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p53 can repress transcription of cell cycle genes through a p21$^{WAF1/CIP1}$-dependent switch from MMB to DREAM protein complex binding at CHR promoter elements

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Abbreviations: CDE, cell cycle-dependent element; CHR, cell cycle genes homology region; DREAM, DP, RB-like, E2F4 and MuvB complex; MMB, MYB-MuvB complex; ChIP, chromatin immunoprecipitation; cdk, cyclin-dependent kinase

The tumor suppressor p53 plays an important role in cell cycle arrest by downregulating transcription. Many genes repressed by p53 code for proteins with functions in G_2/M. A large portion of these genes is controlled by cell cycle-dependent elements (CDE) and cell cycle genes homology regions (CHR) in their promoters. *Cyclin B2* is an example of such a gene, with a function at the transition from G_2 to mitosis. We find that p53-dependent downregulation of *cyclin B2* promoter activity is dependent on an intact CHR element. In the presence of high levels of p53 or p21$^{WAF1/CIP1}$, protein binding to the CHR switches from MMB to DREAM complex by shifting MuvB core-associated proteins from B-Myb to E2F4/DP1/p130. The results suggest a model for p53-dependent transcriptional repression by which p53 directly activates *p21$^{WAF1/CIP1}$*. The inhibitor then prevents further phosphorylation of p130 by cyclin-dependent kinases. The presence of hypophosphorylated pocket proteins shifts the equilibrium for complex formation from MMB to DREAM. In the case of promoters that do not hold CDE or E2F elements, binding of DREAM and MMB solely relies on a CHR site. Thus, p53 can repress target genes indirectly through CHR elements.

Introduction

The p53 protein is an important tumor suppressor.[1] Furthermore, in recent years, other probably more fundamental functions like its participation in controlling fertility have been discovered.[2,3] Acting as a tumor suppressor, p53 can cause cell cycle arrest and is able to induce apoptosis. In order to carry out these functions, p53 mostly acts as a transcriptional activator or repressor.[4] Transactivation involves DNA binding to a p53 consensus site.[5-9] Importantly, a substantial portion of the genes controlled by p53 are transcriptionally repressed.[9-12] In a genome-wide expression analysis searching for p53 target genes, 38% of 1,425 mRNAs significantly changed in their expression were observed to be downregulated.[13] For most promoters of genes repressed by p53, transcription factor-specific binding sites responsible for the downregulation have not been identified.[11,12] However, in a few cases, mechanisms have been implicated which contribute partially to p53-dependent downregulation of genes like the displacement of activating transcription factors by p53 or by sequestering such transcriptional regulators independent of DNA binding, preventing them from activating genes.[11,14,15] Alternatively, indirect mechanisms without DNA binding by p53 to its downregulated target gene were implicated. The most detailed reports suggested p21$^{WAF1/CIP1}$ as a link.[16-18] An important experiment employing the inhibition of translation by cycloheximide showed that repression of genes like *cyclin B2* depends on protein synthesis, while activated genes as *p21$^{WAF1/CIP1}$* do not.[13] This suggests that p53-dependent activation and repression are controlled by different mechanisms, and that downregulation requires an additional regulatory step, including synthesis of a new protein.

Interestingly, in the collection of genes downregulated by p53 a large portion is transcribed differentially during the cell cycle with promoters controlled through CDE and CHR sites.[19,20] Very recently we identified the DREAM complex to bind the CHR of the *cyclin B2* promoter in G_0. This binding shifts to the B-Myb-containing MMB (MYB-MuvB) complex contacting the CHR independently of the CDE in proliferating cells.[21] DREAM was first discovered in *C. elegans* and flies. In mammals, the complex consists of LIN9, LIN37, LIN52, LIN54 and RBBP4, forming the MuvB core of DREAM, together with E2F4, DP1, p130 and p107.[22-24] The DREAM complex binds to promoters in G_0 and early G_1 and serves to repress transcription. When cells progress

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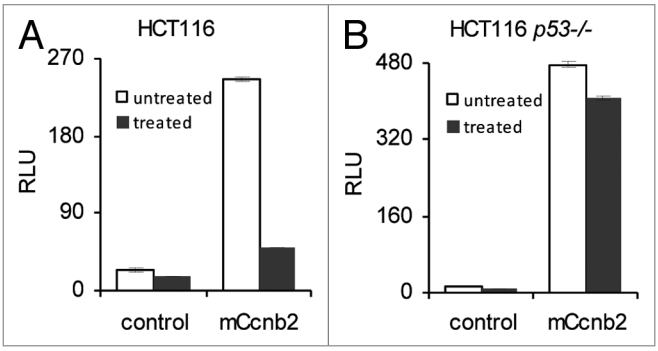

Figure 1. Downregulation of *cyclin B2* promoter activity after DNA damage involves p53. (**A**) HCT116 parental and (**B**) HCT116 *p53-/-* cells were transiently transfected with 250 ng of the mouse *cyclin B2* wild-type luciferase reporter construct (mCcnb2) or empty vector as control (pGL4.10) along with 25 ng Renilla luciferase plasmid (pGL4.70) for monitoring transfection efficiency. Treatment with 0.2 μg/ml doxorubicin was followed by luciferase assays after 48 h.

through the cell cycle, E2F4/DP1 and p130/p107 appear to be released from DREAM, and B-Myb is incorporated into the complex instead to form the MMB (MYB-MuvB) complex, which then can activate gene expression in S phase.[21,23,24,25,26,27] Furthermore, additional proteins like the FOXM1 transcription factor appear to interact and function with MMB or DREAM complexes.[28-30]

In this study, we show that transcriptional repression of *cyclin B2* by p53 requires an intact CHR element in the promoter and p21$^{WAF1/CIP1}$. In response to DNA damage, protein binding to the CHR shifts from the MMB to the DREAM complex.

Results

In an earlier report, we had shown that the tumor suppressor protein p53 can repress transcription of mammalian *cyclin B2*.[31] There is no site in the *cyclin B2* promoter that would resemble the established p53 binding consensus. Thus, downregulation appeared independent from a p53 site in the promoter. Mechanistic aspects regarding the involvement of other proteins, particularly transcription factors and their binding sites, remained unclear. Another question not being addressed was if downregulation of *cyclin B2* after DNA damage, which contributes to G_2/M cell cycle arrest, is dependent on its promoter.

Downregulation of *cyclin B2* promoter activity after DNA damage involves p53. We tested *cyclin B2* promoter downregulation after DNA damage with a wild-type mouse *cyclin B2* (mCcnb2) promoter reporter construct in HCT116 cells. DNA damage was induced by treatment of cells with the chemotherapeutic drug doxorubicin. We observed a decrease in *cyclin B2* promoter activity upon induction of endogenous p53 with doxorubicin in HCT116 cells expressing wild-type p53 (**Fig. 1A**). Downregulation is essentially lost when the experiment is performed in HCT116 *p53-/-* cells (**Fig. 1B**).

p53-dependent *cyclin B2* repression requires p21$^{WAF1/CIP1}$. Next we tested whether p21$^{WAF1/CIP1}$ is essential for p53-dependent repression of the *cyclin B2* promoter. Wild-type mCcnb2 reporter plasmid was transfected into HCT116 and HCT116 *p21-/-* cells, which were subsequently treated with doxorubicin. In contrast to HCT116 cells, HCT116 *p21-/-* cells showed no decrease in *cyclin B2* promoter activity following doxorubicin treatment (**Fig. 2A**). Furthermore, p53-mediated repression of mCcnb2 wt promoter activity upon co-transfection with p53 expression plasmid was nearly absent in HCT116 *p21-/-* cells but not in HCT116 parental and HCT116 *p53-/-* cells (**Fig. 2B**). The 3–4-fold repression in HCT116 and HCT116 *p53-/-* cells was essentially lost in the HCT116 *p21-/-* cells. Thus, these observations indicated that p53-mediated repression of *cyclin B2* transcription depends on p21$^{WAF1/CIP1}$.

As a control, we analyzed cell cycle distribution of the three HCT116 cell variants used in **Figures 1 and 2**. Since lack of functional *p53* or *p21$^{WAF1/CIP1}$* genes affects the cell cycle, it was necessary to show that effects observed on the expression of the *cyclin B2* reporter are not simply due to an altered cell cycle distribution. We noticed that both the *p53-/-* and *p21-/-* cells had a larger G_2/M cell population after doxorubicin treatment when compared with the HCT116 parental cells (**Fig. 2C**). Since *cyclin B2* is expressed in G_2/M, a shift toward these phases generally enhances its expression. Importantly, although this effect strongly compensates a downregulation of G_2/M-expressed genes, still a significant reduction of the *cyclin B2* reporter was observed upon p53 expression in the parental HCT116 and the *p53-/-* cells but not in the *p21-/-* cells (**Fig. 2B**). This argues strongly in favor of requiring p21$^{WAF1/CIP1}$ for repression by p53.

Repression of *cyclin B2* transcription by p53 and p21$^{WAF1/CIP1}$ is dependent on an intact CHR element in the promoter. In the *cyclin B2* promoter, no site that fits the established p53 binding consensus was located.[5,31] Furthermore, p53-repressed genes usually are downregulated by an indirect though largely undefined mechanism. In our earlier report, we observed that regulation is essentially located in the DNA fragment 200 nucleotides upstream of the translational start. However, due to cryptic sites in the pGL3 plasmid contributing non-specific downregulation, we detected only a partial loss of repression after mutating several sites in that fragment (data not shown).[31] To address the question anew which sites control p53-dependent repression, we analyzed wild-type or mCcnb2 mutants in improved pGL4.10 reporter constructs for repression by co-expressed wild-type vs. mutant p53 in HCT116 *p53-/-* cells. The mCcnb2 wt promoter construct was downregulated by wild-type p53 (**Fig. 3A**). Repression was largely lost when the CDE was mutated. In the construct in which the CHR element had been mutated, repression of the *cyclin B2* promoter by p53 was completely abolished. Mutation of both CDE and CHR elements in the same construct also led to a loss of repression but did not result in a further deregulation compared with the CHR mutant (**Fig. 3A**). Taken together, this indicates that the CHR is the main element required for p53-dependent downregulation.

Next, we tested the effect of p21$^{WAF1/CIP1}$ on wild-type and mutant *cyclin B2* promoter constructs in HCT116 *p53-/-* cells. We compared the decrease in reporter activity following overexpression of wild-type p21$^{WAF1/CIP1}$ with expression of a non-functional

p21$^{WAF1/CIP1}$ mutant as a control. Wild-type p21$^{WAF1/CIP1}$ strongly repressed *cyclin B2* promoter reporter activity (**Fig. 3B**). This effect was even greater than the response to p53 expression (**Fig. 3A**). Mutation of the CDE site in the mouse *cyclin B2* promoter construct resulted in an approximately 2-fold loss of repression. The reporter with a mutation in the CHR yielded nearly no remaining repression, and the CDE/CHR double mutant lost all p21$^{WAF1/CIP1}$-dependent downregulation (**Fig. 3B**).

As controls for these experiments, the influence of overexpressed proteins on the cell cycle was tested. Overexpression of p53 wt or p21$^{WAF1/CIP1}$ wt increased the G_1 cell population and lowered fractions in G_2/M phases compared with forced expression of mutant p53 or p21$^{WAF1/CIP1}$, respectively (**Fig. 3C**). However, these small changes in cell cycle distribution are not sufficient to account for alterations in reporter activity. Therefore, downregulation of the *cyclin B2* promoter by p53 or p21$^{WAF1/CIP1}$ is not simply an indirect effect of the partial cell cycle arrest.

Taken together, the results suggest that the CHR is a central element necessary for repression of the *cyclin B2* promoter by p53 and p21$^{WAF1/CIP1}$, whereas the CDE appears to contribute but is not as important as the CHR for repression.

Protein complex binding to the CHR switches from MMB to DREAM after doxorubicin treatment. The loss of repression by mutation of the CHR element prompted us to investigate the recruitment of proteins to the mouse *cyclin B2* promoter. In an earlier report, we showed that the DREAM complex binds to the *cyclin B2* promoter primarily through the CHR in G_0 cells.[21] In order to analyze protein complex composition after DNA damage, we performed DNA affinity purification with biotinylated wild-type, CDE and CHR-mutated *cyclin B2* promoter probes from nuclear extracts of untreated und doxorubicin-treated NIH3T3 cells followed by western blot analysis. A fragment of the mouse *Gapdhs* promoter served as a negative control. Doxorubicin treatment increased binding of p130, p107 and E2f4 to the mCcnb2 probe (**Fig. 4A**). In contrast, a reciprocal reduction of B-myb binding was observed. Interestingly, Lin9 binding was also induced after DNA damage (**Fig. 4A**). Binding of the NF-Y subunit α Nf-ya, which had been shown to bind to CCAAT-boxes of the *cyclin B2* promoter,[32] is independent of the CDE or CHR sites and served as a control for equal pulldown efficiency. Nf-ya binding to all *cyclin B2* probes did not change noticeably following doxorubicin treatment (**Fig. 4A**). When *cyclin B2* promoter probes mutated in the CDE were used, a slight reduction of DREAM protein binding was observed. Mutation of the CHR led to an essentially complete loss of binding of DREAM/MMB complex components to the *cyclin B2* promoter (**Fig. 4A**).

As controls, samples from untreated and doxorubicin-treated cells employed for preparing nuclear extracts were tested for *cyclin B2* mRNA expression and for their cell cycle distribution. Treatment of NIH3T3 cells with doxorubicin for 48 h caused a more than 3-fold decrease in *cyclin B2* mRNA levels (**Fig. 4B**). The cell cycle analysis of the NIH3T3 cells following doxorubicin treatment showed an increase in G_2/M populations (**Fig. 4C**). This shift in cell cycle distribution would lead to an increase of *cyclin B2* expression, and cells should rather shift complex formation from DREAM to MMB after doxorubicin treatment. Since we observed the reverse, it can be concluded that the observations are not simply a result of the overall changes in cell cycle phases. Thus, downregulation of *cyclin B2* expression has to compensate for the resulting increase due to the shift in the cell cycle.

Taken together, these data demonstrate that the CHR is essential for binding of the DREAM/MMB complex to the

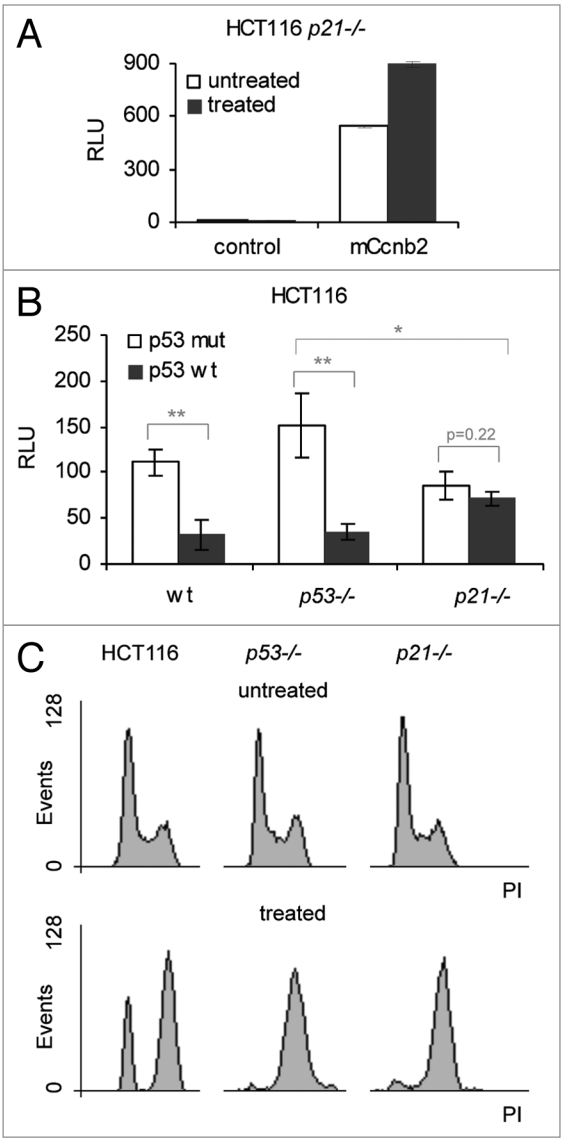

Figure 2. p53-dependent repression of *cyclin B2* requires p21$^{WAF1/CIP1}$. (**A**) HCT116 *p21-/-* cells were transfected with mCcnb2 wt promoter constructs and treated with doxorubicin as described in **Figure 1.** (**B**) HCT116, HCT116 *p53-/-* and HCT116 *p21-/-* cells were transiently co-transfected with 250 ng of the mCcnb2 wt luciferase reporter construct together with 25 ng wt or mutant p53 expression plasmid along with 25 ng Renilla luciferase plasmid (pGL4.70) followed by luciferase assay after 24 h. Differences in transfection efficiencies are compensated by comparison to Renilla luciferase expression (pGL4.70). Mean ± SD; n = 3; *, $p < 0.05$; **, $p < 0.01$; ***. (**C**) FACS analyses of cells cultured in parallel to cells used in **Figures 1 and 2** before and after treatment with doxorubicin. Cells were stained for DNA content with propidium iodide.

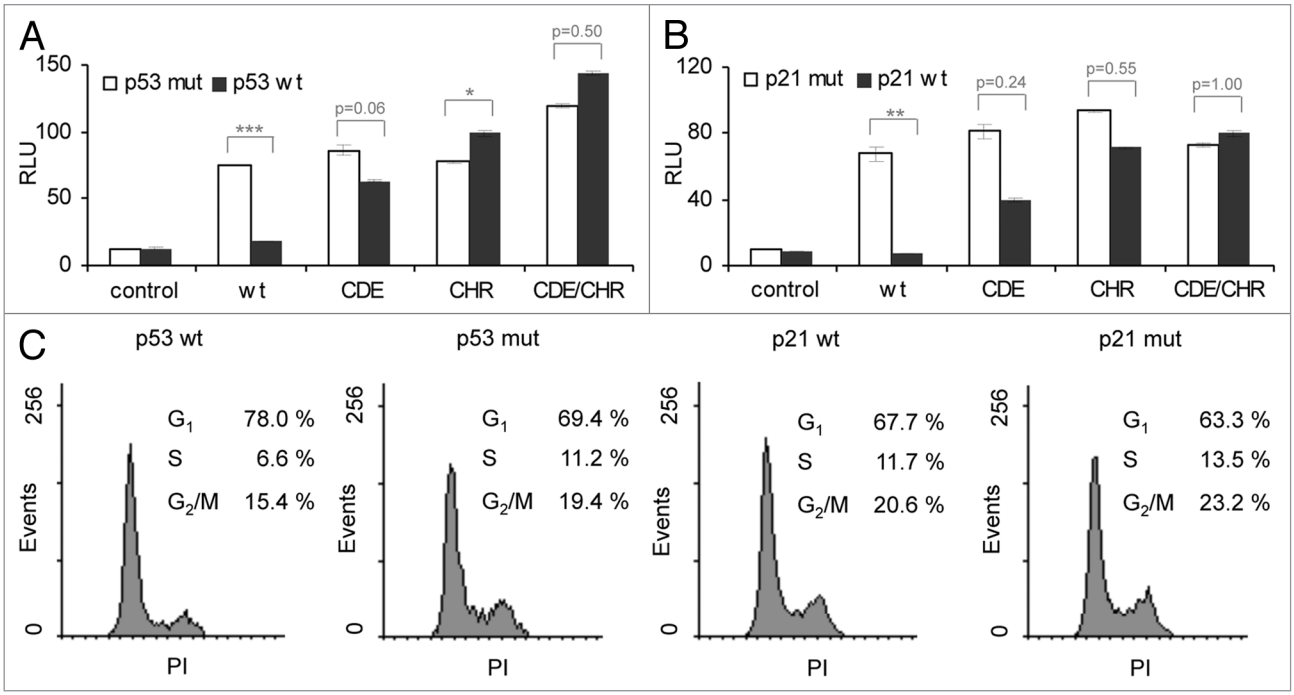

Figure 3. Repression of *cyclin B2* transcription by p53 and p21$^{WAF1/CIP1}$ is dependent on an intact CHR element in the promoter. Luciferase reporter constructs and (**A**) p53 or (**B**) p21 wt and mutant expression plasmids were co-transfected in HCT116 *p53-/-* cells. Luciferase activities of mouse *cyclin B2* wild-type (wt) and mutant promoters (CDE, CHR and CDE/CHR mutants) were measured after 24 h. (**C**) Cell cycle distribution of GFP-expressing cells co-transfected with p53 or p21$^{WAF1/CIP1}$ expression plasmids was determined by FACS. Transfected cells were selected from the total cell pool by the fluorescence originating from expression of the co-transfected GFP plasmid. For FACS analysis of GFP-expressing cells, DNA was stained with Hoechst 33343. Mean ± SD; n = 2; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

cyclin B2 promoter, and protein binding to the CHR switches from MMB to DREAM upon doxorubicin treatment.

p21$^{WAF1/CIP1}$ is required for binding of DREAM components to the CHR element. In order to further elucidate the function of p21$^{WAF1/CIP1}$ in regulating p53-dependent transcriptional repression, we employed the HCT116 cell system for which *p53-* and *p21$^{WAF1/CIP1}$*-deficient mutants are available. Expression of *cyclin B2* mRNA was strongly decreased in HCT116 parental cells but not in HCT116 *p53-/-* and HCT116 *p21-/-* cells following incubation with doxorubicin (**Fig. 4D**). As controls, we analyzed cell cycle distribution before and after doxorubicin treatment. Following incubation with the drug, a substantial shift toward G_2/M cell populations was induced in parental HCT116 cells (as in **Fig. 2C**). Combined with the information that *cyclin B2* is downregulated in cells from the same experiment, it is discernible that negative regulation of *cyclin B2* by doxorubicin treatment was not an indirect effect of a change in cell cycle distribution. In HCT116 *p53-/-* and HCT116 *p21-/-* cells, the shift toward G_2/M cell populations is similar but even more pronounced (**Fig. 2C**). These observations are complementary to results described in **Figure 2B**, and suggest again that p53 and p21$^{WAF1/CIP1}$ appear to be essential to mediate downregulation of *cyclin B2* expression after doxorubicin treatment as well.

In order to directly test whether p21$^{WAF1/CIP1}$ is necessary for the increased binding of p130/E2F4 to the *cyclin B2* promoter, we performed DNA affinity purification with proteins from HCT116 *p21-/-* cells. As controls, parental HCT116 and HCT116 *p53-/-* cells were used. Similar to experiments with extracts from NIH3T3 cells, p130 and E2F4 binding was increased in HCT116 cells after drug treatment (**Fig. 4E**). In contrast, E2F4/p130 binding was not enriched in HCT116 *p21-/-* cell extracts, indicating that p21$^{WAF1/CIP1}$ is necessary for the increased binding of p130/E2F4 to the *cyclin B2* promoter (**Fig. 4E**). Reorganization of the protein complex after DNA damage is also dependent on p53, since we observed that p130/E2F4 binding to the *cyclin B2* promoter was not induced in extracts from HCT116 *p53-/-* cells (**Fig. 4E**).

Taken together, these data indicate that p53 and p21$^{WAF1/CIP1}$ are required after DNA damage to shift the protein complex binding the CHR from the B-Myb-holding MMB to the E2F4/p130-containing DREAM.

In vivo protein binding to CHR-regulated promoters shifts from B-Myb to E2F4/p130 after p53 induction. After finding evidence for a shift from MMB to DREAM binding to CHRs upon p53 activation, we analyzed protein binding in vivo to the mouse and human *cyclin B2* promoters by chromatin immunoprecipitation (ChIP). Chromatin was isolated from untreated und doxorubicin-treated cells and immunoprecipitated with antibodies specific for E2f4, p130, p107, B-myb, Lin9 and p53. In mouse NIH3T3 cells, doxorubicin clearly induced binding of E2f4, p130 and Lin9 (**Fig. 5A**). Recruitment of B-myb was reduced after treatment. As a negative control, these proteins did not bind to the *glyceraldehyd-3-phosphate dehydrogenase* promoter (*Gapdhs*), indicating specific binding in the ChIP assays.

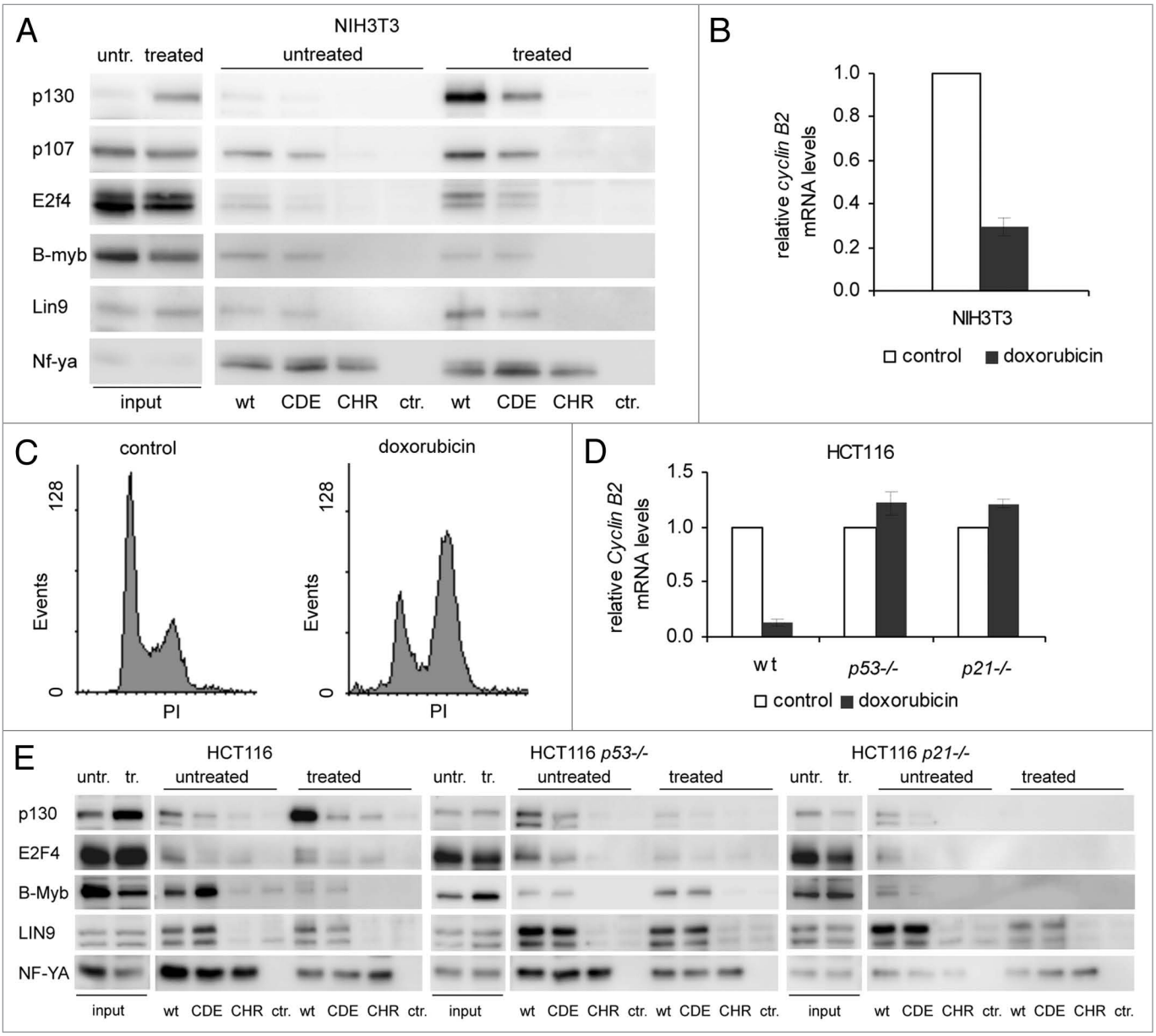

Figure 4. Protein binding to the CHR element switches from MMB to DREAM after doxorubicin treatment, and p21$^{WAF1/CIP1}$ is required for binding of DREAM components to the CHR element. (A) DNA-affinity purification and western analysis of proteins binding to *cyclin B2* wt and mutant promoters. Nuclear extracts from normally proliferating NIH3T3 (**A–C**) and HCT116 cells (**D and E**) untreated or treated with doxorubicin were employed for DNA-affinity purification using biotinylated mouse *cyclin B2* probes. (**A and E**) Bound proteins and input (15 μg) were analyzed by immunoblotting employing antibodies indicated on the left. As a negative control, a fragment of the mouse *Gapdhs* promoter was used. As a protein binding to all *cyclin B2* probes, Nf-ya, the subunit α of the trimeric transcription factor NF-Y, was detected as a control. (**B and D**) *Cyclin B2* expression was analyzed by real-time reverse transcription PCR and normalized to expression of *U6*. (**C**) Cell cycle distribution of untreated and doxorubicin-treated NIH3T3 cells was determined by FACS.

Similar changes in binding pattern of the DREAM/MMB component proteins were observed upon induction of p53 by doxorubicin in human HCT116 cells. It is important to note that while function of the CHR in the mouse *cyclin B2* gene is supported by an adjacent CDE, regulation in the cell cycle and binding of DREAM/MMB in the human *cyclin B2* promoter is solely mediated by a CHR.[21] In the human HCT116 cell system, in vivo binding of E2F4 and p130 was increased, and binding of B-Myb was reduced after treatment with the DNA damaging agent (**Fig. 5B**). The change in binding pattern for E2F4, p130 and B-Myb before and after induction was found reversed when comparing ChIP results from HCT116 *p53-/-* and HCT116 *p21-/-* with HCT116 parental cells (**Fig. 5B**). This indicates that p53 as well as p21$^{WAF1/CIP1}$ are required for the switch from MMB to DREAM in parental HCT116 cells.

The ChIP signal for LIN9 binding was essentially not affected by p53 induction (**Fig. 5B**). This is consistent with the notion that LIN9 is present in the repressive as well as in the activating

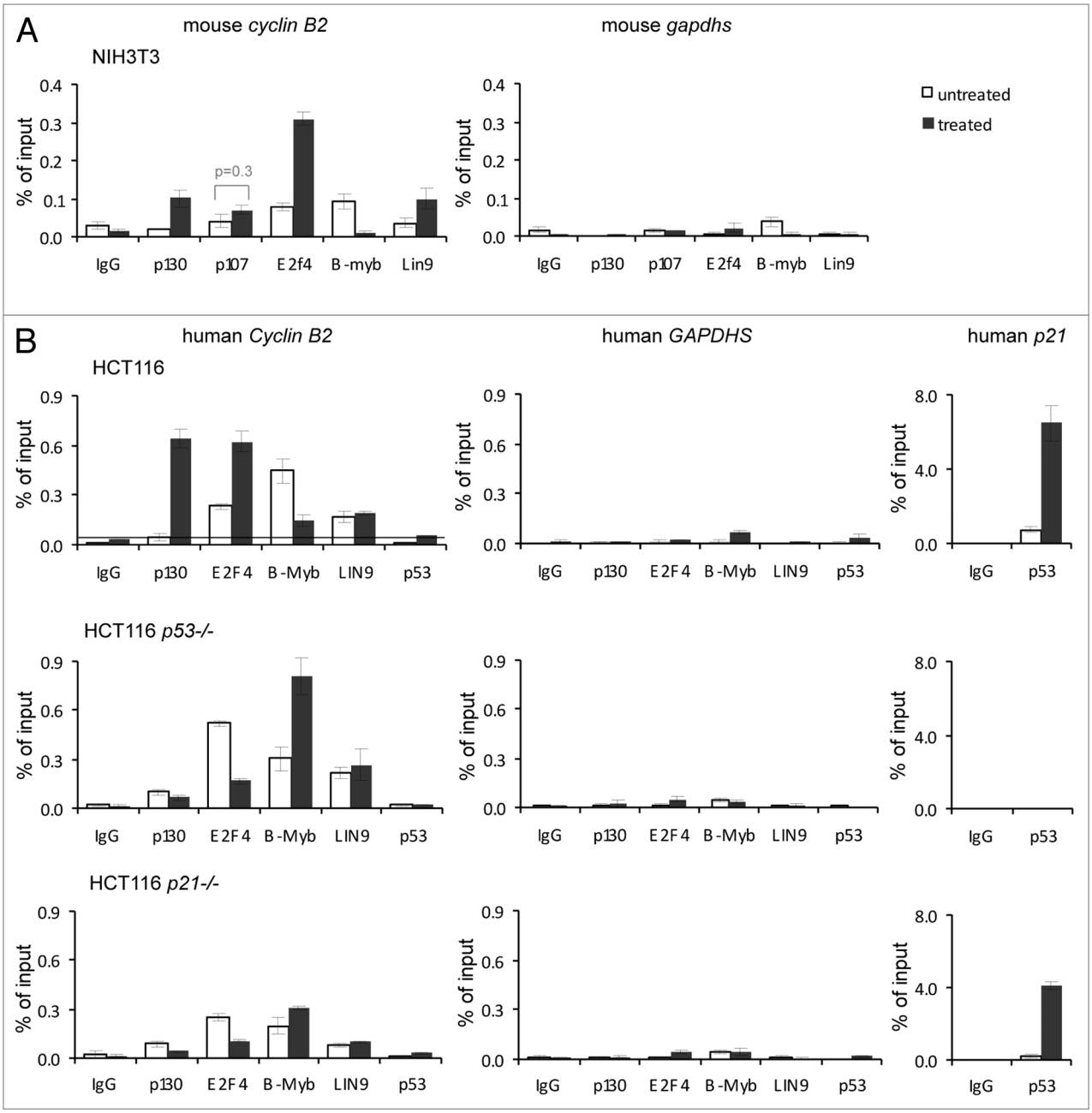

Figure 5. In vivo protein binding to CHR-regulated promoters shifts from B-Myb to E2F4/p130 after p53 induction. Chromatin immunoprecipitations were performed with cross-linked chromatin from untreated or doxorubicin-treated (**A**) NIH3T3 or (**B**) HCT116 cells. Antibodies targeted p130, E2F4, p53, p107, LIN9 or B-Myb. As negative control, rabbit IgG was used. The indicated promoters were detected by real-time PCR. All signals are given relative to the input DNA signal. The horizontal line in (**B**) indicates a threshold under which signals are considered to be background.

complex at the CHR.[21] A significant recruitment of p53 to the *cyclin B2* promoter in untreated or treated cells was not detected, although, as a positive control, p53 binding to the *p21*$^{WAF1/CIP1}$ promoter after induction in HCT116 *p21-/-* and HCT116 parental cells was observed. Protein binding to *Gapdhs* served as negative control (**Fig. 5B**).

In addition to the in vitro protein binding to specific sites in the promoter, we tested in vivo binding of the DREAM/MMB proteins to the CDE and CHR elements by ChIP. To this end, we stably transfected HCT116 cells with luciferase reporter plasmids containing the mouse *cyclin B2* promoter fragments as wild-type, CDE or CHR mutants. These constructs contained the same DNA sequences that we had used as probes in the DNA affinity purification-western analyses. Chromatin was extracted from doxorubicin-treated and untreated cells, and ChIPs were performed for E2F4, p130, B-Myb, LIN9 and p53 binding to the stably transfected promoter. For all proteins, maximal binding appeared at wild-type promoter (**Fig. 6**). Binding of p130 and E2F4 increased after doxorubicin treatment, whereas B-Myb binding decreased. When the CDE in the mouse *cyclin B2*

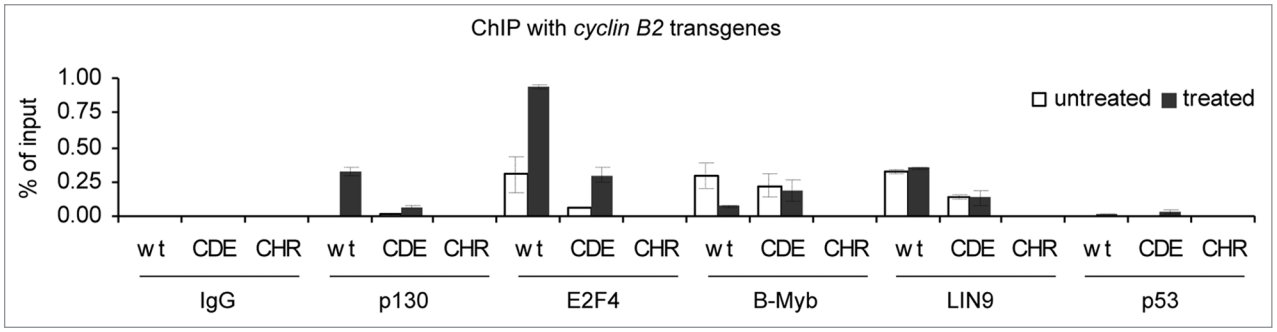

Figure 6. In vivo protein binding switches from MMB to DREAM after DNA damage and is dependent on an intact CHR element. HCT116 parental cells were stably transfected with *cyclin B2* wild-type, CDE or CHR mutant promoter constructs. Nuclear extracts of untreated cells and cells treated with doxorubicin for 24 h were prepared, and ChIPs were performed with antibodies targeting E2F4, p130, B-Myb, p53 and LIN9. As a negative control, a non-targeting rabbit antibody was used. All signals are given relative to the input.

promoter was mutated, a strong decrease of E2F4, p130, B-Myb and LIN9 binding was observed. This observation indicates an important role for the CDE in DREAM binding. In contrast, binding of all proteins was reduced to background level when the CHR was mutated (**Fig. 6**).

p130 is hypophosphorylated as a component of DREAM bound to DNA. We next investigated the phosphorylation pattern of p130 and p107 before and after DNA damage. DNA affinity purification-western experiments showed that only the faster migrating hypophosphorylated form of p130 binds to the mouse *cyclin B2* wild-type probe (**Fig.** 7). For p107, a significant change in intensity or mobility is not observed; after doxorubicin treatment binding of p130 is increased (**Fig. 7A**). A more precise analysis of the phosphorylation status of p130 indicated that just hypophosphorylated p130 is bound to DNA in two cell systems (**Fig. 7B**). This suggests that binding of p130 to the DREAM complex is regulated through p130 hypophosphorylation.

p53 and p21$^{WAF1/CIP1}$ do not bind to their repressed target promoter. As there are reports suggesting that p53 can directly or indirectly contact DNA of its repressed target genes[9,33] and that p21$^{WAF1/CIP1}$ may also form part of a complex binding CDE/CHR promoters,[34] we tested their binding in our assay. While we observed an increase of p53 and p21$^{WAF1/CIP1}$ protein levels in the cell lysates, only weak, non-specific binding of p53 and p21$^{WAF1/CIP1}$, similar to that to a negative-control probe, was detected (**Fig. 8A**).

Hyperphosphorylated cdk2 can form a complex with p130 in solution but not with DREAM bound to target promoter DNA. CDK2 had been observed in precipitates when mammalian DREAM was discovered.[23] We tested if cdk2 is a component of DREAM after p53 induction. Under conditions which allow detection of DREAM components in DNA-affinity purifications, specific binding of cdk2 to the *cyclin B2* promoter was not observed (**Fig. 8A**). It is noteworthy that a non-specific band for cdk2 was detected in all samples, including the negative controls, which establishes that specific cdk2 binding would have been discovered. After looking at DNA-bound proteins, we also tested protein complex formation in solution. The faster migrating band of a doublet specific for cdk2 had been shown to represent the hyperphosphorylated active form carrying a phosphate at Thr 160.[35,36] This cdk2 variant is observed in CoIPs, with p130 in cells before treatment (**Fig. 8B**). None of the two cdk2 forms detected in controls precipitates with p130 after induction of DNA damage.

In addition, p21$^{WAF1/CIP1}$ is not part of the complex containing p130, either before or after doxorubicin treatment. However, p21$^{WAF1/CIP1}$ forms a soluble complex with cdk2 independent of DNA binding (**Fig. 8B**).

Another point we raised is if most p53-target repression depends on cdk inhibition, or whether alternate pathways may contribute significantly to downregulation of p53 targets. We employed the cdk inhibitor roscovitine and compared its response on *cyclin B2* expression to that after doxorubicin treatment. We found that similar downregulation is observed with both reagents (**Fig. 8C**). This effect is not simply due to a shift in cell cycle distribution (**Fig. 8D**). Thus, we conclude that most of the downregulation in this p53-dependent system is due to inhibiting cdks, which in the natural setting is most likely an effect of p21$^{WAF1/CIP1}$ induction.

Taken together, p53 and p21$^{WAF1/CIP1}$ are required after DNA damage to shift the protein complex binding the CHR from the B-Myb-holding MMB to the E2F4/p130-containing DREAM, but p53, cdk2 and p21$^{WAF1/CIP1}$ are not part of the complex contacting the DNA (**Fig. 9**).

Discussion

Many aspects of p53-dependent transcriptional repression have been described as fragments on diverse objects of study before, often controversial, sometimes even contradictory. As an example, specific target sites for p53-dependent repression promoters were either not investigated or could not be confirmed by other groups. In the current report, we provide for the first time a complete set of experiments starting from the induction of DNA damage through the increase in p53 and p21$^{WAF1/CIP1}$ levels, leading to protein complex replacement on a specific DNA response element ultimately resulting in downregulation of a target gene.

Progression through the cell cycle is controlled by a few central regulators. p53 participates in this regulation as an activating transcription factor or by repressing target genes such as *cyclin A,*

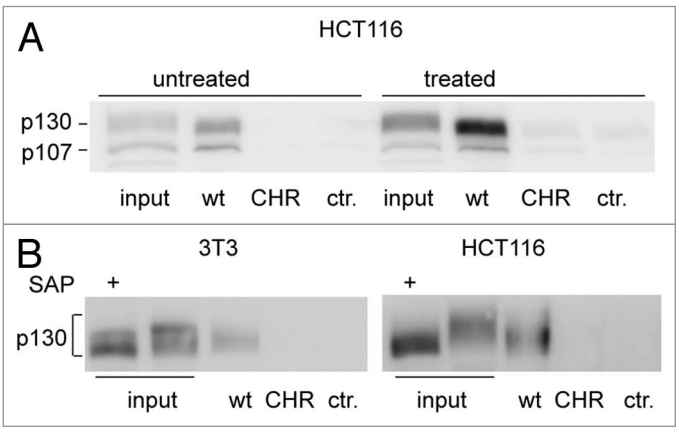

Figure 7. p130 is hypophosphorylated as a component of DREAM bound to DNA. Nuclear extracts from untreated cells or cells treated with doxorubicin for 24 h were employed for DNA-affinity purification using biotinylated mCcnb2 probes. DNA-bound or input (15 μg) protein was analyzed by immunoblotting. As a negative control, a fragment of the mouse *Gapdhs* promoter was used. (**A**) p130 and p107 protein from HCT116 cells from input or bound to promoter DNA. (**B**) Differently phosphorylated p130 in phosphatase-treated or untreated input samples compared with DNA-bound protein. Shrimp alkaline phosphatase (SAP) was used to dephosphorylate input samples. The extracts and eluates were subjected to low-percentage polyacrylamide SDS PAGE.

cyclin B1, cyclin B2, cdc2/cdk1, Cdc25A, Cdc25C, Rad51, PLK1/2 and *BRCA1*.[11,14,15,37-39] From this collection of targets, *cyclin B2* was chosen as an example to study p53-dependent transcriptional repression in detail, since it is required for transition from G_2 to mitosis and is part of the G_2/M checkpoint control;[40] its synthesis is mostly regulated on the transcriptional level and its promoter is well characterized.[21,41]

Our recent discovery with the *cyclin B2* promoter as an example that a protein complex named DREAM can bind solely through the CHR element to promoters[21] led to experiments refining the mechanism of p53-dependent repression by clarifying some aspects or by dismissing others.

The DREAM components were identified to form a complex in human cells.[23,24] While E2F1–3 had been linked to activating functions early in the cell cycle, E2F4 in particular had been implicated to function as a component of the DREAM repressor complex.[23,24,42] The fact that E2F4 and DP1 are part of the complex together with results from ChIP-chip experiments suggested at the time that DREAM binds through E2F4/DP1 to E2F sites in the promoters of cell cycle genes.[23] In contrast to that, we showed that DREAM can regulate cell cycle-dependent repression solely through a CHR element, proving that E2F sites are not required for DREAM binding.[21] More specifically, we have demonstrated that cell cycle-dependent expression of mouse *cyclin B2* is controlled mainly by a CHR with the assistance of a CDE. When looking at protein binding to the mouse *cyclin B2* promoter, we observed that Lin9 and B-myb binding is dependent only on the CHR site, whereas binding of DREAM components E2F4 and p130 also appeared dependent on the CDE site. Importantly, the human *cyclin B2* gene does not contain a CDE. Thus, the DREAM complex binds just through that CHR and represses transcription in G_0. These observations imply that E2F4 and DP1, which are essential components of DREAM, do not bind through an E2F or a CDE site but through other proteins in DREAM to the CHR of such promoters.[21,43]

With this study, investigating the p53 responsiveness of the *cyclin B2* promoter, we show that the CHR with the help of the CDE is responsible for mediating p53-dependent transcriptional repression (**Fig. 3**). When looking at protein binding to these sites in vitro before p53 was induced, we detected members of the MMB complex, in particular B-myb, primarily binding to the CHR. After induction of p53, binding shifts from the B-myb-containing MMB to the DREAM complex, which consists of E2f4/Dp1/p130 instead of B-myb (**Fig. 4**). The same picture is seen when viewing binding in vivo by ChIP in the mouse and human systems (**Fig. 5**). Since the human *cyclin B2* promoter does not contain a functional CDE, in cell cycle regulation as well as in p53-dependent repression, DREAM and MMB binding can depend solely on CHR sites.

Although this binding site preference was not observed before, our results regarding the switch of B-Myb to E2F4/DP1/p130 by changing MMB to DREAM is consistent with a study which showed that B-Myb is required to re-enter the cell cycle after DNA damage-induced G_2 arrest. It was described that after DNA damage or p53 induction, association of B-Myb with LIN9 switches to binding of p130 with LIN9.[44] We describe here that the switch in complex composition happens not only in solution but is observed when protein complexes are located at the CHR site.

It can be assumed that p130 cannot be hyperphosphorylated when it is part of the DREAM complex. In co-immunoprecipitations of the soluble proteins from lysates not bound to DNA, only hypophosphorylated p130 was detected in the complex.[44] We showed that in DREAM bound to DNA, p130 is found in the hypophosphorylated state (**Fig. 7**). For activating E2Fs, hypophosphorylation of RB pocket proteins generally blocks E2Fs, while bound to DNA, from activating transcription and repressing protein complexes, as E2F4/DP1/p130 would also be expected to bind DNA through the E2F component.[45] However, the human *cyclin B2* promoter does not have an E2F or CDE site, and binding of DREAM solely requires the CHR element, which is very different from an E2F site. Thus, it is particularly interesting that E2F4/DP1/p130 as components of DREAM participate in transcriptional repression without contacting the DNA directly through an E2F site. As another difference to the established E2F/RB-pocket protein system, E2F leaves the complex and thereby also the association with DNA after phosphorylation of the pocket protein. Hyperphosphorylation of p130 appears to not only free the pocket protein from DREAM complexes, but also E2F4 and DP1 dissociate from DREAM. Thus, E2F originating from DREAM is not necessarily bound to DNA before or after being set free from pocket protein binding.

A similar concept applies to the B-Myb component of MMB. There is no conserved Myb site in the MMB-binding *cyclin B2* promoter through which the transcription factor would bind DNA. Binding of MMB and thereby also of B-Myb is lost when the CHR is mutated. Consistently, it was observed that

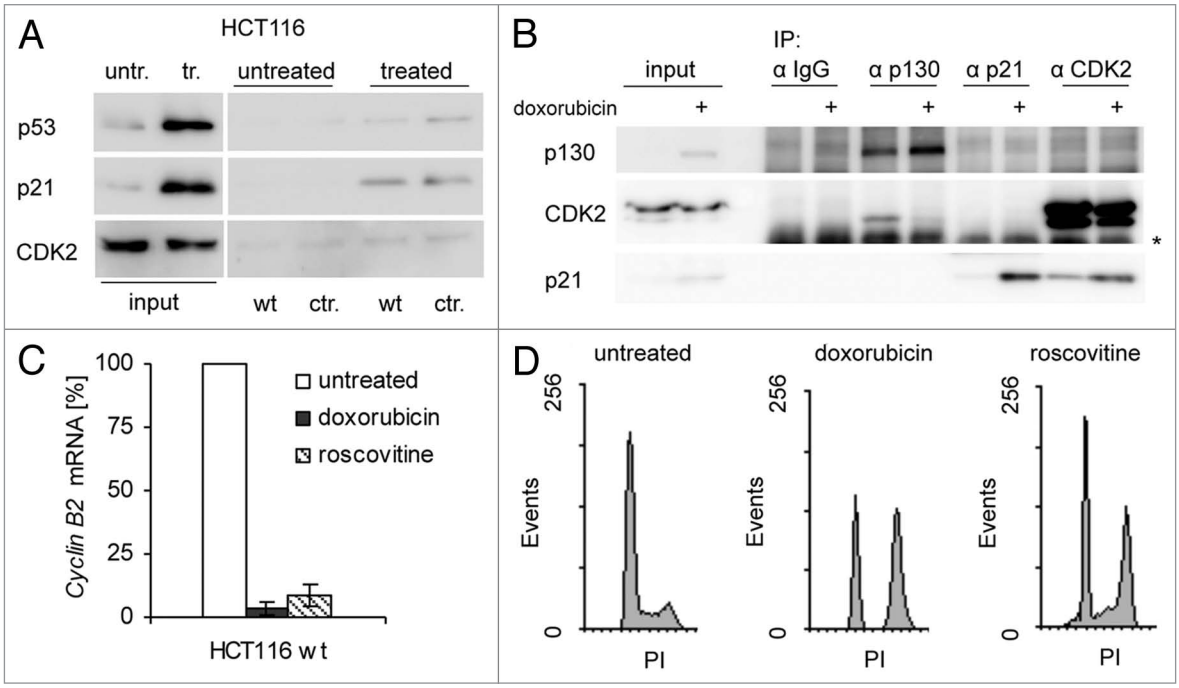

Figure 8. cdk2 interaction with p130 is disrupted after doxorubicin treatment. (**A**) DNA-affinity purification and western analysis was done as described in **Figure 4**. (**B**) HCT116 cells were treated with 0.2 μg/ml doxorubicin for 24 h or left growing in normal media. Lysates were precipitated with the indicated antibodies and bound proteins were detected via immunoblotting employing antibodies indicated on the left. (*; light antibody chain). (**C**) HCT116 wild-type cells were treated with 0.2 μg/ml doxorubicin or 25 μM roscovitine. After 24 h, mRNA was extracted and the relative expression of *cyclin B2* mRNA was measured by qPCR. (**D**) Cell cycle distribution of untreated and treated cells was determined by FACS.

transcriptional activation during the cell cycle is diminished when binding of MMB is lost after mutation of the CHR.[21] Therefore, B-Myb can serve as activator in the MMB complex without itself being directly attached to DNA.

Phosphorylation of p130 and p107 is thought to be controlled by cyclin E(A)-cdk2 and cyclin D-cdk4(6).[46] These cyclin/cdk combinations can be inhibited by $p21^{WAF1/CIP1}$.[47,48] We showed that in cells lacking functional $p21^{WAF1/CIP1}$, p53 is unable to repress its target promoters. Our results are consistent with observations by which $p21^{WAF1/CIP1}$ is necessary and sufficient to downregulate several p53 target genes.[17,18,44] Furthermore, without $p21^{WAF1/CIP1}$, the switch from MMB to DREAM binding to the CHR in vitro and in vivo is no longer possible after p53 induction (**Figs. 4 and 5**). Additionally, we found that expression of $p21^{WAF1/CIP1}$ led to transcriptional repression similar to that observed by p53, which is also mediated mostly through the CHR (**Fig. 3**). Taken together, these observations suggest that $p21^{WAF1/CIP1}$ is required as a signaling link in transcriptional repression downstream from p53 (**Fig. 9**).

Another aspect of cdk function is its participation as a component in complex formation. Very early it has been shown that cdk2 forms a complex with p130/E2F4/DP1.[36] Furthermore, with the initial description of DREAM binding cdk2 in co-immunoprecipitations, it has been implicated that cdk2 is also a component of DREAM.[23] We asked if cdk2 is associated with DREAM when the complex is bound to DNA. We found the hyperphosphorylated and active form of cdk2 to immunoprecipitate with p130 before induction of DNA damage but not with $p21^{WAF1/CIP1}$ (**Fig. 8**). After doxorubicin treatment, no cdk2 was precipitated with either p130 or $p21^{WAF1/CIP1}$. We conclude from these observations that hyperphosphorylated cdk2 forms a complex with p130/E2F4/DP1 in solution but dissociates from the complex when DREAM is bound to promoter DNA.

Some reports implicate that p53 and $p21^{WAF1/CIP1}$ protein binding to target promoters is required for repression. Recently, it had been suggested that $p21^{WAF1/CIP1}$ is tethered to CDE/CHR-regulated promoters.[34] However, from DNA-affinity purification followed by western analysis we have no indication that $p21^{WAF1/CIP1}$ binds to p53-repressed promoters (**Fig. 8**). Furthermore, ChIP results obtained with real-time PCR on the p53-repressed target promoter showed that p53 binds in vivo only at background level in human and mouse cells when compared with the association with the *p21*$^{WAF1/CIP1}$ promoter as a positive control (**Figs. 5 and 6**). This stands in contrast to observations on p53 binding to the *cyclin B2* and other promoters made earlier with slightly differing techniques.[37,49] However, our observations are consistent with genome-wide p53 DNA binding studies.[50,51] In the light of the importance the CHR appears to have for switching MMB/DREAM binding, taken together with the observation that CCAAT-boxes and changes in binding of NF-Y are not necessary and binding of p53 to the target promoter is not observed in p53-dependent repression, we conclude that promoters repressed through CDE/CHR sites appear not to be part of the p53/NF-Y liaison.[33] However, it remains to be elucidated what the function is, besides serving as basal activating sites of the mostly multiple CCAAT-boxes often found in CDE/CHR promoters.[20,33]

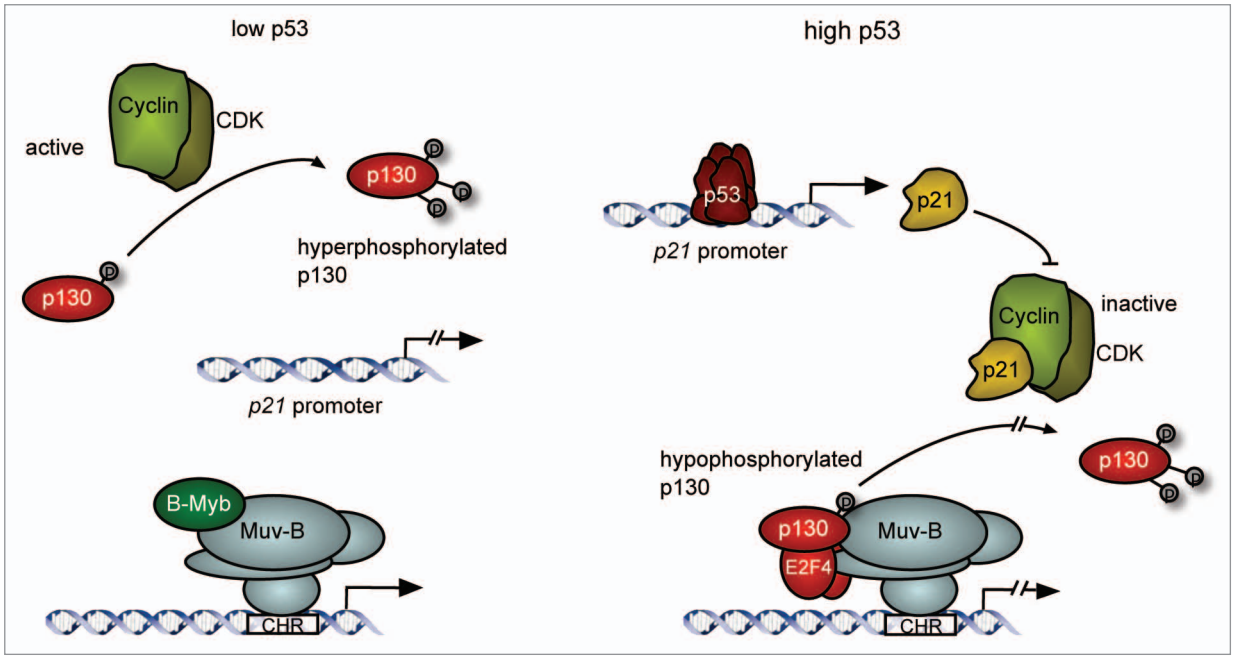

Figure 9. p53 controls transcriptional repression of target genes by an indirect mechanism through CDE and CHR elements. High levels of p53 lead to transcriptional activation of its target *p21*$^{WAF1/CIP1}$. Inhibition of cyclin/cdk complex activity by p21$^{WAF1/CIP1}$ then leads to hypophosphorylation of p130. Hypophosphorylated p130, together with E2F4/DP1, can then form complexes with the MuvB core replacing B-Myb. During the switch from activating MMB to DREAM, the MuvB core remains bound to CHR elements. Binding to CHRs is in some promoters supported by an adjacent CDE site. In summary, p53 activation causes a switch from MMB to DREAM bound to CHR sites turning an activating to a repressing transcriptional complex.

Another aspect not being explained by older models on p53-dependent transcriptional repression is that synthesis of new protein is necessary. With the results presented here, particularly by the step involving synthesis of p21$^{WAF1/CIP1}$ protein, which appears to be amplified by a positive feedback loop by which p21$^{WAF1/CIP1}$ can increase p53 protein levels through reducing mdm2-dependent p53 degradation,[52] we explain the requirement for protein synthesis when genes are repressed by p53.[13]

It is expected that many more promoters are regulated by p53 through a mechanism switching MMB to DREAM binding to CDE/CHRs, since more than 800 genes had been shown by a genome-wide approach to bind DREAM components.[23] Thus, *cyclin B2* likely represents a large fraction of p53-repressed genes displaying the same mechanism for p53-dependent repression. When DREAM was first described, it was open at which sites MMB and DREAM would bind. By identifying the CHR as the transcription factor binding site and the required element for repression, we resolve the quest for a p53 repressor element.[11,12]

In summary, our data are consistent with a new model for p53-dependent transcriptional repression (**Fig. 9**). The induction of p53 directly activates its transcriptional target *p21*$^{WAF1/CIP1}$. The inhibitor then prevents further phosphorylation of p130 by cdks. The presence of hypophosphorylated pocket proteins shifts the equilibrium for complex formation from MMB to DREAM. One factor supporting this shift is the concomitant downregulation of B-Myb by p53.[53] In the case of promoters which do not hold CDE or E2F elements, binding of DREAM and MMB solely relies on a CHR site. Thus, p53 can repress target genes indirectly through CHR cell cycle elements. The elusive p53 repression-responsive region postulated for many genes is likely identified with the CHR.

Materials and Methods

Cell culture, drug treatment and FACS analysis. HCT116 parental, HCT116 *p53-/-* and HCT116 *p21-/-*, kindly provided by Bert Vogelstein,[54] and NIH3T3 cells (ACC59, DSMZ) were grown as described.[55] Stably transfected HCT116 cell lines were created by transfecting promoter-reporter constructs based on the pGL4.14-Hygro vector (Promega) and selected with hygromycin (PAA) at a concentration of 500 mg/ml. Cells were treated with 0.2 μg/ml doxorubicin for 24 or 48 h. FACS analyses were described earlier.[21] Roscovitine (Sigma) was dissolved as a 25 mM stock solution in chloroform and cells were grown for 24 h in the presence of 25 μM roscovitine.

Plasmids and DNA probes. The short mouse *cyclin B2* promoter with a size of 211 bp (nt -210 to +1, named mCcnb2) was produced by PCR amplification and subsequent ligation in pGL4.10 vector (Promega). DNA probes for affinity purification with the same sequence as mCcnb2 and the mouse *Gapdhs* promoter were generated as described earlier.[21]

The human p53 expression plasmids, pcDNA-p53wt and pcDNA-p53mut, were produced by amplifying the insert of pCMV-p53wt and pCMV-p53mut R175H (kindly provided by Bert Vogelstein) and ligation in pcDNA3.1HisC (Invitrogen). Expression plasmids for human p21$^{WAF1/CIP1}$, pCEP-p21wt and pCEP-p21mut were generously provided by Bert Vogelstein.[8]

Transfections and luciferase assays. For measuring p53-dependent promoter activity with luciferase reporter assays, HCT116 cells were plated in 24-well plates (75,000 cells per well) and transfected by lipofection with Fugene 6 (Promega).[56] Cells were cultured overnight before cotransfection of 250 ng of promoter reporter plasmids (pGL4.10, Promega) along with 25 ng of constructs expressing wild-type or mutant p53 or p21 proteins and 25 ng *Renilla* luciferase plasmid (pGL4.70, Promega). After 24 h, cells were collected, and luciferase activity was measured with the Dual-Luciferase Reporter Assay system (Promega).

DNA-affinity purification. DNA-affinity purification of protein complexes with untreated and doxorubicin-treated cells was performed as described before.[21]

RNA extraction, reverse transcription and quantitative real-time PCR. Total RNA was isolated from cell lines using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. One-step reverse transcription and quantitative real-time PCR was performed on a LightCycler instrument (Roche) using QuantiTect SYBRGreen PCR Kit (QIAGEN). *U6* was used as an endogenous control.

SDS PAGE and western blot. SDS PAGE and western blot were performed following standard protocols.[57] For detection of DREAM complex components, the following antibodies were applied: E2F-4 (C-20, Santa Cruz Biotechnology), p130 (C-20, Santa Cruz Biotech.), p107 (C-18, Santa Cruz Biotechnology), LIN9 (ab62329, Abcam), NF-YA (G-2, Santa Cruz Biotechnology). The B-Myb LX015.1 monoclonal antibody was a kind gift from Roger Watson.[58] Antibodies against p53 (Ab-6, DO-1) and p21 (Ab-1, EA10) were purchased from Calbiochem.

Phosphatase treatment. Thirty μg of nuclear extract were mixed with phosphatase buffer. Five units of shrimp alkaline phosphatase (SAP) were added and incubated for 60 min at 37°C.

Co-immunoprecipitations. Cells were lysed in native lysis buffer. For immunoprecipitation, 2 mg of the whole-cell lysate were used and incubated with 2 μg of the desired antibodies for 1 h at 4°C. After addition of 35 μl Protein G Dynabead suspension (Invitrogen), the mixture was incubated for 15 min at room temperature. The beads were washed twice with lysis buffer and three times with washing solution before SDS gel loading buffer was added and heated at 70°C for 5 min. Proteins were immunoprecipitated and analyzed by western blot with antibodies directed against p130 (C-20, Santa Cruz Biotechnology), p21 (Ab-1, EA10, Calbiochem) and cdk2 (M2, Santa Cruz Biotechnolgy).

Chromatin immunoprecipitations. ChIPs were performed as described earlier with the following antibodies: E2F-4 (C-20, Santa Cruz Biotechnology), p130 (C-20, Santa Cruz Biotechnology), p107 (C-18, Santa Cruz Biotechnology), B-Myb (N-19, Santa Cruz Biotechnology), p53 (FL-393, Santa Cruz Biotechnology), p53 (Ab-6, DO-1, Calbiochem) and LIN9 (ab62329, Abcam).[21] Another LIN9 antibody was a kind gift from J.A. DeCaprio. A non-related rabbit antibody was used as a control for non-specific signals. For all precipitations, 1–2 μg of antibody and 20–35 μl of Protein G Dynabead suspension (Invitrogen) were used. ChIP primer sequences can be obtained upon request.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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