Regulation of E2F1 Function by the Nuclear Corepressor KAP1*

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KAP1 is a nuclear corepressor with conserved domains for RING finger, B boxes, leucine zipper α helical-coiled-coil region, plant homeo domain finger, and bromo domain. The plant homeo domain finger and bromo domain of KAP1 cooperatively function as a transcription repression domain by recruiting the histone deacetylase complex NuRD and histone H3 lysine 9-specific methyltransferase SETDB1. Here we report that KAP1 binds the E2F1 transcription factor in a retinoblastoma protein (pRb)-independent fashion and inhibits E2F1 activity. KAP1 stimulates formation of E2F1-HDAC1 complex and inhibits E2F1 acetylation. Ectopic expression of KAP1 represses E2F1 transcription and apoptosis functions independent of pRb. Depletion of endogenous KAP1 in pRb-deficient Saos2 cells by RNA interference increases E2F1 acetylation level, stimulates E2F1 transcriptional activity, and sensitizes apoptosis response to DNA damage. Therefore, KAP1 contributes to the negative regulation of E2F1 and may serve as a partial backup to prevent E2F1-mediated apoptosis in the absence of pRb.

Members of the E2F family, particularly E2F1, play important roles in regulating cell proliferation and apoptosis. E2F1 stimulates the transcription of several genes in the apoptosis pathway (1). Overexpression of E2F1 induces premature S phase entry and often results in apoptosis (2, 3). E2F1 is stabilized and activated by DNA damage in an ATM-dependent manner, resulting in the activation of a subset of E2F1 targets (4, 5). E2F1 also undergoes acetylation on several lysine residues adjacent to the DNA binding domain, which regulates its DNA binding (6). The acetylation level of E2F1 is stimulated by DNA damage, which may regulate its ability to induce pro-apoptotic targets (7). DNA damage-induced apoptosis in p53-deficient cells often require E2F1 activity (8, 9).

The retinoblastoma protein pRb has been established as the principle regulator of E2F1 (10). pRb forms a complex with E2F1 and recruits histone deacetylase 1 (HDAC1) and SUV39H1 to E2F1 target promoters, causing changes in chromatin modification levels and transcriptional silencing (11). Mutation of pRb or hyperphosphorylation by activated cyclin D/E-cyclin-dependent kinase 4 complex in cancer cells presumably leads to deregulation of E2F1 activity, contributing to loss of cell cycle control and increased tendency to undergo apoptosis. However, it is clear that E2F1 activity is controlled by multiple factors in addition to pRb. The TopBP1 protein has been shown to bind E2F1 independent of pRb and recruits Brg1, a central component of the SWI/SNF chromatin-remodeling complex to repress E2F1 target promoters (12, 13). The Class III histone deacetylase SirT1 also interacts with E2F1 and inhibits E2F1 transcriptional and apoptosis function after DNA damage (14). Therefore, the apoptosis activity of E2F1 is likely to be controlled by multiple factors besides pRb to prevent cell death in the absence of pRb. These factors may provide redundancy, promoter specificity, and signal-specific regulation of E2F1.

In this report, we describe the identification of nuclear corepressor KAP1 as a novel E2F1-binding protein. KAP1 (also named TIF1β) was first identified as a factor important for the transcription repression function of Kruppel-associated box domain (15). KAP1 contains conserved domains for RING finger, B boxes, leucine zipper α helical-coiled-coil region, plant homeo domain finger, and bromo domain. The N-terminal RING, B boxes, and coiled-coil sequences (RBCC) are necessary and sufficient for interaction with Kruppel-associated box and formation of KAP1 oligomers (16). The RBCC tripartite motif (also called TRIM) is found in a large family of proteins including PML, TIF1, and Rfp (17). The C-terminal plant homeo domain finger and bromo domain of KAP1 cooperatively function as a transcription repression domain by recruiting the histone deacetylase complex NuRD and histone H3 lysine 9-specific methyltransferase SETDB1 (18, 19). Therefore, KAP1 may function as a corepressor in part by being targeted to DNA by other transcription factors and promotes modification of chromatin structure. KAP1 expression is important for normal development in mice. Germ line homozygous knock out of KAP1 results in embryonic lethality at E5.5 (20).

The physiological targets and functions of KAP1 are still poorly understood. Its interactions with chromatin modification factors such as HDAC1, SETDB1, and HP1 are consistent with interesting activities in the regulation of chromatin structure and heterochromatin formation, resulting in both transient and stable epigenetic silencing of reporter genes in artificial systems (21, 22). Recent studies suggested that KAP1 is regulated by DNA damage and is phosphorylated by ATM on
Ser-824 (23, 24), which then mediates global chromatin decondensation after formation of DNA double-stranded breaks. KAP1 expression and phosphorylation of Ser-824 is important for cell survival after DNA damage through unknown mechanisms. Recently, KAP1 has also been shown to interact with the p53 regulator MDM2 and inhibit p53 activity (25, 26). KAP1 depletion by RNA interference increases p53 activity after DNA damage, which may also contribute to increased apoptosis.

Results described below show that E2F1 and KAP1 interact through the coiled-coil domain of KAP1 and the central domain of E2F1. KAP1 stimulates formation of E2F1-HDAC1 complex and inhibits E2F1 acetylation. Overexpression of KAP1 inhibits E2F1 transcriptional and apoptotic activity. Reduction of endogenous KAP1 level increases E2F1 acetylation level and sensitizes cells to E2F1 activation and apoptosis after DNA damage. Therefore, KAP1 is a novel E2F1-binding protein that contributes to E2F1 functional regulation.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Reagents—H1299, Saos2, HT1080, and 293T cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. KAP1 constructs were described previously (15, 25, 27). Inhibition of KAP1 expression was achieved by hairpin RNA expression using lentiviral vector pLSL-green fluorescent protein (gift from Dr. Peter Chumakov). The following oligos (only 23-mer sense strand was used) were used: K2 (5’-CCAGCCACCCGGAAGATGGA, specific against KAP1) and GT (5’-CCTCTTTCTTATCCTCGTATGT, a control against alternatively spliced isoform of SETDB1). Purified adenovirus expressing p53 was prepared as described previously (28). Acetylated E2F1-specific rabbit antibodies were raised against acetylated E2F1 peptides HPG(Ac-K117)GVKSPG and PGE(Ac-K125)SRYS (GenScript) and affinity-purified using columns conjugated with the acetylated and non-acetylated peptides. Both Ac-K117 and Ac-K125 antibodies produced similar results, with the Ac-K117 antibody being more sensitive.

Western Blot—Cells were lysed in lysis buffer (50 mm Tris-HCl, pH 8.0, 5 mm EDTA, 150 mm NaCl, 0.5% Nonidet P-40, 1 mm phenylmethylsulfonyl fluoride) and centrifuged for 5 min at 10,000 × g; the insoluble debris were discarded. Cell lysate (10–50 μg of protein) was fractionated by SDS-PAGE and transferred to Immobilon P filters (Millipore). The filter was blocked for 1 h with phosphate-buffered saline containing 5% nonfat dry milk, 0.1% Tween 20. E2F1 was detected using monoclonal antibody KH95 or rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology). Antibodies for pRb, p27, cyclin D3, Aplaf-1, and dihydrofolate reductase were purchased from Santa Cruz Biotechnology. KAP1 was detected with rabbit anti-KAP1 antibody (Bethyl). The filter was developed using horse-radish peroxidase-conjugated secondary antibodies and ECL plus reagent (Amersham Biosciences).

Immunoprecipitation-Western Blot Assay—Cells were lysed in lysis buffer and centrifuged for 5 min at 10,000 × g, and the insoluble debris were discarded. Cell lysate (200–500 μg of protein) was immunoprecipitated using 0.5–1 μg of mouse monoclonal antibody against E2F1, KAP1, or epitope tags and protein A-agarose beads for 4 h at 4 °C. The beads were washed extensively with lysis buffer, boiled with SDS sample buffer, fractionated by SDS-PAGE, and analyzed by anti-KAP1 or E2F1 Western blot using rabbit polyclonal antibodies to reduce background from Ig heavy chain.

Luciferase Reporter Assay—Cells (50,000/well) were plated in 24-well plates and transfected with a mixture containing 20 ng of E2F1-responsive luciferase reporter plasmids, 5 ng of CMV-laCZ plasmid, 20 ng of E2F1 expression plasmid, and 50 ng of KAP1 plasmid. Transfection was achieved using Lipofectamine PLUS reagents (Invitrogen), and cells were analyzed for luciferase and β-galactosidase expression after 24 h. The ratio of luciferase/β-galactosidase activity was used as indicator of E2F1 transcriptional activity.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation was carried out using the published procedure. H1299 cells were transiently transfected with E2F1 and KAP1 expression plasmids. E2F1 chromatin immunoprecipitation (ChIP) was performed using KH95 antibody; KAP1 ChIP was performed using a rabbit polyclonal antibody (Bethyl). Coprecipitated chromatin was analyzed by PCR (28–32 cycles) for the presence of E2F1 promoter using primers 5’-AGGAACCGCGCCGTCGTTGTCCCGT and 5’-CTGCCCTGCAAGGTCCGCCGCACTT.

RESULTS

KAP1 Forms a Complex with E2F1 and Regulates E2F1 Activity—E2F1 is regulated by interaction with the retinoblastoma protein pRb, which recruits histone deacetylase and methylase to suppress E2F1 target promoters. The KAP1 corepressor has been shown to repress Kruppel-associated box family transcription factors and recently found to inhibit p53 activity through interaction with MDM2. However, relatively few physiological targets or partners of KAP1 have been identified to date. Recent studies implicated KAP1 playing a role in the inhibition of apoptosis after DNA damage, and KAP1 is itself a target of DNA damage-induced phosphorylation. In addition to p53, E2F1 is an important regulator of apoptosis in transformed or DNA-damaged cells. Therefore, we tested whether E2F1 interacts with KAP1. When 293T cells were immunoprecipitated using E2F1 antibody KH95, coprecipitation of endogenous KAP1 was detected by Western blot (Fig. 1a). Transfection of additional KAP1 strongly increased the level of coprecipitation with endogenous E2F1 (Fig. 1a). Because 293T cells express high levels of p53 and SV40 T antigen that may cause formation of new complexes, the experiment was repeated using H1299 cells devoid of p53 and viral proteins. The results also confirmed the coprecipitation of endogenous E2F1 and KAP1 in this cell line (Fig. 1b).

To test whether KAP1 can regulate E2F1 activity, H1299 cells were transfected with E2F1 expression vector and several E2F1-responsive promoters (SirT1, Cyclin D3, p27, E2F1) (14, 29–31). Overexpression of KAP1 significantly inhibited E2F1 transcription activation function in such assays (Fig. 1c). In 293T cells which can be transfected at near 100% efficiency, cotransfection of KAP1 and pRb showed cooperative effect in the inhibition of endogenous E2F1 target expression (Fig. 1d). To further test whether endogenous KAP1 has a role in regulating E2F1, the E2F1 reporter constructs were cotransfected...
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with E2F1 into U2OS cell lines with stable knock down of endogenous KAP1. E2F1 activation of the reporters was also higher (up to 2-fold) in cells with reduced KAP1 level (Fig. 1e), suggesting that endogenous level of KAP1 contributes to inhibition of E2F1 activity.

E2F1 recruits pRb to target gene promoters and directly represses transcription from the promoter. Because KAP1 can also repress transcription after being targeted to promoters, its recruitment by E2F1 to DNA was tested by ChIP assay. In untransfected H1299 cells, low levels of E2F1 and KAP1 binding to the E2F1 promoter were detected, possibly from endogenous proteins. Transfection of E2F1 alone resulted in increased KAP1 binding to the promoter, suggesting that high level E2F1 expression recruited the endogenous KAP1 to DNA (Fig. 1f). Transfection of KAP1 alone also increased KAP1 ChIP signal, presumably mediated by endogenous E2F1 or other DNA binding factors. Moderate increase of KAP1 DNA binding was observed after E2F1 and KAP1 cotransfection. E2F1 ChIP signal was significantly increased after transfection of exogenous E2F1 alone as expected, but the signal was partially reduced by cotransfection with KAP1 (Fig. 1f). This inhibitory effect from KAP1 may be due to its ability to reduce the level of E2F1 acetylation (see below), thus reducing E2F1 DNA binding. These results suggested that KAP1 regulates E2F1 by a combination of DNA binding inhibition and promoter recruitment mechanisms.

Mapping of KAP1-E2F1 Interaction Domains—To identify the regions on E2F1 and KAP1 involved in complex formation, FLAG and Myc-tagged KAP1 deletion mutants were cotransfected with E2F1 into 293T cells. Coprecipitation of E2F1 with the KAP1 mutant was determined by KAP1 immunoprecipitation (IP) using epitope-specific antibodies followed by E2F1 Western blot. The results showed that the coiled-coil region of KAP1 from 191–419 was necessary and sufficient for binding E2F1 (Fig. 2a). This region was also involved for binding to MDM2 in a previous study (25).

To map the KAP1 binding domain on E2F1, a panel of FLAG-tagged E2F1 deletion mutants was cotransfected with KAP1 into 293T cells. E2F1 mutants were precipitated with FLAG antibody, and the coprecipitation of KAP1 was detected by Western blot. The results showed that the region between residue 192–382 of E2F1 was required for binding to KAP1 (Fig. 2b). This region includes the “Marked Box” that is implicated in protein-protein interactions in other E2F members and is critical for the induction of apoptosis by E2F1 (32). Therefore, the KAP1 binding region on E2F1 does not overlap with the C-terminal pRb binding and transcription activation region, suggesting that KAP1-E2F1 binding is independent of pRb, which was further tested below.

KAP1-E2F1 Physical and Functional Interaction Is Independent of pRb—A critical regulator of E2F1 is the tumor suppressor pRb. To determine whether E2F1–KAP1 interaction is dependent on pRb or regulated by pRb, Saos2 cells which are deficient for both p53 and pRb were examined. IP-Western blot assay showed interaction of endogenous E2F1 and KAP1 in Saos2 cells similar to the pRb wild type H1299 cells (Fig. 3a), suggesting that E2F1–KAP1 interaction can occur in the absence of pRb.

To test whether pRb regulates K2F1-KAP1 binding, Saos2 cells were transiently transfected with E2F1, KAP1, and pRb expression plasmids and analyzed by E2F1 IP-KAP1 Western blot. Coprecipitation between E2F1 and KAP1 was not affected by overexpression of pRb in such assay (Fig. 3b), suggesting that pRb does not regulate the interaction between E2F1 and KAP1. This result is also consistent with the domain-mapping result showing that KAP1 interacts with a region on E2F1 distinct from the pRb binding site.

To test whether KAP1 regulates E2F1 in the absence of pRb, Saos2 cells were transfected with E2F1-responsive reporters and E2F1 expression plasmid. Compared with H1299 cells KAP1 showed similar ability to repress E2F1 transcription activation (Fig. 3c). Coexpression of KAP1 and pRb showed moderate cooperation in the repression of E2F1 activity (Fig. 3d). These results showed that KAP1 regulation of E2F1 is independent of pRb and probably represents an additional mechanism of control over E2F1 activity.
KAP1 Promotes E2F1-HDAC1 Interaction and Inhibits E2F1 Acetylation—E2F1 inhibition by pRb involves recruitment of HDAC1 and SUV39H1 to E2F1. KAP1-mediated transcriptional repression also involves recruitment of HDAC1 and histone methyltransferase SETDB1. To determine whether KAP1 regulates E2F1 interaction with HDAC1, H1299 cells transiently transfected with E2F1 and FLAG-HDAC1 were analyzed by E2F1 IP and FLAG Western blot. Coexpression of KAP1 significantly stimulated E2F1 coprecipitation with HDAC1 (Fig. 4a), suggesting that KAP1 recruits HDAC1 to E2F1. To test the role of endogenous KAP1 in E2F1-HDAC1 interaction, HT1080 cells with stable KAP1 knock down were examined by E2F1 IP and HDAC1 Western blot. The coprecipitation of endogenous E2F1 and HDAC1 was also reduced in cells depleted of KAP1 (Fig. 4b), suggesting that KAP1 is responsible for a fraction of E2F1-HDAC1 binding in this cell line. In reporter assays, KAP1 deletion mutant without the C-terminal plant homeo domain and bromo domains failed to inhibit E2F1 transactivation function (Fig. 4c), suggesting that recruitment of HDAC1 is important for the repression of E2F1 activity.

E2F1 is acetylated at several lysine residues adjacent to the DNA binding domain (Lys-117, -120, -125), which stimulates its binding to DNA. E2F1 acetylation is stimulated by stress such as DNA damage and is inhibited by pRb. However, previous studies of E2F1 acetylation used pan-specific anti-acetyl-lysine antibodies, which leaves uncertainty about the site of acetylation and specificity. To test whether KAP1 binding and recruitment of HDAC1 results in the reduction of E2F1 acetylation level, we generated an acetylated peptide-specific antibody against the Lys-117 site of E2F1. In specificity tests, the antibody did not cross-react with E2F1 expressed in Escherichia coli, as expected from lack of modifications to E2F1 (Fig. 4d). However, E2F1 cotransfected with the CBP coactivator and acetyltransferase in H1299 cells was strongly reactive to the Ac-K117 antibody (the signal was marginal without immunoprecipitation), suggesting that the antibody was specific for acetylated E2F1 (Fig. 4d). Expression of KAP1 significantly reduced the level of E2F1 acetylation by CREB-binding protein (CBP), which was reversed by treatment with the HDAC inhibitor Tri-
chostatin A (Fig. 4e). Overexpression of HDAC1 also strongly suppressed E2F1 acetylation by CBP.

To further test the specificity and mechanism of E2F1 deacetylation by KAP1, sub-optimal levels of KAP1 and HDAC1 were coexpressed with E2F1 and CBP. Full-length KAP1 and KAP1 mutant (239–835) capable of binding both E2F1 and HDAC1 showed strong cooperation with HDAC1 in blocking E2F1 acetylation without affecting the expression level of CBP. In contrast, KAP1 mutant without the HDAC1 binding site (20–419) failed to cooperate with HDAC1 in E2F1 deacetylation (Fig. 5a). Therefore, KAP1 recruitment of HDAC1 to E2F1 is necessary for the inhibition of E2F1 acetylation, suggesting that the effect was due to active deacetylation by HDAC1.

To test the role of endogenous KAP1 in regulating E2F1 acetylation level, U2OS and Saos2 cells with stable knockdown of KAP1 were examined by Western blot. Coexpression of endogenous E2F1 and HDAC1 was reduced in cells depleted of KAP1. c, Saos2 cells transfected with E2F1-response reporter, E2F1, and KAP1 deletion mutants were analyzed for luciferase expression. KAP1 mutants without the C-terminal plant homeo domain and bromo domain failed to inhibit E2F1 transactivation function. d, validation of E2F1 Ac-K117 antibody. FLAG-E2F1 was transfected with hemagglutinin-CBP and hemagglutinin-PCAF into H1299 cells, precipitated using KH95 antibody, and blotted with a rabbit antibody against E2F1 peptide with acetylated Lys-117. E2F1 expressed in E. coli was used as negative control. The lower panel shows the relative levels of E2F1 loading in a duplicate gel. e, H1299 cells were transiently transfected with E2F1 and hemagglutinin-CBP. E2F1 was precipitated using KH95 antibody and blotted with Ac-K117 antibody. Expression of KAP1 reduced E2F1 acetylation, which was reversed by 6 h of treatment with 500 nm Trichostatin A. The lower panel shows the relative levels of E2F1 loading in a reprobe of the same membrane.

Knock Down of KAP1 Increases E2F1-mediated Gene Expression and Apoptosis—To test whether KAP1 regulates the activity of endogenous E2F1, Saos2 cells with stable knockdown of KAP1 was examined for the expression of several E2F1 target genes. Reduction of KAP1 level resulted in significantly increased expression of cyclin D3, p27, and dihydrofolate reductase in Saos2 cells (Fig. 6a). These results are consistent with increased levels of endogenous E2F1 activity. In transient transfection assays, E2F1 induced apoptosis in the p53-null H1299 cells and overexpression of KAP1 significantly inhibited E2F1-induced apoptosis (Fig. 6b).

E2F1 has been shown to be critical for etoposide-induced apoptosis in cells devoid of p53. In Saos2 cells, knockdown of KAP1 also increased sensitivity to etoposide by ∼2-fold (Fig. 6c). Infection with adenovirus vector expressing E2F1 resulted in moderate cooperation with etoposide in Saos2
cells, and the effect was significantly enhanced after knock down of KAP1 (Fig. 6c). These results suggest that KAP1 plays a role in regulating the apoptotic function of E2F1 independent of p53 and pRb. However, KAP1 has recently been shown to be phosphorylated by ATM pathway and to function in cell survival after DNA damage, possibly by modulating chromatin conformation in a global scale. Therefore, our results do not rule out the possibility of indirect cooperation between E2F1 activation and KAP1 knock down on cell survival.

**DISCUSSION**

The results presented above showed that the nuclear corepressor protein KAP1 is involved in the regulation of E2F1 in a pRb-independent fashion. KAP1 binds to E2F1 and recruits HDAC1 to E2F1. This recruitment is responsible for inhibi-
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The inhibition of E2F1 acetylation, which may reduce the DNA binding affinity of E2F1. Furthermore, KAP1 can be recruited to DNA by E2F1, which may in turn repress transcription from E2F1 target promoters directly. Therefore, the mechanism of KAP1 function is similar to pRb. However, unlike E2F1–pRb binding that is regulated in a cell cycle-dependent fashion by cyclin-dependent kinase 4 phosphorylation of pRb, KAP1 level is not known to undergo cell cycle changes and there is no direct evidence that it undergoes cell cycle-dependent changes in phosphorylation. KAP1 expression level is not regulated by E2F1, and KAP1–E2F1 binding is not affected by cell cycle status (data not shown). Therefore, KAP1 is likely to play a different regulatory role on E2F1 compared with pRb. KAP1 binds to a region on E2F1 distinct from the C-terminal pRb binding site, and the two interactions are not mutually exclusive. Therefore, KAP1 and pRb can potentially regulate E2F1 in a cooperative or additive fashion. Interestingly, the KAP1 binding domain of E2F1 includes the Marked Box that was implicated in protein interaction, regulation of transcriptional activity and promoter selectivity, and induction of apoptosis by E2F1 (32).

KAP1 was found as a corepressor protein for the Kruppel-associated box domain transcription repressors and has shown interesting activity in inducing heritable gene silencing and epigenetic modifications of target DNA through recruitment of HDAC, SETDB1, and HP1 proteins (22). Germ line knock out of KAP1 results in early embryonic lethality (20), and tissue-specific conditional knock out in mouse testes disrupts spermatogenesis, possibly due to excessive cell death (33). Its interaction with HP1 is also important for regulating embryonic stem cell differentiation in response to retinoic acid (34). Interestingly, recent studies revealed important functions of KAP1 in cellular response to DNA damage in mediating chromatin conformational changes on both a global level and at the level of p53 regulation (23, 25). KAP1 interacts with the p53 regulator MDM2 through the coiled-coil region and cooperates with MDM2 to deacetylase p53 and inhibit p53 activity (25). KAP1 knock down leads to increased basal p53 activity and enhanced p53 activation after ionizing irradiation and low dose actinomycin D treatment, which activates p53 without inducing DNA damage (25, 26). Therefore, KAP1 is involved in regulating basal and stress-activated p53 functions.

KAP1 inhibition of E2F1 showed several similarities to p53 regulation. The same region of KAP1 (coiled-coil) was involved in binding E2F1, and KAP1 also recruits HDAC to E2F1 and causes inhibition of E2F1 acetylation. KAP1 knock down also increased basal E2F1 activity and increased E2F1-mediated apoptosis after DNA damage in p53-null cells. These results established KAP1 as a novel regulator of E2F1 independent of pRb and p53. E2F1 and other family members have both oncogenic and tumor suppressor functions. The role of KAP1 in the function and regulation of this complex family remains to be further investigated. The ATM-mediated phosphorylation of KAP1 after DNA damage and the involvement of KAP1 in regulating two DNA damage-inducible proteins (p53 and E2F1) suggest that KAP1 is an important player in cellular response to damage and stress. These connections also suggest that KAP1 may be involved in the development of cancer as an oncogene. The DNA-damage sensitization by KAP1 knock down may be due to both E2F1- and p53-mediated effects. Therefore, targeting the key biochemical activities of KAP1 may be useful for cancer treatment.

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