Histone Demethylase KDM5B Collaborates with TFAP2C and Myc To Repress the Cell Cycle Inhibitor $p21^{cip}$ (CDKN1A)

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The TFAP2C transcription factor has been shown to downregulate transcription of the universal cell cycle inhibitor $p21^{cip}$ (CDKN1A). In examining the mechanism of TFAP2C-mediated repression, we have identified a ternary complex at the proximal promoter containing TFAP2C, the oncoprotein Myc, and the trimethylated lysine 4 of histone H3 (H3K4me3) demethylase, KDM5B. We demonstrated that while TFAP2C and Myc can downregulate the CDKN1A promoter independently, KDM5B acts as a corepressor dependent on the other two proteins. All three factors collaborate for optimal CDKN1A repression, which requires the AP-2 binding site at −111/−103 and KDM5B demethylase activity. Silencing of TFAP2C-KDM5B-Myc led to increased H3K4me3 at the endogenous promoter and full induction of CDKN1A expression. Coimmunoprecipitation assays showed that TFAP2C and Myc associate with distinct domains of KDM5B and the TFAP2C C-terminal 270 amino acids (aa) are required for Myc and KDM5B interaction. Overexpression of all three proteins resulted in forced S-phase entry and attenuation of checkpoint activation, even in the presence of chemotherapy drugs. Since each protein has been linked to poor prognosis in breast cancer, our findings suggest that the TFAP2C-Myc-KDM5B complex promotes cell cycle progression via direct CDKN1A repression, thereby contributing to tumorigenesis and therapy failure.

The activation factor 2 (TFAP2) family consists of five homologous, developmentally regulated transcription factors, TFAP2A to -E, each encoded by a separate gene. Structurally, TFAP2 proteins contain a highly conserved C-terminal helix-span-helix motif required for dimerization, a basic DNA binding domain, and a third, less-conserved region toward the N terminus which contains a proline and glutamine-rich activation domain. These factors have been shown to bind to palindromic GC-rich DNA recognition sequences as either homo- or heterodimers and act as transcriptional activators or repressors in a promoter-specific manner (9).

Although studies in knockout mice (reviewed in reference 9) and of phenotypically related inherited human traits (25, 32) have shown that these factors have important functions during embryogenesis, they are minimally expressed in most adult tissues. However, expression of the TFAP2A and TFAP2C proteins has been demonstrated in a variety of solid tumors, including breast cancer and melanoma (reviewed in reference 24). TFAP2A expression in breast tumors has been associated with a favorable outcome and shows a positive correlation with expression of estrogen receptor α (ERα) and CDKN1A (14), while elevated expression of TFAP2C has generally been correlated with an adverse phenotype and resistance to hormone therapy (13, 16). Studies *in vitro* have shown that direct AP-2 transcriptional targets include many genes involved in tumor progression. Of particular interest is the regulation of the universal cell cycle inhibitor $p21^{cip}$ (CDKN1A), which can be activated through p53-dependent and -independent pathways to mediate cell cycle arrest (1). AP-2 factors bind a specific sequence in the proximal region of the promoter, and while TFAP2A positively regulates CDKN1A expression (27, 41), TFAP2C has been shown to repress its expression in breast cancer cells (36). These opposing activities at the CDKN1A locus may contribute to the contrasting phenotypes associated with tumors expressing these two AP-2 factors.

When mediating transcriptional activation, it has been shown that AP-2 factors associate with the CITED family of adapter proteins (Cited 2 and 4), which in turn recruit the histone acetyltransferases (HATs) CBP/p300 (2, 7). To date, no clear AP-2 corepressor proteins have been identified, although modification through sumoylation may be required for repressor activity (4, 10). TFAP2A has also been reported to interact with other nuclear factors, including Myc, pRB, and p53 (reviewed in reference 9), to regulate the transcription of target genes. The proto-oncogene *MYC* controls cellular growth, differentiation, and apoptosis, and its deregulation contributes to the development of a variety of cancers, including breast cancer. It encodes a basic region-helix-loop-helix-zipper (bHLHZ) transcription factor which forms a specific heterodimer with the small bHLHZ protein, Max. Myc-Max heterodimers bind with high affinity to the palindromic DNA sequence CACGTG (E box) but can also bind several other related sequences, leading to interaction at up to 15% of all promoters. While the genes activated by Myc include those required for cell growth and cell cycle progression, most of the genes it downregulates are involved in cell cycle arrest, and they notably include $p21^{cip}$ (CDKN1A) (11, 17). As with other activators of transcription, Myc is able to recruit HAT-containing coactivator complexes to its target genes (reviewed in reference 8), but the precise mechanism behind Myc-mediated gene repression is still disputed: Myc interaction occurs close to the transcriptional start

site (TSS), but direct DNA binding, at least at the *CDKN1A* gene, is not required (12, 17).

The reported colocalization of TFAP2C and Myc at the proximal promoter of $p21^{cip}$ (*CDKN1A*) prompted us to investigate the possibility of a functional link between these two transcription factors in the repression of this gene in breast tumor cells. Moreover, recent studies have suggested that Myc forms a complex with the transcriptional corepressor KDM5B (lysine-specific demethylase 5B; also known as Jarid1B/PLU-1). Like TFAP2C, the KDM5B protein is overexpressed in breast cancer lines and tumors (22). Its proven ability to demethylate H3K4me3 (trimethylated lysine 4 of histone H3), an epigenetic marker particularly associated with the TSS of active promoters, is consistent with the transcriptional repressor role associated with this protein, and it has been proposed that KDM5B may promote tumorigenesis by downregulating expression of oncosuppressor genes, such as *BRCA1* and *HOXA5* (26, 39). In this work, we have investigated whether KDM5B regulates the expression of *CDKN1A* in cooperation with the TFAP2C and Myc proteins. Our data show that TFAP2C, Myc, and KDM5B form a functional protein complex in the vicinity of the TSS of the *CDKN1A* gene and corepress its expression in proliferating and drug-treated breast cancer cells, thus promoting cell cycle progression.

MATERIALS AND METHODS

Cell culture, transient transfection, and antibodies. MCF-7, HepG2, and H1299 cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in 10% CO2 at 37°C. The shTFAP2C-MCF-7 line (36) was induced using 1 μg/ml doxycycline (Sigma). Transfection reagents were Genejuice (Merck) for plasmid DNA and Interferin (Polyplus) for small interfering RNA (siRNA), used according to the manufacturer's directions. Subconfluent MCF-7 or shTFAP2C-MCF-7 cells were transiently transfected with 20 nM nonsilencing control siRNA (no. 1022076; Qiagen) or with validated siRNA targeting KDM5B (J-009899-06; Darmacon) or Myc (6341; Cell Signaling). The following antibodies for Western blotting (Santa Cruz unless stated otherwise) were used: KDM5B (3273; Cell Signaling), Myc tag (2276; Cell Signaling), p53 (p53-DO-1, sc-126), TFAP2C (6E4, sc-53162), $p21^{cip}$ (2946; Cell Signaling), Myc (sc-788), β-actin (C-2, sc-8432), lamin A/C (4477; Cell Signaling), green fluorescent protein (GFP) (sc-8334), and Hsc70 (sc7298). Horseradish peroxidase-conjugated secondary antibodies were from DakoCytomation.

Plasmids and reporter assays. The pcDNA3.1-based TFAP2C and KDM5B expression plasmids, the pGL-3-CDKN1A luciferase reporter, and pGL-3-Mut4/Mut5 constructs have been previously described (26, 27). The fusion protein GFP-TFAP2C was generated by cloning the TFAP2C cDNA sequence in frame into the pEGFP-C1 vector (Clontech). The C-terminal deletion mutant (ΔC) was truncated at the internal SalI restriction site, retaining amino acids 1 to 180. The N-terminal deletion mutant (ΔN) fused sequences C-terminal of the SalI site (amino acids 181 to 450) to EGFP and retains the basic region and the helix-span-helix dimerization domains (5). To ensure efficient nuclear translocation of all fusion proteins, an oligonucleotide (5′-GA TCT CCA AAA AAG AAG AGA AAG GTT CA-3′), encoding a recognized nuclear localization signal (15), was ligated in frame between the GFP and wild-type (wt) or truncated TFAP2C sequences. The KDM5B deletion mutant constructs were as described previously (38). The pcDNA3-Myc expression plasmid was from Addgene. Reporter assays were controlled by cotransfection with the pSV-β-galactosidase control vector (Promega). Cells were harvested after 48 h and processed for Western blotting or assayed for luciferase and β-galactosidase activity (Promega).

RNA analysis. Total RNA was extracted from subconfluent cells (RNeasy minikit; Qiagen), and 500 ng was reverse transcribed using the high-capacity

reverse transcription kit (Applied Biosystems). The cDNA was analyzed by quantitative real-time PCR (q-RT-PCR) using TaqMan reaction mix and the StepOne real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. TaqMan primer/probe sets were used to analyze the expression of TFAP2C (Hs00231476_m1), CDKN1A (Hs00355782_m1), KDM5B (Hs00366783_m1) and Myc (Hs0095030_m1), while a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan probe (no. 402869) was used as an internal control (Applied Biosystems). Analysis of q-RT-PCR data was carried out using the comparative $\Delta\Delta C_T$ method. For each sample, the intensity of the amplimer was normalized against that of the GAPDH control, and the data were depicted as fold changes ($2^{-\Delta\Delta CT}$) with respect to the RNA level observed in the control experiments.

ChIP assay. Subconfluent MCF-7 or shTFAP2C-MCF-7 cells were cross-linked by addition of formaldehyde directly to the culture medium to a final concentration of 1% for 10 min and assayed using the ChIP-IT Express chromatin immunoprecipitation (ChIP) kit (Active Motif). Chromatin was sheared to an average size of 500 bp using a Sonics Vibracell VCX500 sonicator and immunoprecipitated with ChIP-validated antibodies against KDM5B (3), TFAP2C (H77, sc-8977; Santa Cruz), Myc (sc-764), histone H3 (ab1791; Abcam), monomethylated lysine 4 of histone H3 (H3K4me1) (ab8895; Abcam), dimethylated lysine 4 of histone H3 (H3K4me2) (ab7766; Abcam) or H3K4me3 (ab8580; Abcam), and 2 μg purified rabbit IgG (sc2027). Quantification of immunoprecipitated DNA was carried out in triplicate using the StepOne real-time PCR system with SYBR green PCR master mix (Applied Biosystems). Primer sequences and calculation of values as fold enrichment compared with results for the IgG control were as detailed previously (36).

Coimmunoprecipitation assays. Immunoprecipitation was carried out according to the manufacturer's protocol using the Universal Magnetic coimmunoprecipitation kit (Active Motif). Cells were lysed in nuclear extraction buffer supplemented with 5 μM trichostatin A, 1 μM phenylmethylsulfonyl fluoride (Sigma), and protease inhibitor cocktail (Roche). Nuclear extract (0.5 mg) was incubated with 50 μl of protein G-coated magnetic beads and 2 μg of either KDM5B (ab27689; Abcam), TFAP2C (6E4, H77 or AP25H01, sc-81181), Myc (sc-788), Myc tag (no. 2276; Cell Signaling), or control IgG (sc-2027) antibody overnight at 4°C. Immunoprecipitates were washed three times with the supplied wash buffer for 5 min at 4°C and resuspended in 45 μl of 2× SDS Western loading buffer. For GFP-tagged proteins, immunoprecipitation was carried out using the GFP-Trap kit (ChromoTek). Samples were resolved via SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes for Western blot analysis. Additional Western blots established that the ΔC mutant is recognized only by the H77 antibody while the ΔN mutant interacts with all three TFAP2C antibodies.

Cell cycle analysis. Asynchronous HepG2 cells were transiently transfected with expression vectors and treated with 1 mM hydroxyurea, 6 nM vinblastine, or vehicle alone (control). After incubation at 37°C for 16 h, cells were trypsinized, washed in phosphate-buffered saline, and fixed using ice-cold 70% ethanol. Fixed cells were incubated with 50 μl of 100 μg/ml RNase and stained with 300 μl of 50-μg/ml propidium iodide (Invitrogen) for 30 min at room temperature. Flow cytometry and cell cycle analysis were performed as previously described (36).

RESULTS

***CDKN1A* repression by TFAP2C, KDM5B, and Myc.** To study the relative activities of TFAP2C, KDM5B, and Myc in the repression of *CDKN1A*, we used the estrogen receptor-positive (ER$^+$) MCF-7 breast cancer line, which expresses all three factors. We have previously described an MCF-7 derivative line with doxycycline-inducible short hairpin RNA (shRNA) targeting TFAP2C (shTFAP2C cells); induction of TFAP2C silencing leads to upregulation of *CDKN1A* and p53-independent G₁/S arrest (36). Here, transient transfection of this line with validated specific siRNA against Myc and KDM5B allowed us to examine the expression level of the endogenous *CDKN1A* transcript and protein

(Fig. 1A and B) after silencing each factor either individually or in combination. TFAP2C and Myc silencing, alone or together, induced expression of the *CDKN1A* transcript, while knockdown of KDM5B alone or with either TFAP2C or Myc had little effect. However, silencing all three proteins significantly increased *CDKN1A* mRNA induction (Fig. 1A, compare last 2 columns), indicating that KDM5B can collaborate with the two transcription factors to repress *CDKN1A* transcription in cycling cells. We observed a similar pattern of induction at the protein level (Fig. 1B and C).

Reporter assays were used to confirm that *CDKN1A* repression by these factors occurs at the promoter level. Initially, HepG2 cells (which do not express AP-2 factors or KDM5B) were transiently transfected with a luciferase reporter construct containing the *CDKN1A* promoter (−2325/+8), and escalating amounts of an expression vector for each test factor individually. As shown previously, independent overexpression of either TFAP2C or Myc repressed *CDKN1A* promoter activity in a dose-dependent manner; however, addition of KDM5B alone did not significantly alter reporter activity (Fig. 2A). In contrast, when a constant, suboptimal dose of the TFAP2C construct was cotransfected with increasing amounts of the KDM5B expression construct, KDM5B corepressor activity was observed, since reporter activity declined as the dose of KDM5B plasmid increased (Fig. 2B). A similar result was also seen with a constant level of the Myc expression construct (data not shown). Moreover, by optimizing transfection input levels for all the constructs, we could demonstrate that coexpression of all three proteins significantly further repressed *CDKN1A* promoter activity (Fig. 2C), thus mirroring the induction of endogenous *CDKN1A* mRNA observed after cosilencing TFAP2C, KDM5B, and Myc in MCF-7 cells (Fig. 1A).

TFAP2C, Myc, and KDM5B form a nuclear complex in MCF-7 cells. To address if negative regulation of *CDKN1A* by TFAP2C, Myc, and KDM5B occurs through complex formation between these factors, we first used confocal microscopy to show that all three proteins colocalize in the nuclear compartment of MCF-7 cells, and TFAP2C silencing did not alter the localization of KDM5B or Myc (see Fig. S1 in the supplemental material).

Next, we looked for physical interaction between these factors, examining endogenous proteins present in MCF-7 nuclear extracts by coimmunoprecipitation (coIP) assays. As seen in Fig. 3A, Myc and TFAP2C were both immunoprecipitated using an antibody specific for KDM5B (lane 5), and TFAP2C and KDM5B were also both detected in anti-Myc immunoprecipitates (lane 6). In order to detect endogenous KDM5B or Myc in TFAP2C immunoprecipitates, we tested antibodies specific for different regions along the 450-amino-acid (aa) protein. The H77 polyclonal antibody was raised against a peptide corresponding to amino acids 145 to 221; 6E4 recognizes an epitope within the C-terminal 20 aa (13), while AP25H01 was raised against a recombinant protein corresponding to amino acids 50 to 200 of TFAP2C. Interestingly, Myc was coprecipitated with both H77 and 6E4, while KDM5B was precipitated only with 6E4 (Fig. 3A, compare lanes 3 and 4). Although AP25H01 efficiently precipitated TFAP2C, it did not bring down either KDM5B or Myc (lane 2), suggesting that the epitope recognized by this antibody is not accessible when TFAP2C interacts with KDM5B and/or Myc. These assays confirm that endogenous TFAP2C, Myc, and KDM5B interact physically with each other to form a complex.

Protein domains required for interaction and KDM5B corepressor activity. KDM5B is a 1,544-aa protein which comprises several conserved domains, including an ARID (A/T-rich interacting domain) domain that, in the case of KDM5 family members, binds GC-rich DNA sequences (26, 34), a C5HC2 zinc finger (Zf), three plant homeobox (PHD) domains, a Jumonji N (JmjN) domain, and the Jumonji C (JmjC) demethylase catalytic domain (Fig. 3B) (38). Previous studies of the KDM5B *Drosophila melanogaster* ortholog, Lid, and mammalian KDM5A/B established that Myc can interact independently with both the JmjC and Zf domains (20, 28). To determine which KDM5B domains mediate interaction with TFAP2C, we expressed wild-type TFAP2C together with a series of tagged KDM5B deletion mutants (Fig. 3B) (38) in H1299 cells, which do not express either factor. After 48 h, nuclear lysates were prepared for coIP with the TFAP2C antibody, 6E4. Proteins lacking the JmjN, Arid, JmjC, Zf, or PHD1 domain still bound TFAP2C, while removal of the two C-terminal PHD domains abolished TFAP2C interaction (Fig. 3C, mutant ΔC). Thus, distinct KDM5B domains are required for its interaction with TFAP2C and Myc.

For the reciprocal experiment, GFP fusion proteins with full-length or truncated TFAP2C sequences were generated (see Materials and Methods): deletion of the C-terminal region (ΔC) retained the proline-rich activation domain, while ΔN retained just 40 amino acids of N-terminal sequence plus the C-terminal basic DNA binding and helix-span-helix dimerization domains. Each fusion protein was expressed in H1299 cells, together with full-length, tagged KDM5B, and nuclear extracts were assayed for interacting proteins using the GFP-trap system. As shown in Fig. 3D, only the full-length and ΔN fusion proteins bound KDM5B and endogenously expressed Myc. Assessing this finding together with the coIP data with TFAP2C antibodies (Fig. 3A) suggests that the basic-helix-span-helix domains and the 40 amino acids immediately N-terminal of this region are necessary for TFAP2C interaction with both Myc and KDM5B.

To identify the KDM5B domains required for corepressor function, we transiently transfected HepG2 cells with the (−2325/+8) *CDKN1A* luciferase reporter construct and the series of KDM5B deletion mutants (Fig. 3B) either with or without a TFAP2C expression vector. As before, expression of KDM5B alone had no effect on reporter activity but was able to cooperate with TFAP2C to repress the *CDKN1A* promoter (Fig. 4A, compare first and second black columns). In this assay, the majority of the KDM5B mutants also showed significant, wt levels of corepression activity (denoted by asterisks; Fig. 4A). The exceptions were constructs lacking either the C-terminal PHD domains (ΔC) or the JmjC domain (ΔJmjC) (Fig. 4A; nonsignificant [n.s.] difference from the control). Since it was unclear whether the demethylase activity itself or the physical requirement of the JmjC domain for protein-protein interactions was essential for corepressor activity, we additionally tested a point mutation, H499A, known to abrogate demethylase activity (38). Compared to wt KDM5B, the H499A mutant and the JmjC mutant both showed significantly reduced corepressor activity when expressed with either TFAP2C or Myc or both factors (Fig. 4B). Together these data suggest that KDM5B-mediated repression at the *CDKN1A* promoter requires its functional interaction with TFAP2C/Myc proteins plus an intact, functional JmjC domain.

AP-2 binding at the *CDKN1A* promoter is critical for repression by the TFAP2C/Myc/KDM5B ternary complex. We have previously identified an AP-2 DNA binding site (GCCCCGGGG) at −111/−103 in the *CDKN1A* promoter (27). In contrast, there is
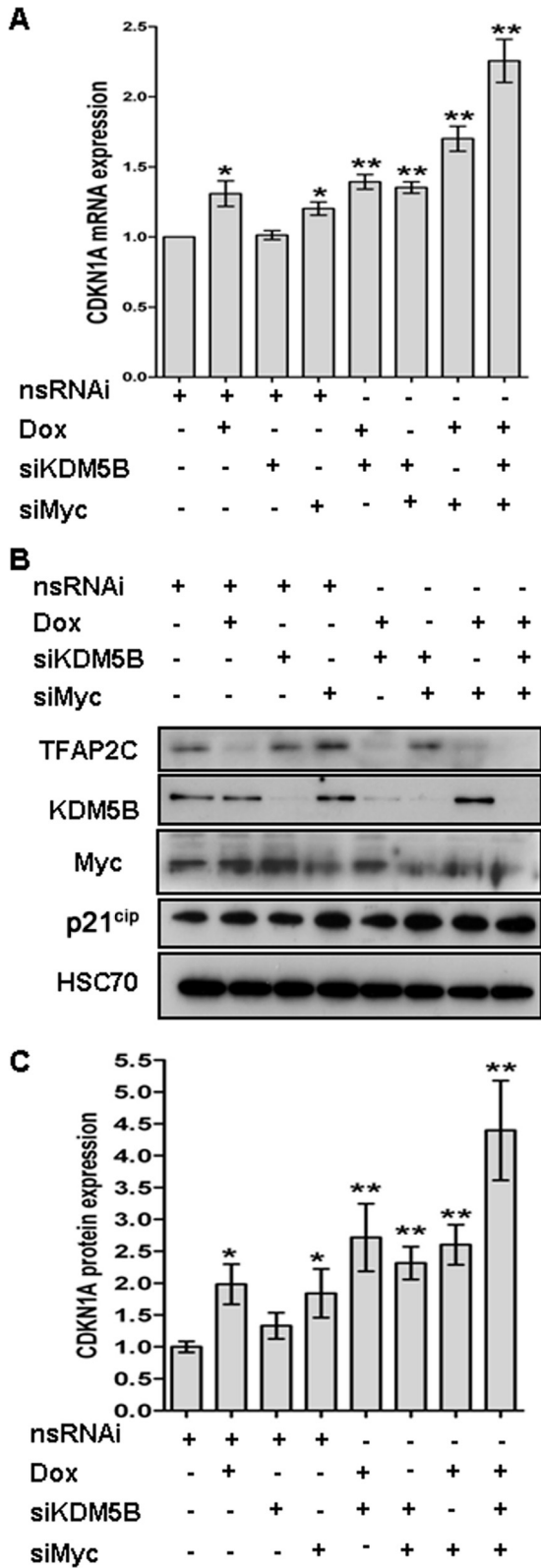
FIG 1 TFAP2C, Myc, and KDM5B collaborate to repress *CDKN1A* expression. shTFAP2C-MCF-7 cells were transiently transfected with the indicated siRNA and/or treated with doxycycline (Dox) to induce TFAP2C silencing. All samples were additionally transfected with a nonsilencing control (nsRNAi); the amount was adjusted to keep the total level of siRNA constant across all samples. Cells were harvested 72 h after addition of siRNA/Dox. (A) Total
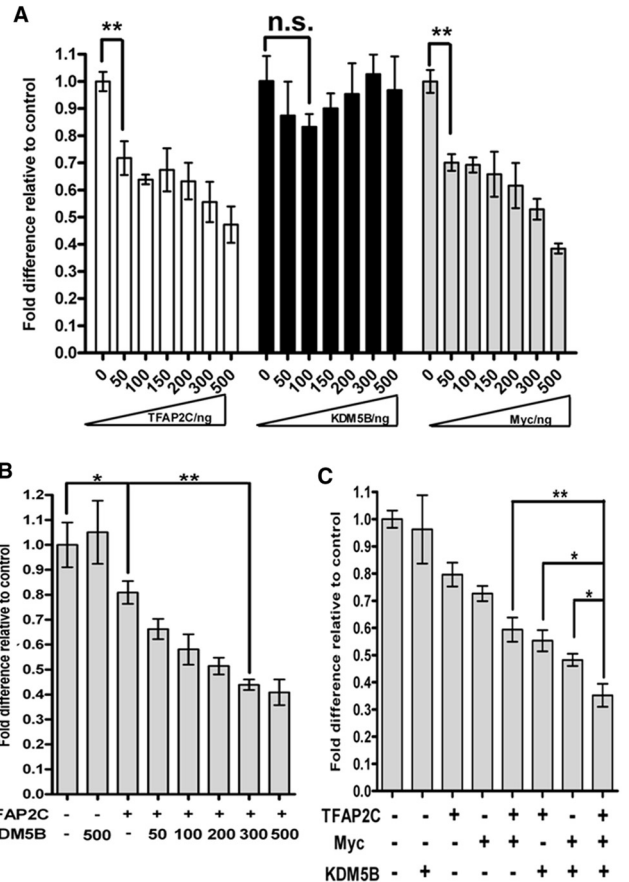

FIG 2 TFAP2C, Myc, and KDM5B corepress *CDKN1A* promoter activity. (A) HepG2 cells were transfected with a *CDKN1A*-luc reporter construct (containing genomic sequences from −2325 to +8 relative to the TSS; 150 ng) plus increasing amounts of TFAP2C, KDM5B, or Myc expression vectors as indicated. (B) As in panel A, with TFAP2C fixed (50 ng) and/or KDM5B (escalating dose; ng) where indicated. (C) As in panel B, with fixed levels of TFAP2C (50 ng), KDM5B (300 ng), and/or Myc (50 ng) expression constructs as indicated. Luciferase values were corrected for transfection efficiency and are expressed as fold activity compared to the vector-alone (VA) control (first column), set at 1, using data from 3 independent experiments, each performed in triplicate; error bars indicate the standard errors. Student's *t* test was used to compare data from the indicated samples: ∗, $P < 0.05$; ∗∗, $P < 0.001$. n.s., not significant.

no consensus Myc binding site within the proximal promoter, and it is thought that Myc is recruited to the locus by "piggy-backing" on other factors, such as Miz-1 or Sp1, that bind to sites within 100 bp of the *CDKN1A* TSS (17, 29). To investigate the importance of the AP-2 binding site for repression by the TFAP2C/Myc/KDM5B complex, we performed further reporter assays using additional

RNA was analyzed by quantitative PCR (qPCR) and normalized to GAPDH. *CDKN1A* data are represented as fold change ($2^{-\Delta\Delta CT}$) from results for control cells (first column), averaged from 3 independent experiments ± standard error. ∗, $P < 0.05$; ∗∗, $P < 0.01$ (Student's *t* test). TFAP2C, Myc, and KDM5B silencing was confirmed by qPCR (not shown). Whole-cell lysates from each experiment were analyzed by Western blotting to monitor changes in protein levels; Hsc70 was used as a loading control. One experiment is shown in panel B; in panel C, the p21[cip] (CDKN1A) blots from all the experiments were scanned and normalized to the Hsc70 signal, and each condition is expressed as fold induction over control results (first column), as in panel A.
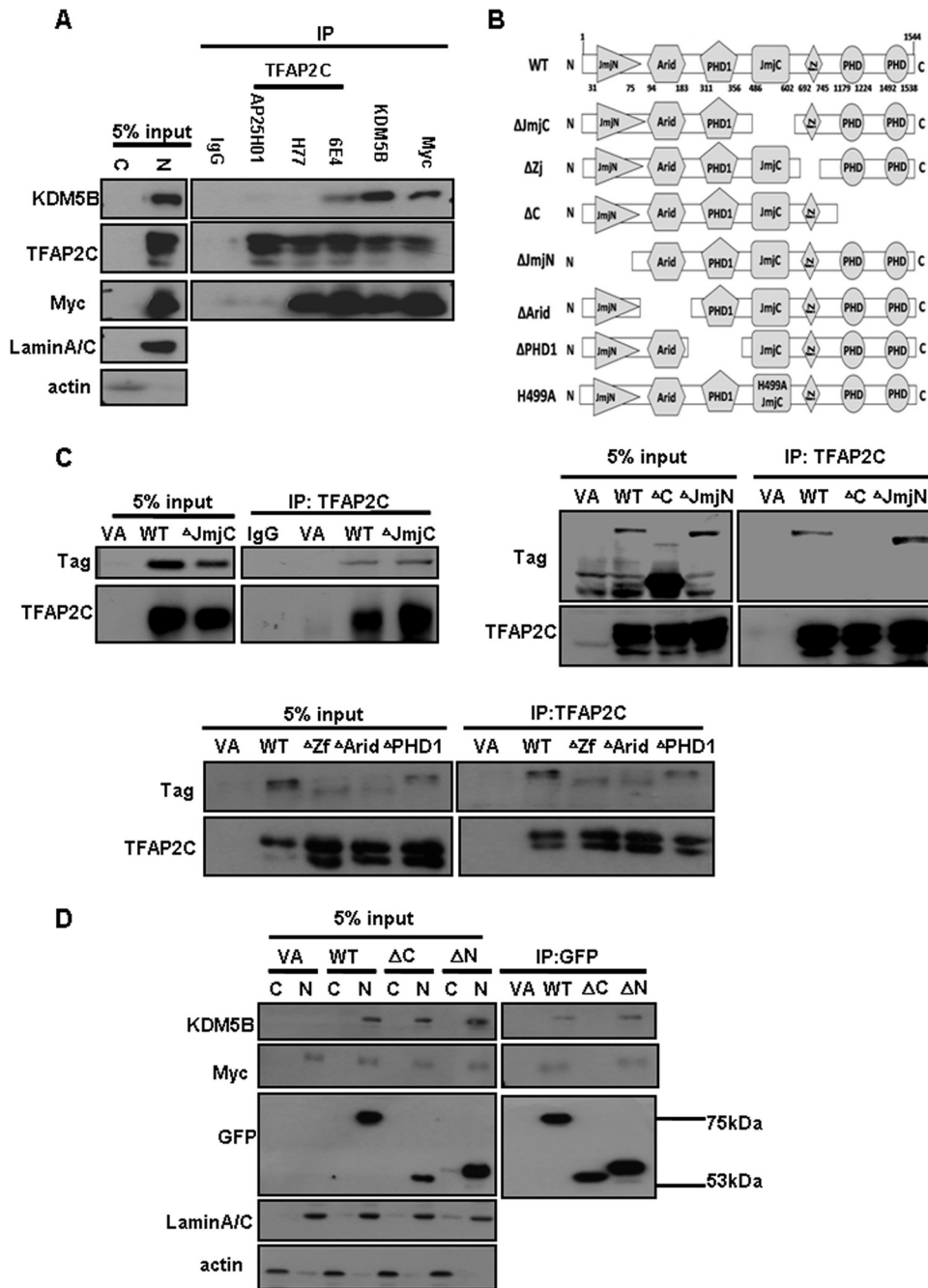
FIG 3 KDM5B forms a complex with TFAP2C and Myc. (A) Cytoplasmic (C) and nuclear (N) extracts were prepared from MCF-7 cells (see Materials and Methods). The nuclear extracts were subjected to immunoprecipitation (IP) with antibodies against Myc, KDM5B, or TFAP2C (6E4, H77, or AP25H01, as marked) prior to Western blotting (WB) for the indicated proteins (lanes 1 to 6). C and N proteins, equivalent to 5% of the amount used for IP, were also analyzed by WB to control for protein size and extract integrity. (B) Schematic representation of the domain structure of KDM5B and the series of peptide-tagged deletion mutants. (C) H1299 cells (lacking TFAP2C and KDM5B expression) were cotransfected with TFAP2C and tagged wt or mutant KDM5B proteins or vector alone (VA) as a control. Nuclear extracts were subjected to IP for TFAP2C (6E4) followed by WB for TFAP2C or the peptide Tag to reveal mutant KDM5B proteins, as indicated. (D) GFP-tagged TFAP2C wild-type (WT) or a ΔN or ΔC deletion mutant was cotransfected with KDM5B, and nuclear extracts were immunoprecipitated using the GFP-trap system prior to WB to detect each protein plus endogenously expressed Myc. Additional blotting for actin and lamin A/C was used to confirm the integrity of the cytoplasmic and nuclear extracts, respectively; the 6E4 antibody was used for all TFAP2C blots.

CDKN1A luciferase constructs mutated to abolish AP-2 binding (mut4 and mut5) (27). Mutation of the AP-2 binding site clearly abrogated repression by TFAP2C alone and with KDM5B (Fig. 5, column groups 3 and 6). Myc-mediated repression was less af-fected by mutation of the AP-2 binding site (Fig. 5, column groups 4 and 7), but intriguingly, the degree of repression was signifi-cantly attenuated on mutant promoters when Myc was coex-pressed with TFAP2C, either alone or with KDM5B (Fig. 5, col-
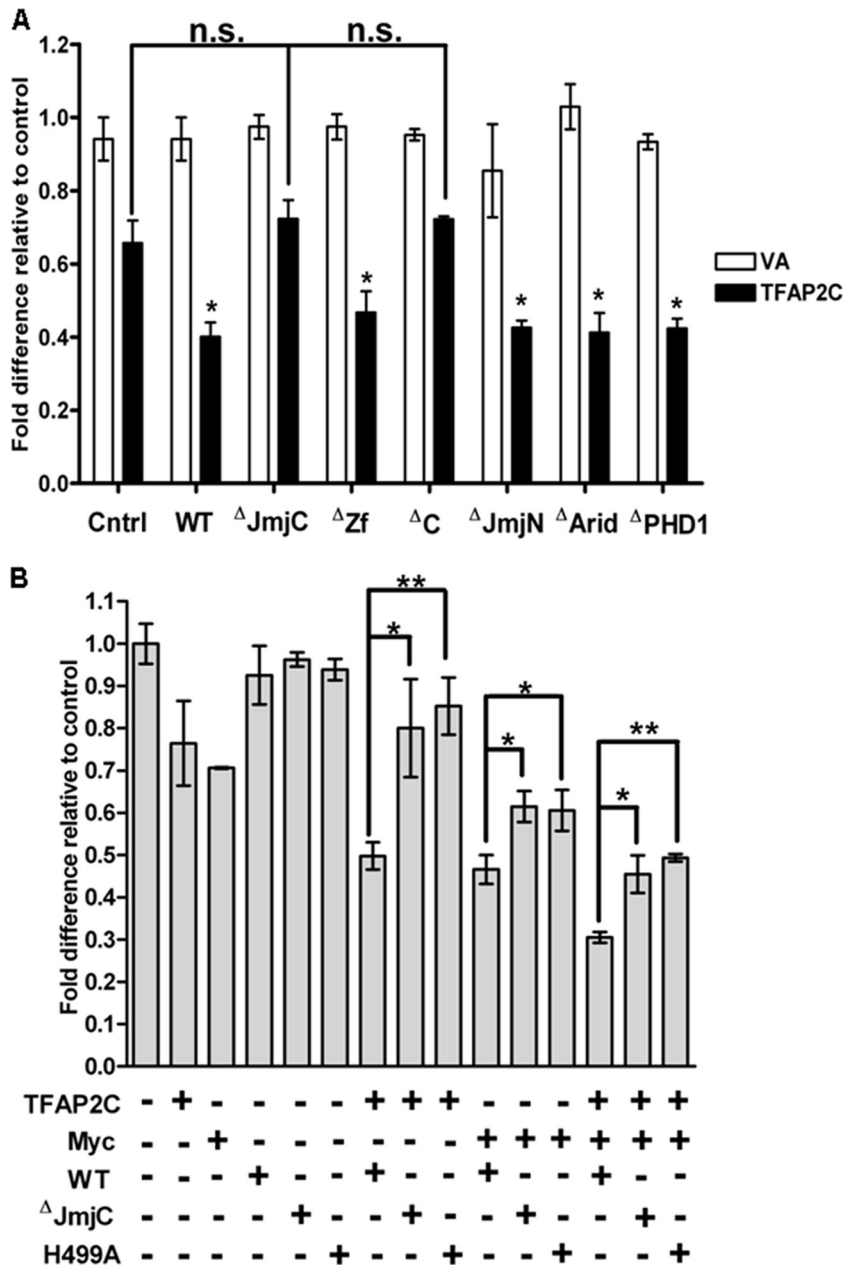
FIG 4 Identification of KDM5B domains required for corepressor activity. (A) HepG2 cells were cotransfected with 150 ng of *CDKN1A*-luc and 50 ng TFAP2C expression plasmid or vector-alone (VA) control, as indicated, plus 300 ng of wt or mutant KDM5B, as labeled. (B) As in panel A, with TFAP2C and/or Myc (50 ng) in combination with wt or KDM5B JmjC domain mutants (see Fig. 3B), as indicated. Efficient expression of all KDM5B constructs was assessed by blotting (see Fig. S2A in the supplemental material). Luciferase values, corrected for transfection efficiency, are expressed as fold activity compared to that for the vector-alone control, set at 1, using data from 3 independent experiments, each performed in triplicate; error bars indicate the standard errors. Student's *t* test was used to compare data from the indicated samples: n.s., not significant; ∗, $P < 0.05$; ∗∗, $P < 0.001$.

umn groups 5 and 8). This suggests, therefore, that while Myc does not require an intact AP-2 site to interact with and repress the *CDKN1A* locus, when TFAP2C is present, its binding at −111/−103 allows optimal recruitment of Myc and KDM5B to the proximal promoter and more-efficient repression.

We next used ChIP assays to verify the colocalization of TFAP2C, Myc, and KDM5B at the endogenous *CDKN1A* locus. In agreement with results of the reporter assays described above, the proximal region of the *CDKN1A* promoter (primers at −21/

+44), but not regions further upstream (−2290/−2185 or −935/−864), was immunoprecipitated specifically using antibodies to TFAP2C, KDM5B, or Myc (Fig. 6A). Functional interrelationships between these factors at the *CDKN1A* locus were tested by comparing ChIP assays with control cells and those silenced for each factor in turn. Treatment with doxycycline for 72 h reduced TFAP2C binding, as expected; however, the localization of Myc and KDM5B at the promoter was also reduced to background levels in TFAP2C-silenced cells (Fig. 6A). Similar results were ob-
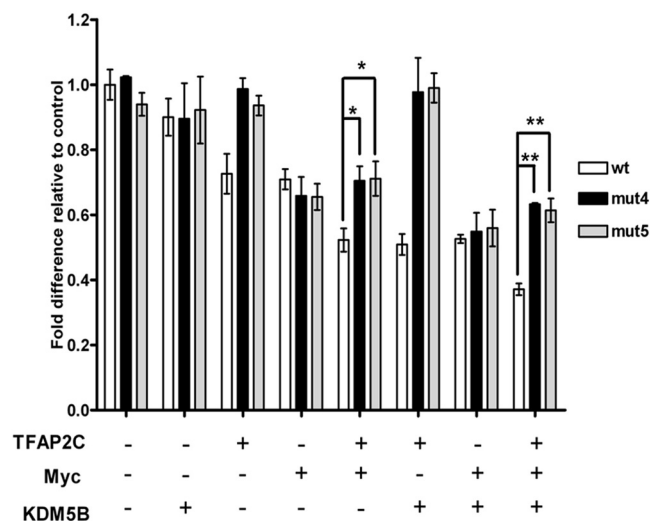
FIG 5 The AP-2 binding site at −111/−103 is required for optimal *CDKN1A* repression by the ternary complex. HepG2 cells were transfected, as described for Fig. 5, with the wt or versions of the *CDKN1A*-luc reporter construct mutated within the AP-2 binding site (mut4 and mut5) (27), plus expression constructs for TFAP2C, Myc, and KDM5B, as indicated. Luciferase values, corrected for transfection efficiency, are expressed as fold activity compared to that for the vector-alone control, set at 1, using data from 3 independent experiments, each performed in triplicate; error bars indicate the standard errors. Student's *t* test was used to compare data from the indicated samples; *, $P < 0.05$; **, $P < 0.001$.

tained when cells were treated for 24 or 120 h (data not shown). These data cannot be explained by changes in the Myc or KDM5B protein level, since these remained constant in doxycycline-treated cells (Fig. 1B). siRNA-mediated silencing of Myc also reduced binding of both TFAP2C and KDM5B to the *CDKN1A* proximal promoter, while knockdown of KDM5B did not influence interaction by the other two factors, although KDM5B localization to the promoter was reduced as expected (Fig. 6A). Therefore, not only does TFAP2C assist the binding of Myc to this locus, as suggested by the reporter assays with mutated AP-2 binding sites (Fig. 5), but Myc also facilitates TFAP2C binding.

Since KDM5B is a specific di- and trimethyl-H3K4 demethylase (39), we tested whether silencing of *TFAP2C* or *MYC*, with the resulting reduction in KDM5B localization at the *CDKN1A* locus, would lead to changes in H3K4 methylation status within the proximal promoter. Silencing individual factors or pairs of factors did not significantly alter the H3K4 methylation level; in particular, loss of KDM5B alone did not promote H3K4 trimethylation, since this would require the recruitment of a COMPASS-like methyltransferase complex, which can occur only during transcriptional activation (31). However, when all three factors were cosilenced (a situation that does lead to *CDKN1A* activation [Fig. 1]), we observed a robust increase in the H3K4me3 signal (Fig. 6B). In contrast, levels of H3K4me1 did not alter significantly, while there was a general decline in the H3K4me2 signal (data not shown). Taken together, these results demonstrate that TFAP2C and Myc, but not KDM5B, are required for the localization and stabilization of the complex at the *CDKN1A* proximal promoter. Furthermore, while TFAP2C and Myc can downregulate *CDKN1A*, the demethylase activity of KDM5B is required for maximal repression of both transiently expressed reporter con-

structs and the endogenous gene, and coordinate loss of all three factors led to increased H3K4me3 levels, in keeping with the activation of *CDKN1A* expression (Fig. 1A).

Effect of overexpression of the TFAP2C-Myc-KDM5B complex on cell cycle progression and checkpoint activation. Since *p21^{cip}* (*CDKN1A*) functions mainly as a regulator of cell cycle progression by inhibiting CDK activity and is required for activation of G_1/S and G_2/M checkpoints when cells are exposed to DNA damage or replication or mitotic stress, we questioned whether manipulating levels of TFAP2C, Myc, and/or KDM5B could affect cell cycle distribution and activation of specific checkpoints. HepG2 cells, which express wild-type *CDKN1A*, were transiently transfected with the TFAP2C, Myc, and/or KDM5B expression vectors and 48 h later were subjected to cell cycle analysis. Western blotting was used to confirm expression of exogenous proteins. Overexpression of KDM5B alone had little effect on *CDKN1A* expression but enhanced repression when cells were cotransfected with TFAP2C and/or Myc (Fig. 7, top left panel, vehicle treated), consistent with the reporter assays described above (Fig. 2). Cell cycle distribution analysis showed that transient overexpression of TFAP2C or Myc but particularly both proteins together forced a higher percentage of cells into S phase. Overexpression of KDM5B alone had a minimal effect on cell cycle distribution; however, cotransfection with TFAP2C and/or Myc led to a significant shift from G_1 to S phase (Fig. 7, lower left panel).

Since overexpression of the TFAP2C-Myc-KDM5B complex induced G_1/S transition in cycling cells, we next investigated whether the complex could also affect activation of *CDKN1A* at the S-phase or G_2/M checkpoints induced by treatment with hydroxyurea (HU) or vinblastine, respectively. We first assayed the effects on endogenous protein expression after drug treatment for 24 and 48 h in MCF-7 cells. Induction of *CDKN1A* mRNA and protein was observed with both drugs in concert with upregulation of p53 levels (Fig. 8A and B). Expression of TFAP2C and Myc was generally downregulated following treatment with vinblastine or HU, both at the mRNA and protein levels (Fig. 8A and data not shown), although levels of KDM5B remained more constant. The binding of each protein to the proximal region of the *CDKN1A* promoter was assessed by ChIP assays with drug-treated cells and showed that treatment for 24 or 48 h reduced binding of all three factors to the *CDKN1A* locus (Fig. 8C). This demonstrates that drug activation of the endogenous *CDKN1A* gene was accompanied by reduced expression of Myc and TFAP2C and coordinate release of these transcriptional repressors from the proximal promoter.

Since drug induction of *CDKN1A* was accompanied by loss of the TFAP2C-Myc-KDM5B complex, we investigated whether forced overexpression of these proteins could repress *CDKN1A* expression and thereby attenuate the cytostatic effect induced by either HU or vinblastine treatment. Arrest in S or G_2/M phase was clearly seen after cell cycle analysis of control cells treated with HU or vinblastine, respectively (Fig. 7, bottom panels, compare the first group of columns for each condition). However, overexpression of TFAP2C and/or Myc decreased the extent of the S or G_2/M block, allowing a proportion of cells to reenter the cycle. Overexpression of KDM5B alone did not affect HU- or vinblastine-induced checkpoints, but KDM5B was able to cooperate with either TFAP2C or Myc to relieve the cell cycle block, with the most significant effects being observed when all three proteins were expressed together, particularly for HU-treated cells, where the cell
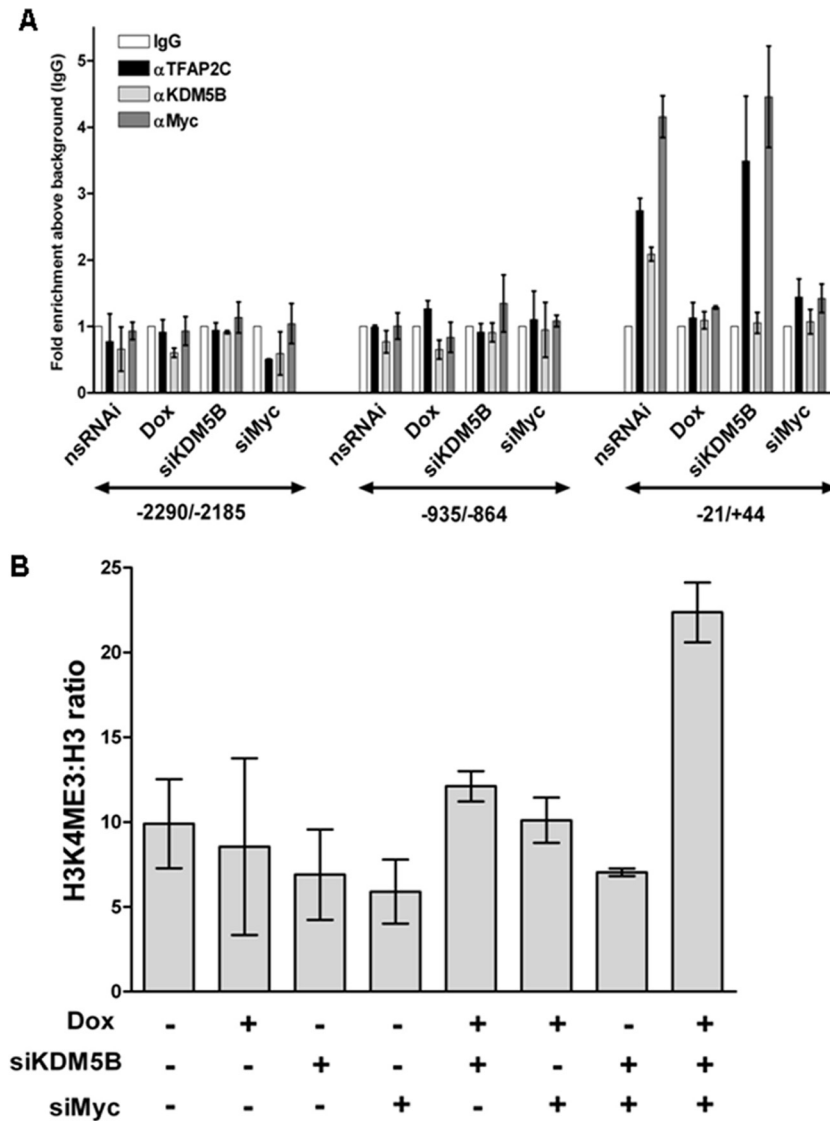
FIG 6 Colocalization and functional interrelation between endogenous TFAP2C, Myc, and KDM5B at the *CDKN1A* locus. (A) shTFAP2C-MCF-7 cells were transfected with nonsilencing control siRNA (nsRNAi) or siRNA against Myc (siMyc) or KDM5B (siKDM5B) or treated with doxycycline (Dox) to induce shTFAP2C and harvested 72 h later for use in ChIP with antibodies against IgG (control), TFAP2C, (KDM5B, or Myc. Precipitates were analyzed by qPCR using primer pairs specific to 3 distinct regions ($-2290/-2185$, $-935/-864$, and $-21/+44$) across the *CDKN1A* 5′ sequence, as indicated by the double-headed arrows. (B) ChIP was performed as described for panel A using antibodies specific for histone 3 and H3K4-me3. qPCRs were performed using the $-21/+44$ *CDKN1A* proximal promoter-specific primers. Results are shown as fold enrichment above the IgG (background) control (A) or as an H3K4me3/H3 ratio (B). Data were averaged from three independent experiments, $\pm$ standard errors.

cycle profiles resembled those of untreated cells (Fig. 7, compare the last series of columns for the HU-treated condition with the first series of columns for the control condition). For each treatment, repression of p21$^{\text{cip}}$ expression was maximal when all three proteins were coexpressed (Fig. 7, upper panels, last lane).

In summary, since its overexpression is sufficient to induce entry into S phase of the cell cycle and activate proliferation of normal cycling cells, TFAP2C resembles Myc in these assays and acts as an oncogene. KDM5B has little effect alone but can amplify responses to the other two factors. Activation of S or G$_2$/M checkpoints is attenuated when TFAP2C or Myc is overexpressed, with and without KDM5B overexpression, suggesting that elevated levels of these proteins in cancer cells may collaborate to confer re-

sistance to genotoxic or mitotic stress via direct downregulation of the universal cell cycle inhibitor *CDKN1A*.

DISCUSSION

As with TFAP2C (13, 16) and Myc (37), KDM5B overexpression has been associated with a poorer outcome in breast cancer patients (22), and all three proteins have been shown to promote cell proliferation by facilitating G$_1$/S transition (36, 39), a checkpoint particularly sensitive to cellular levels of p21$^{\text{cip}}$. Repression of *CDKN1A*, mediated by Myc or TFAP2C, has been documented previously (see the introduction), and the related KDM5A demethylase was shown to regulate H3K4 methylation status at this locus in gastric tumor cells, although its site and mode of binding
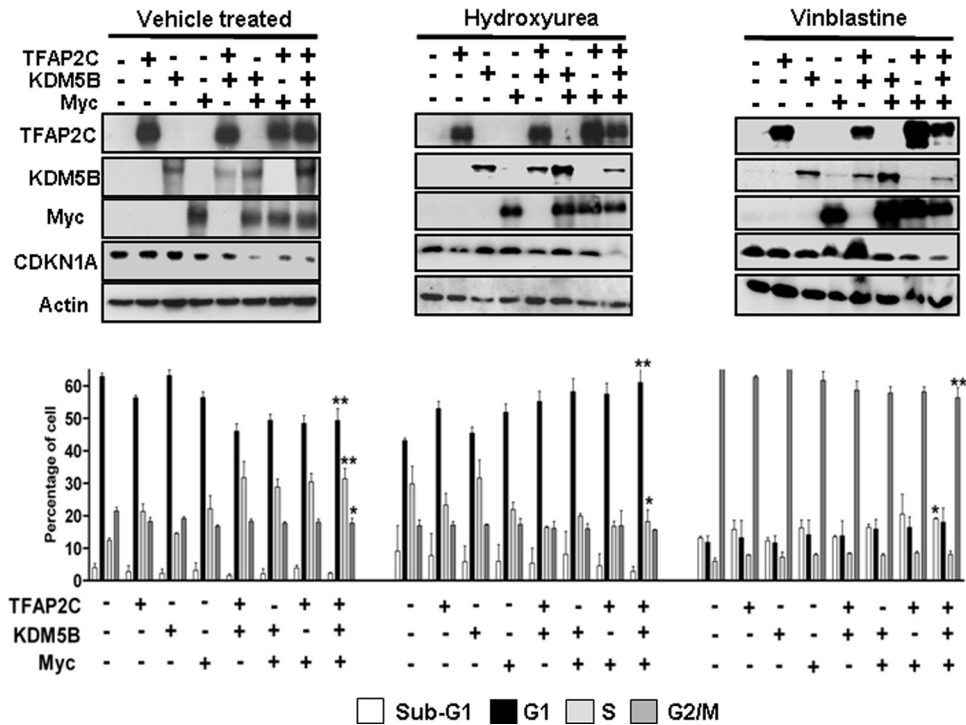
FIG 7 TFAP2C/KDM5B/MYC overexpression attenuates drug-activated checkpoints and forces cells to enter S phase. HepG2 cells were transfected with TFAP2C, KDM5B, or Myc expression vectors in combination, as shown. After 24 h, transfected cells were treated with normal medium (vehicle treated, LH panels), hydroxyurea (middle panels), or vinblastine (RH panels) for a further 16 h. Cells were then either lysed and assayed by Western blotting for the proteins indicated (upper panels) or ethanol fixed and stained with propidium iodide for cell cycle analysis (lower panels). Graphs show percentages of cells from each condition in sub-G_1, G_1, S, or G_2/M phase. Representative experiments from 3 repeats (each done in triplicate) are shown; $*$, $P < 0.05$; $**$, $P < 0.01$ (Student's t test; significant differences between control [1st group of columns] and 3-way-transfected cells [last group of columns] for cycling [vehicle treated] or drug-treated cells).

to the promoter were not defined (40). Our data suggest that in breast tumor cells, where it is expressed predominantly (19), KDM5B is the key H3K4me3 demethylase which acts in a ternary complex with Myc and TFAP2C to regulate *CDKN1A* expression, thus uniting many separate observations and providing a mechanistic rationale for how deregulated expression of these factors may permit breast cancer cells to sustain their proliferation and survival.

Since Myc repression of *CDKN1A* does not involve heterodimerization with Max or direct DNA binding (12), a number of studies have looked at the requirement of Myc to access the promoter through interaction with other DNA binding proteins, particularly Sp1 and Miz-1. The precise mechanism remains unclear, possibly because more than one pathway is active, with cell type-specific factors undoubtedly playing a role (reviewed in reference 17). Here we have explored the interdependence of Myc and TFAP2C. Investigating the binding of these proteins at the endogenous *CDKN1A* gene in silenced MCF-7 cells by ChIP (Fig. 6) revealed that each factor appeared to stabilize and/or facilitate the binding of the other to the proximal promoter. This was partly recreated using reporter assays where maximal repression by all three factors could be achieved only on promoter constructs with an intact AP-2 binding site (Fig. 5). Taking the ChIP and reporter assay data together, therefore, this suggests that TFAP2C and Myc interact with the *CDKN1A* locus at separate sites, with only the AP-2 site at −111/−103 for TFAP2C defined, and together they recruit the histone demethylase KDM5B to form a repressive ternary complex required for optimal downregulation of *CDKN1A* (Fig. 9, diagram). Interestingly, a similar mutual interdependence has recently been suggested to allow the stable binding of both TFAP2C and FoxA1 to specific chromatin sites, leading to the recruitment of ERα and estrogen-responsive gene expression in breast cell lines (33).

The majority of studies examining the activity of the KDM5 family of histone demethylases have relied on the analysis of global H3K4 methylation status, which suggested that these enzymes can act on a broad range (tri, di, mono) of methylation states at this site. However, a small number of specifically regulated genes have been identified which, as shown here for *CDKN1A*, all exhibited KDM5B binding close to their TSS. Moreover, modulation of KDM5B expression led to consistent alterations in H3K4me3 levels at these loci, but dimethylation (me2) and monomethylation (me1) levels remained relatively constant (39). This again mirrors our ChIP results at the *CDKN1A* locus, where cosilencing of the ternary complex proteins led to induction of gene transcription (Fig. 1) and a concomitant increase in H3K4me3 but little alteration in me2 or me1 levels (Fig. 6B and data not shown). This suggests that the observations made on global H3K4 methylation status may not always reflect the activities of KDM5 demethylases bound at the promoters of their directly regulated genes.

The C-terminal region of KDM5B contains two PHD fingers, one of which has been shown to bind H3K4me3 and contribute to binding to active genes (reviewed in reference 18). This C-terminal domain was also found here to be required for interaction with
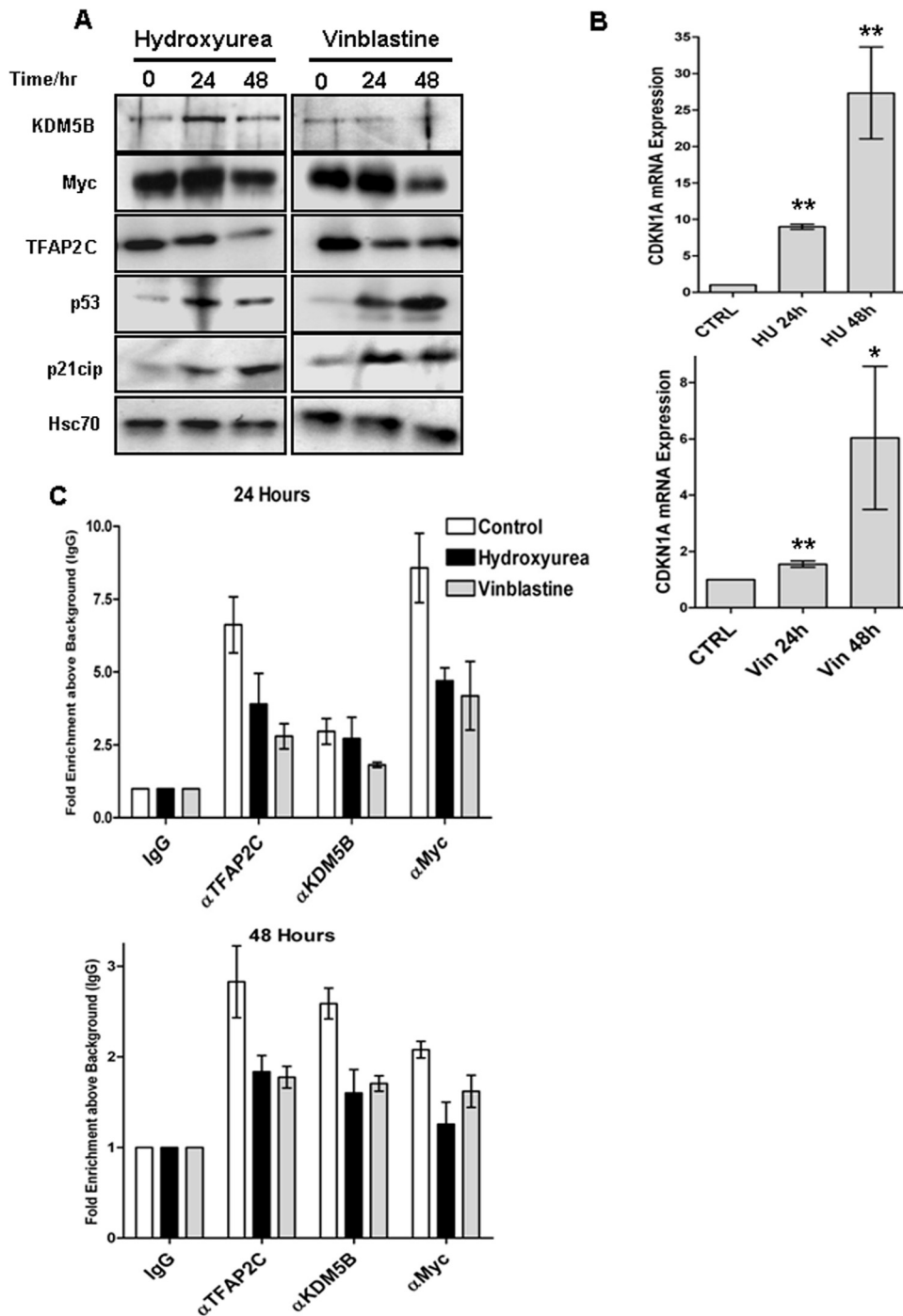
FIG 8 HU and vinblastine induce *CDKN1A* and reduce TFAP2C/KDM5B/Myc occupancy at the proximal promoter. MCF-7 cells were treated with hydroxyurea (HU) or vinblastine (Vin) or vehicle control (CTRL) for 24 and 48 h. (A) Whole-cell extracts from treated or control cells, blotted for the indicated proteins. (B) q-RT-PCR analysis of *CDKN1A* expression in control and treated cells. Data represent the means and standard errors for 3 independent experiments. Significant differences (*, $P < 0.05$; **, $P, 0.01$) between induced and uninduced cells are given (Student's *t* test). (C) ChIP assay for TFAP2C, KDM5B, and Myc factors was performed as for Fig. 6 using primers to the −21/+44 region of *CDKN1A*. Values are averages for three independent ChIP experiments; error bars represent standard errors between the repeats.

TFAP2C (Fig. 3). Studies primarily of the *Drosophila* KDM5 homologue Lid have shown that the JmjC and Zf motifs were independently required for Myc interaction (20, 28), and thus these two factors interact with distinct domains of KDM5B. In addition, analysis of TFAP2C deletion mutants plus data from coIP with a range of TFAP2C antibodies (Fig. 3) defines amino acids 181 to 430 as the region of TFAP2C able to bind KDM5B and Myc. This includes the basic-helix-span-helix domain, suggesting that TFAP2C dimerization may be required for factor interaction, although actual binding may be mediated by a 40-aa region imme-
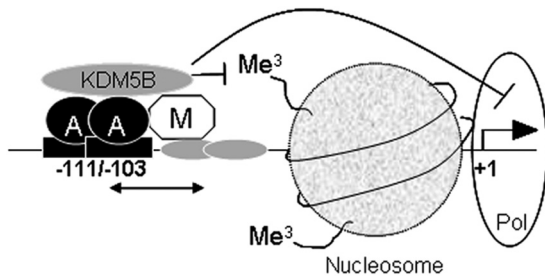
FIG 9 Schematic diagram of *CDKN1A* repression by TFAP2C/Myc/KDM5B. The TSS (+1) and proximal promoter region of *CDKN1A* are represented with the AP-2 site at −111/−103, bound by a TFAP2C dimer (A). Myc (M) is shown binding adjacent regions through interaction with another undefined DNA binding factor (shown as gray ovals). Myc and TFAP2C stabilize each other's binding to the promoter (double-headed arrow) and together recruit KDM5B, which has a negative effect on chromatin structure via demethylation of H3K4me3, leading to suppression of RNA polymerase II (Pol)-mediated transcription of the gene.

diately N-terminal of the basic domain. The sequences required for Myc/KDM5B interaction therefore overlap those previously shown to mediate TFAP2C binding to CITED2/p300 (6) during gene activation, suggesting that competition by distinct complexes for an overlapping interaction domain may determine whether TFAP2C will activate or repress transcription.

Reporter assays confirmed the requirement for the C-terminal KDM5B PHD fingers for corepressor activity with TFAP2C. An intact JmjC domain was also essential, with even a single amino acid substitution (H499A), known to prevent demethylase activity, neutralizing corepressor function (Fig. 4B). Superficially this suggests that demethylase activity is required for corepression, which would agree with our ChIP data on histone methylation status at the *CDKN1A* locus (Fig. 6). However, previously reported domain structure analyses for KDM5B, again using the global H3K4 methylation level as the readout, concluded that disruption of any of the domains within the N-terminal half of the protein will ablate demethylase activity. This is thought to be due to spatial constraints on the folding of the JmjN and JmjC domains to form a functional enzyme (38, 39). Since other domains were not found to be required for corepressor activity with TFAP2C, it suggests either that there are differences between demethylase activity monitored on total histone versus observations at gene-specific sites or the intact JmjC domain alone is required for optimal localization of KDM5B at the *CDKN1A* promoter and even the H499A mutation is sufficient to disrupt this.

In *Drosophila*, the interaction between dMyc and the JmjC domain of Lid was shown to negatively regulate demethylase activity and thereby activate gene expression (20, 28). The increased level of H3K4me3 we observe at the *CDKN1A* locus upon silencing of TFAP2C/Myc/KDM5B suggests, however, that here TFAP2C and Myc recruit active demethylase that contributes to repression by removal of the H3K4me3 epigenetic mark. This agrees with many other studies confirming a role in dynamic gene silencing for the KDM5 family; notably, the recruitment of KDM5A by the core transcription factor RBP-J to silence target genes during Notch signaling has several parallels with our findings here (21). Nevertheless, repression by the ternary complex is likely to involve additional mechanisms, since we have previously reported that loss of histone deacetylases (HDACs) and increased histone acetyla-

tion also occur during induction of *CDKN1A* upon TFAP2C silencing in MCF-7 cells (36). However, KDM5B has also been shown to interact with HDACs through its PHD fingers (3), and the homologue KDM5A has been purified in HDAC corepressor complexes (35).

We have extended our examination of the interaction of TFAP2C/Myc/KDM5B with each other and the *CDKN1A* locus by using functional experiments to demonstrate that overexpression of these factors can perturb normal cell cycle control, both in cycling cells and in those incubated with drugs to activate p53-dependent checkpoints. In each case, the most efficient reversal of drug-induced *CDKN1A* activation occurred when all three ternary complex proteins were coexpressed, and this was sufficient to force arrested cells back into cycle (Fig. 7). Moreover, both *TFAP2C* and *MYC* are estrogen-responsive genes, and loss of this regulation in breast cancer has been associated with the lack of *CDKN1A* induction in response to antioestrogen therapy, thus contributing to resistance (13, 23, 36). Significantly, the ubiquitously expressed demethylase homologue KDM5A was found to be upregulated in drug-tolerant subpopulations of cells from several tumor origins treated with a range of anticancer agents (30). Thus, this family of histone demethylases may be particularly implicated in the epigenetic heterogeneity within cancer cell populations that can contribute to the development of treatment resistance and tumor progression. This suggests, therefore, that the association of TFAP2C, KDM5B, and Myc allows these proteins to cooperate when overexpressed in breast tumors, leads to a poor patient prognosis, and provides a rationale for the further investigation of histone demethylase activity as a therapeutic target in this disease.

ACKNOWLEDGMENTS

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