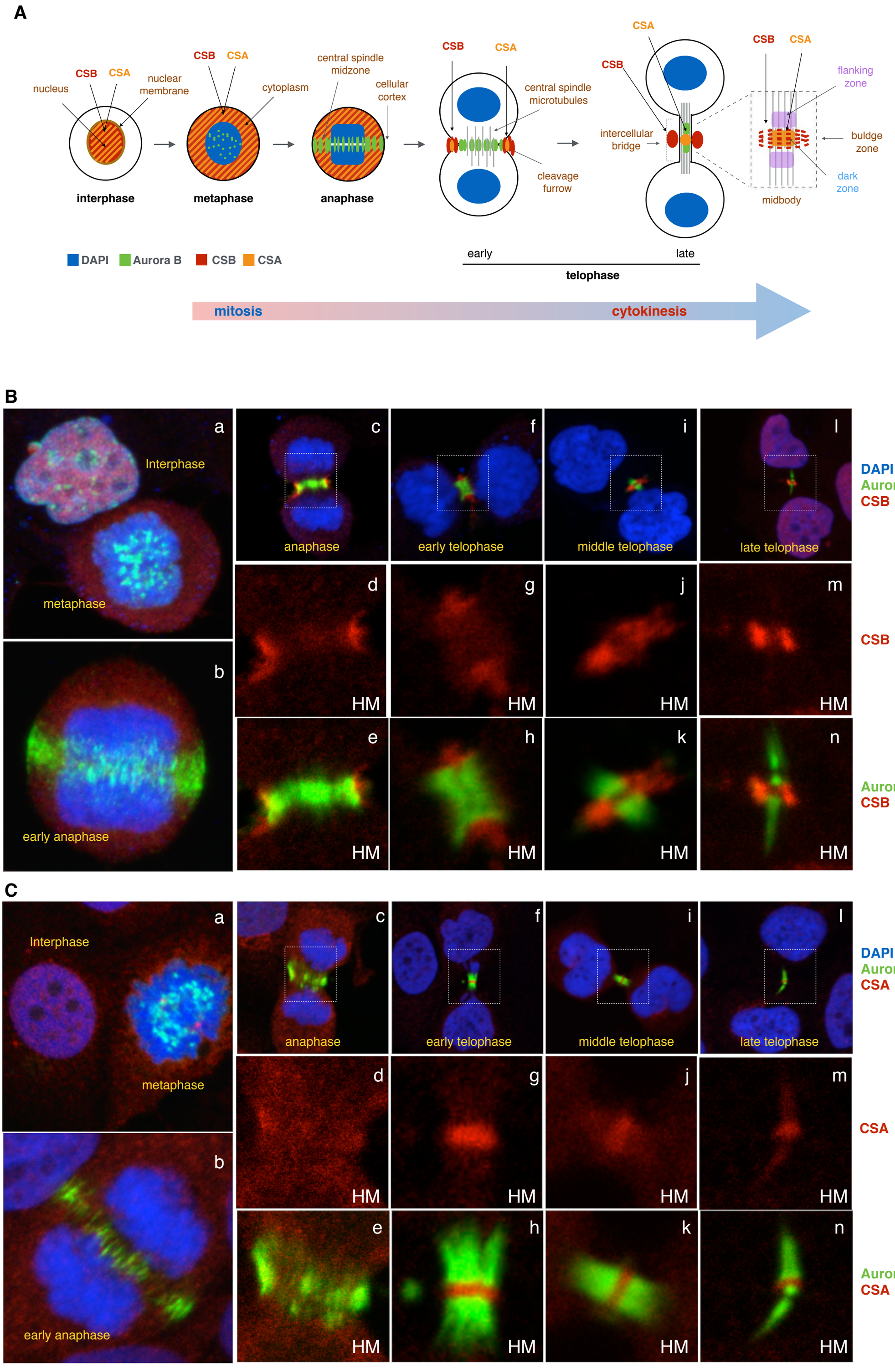
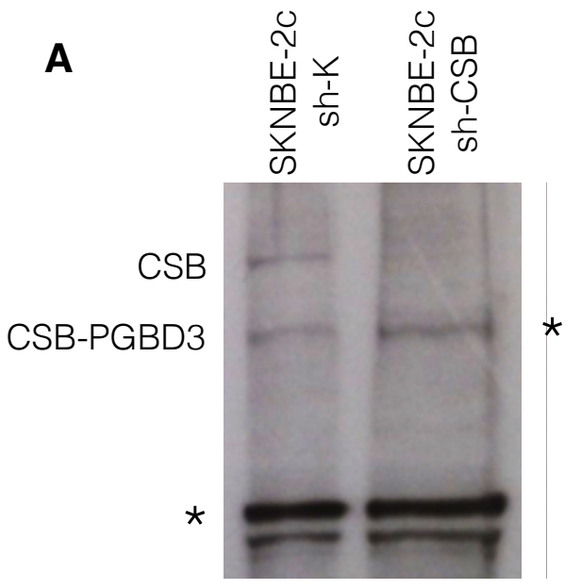
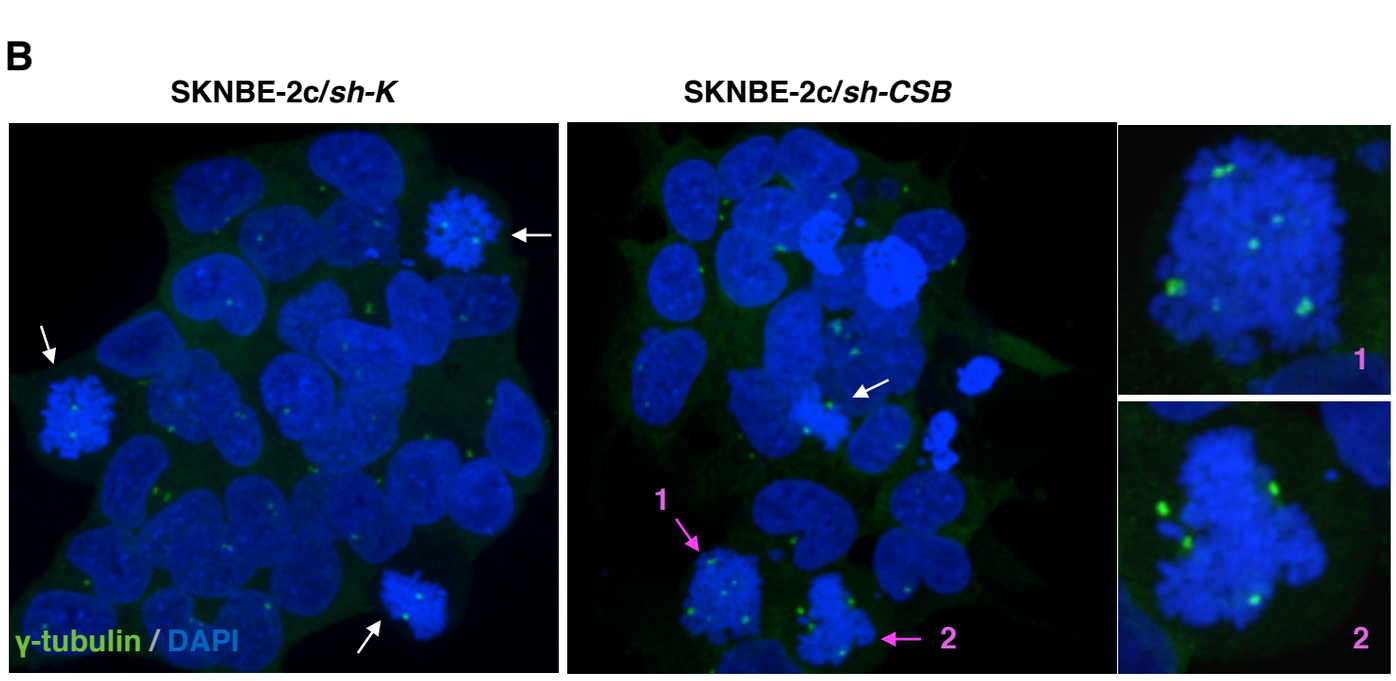
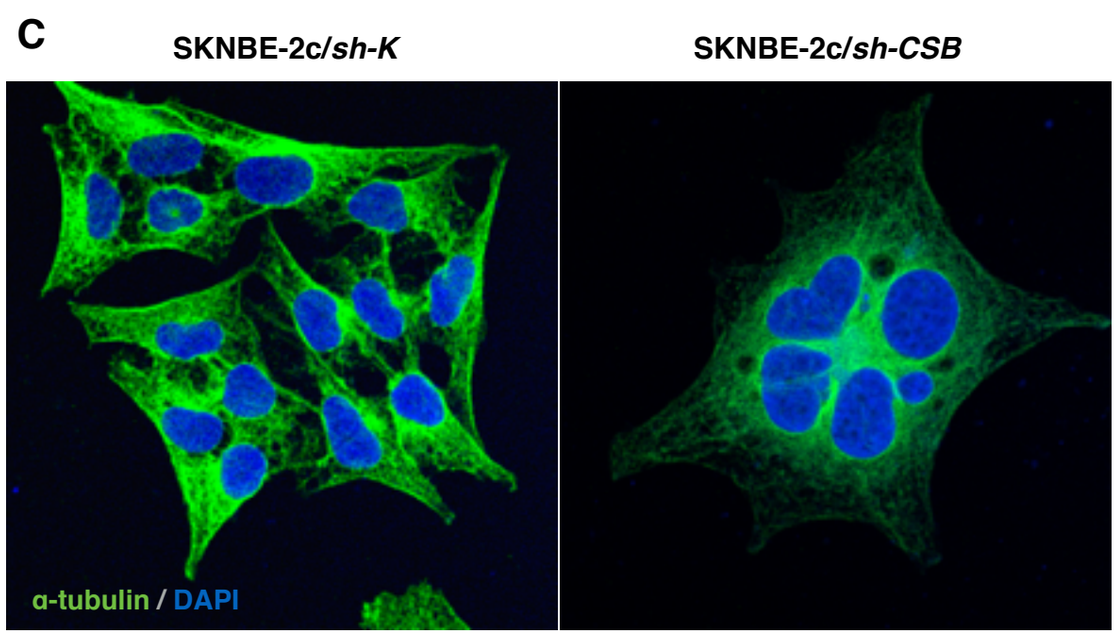
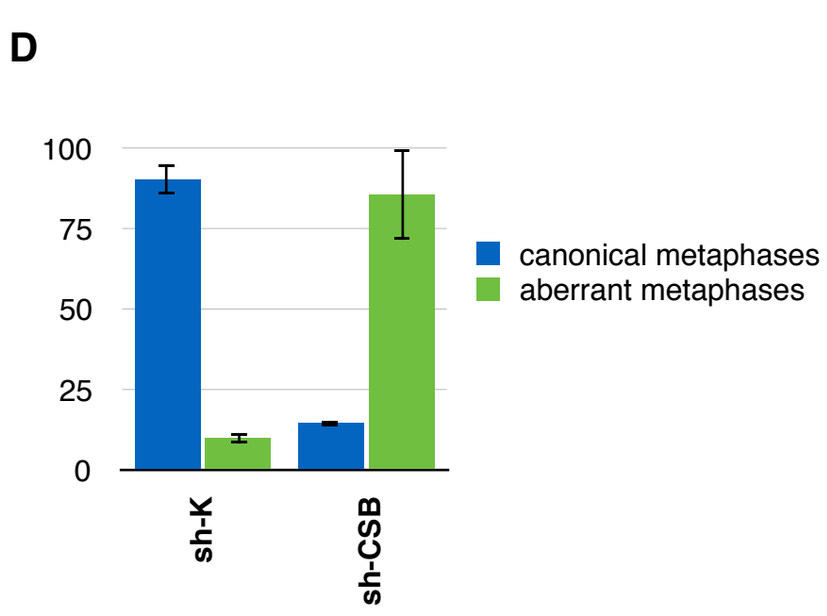

A
nucleus
CSB
CSA
nuclear membrane
interphase
cytoplasm
metaphase
central spindle midzone
cellular cortex
anaphase
central spindle microtubules
cleavage furrow
intercellular bridge
flanking zone
buldge zone
dark zone
midbody
DAPI
Aurora B
CSB
CSA
early
late
telophase
mitosis
cytokinesis
B
Interphase
metaphase
early anaphase
anaphase
early telophase
middle telophase
late telophase
HM
DAPI
Aurora B
CSB
CSB
Aurora B
CSB
C
Interphase
metaphase
early anaphase
anaphase
early telophase
middle telophase
late telophase
HM
DAPI
Aurora B
CSA
CSA
Aurora B
CSA

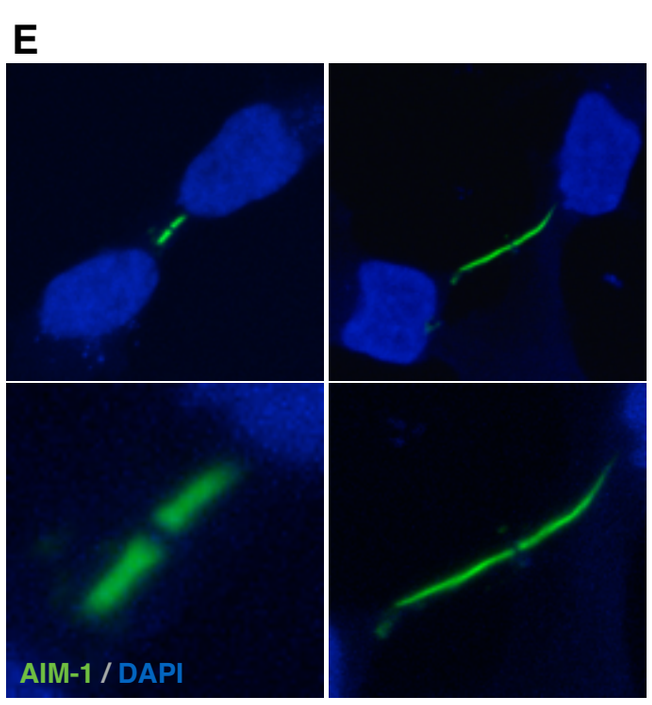
Figure 3

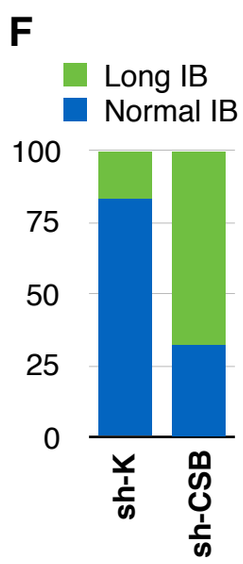

A
SKNBE-2c sh-K
SKNBE-2c sh-CSB
CSB
CSB-PGBD3
*
*


B
SKNBE-2c/sh-K
SKNBE-2c/sh-CSB
γ-tubulin / DAPI
1
2
1
2


C
SKNBE-2c/sh-K
SKNBE-2c/sh-CSB
α-tubulin / DAPI


D
100
75
50
25
0
sh-K
sh-CSB
canonical metaphases
aberrant metaphases


E
AIM-1 / DAPI
normal intercellular bridge (Normal IB)
long intercellular bridge (Long IB)


F
Long IB
Normal IB
100
75
50
25
0
sh-K
sh-CSB

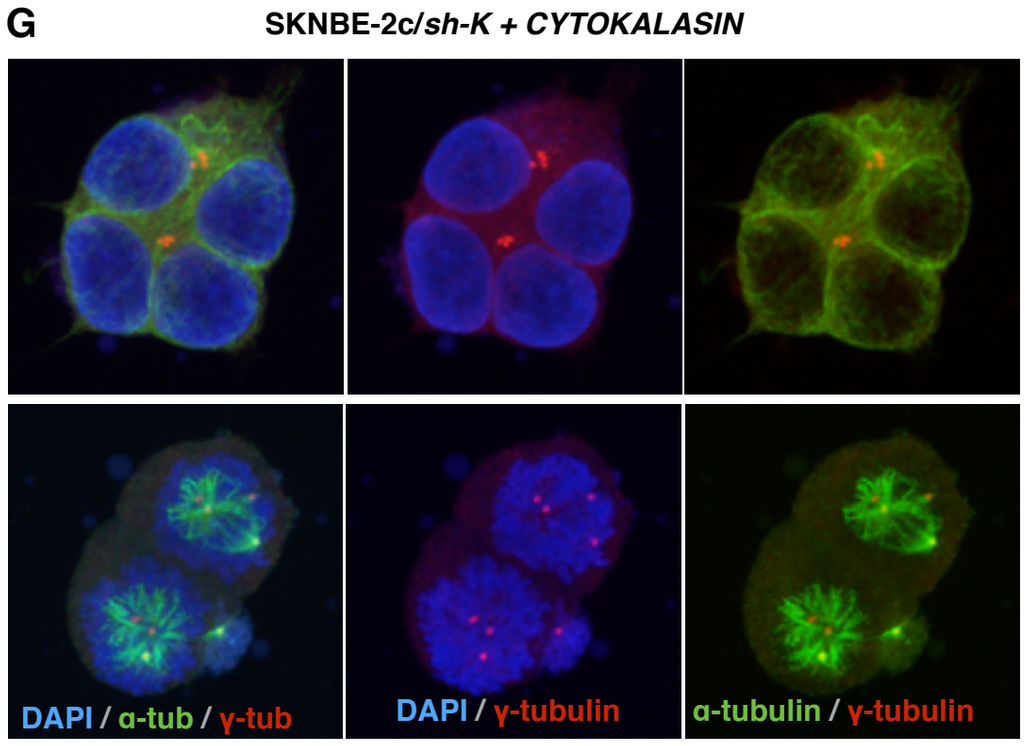

G
SKNBE-2c/sh-K + CYTOKALASIN
DAPI / α-tub / γ-tub
DAPI / γ-tubulin
α-tubulin / γ-tubulin

Figure 4

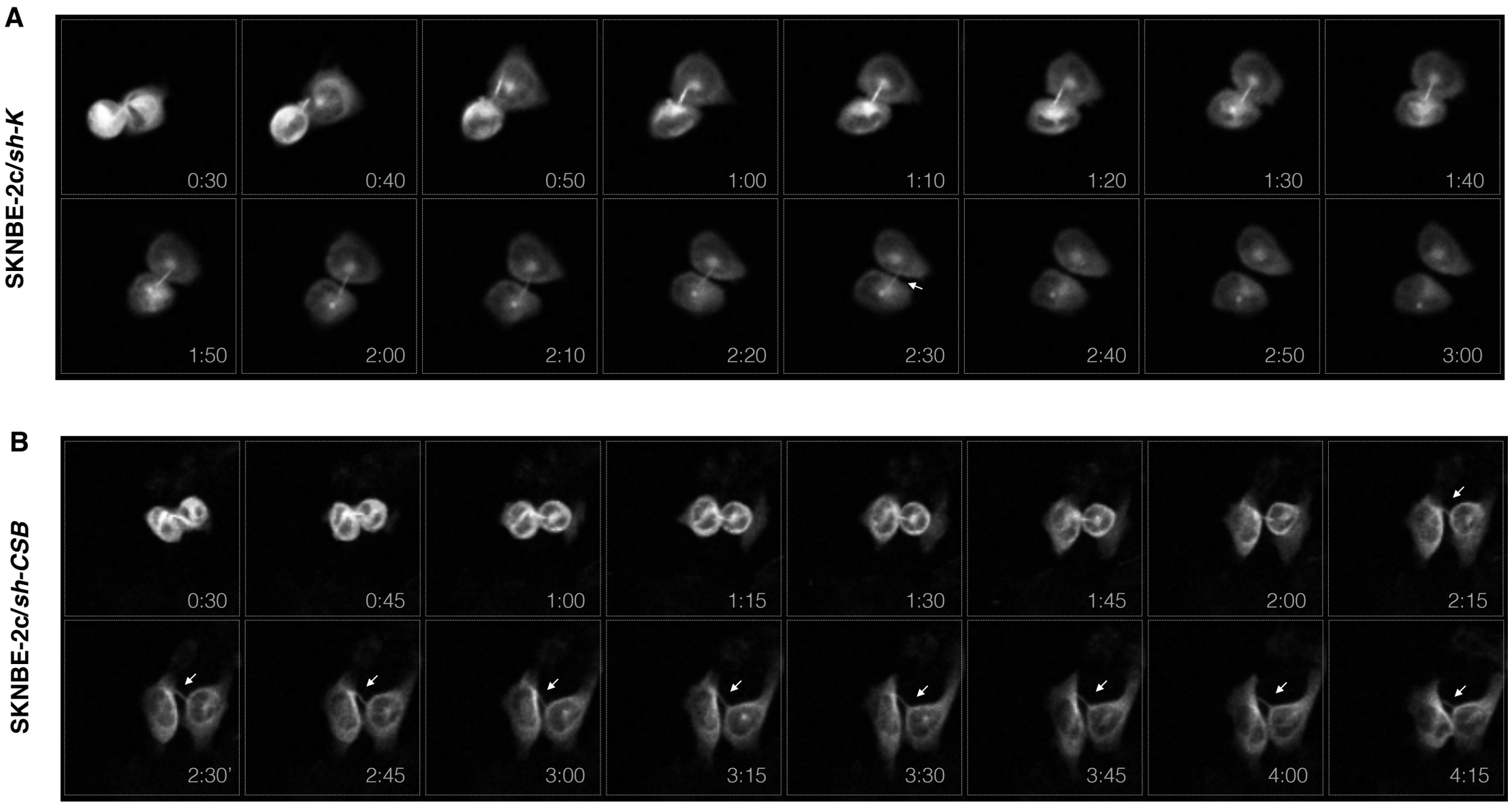

A
SKNBE-2c/sh-K
0:30
0:40
0:50
1:00
1:10
1:20
1:30
1:40
1:50
2:00
2:10
2:20
2:30
2:40
2:50
3:00
B
SKNBE-2c/sh-CSB
0:30
0:45
1:00
1:15
1:30
1:45
2:00
2:15
2:30'
2:45
3:00
3:15
3:30
3:45
4:00
4:15

Figure 5

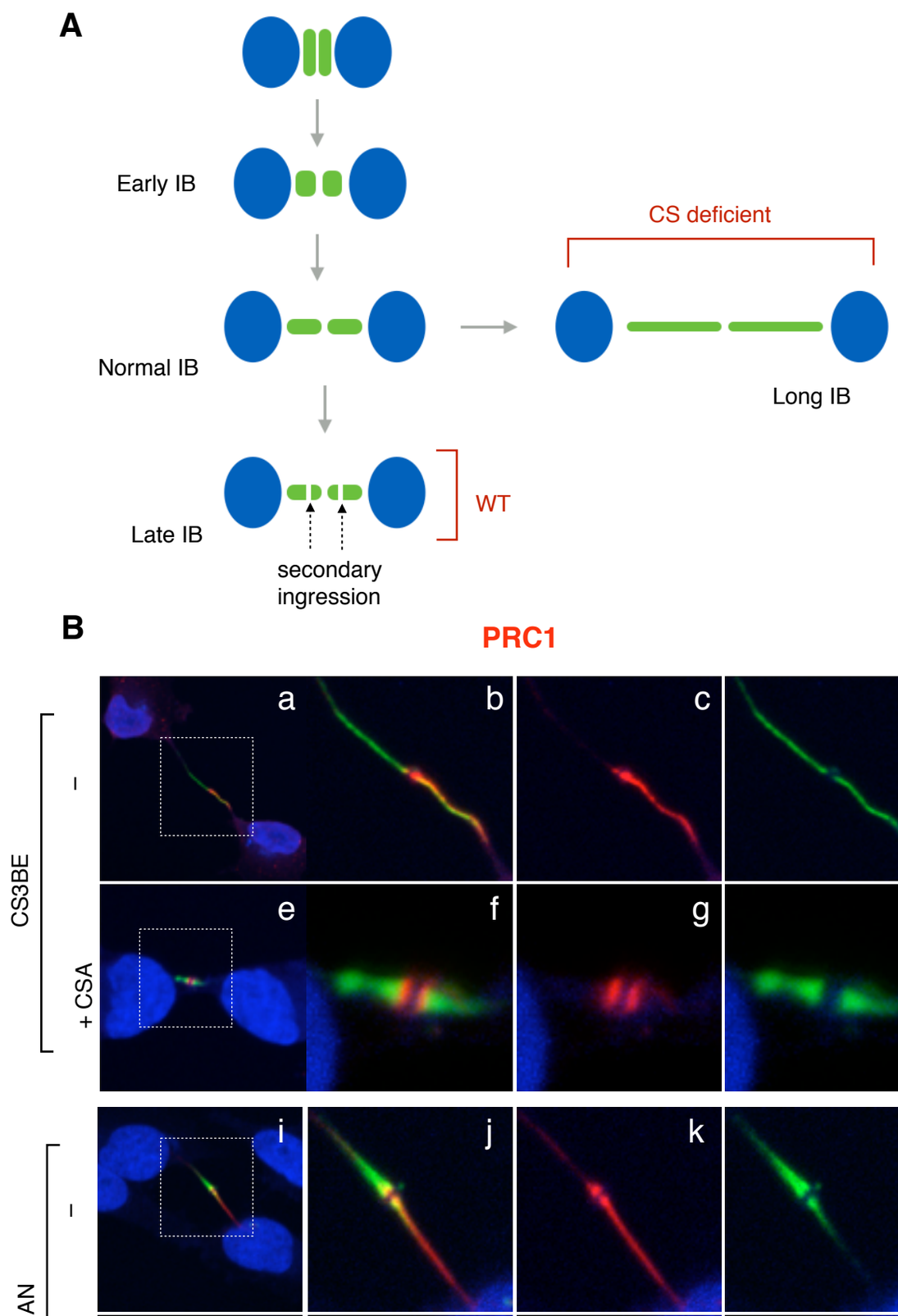
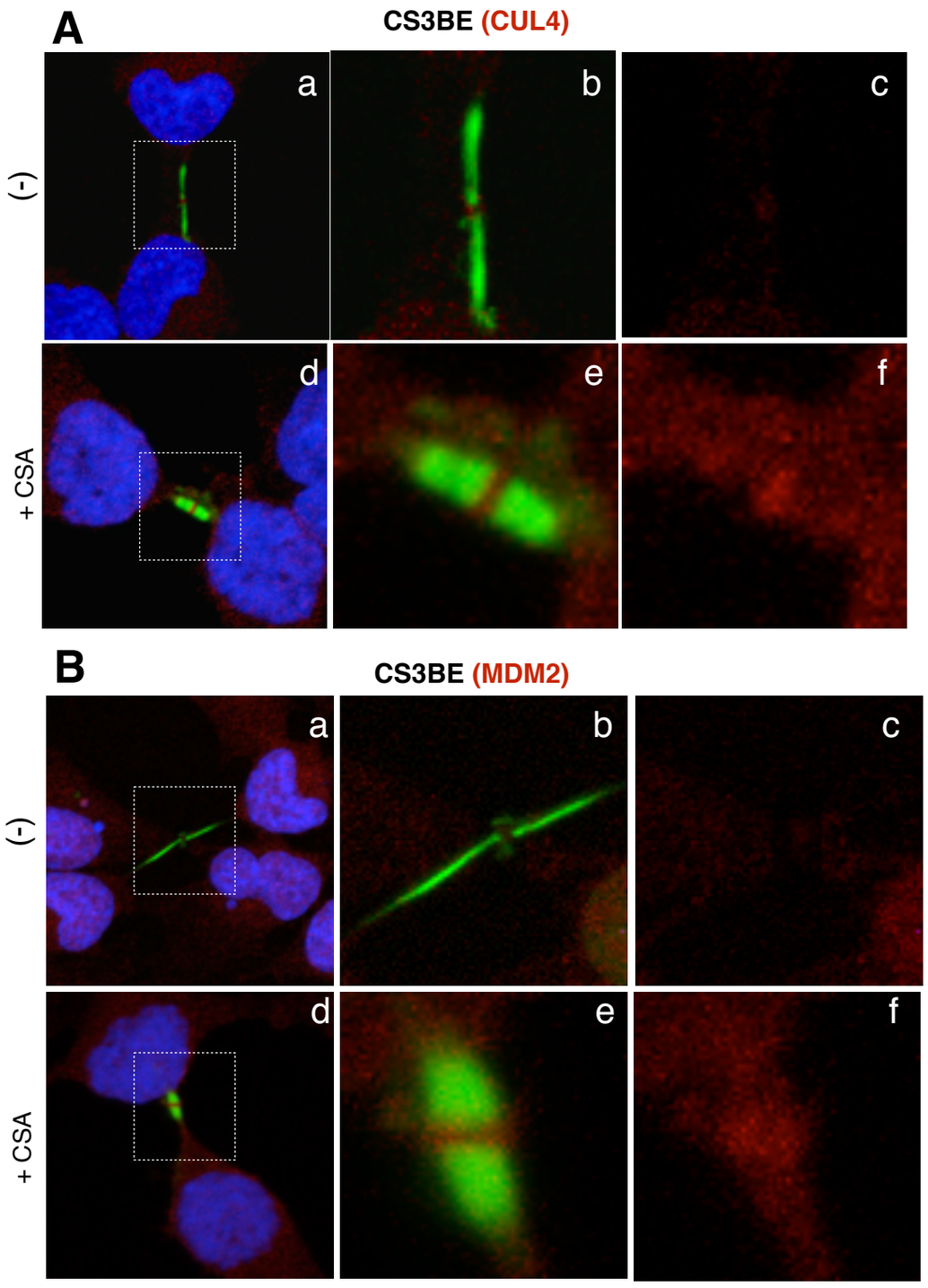
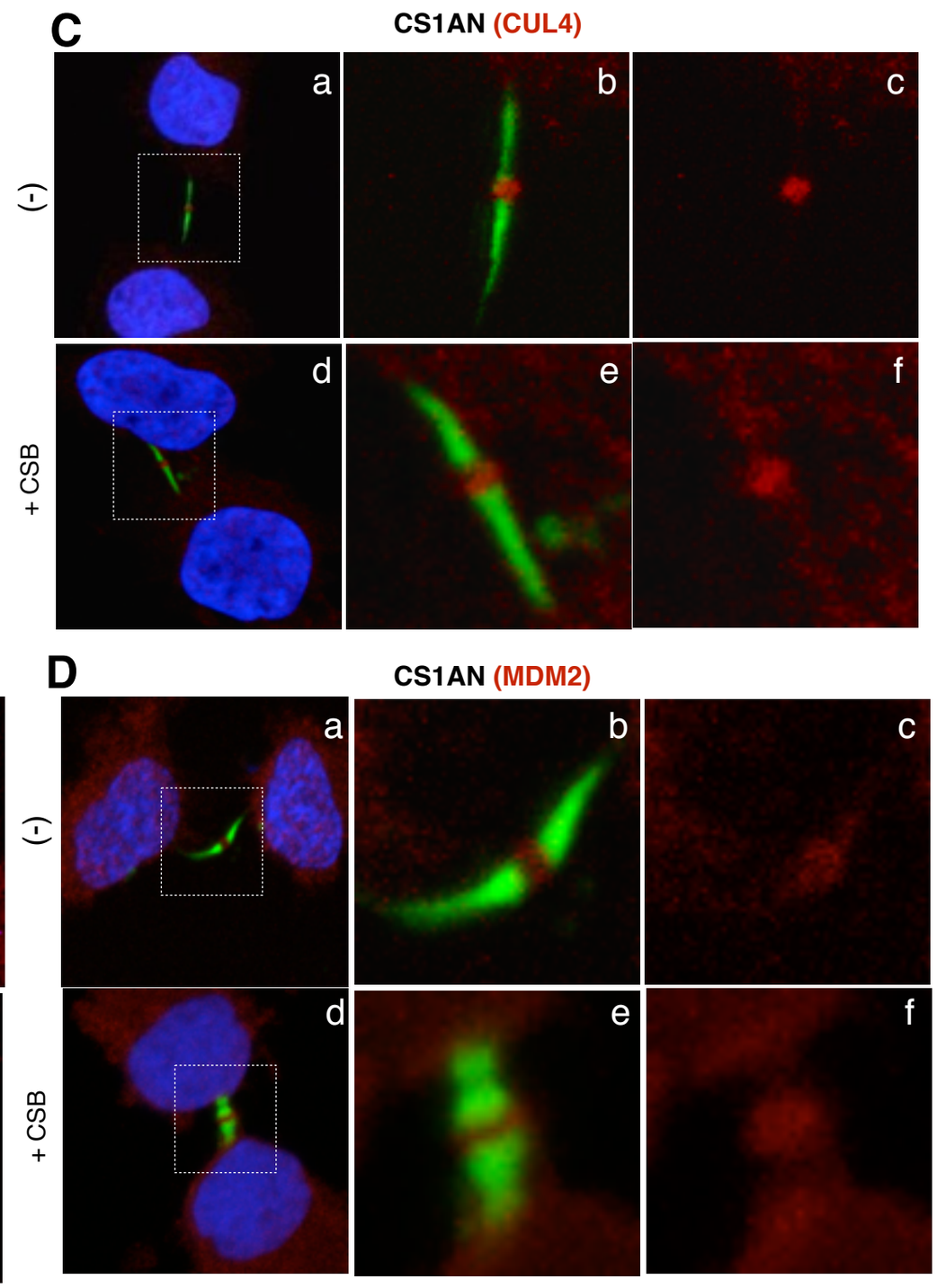

A
Early IB
Normal IB
Late IB
secondary ingression
CS deficient
Long IB
WT
B
PRC1
CS3BE
–
+ CSA
CS1AN
–
+ CSB
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
C
PLK1
CS3BE
–
+ CSA
CS1AN
–
+ CSB
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

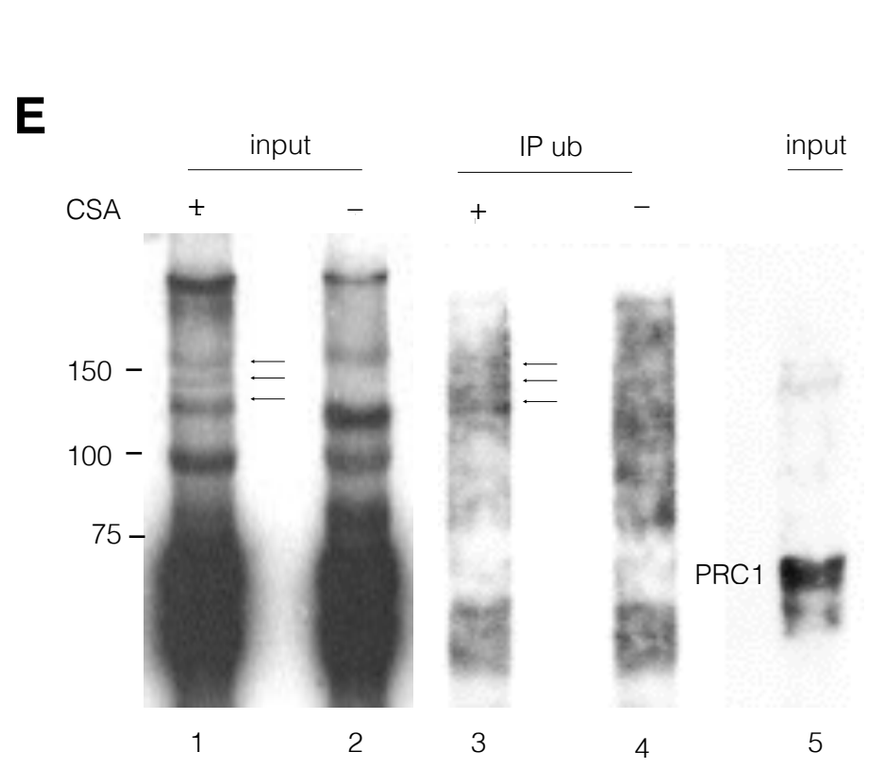
Figure 6

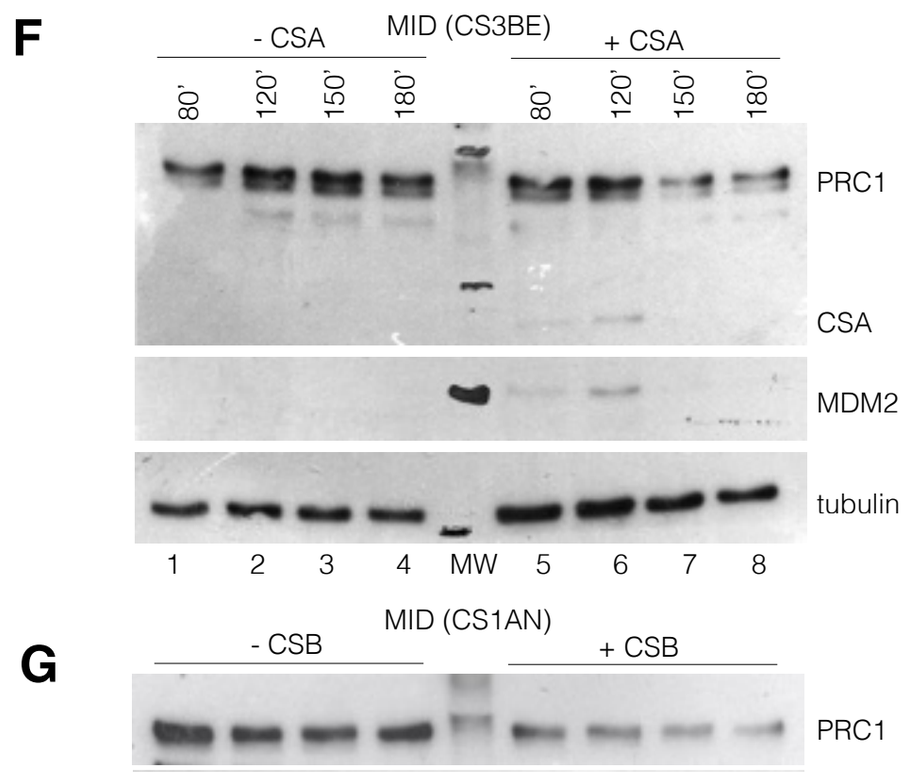

A
CS3BE (CUL4)
(-)
+ CSA
a
b
c
d
e
f

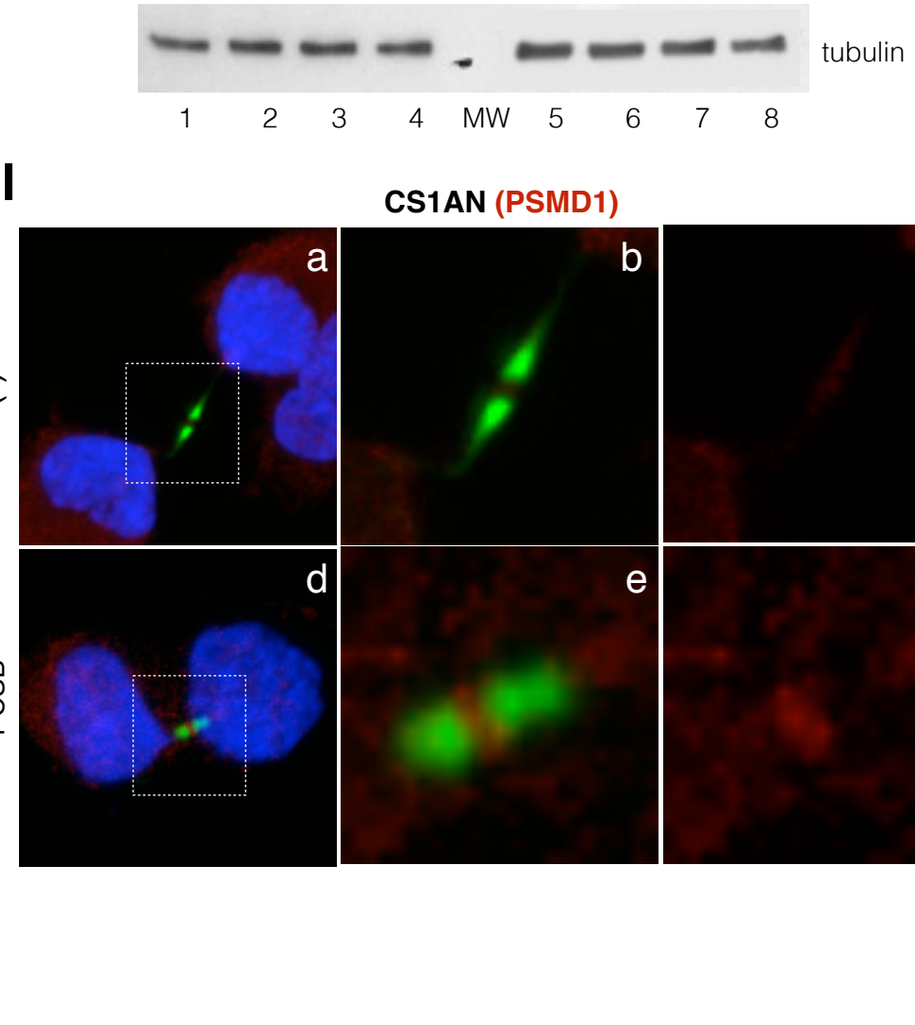

C
CS1AN (CUL4)
(-)
+ CSB
a
b
c
d
e
f

B
CS3BE (MDM2)
(-)
+ CSA
a
b
c
d
e
f

D
CS1AN (MDM2)
(-)
+ CSB
a
b
c
d
e
f


E
input
IP ub
input
CSA
+
–
+
–
150
100
75
PRC1
1
2
3
4
5


F
MID (CS3BE)
- CSA
+ CSA
80'
120'
150'
180'
80'
120'
150'
180'
PRC1
CSA
MDM2
tubulin
1
2
3
4
MW
5
6
7
8


G
MID (CS1AN)
- CSB
+ CSB
PRC1
tubulin
1
2
3
4
MW
5
6
7
8

H
CS3BE (PSMD1)
(-)
+ CSA
a
b
c
d
e
f

I
CS1AN (PSMD1)
(-)
+ CSB
a
b
c
d
e
f

Figure S1

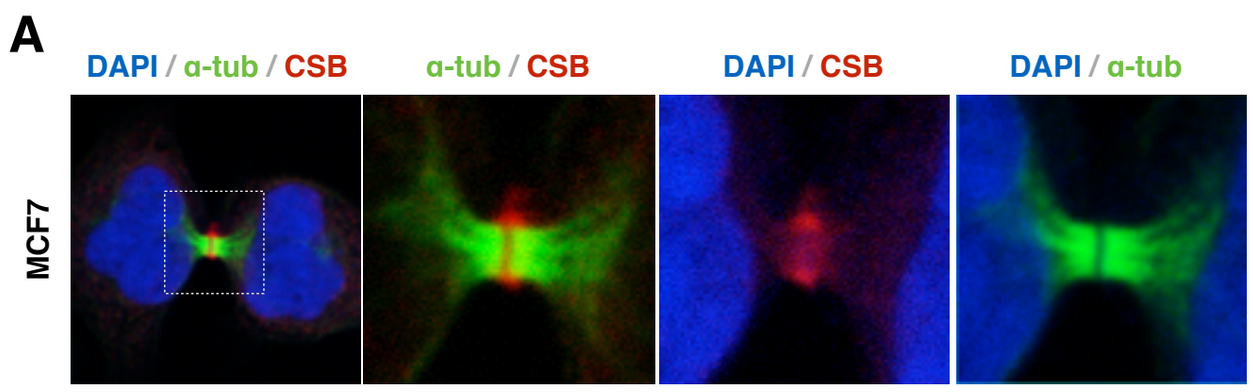


Figure S2

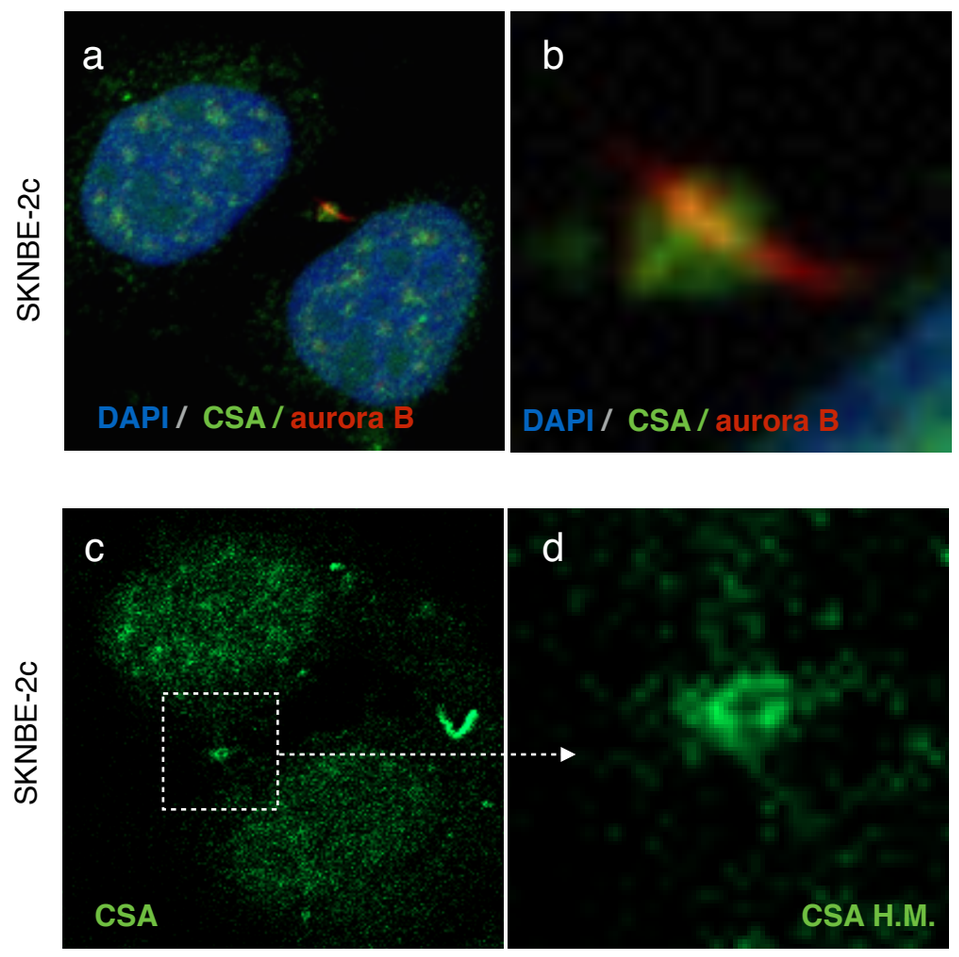


Figure S3

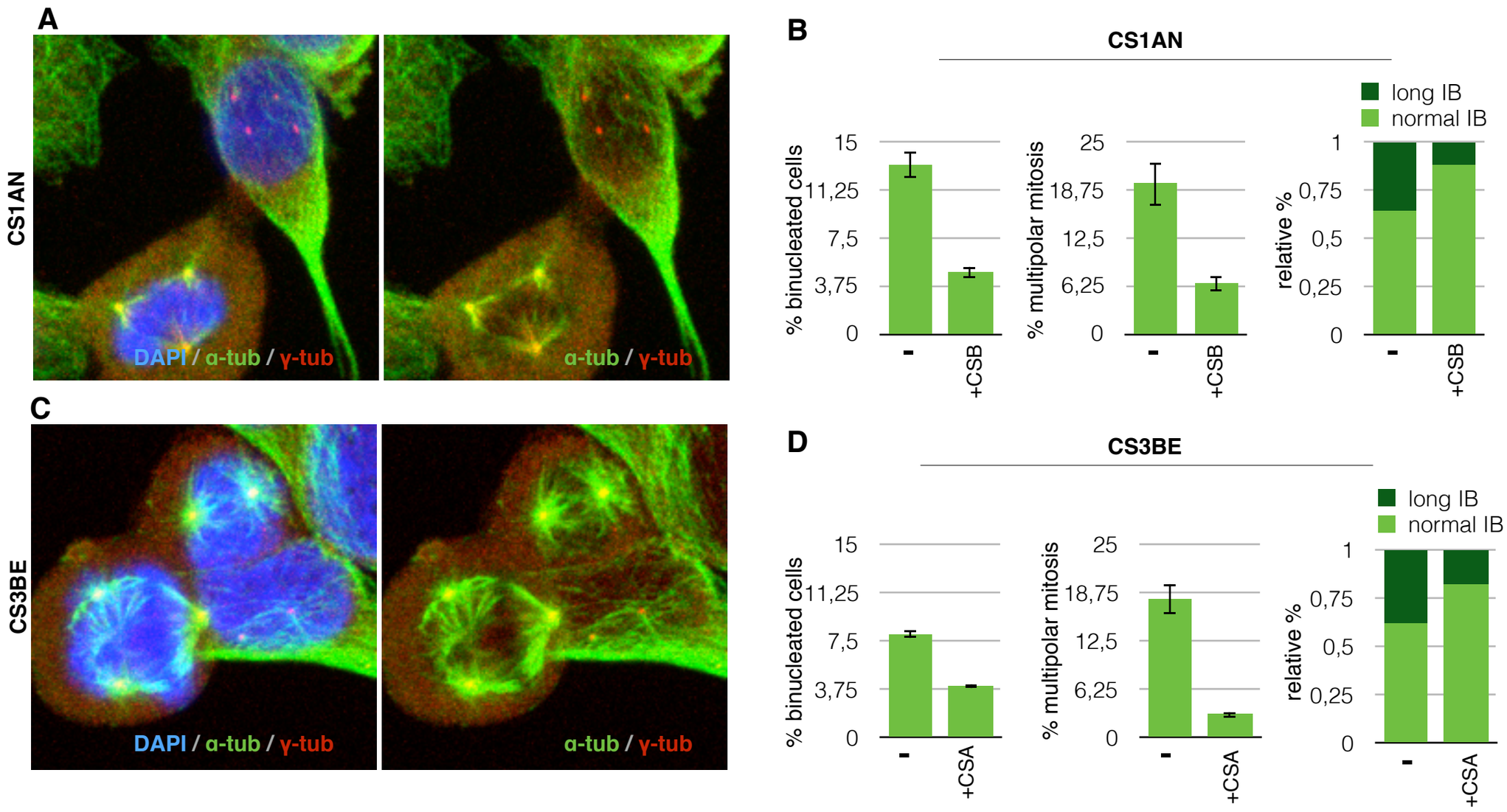
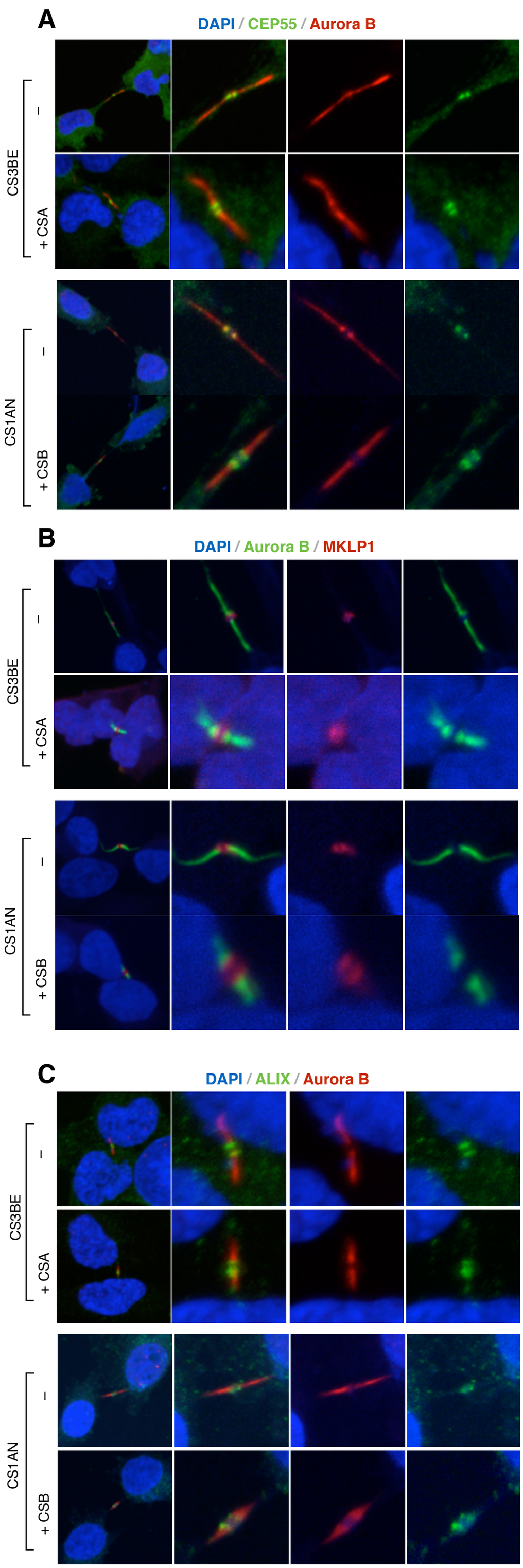

A
CS1AN
DAPI / α-tub / γ-tub
α-tub / γ-tub
C
CS3BE
DAPI / α-tub / γ-tub
α-tub / γ-tub
B
CS1AN
% binucleated cells
15
11,25
7,5
3,75
0
-
+CSB
% multipolar mitosis
25
18,75
12,5
6,25
0
-
+CSB
relative %
long IB
normal IB
1
0,75
0,5
0,25
0
-
+CSB
D
CS3BE
% binucleated cells
15
11,25
7,5
3,75
0
-
+CSA
% multipolar mitosis
25
18,75
12,5
6,25
0
-
+CSA
long IB
normal IB
relative %
1
0,75
0,5
0,25
0
-
+CSA

Figure S4


A
DAPI / CEP55 / Aurora B
CS3BE
–
+ CSA
CS1AN
–
+ CSB
B
DAPI / Aurora B / MKLP1
CS3BE
–
+ CSA
CS1AN
–
+ CSB
C
DAPI / ALIX / Aurora B
CS3BE
–
+ CSA
CS1AN
–
+ CSB

Conclusions

During my PhD research activity I have investigated about CS proteins role in neural differentiation and cytokinesis control, thus demonstrating that they not only participate in DNA repair and / or adjust the trigger of apoptosis but also regulate cell fate decision by taking part in the last step of mitosis, cytokinesis, or cell differentiation.

In the first work, in fact, we have demonstrated 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* (*Microtubule-associated Protein 2*) accompanied by impaired cell polarization and neuritogenesis, thus explaining the molecular basis of at least some of the neurological symptoms reported in CS patients.

Rather, in my second work, I've showed a new and previously unpredicted extranuclear role for both CSA and CSB protein in the abscission control, through the ubiquitination and degradation of *PRC1* (*Protein Regulator of Cytokinesis 1*) at the midbody.

Cellular polarization and cytoskeletal modifications are both involved in cellular division and differentiation; in absence of CSB and CSA we have found an enlarged cell shape, with a ratio nucleus/cytoplasm in favor of this one, which is typical of senescent cells. CSA and CSB regulate the ubiquitination of the microtubule binding protein PRC1 during cytokinesis, while during neural differentiation CSB transcriptionally regulates the expression of another microtubule binding protein, MAP2. So it seems that CSA and CSB, rather considered merely nuclear proteins, have an active role in the regulation of cytoskeletal related proteins, acting directly or at transcriptional level on them.

In addiction, both processes of cellular division and differentiation are dependent on cell-cycle control. As preliminary data, we have found that CSA protein localizes at the centrosomes during *prometaphase*. Thus, we hypothesize that CSA and CSB could be involved in the regulation of mitotic entry, acting as controllers in a *check-point* for genomic integrity assurance. In this contest, they may not only be involved in DNA repair, but also in the correct segregation of genetic material during cellular division.

These findings open a new scenario in the understanding of Cockayne syndrome etiopathology and of CS proteins role in cell physiology, assigning them a key role in cell fate decisions.

References

Weidenheim KM, Dickson DW, Rapin I. Neuropathology of Cockayne syndrome: evidence for impaired development, premature aging, and neurodegeneration. Mech Ageing Dev 2009; 130: 619–636.

Laugel V, Dalloz C, Durand M, Sauvanaud F, Kristensen U, Vincent MC et al. Mutation update for the CSB/ERCC6 and CSA/ERCC8 genes involved in Cockayne syndrome. Hum Mutat 2010; 31: 113–126.

Natale V. A comprehensive description of the severity groups in Cockayne syndrome. Am J Med Genet 2011; 155A: 1081–1095.

Henning KA, Li L, Iyer N, McDaniel LD, Reagan MS, Legerski R et al. The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. Cell 1995; 82: 555–564.

Troelstra C, van Gool A, de Wit J, Vermeulen W, Bootsma D, Hoeijmakers JH. ERCC6, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. Cell 1992; 71: 939–953.

Lehmann AR. Three complementation groups in Cockayne syndrome. Mutat Res 1982; 106: 347–356.

Venema J, Mullenders LH, Natarajan AT, van Zeeland AA, Mayne LV. The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. Proc Natl Acad Sci USA 1990; 87: 4707–4711.

Licht CL, Stevnsner T, Bohr VA. Cockayne syndrome group B cellular and biochemical functions. Am J Hum Genet 2003; 73: 1217–1239.

Laine JP, Egly JM. When transcription and repair meet: a complex system. Trends Genet 2006; 22: 430–436.

Sarasin A, Stary A. New insights for understanding the transcription-coupled repair pathway. DNA Repair (Amst) 2007; 6: 265–269.

MayneLV, LehmannAR. Failure of RNA synthesist or recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum. Cancer Res 1982; 42: 1473–1478.

Balajee AS, Proietti-De-Santis L, Brosh RM, Selzer R, Bohr VA. Role of the ATPase domain of the Cockayne syndrome group B protein in UV induced apoptosis. Oncogene 2000; 19: 477–489.

Aamann MD, Muftuoglu M, Bohr VA, Stevnsner T. Multiple interaction partners for Cockayne syndrome proteins: implications for genome and transcriptome maintenance. Mech Ageing Dev 2013; 134: 212–224.

Caputo M, Frontini M, Velez-Cruz R, Nicolai S, Prantera G, Proietti-De-Santis L. The CSB repair factor is overexpressed in cancer cells, increased apoptosi resistence, and promotes tumor growth. DNA Repair 2013; 12: 293–299.

Brooks PJ. Blinded by the UV light: how the focus on transcription-coupled NER has distracted from understanding the mechanisms of Cockayne syndrome neurologic disease. DNA Repair 2013; 12: 656–671.

Balajee AS, May A, Dianov GL, Friedberg EC, Bohr VA. Reduced RNA polymerase II transcription in intact and permeabilized Cockayne syndrome group B cells. Proc Natl Acad Sci USA 1997; 94: 4306–4311.

Selby CP, Sancar A. Cockayne syndrome group B protein enhances elongation by RNA polymerase II. Proc Natl Acad Sci USA 1997; 94: 11205–11209.

Tantin D, Kansal A, Carey M. Recruitment of the putative transcription-repair coupling factor CSB/ERCC6 to RNA polymerase II elongation complexes. Mol Cell Biol 1997; 17: 6803–6814.

van Gool AJ, van der Horst GT, Citterio E, Hoeijmakers JH. Cockayne syndrome: defective repair of transcription? EMBO J 1997; 16: 4155–4162.

Bradsher J, Auriol J, Proietti de Santis L, Iben S, Vonesch JL, Grummt I et al. CSB is a component of RNA pol I transcription. Mol Cell 2002; 10: 819–829.

Yuan X, Feng W, Imhof A, Grummt I, Zhou Y. Activation of RNA polymerase I transcription by cockayne syndrome group B protein and histone methyltransferase G9a. Mol Cell 2007; 27: 585–595.

Brooks PJ, Cheng TF, Cooper L. Do all of the neurologic diseases in patients with DNA repair gene mutations result from the accumulation of DNA damage? DNA Repair 2008; 7: 834–848.

Proietti-De-Santis L, Drane P, Egly JM. Cockayne syndrome B protein regulates the transcriptional program after UV irradiation. EMBO J 2006; 25: 1915–1923.

Filippi S, Latini P, Frontini M, Palitti F, Egly JM, Proietti-De-Santis L. CSB protein is (a direct target of HIF-1 and) a critical mediator of the hypoxic response. EMBO J 2008; 27: 2545–2556.

Frontini M, Proietti-De-Santis L. Cockayne syndrome B protein (CSB): linking p53, HIF-1 and p300 to robustness, lifespan, cancer and cell fate decisions. Cell Cycle 2009; 8: 693–696.

Newman JC, Bailey AD, Fan HY, Pavelitz T, Weiner AM. An abundant evolutionarily conserved CSB-PiggyBac fusion protein expressed in Cockayne syndrome. PLoS Genet 2008; 4: e1000031.

Velez-Cruz R, Egly JM. Cockayne syndrome group B (CSB) protein: at the crossroads of transcriptional networks. Mech Ageing Dev 2013; 134: 234–242.

Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C et al. Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 2007; 315: 1243–1249.

Sanai N, Berger MS, Garcia-Verdugo JM, Alvarez-Buylla A. Comment on 'Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension'. Science 2007; 318: 393.

Tau GZ, Peterson BS. Normal development of brain circuits. Neuropsychopharmacology 2010; 35: 147–168.

Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai HH, Wong M et al. Corridors of migrating neurons in the human brain and their decline during infancy. Nature 2011; 478: 382–386.

Bonfanti L, Peretto P. Adult neurogenesis in mammals—a theme with many variations. Eur
J Neurosci 2011; 34: 930–950.

Spreen O, Risser AH, Edgell D. Developmental Neuropsychology. Oxford University press:
New York, 1995.

Cunningham CC, Leclerc N, Flanagan LA, Lu M, Janmey PA, Losik KS. Microtubule-associated protein 2c reorganizes both microtubules and microfilaments into distinct cytological structures in an actin-binding protein-280-deficient melanoma cell line. J Cell Biol 1997; 136: 845–857.

Tantin D. RNA polymerase II elongation complexes containing the Cockayne syndrome group B protein interact with a molecular complex containing the transcription factor IIH components xeroderma pigmentosum B and p62. J Biol Chem 1998; 273: 27794–27799.

Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Se´raphin B. A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol 1999; 17: 1030–1032.

Rumpf C, Cipak L, Dudas A, Benko Z, Pozgajova M, Riedel CG et al. Casein kinase 1 is required for efficient removal of Rec8 during meiosis I. Cell Cycle 2010; 9: 2657–2662.

Rumpf C, Cipak L, Schleiffer A, Pidoux A, Mechtler K, Tolic´-Nørrelykke IM et al. Laser microsurgery provides evidence for merotelic kinetochore attachments in fission yeast cells lacking Pcs1 or Clr4. Cell Cycle 2010; 9: 3997–4004.

Cipak L, Spirek M, Novatchkova M, Chen Z, Rumpf C, Lugmayr W et al. An improved strategy for tandem affinity purification-tagging of Schizosaccharomyces pombe genes. Proteomics 2009; 9: 4825–4828.

Gregan J, Riedel CG, Petronczki M, Cipak L, Rumpf C, Poser I et al. Tandem affinity purification of functional TAP-tagged proteins from human cells. Nat Protoc 2007; 2: 1145–1151.

Ho L, Crabtree GR. Chromatin remodelling during development. Nature 2010; 463: 474–484.

Hong EJ, West AE, Greenberg ME. Transcriptional control of cognitive development. Curr Opin Neurobiol 2005; 15: 21–28.

Hsieh J, Gage FH. Chromatin remodeling in neural development and plasticity. Curr Opin Cell Biol 2005; 17: 664–671.

Goldberg AD, Allis CD, Bernstein E. Epigenetics: a landscape takes shape. Cell 2007; 128: 635–638.

Wang W, Xue Y, Zhou S, Kuo A, Cairns BR, Crabtree GR. Diversity and specialization of mammalian SWI/SNF complexes. Genes Dev 1996; 10: 2117–2130.

Wu JI, Lessard J, Olave IA, Qiu Z, Ghosh A, Graef IA et al. Regulation of dendritic development by neuron-specific chromatin remodeling complexes. Neuron 2007; 56: 94–108.

Chatterjee S, Mizar P, Cassel R, Neidl R, Selvi BR, Mohankrishna DV et al. A novel activator of CBP/p300 acetyltransferases promotes neurogenesis and extends memory duration in adult mice. J Neurosci 2013; 33: 10698–10712.

Laposa RR, Huang EJ, Cleaver JE. Increased apoptosis, p53 up-regulation, and cerebellar neuronal degeneration in repair-deficient Cockayne syndrome mice. Proc Natl Acad Sci USA 2007; 104: 1389–1394.

Brace LE, Vose SC, Vargas DF, Zhao S, Wang XP, Mitchell JR. Lifespan extension by dietary intervention in a mouse model of Cockayne syndrome uncouples early postnatal development from segmental progeria. Aging Cell 2013; 12: 1144–1147.

Sacco R, Tamblyn L, Rajakulendran N, Bralha FN, Tropepe V, Laposa RR. Cockayne syndrome b maintains neural precursor function. DNA Repair (Amst) 2013; 12: 110–120. 51. Walton RM. Postnatal neurogenesis: of mice, men, and macaques. Vet Pathol 2012; 49: 155–165.

Yang Z, Ming GL, Song H. Postnatal neurogenesis in the human forebrain: from two migratory streams to dribbles. Cell Stem Cell 2011; 9: 385–386.

Feliciano DM, Bordey A. Newborn cortical neurons: only for neonates? Trends Neurosci 2013; 36: 51–61.

Robbins JH, Brumback RA, Mendiones M, Barrett SF, Carl JR, Cho S et al. Neurological disease in xeroderma pigmentosum. Documentation of a late onset type of the juvenile onset form. Brain 1991; 114: 1335–1361.

Przedborski S, Vila M, Jackson-Lewis V. Neurodegeneration: what is it and where are we? J Clin Invest 2003; 111: 3–10.

Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol 2006; 91: 807–819.57. Aamann MD, Sorensen MM, Hvitby C, Berquist BR, Muftuoglu M, Tian J et al.

Cockayne syndrome group B protein promotes mitochondrial DNA stability by supporting the DNA repair association with the mitochondrial membrane. FASEB J 2010; 24: 2334–2346.

Scheibye-Knudsen M, Ramamoorthy M, Sykora P, Maynard S, Lin PC, Minor RK et al. Cockayne syndrome group B protein prevents the accumulation of damaged mitochondria by promoting mitochondrial autophagy. J Exp Med 2012; 209: 855–869.

Berquist BR, Canugovi C, Sykora P, Wilson DM, Bohr VA. Human Cockayne syndrome B protein reciprocally communicates with mitochondrial proteins and promotes transcriptional elongation. Nucleic Acids Res 2012; 40: 8392–8405.

Hu CK, Coughlin M, and Mitchison TJ. (2012) Midbody assembly and its regulation during cytokinesis. Mol. Biol. Cell 23:1024–1034.

Neef R, Gruneberg U, Kopajtich R et al. Choice of Plk1 docking partners during mitosis and cytokinesis is controlled by the activation state of Cdk1. Nature Cell Biol 2007; 9:436-U132.

Megan Fabbro, Bin-Bing Zhou, Mikiko Takahashi, Boris Sarcevic, Preeti Lal, Mark E. Graham, Brian G. Gabrielli, Phillip J. Robinson, Erich A. Nigg, Yoshitaka Ono, Kum Kum Khanna Cdk1/Erk2- and Plk1-Dependent Phosphorylation of a Centrosome Protein, Cep55, Is Required for Its Recruitment to Midbody and Cytokinesis, Dev Cell. 2005 Oct;9(4):477-88.

Zhao, W., Seki, A., & Fang, G. (2006). Cep55, a Microtubule-bundling Protein, Associates with Centralspindlin to Control the Midbody Integrity

and Cell Abscission during Cytokinesis. Molecular Biology of the Cell, 17(9), 3881–3896. http://doi.org/10.1091/mbc.E06-01-0015

Jez G. Carlton, Monica Agromayor, and Juan Martin-Serrano, Differential requirements for Alix and ESCRT-III in cytokinesis and HIV-1 release. vol. 105 no. 30 Jez G. Carlton, 10541–10546, doi: 10.1073/pnas.0802008105

Ruchaud, S., Carmena, M., and Earnshaw, W. C. (2007). Chromosomal passengers: conducting cell division. Nature reviews molecular cell biology 8, 798–812.

Skop AR, Liu H, Yates J et al. Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms. Science 2004; 305:61-66.

Echard A, Phosphoinositides and cytokinesis: the "PIP" of the iceberg, Cytoskeleton (Hoboken). 2012 Nov;69(11):893-912. doi: 10.1002/cm. 21067. Epub 2012 Sep 25.

Guizetti J, Schermelleh L, Mäntler J, Maar S, Poser I, Leonhardt H, Müller-Reichert T, Gerlich DW Cortical constriction during abscission involves helices of ESCRT-III-dependent filaments.Science. 2011 Mar 25; 331(6024):1616-20.

Jinghe Liu,Gregory D. Fairn,Derek F. Ceccarelli,Frank Sicheri,Andrew Wilde, Cleavage Furrow Organization Requires PIP2-Mediated Recruitment of Anillin, Current Biology Volume 22, Issue 1, 10 January 2012, Pages 64–69, doi:10.1016/j.cub.2011.11.040.

Amel Kechad, Silvana Jananji, Yvonne Ruella,Gilles R.X. Hickson, Anillin Acts as a Bifunctional Linker Coordinating Midbody Ring Biogenesis during Cytokinesis, Current Biology 3 7 February 2012, Pages 197–203, doi:10.1016/j.cub.2011.11.062.

Gruneberg U1, Neef R, Li X, Chan EH, Chalamalasetty RB, Nigg EA, Barr FA. KIF14 and citron kinase act together to promote efficient cytokinesis. J Cell Biol. 2006 Jan 30;172(3):363-72. Epub 2006 Jan 23.

Echard A, Hickson GR, Foley E et al. Terminal cytokinesis events uncovered a er an RNAi screen. Curr Biol 2004; 14:1685-1693.

Piel M, Nordberg J, Euteneuer U et al. Centrosome-dependent exit of cytokinesis in animal cells. Science 2001; 291:1550-1553.
Gromley, A. et al. (2005) Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. Cell 123, 75–87

Pohl, C. and Jentsch, S. (2009) Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. Nat. Cell Biol. 11, 65–70

Schweitzer, J.K. and D'Souza-Schorey, C. (2004) Finishing the job: cytoskeletal and membrane events bring cytokinesis to an end. Exp. Cell Res. 295, 1–8

Benink, H.A. and Bement, W.M. (2005) Concentric zones of active RhoA and Cdc42 around single cell wounds. J. Cell Biol. 168, 429–439

Darenfed, H. and Mandato, C.A. (2005) Wound-induced contractile ring: a model for cytokinesis. Biochem. Cell. Biol. 83, 711–720

Elia N, Sougrat R, Spurlin TA, Hurley JH, Lippincott-Schwartz J. Dynamics of endosomal sorting complex required for transport (ESCRT) machinery during cytokinesis and its role in abscission. Proc. Natl. Acad. Sci. U.S.A. 2011; 108:4846–4851.

Connell, J.W. et al. (2009) Spastin couples microtubule severing to membrane traffic in completion of cytokinesis and secretion. Traffic 10, 42–56

Hélia Neto, Gwyn W. Gould The regulation of abscission by multi-protein complexesJ Cell Sci 2011 124: 3199-3207; doi: 10.1242/jcs.083949

Ryogo Kuriyama, Guy Keryer and Gary G. Boris The mitotic spindle of chinese hamster ovary cells isolated in taxol-containing medium. J. Cell Set. 66, 265-275 (1984)

Yueyuan Zheng, Junjie Guo, Xu Li, Yubin Xie, Mingming Hou, Xuyang Fu, Shengkun Dai, Rucheng Diao, Yanyan Miao and Jian Ren, An integrated overview of spatiotemporal organization and regulation in mitosis in terms of the proteins in the functional supercomplexes, Frontiers in Microbiology, October 2014 | Volume 5 | Article 573

Adi Tamir, Nadav Elad and Ohad Medalia, Assembly and breakdown of microtubules within the midbody, Communicative & Integrative Biology Volume 4 Issue 5 DOI: 10.4161/cib.4.5.16050

Maike S. van der Waal, Rutger C.C. Hengeveld, Armando van der Horst, Susanne M.A. Lens, Cell division control by the Chromosomal Passenger Complex, 2012 Elsevier Inc. doi:10.1016/j.yexcr.2012.03.015

Douglas R. Mackay and Katharine S. Ullman,Coordinating postmitotic nuclear pore complex assembly with abscission timing, Nucleus 2:4, 283-288; July/August 2011; 2011 Landes Bioscience

Michael Glotzer, Cytokinesis: progress on all fronts, Current Opinion in Cell Biology 2003, 15:684–690

Francis A. Barr and Ulrike Gruneberg, Cytokinesis: Placing and Making the Final Cut, Cell *131*, November 30, 2007 DOI 10.1016/j.cell.2007.11.011

Patricia Wadsworth, Cytokinesis: Rho Marks the Spot, Current Biology Vol 15 No 21 DOI: 10.1016/j.cub.2005.10.021

Alisa Piekny, Michael Werner and Michael Glotzer, Cytokinesis: welcome to the Rho zone, TRENDS in Cell Biology Vol.15 No.12 December 2005 doi:10.1016/j.tcb.2005.10.006

G. Ekin Atilla-Gokcumen› Adam B. Castoreno, Sofia Sasse, and Ulrike S. Eggert, Making the cut: The chemical biology of cytokinesis, *ACS Chem Biol*. 2010 January 15; 5(1): 79–90. doi:10.1021/cb900256m.

Patrick Steigemann and Daniel W. Gerlich, Cytokinetic abscission: cellular dynamics at the midbody, Trends in Cell Biology Vol.19 No.11, doi: 10.1016/j.tcb.2009.07.008

Kazuhiro Kishi, Marcel A. T. M. van Vugt, Ken-ichi Okamoto, Yasunori Hayashi, and Michael B. Yaffe, Functional Dynamics of Polo-Like Kinase 1 at the Centrosome, MOLECULAR AND CELLULAR BIOLOGY, June 2009, p. 3134–3150 Vol. 29, doi:10.1128/MCB.01663-08

Cinzia Rinaldo, Alice Moncada, Alessandra Gradi, Laura Ciuffini, Donatella D'Eliseo, Francesca Siepi, Andrea Prodosmo, Alessandra Giorgi, Giovanna M. Pierantoni, Francesco Trapasso, Giulia Guarguaglini, Armando Bartolazzi, Enrico Cundari, M. Eugenia Schinina, Alfredo Fusco and Silvia Soddu, HIPK2 Controls Cytokinesis and Prevents Tetraploidization by Phosphorylating Histone H2B at the Midbody, Molecular Cell *47*, 87–98, July 13, 2012 DOI 10.1016/j.molcel.2012.04.029

MICHAEL MULLINS and J. RICHARD McINTOSH, Isolation and initial characterization of the mammalian midbody, THE JOURNAL Of CELt BIOLOGY • VOLUMI. 94 SEPTEMBER 1982 654-661

Ulrike Gruneberg, Rüdiger Neef, Xiuling Li, Eunice H.Y. Chan, Ravindra B. Chalamalasetty, Erich A. Nigg, and Francis A. Barr, KIF14 and citron kinase act together to promote efficient cytokinesis, The Journal of Cell Biology, Vol. 172, No. 3, January 30, 2006 363–372

Monica Agromayor and Juan Martin-Serrano, Knowing when to cut and run: mechanisms that control cytokinetic abscission, Trends in Cell Biology, September 2013, Vol. 23, No. 9

G. Ekin Atilla-Gokcumen, Adam B. Castoreno, Sofia Sasse, and Ulrike S. Eggert, Making the cut: The chemical biology of cytokinesis, *ACS Chem Biol*. 2010 January 15; 5(1): 79–90. doi:10.1021/cb900256m.

Victoria E. Foe and George von Dassow,Stable and dynamic microtubules coordinately shape the myosin activation zone during cytokinetic furrow formation J. Cell Biol. Vol. 183 No. 3 457–470

Sandrine Ruchaud, Mar Carmena, and William C. Earnshaw, The Chromosomal Passenger Complex: One for All and All for One, Cell *131*, October 19, 2007 DOI 10.1016/j.cell.2007.10.002

Arnaud Echard and Patrick H. O'Farrell, The Degradation of Two Mitotic Cyclins Contributes to the Timing of Cytokinesis, *Curr Biol*. 2003 March 4; 13(5): 373–383.

Guillaume Normand and Randall W. King , Understanding Cytokinesis Failure, *Polyploidization and Cancer*, edited by Randy Y.C. Poon. ©2010 Landes Bioscience and Springer Science+Business Media.

Lai Kuan Dionne, Xiao-Jing Wang and Rytis Prekeris, Midbody: from cellular junk to regulator of cell polarity and cell fate, Current Opinion in Cell Biology 2015, 35:51–58

Carmena M. 2012 Abscission checkpoint control: stuck in the middle with Aurora B. Open Biol 2: 120095. http://dx.doi.org/10.1098/rsob.120095

Yasuhiko Terada, Masaaki Tatsuka, Fumio Suzuki, Yuko Yasuda, Setsuya Fujita and Masayuki Otsu, AIM-1: a mammalian midbody-associated protein required for cytokinesis, The EMBO Journal Vol.17 No.3 pp.667–676, 1998

Hochegger H, He´garat N, Pereira-Leal JB. 2013 Aurora at the pole and equator: overlapping functions of Aurora kinases in the mitotic spindle. Open Biol 3: 120185. http://dx.doi.org/10.1098/rsob.120185

Patrick Steigemann, Claudia Wurzenberger, Michael H.A. Schmitz, Michael Held, Julien Guizetti, Sandra Maar and Daniel W. Gerlich, Aurora B-Mediated Abscission Checkpoint Protects against Tetraploidization, Cell *136*, 473–484, February 6, 2009 DOI 10.1016/j.cell.2008.12.020

Armando van der Horst & Susanne M.A. Lens, Cell division: control of the chromosomal passenger complex in time and space, Chromosoma (2014) 123:25–42 DOI 10.1007/s00412-013-0437-6

Pohl C, Jentsch S. (2008). Final Stages of Cytokinesis and Midbody Ring Formation are Controlled by BRUCE. Cell, **132**, 832-845.

Musab S. Bhutta, Christopher J. McInerny * and Gwyn W. Gould, ESCRT Function in Cytokinesis: Location, Dynamics and Regulation by Mitotic Kinases, Int. J. Mol. Sci. 2014, 15, 21723-21739; doi:10.3390/ ijms151221723

Jeremy G Carlton, Anna Caballe, Monica Agromayor, Magdalena Kloc, and Juan Martin-Serrano, ESCRT-III Governs the Aurora B-Mediated Abscission Checkpoint Through CHMP4C, *Science*. 2012 April 13; 336(6078): 220–225. doi:10.1126/science.1217180.

Yasuhiro Kurasawa, William C Earnshaw, Yuko Mochizuki, Naoshi Dohmae and Kazuo Todokoro, Essential roles of KIF4 and its binding partner PRC1 in organized central spindle midzone formation, The EMBO Journal (2004) 23, 3237–3248

Jeffrey K. Bailey, Alexander T. Fields, Kaijian Cheng, Albert Lee, Eric Wagenaar, Remy Lagrois, Bailey Schmidt, Bin Xia, and Dzwokai Ma, WD Repeat-containing Protein 5 (WDR5) Localizes to the Midbody and Regulates Abscission, THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL.290,NO.14,pp.8987–9001,April3,2015

Kerry Bloom, NoCut: Cytokinesis in Check, Cell *125*, April 7, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.cell.2006.03.016

Chi-Kuo Hu, Nurhan Özlü, Margaret Coughlin, Judith J. Steen, and Timothy J. Mitchison, Plk1 negatively regulates PRC1 to prevent premature midzone formation before cytokinesis, Molecular Biology of the Cell, doi/10.1091/ mbc.E12-01-0058) on May 23, 2012.

Cristiana Mollinari, Jean-Philippe Kleman, Wei Jiang, Guy Schoehn, Tony Hunter, and Robert L. Margolis, PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone, The Journal of Cell Biology, Volume 157, Number 7, June 24, 2002 1175–1186

Special Thanks

Per una persona come me è piuttosto difficile, se non impossibile, scindere la vita privata da quella lavorativa; mi sembra quindi doveroso ringraziare qui tutti coloro che mi sono stati vicino, dentro e fuori dal laboratorio, contribuendo alla mia riuscita.

Il primo, speciale ringraziamento va alla mia famiglia: i miei genitori, Fabio e Silvia, che mi hanno sopportata e supportata in questi tre anni. Babbo e mamma, senza il vostro aiuto non avrei potuto compiere questo percorso; avrei voluto essere una figlia migliore per voi.

A Luca, mentore e tutor, che ha accolto nel suo laboratorio una timida studentessa e le ha dato fiducia, consentendole di crescere sia dal punto di vista professionale che umano. Se sono arrivata fin qui, lo devo a te.

A Michele, il collega ideale, compagno di confidenze, la metà del mio progetto: nessuno sarà mai alla tua altezza, mio caro Watson.

Un ringraziamento speciale ad Alessio, collega prezioso e competente sul lavoro, impagabile amico nella vita; il tuo sostegno, la tua modesta ma grande saggezza, il tuo sorriso dolce, sono stati per me in quest'ultimo anno una vera ancora di salvezza. L'augurio che ti faccio è di imparare ad apprezzarti, un giorno, per quanto veramente vali.

Un grazie a Manuela, presenza costante in questi anni, per avermi insegnato le basi e l'amore per il mio lavoro.

A Chiara e Davide, cui ho cercato a mia volta di trasmettere questo amore, malgrado la mia goffaggine. Il vostro aiuto è stato prezioso, la vostra compagnia il regalo più bello. Vi voglio bene.

A Laura e Claudia il mio ringraziamento per la collaborazione sul lavoro e per il bel rapporto nato tra noi.

Al Professor Prantera ed alla Professoressa Soddu per le preziose consulenze di questi anni: avere la possibilità di confrontarmi con persone

così esperte e piene di conoscenza mi ha profondamente arricchita e lusingata.

A Chiara, il mio spirito affine, la mia parte razionale. Quando era tutto buio tu mi hai presa per mano e guidata verso la luce; non avrei potuto desiderare una compagna di vita migliore, *Laotong*.

A Veronica, sorellina preziosa, presenza costante nel mio passato, presente e spero futuro.

Ad Andrea, Iris, Sabrina, Giorgia, Ilaria, Maria Grazia, Giulia, Marco e David, grazie per aver riempito la mia vita con la vostra amicizia.

A Te, di nuovo.

A tutti voi che siete qui con me in un giorno così importante per la mia carriera, grazie dal profondo del cuore.

Elena