CSB protein is (a direct target of HIF-1 and) a critical mediator of the hypoxic response

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Cockayne syndrome (CS) is a rare genetic disease characterized by neurological problems, growth failure and premature ageing. Many of these features cannot simply be ascribed to the defect that CS cells display during transcription-coupled repair. Here, we show that CSB mutant cells are unable to react to hypoxic stimuli by properly activating the hypoxia-inducible factor-1 (HIF-1) pathway, a defect that is further enhanced in the event of a concomitant genotoxic stress. Furthermore, we show that CSB expression is under the control of HIF-1 and has a critical function during hypoxic response by redistributing p300 between HIF-1 and p53. Altogether, our data demonstrate that CSB is part of a feedback loop mechanism that modulates the biological functions of p53. The outcome of this study provides new insights into the understanding of the molecular basis of the CS phenotype and the involvement of the CSB protein in the hypoxic response pathway.

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Introduction

Cockayne syndrome B (CSB) is a 168-kDa protein that belongs to the SWI2/SNF2 family of chromatin remodellers (Matson *et al*, 1994). It exhibits ATPase activity (Selby and Sancar, 1997; Citterio *et al*, 2000; Beerens *et al*, 2005) and has conserved helicase motifs (Troelstra *et al*, 1992). The emerging view is that CSB is a multifunctional protein with different modes of action, depending on the functional context and the operative site. CSB has been shown to have a function in the transcription-coupled repair subpathway of nucleotide excision repair,

which rapidly corrects certain transcription-blocking lesions located on the transcribed strand of active genes (Sarker *et al*, 2005; Fousteri *et al*, 2006; Laine and Egly, 2006). In addition, CSB has been shown to interact and stimulate transcriptional protein complexes of all three classes of nuclear RNA polymerases (Selby and Sancar, 1997; Tantin *et al*, 1997; van Gool *et al*, 1997; Bradsher *et al*, 2002; Yuan *et al*, 2007). Indeed, CSB-deficient cells exhibit *in vivo* defects in transcription initiation and elongation (Balajee *et al*, 1997; Proietti-De-Santis *et al*, 2006).

Mutations in CSB gene give rise to Cockayne syndrome (CS), an autosomal recessive disorder that affects the growth, development and maintenance of a wide range of tissues and organs. Among these, progressive neurological abnormalities (including demyelination, ataxia and cerebellar atrophy) are key phenotypes (Nance and Berry, 1992). Impaired repair of oxidative damage may account for some of the neurological symptoms (Licht *et al*, 2003). Unexpectedly, given the defect exhibited in DNA repair, CS patients do not present an increased risk of cancer. However, patients with the classical form (CS type 1) display normal intrauterine somatic and brain growth with developmental crisis in the first or second year of life. This led us to hypothesize the existence of a coordinated mechanism that is functional in the fetus (in uterus) but that is deficient in the infant. Inadequate respiration and oxygen homeostasis could represent one of the Achilles' heel of CS patients during extrauterine life, when the infant is no longer sustained by maternal placenta for what concerns angiogenic growth factors and gaseous oxygen. Accordingly, among the tissues that degenerate in CS, some appear to be very sensitive to oxygen levels, including the Purkinje cells and oligodendrocytes (Chen *et al*, 2003; Liu *et al*, 2006; Laposa *et al*, 2007).

Oxygen deprivation (hypoxia) occurs in tissues when O₂ supply through the cardiovascular system fails to meet the cellular demand. Hypoxia occurs in physiological as well as in pathological conditions (inflammation and solid tumour formation).

Hypoxic stress has been shown to exert an effect through p53 stabilization, predominantly by post-translational modification (Harris, 2002). As a consequence, p53 becomes active and can initiate cell demise not only by promotion of the transcription of cell cycle-regulating genes such as *p21* but also of the genes involved in apoptotic events such as *bax*. Alternatively, a large number of molecular events can prevent cell death by inducing adaptive responses that, in turn, facilitate cell proliferation and survival (Pugh and Ratcliffe, 2003). The regulation of most proteins required for hypoxic adaptation occurs at the gene level and involves transcriptional induction through the binding of the hypoxia-inducible factor-1 (HIF-1) to the hypoxia-responsive element (HRE) at the promoter of the regulated genes. HIF-1 is a heterodimer that consists of the inducible HIF-1 α subunit and the constitutively expressed HIF-1 β subunit (Semenza, 2003). To activate transcription, HIF-1 must recruit the transcriptional

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cofactor/histone acetyltransferase protein p300 to the target gene promoters (Arany *et al*, 1996).

In this study, we showed that CSB mutant cells are not able to properly react to hypoxia. Despite the correct HIF-1 recruitment on the promoters of genes involved in the hypoxic response, such as vascular endothelial growth factor (VEGF) and GADPH, downstream events such as RNA polymerase II, TFIIB and p300 recruitments do not occur correctly, thereby leading to an insufficient hypoxic response.

Results

CSB expression is under the control of HIF factor

Sequence analysis of the human CSB promoter revealed two potential HREs matching the HIF-1-binding consensus sequence BACGTSSK (B = G/C/T, S = G/C and K = G/T) in all eight bases (Figure 1A). Using chromatin immunoprecipitation (ChIP), we examined the kinetic of HIF-1 occupancy at the HREs of the CSB promoter of normal human primary fibroblasts FB789 (hereinafter called as WT), after either oxygen deprivation or hypoxic mimetic drug exposure. We employed compounds such as desferrioxamine (DFO) and cobalt chloride (CoCl₂), which have been demonstrated to induce HIF-1 stabilization and overexpression of its target genes (An *et al*, 1998; Figure 1C).

The occupancy of HIF-1 to the CSB promoter progressively increased in WT cells after incubation with either DFO or CoCl₂ as well as during oxygen deprivation (1% O₂) (Figure 1B).

RT-PCR and western blotting confirmed that the increased binding of HIF-1 to the CSB promoter results in increased CSB messenger RNA synthesis (Figure 1D) and CSB protein accumulation (Figure 1E). The pattern of HIF-1-mediated CSB expression was similar regardless of whether the cells had been oxygen-deprived or exposed to either DFO or CoCl₂.

HIF-1 pathway is impaired in CSB mutant cells

As the CSB protein is induced in normal cells by hypoxic stimuli through the HIF/HRE mechanism, we wondered whether CSB was required for the proper activation of the HIF-regulated response. We therefore analysed whether the hypoxia response pathway was affected in CSB mutant cells. CS548VI (hereinafter called as CS-B) is a human primary fibroblast line derived from a CS patient with a deletion spanning the promoter region (LPDS, unpublished data) and the exon 1 of the CSB gene resulting in the complete absence of CSB protein (Laugel *et al*, 2008). CS1PV (hereinafter called as CS-B I) is a human primary fibroblast line derived from a Cockayne patient homozygous for a mutation that converts Arg453 to a stop codon leading to a truncated protein (Colella *et al*, 1999).

First, we monitored the expression of the HIF-1-inducible gene VEGF by quantitative RT-PCR. VEGF mRNA levels were induced by the exposure to DFO (Figure 2A). In normal fibroblasts (either FB789-WT or C3PV-WT I), the induction started at 4 h (~5 fold) and reached its maximum at 16 h (~30-fold induction). In contrast, VEGF expression was consistently reduced in CS-B patients' derived cells (~1.5- and ~12-fold induction, at 4 and 16 h, respectively). It is noteworthy that CSB siRNA treatment in WT cells resulted in a similar defect in HIF-dependent transcription, highlighting the essential role of CSB protein in this pathway.

Figure 1 (A) Promoter region of human CSB gene, stretching from position -1000. The two putative HREs, matching the canonical HRE sequence BACGTSSK are highlighted in cyan. The CACAG element important for the functionality of various HREs is highlighted in grey. (B) Kinetics of HIF-1α occupancy at the promoter of CSB gene after oxygen deprivation (1% O₂) or either DFO (1 mM) or CoCl₂ (1 mM) stimulation. Soluble chromatin was prepared from normal cells at indicated times after hypoxic treatment and subjected to ChIP assay using an antibody against HIF-1α. Real-time PCR using specific primers were performed to test the relative enrichment for the promoter region of the gene compared with the immunoprecipitated DNA compared with the input. (C) A representative western blot result shows HIF-1α protein levels in normal cells exposed to DFO, CoCl₂ and 1% O₂, for 24 h. (D) Increased mRNA expression of CSB in normal cells exposed to DFO, CoCl₂ and 1% O₂. mRNA levels of CSB determined by real-time RT-PCR, normalized by 18S RNA levels are shown. (E) A representative western blot result shows CSB and β-tubulin protein levels in normal cells exposed to DFO, CoCl₂ and 1% O₂, for 24 h. *Aspecific band.

The pattern of HIF-1-mediated VEGF expression and the defect exhibited by CS-B cells in properly responding to hypoxic stimuli was confirmed by exposing the cells either to oxygen deprivation or CoCl₂ (Figure 2B).

Figure 2 (A–C) Increased mRNA expression of VEGF in cells exposed to DFO (1 mM), CoCl₂ (1 mM) and oxygen deprivation (1% O₂). mRNA levels of VEGF determined by real-time RT-PCR, relative to cells derived from healthy subjects (WT: FB789; WT I: C3PV) or CS-B patients (CS-B: CS548VI; CS-B I: CS8PV) are shown. Where indicated, the cells were co-exposed to KBrO₃ (1 mM) or UV irradiation (5 J/m²). mRNA levels have been normalized by 18S RNA levels. (D) Western blot shows HIF-1α and β-tubulin protein levels upon DFO treatment with or without co-exposure to genotoxic agents KBrO₃ or UV irradiation.

The response to DFO was also analysed in combination with DNA-damaging agents such as KBrO₃ or UV irradiation as in CS-B mutant cells DNA damage has an inhibitory effect on the activation of certain genes (Proietti-De-Santis *et al*, 2006). The DFO-associated induction of VEGF was further reduced when CS-B cells were simultaneously exposed to either KBrO₃ or UV irradiation (Figure 2C).

HRE-dependent transcription can be modulated through the availability of HIF-1 (Rapisarda and Melillo, 2007). To investigate this possibility, we analysed the amount of HIF-1α protein in both WT and CS-B cells after DFO stimulation by western blotting (Figure 2D). As expected, HIF-1α is undetectable in both WT and CS-B untreated cells, but it is readily stabilized after DFO treatment. The induction was similar in both the cell lines and did not appear to be affected by DNA damage.

We next investigated whether the decreased response to hypoxic stimuli detected in CS-B cells was due to a defect in the assembly of the transcriptional apparatus. ChIP experiments showed a significant increase in both RNA pol II and TFIIB occupancy on the *VEGF* promoter in WT cells (Figure 3A and B, respectively), most likely the result of the transcriptional activation due to the stimulation operated by HIF-1 binding at the promoter (Figure 3C). Conversely, we found that both RNA pol II and TFIIB were not recruited to the same extent on the *VEGF* promoter in

CS-B cells after DFO treatment. This approach also highlights that the defective response to hypoxic stimuli is accentuated when CS-B cells are exposed to DFO in combination with genotoxic stress. The reduced ability of CS-B cells to respond to DFO stimulation is not due to a decreased recruitment of HIF-1 at the cis-acting (HRE) region of the promoter (Figure 3C).

To gain insight into the chromatin modifications generated upon gene activation, we investigated the level of nucleosomal histone acetylation around the promoter region. We found a remarkable defect in H4 acetylation at the *VEGF* promoter after DFO treatment in CS-B cells compared with that observed in WT cells (Figure 3D). The analysis also showed that in CS-B cells, simultaneous genotoxic attack exacerbated the histone acetylation defect. The lower level of H4 acetylation, as observed in CS-B cells after DFO stimulation, correlates with the malfunctioning recruitment of the p300 histone acetyltransferase at the promoter of *VEGF* (Figure 3E). p300 is known to function as an essential cofactor of the HIF activation pathway.

Time course experiments on the housekeeping gene *GAPDH*, known to be induced by HIF-1 (Graven *et al*, 1999), revealed a similar trend: a rapid increase in the mRNA level of *GAPDH* after DFO treatment in WT but not in CS-B cells (Figure 4A). Also, ChIP experiments revealed that the absence of a functional CSB protein prevents the

Figure 3 Kinetics of (A) RNA pol II, (B) TFIIB, (C) HIF-1 α , (D) acetylated H4 and (E) p300 occupancy at the promoter of *VEGF* gene determined by ChIP at indicated times.

recruitment of RNA pol II at the *GAPDH* promoter (Figure 4B), which correlates with a defect in both histone H4 acetylation and p300 enrichment around the promoter region of the gene (Figure 4D and E, respectively). Again, the lack of a functional CSB protein did not prevent the correct recruitment of HIF-1 at the HRE of the promoter (Figure 4C). The deficiency in the induction of *GAPDH*, as relieved in CS-B cells, was further exacerbated in case of simultaneous DFO and genotoxic (KBrO_3) attack.

Altogether, these data demonstrated that CSB mutant cells fail to activate the hypoxic response pathway controlled through HIF-1 upon hypoxic stimulus.

p53 pathway is massively activated in CSB mutant cells

It is well known that p53-responsive genes are activated upon hypoxia or hypoxia mimic agents (Pan *et al*, 2004). This led us to investigate whether CSB deficiency would also affect the hypoxia-induced activation of p53 target genes.

Western blot performed with extracts of WT and CS-B cells showed an increase in the p53 protein in the DFO-treated cells with respect to the untreated cells (Figure 5A). Interestingly, the induction of p53 protein was remarkable in DFO-treated CS-B cells and further enhanced when co-exposed to the DNA-damaging agents KBrO_3 or UV irradiation.

Figure 4 (A) Increased mRNA expression of GAPDH in cells exposed to DFO (1 mM). mRNA levels of GAPDH determined by real-time RT-PCR, relative to WT and CS-B cells, normalized with 18S RNA levels are shown. Where indicated, the cells were co-exposed to KBrO₃ (1 mM). Kinetics of (B) RNA pol II, (C) HIF-1 α , (D) acetylated H4 and (E) p300 occupancy at the promoter of *GAPDH* gene determined by ChIP at indicated times.

We next analysed the expression of the p53-responsive *p21* gene, already shown to be implicated in the control of cell cycle progression. *p21* mRNA analysis performed by quantitative RT-PCR revealed a notable increased expression of the *p21* gene in CS-B cells after DFO stimulation, either in the presence or absence of genotoxic attack (Figure 5B). This increase in *p21* messenger RNA correlates with a remarkable increase in RNA pol II recruitment at its promoter (Figure 5C) and correlates also with an increased recruitment of p53 at the cis-acting responsive regions of the gene,

located 2.3 kb upstream of the transcription initiation site (Figure 5D).

In contrast to HIF-regulated promoters, histone H4 acetylation and recruitment of p300 occurred properly at the *p21* promoter, regardless of the presence of the CSB protein (Figure 5E and F).

Similar to the *p21* gene, the expression of another p53-dependent gene with pro-apoptotic function, *Bax*, was enhanced in CS-B cells when treated with DFO as compared with the normal ones (Supplementary Figure 1).

Figure 5 (A) Western blot shows p53 protein levels upon DFO (1 mM) treatment with or without co-exposure to genotoxic agents such as KBrO₃ (1 mM) or UV irradiation (5 J/m²) in WT and CS-B cells. (B) mRNA expression of *p21* in cells exposed to DFO. mRNA levels determined by real-time RT-PCR and normalized with 18S RNA levels are shown. Kinetics of (C) RNA pol II, (D) p53, (E) acetylated H4 and (F) p300 occupancy at the promoter of *p21* gene determined by ChIP at indicated times. (G) Cell cycle progression analysis of both WT and CS-B cells exposed to different concentrations of DFO (1 and 5 mM) for 24 h. (H) Apoptotic analysis of both normal and CS-B cells exposed to DFO (1 and 5 mM) for 24 h and analysed 48 h later (72 h after the beginning).

The notable upregulation of the p53 pathway, observed in CS-B cells after DFO exposure, resulted in a consistent arrest in the G1/S boundary of the cell cycle, whereas WT cells showed a profile similar to that observed in untreated cells (Figure 5G). Also, morphological analysis of DFO-induced apoptosis, by DAPI staining, showed a dramatic enhancement of cell death in CS-B cells (Figure 5H). Consistently, colony survival assay has shown an increased cellular sensitivity to DFO of CS-B cells (Supplementary Figure 1F).

Altogether, these data demonstrated that in CSB mutant cells, the hypoxic-induced p53 response is more prominent than the HIF-1 one and gives rise to cell demise, by promoting cell cycle arrest and apoptosis.

CSB limits the overactivation of the p53 response

A number of reports suggested that p53 might compete with HIF-1 for the limiting amount of the transcriptional co-activator p300 (Schmid *et al*, 2004). We then investigated whether the strong activation of p53 protein observed in CS-B cells might exert any inhibitory effect on the activation of HIF target genes by depleting the HIF-responsive promoter of p300.

Therefore, we ablated p53 by RNA interference in CS-B cells. Western blotting (Figure 6A) confirmed that there was substantial suppression of p53 in CS-B cells transfected with RNA oligonucleotides targeting p53 mRNA (CS-B⁺ si-p53). ChIP experiments evidenced that p53 knockdown indeed

Figure 6 (A) Western blotting analysis of p53 and β -tubulin protein amount from whole cellular extracts of WT and CS-B cells transfected with either control RNA oligonucleotides (CS-B⁺ si-ctrl) or targeting p53 mRNA (CS-B⁺ si-p53). Protein extracts of cells, treated as indicated at the bottom of the panel, were collected at the indicated times. (B) Kinetics of RNA pol II and (C) p300 occupancy at the promoter of VEGF gene of WT, CS-B⁺ si-ctrl and CS-B⁺ si-p53 cells. (D) Increased mRNA expression of VEGF in cells exposed to DFO (1 mM).

rescued the recruitment of both RNA pol II and p300 at the VEGF promoter after DFO stimulation (Figure 6B and C) and mRNA VEGF expression (Figure 6D), regardless of whether or not the cells had been exposed to KBrO₃ agent, demonstrating that excessive p53 response interferes with the HIF-1-induced gene activation, in CS-B cells.

The preferential induction of p53 versus HIF response in CS-B cells and the competition for the critical recruitment of p300 to their respective promoters led us to investigate the role of CSB in this process. We first highlighted that transient expression of wild-type CSB gene in CS-B cells restored the efficiency of the HIF/HRE transcriptional response (i.e. the recruitment of both RNA pol II and p300 at the VEGF promoter; Figure 7A and B).

Intriguingly, ChIP performed in WT cells, using antibody against CSB, indicates no binding of the CSB protein to the VEGF promoter (Supplementary Figure 2), in contrast to that shown earlier for other genes (Proietti-De-Santis *et al*, 2006). Nevertheless, CSB appears to be indispensable for the correct activation of the HIF/HRE mechanism pointing to an additional role of CSB during the transcription initiation process, apart from its promoter binding-associated function.

Knowing that p53 competes with HIF-1 for p300 binding and that either p53 silencing or CSB re-expression in CS-B cells restores the HIF/HRE mechanisms, we questioned whether the interaction between p53 and p300 was destabilized by CSB. Indeed, it has been previously reported that p300–p53 binding is involved in both the stabilization and the transactivation activity of p53 (Yuan *et al*, 1999).

Therefore, we expressed recombinant p53, p300 and CSB proteins in Sf9 insect cells infected with the corresponding baculoviruses and after incubation of the appropriate extracts, we examined protein–protein interaction through immunoprecipitation (Figure 7C). Following this procedure, we incubated limiting concentrations of p53 extracts with excess amounts of p300 and/or CSB extracts. The concentrations of the p53 antibodies used for the immunoprecipitations were adequately titrated to fully precipitate the amounts of p53 protein-containing extracts. We showed that p53 co-precipitate with either p300 (lane 1) or CSB (lane 3) when the respective proteins were singularly incubated with p53. However, we found that p300 did not co-precipitate with p53 in the presence of CSB (lane 5), illustrating that CSB competes with p300 for the binding site on p53.

To determine the respective role of CSB in promoting the transcription of HIF-1-regulated genes versus those regulated by p53, we designed the following experiment: either p53, HIF-1 α , p300 or CSB expression vectors were transfected into WT cells, together with reporter vectors under the control of either MDM2 (pMDM2-Firefly-Luc) or VEGF (pVEGF-Renilla-Luc) promoters (Figure 7D). As expected, we found that the expression of p53 upregulated the activity of pMDM2-Firefly-Luc (group 2), whereas HIF-1 α selectively stimulated the activity of pVEGF-Renilla-Luc (group 3). Co-expression of both p53 and HIF-1 α resulted in the inhibition of pVEGF-Renilla-Luc (group 4), which was rescued by the simultaneous co-expression of either p300 (group 5) and/or CSB (group 6). However, it should be noted that there was a slight inhibition of pMDM2-Firefly-Luc when CSB was expressed.

Altogether, these data highlight the role of p300 in the regulation of the expression of a specific set of genes, and clearly demonstrate that p53-responsive genes, when over-

activated, could be toxic as they titrate away essential transcription factors such as p300. In this regard, CSB seems to have an essential function by interacting with p53 and thereby competing away the essential factor p300 from p53.

Discussion

The HIF-1 acclimatizing response is impaired in CSB mutant cells

In this paper, we have investigated the ability of CSB mutant cells to deal with hypoxic stimuli by triggering the HIF-1-controlled pathway. HIF-1 is implicated in the regulation of a vast array of genes that control multiple cellular functions such as angiogenesis, glucose metabolism and cell survival. These metabolic programmes are important under physiological conditions and also in the development and progression of tumours (Semenza, 2006). Here, we have shown that CSB is a direct target of HIF-1 in response to hypoxia. Furthermore, we have demonstrated that CSB mutant cells respond to hypoxic stimuli poorly through the HIF-1 pathway when compared with normal cells. Also, genotoxic attacks, including oxidative damage (KBrO₃), exacerbated the defect exhibited by CSB mutant cells. This is particularly interesting because it is well known that hypoxia impairs the mitochondrial respiratory chain, therefore leading to an overproduction of ROS (Guzy and Schumacker, 2006). This would then start a very dangerous chain of events in which hypoxia increases the damage, and the damage in turn leads to hypoxia resulting in cell death.

ChIP studies revealed that despite the normal recruitment of HIF-1 to the responsive promoters such as VEGF and GADPH, the recruitment of the acetyltransferase p300 to these promoters is deficient and thereby the chromatin in the surrounding regions was poorly acetylated. Consequently, the pre-initiation complex (PIC) fails to assemble on these loci. This phenomenon appears to be directly correlated with p53 protein levels, which are exceptionally elevated in CSB cells upon hypoxic stimuli.

Hypoxic stress is known to upregulate p53 (Graeber *et al*, 1994). Recent studies, including ours, have shown that p53 has an antagonistic function on the HIF-1 pathway; p53 competes with HIF-1 for binding to the shared co-activator p300. As a consequence, once p53 becomes activated it attenuates the HIF-1-controlled adaptive response (Schmid *et al*, 2004). Accordingly, we showed that overexpression of p300 relieves the inhibition of HIF-1-controlled activity caused by the elevated levels of p53. A similar result was obtained by overexpressing CSB (Figure 7D). CSB protein did not promote VEGF transcription by exerting a cis-acting action on its promoter, as instead shown with other gene promoters (Proietti-De-Santis *et al*, 2006). This raises the intriguing question of a possible trans-acting role for CSB.

The interactions of CSB and p300 with p53 are mutually exclusive

By analysing protein–protein interaction with recombinant polypeptides, we revealed a competitive binding of CSB and p300 to p53 (Figure 7C). As a result, the interaction between p53 and p300 as well as p53 transactivating activity was significantly reduced by CSB expression. It is important to note that binding of p300 to p53 is fundamental for p53 transactivating activity: both for the function of p300 as a

Figure 7 Kinetics of (A) RNA pol II and (B) p300 occupancy at the promoter of *VEGF* gene of WT and CS-B cells either untransfected or transfected with an expression vector coding for wild-type CSB protein (CS-B⁺ wt-csb). (C) Competitive binding of CSB and p300 to p53: baculovirus-infected insect cell lysates containing p53, p300 or CSB proteins were mixed as indicated at the top of each image, and immunoprecipitated with Ab-p53 antibodies crosslinked to agarose beads. The immunoprecipitated (IP) and the flowthrough (FT) fractions were analysed by western blotting. (D) p53 represses HIF-1-stimulated transcription from VEGF promoter construct: WT cells were co-transfected with both pVEGF-Renilla-Luc (0.1 µg) and pMDM2-Firefly-Luc (0.1 µg) constructs in combination with either empty vector or plasmids expressing wt-p53 (0.5 µg), wt-p300 (0.5 µg), wt-HIF-1α (0.5 µg), wt-CSB (0.5 µg) proteins as indicated at the bottom of the histogram. Total amount of DNA was maintained at 3 µg with the appropriate amount of empty vector. Cells were transiently transfected for 24 h before harvesting for luciferase activity. The results are expressed as fold activation relative to the cells transfected only with the pVEGF-Renilla-Luc and pMDM2-Firefly-Luc indicator plasmids.

Figure 8 CSB is part of a feedback loop mechanism to modulate p53 protein transactivation activity. Hypoxic stress is known to upregulate either HIF-1-controlled response, by the stabilization of HIF-1 α subunit, or p53 response. Our hypothesis is that CSB, the expression of which is under the control of HIF-1, by decreasing p53 activity after cellular stress would re-equilibrate the physiological response towards cell proliferation and survival instead of cell cycle arrest and cell death.

transcriptional co-activator of p53 target genes (Lill *et al*, 1997) and for the stability of p53, by slowing down Mdm2-dependent p53 degradation (Ito *et al*, 2001). The upregulation of CSB during hypoxic stress may describe a role for CSB as part of a feedback loop mechanism to decrease both p53 protein amount and its transactivation activity.

It is likely that the CSB protein, by modulating p53 activity after cellular stress would re-equilibrate the physiological response towards cell proliferation and survival instead of cell cycle arrest and cell death (Figure 8). In contrast, the absence of CSB would increase the binding of p53 to p300 causing the stabilization of p53 and the activation of its target genes, including the ones involved in the apoptotic commitment.

Altogether, our data highlight the role of p300 in the regulation of the expression of a specific set of genes, and demonstrate that an overactivated p53 response is toxic because this protein titrates away essential transcription factors such as p300, which are also required for an anti-apoptotic transcriptional response. In this regard, CSB would have an essential function by interacting with p53 and therefore releasing the essential factor p300 from p53. This hypothesis would also provide an explanation as to why in CSB cells p53 induction is higher both in intensity and duration. Whether CSB/p53 interaction could have any function in the ubiquitylation and proteosomal degradation of p53, as previously envisaged for RNA pol II (Licht *et al*, 2003) remains an open question.

We speculate that CSB, besides its role during DNA repair and transcription (Sarasin and Stary, 2007), functions as a master switch factor that can selectively influence the transcription of a set of genes, after DNA damage or cellular stress, by modulating the biological functions of p53.

Explaining the phenotype of CS

This study provides important information for the understanding of the neurodegenerative phenotype of CS. Among the genes that were not properly activated in CSB mutant

cells, there are VEGF and GAPDH, key players in angiogenesis and glucose metabolism, respectively.

Structural and functional integrity of brain function profoundly depends on regular oxygen and glucose supply (Carmeliet, 2003). Any disturbance of this supply becomes life threatening and may result in severe loss of brain functions. Because of the fact that oxygen may also be dangerous/toxic, there are mechanisms that allow the brain to exist under low oxygen conditions (LaManna *et al*, 2004). Hypoxic acclimatizing responses involve metabolic and vascular processes that are mediated by HIF-1 and entail the induction of enzymes or growth factors inducing angiogenesis (such as VEGF) and anaerobic glycolysis (such as GAPDH). Therefore, the general inhibition of HIF-controlled gene expression, as observed in CSB mutant cells, could be at the basis of the neurodegenerative phenotype exhibited by CS patients.

It is equally possible that the elevated expression of p53 has a part in the premature ageing phenotype seen in CS, as already evidenced concerning other disorders such as Parkinson's disease, Alzheimer's disease and Huntington's disease (Jacobs *et al*, 2006).

The reason for a reduced cancer incidence in CS patients, in spite of the defect exhibited in DNA repair, is currently not known.

Several studies have associated HIF-1 function with human cancer progression (Harris, 2002). Hypoxia occurs in the early stages of tumour development (before metastasis) when the tumoral mass becomes larger than 2 mm and is no longer sustained by the pre-existent vascularization. The ability to survive under hypoxic conditions is one of the fundamental physiological differences between tumour cells and normal cells. Accordingly, hypoxia-inducible genes regulate several biological processes, including cell proliferation, angiogenesis, metabolism, apoptosis, immortalization and migration that overlap the ones required by cancer cells (Hanahan and Weinberg, 2000). As many of the HIF-1-controlled actions promote cell survival, the question arose whether HIF-1 can be considered as an antiapoptotic factor. The idea that HIF-1 may protect affected tissue/cells during low oxygen supply is supported by reports showing that pro-survival genes, such as nucleophosmin for instance, are induced by hypoxia in an HIF-1-dependent manner, thus inhibiting p53 activation and maintaining cell survival (Li *et al*, 2004). In this regard, we observed that CSB gene is also HIF-1 responsive. Potential HREs have been found in the promoter region of the CSA gene, as well. Further studies will be necessary to value the role, if any, of the CSA protein in the pathway that we unveiled here. One plausible hypothesis is that the inability of CS mutant cells to adequately deal with hypoxia, a consequence of malignant growth, prevents the development of cancers in CS patients.

Materials and methods

Cell lines

The normal (FB789 and C3PV) and CS-B (CS548VI and CS1PV) primary human fibroblasts, kindly provided by M Stefanini (Pavia) and A Sarasin (Paris), were grown in minimal essential medium containing 15% fetal calf serum and 40 mg/ml gentamycin.

Antibodies

The monoclonal antibodies against RPB1-RNA pol II (7C2), Ac-H4 and CSB (3H8 and 1A11) were produced by the IGBMC facility. The

monoclonal antibody against p53 (DO-1) and polyclonal antibodies against CSB (H300), p300 (N15), HIF-1 α (H206) and TFIIB (C18) were purchased from Santa Cruz.

Retrotranscription and real-time quantitative PCR

RNA was isolated by using Nucleospin RNAII kit (Macherey Nagel). cDNA synthesis was performed by using random hexanucleotides and AMV reverse transcriptase (Sigma). Real-time quantitative PCR was carried out with the QuantiTect SYBR Green PCR kit (Qiagen) and the LC480 system (Roche). Results were normalized to 18S. Primer sequences are available on request.

Western blot analysis

Cells were lysed for 10 min on ice in RIPA buffer. The cell lysates were centrifuged at 13 000 r.p.m. for 5 min and the supernatant containing the proteins was recovered. Protein concentration was determined by Bradford protein assay kit (Bio-Rad). Proteins (50 μ g) were fractionated on polyacrylamide gradient gel electrophoresis and blotted onto PVDF membrane (Amersham) following the standard protocols. The membrane was incubated with TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.2% Tween-20) buffer containing 5% NFDm for 60 min at RT and subsequently incubated with primary antibodies and HRP-conjugated secondary antibody (Vector). The signal was detected using the enhanced chemiluminescence method following the manufacturer's instructions (Amersham).

ChIP

Cells were crosslinked with a 1% formaldehyde solution for 10 min at RT. Crosslinking was stopped by the addition of glycine to 125 mM final concentration. Samples were sonicated to generate DNA fragments below 500 bp. For immunoprecipitations, 1 mg of protein extract was precleared for 2 h with 50 ml of a 50% slurry of 50:50 protein A/G-sepharose before addition of the indicated antibodies. Then, 2 mg of each antibody was added to the reactions and incubated overnight 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 crosslinking was reversed by adjusting to 200 mM NaCl and incubating for 5 h at 65°C. Further proteinase-K digestion was performed for 2 h at 42°C. DNA was purified by using Qiagen columns (QIAquick PCR purification Kit). Immunoprecipitated DNA was quantified by real-time quantitative PCR. Primer sequences are available on request.

RNA interference

A pool of four RNA oligonucleotides (Dharmacon) forming a 19 base duplex core, specifically designed to target p53 mRNA, was transfected in CS-B cells at the concentration of 50 nM. A pool of RNA oligonucleotides, without any target mRNA, was used as a control. RNA transfection was performed by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

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Cell cycle progression and apoptosis analysis

Normal and CS-B cells were fixed with 70% ethanol at 24 h after DFO treatment (1 and 5 mM) and processed for flow cytometry. Samples were resuspended in 20 mg/ml propidium iodide and analysed on a Becton Dickinson FACSCalibur. Histograms were generated using WinMDI software.

Normal and CS-B cells were treated with DFO (1 and 5 mM) to determine their apoptotic response. A combination of fluorescein diacetate (FDA; 5 μ g/ml), propidium iodide (PI, 0.5 μ g/ml) and Hoechst (HO, 1 μ g/ml) dyes were used to identify the apoptotic and necrotic cells from viable cells. FDA and HO are vital dyes that stain the cytoplasm and nucleus of the viable cells, respectively. The necrotic and the late stage of apoptotic cells are readily identified by PI staining. Cells in the early phase (viable–HO stained) and late phase (dead–PI stained) of apoptosis displayed the characteristic pattern of chromatin fragmentation. Approximately 2000 randomly chosen cells were microscopically analysed for apoptosis at 72 h after DFO exposure.

Transfections and luciferase reporter assays

HeLa cells (2.5×10^5) were plated in six-well plates and transfected with either pVEGF-Renilla luciferase (made by substituting the firefly luciferase gene of the pGL2-VEGF plasmid, kindly provided by Dr Ellis from Anderson Cancer Center, Houston, USA, with the Renilla luciferase gene), pMDM2-Firefly luciferase, pCH110 (from Invitrogen coding for β -galactosidase) reporter plasmids and either p300, p53, CSB and HIF-1 α (the latter kindly furnished by Dr Kung from Dana Farber Cancer Institute, Boston, USA) protein expressing plasmid at different concentrations. After 48 h, the cells were harvested and screened for galactosidase and luciferase activity. Cells were transfected using the Jet-Pei transfection reagent (Polyplus transfection). Each transfection was repeated four times. Measurement of stable luminescence from the two luciferase reporter genes in a single sample was performed using Dual-Glo™ Luciferase Assay System (Promega).

Supplementary data

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

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