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ORIGINAL ARTICLE

KDM2B/FBXL10 targets c-Fos for ubiquitylation and degradation in response to mitogenic stimulation

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KDM2B (also known as FBXL10) controls stem cell self-renewal, somatic cell reprogramming and senescence, and tumorigenesis. KDM2B contains multiple functional domains, including a JmjC domain that catalyzes H3K36 demethylation and a CxxC zinc-finger that recognizes CpG islands and recruits the polycomb repressive complex 1. Here, we report that KDM2B, via its F-box domain, functions as a subunit of the CUL1-RING ubiquitin ligase (CRL1/SCF^{KDM2B}) complex. KDM2B targets c-Fos for polyubiquitylation and regulates c-Fos protein levels. Unlike the phosphorylation of other SCF (*S*KP1-*C*UL1-*F*-box)/CRL1 substrates that promotes substrates binding to F-box, epidermal growth factor (EGF)-induced c-Fos S374 phosphorylation dissociates c-Fos from KDM2B and stabilizes c-Fos protein. Non-phosphorylatable and phosphomimetic mutations at S374 result in c-Fos protein which cannot be induced by EGF or accumulates constitutively and lead to decreased or increased cell proliferation, respectively. Multiple tumor-derived KDM2B mutations impaired the function of KDM2B to target c-Fos degradation and to suppress cell proliferation. These results reveal a novel function of KDM2B in the negative regulation of cell proliferation by assembling an E3 ligase to targeting c-Fos protein degradation that is antagonized by mitogenic stimulations.

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INTRODUCTION

The activator protein-1 (AP-1) transcriptional factor complex is central to many biological processes.[1,2] Activator protein-1 complexes perform such diverse functions by potentially forming a large number of homo- or heterodimeric complexes through combinatorial interaction between members of Fos, Jun, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families. Proto-oncogene *c-Fos* is one of the first genes identified to be induced by mitogenic stimulation.[3] c-Fos forms a dimeric complex with c-Jun as the first identified activator protein-1.[4–7] The regulation of c-Fos has been extensively studied and serves as paradigm for the tight, dynamic and multiple level regulation of stress and growth factor response.[8] c-Fos is typically expressed at a very low level in both cells cultured *in vitro* and *in vivo*. Mitogenic stimulation, particularly the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway, rapidly induces *c-Fos* mRNA.[9,10] The c-Fos protein is intrinsically unstable because of degradation by the 26S proteasome and is protected by phosphorylation.[11,12] Multiple putative phosphorylation sites, mostly in the C-terminal region of the c-Fos protein, have been reported to regulate c-Fos protein stability. In particular, two residues—Ser362 and Ser374—were found to be phosphorylated by RSK1/2 and ERK1/2, respectively,[13] and their phosphorylation stabilizes c-Fos protein.[11] Genetic studies using knock-in mutation demonstrated that phosphorylation on these two residues have important roles for cell differentiation, cytokine response and tumorigenesis *in vitro*.[14] In contrast, the identity of the E3 ubiquitin ligase that targets c-Fos degradation and that antagonized by ERK1/2-mediated phosphorylation has not been established. UBR1, a member of the N-end rule family E3 ligase, has been linked to c-Fos degradation in the cytoplasm, which is protected by ERK5-mediated phosphorylation at two separate sites, Thr232, which blocks c-Fos nuclear export, and Ser32, which disrupts the interaction between c-Fos and UBR1.[15] The physiological significance of ERK5-mediated protection of c-Fos from UBR1-promoted degradation is currently unclear.[16]

KDM2B (also known as FBXL10, NDY1, JHDM1B and CxxC2) controls stem cell self-renewal,[17] somatic cell reprogramming,[18] cell senescence[19,20] and tumorigenesis.[21–23] KDM2B/FBXL10 is a protein of multidomains, including a JmjC domain situated at the N-terminal region of the protein, followed by a CxxC domain, a PHD domain, a F-box motif and seven leucine-rich repeats (LRRs, see Supplementary Figure S2A). The JmjC domain catalyzes H3K36 demethylation[24] and the CxxC zinc-finger domain recognizes CpG islands and recruits polycomb repressive complex 1 (PRC1) to target genes.[17,25–28] KDM2B was also found to interact with SKP1 via its F-box domain,[27,28] a linker protein involved in the assembly of the SCF (*S*KP1-*C*UL1-*F*-box) E3 ubiquitin ligase complex, raising the possibility that KDM2B could additionally contain an E3 ligase function. The substrate of this putative KDM2B E3 ligase, however, has not been identified. Intriguingly, KDM2B has been reported to repress the transcription mediated by either c-Jun or c-Fos through a mechanism not completely understood.[14,28] In this study, we explore the possibility that a KDM2B-containing E3 ligase targets c-Fos for ubiquitination and degradation.

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RESULTS

KDM2B/FBXL10 and CUL1 destabilize c-Fos protein

To determine whether KDM2B regulates c-Fos protein level, as well as its transcription, we first generated HEK293 cells with stable knockdown of *KDM2B*. KDM2B expresses multiple isoforms produced by alternative splicing and differential transcriptional initiation. KDM2B-1 (NM_032590) is the longest and likely the full-length isoform and carries the histone demethylase JmjC domain, whereas KDM2B-2 (NM_013910) is translated from an mRNA initiated at an internal promoter of the KDM2B-1 transcript and lacks the JmjC domain (Supplementary Figure S1A). We first determined the subcellular localization and found that both long and short isoforms of KDM2B localized in the nucleus (Supplementary Figure S1B). We then characterized three short hairpin RNAs (shRNAs) targeting different regions of *KDM2B*. We found that sh*KDM2B* no. 1 targeted *KDM2B-1* (slow-migrating) only and sh*KDM2B* no. 2 targeted both of *KDM2B-1* and *KDM2B-2* effectively (Figure 1a). Notably, we found an increase of c-Fos protein levels in cells stably expressing sh*KDM2B* no. 1 and an even more marked increase of c-Fos protein levels in cells stably expressing sh*KDM2B* no. 2. This result suggested that KDM2B negatively regulated c-Fos protein level and this function of KDM2B did not require its JmjC domain-encoded histone demethylase activity. We next examined whether KDM2B controls the stability of c-Fos protein. Owing to the extremely low basal level expression of c-Fos protein in unstimulated cells, we established HEK293 cells stably expressing FLAG-tagged c-Fos (Supplementary Figure S1C). FLAG-c-Fos has a half-life ($t_{1/2}$) of ~18 min (Supplementary Figure S1D), confirming that c-Fos is a very short-lived protein undergoing rapid turnover. Treatment of cells with MG132, a 26S proteasome inhibitor, effectively blocked c-Fos degradation (Supplementary Figures S1E and S1F), confirming previous reports that c-Fos is degraded by the proteasome pathway.[11,12] Importantly, knockdown of *KDM2B* effectively stabilized FLAG-c-Fos, extending its half-life from 18 min beyond 40 min of experimental duration (Figure 1b).

To determine the mechanism by which KDM2B negatively regulates c-Fos, we tested the possibility that KDM2B promotes c-Fos degradation by binding to SKP1 and CUL1 through its F-box. We first determined whether CUL1 affects c-Fos protein level. We found that knockdown of *CUL1* in HEK293 cells with two different small interference RNAs (siRNAs) resulted in an ~6-fold increase of c-Fos protein levels without significant change in *c-Fos* mRNA levels (Figure 1c). Similarly depletion of *KDM2B* or *CUL1* in normal human fibroblast strains 1 (NHF1) also resulted in the accumulation of c-Fos (Figure 1d). Furthermore, codepletion of both *KDM2B* and *CUL1* did not cause any additional increase of c-Fos protein level (Figure 1d), suggesting that KDM2B and CUL1 act in the same pathway for controlling c-Fos. Taken together, these results demonstrate that KDM2B has a major role to target c-Fos protein degradation by a CUL1-based E3 ligase. Following commonly accepted nomenclature, we refer to the KDM2B-CUL1 E3 ligase as SCF/CRL1^{KDM2B} where the substrate recruiter F-box protein KDM2B is in superscript.

c-Fos is a substrate of SCF$^{KDM2B/FBXL10}$ E3 ubiquitin ligase

To demonstrate that KDM2B assembles the SCF^{KDM2B} ligase complex to target c-Fos ubiquitylation, we examined its interaction with endogenous SCF E3 ligase complex components. Co-immunoprecipitation (Co-IP) assays demonstrated that FLAG-tagged KDM2B interacted with endogenous SKP1, CUL1 and ROC1 (Figure 2a). Meanwhile, we also detected the interaction between FLAG-KDM2B and endogenous c-Fos (Figure 2a). The F-box domain in F-box proteins is essential for its binding to SKP1 and thus the assembly of SCF complex.[29–31] To determine the CUL1/SKP1-binding region in KDM2B, we constructed various deletion mutants of KDM2B (Supplementary Figure S2A). Co-IP assays revealed that deletion of both LRRs and F-box domains from KDM2B (ΔLRF), but not the LRRs only (ΔLRR), completely disrupted its binding to CUL1 (Supplementary Figure S2B). Conversely, the C-terminal of KDM2B (C300) containing the F-box and LRRs strongly interacted with CUL1 (Supplementary Figure S2B), suggesting that the F-box domain of KDM2B is essential for its binding to CUL1. Structural analysis has previously shown that the N-terminal region of F-box domain in SKP2 is essential for its binding to SKP1.[32] Similar to SKP2, deletion of 23 amino acids in the N-terminal half of the F-box domain from KDM2B (residues 1056–1078, referred to as ΔF23) substantially reduced its binding to CUL1, and deletion of 48 residues containing the entire F-box domain (residues 1057–1105, ΔF48) completely abolished its binding to CUL1 (Figure 2b). Two hydrophobic helical surfaces in the N-terminal tip of CUL1, H2 and H5, pack with hydrophobic and polar residues from SKP1 to form a large interface.[32] Mutation of either helix in CUL1 significantly disrupted its binding to KDM2B (Figure 2c). Taken together, these results demonstrate that KDM2B, via its F-box domain, assembles a *bona fide* SCF-type E3 ubiquitin ligase complex.

To demonstrate that endogenously expressed c-Fos binds to KDM2B *in vivo*, HEK293 cells were treated with MG132 followed by immunoprecipitation using an antibody against c-Fos. The interactions between c-Fos and KDM2B, two isoforms translated by different promoters, were readily detected (Figure 2d and Supplementary Figure S2C). A fragment containing the C-terminal 300 residues of KDM2B that includes the LRRs and F-box could bind c-Fos, and conversely, deletion of the LRRs substantially reduced the binding of KDM2B with c-Fos (Supplementary Figure S2D). Deletion of the F-box from KDM2B (ΔF48) also reduced the interaction between KDM2B and c-Fos (Figure 2e). These results indicate that the LRRs domain in KDM2B is mainly responsible for the binding with the substrate c-Fos and that the F-box domain may also contribute to the binding.

To demonstrate the ubiquitylation of c-Fos by the SCF^{KDM2B} E3 ligase, we carried out both *in vivo* and *in vitro* ubiquitylation assays. We first knocked down either *CUL1* or *KDM2B* and found that knocking down either gene reduced the ubiquitylation of endogenous c-Fos (Figure 2f). An *in vitro* ubiquitylation assay demonstrated that the CUL1 E3 immunocomplexes efficiently ubiquitylated c-Fos, converting nearly all c-Fos into ubiquitylated form (Figure 2g). The ubiquitylation of c-Fos was dependent on the addition of E1 and E2, CUL1 E3 complex, substrate receptor KDM2B and substrate c-Fos. We therefore conclude that c-Fos is a substrate of SCF^{KDM2B} E3 ubiquitin ligase.

EGF stabilizes c-Fos by dissociating c-Fos from KDM2B/FBXL10

The MEK/ERK signaling pathway activates c-Fos transcription in response to extracellular growth factors such as EGF.[9] Supporting this conclusion, EGF stimulation markedly increased the endogenous c-Fos protein as early as within 20 min, which was compromised by the classical MEK1/2 (the ERK1/2-activating kinases) inhibitor U0126 (Supplementary Figures S3A–C). In addition to the transcriptional activation, MEK/ERK pathway has also been reported to increase c-Fos level by stabilizing c-Fos protein through an undefined mechanism that involves phosphorylation at Ser362 and Ser374.[11] We therefore examined whether EGF stabilizes c-Fos protein by regulating SCF^{KDM2B}-mediated c-Fos ubiquitylation. To separate from the intrinsically transcriptional regulation of *c-Fos*, we took advantage of HEK293 cells that stably express ectopic FLAG-tagged c-Fos. EGF treatment induced FLAG-c-Fos protein accumulation as early as within 20 min and continuously in a time-dependent manner without significant change in *FLAG-c-Fos* mRNA levels (Figure 3a). Consistently, EGF treatment stabilized FLAG-c-Fos in HEK293 cells, extending its half-life from 15 to 45 min (Figure 3b). Moreover,

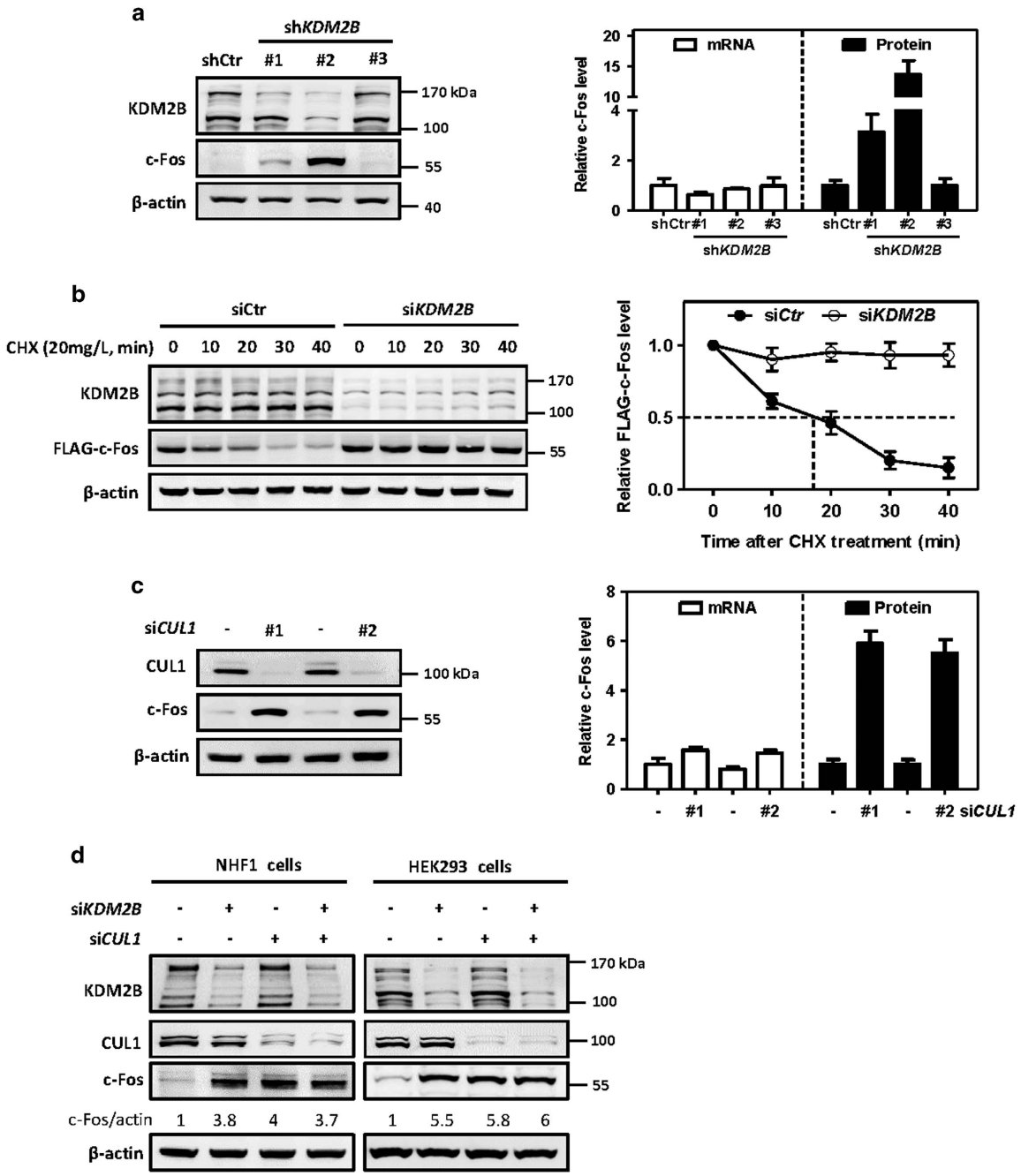
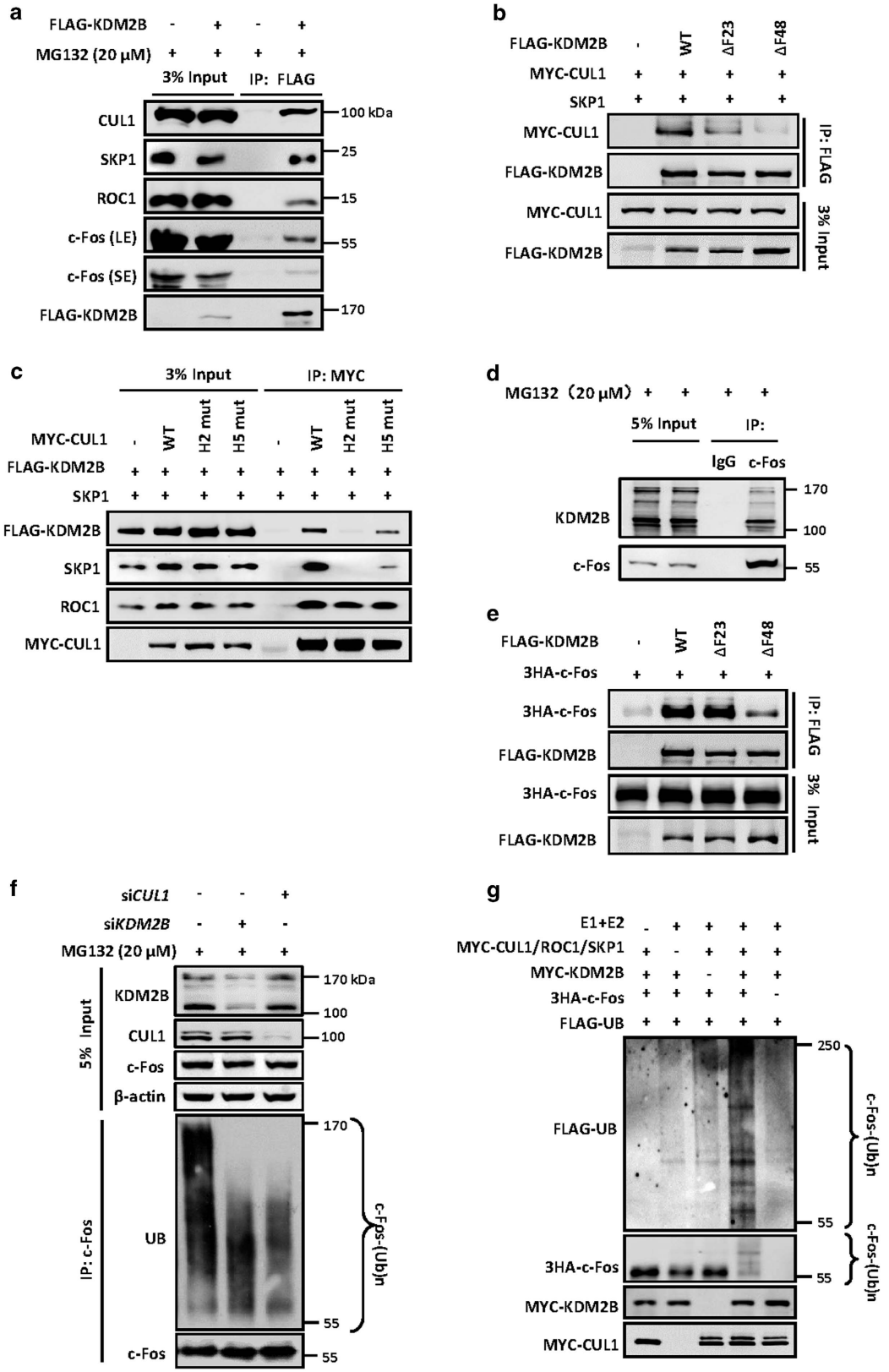


Figure 1. KDM2B/FBXL10 and CUL1 destabilize c-Fos protein. (**a**) Stable knockdown of *KDM2B* increases endogenous c-Fos protein level. Two distinct bands of KDM2B (170 and 120 kDa, respectively) were observed in HEK293 cells, representing the two isoforms. The protein and mRNA levels of c-Fos were determined by western blot and qRT–PCR, respectively, and normalized against β-actin. Error bars represent ±s.d. for triplicate experiments. (**b**) Ectopically expressed c-Fos is stabilized by knockdown of *KDM2B*. HEK293 cells stably expressing FLAG-c-Fos were transfected with siRNA oligonucleotides targeting *KDM2B* or control siRNA. At 72 h after siRNA transfection, the half-life of FLAG-c-Fos protein levels was determined by CHX (20 mg/l) chase assay and normalized against β-actin. Error bars represent ±s.d. for triplicate experiments. (**c**) Knockdown of *CUL1* increases c-Fos protein level. HEK293 cells were transfected with two different siRNAs targeting *CUL1* or a control siRNA. At 72 h after transfection, the protein and mRNA levels of c-Fos were determined by western blot and qRT–PCR, respectively, and normalized against β-actin. Error bars represent ±s.d. for duplicate experiments. (**d**) Knockdown of either *KDM2B* or *CUL1* or combination increases c-Fos protein to a similar level. NHF1 and HEK293 cells were transfected with siRNA oligonucleotides targeting either *KDM2B* or *CUL1* individually or in combination. The protein and mRNA levels of c-Fos were determined by western blot and qRT–PCR, respectively, and normalized against β-actin. Error bars represent ±s.d. for triplicate experiments.

EGF-induced c-Fos accumulation was blocked by the MEK1/2 inhibitor, U0126, and an ERK1/2 inhibitor, SCH772984 (Figure 3c). Inhibition of translation by the treatment of cells with cycloheximide (CHX) reduced the steady-state level of c-Fos protein, but this reduction was not seen in cells treated with EGF (Figure 3d), supporting a post-translational mechanism by which EGF stabilizes c-Fos. Notably, the c-Fos protein accumulated in EGF-treated cells was highly phosphorylated, as indicated by the appearance of a

discrete slower migrating band (Figures 3a–d), supporting a previous notion that the phosphorylated form c-Fos is resistant to the degradation.

To determine whether KDM2B is involved in the regulation of c-Fos by EGF, we first examined the effect of KDM2B knockdown on EGF-induced c-Fos protein levels and found that knockdown of *KDM2B* prolonged the high level of c-Fos protein following EGF stimulation (Figure 3e). To determine how EGF stabilizes c-Fos, we next examined the association between c-Fos and KDM2B in EGF-treated cells and found that EGF treatment dissociated their binding in a time-dependent manner (Figure 3f). The reduction of c-Fos and KDM2B interaction by EGF was completely blocked by



U0126 (Figure 3g), a highly selective inhibitor of both MEK1 and MEK2, suggesting the involvement of MEK/ERK signaling pathway in the regulation of c-Fos association with KDM2B. TPA (12-O-tetradecanoylphorbol-13-acetate) is a potent tumor promoter that activates PKC signaling pathway (PKC-Ras-Raf-MEK-ERK), which causes phosphorylation of c-Fos and dissociates c-Fos from KDM2B. Consistently, we found that TPA treatment decreased the association of KDM2B with c-Fos (Supplementary Figure S3F) and accumulated c-Fos protein (Supplementary Figure S3D). Importantly, we found that knockdown of *KDM2B* stabilized c-Fos protein in the presence of TPA (Supplementary Figure S3E). Taken together, these results demonstrate that c-Fos level is regulated at both transcriptional and post-translational levels and that c-Fos protein is stabilized by EGF-promoted phosphorylation, which dissociates c-Fos from its E3 ligase, KDM2B.

EGF-mediated phosphorylation at S374 stabilizes c-Fos and promotes cell proliferation

It has been previously reported that c-Fos can be stabilized by phosphorylation at several sites, including S32, S362 and S374 sites,[11,15] which are stimulated by ERK5 and ERK1/2 pathways. To determine which of these sites is involved in the ubiquitylation of c-Fos by the SCF^{KDM2B} E3 ligase, we established HEK293 cells stably expressing FLAG-c-Fos mutants targeting individual serine residues. We found that a non-phosphorylatable mutation at S374 (S374A) completely abolished EGF-induced c-Fos stabilization and protein accumulation, whereas S362A and S32A mutations had mild or no effect on EGF-induced increase of c-Fos stability and steady-state c-Fos level (Figure 4a and Supplementary Figure S4A). In contrast to S374A mutation, a phosphomimetic mutation of S374 (S374D) markedly extended the half-life of FLAG-c-Fos from ~18 min to more than 1 h of the experimental duration (Figure 4b), whereas neither S32D nor S362D mutation had significant effect on c-Fos stability (Supplementary Figure S4B). Collectively, these results indicate that S374 is a major phosphorylation site that contributes to c-Fos stabilization in response to EGF stimulation.

We next determined whether EGF-promoted c-Fos dissociation from KDM2B is mediated by S374 phosphorylation. Western blot showed that the S374-phosphorylated form is closely associated with the appearance of the slow-migrating form of c-Fos in EGF-treated cells that are resistant to KDM2B binding (Figures 3a and e and Supplementary Figure S3A). We then expressed wild-type and S374A mutant c-Fos and found that S374A mutation inhibited EGF-induced dissociation of c-Fos from KDM2B (Figure 4c). Notably, S374-phosphorylated c-Fos, while significantly induced by EGF, was not detected in the KDM2B immunocomplex (Figure 4d). Consistently, S374D mutation of c-Fos reduced its binding to KDM2B (Figure 4e). EGF-induced cell proliferation was partially compromised by the expression of S374A mutant of c-Fos and promoted by S374D mutation (Figures 4f and g). Consistently, c-Fos S374D mutant that is resistant to KDM2B-mediated degradation is more potent than wild-type in activating target genes (Supplementary Figure S4C). Taken together, these results demonstrate that EGF-induced phosphorylation at S374 in c-Fos disassociates it from KDM2B, resulting in increased stability and level of c-Fos and contributing to cell proliferation.

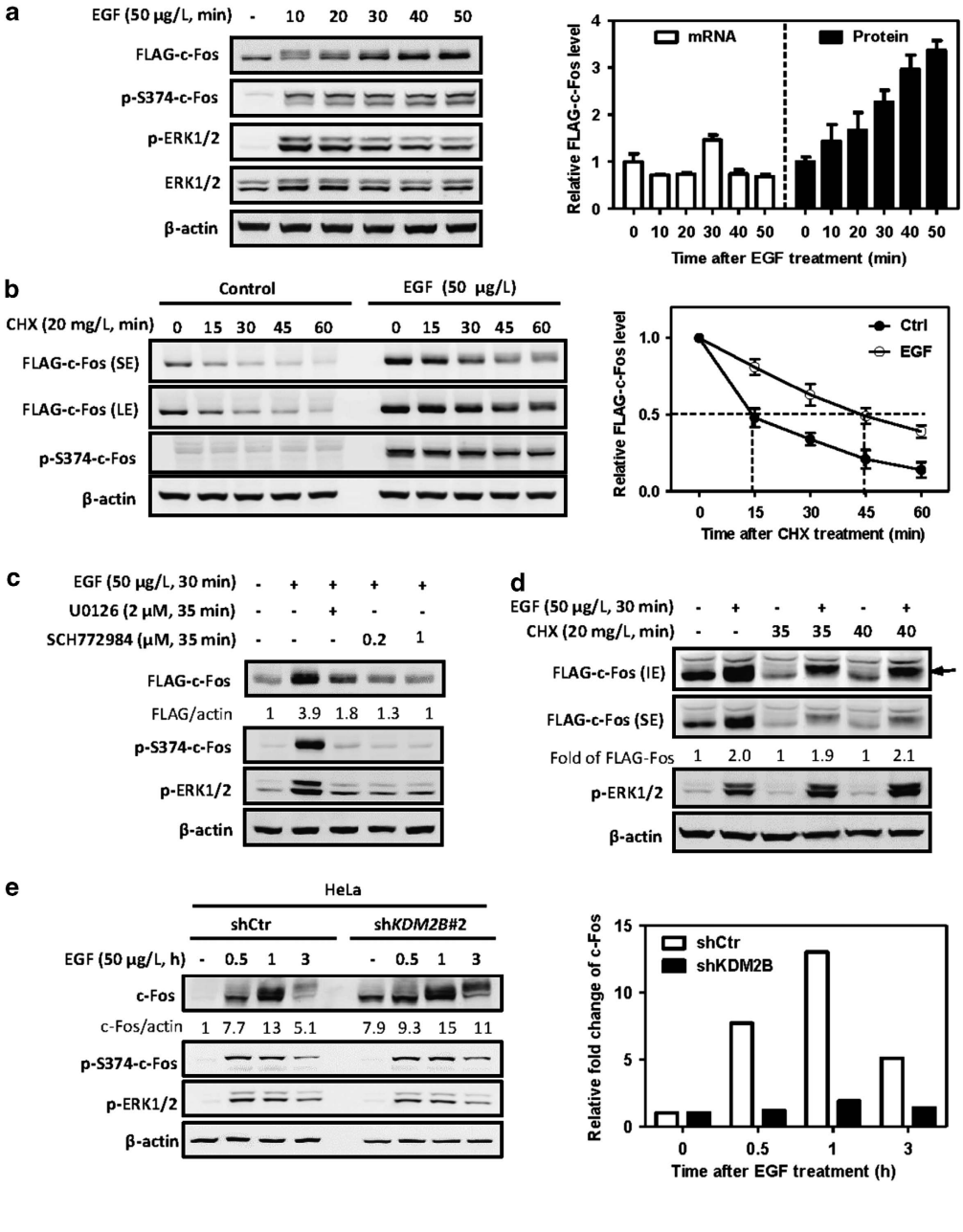
KDM2B/FBXL10 inhibits cell proliferation via promoting c-Fos degradation

To determine the effect of KDM2B on cell proliferation, we first generated HeLa cells with stable knockdown of *KDM2B*, which resulted in increased c-Fos protein levels (Figure 5a, left panel). Reintroduction of wild-type, but not ΔF-box mutant (ΔF48), KDM2B reduced the c-Fos protein level back to that similar in the parental HeLa cells (Figure 5a, right panel). Consistent with the increased level of c-Fos protein, ectopic expression of ΔF-box mutant, but not the wild-type KDM2B, increased the rate of cell proliferation, and this effect was not seen when *c-Fos* was knocked down (Figure 5b). EdU (5-ethynyl-2′-deoxyuridine) cell proliferation assay also showed that KDM2B ΔF-box mutant lost its ability to suppress cell proliferation and knockdown of *c-Fos* reduced cell proliferation back to the rate similar to that seen in control cells (Figure 5c and Supplementary Figure S5). We also examined several target genes of c-Fos linked to cell proliferation and growth, including cyclin D1 (*CCND1*) involved in G1 cell cycle control, matrix metallopeptidase 1 (*MMP1*) linked to embryonic development and tissue remodeling and *CD44* linked to cell–cell interactions, cell adhesion and migration. We found that the accumulation of c-Fos resulting from reduced expression or dysfunction of KDM2B increased the transcriptional level of these genes (Figure 5d). Taken together, these results demonstrate that KDM2B-promoted c-Fos degradation contributes to its function in the negative regulation of cell proliferation.

Tumor-derived mutations impair the function of KDM2B/FBXL10 to target c-Fos degradation and suppress cell proliferation

Recent cancer genome sequencing efforts have identified a number of mutations targeting KDM2B. For example, the COSMIC (Catalog of Somatic Mutations in Cancer) database reports 16 deletions and 7 nonsense mutations of KDM2B in different type of human cancers (Supplementary Figure S6). A loss of function of KDM2B could be viewed consistent with its function in suppressing cell proliferation. Among these reported mutations are three, C1085fs*14, W1161*, H1297fs*4, that predict to destroy the F-box or/and the leucine-rich repeats (LRRs) domains of KDM2B

Figure 2. c-Fos is a substrate of $SCF^{KDM2B/FBXL10}$ E3 ubiquitin ligase. (**a**) Ectopically expressed KDM2B binds to endogenous CUL1/SKP1/ROC1 E3 ligase. HEK293T cells were transfected with plasmid expressing FLAG-tagged KDM2B and treated with 20 μM MG132 for 6 h. The interactions between KDM2B and endogenous CUL1, SKP1, ROC1 and c-Fos proteins were determined by Co-IP assay. LE and SE refer to long exposure and short exposure, respectively. (**b**) The F-box domain of KDM2B is essential for its binding to CUL1. HEK293T cells were transfected with plasmids expressing indicated proteins. The interactions between wild-type (WT) or mutant KDM2B and CUL1 were determined by Co-IP assay. (**c**) The H2 and H5 helixes of CUL1 are essential for its binding to KDM2B. HEK293T cells were transfected with plasmids expressing indicated proteins. The interactions between KDM2B and WT or mutant CUL1 were determined by Co-IP assay. (**d**) Endogenous interactions between c-Fos and KDM2B two isoforms determined by Co-IP. HEK293 cells were treated with 20 μM MG132 for 6 h and cell lysates were used for IP with anti-IgG or c-Fos antibody. (**e**) The C-terminal of F-box domain in KDM2B is required for its binding to c-Fos. HEK293T cells were transfected with plasmids expressing indicated proteins. The interactions between WT or mutant KDM2B and c-Fos were determined by Co-IP. (**f**) Knockdown of either *KDM2B* or *CUL1* decreases c-Fos ubiquitylation *in vivo*. HEK293 cells were transfected with siRNA oligonucleotides targeting indicated genes and treated with 20 μM MG132 for 6 h. Endogenous c-Fos was immunoprecipitated and immunoblotted with an antibody-specific against ubiquitin. (**g**) *In vitro* ubiquitylation of c-Fos by SCF^{KDM2B} E3 ubiquitin ligase. Purified c-Fos protein was incubated with immunopurified E3 CUL1 complex and KDM2B individually or in combination in the presence or absence of E1, E2, ATP and FLAG-ubiquitin *in vitro* for 1 h. The reaction mixture was resolved by SDS–PAGE and blotted using antibodies (from top to bottom panels) recognizing FLAG, HA and MYC.



(Figure 6a). We therefore set to determine whether these tumor-derived mutations affect the activity of KDM2B in promoting c-Fos degradation and suppressing cell proliferation. We recreated all these three mutations and first examined their effect on the binding of KDM2B with c-Fos. We found that all three mutations reduced the binding of KDM2B with c-Fos when compared with the wild-type KDM2B expressed at a similar level (Figure 6b). We next carried out *in vivo* ubiquitylation assay and demonstrated that all three tumor-derived mutations in KDM2B evidently reduced the activity of KDM2B in promoting c-Fos ubiquitylation (Figure 6c). Knockdown-replacement experiment demonstrated that these three mutations also abolished the ability to decrease c-Fos protein (Figure 6d). We further demonstrated that all three tumor-derived mutants lost the function of KDM2B in suppressing cell proliferation (Figure 6e). Taken together, these results identify the first functional consequence linked to the tumor mutations in KDM2B.

DISCUSSION

This paper reports two key findings. First, KDM2B/FBXL10 functions as a *bona fide* F-box protein and assembles into an activate SCF/CRL1-type E3 ubiquitin ligase. KDM2B contains a JmjC domain that catalyzes histone H3K36 demethylation. Our finding identifies a second enzymatic activity to this multidomain protein. Of 32 human histone demethylases proteins, KDM2B, and its closest homolog, KDM2A/FBXL11, are the only two KDMs that contain an F-box domain. The F-box domains encode by KDM2B and KDM2A are highly related (78%). Our results suggest that KDM2A, which, similar to KDM2B, also catalyzes H3K36 demethylation and recognizes CpG through its JmjC and CxxC domains,[33] is likely to form a functional SCF E3 ligase. It remains to be determined whether and how these two enzymatic activities are mechanistically linked in gene regulation. It will also be interesting to determine whether KDM2B catalyzes ubiquitylation of any subunit of polycomb repressive complex 1 complex or a histone.

Second, c-Fos is robustly ubiquitylated and degraded by SCF^{KDM2B} E3 ligase. As an immediate-early gene, c-Fos level is rapidly accumulated by both transcriptional activation and protein stabilization, thereby conferring cells the ability to regulate rapidly activator protein-1 target genes during stress and mitogenic responses. Our finding provides a mechanism—dissociating c-Fos from its E3 ligase by EGF-promoted phosphorylation at S374—for cells to stabilize c-Fos protein in response to mitogenic stimulation. Our observations also provide a plausible explanation for the previously observed inhibition of c-Jun- and c-Fos-mediated transcriptional activity by KDM2B.[14,28] Conversely, this mechanism allows cells to maintain the c-Fos at a very low level after withdrawal of the mitogenic stimulation through turning off the c-Fos transcription and constitutive degradation of c-Fos protein once the S374 is dephosphorylated. Many other SCF substrates are also regulated by phosphorylation, which targets a small motif, known as degron, and promotes the substrate to bind with an F-box protein and be degraded by the SCF ligase. Linking signal-dependent phosphorylation to ubiquitylation provides cells an efficient mechanism to regulate protein function through crosstalk between different post-translational modifications. The finding reported here further expands the spectrum of this crosstalk.

MATERIALS AND METHODS

Plasmids and chemicals

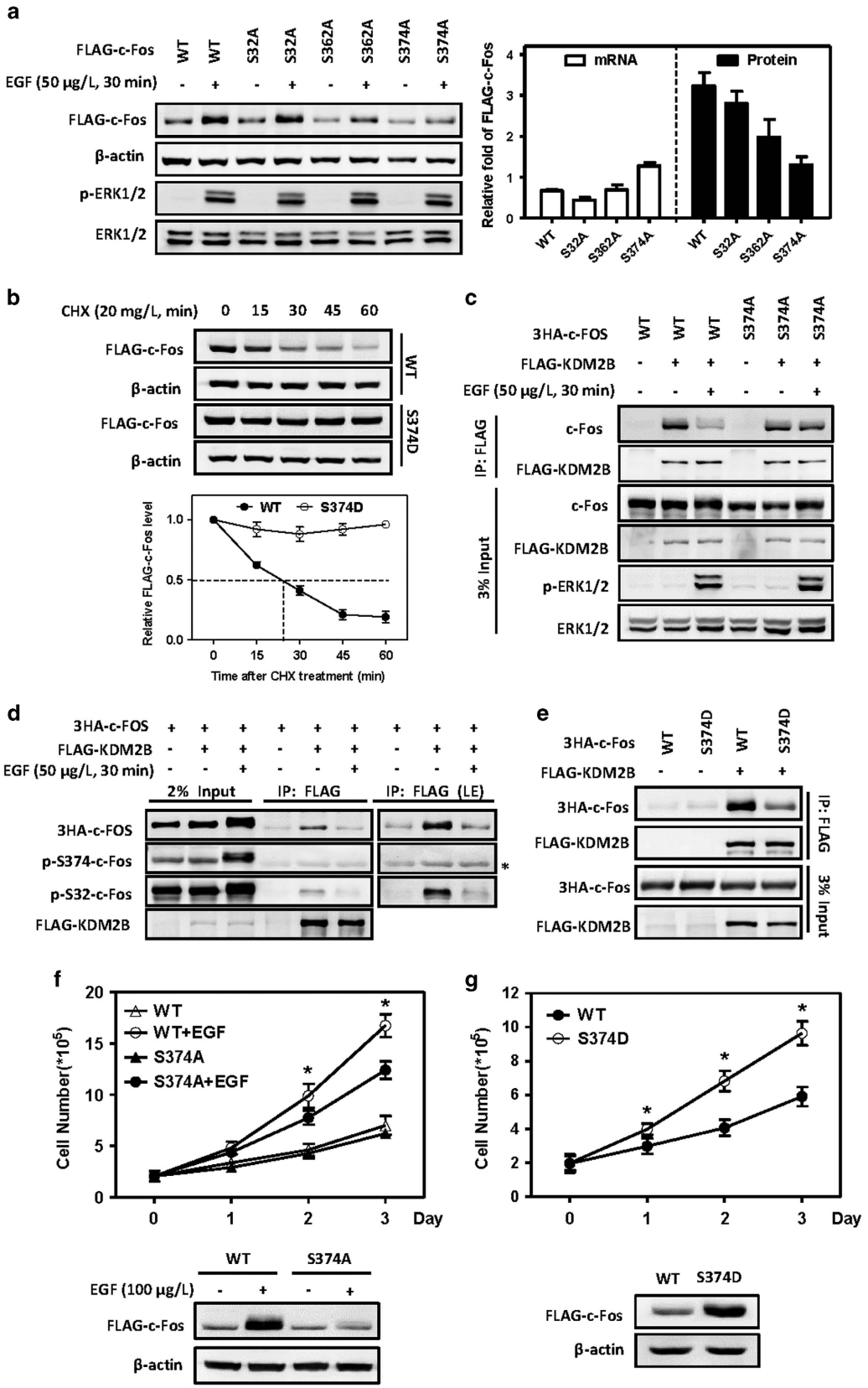
Full-length human KDM2B cDNA was a kind gift from Michele Pagano of New York University (New York, NY, USA). Wild-type and mutant KDM2B were constructed into pRK7-N-FLAG vector for transient expression or pQCXIH-N-FLAG vector for stable transduction by retrovirus infection. Wild-type and mutant CUL1 were constructed into pRK7-N-MYC vector for transient expression. c-Fos cDNA was amplified from a cDNA library of HEK293 cells. Wild-type and mutant c-Fos were constructed into pCDNA3-N-3HA vector for transient expression or pQCXIH-N-FLAG vector for stable transduction by retrovirus infection. The lentivirus packaging plasmids containing sh*KDM2B* were purchased from Shanghai Genechem Company (Shanghai, China). The target sequences of *shKDM2B* are as follows: *shKDM2B* no. 1, 5′-TGAGCGTGAAAGGTTGTTT-3′; *shKDM2B* no. 2, 5′-GCC TTTACAAGAAGACATT-3′; *shKDM2B* no. 3, 5′-TTCTTCAAACGCTGTGGAA-3′. MG132 (C2211; Sigma-Aldrich, St Louis, MO, USA), CHX (94271; Amresco, Solon, OH, USA), EGF (AF-100-15; PeproTech, Rocky Hill, NJ, USA), U0126 (S1102; Selleckchem, Shanghai, China) and SCH772984 (S7101; Selleckchem) were purchased commercially.

Cell culture, transfection and proliferation assay

HEK293, HEK293T and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). NHF1 was a kind gift from Dr William K Kaufmann of University of North Carolina (Chapel Hill, NC, USA). NHF1 was derived from neonatal foreskins and established in secondary culture according to established methods.[34] Mycoplasma contamination was not detected by PCR. HEK293, HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% newborn calf serum, 8 mM L-glutamine, 50 μg/ml penicillin and streptomycin (Gibco, Grand Island, NY, USA). NHF1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HEK293 and HeLa cells with

Figure 3. EGF stabilizes c-Fos by dissociating c-Fos from KDM2B/FBXL10. (**a**) EGF induces c-Fos protein accumulation in a time-dependent manner. HEK293 cells stably expressing FLAG-tagged c-Fos were deprived of serum for 8 h, and then treated with 50 μg/l EGF for the indicated length of time. The protein and mRNA levels of FLAG-c-Fos were determined by western blot and qRT–PCR, respectively, and normalized against β-actin. Error bars represent ± s.d. for triplicate experiments. (**b**) EGF stabilizes c-Fos. HEK293 cells stably expressing FLAG-tagged c-Fos were pretreated with or without EGF (50 μg/l) for 30 min, followed by CHX treatment (20 mg/l) for the indicated time. The protein levels of FLAG-c-Fos were determined by western blot and normalized against β-actin. Error bars represent ± s.d. for triplicate experiments. (**c**) EGF-induced c-Fos accumulation is blocked by MEK1/2 inhibitor U0126 and an ERK1/2 inhibitor (SCH772984). HEK293 cells stably expressing FLAG-c-Fos were deprived of serum for 8 h, followed by treatment with U0126 (2 μM) or SCH772984 (0.2 or 1 μM) for 5 min. EGF (50 μg/l) was then added for additional 30 min. The protein levels of c-Fos were determined by western blot and normalized against β-actin. (**d**) EGF-induced c-Fos accumulation is not affected by inhibition of protein synthesis. HEK293 cells stably expressing FLAG-tagged c-Fos were treated with CHX (20 mg/l) for 5 min or 10 min. Solvent or EGF (50 μg/l) was then added for further treatment for 30 min. The protein levels of FLAG-c-Fos were determined by western blot and normalized against β-actin. Data are shown as relative fold change over protein levels in cells without EGF treatment. The arrow represents highly phosphorylated c-Fos. (**e**) Knockdown of *KDM2B* prolongs the high level of c-Fos protein following EGF stimulation. HeLa cells with stable knockdown of *KDM2B* were deprived of serum for 8 h, followed by treatment with EGF (50 μg/l) for the indicated length of time. The protein levels of c-Fos were determined by western blot and normalized against β-actin. Data are shown as relative fold change over cells without EGF treatment (right panel). (**f**) EGF treatment induces c-Fos S374 phosphorylation and concomitantly reduces the interaction between c-Fos and KDM2B. HEK293 cells were transfected with plasmids expressing indicated proteins and then treated with EGF (50 μg/l) for the indicated length of time. The interactions between c-Fos and KDM2B were determined by Co-IP. (**g**) The reduction of c-Fos and KDM2B interaction by EGF is blocked by U0126. FLAG-KDM2B and 3HA-c-Fos were co-transfected into HEK293 cells. Cells were pretreated with U0126 (2 μM) for 5 min and then treated with EGF (50 μg/l) for another 30 min. The interactions between c-Fos and KDM2B were determined by Co-IP.

stable knockdown or overexpression were established by lentivirus or retrovirus transduction, respectively, selected and maintained in medium containing 1 μg/ml puromycin or 50 μg/ml hygromycin B (Amresco). Cells were cultured in a 37 °C incubator with 5% CO_2. Cell transfection was performed using Lipofectamine 2000 (Life Technologies) for plasmid DNA or Lipofectamine RNAi MAX (Life Technologies) for siRNAs following the manufacturer's instructions. Cells were harvested at 48–72 h post-transfection for protein analyses.



For cell proliferation assay, stable HEK293 cells were triply seeded in 6-well plates at a density of 2×10^5 cells per well and deprived of serum for indicated length of time after adhering for 12 h. Stable HeLa cells were triply seeded in 6-well plates at a density of 4×10^4 cells per well. Cell numbers were counted daily by using Countstar IC1000 (Alit International Trade Co., Ltd., Shanghai, China). For EdU cell proliferation assay, HeLa cells were labeled with 10 μM EdU for 1 h, and then collected with 1% bovine serum albumin in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS. After incubation for 20 min, cells were washed with 1% bovine serum albumin in PBS and permeabilized in 0.5% Triton X-100 for 20 min. Cells were washed and resuspended in the cocktail (250 μl for each sample: PBS 215 μl, $CuSO_4$ 10 μl, sodium ascorbate 25 μl and Azide Alexa Fluor (A10266; Life Technologies) 0.6 μl for 30 min. Cells were washed and resuspended in 500 μl 1% bovine serum albumin in PBS. The percentage of EdU-positive cells was determined by using BD ACCURI C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Antibodies and immunological procedures

Antibodies against FLAG (SG4110-16; Shanghai Genomics Technology, Shanghai, China), MYC (SG4110-18; Shanghai Genomics Technology), β-actin (A00702; GenScript, Shanghai, China), HA (sc-7392; Santa Cruz, Dallas, TX, USA), KDM2B (09-864; Millipore, Billerica, MA, USA), CUL1 (2436-1; Epitomics, Shanghai, China), SKP1 (2538-1; Epitomics), ROC1 (5296-1; Epitomics), ubiquitin (Z045801-5; Dako, Carpinteria, CA, USA), phospho-ERK1/2(Thr202/Tyr204) (4370S; Cell Signaling Technology, Shanghai, China), ERK1/2 (4695P; Cell Signaling Technology), c-Fos (3620-1 (Epitomics) and sc-52 (Santa Cruz)), phospho-c-Fos (Ser32) (5348S; Cell Signaling Technology) and phospho-c-Fos (Ser374) (sc-81485; Santa Cruz) were purchased commercially.

For immunoprecipitation experiments, cells were washed with cold PBS once and lysed in a NP-40 lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 0.5% NP-40 and supplemented with protease inhibitors and phosphatase inhibitors) for 1 h at 4 °C with gentle shaking. Ten percent of cell lysates were mixed directly with 5x Laemmli sample buffer and used as input on western blot. The rest were incubated with specific antibody for 3 h at 4 °C, followed by the addition of protein A-conjugated beads for 1 h at 4 °C. Immunoprecipitates were washed three times with lysis buffer, and proteins were eluted from beads with 50 μl 1x Laemmli loading buffer.

For western blotting, cells were washed with cold PBS once and lysed in 1x Laemmli loading buffer directly or in NP-40 buffer if used for a subsequent immunoprecipitation. Lysates were heated at 99 °C for 5 min, and resolved on 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membrane. Membrane was blocked in 5% milk in Tris-buffered saline and Tween-20 for 1 h at room temperature, followed by incubation with a primary antibody overnight at 4 °C, and a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membrane was imaged either by a Typhoon Scanner (GE Healthcare, Lafayette, CO, USA) or a LAS 4000 Imager system (GE Healthcare).

In vivo and *in vitro* ubiquitination assay

The procedures for both assays were modified from our previously published report.[35] Briefly, for *in vivo* ubiquitylation assay, at 36–48 h after transfection, cells were treated with proteasome inhibitor, MG132 (20 μM) for 6 h to accumulate polyubiquinated c-Fos and thus increase the sensitivity of detection. Cells were collected and lysed in 1% SDS lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA and 1 mM DTT) and boiled for 10 min. For immunoprecipitation, the clarified SDS lysates were diluted 10-fold in 0.5% NP-40 lysis buffer, 10% of total lysates were mixed directly with 5x Laemmli sample buffer and used as input on western blot. The rest were incubated with anti-c-Fos antibody for 3 h at 4 °C, followed by the addition of protein A-agarose for 1 h at 4 °C. Immunoprecipitates were washed three times with lysis buffer and boiled in 1x Laemmli loading buffer before SDS–PAGE. The ubiquitylation levels of c-Fos were determined by immunoblot with anti-Ub antibody.

For *in vitro* ubiquitylation assay, plasmids expressing different genes, as indicated in the figures, were transfected into HEK293T cells. At 48 h after transfection, cells were lysed in NP-40 lysis buffer supplemented with a cocktail of protease inhibitors and phosphatase inhibitors, followed by immunoprecipitation using anti-c-MYC agarose (A7470; Sigma-Aldrich) or anti-HA Sepharose (sc-7392 AC; Santa Cruz). Immunocomplexes were washed with the lysis buffer three times and eluted with MYC (EQKLISEEDL) or HA (YPYDVPDYA) antigen peptides, respectively. 3HA-c-Fos protein was immunopurified and used as the substrate. CUL1-based E3 ligase complex and substrate receptor KDM2B were derived from HEK293T cells co-transfected with plasmids expressing MYC-CUL1, ROC1 and SKP1 or singularly transfected with plasmid expressing MYC-KDM2B, respectively. *In vitro* ubiquitylation was initiated by mixing the substrate with immunopurified E3 complex and KDM2B in a ubiquitin ligation buffer (50 mM Tris-HCl at pH 7.4, 5 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 10 nM okadaic acid (Beyotime Biotechnology, Shanghai, China), 0.6 mM DTT, 12 μg of bovine ubiquitin (Sigma-Aldrich), 1 μg of FLAG-tagged ubiquitin (Sigma-Aldrich), 60 ng of E1 (E301; Boston Biochem, Cambridge, MA, USA), 500 ng of E2 (human Ubc5c), final volume: 30 μl). The reactions were incubated at 37 °C for 1 h on a rotator with gentle shaking, then terminated with SDS sample buffer and boiled at 99 °C for 5 min before SDS–PAGE. The ubiquitylation levels of c-Fos were determined by immunoblot with either anti-FLAG or anti-HA antibody.

RNA interference

RNAi-mediated downregulation of human *KDM2B*, *CUL1*, was performed by transfecting siRNAs into cells in accordance with the manufacturer's instructions of Lipofectamine RNAi MAX (Life Technologies). A non-targeting control siRNA duplex (sense, 5′-UUCUCCGAACGUGUCACGUTT-3′) was included as a negative control. The knockdown efficiency was assessed 72 h after transfection by western blot.

The siRNAs targeting *KDM2B* (siGENOME SMARTpool M-014930-01-0005) were purchased from Dharmacon (Lafayette, CO, USA), which was a mixture of four si*KDM2B* oligonucleotides. The siRNAs targeting *CUL1* (Stealth siRNAs HSS112311 and HSS112310) were purchased from Life

Figure 4. EGF-mediated phosphorylation at S374 stabilizes c-Fos and promotes cell proliferation. (**a**) Mutation of S374 site to alanine abolishes EGF-induced c-Fos protein accumulation. HEK293 cells stably expressing FLAG-tagged c-Fos were deprived of serum for 8 h, and then treated with EGF (50 μg/l) for 30 min. The protein and mRNA levels of FLAG-c-Fos were determined by western blot and qRT–PCR, respectively, and normalized against β-actin. Data are shown as relative fold change over cells without EGF treatment. Error bars represent ± s.d. for duplicate experiments. (**b**) Mutation of S374 site to aspartic acid renders c-Fos resistant to degradation. HEK293 cells stably expressing FLAG-tagged c-Fos WT or S374D mutant were treated with 20 mg/l CHX for the indicated length of time. The half-life of c-Fos protein levels were determined by western blot and normalized against β-actin (bottom). Error bars represent ± s.d. for triplicate experiments. (**c**) The reduction of c-Fos and KDM2B interaction by EGF is inhibited by c-Fos S374A mutant. HEK293 cells were co-transfected with plasmids expressing indicated proteins and then treated with or without EGF (50 μg/l) for 30 min. The interactions between c-Fos and KDM2B were determined by Co-IP. (**d**) KDM2B preferentially interacts with S374 non-phosphorylatable c-Fos. HEK293 cells were co-transfected with plasmids expressing the indicated proteins and then treated with or without EGF (50 μg/l) for 30 min. The FLAG-KDM2B was immunoprecipitated and western blot was performed to detect the co-precipitated c-Fos with indicated antibodies. *The heavy-chain background around 55 kDa. (**e**) S374D mutation of c-Fos hinders its binding to KDM2B. HEK293 cells were co-transfected with plasmids expressing indicated proteins, and the interactions between c-Fos and KDM2B were determined by Co-IP. (**f**) EGF-induced cell proliferation is compromised by S374A mutant of c-Fos. HEK293 cells stably expressing FLAG-c-Fos WT or S374A mutant were cultured in the absence of serum and treated with or without EGF (100 μg/l) for 0, 1, 2 or 3 days, as indicated. EGF was replenished every day. Cell numbers were counted each day. Western blot was performed to show FLAG-c-Fos protein levels on the third day. *The $P < 0.05$ for cells stably expressing S374A mutant versus WT c-Fos under EGF treatment. Error bars represent ± s.d. for triplicate experiments. (**g**) c-Fos S374D mutant is more potent than WT in promoting cell proliferation. HEK293 cells stably expressing FLAG-c-Fos WT or S374D mutant were cultured in the absence of serum for 0, 1, 2 or 3 days, as indicated. Cell numbers were counted each day. Western blot was performed to show FLAG-c-Fos protein levels on the third day. *$P < 0.05$ for cells stably expressing S374D mutant versus WT c-Fos. Error bars represent ± s.d. for triplicate experiments.

Technologies. The sequences of all siRNAs used in this study are shown as follows: si*KDM2B* no. 1, 5′-CAGCAUAGACGGCUUCUCU-3′; si*KDM2B* no. 2, 5′-GGGAGUCGAUGCUUAUUGA-3′; si*KDM2B* no. 3, 5′-GACCUCAGCUGGACCAAUA-3′; si*KDM2B* no. 4, 5′-GCAAUAAGGUCACUGAUCA-3′; si*CUL1* no. 1, 5′-GGCCACUGAAUAAACAGGUAACAAA-3′; si*CUL1* no. 2, 5′-GGAGCUCAGUUUGUUGGCCUGGAAU-3′; si*Fos*, 5′-GGGAUAGCCUCUCUUACUA-3′.

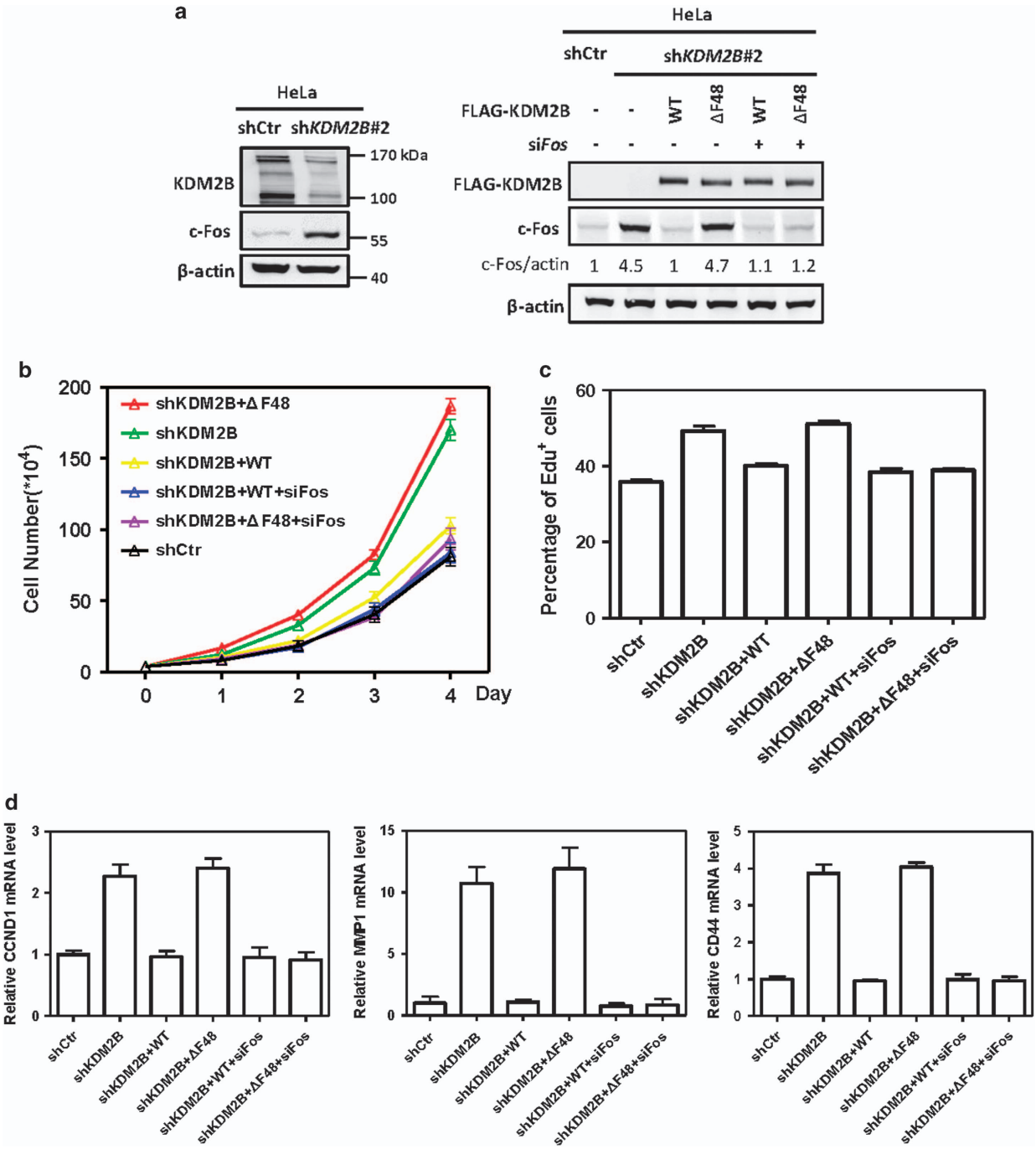


Figure 5. KDM2B/FBXL10 inhibits cell proliferation via promoting c-Fos degradation. (**a**) KDM2B ΔF-box mutant abolished its function in promoting c-Fos degradation. HeLa cells with stable knockdown of *KDM2B* were established by lentivirus transduction. FLAG-tagged WT or ΔF-box mutant of KDM2B was then stably expressed in these cells. siRNA targeting *Fos* or control siRNA was transfected into stable cells as indicated. The protein levels of c-Fos were determined by western blot and normalized against β-actin. (**b**) ΔF-box mutant losses the function of KDM2B in suppressing cell proliferation, and this effect is compromised by c-Fos knockdown. Stable HeLa cells identified as in Figure 5a were transfected with siRNA targeting *Fos*. Cell numbers were counted each day. Error bars represent ±s.d. for triplicate experiments. (**c**) ΔF-box mutant losses the function of KDM2B in inhibiting DNA synthesis, and this effect is compromised by c-Fos knockdown. Stable HeLa cells identified as in Figure 5a were transfected with siRNA targeting *Fos* and labeled with 10 μM EdU for 1 h. The percentage of EdU-positive cells was determined by flow cytometry, indicating the percentage of S-phase cells in the population. Error bars represent ±s.d. for triplicate experiments. (**d**) Knockdown of *KDM2B* increases the transcriptional level of c-Fos-targeting genes, which is compromised by put-back of wild-type, but not ΔF-box mutant KDM2B. Stable HeLa cells identified as in Figure 5a were transfected with siRNA targeting *Fos*. The mRNA levels of genes were determined by qRT–PCR respectively and normalized against β-actin. Error bars represent ±s.d. for triplicate experiments.

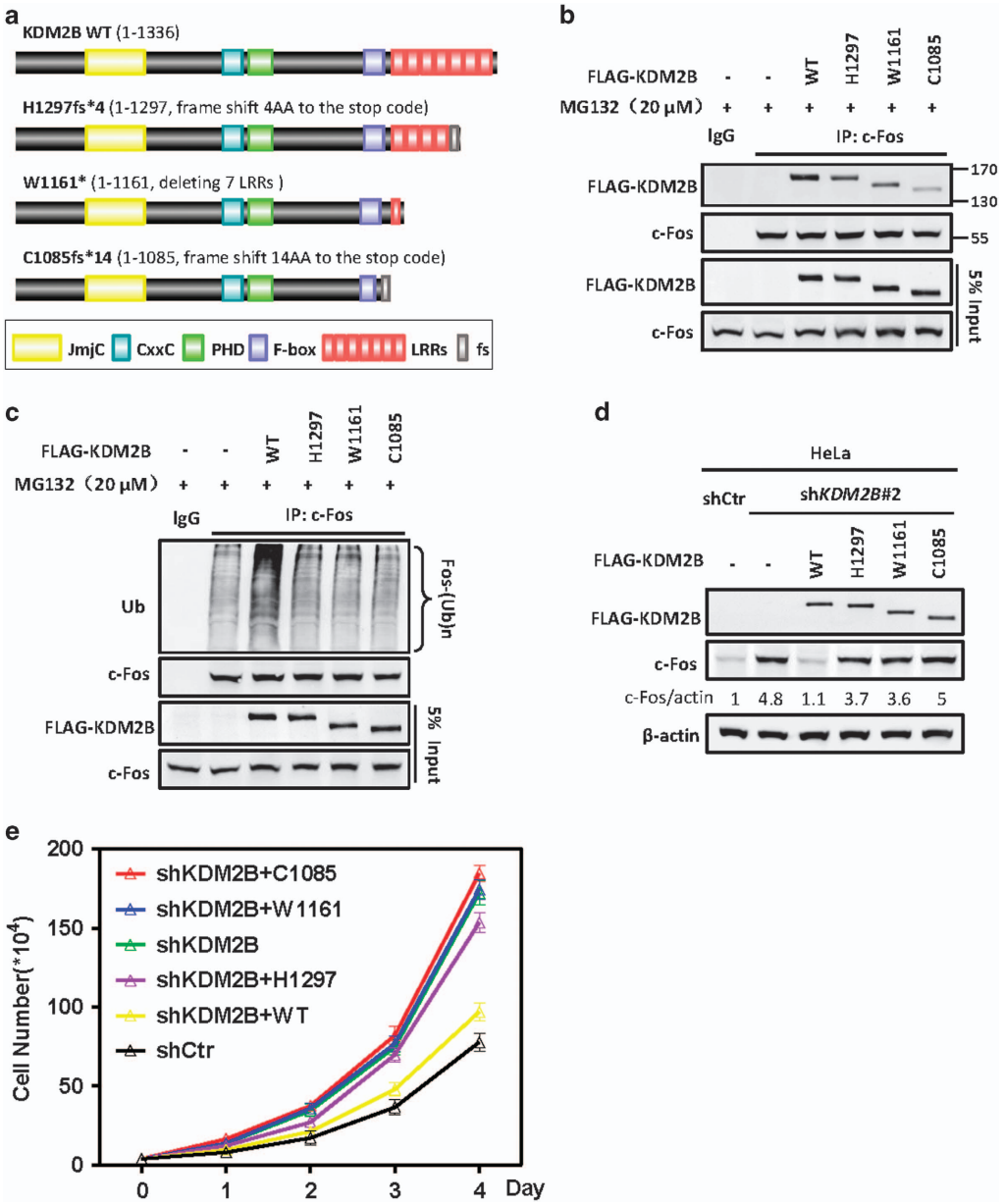


Figure 6. Tumor-derived mutations impair the function of KDM2B/FBXL10 to target c-Fos degradation and suppress cell proliferation. (**a**) Schematic representations of three tumor-derived mutants of KDM2B examined in this study. Additional tumor-derived mutations in KDM2B are shown in Supplementary Figure S6. (**b**) Tumor-derived mutations in KDM2B impair their binding to c-Fos. HEK293 cells were transfected with plasmids expressing indicated proteins and treated with 20 μM MG132 for 6 h. The interactions between WT or mutant KDM2B and c-Fos were determined by Co-IP. (**c**) Tumor-derived mutations in KDM2B impair their ability to ubiquitylate c-Fos *in vivo*. HEK293 cells were transfected with plasmids expressing indicated proteins and treated with 20 μM MG132 for 6 h. Endogenous c-Fos was immunoprecipitated and immunoblotted with an antibody specific against ubiquitin. (**d**) Tumor-derived mutants of KDM2B abolish their function in promoting c-Fos degradation. FLAG-tagged WT or tumor-derived mutants KDM2B was stably expressed in HeLa cells with stable knockdown of *KDM2B*. The protein levels of c-Fos were determined by western blot and normalized against β-actin. (**e**) Tumor-derived mutants lose the function of KDM2B in suppressing cell proliferation. Stable HeLa cells were identified as in (**d**). Cell numbers were counted each day. Error bars represent ±s.d. for triplicate experiments.

RNA isolation and qRT–PCR analysis

Total RNA was isolated from cultured cells using Trizol reagent (Life Technologies) following the manufacturer's instructions. Total RNA (2–5 μg) was reversely transcribed with oligo-dT primers and preceded to quantitative reverse transcription–PCR (qRT–PCR) with gene-specific primers in the presence of SYBR Premix Ex Taq (TaKaRa, Dalian, China). All data were performed in triplicate and β-actin (ACTB) was used as a housekeeping control. Relative abundance of mRNA was determined in 7500 real-time PCR system (Applied Biosystems, Grand Island, NY, USA). Primers (*FLAG-c-Fos*-F/R) were designed to detect specifically the ectopic FLAG-tagged c-Fos, but not endogenous c-Fos. Primer sequences are as follows: *ACTB*-Forward, 5′-GCACAGAGCCTCGCCTT; *ACTB*-Reverse, 5′-GTTGTCGACGACGAGCG-3′; *c-Fos*-F, 5′-CTACCACTCACCCGCAGACT-3′; *c-Fos*-R, 5′-GTGGGAATGAAGTTGGCACT-3′; *FLAG-c-Fos*-F, 5′-GGTAGGCCTCGTACGCTTAAT-3′; *FLAG-c-Fos*-R, 5′-AGGATGACGCCTCGTAGTCT-3′; *CCND1*-F, 5′-GCTGCGAAGTGGAAACCATC-3′; *CCND1*-R, 5′-CCTCCTTCTGCACACATTTGAA-3′; *MMP1*-F, 5′-TTCGGGGAGAAGTGATGTTC-3′; *MMP1*-R, 5′-TCTCTGTCGGCAAATTCGTA-3′; *MMP9*-F, 5′-TGTACCGCTATGGTTACACTCG-3′; *MMP9*-R, 5′-GGCAGGGACAGTTGCTTCT-3′; 5′-*MMP13*-F, TCCTGATGTGGGTGAATACAATG-3′; *MMP13*-R, 5′-GCCATCGTGAAGTCTGGTAAAAT-3′; *CD44*-F, 5′-GACAAGTTTTGGTGGCACG-3′; *CD44*-R, 5′-CACGTGGAATACACCTGCAA-3′; *PTGS2*-F, 5′-GGCGCTCAGCCATACAG-3′; *PTGS2*-R, 5′-CCGGGTACAATCGCACTTAT-3′.

Statistical analysis

Statistical analyses were performed with a paired, two-tailed Student's *t*-test. All data shown represent the results obtained from triplicated independent experiments with standard errors of the mean (mean ± s.d.). The values of $P < 0.05$ were considered statistically significant. No sample was excluded from the analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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