KAP1 promotes proliferation and metastatic progression of breast cancer cells

Joseph B. Addison[1], **Colton Koontz**[1], **James H. Fugett**[1], **Chad J. Creighton**[2], **Dongquan Chen**[3], **Mark K. Farrugia**[1], **Renata R. Padon**[1], **Maria A. Voronkova**[1], **Sarah L. McLaughlin**[1], **Ryan H. Livengood**[4], **Chen-Chung Lin**[1], **J. Michael Ruppert**[1], **Elena N. Pugacheva**[1], and **Alexey V. Ivanov**[1,#]

[1]Mary Babb Randolph Cancer Center and Department of Biochemistry, West Virginia University, Morgantown, WV 26506

[2]Department of Medicine and Dan L. Duncan Cancer Center Division of Biostatistics, Baylor College of Medicine, Houston, Texas, TX 77030

[3]Division of Preventive Medicine and UAB Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35205

[4]Department of Pathology, West Virginia University, Morgantown, WV 26506

Abstract

KAP1 (TRIM28) is a transcriptional regulator in embryonic development that controls stem cell self-renewal, chromatin organization and the DNA damage response, acting as an essential co-repressor for KRAB family zinc finger proteins (KRAB-ZNF). To gain insight into the function of this large gene family, we developed an antibody that recognizes the conserved zinc fingers linker region (ZnFL) in multiple KRAB-ZNF. Here we report that the expression of many KRAB-ZNF along with active SUMOlyated KAP1 is elevated widely in human breast cancers. KAP1 silencing in breast cancer cells reduced proliferation and inhibited the growth and metastasis of tumor xenografts. Conversely, KAP1 overexpression stimulated cell proliferation and tumor growth. In cells where KAP1 was silenced, we identified multiple downregulated genes linked to tumor progression and metastasis, including EREG/epiregulin, PTGS2/COX2, MMP1, MMP2 and CD44, along with downregulation of multiple KRAB-ZNF proteins. KAP1-dependent stabilization of KRAB-ZNF required direct interactions with KAP1. Together, our results show that KAP1-mediated stimulation of multiple KRAB-ZNF contributes to the growth and metastasis of breast cancer.

Keywords

breast cancer; KAP1; KRAB-ZNF; degradation

#Corresponding author: Alexey Ivanov, MBR Cancer Center and Department of Biochemistry, 1 Medical Center Drive, West Virginia University, Morgantown, WV, 26506-9300. Phone:(304)293-4936; Fax:(304)293-4667; aivanov@hsc.wvu.edu.

Conflicts of interest: The authors disclose no potential conflicts of interest.

Introduction

Approximately 2000 sequence-specific transcription factors are encoded in the human genome, including ~800 C_2H_2-type zinc finger proteins (ZNFs) (1). About half of these ZNFs contain the highly conserved KRAB repression domain (2, 3). KRAB-ZNFs and their co-repressor KAP1 have co-appeared in evolution in tetrapods (4). KRAB-ZNFs underwent unprecedented expansion in mammalian genomes, and KRAB motif represents one of the most rapidly evolving domains (5). Despite the large size of this family, only a few KRAB-ZNFs have been studied in sufficient detail to establish their biological and molecular functions (6–9).

Most KRAB-ZNFs adopt simple protein architecture where the conserved N-terminal KRAB domain is linked with the sequence-specific DNA binding domain comprised of tandem arrays of the C_2H_2 type zinc fingers (2, 10). At the molecular level, the KRAB domain directly binds to KAP1, and this interaction is essential for KRAB-ZNFs-mediated repression (11, 12). KAP1 is recruited to chromatin by KRAB-ZNFs (6, 13), and in turn functions as a scaffolding protein for histone- and DNA-modifying enzymes involved in establishing the silenced state of a gene (14, 15). In this process, KAP1 acts as an E3 SUMO ligase and undergoes auto-SUMOylation, which promotes its interaction with the repression machinery (16, 17).

Genetic knockout of KAP1 has revealed its multifaceted role in many organismal processes such as development, reproduction and immune response. KAP1 is essential for differentiation of mouse stem cells *in vivo* (18, 19). Although the role of KAP1 in development could be attributed to the establishment of imprinting methylation patterns (19, 20) and the control of endogenous retroviral elements (7, 21), its function in adult tissues appears to be distinct (21–23).

KAP1 is a ubiquitously expressed nuclear protein, and its role in cancer is just beginning to emerge. Analysis of tissue microarrays demonstrated that KAP1 expression is increased during the clinical progression of 39% of invasive breast carcinomas *in situ* to metastasis in lymph nodes (24). High KAP1 mRNA expression has been found to be an independent prognostic factor for peritoneal carcinomatosis (25). Given the relevance of developmental cell fate regulators and stem cell pluripotency to cancer pathogenesis, understanding how KAP1 functions in cancer cells might be critical for developing future therapeutic strategies.

Overexpression of specific KRAB-ZNF genes in cancer has been documented (10). Several KRAB-ZNFs have been implicated in regulation of oncogenes and tumor suppressors in cell culture models, including p53 (26), MDM2 (27), Rb (28), BRCA1 (29) and pVHL (30). In breast cancer, three *KRAB-ZNFs* undergo gene amplification (31). High expression of 18 KRAB-ZNF genes have been associated with increased resistance of GIST tumors to imatinib treatment (32). However, the expression patterns and functions of the majority of KRAB-ZNFs in breast cancer are still unknown.

Here, we showed that KAP1 and certain KRAB-ZNFs are frequently overexpressed in breast tumors at both mRNA and protein levels. Knockdown of KAP1 in breast cancer cells led to inhibition of cell proliferation, tumor growth and metastasis. Mechanistically, we showed

that KAP1 depletion results in decreased expression of multiple KRAB-ZNF proteins and deregulation of many cancer and metastasis-associated genes. These findings demonstrate that KAP1 and KRAB-ZNFs may play an important role in breast cancer and could be explored as targets for therapeutic intervention.

Materials and Methods

Generation of ZnFL antibody

The rabbit polyclonal ZnFL antibody was raised against a mixture of Z1 and Z2 peptides. Z1 (Ac-CGGH[Q/K/E]RIHTGEKPY[K/E]-amide) and Z2 (Ac-GH[Q/K/E]RIHTGEKPY[K/E]C-amide) peptides were synthesized and used for rabbit immunization and affinity purification of ZnFL antibody.

Cell lines and constructs

MDA-MB-231-luc-D3H2LN (MDA-MB-231LN) cells were purchased from Caliper Life Science. Primary HMECs were purchased from Lonza and Invitrogen. HMLE cells were kindly provided by Dr. Robert Weinberg (MIT, Cambridge, MA), S2 cells by Dr. Alexei Tulin (FCCC, Philadelphia, PA), AB9 and XTC cells by Dr. Neil Hukriede (UPitt, Pittsburgh, PA), CEF and MEF cells by Dr. Daniel Flynn (WVU), KAP1 knockout MEFs (21) by Dr. Didier Trono (EPFL, Lausanne, Switzerland). The other cell lines were purchased and authenticated by ATCC. shRNAs were expressed from pTRIPZ vector and induced by addition of 0.5μg/ml doxycycline for 7 days. FLAG-KAP1 WT and mutants (16) were expressed from pLU vector. ZNF10, 224, 317, 350 were expressed from pcDNA3-6HA.

Cell proliferation assay

2×10^4 cells were plated in triplicates in 6w plates, cultured for 2, 4, 6 and 8 days, trypsinized and counted on Countess (Invitrogen).

Quantitative RT-PCR

Total RNA was extracted using mirVana miRNA Isolation Kit in triplicates. 2μg of total RNA were reverse transcribed using SuperScriptIII and dT_{20} primer. qPCR was performed in an ABI-7500 Real-Time PCR Cycler and analyzed using ABI SDS2.06 software. Gene expression levels were normalized by the geometrical mean of UBC, RPL13A, PCNA and tubulin genes relative to control. The primers and shRNAs are described in the Supplementary Methods.

Western blotting and immunoprecipitation

Cells were lysed in non-reducing Laemmli buffer and total protein was quantified by BCA assay. ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas) was used for subcellular fractionation for Fig. 1C. Lysates with equal amount of total protein were separated on 4–12% Bis-Tris gels and transferred to a PVDF membrane. Protein bands were detected using standard chemiluminescence techniques and quantified by GeneTools software (Syngene). Breast OncoPair INSTA-Blot™ was purchased from Imgenex, cat.

IMB130a. Immunoprecipitation was performed in mIP (Fig. 1D) or RIPA buffer (Fig. 1E, 3E) as described (16). The antibodies are described in the Supplementary Methods.

Animal studies and bioluminescence imaging (BLI)

NOD.Cg-Prkdc[scid] Il2rg[tm1Wjl]/SzJ (NSG) mice were purchased from the Jackson Laboratory and fed doxycycline-containing diet (Bio-Serv) to maintain shRNA expression. Mice were injected with MDA-MB-231LN cells and imaged as described (33). Primary tumor and organs with metastases were collected at the end point of study and analyzed as described (33).

Microarray, proteomics and bioinformatics analyses are described in the Supplementary Methods.

Statistical analysis

Statistical analyses were performed with Prism5 software (GraphPad) using two-tailed Student's *t* test.

Results

Characterization of a ZnFL polyclonal antibody

The limited availability of reagents has hindered analysis of KRAB-ZNF proteins to date. Perhaps as few as four antibodies capable of detecting endogenous levels of individual KRAB-ZNF proteins have been reported (6, 34, 35). After testing multiple commercial antibodies to many KRAB-ZNFs, we were able to detect endogenous levels of ZNF192 and ZNF274. Difficulty in detection of KRAB-ZNFs is likely due to their relatively low expression levels, a characteristic of many transcription factors (1). To overcome this problem and to analyze expression of the majority of KRAB-ZNFs simultaneously, we developed a pan-ZNF-specific antibody, referred to as the ZnFL antibody thereof. A unique structural feature of most C_2H_2-type ZNFs is a short and remarkably conserved <u>zinc</u> <u>f</u>inger <u>l</u>inker (ZnFL) sequence, TGEKPYK/E, that connects two adjacent zinc fingers (Fig. 1A). More than 90% of all individual zinc fingers in human C_2H_2-type ZNFs, including ~350 KRAB-ZNFs, contain this or highly similar sequence (Fig. S1, (4)). The ZnFL is present in each KRAB-ZNF with the frequency typically equal to the number of zinc fingers minus one (Fig. 1B). The median number of zinc fingers in human KRAB-ZNFs is 12 (2), providing multiple ZnFL epitopes in each KRAB-ZNF.

A rabbit polyclonal ZnFL antibody was raised using ZnFL consensus peptides as antigens, affinity purified and validated by Western blotting (Fig. 1C&S3). Nuclear and cytoplasmic fractions from cells from several species representing different clades of the eukaryotes were analyzed. As expected, ZnFL antibody did not detect ZNFs in yeast and reacted with only a few proteins in Drosophila cell lysates. However, strong ZnFL-specific signals were observed in the nuclear fractions from all vertebrate cells. The stronger signal in zebrafish lysates is likely due to the tetraploid nature of these cells and a higher content of nuclear proteins as evidenced by the strong histone H3 signal. Interestingly, only three weak ZnFL-reactive bands were detected in chicken cell lysates, suggesting that the avian ZNFs have

diverged significantly in the structure of the zinc finger linker. Indeed, alignment of zinc finger sequences across species revealed divergence of the avian sequence from the sequence of other species at the fourth and sixth positions (Fig. S2). ZnFL-reactive protein bands were primarily in the nucleus, consistent with the notion that ZNFs are transcription factors. The monoclonal antibody raised to human KAP1 protein detected KAP1 in human, mouse and Xenopus cells (Fig. 1C). It did not recognize chicken KAP1 likely due to sequence divergence between the species. Consistent with the expected range of sizes for human ZNFs (4), the ZnFL antibody detected multiple protein bands in the range of 40–150 kDa in total cellular lysates from all human cell lines tested (Fig. S3).

The interaction of ZnFL-reactive proteins with KAP1 was confirmed using co-immunoprecipitation (IP). Proteins from human mammary epithelial HMLE cells expressing FLAG-KAP1 were immunoprecipitated with the FLAG or control HA antibody (Fig. 1D). The ZnFL antibody detected many proteins in the FLAG immune complex but not in the control IP. Endogenous KRAB-ZNFs, ZNF192 and ZNF274, were specifically detected in the FLAG immune complex and served as positive controls. Most importantly, IP with FLAG antibody recovered the majority of the ZNF protein bands (Fig. 1D, compare lanes 4&8), suggesting that these correspond to ZNFs with the KRAB domain, i.e. KRAB-ZNFs. The reciprocal IP using the ZnFL antibody or control normal rabbit IgG was also performed. As expected, endogenous KRAB-ZNFs, ZNF192 and ZNF274, were present in the ZnFL IP but not the control IP. KAP1 was also detected in the ZnFL immune complex but not the control IP. Further, the ZnFL antibody efficiently precipitated three randomly selected HA-tagged KRAB-ZNFs: ZNF224, ZNF317 and ZNF350 (Fig. 1E), indicating the potential to recognize most KRAB-ZNFs.

The identity of ZnFL antibody-recognized proteins was confirmed by mass spectrometry of control IgG and ZnFL immunoprecipitates. From 37 proteins specific to the ZnFL IP from MDA-MB-231LN breast cancer cells, 22 were ZNFs (Table 1&Suppl. File1) and 15 were abundant non-nuclear contaminants. Both, ZNF192 and ZNF274 are expressed in MDA-MB-231LN cells (Fig. 3B). Absence of ZNF274 in the mass spectrometry data suggested that the identified ZNFs are likely the most abundant or/and amenable to LC-MS/MS and the total number of 22 expressed ZNFs in these cells is an underestimation.

These results clearly demonstrate that ZnFL antibody detects proteins consistent with sequence, expected size, subcellular localization and expression pattern of ZNFs. Notably, the majority of the ZnFL-detected proteins co-immunoprecipitated with KAP1 (Fig. 1D), indicating that significant proportion of ZnFL-recognized bands represent KRAB-ZNFs.

KAP1 and KRAB-ZNFs are overexpressed in human breast tumors

TRIM28/KAP1 and KRAB-ZNFs expression in human breast cancer was first analyzed at the mRNA level. KAP1 was modestly overexpressed (1.3–2.1-fold) in breast carcinoma compared to normal tissue in several public array datasets (Fig. S4). KAP1 was up-regulated in TCGA breast tumor data, with 525 samples profiled by Agilent mRNA expression arrays and 896 samples analyzed by RNA-Seq (Fig. 2A&S4). The KAP1 level was higher in all four intrinsic breast cancer subtypes and in breast cancer metastases (Fig. 2A&B). Analysis of a mouse dataset comprised of 108 tumors from 13 different mouse models of breast

cancer revealed that KAP1 was up-regulated in all molecular tumor subtypes compared to normal tissue, 1.4-fold on average (Fig. S5). KAP1 up-regulation in breast tumors was consistent across multiple datasets but relatively modest in absolute fold change. Noteworthy, the basal level of KAP1 mRNA in normal breast tissue is relatively high and is comparable to the levels of such housekeeping genes as TUBA1A, PCNA and HPRT1.

To determine if KRAB-ZNFs, which utilize KAP1 as a co-repressor, are also overexpressed in breast cancer, we analyzed their expression in the two TCGA breast datasets. Of 365 annotated KRAB-ZNF genes, 343 were detected in the RNA-Seq dataset and 289 in the array dataset. Most KRAB-ZNF genes common for both datasets were regulated in tumors concordantly, though fold changes for up-regulated genes in the RNA-Seq dataset were lower. Thus, 28 genes were up-regulated >1.35-fold in the RNA-Seq dataset and 70 genes in the array dataset, and 48 of the up-regulated genes were common to both datasets (>1.35-fold in at least one dataset, $P<0.05$ in both). 81 genes were down-regulated >1.35-fold in the RNA-Seq dataset and 70 genes in the array dataset, and 64 down-regulated genes were common to both datasets by the same criteria (Fig. 2C & Suppl. File2). Only 10 KRAB-ZNF genes were up-regulated in one dataset and down-regulated in the other or vice-versa. Less than a quarter of the differentially regulated KRAB-ZNFs, however, showed changes of more than 2-fold, suggesting that deregulation of rather multiple KRAB-ZNF genes simultaneously but at a modest level occurs in breast tumors. We also analyzed expression of a more diverse superfamily of non-KRAB-ZNF genes and found similar general trend (Fig. S6, Suppl. File2).

KAP1 protein expression was compared in surgical samples of matched normal-tumor pairs. The vast majority of the tumor samples demonstrated 2–10-fold higher level of KAP1 protein compared to their normal controls (Fig. 2D&S7). Immunohistochemical analysis has documented elevated KAP1 levels in significant proportions of breast, lung, liver, gastric and prostate tumors (Fig. 2E and see TRIM28 at www.proteinatlas.org), indicating that KAP1 overexpression is a common feature of many epithelial cancers.

To analyze KRAB-ZNF proteins expression in breast tumors, the same matched normal-tumor samples were probed with the ZnFL antibody. Interestingly, substantially higher levels of multiple KRAB-ZNFs were detected in most tumors compared to normal controls, and they correlated with KAP1 protein levels (Fig. 2D). These results indicate that KAP1 and multiple KRAB-ZNFs are frequently overexpressed in breast tumors at the mRNA level, but most profoundly at the protein levels.

KAP1, KAP1 SUMOylation and KRAB-ZNFs are up-regulated in human breast cancer cell lines

Analysis of gene expression in tissue samples is confounded by the complexity of tissue composition made up of multiple cell types and their relative contribution to the sum readout signal. To analyze pure cell populations, we compared KAP1 levels in two independent isolates of primary human mammary epithelial cells (HMECs), two immortalized mammary cell lines, HMLE and MCF10A, and a panel of 17 breast cancer cell lines representing luminal, basal and claudin-low breast cancer subtypes. Similarly to the human patient data,

KAP1 mRNA levels were elevated 1.5–3-fold in breast cancer cell lines compared to primary HMECs (Fig. 3A).

KAP1 protein was up-regulated in most cancer cell lines by 2–4-fold when normalized to tubulin, with higher levels observed in luminal cells (Fig. 3B&C). For a comparison, we analyzed expression of established breast cancer oncogenes in the same cells. Interestingly, KAP1 protein was overexpressed at least as often as β-Catenin (36) and more often than c-Met (37) (Fig. 3B).

The most striking difference was detected in the levels of SUMOylated KAP1 (Fig. 3B,D&E, S8). We have previously shown that conjugation of each SUMO moiety to the six SUMOylation sites in KAP1 adds ~15 kDa to the 100 kDa unmodified KAP1 protein and results in the appearance of a ladder of discrete higher molecular weight KAP1-SUMO conjugates (16). SUMO modification of KAP1 can only be detected in cells lysed in buffers containing SDS or NEM, an inhibitor of de-SUMOylating enzymes (Fig. S9, (16)). To confirm that the observed KAP1*S bands represent SUMOylated KAP1, we performed KAP1 IP and WB with SUMO2/3 antibody (Fig. 3E). When calculated as a fraction of total KAP1 signal, levels of SUMOylated KAP1 varied from 4% in HMECs up to 35% in MDA-MB-453 cells, with majority of cancer cell lines within 10–20% range (Fig. S8). When normalized to tubulin, SUMOylated KAP1 was 5–30-fold higher in luminal and basal cancer cell lines compared to HMECs and immortalized cells (Fig. 3D). KAP1 SUMOylation level is an indicator of KAP1 co-repressor activity (16, 17). These results suggest that not only KAP1 level, but also its activity is markedly increased in breast cancer cells.

Most breast cancer cells showed an increase in total ZnFL-specific signal compared to primary HMECs, with higher intensity bands observed in cells of luminal and basal subtypes (Fig. 3B), suggesting that, similarly to KAP1, KRAB-ZNF proteins are broadly up-regulated in breast cancer cell lines.

Genomic alterations of KAP1 and KRAB-ZNFs in human breast tumors

We analyzed frequencies of genomic alterations for KAP1 and all ZNF genes in the TCGA breast dataset (Suppl. File3). Interestingly, only four missense mutations were identified in KAP1, which are likely passenger mutations. We identified five KRAB-ZNF genes mutated in more than 1% of cases and three of them were also down-regulated in tumors at the mRNA level (ZNF208, ZNF41 and ZNF540). Some of the mutations in these three KRAB-ZNFs were nonsense mutations, while approximately half of the missense mutations were located in the zinc finger DNA binding domain, suggesting that they are likely functionally inactivating mutations.

We identified six KRAB-ZNF genes at three separate loci that have undergone genomic deletions with frequencies between 8.3% and 43.9% of cases, and five of them were also down-regulated in tumors at the mRNA level (ZNF778, ZNF595, ZNF141, ZNF721 and ZNF658) (Suppl. File3), suggesting that these are likely loss-of-function events. We identified a cluster of five KRAB-ZNF genes at 1q44 that has undergone genomic amplification in 45.3% of cases, and three of these genes were also up-regulated in tumors at the mRNA level (ZNF695, ZNF669 and ZNF124), suggesting that these are likely gain-of-

function events. In general, non-KRAB-ZNF genes followed similar patterns of mutations and gains/losses to those of KRAB-ZNFs (Suppl. File3).

These results indicate that a small number of KRAB-ZNF genes are genetically altered in breast cancer. The identified genes should be functionally validated as cancer gene candidates in the future.

KAP1 positively regulates the protein levels of multiple KRAB-ZNFs

A positive correlation between KAP1 and KRAB-ZNF protein levels was consistently observed (Fig. 2D,3B, S3). To evaluate if KAP1 controls the expression of KRAB-ZNFs, KAP1 was inhibited using multiple shRNAs in a set of breast cancer cell lines of luminal (ZR75-1), basal (MDA-MB-468) and claudin-low (MDA-MB-231LN) subtypes as well as in 'normal' mammary epithelial cells (HMLE) (Fig. 4A&S10). Surprisingly, we found that expression of multiple KRAB-ZNF proteins decreased significantly in KAP1-depleted cells and in *Kap1* knockout fibroblasts, and conversely increased in cells overexpressing exogenous KAP1. The increase in KRAB-ZNFs levels was also induced by the repression-impaired KAP1 M2 mutant, suggesting that the up-regulation likely occurs at post-transcriptional level (Fig. 4A).

Microarray expression profiling revealed only modest changes in expression of a few KRAB-ZNF genes in KAP1-depleted MDA-MB-231LN cells (Suppl. File4). Previous microarray experiments conducted in multiple cell lines with KAP1 knockdown or in mouse tissues with Kap1 knockout yielded similar findings – marginal (<2-fold) changes in expression of a limited number of KRAB-ZNFs (22, 23, 38). Furthermore, ZNF192 protein decreased significantly upon KAP1 depletion in HMLE and ZR75-1 cells without changes in ZNF192 mRNA (Fig. 4B). These results suggested that the decrease of multiple KRAB-ZNF proteins following KAP1 knockdown is largely post-transcriptional.

To analyze if KRAB-ZNF proteins are degraded faster in the absence of KAP1, we treated cells with the proteasome inhibitor MG-132. Indeed, the expression level of multiple KRAB-ZNFs was elevated in KAP1-depleted cells following inhibition of proteosomal degradation to the same level as in control cells. The level of ZNF192 protein was similarly rescued (Fig. 4C, compare lanes 2&4). At the same time, the levels of KAP1 and control GAPDH proteins decreased slightly (Fig. 4C). These results suggest that KAP1 regulates KRAB-ZNF proteins stability.

Interaction with KAP1 protects the KRAB-ZNFs from proteasomal degradation

KAP1 binds directly to the KRAB domain (12) and can potentially stabilize KRAB-ZNFs through direct protein-protein interaction. In rescue experiments, we infected HMLE shKAP1 cells with lentiviruses expressing shRNA-resistant KAP1 cDNAs. As expected, re-expression of wild type KAP1 resulted in elevated expression of multiple KRAB-ZNFs (Fig. 5A). Expression levels of KRAB-ZNFs were also rescued by reconstitution with KAP1 mutants that are impaired in repression function, i.e. the M2 mutant, which is deficient in interaction with HP1 (39), and the K6R mutant, which is SUMOylation deficient (16) (Fig. 5A&S11). This result confirmed that KAP1-dependent regulation of KRAB-ZNF protein

levels was post-transcriptional. However, re-expression of a KAP1 mutant deficient in KRAB binding, the C2 mutant (16, 40), failed to restore KRAB-ZNFs expression levels, indicating that direct KAP1 binding to the KRAB domain is required for KRAB-ZNFs protein stabilization. We obtained similar results by transfection of KAP1 mutants into *Kap1* –/– MEFs (Fig. S12).

In a complementary experiment, two different KRAB-ZNFs (ZNF10/KOX1 and ZNF350/ ZBRK1) were transiently transfected into control and KAP1-depleted cells. Both wild type ZNF10 and ZNF350 were expressed at significantly lower levels in KAP1-depleted cells compared to control cells (Fig. 5B), indicating that KRAB-ZNFs expression depends on the level of endogenous KAP1. The difference in KRAB-ZNFs levels was not due to variation in transfection efficiency since the expression of co-transfected GFP was similar in both cell lines. To further establish mechanism, ZNF10 and ZNF350 mutants were analyzed. The DV point mutation (DV->AA) in the KRAB domain impairs interaction with KAP1 and its repression activity (11, 12). The DV mutants were expressed at much lower levels than the corresponding wild type KRAB-ZNFs in control cells, indicating that binding of KRAB-ZNFs to KAP1 is critical for expression. A KRAB-only variant of ZNF10 (KRAB) could be expressed in control cells, but was almost undetectable in the absence of KAP1. Expression of a mutant of ZNF350 lacking the KRAB domain (dK) did not significantly differ between control and KAP1-depleted cells. These results strongly support the conclusion that the presence of the KRAB domain, which is essential for KAP1 binding, is required for efficient KRAB-ZNFs expression.

To confirm that the observed positive regulation of multiple KRAB-ZNFs by KAP1 was due to changes in KRAB-ZNFs protein stability, HMLE cells were treated with the protein synthesis inhibitor cyclohexemide (CHX) and KRAB-ZNFs degradation rate was analyzed. Levels of multiple KRAB-ZNFs decreased ~35% in control cells and ~60% in KAP1-depleted cells 4 hours following CHX addition. The levels of endogenous ZNF192 protein decreased in a similar fashion (Fig. 5C&D), indicating that KRAB-ZNFs degrade faster in the absence of KAP1. The level of KAP1 and control GAPDH did not change significantly. In sum, these results indicate that KRAB-ZNF proteins undergo rapid proteasome-dependent turnover characteristic to transcription factors and that direct binding of KAP1 to KRAB-ZNFs protects from proteosomal degradation.

KAP1 promotes cell proliferation, tumor growth and metastasis

To investigate if elevated KAP1 level in tumors is functionally linked to the malignant characteristics of cancer cells, the effect of KAP1 knockdown was analyzed. KAP1 depletion led to a decrease in cell proliferation in all cell lines examined. Strong growth inhibition was also observed in *Kap1* knockout MEFs (Fig. 6A). Conversely, overexpression of exogenous wild type KAP1, but not the functionally impaired KAP1 M2 mutant stimulated cell growth (Fig. 6B), indicating that increased KAP1 levels and its repression activity promote cell proliferation.

To assess the role of KAP1 in tumorigenicity *in vivo*, MDA-MB-231LN cells were used in orthotopic xenograft mouse model. Injection of cells expressing control shRNA into the mammary fat pad of NSG mice resulted in rapid tumor growth as reported previously (33).

Inhibition of KAP1 expression using shRNA reduced tumor growth significantly (Fig. 7A&S13). Conversely, overexpression of exogenous KAP1 resulted in a stimulatory effect on tumor growth (Fig. 7B). These results indicate that KAP1 is a critical component promoting growth of tumor cells *in vivo*.

The number of lung metastases was scored in tumor bearing mice. KAP1 knockdown tumors produced fewer metastatic colonies and KAP1 overexpressing tumors produced more metastatic colonies than controls (Fig. 7C-D). Since metastasis in these experiments simply correlated with tumor growth, an independent experiment to determine if KAP1 affects lung colonization following tail vein injection was performed. There was a significant decrease in lung bioluminescence in mice xenografted with KAP1 knockdown cells compared to control cells (Fig. 7E-F). These findings demonstrate that KAP1 depletion also inhibits metastatic outgrowth in lungs.

KAP1 regulates expression of genes promoting tumor growth and metastasis

To investigate the mechanisms by which KAP1 potentially promotes tumor growth and metastasis, we analyzed our microarray data from KAP1-depleted MDA-MB-231LN cells (Suppl. File4). The genes for PTGS2/COX2, EREG/epiregulin, MMP1 and MMP2 have been previously identified in the lung metastasis gene signature derived from MDA-MB-231LM2 cells as essential factors of aggressive malignant and metastatic behavior (41, 42). Using RT-qPCR we further confirmed their down-regulation in MDA-MB-231LN and HMLE cells following KAP1 knockdown. Conversely, we found up-regulation of some of these genes in cells overexpressing wild type KAP1 (Fig. 7G-H), suggesting that their expression is specifically influenced by KAP1. Changes of COX2 and CD44 expression were confirmed by Western blot (Fig. 7I). Together, these data indicate that KAP1 regulates expression of multiple genes promoting tumor growth and metastasis.

Discussion

Elevated expression of KAP1 mRNA has been reported in lung and gastric cancers (25, 43). Herein, we demonstrate that KAP1 is overexpressed in the majority of breast tumors and breast cancer cell lines, at both the mRNA and protein levels (Fig. 2&3, S4&S5). Interestingly, though KAP1 mRNA is consistently but modestly overexpressed in cancer cells, KAP1 protein is up-regulated at significantly higher levels (Fig. 2D,2E&S7), suggesting that post-transcriptional mechanism(s) could also be at play. In this regard, KAP1 SUMOylation, which is a major indicator of KAP1 repression activity (16, 17), is dramatically increased in cancer cells (Fig. 3&S8) and may potentially account for higher KAP1 protein levels. In general, we observed a direct correlation between KAP1 protein and its SUMOylation levels (Fig. 3C,3D;S3). SUMOylation can compete for the same lysine residues with ubiquitynation and thus may inhibit ubiquitin-dependent protein degradation (44).

KRAB-ZNF genes constitute one fifth of all human transcription factors (1, 3). Lack of reagents for detection of endogenous levels of KRAB-ZNF proteins has significantly impeded elucidation of their functions. We have developed and characterized a potent ZnFL antibody directed to a highly conserved TGEKPY zinc finger linker and demonstrated that

this antibody is capable of detecting multiple ZNF proteins. A similar approach was recently used to develop an antibody to a phosphorylated form of the TGEKPY motif (45). We confirmed the sensitivity and specificity of the ZnFL antibody using multiple approaches (Fig. 1, Table 1).

Overexpression of certain KRAB-ZNF genes in cancer has been documented (10, 31, 32). Here, we analyzed two large TCGA breast datasets and found that dozens of KRAB-ZNFs were up-regulated, while dozens others were down-regulated in breast tumors. Expression of a much smaller number of KRAB-ZNFs changed markedly (>2-fold) (Fig. 2C). At the same time, multiple KRAB-ZNF proteins detected by the ZnFL antibody were overexpressed dramatically in breast tumors (Fig. 2D), suggesting that they could be up-regulated at the protein rather than mRNA level.

Stabilization of individual proteins via assembly into functional multi-protein complexes is a common phenomenon. Stabilization of a sequence-specific transcription factor upon binding to its co-repressor has been documented for E2F-Rb (46), p53-Sin3a (47) and RUNX1-Sin3a (48) interactions. Stabilization is typically achieved via inhibition of ubiquitin-dependent proteasome mediated degradation. Herein, we showed that multiple KRAB-ZNFs undergo rapid proteasome-dependent turnover and are stabilized upon binding to their co-repressor KAP1 (Fig. 4&5). Therefore, significant up-regulation of KRAB-ZNFs observed in breast tumors (Fig. 2D&E) is likely due to their stabilization by elevated KAP1. Dramatic decrease in the expression of dozens of KRAB-ZNF transcriptional repressors could also explain constitutive chromatin relaxation and reactivation of endogenous retroviral repeats observed in KAP1-deficient cells (21, 49).

Previous analyses of the KAP1 role in cancer cell proliferation yielded contradictory results (25, 43). Here, we used multiple shRNAs to knockdown KAP1 in four cell lines and showed that loss of KAP1 inhibited cell proliferation in all cases. Concordantly, overexpression of KAP1 yielded a complementary growth-stimulatory effect (Fig. 6). Consistent with the *in vitro* studies, inhibition of KAP1 slowed tumor growth and metastasis of MDA-MB-231LN cell xenografts *in vivo* (Fig. 7).

The mechanism by which KAP1 promotes cell proliferation and tumor growth is unknown but likely involves the action of KAP1 regulated genes. Here, we showed that KAP1 affects expression of many genes implicated in tumor progression and metastasis, particularly PTGS2/COX2, EREG/epiregulin, MMP1 and MMP2 that were previously linked to aggressive tumor behavior (41, 42) (Fig. 7). Identity of individual KRAB-ZNFs involved in regulation of these genes and whether it occurs through direct or indirect mechanism remain to be determined in future studies.

Altogether, we showed that KAP1 is an important positive regulator of cell proliferation and tumor growth. This study suggests that anti-KAP1 drugs should be developed for anticancer therapy. An inhibitor disrupting transcription factor BCL6 with its co-repressor SMRT has been shown to be effective against diffuse large B-cell lymphoma *in vivo* (50). This opens the possibility that small molecule inhibitors of the KRAB-KAP1 interaction could be developed and potentially be used therapeutically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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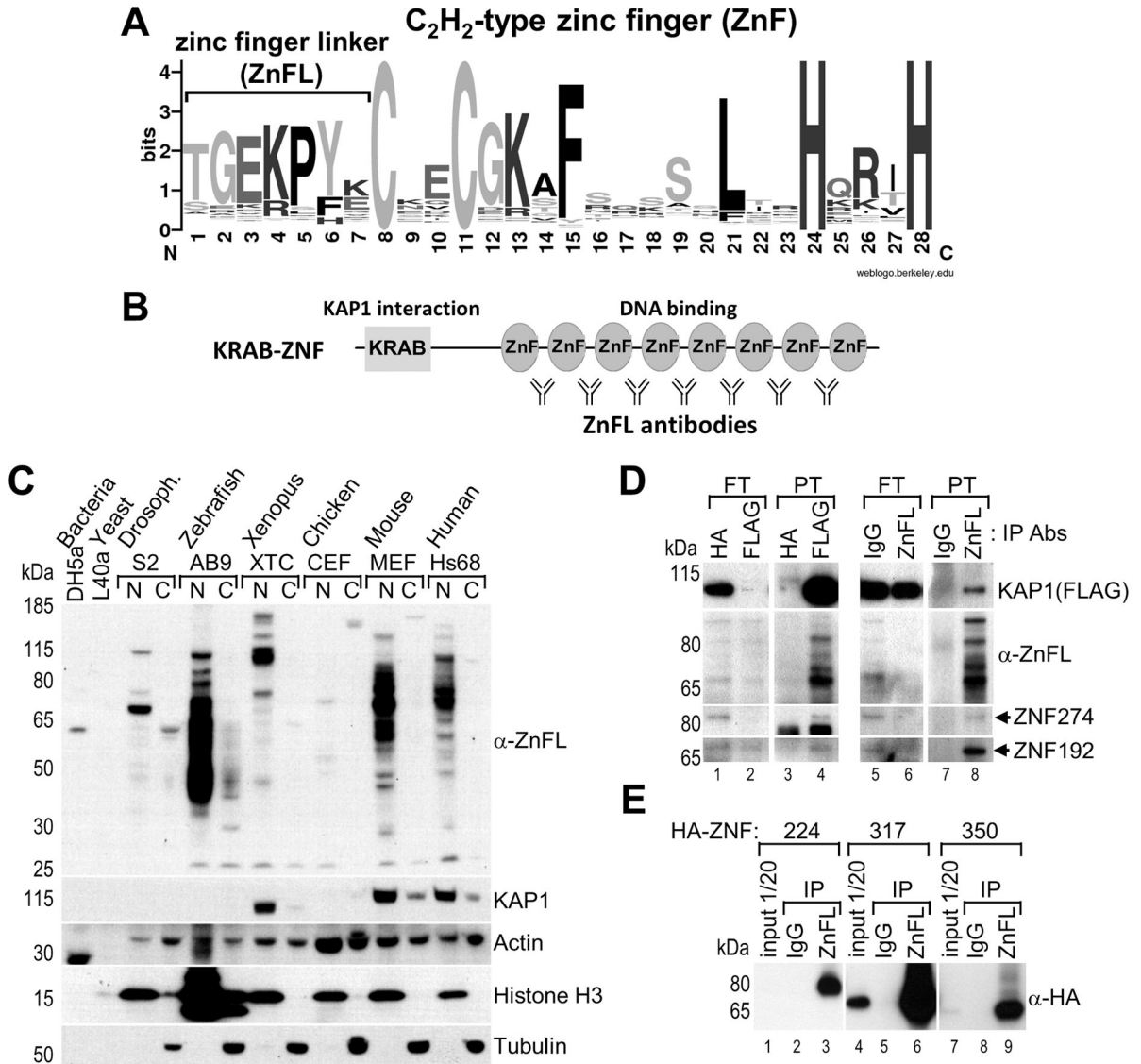
Figure 1. Characterization of ZnFL antibody

A, Logo representation of amino acid conservation in zinc fingers of human C_2H_2-type ZNFs. A high bit score reflects invariant residues C, C, H and H. 7-aa zinc finger linker sequence TGEKPYK/E is highly conserved. **B,** Domain structure of a typical KRAB-ZNF protein. ZnF – individual zinc finger. ZnFL antibody binds between ZnFs. **C,** WB analysis with ZnFL and the specified antibodies. The indicated cells were fractionalized into nuclear (N) and cytoplasmic (C) fractions. Histone H3 and tubulin are markers for nuclear and cytoplasmic fractions, respectively. Actin and sc-10809 - loading controls. **D,** KAP1 and KRAB-ZNFs co-immunoprecipitation. HMLE cells expressing FLAG-KAP1 were immunoprecipiated with control HA or FLAG antibody and control rabbit IgG or ZnFL antibody. FT - flow through, PT - pellet. **E,** IP-WB analysis of transfected HA-KRAB-ZNFs: ZNF224, ZNF317 and ZNF350.
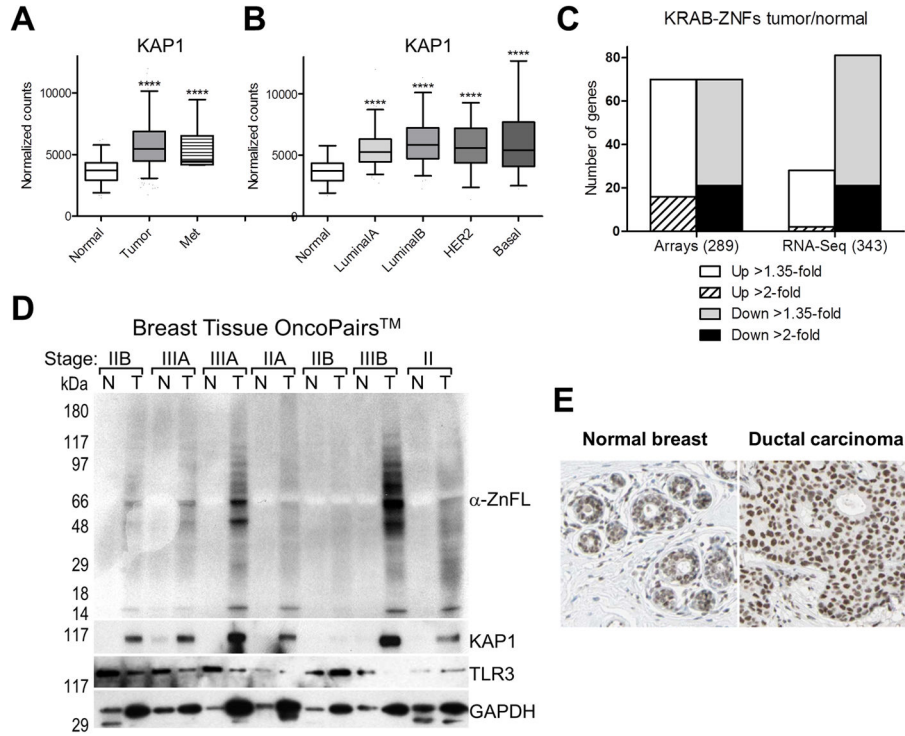
Figure 2. KAP1 and KRAB-ZNFs are overexpressed in breast tumors

A & B, Analysis of KAP1 in TCGA Breast RNA-Seq data from 105 normal and 798 tumor samples of different subtypes and metastases. Data shown as mean and 5–95% percentile. **** - $P < 0.0001$, two-tailed t test. **C,** Analysis of KRAB-ZNFs expression in TCGA Breast Array (n=527) and RNA-Seq (n=896) datasets, $P < 0.05$. **D,** WB analysis of KAP1 and KRAB-ZNFs in biopsy samples of matched normal(N)–tumor(T) pairs. TLR3 and GAPDH are shown for comparison. **E,** Representative immunohistochemistry for KAP1 in normal breast and in invasive ductal carcinoma. Images from Human Protein Atlas: www.proteinatlas.org.
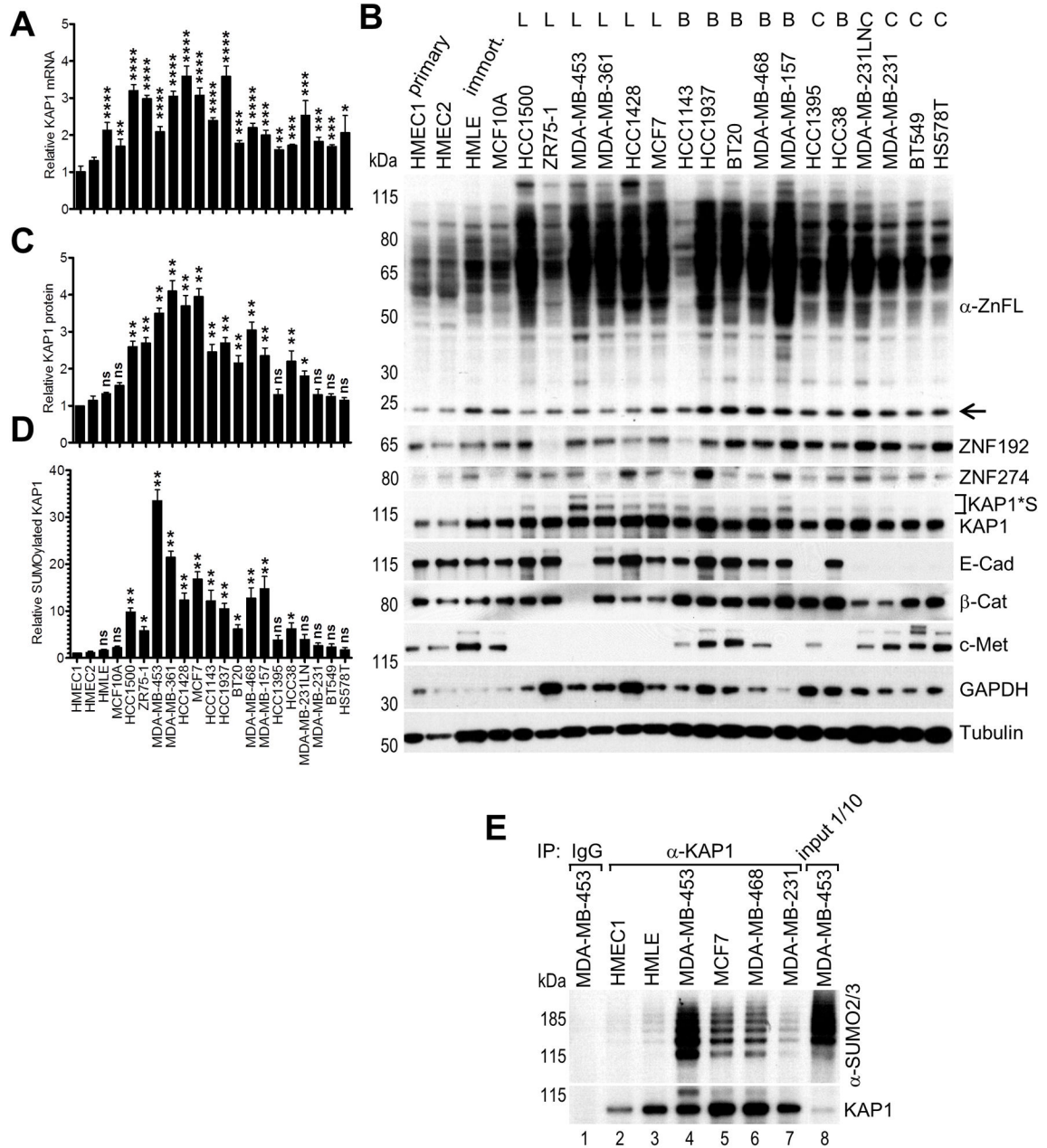
Figure 3. KAP1 mRNA, protein and SUMOylation are up-regulated in breast cancer cell lines
The indicated cell lines representing luminal (L), basal (B) and claudin-low (C) breast cancer subtypes were analyzed for (**A**) KAP1 mRNA by RT-qPCR, (**B&C**) KAP1 protein and (**B&D**) KAP1 SUMOylation by WB. Arrow points to a ~20kDa protein band, which serves as loading control. E-cadherin is shown as a reference for luminal and basal cells and as additional loading control. Expression of β-catenin and c-Met oncoproteins as well as GAPDH and tubulin is elevated in cancer cells. KAP1*S – SUMOylated forms of KAP1. Graphs in C&D show densitometric quantification of (B) normalized by tubulin. In A,C&D, levels for HMEC1 cells were assigned the relative value of 1. **E**, Immunoprecipitation with

control IgG and KAP1 antibody and WB for SUMO2/3 and KAP1. Data shown as mean ±SD. * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$, **** - $P < 0.0001$, two-tailed t test.
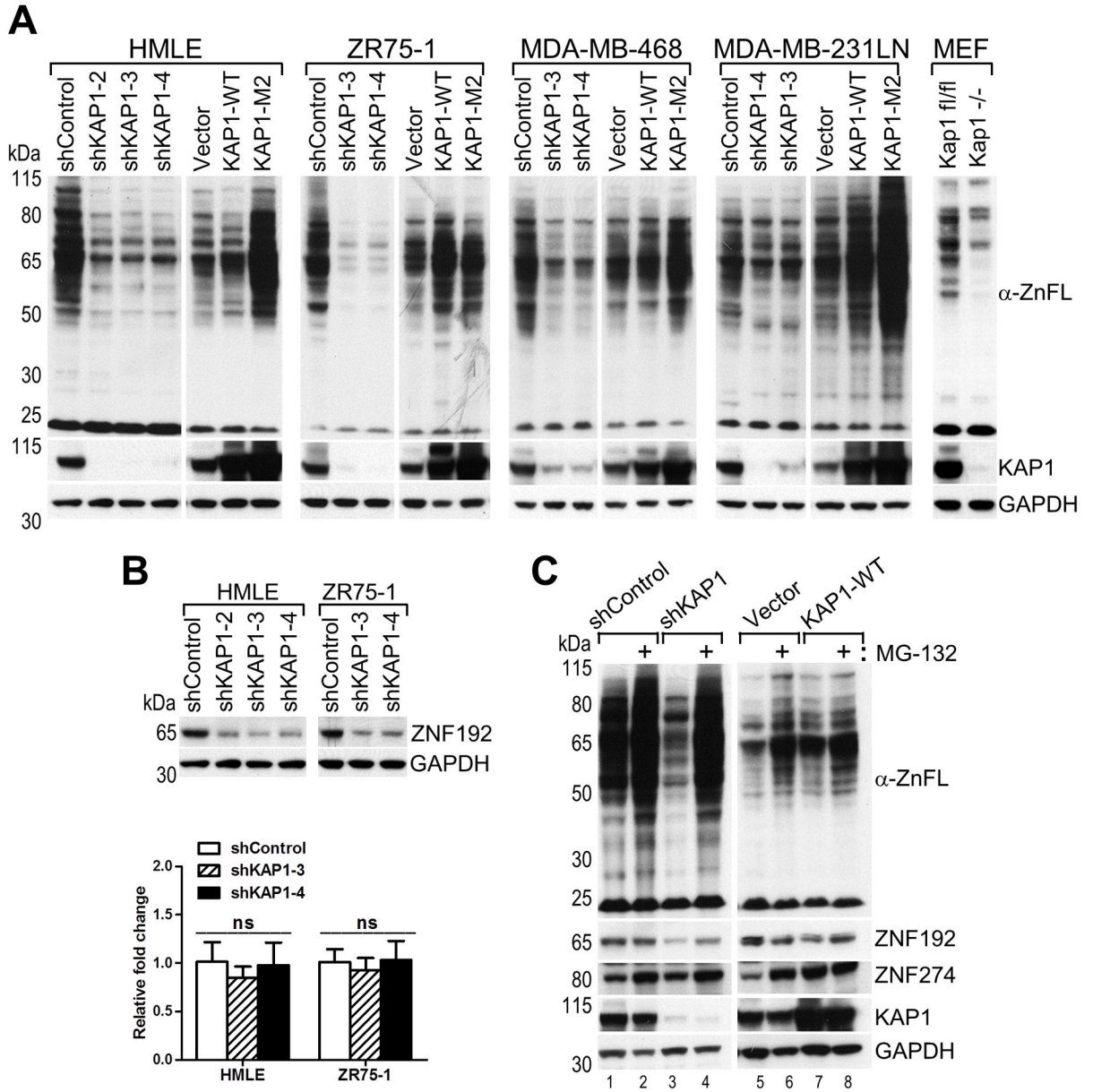
Figure 4. KAP1 positively regulates expression of multiple KRAB-ZNF proteins
A, WB of KRAB-ZNFs and KAP1 in the indicated cell lines with KAP1 knockdown and overexpression, and in mouse embryonic fibroblasts (MEFs). fl/fl – flox/flox, −/− - KAP1 knockout alleles. **B,** WB and RT-qPCR of ZNF192 in HMLE and ZR75-1 cells shown in (A). Data shown as mean ±SEM. **C,** WB of the indicated HMLE cell lines treated with vehicle or 10μM MG-132 for 8 hours. GAPDH - loading control.
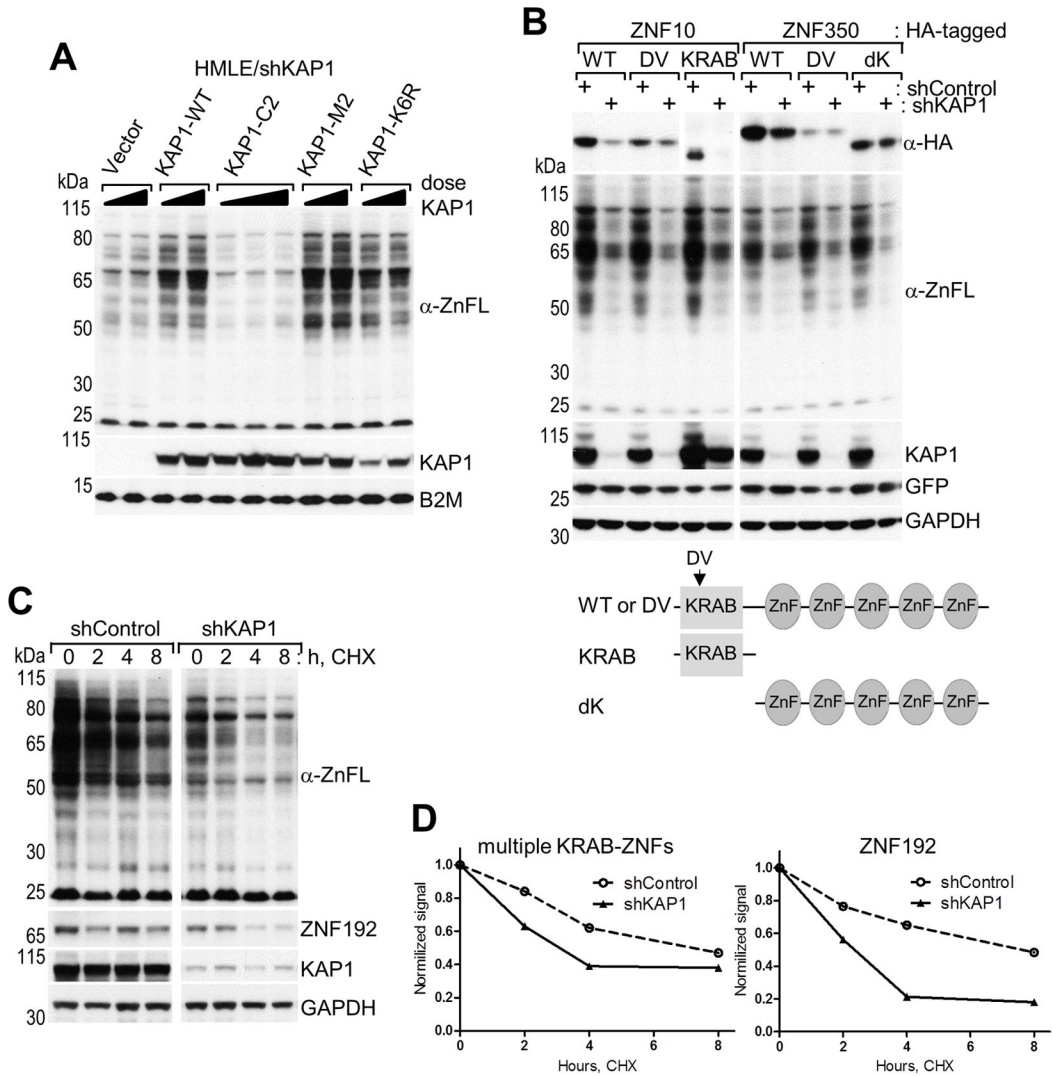
Figure 5. Expression of KRAB-ZNF proteins depends on their ability to interact with KAP1 and this interaction protects the KRAB-ZNFs from proteasomal degradation
**A**, WB of KAP1 and KRAB-ZNFs in HMLE/shKAP1 cells reconstituted with vector, wild type and the indicated KAP1 mutants. Increasing doses of KAP1 expressing lentiviruses were used. B2M - loading control. **B,** WB of H1299 cell lines transfected with HA-tagged ZNF10 and ZNF350. Below is a diagram of the constructs used. WT- wild type, DV – mutant deficient in KAP1 binding, KRAB – KRAB domain only, dK – deleted KRAB domain. GAPDH - loading control. **C,** WB analysis of HMLE cells treated with 50µg/ml cycloheximide (CHX) for 0, 2, 4 and 8 hours. **D**, Quantification of (C) normalized to GAPDH.
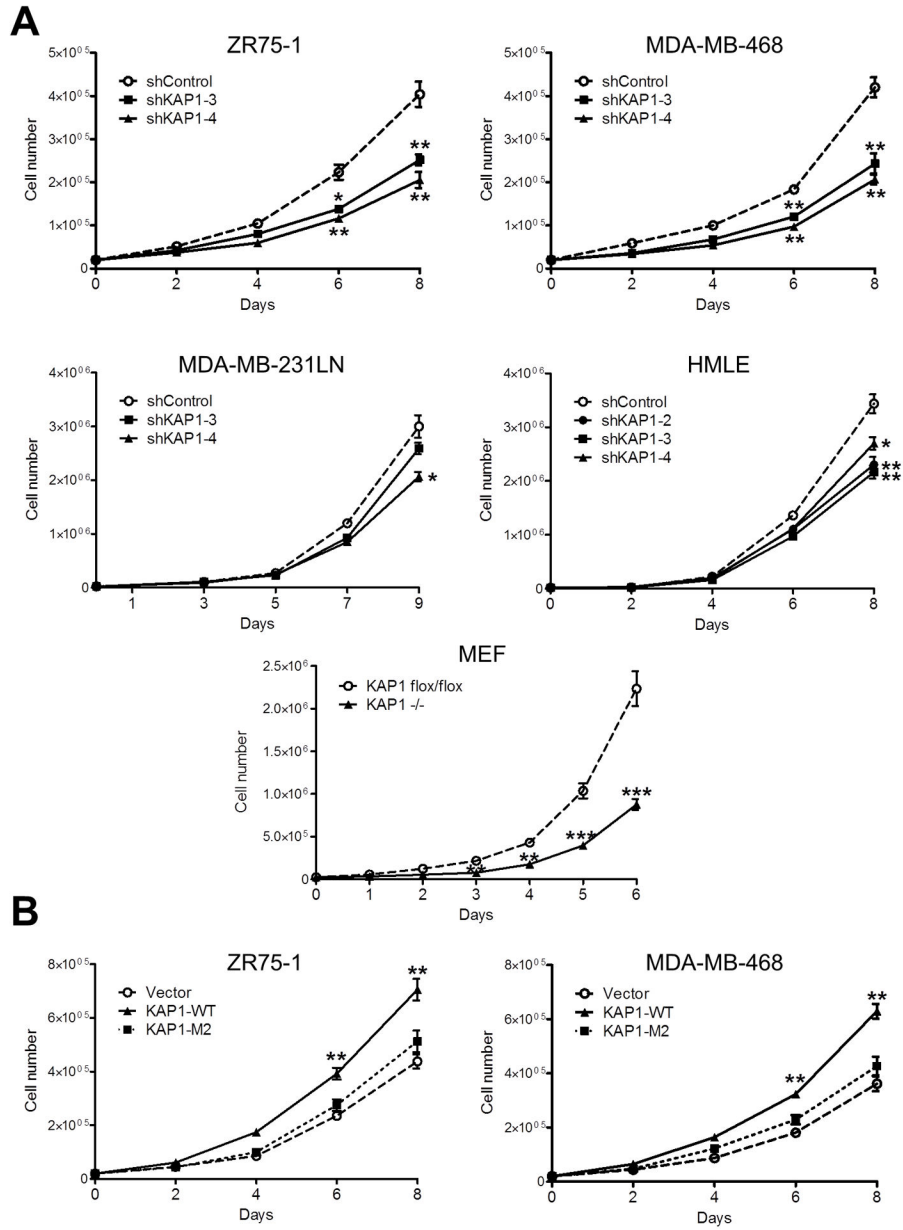
Figure 6. KAP1 promotes cell proliferation *in vitro*
A&B, Growth curves for the cell lines characterized in Fig. 4A. Data shown as mean ±SEM.
* - *P* < 0.05, ** - *P* < 0.01 *** - *P* < 0.001, two-tailed *t* test.
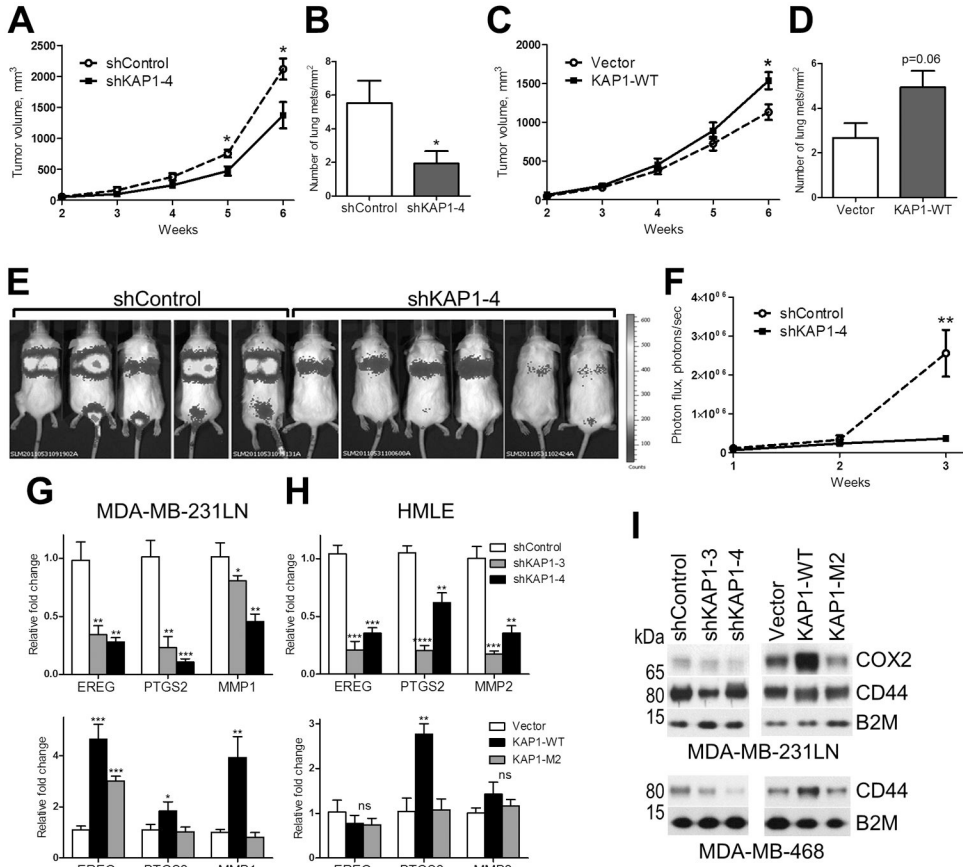
Figure 7. KAP1 promotes tumor growth and metastasis and regulates expression of cancer-associated genes

A&B, Tumor growth curves of orthotopically injected MDA-MB-231LN cells: shControl (n=4), shKAP1-4 (n=5), vector control (n=4), KAP1-WT (n=5). **C&D**, Quantification of metastases in lungs of the animals in (A) and (B). **E,** Bioluminescent images of mice injected intravenously with the indicated MDA-MB-231LN cell lines at 3 weeks post-injection. **F,** Quantification of (E). **G&H,** RT-qPCR of genes promoting tumor growth and metastasis in the indicated cell lines. **I,** WB of COX2 and CD44 in the indicated cell lines. B2M - loading control. Data shown as mean ±SEM, * - $P < 0.05$, ** - $P < 0.01$, two-tailed t test.

Table 1

Mass spectrometry identification of ZNFs immunoprecipitated by control IgG and ZnFL antibody.

Accession Number	SpC IgG	SpC ZnFL	KRAB domain	SCAN domain	Number of ZFs
Q15072|OZF/ZNF146	0	17			10
Q5VV52|ZNF691	0	16			7
Q9H5H4|ZNF768	0	12			10
Q7L3S4|ZNF771	0	10			8
Q96N38|ZNF714	0	9	+		14
Q86W11|ZSCAN30	0	8		+	7
Q15776|ZNF192/ZKSCAN8	0	8	+	+	9
Q32CT1|ZNF260	0	8			13
Q96CX3|ZNF501	0	8			9
P17026|ZNF22	0	7			5
P17028|ZNF24	0	7		+	4
Q9Y2G7|ZFP30	0	4	+		13
Q86UQ0|ZNF589	0	4	+		4
Q969J2|ZKSCAN4	0	4	+	+	7
P17029|ZKSCAN1	0	3	+	+	6
Q9BRR0|ZKSCAN3	0	3	+	+	7
P17041|ZNF32	0	2			7
Q14592|ZNF460	0	2	+		11
Q96MX3|ZNF48	0	2			12
P36508|ZNF76	0	2			7
Q6DD87|ZNF787	0	2			7
P25490|YY1	0	2			4

SpC - spectral counts.