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SKP2 loss destabilizes EZH2 by promoting TRAF6-mediated ubiquitination to suppress prostate cancer

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Abstract

EZH2 is crucial for the progression of prostate cancer (PCa) and castration-resistant prostate cancer (CRPC) through upregulation and activation of progenitor genes, as well as androgen receptor (AR)-target genes. However, the mechanisms by which EZH2 is regulated in PCa and CRPC remain elusive. Here we report that EZH2 is post-transcriptionally regulated by SKP2 *in vitro* in cultured cells and *in vivo* in mouse models. We observed aberrant upregulation of Skp2, Ezh2 and histone H3 lysine 27 trimethylation (H3K27me3) in both *Pten* null mouse embryonic fibroblasts (MEFs) and *Pten* null mouse prostate tissues. Loss of Skp2 resulted in a striking decrease of Ezh2 levels in *Pten/Trp53* double-null MEFs and in prostate tumors of *Pten/Trp53* double-null mutant mice. SKP2 knockdown decreased EZH2 levels in human PCa cells through upregulation of TRAF6-mediated and lysine(K) 63-linked ubiquitination of EZH2 for degradation. Ectopic expression of TRAF6 promoted the K63-linked ubiquitination of EZH2 to decrease EZH2 and H3K27me3 levels in PCa cells. In contrast, TRAF6 knockdown resulted in a reduced EZH2 ubiquitination with an increase of EZH2 and H3K27me3 levels in PCa cells. Furthermore, the catalytically dead mutant TRAF6 C70A abolished the TRAF6-mediated polyubiquitination of recombinant human EZH2 *in vitro*. Most importantly, a concurrent elevation of Skp2 and Ezh2 was found in CRPC tumors of *Pten/Trp53* mutant mice, and expression levels of SKP2 and EZH2 were positively correlated in human PCa specimens. Taken together, our findings revealed a novel mechanism on EZH2 ubiquitination and an important signaling network of SKP2-TRAF6-EZH2/H3K27me3, and targeting SKP2-EZH2 pathway may be a promising therapeutic strategy for CRPC treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTRODUCTION

Aberrant histone modification is an important epigenetic event associated with the development and progression of various human cancers including PCa.[1,2] Mechanistic explanations on the dysregulation of histone modifications are needed in order to precisely target the key epigenetic regulators for PCa treatment. S-phase kinase-associated protein 2 (SKP2), a member of the F-box protein family, forms SKP2–SCF complex with SKP1, Cullin-1 and RBX1 to act as a ubiquitin ligase for protein ubiquitination and degradation of tumor suppressors and other proteins.[3,4] More importantly, SKP2 is a proto-oncogene involved in cancer progression and metastasis in human cancers including PCa.[5–7] SKP2 elevation is correlated with loss of tumor suppressor PTEN,[8] and is regulated through AKT-dependent phosphorylation and cytosolic accumulation of SKP2 in human PCa.[6,9] SKP2 contributes to cancers through its E3 ligase activity to degrade downstream targets such as p27 and androgen receptor (AR).[6,10,11] SKP2 also has functions independent of an E3 ligase to regulate oncoproteins such as ATF4,[5] RhoA and Miz1,[7] and C-Myc in PCa.[12,13] We recently reported that SKP2 ablation elevates the ubiquitination and activity of JARID1B, a histone modification demethylase, to decrease histone H3 lysine 4 trimethylation (H3K4me3) levels in suppression of PCa tumorigenesis.[2] That study provided a clue on the role of SKP2 on histone modifications and the epigenetic alterations in PCa. Although levels of SKP2 and Enhancer of zeste homolog 2 (EZH2) are highly associated with aggressive features of human PCa,[1,14] it remains unclear whether SKP2 determines the levels of histone H3 lysine 27 trimethylation (H3K27me3) state by modulating EZH2.

EZH2 is a catalytic subunit of polycomb repressive complex 2 (PRC2) that globally silences gene expression through H3K27me3.[15] For example, EZH2 may function as a transcriptional repressor to downregulate tumor suppressors such as ADRB2 and DAB2IP, resulting in malignant progression of PCa.[16,17] In addition, EZH2 activates multiple oncogenes such as Ras, NF-κB and AR in metastatic PCa and CRPC.[18–20] The posttranslational modifications of EZH2 such as ubiquiti-nation, SUMOylation, phosphorylation and glycosylation not only determine its stability and oncogenic activity but also regulate the biological functions of PRC2 in cancer cells.[21] The form and status of EZH2 ubiquitination are critical to remain its interaction with other subunits of PRC2. Studies reveal that Smurf2, β-TrCP and Praja1 can function as ubiquitin E3 ligases for EZH2 in neuron differentiation,[22] lymphoma[23] and breast cancer,[24,25] respectively. However, molecular mechanisms underlying the aberrant elevation of EZH2 in PCa including CRPC are poorly understood, and the relationship between EZH2 and SKP2 is still unknown. In this study, we found that SKP2 stabilizes EZH2 through TRAF6-mediated ubiquitination in human PCa, and that SKP2 deletion decreases EZH2 to suppress prostate tumorigenesis. Our findings revealed that SKP2 is a novel epigenetic regulator, and targeting SKP2–EZH2 pathway may be a promising approach for PCa treatment.

RESULTS

Aberrant elevation of Skp2 and Ezh2 upon Pten loss in MEFs *in vitro* and mouse prostates *in vivo*

To explore the correlation between Skp2 (SKP2 in human and Skp2 in mouse) and Ezh2 (EZH2 in human and Ezh2 in mouse), as well as their possible regulation upon oncogenic insults, we took advantage of the well-defined cancer biology and genetic systems, *Pten*-null mouse embryonic fibroblasts (MEFs) and mouse models. We first examined the protein levels of Skp2 and Ezh2 *in vitro* in *Pten*-null ($Pten^{-/-}$) MEFs. As shown in western blot, Pten expression levels were completely abrogated in $Pten^{-/-}$ MEFs with a striking increase in the activated form of AKT (pAKT-S473) (Figure 1a and Supplementary Figure S1). In agreement with previous reports,[26,27] *Pten* loss resulted in a 30-fold increase of Skp2 in $Pten^{-/-}$ MEFs, as compared with *WT* MEFs (Figure 1a). Interestingly, *Pten* loss also led to an ~ 25-fold increase in Ezh2 levels, and Ezh2 elevation had functional consequences as indicated by a 6-fold increase of H3K27me3 levels, as compared with WT MEFs. Importantly, loss of one allele of *Pten* gene ($Pten^{+/-}$) also resulted in the significant increase of Skp2 and Ezh2 levels ($P<0.001$), suggesting that overall cellular Pten levels may concomitantly determine Skp2 and Ezh2 expression. We next performed immunohistochemical (IHC) staining of Skp2, Ezh2 and H3K27me3 to examine protein levels *in vivo* in prostate tumors of *Pten*-null ($Pten^{pc\ -/-}$) mice. Consistently, IHC results revealed protein levels of Skp2, Ezh2 and H3K27me3 were strikingly increased in $Pten^{pc\ -/-}$ mice, as compared with that of *WT* mice (Figures 1b and c). Moreover, both Skp2 and Ezh2 were also found in the cytoplasm of some tumor cells, in addition to their nuclear localizations in $Pten^{pc\ -/-}$ mice. These results for the first time demonstrated a Skp2–Ezh2/H3K27me3 signaling pathway in which Ezh2 may be closely associated with Skp2, at least in the context of *Pten* loss.

Skp2 deficiency decreases levels of Ezh2 and H3K27me3 *in vitro* and *in vivo*

We previously reported that SKP2 loss contributes to the suppression of prostate tumorigenesis in part through the regulation of JARID1B/H3K4me3.[2] Since the epigenetic switches on H3K4me3/H3K27me3 were frequently found in cancers,[28] we reasoned that Skp2 loss may also downregulate H3K27me3 to suppress prostate tumorigenesis. To test the hypothesis, we evaluated the effects of *Skp2* deficiency on Ezh2 and H3K27me3 in *Pten/Trp53* double-null MEFs and in $Pten^{pc-/-}; Trp53^{pc-/-}$ mutant mice (a fast PCa progression mouse model). *Pten/Trp53* double-null ($Pten^{-/-}; Trp53^{-/-}$) and *Pten/Trp53/Skp2* triple-null ($Pten^{-/-}; Trp53^{-/-}; Skp2^{-/-}$) MEFs, as well as $Pten^{pc-/-}; Trp53^{pc-/-}$ and $Pten^{pc-/-}; Trp53^{pc-/-}; Skp2^{-/-}$ mutant mice were prepared as previously reported.[29] Western blot analysis showed that levels of both Ezh2 and H3K27me3 in *Pten/Trp53/Skp2* MEFs were markedly decreased as compared with that in *Pten/Trp53* MEFs (Figure 2a). Quantification analysis revealed that *Skp2* deletion resulted in a two-fold reduction of Ezh2 and H3K27me3 (Figure 2b), underscoring an essential role of Skp2 on the regulation of Ezh2 and H3K27me3. Our data were in agreement with the literature showing that the protein levels of EZH2, a specific histone methyltransferase of H3K27me3/2, are positively correlated with H3K27me3 levels during malignancy progression.[19] In addition, our results support the notion that Skp2 modulates Ezh2 thereby to regulate H3K27me3 levels in cells, suggesting

that SKP2–EZH2/H3K27me3 signaling may in part determine the malignant features of cancer cells.

Given the impact of *Skp2* deficiency on the inhibition of cell proliferation and the suppression of prostate tumorigenesis, we then examined whether *Skp2* deficiency similarly decreases Ezh2 and H3K27me3 *in vivo*. Consistent with the findings in MEFs, both Ezh2 and H3K27me3 levels were markedly decreased in prostate tumors of $Pten^{pc-/-}; Trp53^{pc-/-}; Skp2^{-/-}$ mice as compared with that of $Pten^{pc-/-}; Trp53^{pc-/-}$ mice (Figure 2c and Supplementary Figure S2). As shown, Ezh2 and H3K27me3 were strikingly elevated in all epithelial cells of $Pten^{pc-/-}; Trp53^{pc-/-}$ tumors, but mildly increased only in some epithelial cells of $Pten^{pc-/-}; Trp53^{pc-/-}; Skp2^{-/-}$ tumors. Quantification analysis revealed that *Skp2* loss resulted in a 30% decrease of Ezh2 with a consequent 30% reduction of H3K27me3 in $Pten^{pc-/-}; Trp53^{pc-/-}; Skp2^{-/-}$ mice, when compared with $Pten^{pc-/-}; Trp53^{pc-/-}$ mice (Figure 2d). Our data provided evidence upon the elevation of Ezh2 and H3K27me3 on oncogenic stresses and their concomitant downregulation by knockout of one oncogene in mouse models. Our *in vitro* and *in vivo* results suggest that, in addition to JARID1B-directed H3K4me3,[2] SKP2 may also contribute to PCa tumorigenesis through EZH2–H3K27me3 for the epigenetic regulation of chromatin modifications.

EZH2 stability is controlled by SKP2 and TRAF6 in human prostate cancer cells

We next sought to understand the mechanisms underlying the regulation of EZH2/H3K27me3 by SKP2 using human PCa cells. Since we have established a stable PC3-shSKP2 cell line in that *SKP2* is knocked down by shRNA,[2,10] we examined the effects of *SKP2* knockdown on EZH2 and H3K27me3 levels in human PCa cells. Western blot results revealed that *SKP2* knockdown resulted in a 50% decrease of both EZH2 and H3K27me3 levels in PC3-shSKP2 cells, as compared with the control (PC3-scrambled cells) (Figure 3a). The results were in agreement with our discoveries in MEFs (Figure 2a). To find out whether *SKP2* affects *EZH2* levels at the transcriptional or posttranscriptional level, or both, we performed RT-qPCR to examine the mRNA levels of *EZH2* upon *SKP2* knockdown. Our results showed that mRNA levels of *EZH2* in PC3-shSKP2 cells were comparable to that in PC3-scrambled cells (Figure 3b), suggesting that the reduction of EZH2 levels upon *SKP2* loss occurred at the posttranslational level. Consistently, EZH2 levels in PC3-shSKP2 cells were decreased by ~ 70% at 1 h, when compared with the ~ 10% decrease in PC3-scrambled cells, indicating that SKP2 modulates the steady-state levels of EZH2 in human PC3 PCa cells (Figure 3c). By contrast, a restoration of *SKP2* levels in PC3-shSKP2 cells by ectopic expression of *SKP2* enhanced the steady-state levels of EZH2 (Supplementary Figure S3). To further understand the role of SKP2 in EZH2 regulation, we ectopically overexpressed *SKP2* in C4-2B cells, an AR-positive PCa cell line. In contrast to *SKP2* knockdown, *SKP2* overexpression indeed resulted in a dramatic increase of EZH2 levels (Figure 3d). Furthermore, a decrease of SKP2 by compound C25, a small-molecule inhibitor of SKP2,[30] significantly reduced EZH2 levels in both C4-2B and PC3 PCa cells (Supplementary Figure S4). Together, these results strongly supported an affirmative correlation between SKP2 and EZH2 in human PCa cells.

Since SKP2 is an E3 ligase, we wondered whether SKP2 could modulate EZH2 stability through the ubiquitination machinery. Therefore, we performed *in vivo* ubiquitination assays. However, SKP2 decreased the poly-ubiquitination of EZH2 in HEK293T cells, indicating that EZH2 is not a substrate of SKP2 E3 ligase (Supplementary Figure S5). As TRAF6, a ubiquitin E3 ligase involved in chromatin modification, is upregulated upon *SKP2* loss in PCa cells,[2] we then turned our focus of the investigation to EZH2 levels being regulated by TRAF6. Western blot analysis revealed that the ectopic expression of *SKP2* strikingly decreased TRAF6 levels in C4-2B cells (Figure 3d), supporting our previous findings that *SKP2* knockdown increased TRAF6 levels in PCa cells.[2] These data prompted us to reason that EZH2 levels may be determined by TRAF6 in a SKP2-dependent fashion. We then examined the effects of *TRAF6* knockdown and overexpression on the changes of EZH2 levels in PCa cells. Indeed, *TRAF6* knockdown by two different sets of siRNAs markedly increased both EZH2 and H3K27me3 levels in PC3 cells (Figure 3e). In contrast, ectopic overexpression of *TRAF6* decreased both EZH2 and H3K27me3 levels in PC3 cells (Figure 3f). The TRAF6-mediated regulation of EZH2 was further observed in C4-2B cells (Supplementary Figure S6). Taken together, our results strongly suggest that EZH2 and H3K27me3 levels may be directly controlled by TRAF6, and that SKP2 may affect EZH2 by modulating TRAF6 levels in human PCa cells.

In silico analysis predicted that EZH2 contains three potential TRAF6 binding motifs (Supplementary Figure S7). The PEEREE, one of the TRAF6 binding motifs on EZH2, is identical to the known TRAF6 binding site of AKT, [31] strongly suggesting that EZH2 may be a substrate for TRAF6-mediated ubiquitination. To examine the interaction between TRAF6 and EZH2 in PCa cells, we performed co-immunoprecipitation (co-IP) assays with anti-TRAF6 antibody, using cell lysates of PC3 cells expressing these two endogenous proteins. The results indeed revealed a physical binding of TRAF6 and EZH2 in human PCa cells (Figure 3g). Immunofluorescence (IF) staining demonstrated a co-localization of TRAF6 and EZH2 in nucleus of PC3 cells, supporting the notion that they may physically interact (Figure 3h).

EZH2 is decreased by TRAF6 through lysine(K)63-linked polyubiquitination

We then asked ourselves whether TRAF6 is a ubiquitin E3 ligase for EZH2 ubiquitination and degradation. To this end, we first performed *in vivo* ubiquitination assay in HEK293T cells trans-fected with EZH2, TRAF6, ubiquitin wild type (WT) and mutation plasmids (K48-only or K63-only mutants). As expected, TRAF6 overexpression markedly increased the levels of EZH2 polyubi-quitination, which likely causes the decrease of EZH2 levels (Figure 4a). Interestingly, EZH2 polyubiquitination enhanced by TRAF6 was mainly extended through K63-linkage instead of K48-linkage. We then examined the impact of TRAF6 decrease on the ubiquitination of endogenous EZH2 in human PCa cells. Indeed, *TRAF6* knockdown by siRNA resulted in a striking reduction of the K63-linked ubiquitination of endogenous EZH2 in PC3 cells, while EZH2 levels in whole cell lysates increased (Figure 4b). In contrast, ectopic overexpression of *TRAF6* resulted in a noticeable increase of the K63-linked ubiquitination of endogenous EZH2 in C4-2B cells (Figure 4c). Most importantly, the TRAF6 dead mutant C70A also resulted in a dramatic reduction of the K63-linked ubiquitination of endogenous EZH2 as compared with TRAF6 WT, indicating

that EZH2 is a substrate of TRAF6-mediated ubiquitination (Figure 4d). To further confirm whether TRAF6 is a direct ubiquitin E3 ligase for EZH2, we performed *in vitro* ubiquitination assay with recombinant human Flag-EZH2 plus recombinant human GST-TRAF6 or GST-TRAF6 C70A mutant (Supplementary Figure S8). Remarkably, GST-TRAF6 WT catalysed the polyubiquitination of EZH2, whereas GST-TRAF6 C70A mutation completely abrogated EZH2 polyubiquitination (Figure 4e). These lines of evidence demonstrated for the first time that TRAF6 is the E3 ligase for EZH2 ubiquitination and EZH2 levels are decreased by TRAF6 through the K63-linked polyubiquitination. Together, our results revealed a novel signal pathway of SKP2-TRAF6-EZH2 in which SKP2 may contribute to EZH2 stability through the regulation of TRAF6-mediated ubiquitination of EZH2 in PCa.

EZH2 correlates with the recurrent growth of prostate tumors in *Pten/Trp53* mice

Dysregulation of histone modifications is associated with the development of advanced PCa including CRPC growth in humans. We previously reported H3K4me3 upregulation in recurrent prostate tumors in castrated *Pten/Trp53* mutant mice,[2] providing *in vivo* evidence on the aberrant histone modifications in CRPC. In this study, we wished to obtain new insights into the role of EZH2/H3K27me3 coupling on CRPC growth with application of *Pten/Trp53* mouse models. To appreciate the expression profiles of EZH2 and H3K27me3 on CRPC, we used regressive or recurrent lesions within prostate tissues of mice to perform IHC staining.[2] Consistent with previous results, Skp2 accumulation was noticeably increased in recurrent tumors as compared with that in regressive tumors (Figures 5a and b), highlighting a crucial role of Skp2 on CRPC growth. Importantly, both Ezh2 and H3K27me3 were strikingly elevated in recurrent tumors of *Pten/Trp53* mice, along with Skp2 elevation, compared with that in regressive tumors. The expression patterns on Ezh2 and H3K27me3 elevation for CRPC growth in mice are in agreement with the reports on EZH2/H3K27me3 in human CRPC specimens.[19,32,33] These *in vivo* results strongly support the notion that dysregulation of SKP2 and EZH2/H3K27me3 can drive CRPC growth in humans.

EZH2 is positively correlated with SKP2 expression in human prostate cancer

To understand the correlation between SKP2 and EZH2, as well as the relevance on PCa progression in humans, we performed IHC staining of SKP2 and EZH2 in human prostate tissue microarrays (TMA) consisting of cancer and normal samples. The elevation of SKP2 was found in both cytoplasm and nucleus of malignant cells, while EZH2 elevation was primarily accumulated in nucleus of cancer cells (Figure 5c). Lesions with elevated SKP2 expression were also showing the increased EZH2 based on the IHC staining from adjacent tissue sections of PCa TMA. We further examined the expression profile of SKP2 and EZH2 in the same specimens. Notably, cancer cells with a high level of SKP2 normally showed a high level of EZH2 (Supplementary Figure S9). In addition, we evaluated the correlation between SKP2 and EZH2 levels by performing a Pearson Correlation test with scores taken as continuous variables, which was further validated by Chi-square tests. Both statistical analyzes indicated that SKP2 levels were significantly correlated with EZH2 (Pearson correlation coeffi-cient = 0.84, $P<0.00000002$; χ^2 test, $P = 0.0001$) (Figure 5d and

Supplementary Table S2). These results indicate that SKP2 dysregulation plays a critical role on EZH2 alteration in human PCa.

DISCUSSION

Histone modification enzymes, including histone demethylase JARID1B[34] and histone methyltransferase EZH2,[19] have been reported to be aberrantly upregulated in various human cancers including PCa. Elevation of JARID1B and EZH2 is associated with metastatic PCa and CRPC,[18,19] yet their regulation remains poorly understood. We previously demonstrated that SKP2 deletion decreases prostate tumorigenesis through involving the K63-linked ubiquitination of JARID1B by TRAF6. [2] In this study, we uncovered from MEFs, mouse models, human PCa cells and TMA specimens, that SKP2 is essential for the elevation of EZH2 and H3K27me3 in PCa including CRPC. We found a positive correlation between SKP2 and EZH2, and a novel mechanism by which SKP2 stabilizes EZH2 by reducing the TRAF6-mediated and K63-linked ubiquitination of EZH2. This is the first report that SKP2 is linked with the epigenetic modification of H3K27me3 through EZH2 in PCa. Our results indicate that SKP2 and EZH2 synergistically drive the progression of PCa and CRPC by increasing their oncogene potentials in both genetic and epigenetic functions (polycomb-dependent).

One of the most interesting discoveries in this study is that TRAF6-mediated ubiquitination determines EZH2 stability in PCa cells. As a global regulator through H3K27me3 and a coactivator for key transcriptional factors such as AR, EZH2 exerts a driving role on PCa progression, distant metastasis and CRPC.[18–20] However, the regulation of EZH2 ubiquitination and stability, as well as the relevance on PCa and CRPC has remained elusive. We demonstrated for the first time that TRAF6 is a direct ubiquitin E3 ligase for EZH2 degradation, thus regulating the stability of EZH2 in PCa. The activity and stability of EZH2 are largely affected by a variety of posttranslational modifications, which consequently alters the biological functions of PRC2 in cells.[21] For example, EZH2 phosphorylation at Serine 21 by AKT inhibits its enzymatic activity to decrease H3K27me3 levels, which is required for its polycomb-independent function in PCa,[20,35] EZH2 glycosylation at Serine 75 by O-linked N-acetylglucosamine transferase (OGT) promotes EZH2 stability.[36] Smurf2 and β-TrCP were recently reported to be E3 ligases for EZH2 through the polyubiquitination and proteasome-dependent degradation procedure in neurons and lymphoma.[22,23] The ubiquitination and stability of EZH2 are co-regulated by E3 ligase Praja1 and FOXP3 in breast cancer,[24,25] indicating the complexity of EZH2 regulation. Our results revealed that TRAF6 catalyses K63-linked polyubiquitination of EZH2 for protein degradation, which is blocked upon SKP2 elevation in PCa. Our discoveries suggest a novel oncogenic pathway of SKP2-TRAF6-EZH2/H3K27me3 in PCa and CRPC, as well as the implication of therapeutic targeting of this pathway for inhibition of PCa growth.

K63-linked ubiquitination of proteins involves a variety of functions such as kinase activation and protein trafficking between cellular compartments, independent of proteasome-mediated degradation machinery.[37] However, this type of ubiquitination can promote or aid in the lysosomal-mediated degradation of substrate proteins, including transmembrane proteins such as LDL receptor and Class I molecule,[38–40] cytosolic proteins

such as α-synuclein,[41] and nuclear proteins such as HIF1A.[42] Although EZH2 contains the minimum sequence of YXXØ motif (for lysosomal-mediated degradation of transmembrane proteins) including YAKV and YVGI at the C-terminal (data not shown), it is deficient of the pentpeptide sequence KFERQ, a sorting signal for lysosomal-mediated degradation of cytosolic proteins.[43] Further investigation will be needed to elucidate whether the lysosome-dependent degradation is involved in SKP2-TRAF6-dependent EZH2 stability. Alternatively, the K63-linked polyubiquitination of EZH2 may serve to provide a substrate to be targeted by other E3 ligases for EZH2 degradation.

Emerging evidence shows that H3K27me3 levels contribute to the development of human cancers, and correlate with aggressive features of PCa.[1,14,32] However, EZH2 can activate the expression of multiple transcriptional factors including AR, by Ser21 phosphorylation of EZH2 and methylation of AR, independent of EZH2 regulation of H3K27me3 in CRPC.[20] Our results show a marked upregulation of Skp2, Ezh2 and H3K27me3 in a concurrent fashion in *Pten/Trp53* mice. Given the striking impact of SKP2 on EZH2 and H3K27me3, it is likely that SKP2 may contribute to the elevated expression of EZH2 and H3K27me3 in human CRPC, indicating a critical role for H3K27me3 on human CRPC, as exhibited in our *Pten/Trp53* mouse model (Figure 5a). Furthermore, the concomitant upregulation of SKP2, EZH2 and H3K27me3 underscores that the activation of SKP2–EZH2/H3K27me3 is an important oncogenic pathway in CRPC growth. Our findings, consistent with literature, suggest complicate roles of EZH2/ H3K27me3 in CRPC as follows. (1) Coexistence of polycomb-dependent and -independent function of EZH2 under *PTEN* and *TP53* loss. The non-linear correlation between EZH2 and H3K27me3 in the Varambally cohort may be caused by genetic alterations independent of *PTEN* and *TP53*.[19,20] (2) H3K27me3 levels may be regulated in a both EZH2-dependent and -independent manner, through other histone modification enzymes such as JMJD3.[44] Certainly, further investigation is needed to understand H3K27me3 regulation in CRPC with heterogeneity.

In conclusion, our results revealed a novel mechanism in which EZH2 stability is regulated by SKP2 through the TRAF6-mediated and K63-linked ubiquitination, which contributes to elevated levels of H3K27me3 during prostate tumorigenesis and CRPC growth. Our findings indicate that SKP2 influences PCa progression by serving as an epigenetic regulator of the global transcriptional regulator EZH2. Therefore, targeting SKP2–EZH2 pathway may represent a promising therapeutic strategy to control the growth of PCa.

MATERIALS AND METHODS

Mice and tumor analysis

Pten, Pten/Trp53 and *Pten/Trp53/Skp2* mutant mice (in a mixed genetic background of C57BL/6 J × 129sv × DBA2) were generated as previously described.[2,5,29] Animal maintenance and experiments were conducted in accordance with an IACUC-approved protocol at Meharry Medical College. Prostate tissues from mice with indicated genotypes were used for preparation of paraffin sections according to standard protocols.[2,5,29] Cell culture, siRNA, shRNA and real-time reverse transcription PCR PC3 (ATCC) and C4-2B (MD Anderson, Houston, TX, USA) cells were grown in RPMI 1640 medium with 10% FBS and 1% Pen/Strep in an incubator at 37 °C with 5% CO_2. For transient expression or

knockdown, cells were transfected with desired plasmids or two TRAF6 siRNAs (Supplementary Table S1) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the expression of target genes was determined 48–72 h post-transfection using real-time qPCR or western blotting. *SKP2* knockdown by shRNA was performed as previously described.[2,45] Real-time reverse transcription qPCR with the forward and reverse primers (Supplementary Table S1) was conducted in triplicate as previously reported.[2,45–47]

Western blotting and half-life determination

Cell lysates were prepared in RIPA buffer (1 × PBS, 1% Nonidet P40/Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, with or without 0.1% SDS).[2] Antibodies used were: anti-H3K27me3 (1:10 000, 07-499, Millipore, Billerica, MA, USA), anti-H3 (1:10 000, Ab1791, Abcam, Cambridge, MA, USA), anti-Skp2 (1:500, sc-7164, Santa Cruz, Dallas, TX, USA), anti-EZH2 (1:200, 07-1583, Millipore), anti-EZH2 (1:1000, 5246, Cell Signaling, Danvers, MA, USA), anti-EZH2 (1:1000, 612666, BD Biosciences, San Jose, CA, USA), anti-β-actin (1: 10 000, A5316, Sigma, St Louis, MO, USA), ant-Flag M2 affinity gel (A2220, Sigma), anti-Flag M2 antibody (1:1000, F1804, Sigma), anti-C-Myc (1:1000, sc-40, Santa Cruz), anti-HA.11 (1:1000, MMS-101 P, Covance, San Diego, CA, USA), anti-TRAF6 (1:1000, sc-8409, Santa Cruz), and anti-TRAF6 (1:1000, 8028, Cell Signaling). To determine the stability of EZH2, cells were cultured in starvation medium with cycloheximide (CHX, 100 μg/ml). After that, cell lysates were harvested at indicated time points, and subject to western blotting analysis using EZH2 antibody.[2]

In vivo and *in vitro* ubiquitination assays

For *in vivo* ubiquitination assay, HEK293T cells were transfected with Flag-tagged EZH2,[48] HA-tagged ubiquitin WT or ubiquitin mutants (K48-only and K63-only),[49] Myc-tagged TRAF6[50,51] or its mutant C70A,[51] along with or without Myc-tagged SKP2 plasmids as indicated for 24 h, and then treated with 10 μM MG132 for additional 6 h. The treated cells were subject to *in vivo* ubiquitination assays.[2,52] His-tagged pull-down assay was performed as previously described.[10] For *in vitro* ubiquitination assay, BL21 competent cells were transformed with pGEX-4X1, pGEX-4X1-TRAF6 and pGEX-4X1-TRAF6 C70A plasmids,[31] and induced with 0.1 mM IPTG for 3 h at 37 °C. Proteins were purified from *Escherichia coli* lysates with glutathione Sepharose 4B beads (GE healthcare, Pittsburgh, PA, USA) according to the manufacturer's instruction. *In vitro* ubiquitination reaction consisted of the following components: ~ 1 μg purified GST-TRAF6 or its mutant C70A, 2 μg recombinant Flag-EZH2 (Origene, Rockville, MD, USA), 100 μM HA-ubiquitin, 100 nM E1, 1 μM of Ubc13/UeV1a (Boston Biochem, Cambridge, MA, USA), in a volume of 50 μl reaction buffer (25 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT and 1 mM ATP). The reaction was run for 3 h at 37 °C. After that, the reaction volume was adjusted to 150 μl, dissociated by heating at 95 °C in 1% SDS(v/v), and diluted 1:10 with IP buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, protease inhibitor cocktail (Roche, Indianapolis, IN, USA)).[53] The diluted reactions were precleared with Protein A/G plus beads then immunoprecipitated with 1 μg mouse anti-Flag antibody and 20 μl Protein A/G plus beads overnight at 4 °C. After six times of washes with IP buffer, the beads were subject to western blotting analysis.

Immunofluorescence (IF) and immunohistochemistry (IHC)

IF staining on PC3 cells was performed with anti-TRAF6 (1:100, D-10, Santa Cruz) and anti-EZH2 (1:100, D2C9, Cell Signaling) antibodies.[2,45] IHC staining on randomly-selected mouse tissue sections was conducted as previously reported.[2] The antibodies used were: anti-Skp2 (1: 50, H-435, Santa Cruz), anti-EZH2 (1:200, 07-1583, Millipore), anti-H3K27me3 (1:2000, 07-499, Millipore), anti-Ki67 (1:200, 16667, Abcam). The immunostained slides were scanned by Leica SCN400 at Digital Histology Shared Resource in Vanderbilt University. The stained cells in annotated tumor regions were counted at × 20 magnification and analyzed by Tissue IA 2.0 analysis software.

Human prostate tissue microarray (TMA) slides were purchased from Biomax (PR483b, Rockville, MD, USA) consisting of 35 cancer cases and 5 normal cases in 80 cores. Antigen retrieval was performed for 20 min at 98 °C using Labvision PT module in 10 mM citrate buffer, pH 6.0 for EZH2, or 1 mM EDTA buffer, pH 8.0 for SKP2. Immunostaining was mechanically performed on the Labvision Autostainer using the Ultravision Quanto (HRP polymer) Detection System (Thermo Scientific, Waltham, MA, USA). Standard incubation times were used, except that the primary antibody was incubated for 60 min followed by a stringent 5 min wash in TBS containing 0.1% Tween20. Tissue sections were probed with anti-SKP2 (1:250, 2C8D9, Invitrogen) or anti-EZH2 (1:50, D2C9, Cell Signaling) in OP Quanto antibody diluent (Thermo Fisher, Nashville, TN, USA). The scores of SKP2 and EZH2 were blindly graded as: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) positivity according to the staining intensities.

Statistical analysis

Statistical analysis was conducted with two-tailed Student's *t*-test. Pearson correlation test and chi-square test were performed for correlation analysis. The values of $P<0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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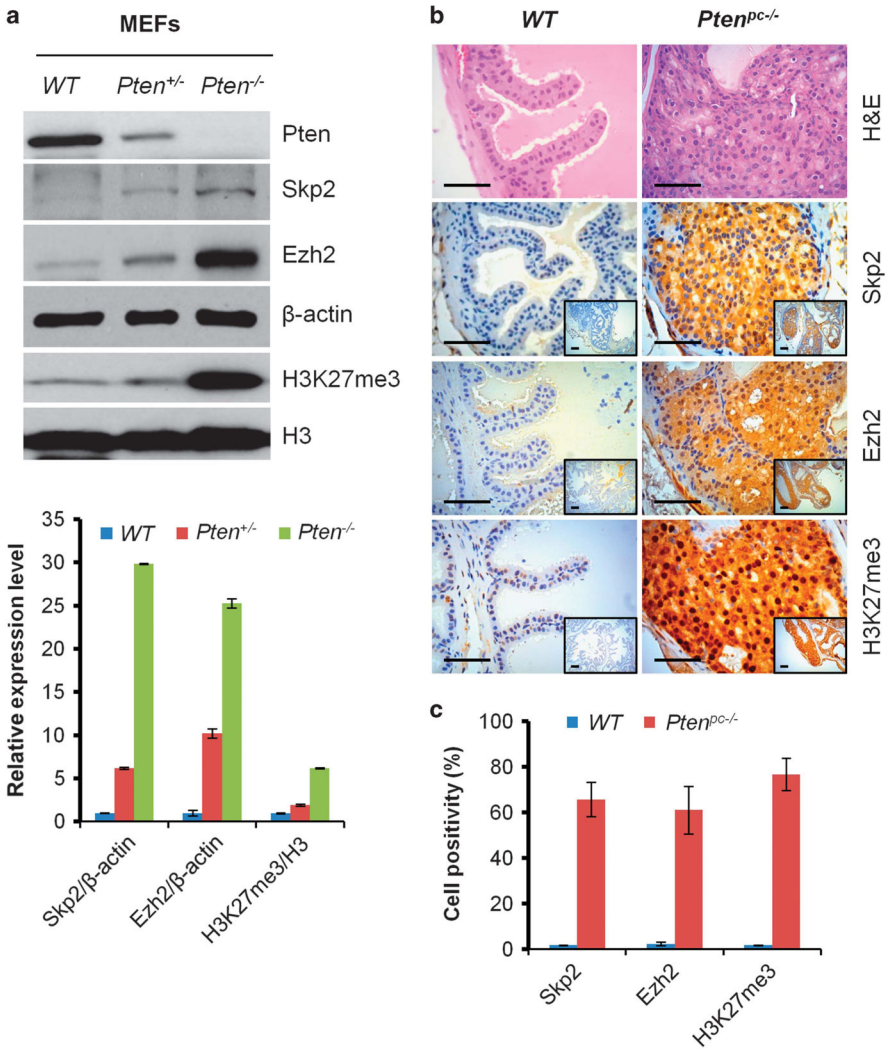

Figure 1.

Aberrant elevation of Skp2 and Ezh2 upon Pten loss *in vitro* in MEFs and *in vivo* in mouse tissues. (**a**) Top panel: Western blot analysis to show the increases of protein levels of Skp2, Ezh2 and H3K27me3 upon Pten loss in MEFs. Bottom panel: Quantification analysis of protein levels for Skp2, Ezh2 and H3K27me3 from (**a**). Error bars represent means ±s.d. (P<0.001). (**b**) Immunohistochemical (IHC) staining on the aberrant elevation of Skp2, Ezh2 and H3K27me3 in prostate tumors of *Pten* mutant (*Pten*$^{pc-/-}$) mice (n = 3) at 6 months of age. Prostate tissues of age-matched *WT* mice were used as the control. Hematoxylin and eosin (H&E) staining showed the morphology of prostate glands in mice, and scale bars represent 50 μm (inset 100 μm). (**c**) Quantification analysis of protein levels for Skp2, Ezh2 and H3K27me3 in prostate tumors of mice from (**b**).

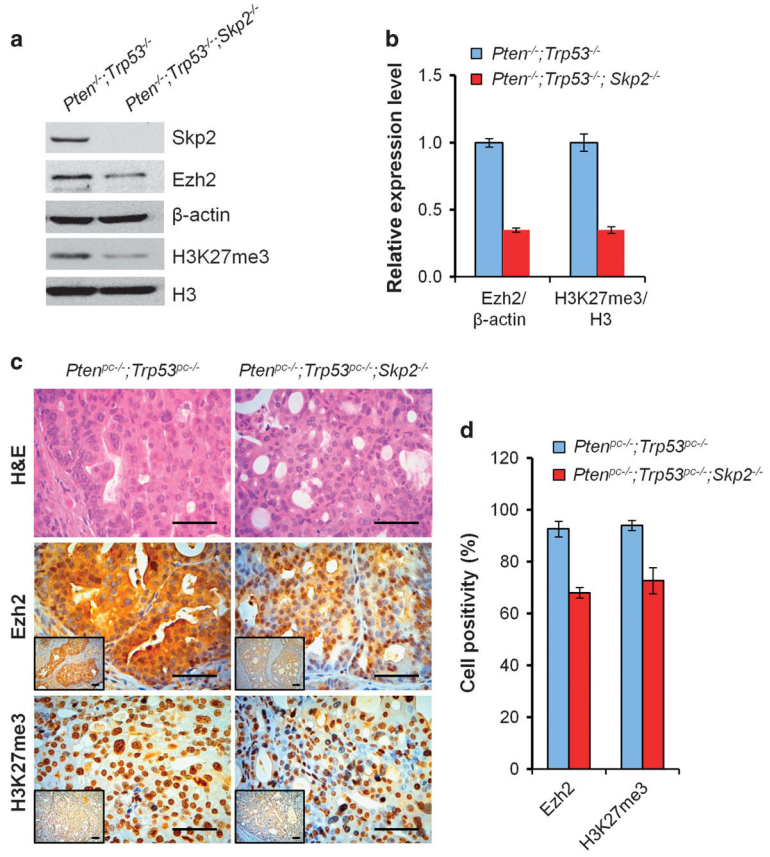

Figure 2.

Skp2 deficiency decreases the levels of Ezh2 and H3K27me3 *in vitro* and *in vivo*. (**a**) Western blot analysis of protein levels of Ezh2 and H3K27me3 in *Pten/Trp53* double-null and *Pten/Trp53/Skp2* triple-null MEFs. (**b**) Quantification analysis of protein levels for Ezh2 and H3K27me3 in MEFs upon Skp2 inactivation from (**a**). Error bars represent means ±s.d. (**c**) IHC staining of Ezh2 and H3K27me3 in prostate tumors of *Pten/ Trp53* and *Pten/Trp53/ Skp2* mutant mice ($n = 3$) at 4 months of age. Scale bars represent 50 μm (inset 100 μm). (**d**) Quantification analysis of tumor cells positive for Ezh2 and H3K27me3 from (**c**). Error bars represent means ±s.d. from three mice for each group.

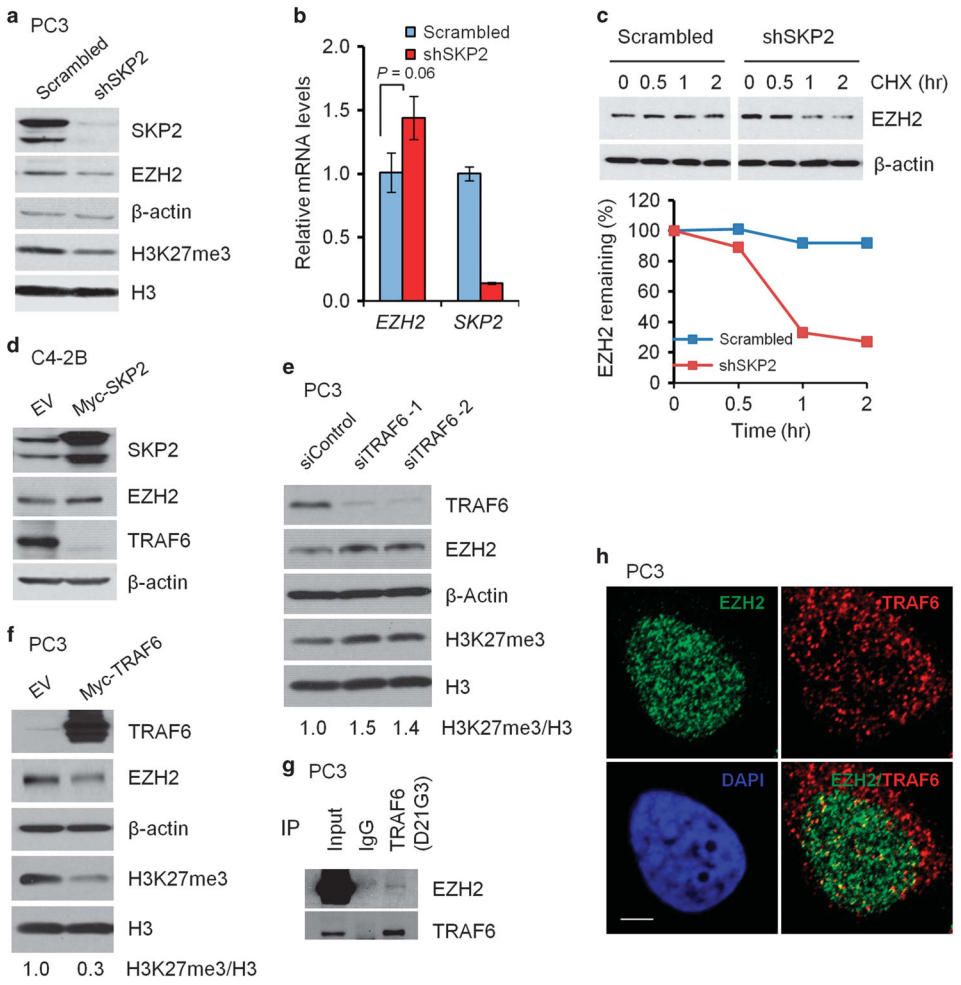

Figure 3.

EZH2 stability is co-regulated by SKP2 and TRAF6 in human prostate cancer cells. (**a**) Western blot analysis of EZH2 and H3K27me3 in PC3-scrambled and PC3-shSKP2 PCa cells. (**b**) Quantitative RT-qPCR analysis of the mRNA levels of *EZH2* in PC3 cells upon *SKP2* knockdown. Error bars represent means ±s.d. of triplicates. (**c**) *SKP2* knockdown shortens the half-life of EZH2. Top panel: Western blot analysis of the half-life of EZH2 upon *SKP2* knockdown in PC3 cells. Cells were treated with cycloheximide (CHX, 100 μg/ml), and then levels of EZH2 were detected at defined time points. Bottom panel: Quantification of EZH2 normalized to β-actin from the top panel. (**d**) *SKP2* overexpression inhibits TRAF6 but upregulates EZH2 in C4-2B cells. EV, Empty vector. (**e**) *TRAF6* knockdown upregulates EZH2 and H3K27me3 in PC3 cells. Two different sets of siTRAF6 oligos were used. (**f**) *TRAF6* overexpression decreases EZH2 and H3K27me3 in PC3 cells. (**g**) Co-immunoprecipitation analysis indicates a physical interaction between endogenous EZH2 and TRAF6 proteins in PC3 cells. (**h**) Immunofluorescence images show a co-localization of endogenous EZH2 and TRAF6 in PC3 cells. Scale bar represents 5 μm.

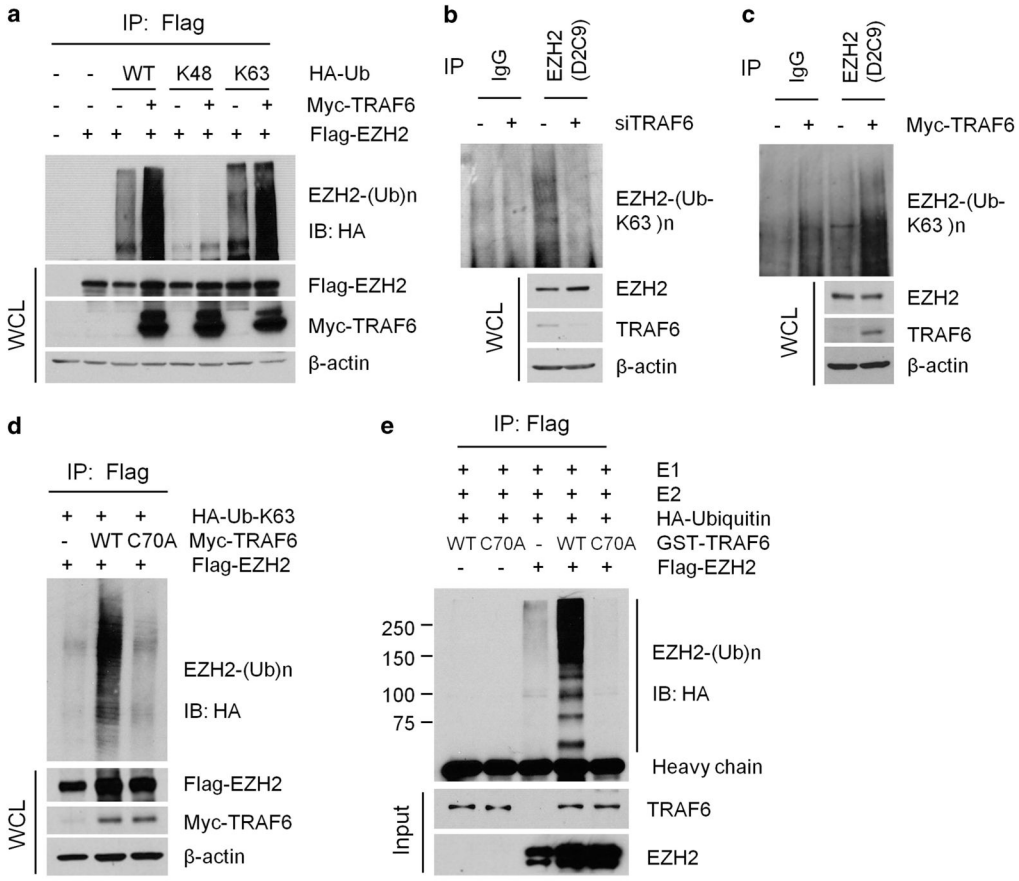

Figure 4.

TRAF6 is an E3 ubiquitin ligase for EZH2 ubiquitination through lysine 63-linkage. (**a**) *In vivo* ubiquitination assay showed that TRAF6 promotes the K63-linked polyubiquitination of EZH2 in HEK293T cells. Cells were transfected with Flag-EZH2, Myc-TRAF6, along with various HA-ubiquitin (HA-Ub) constructs. K48 and K63 represent HA-Ub-K48-only and HA-Ub-K63-only, respectively. WCL represents whole cell lysates. (**b**) Endogenous IP assay demonstrated that TRAF6 knockdown results in a reduction of EZH2 polyubiquitination in PC3 cells. (**c**) TRAF6 overexpression increases EZH2 polyubiquitination in C4-2B cells. (**d**) TRAF6 mutation at C70A dramatically decreases the TRAF6-mediated polyubiquitination of EZH2 *in vivo*. HEK293T cells were transfected with Flag-EZH2, HA-Ub-K63 only, along with TRAF6 WT or TRAF6 C70A (catalytically dead mutant). (**e**) TRAF6 mutation at C70A abolishes the TRAF6-mediated polyubiquitination of EZH2 *in vitro*. Flag-EZH2 proteins were incubated with adenosine triphosphate, HA-Ub, E1, and E2 (Ubc13/Uve1a), along with GST, GST-TRAF6, or GST-TRAF6-C70A proteins for *in vitro* ubiquitination of EZH2.

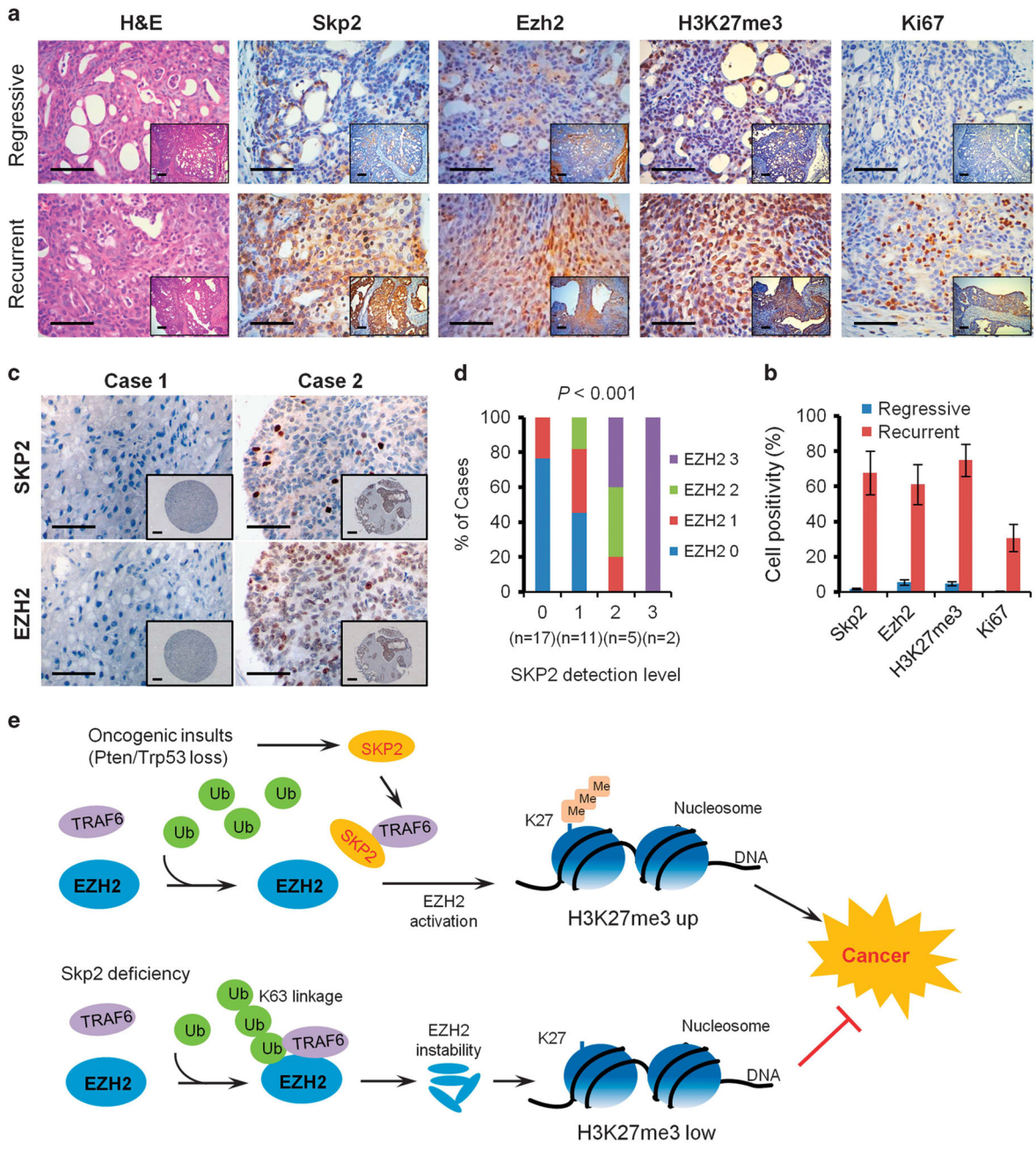

Figure 5.

Skp2 and Ezh2 are increased in recurrent prostate tumors of *Pten/Trp53* mice, and SKP2 is correlated with EZH2 in human prostate cancer. (**a**) IHC staining of Skp2, Ezh2, H3K27me3 and Ki67 in regressive and recurrent lesions of prostate tumors of castrated *Luc/Pten/Trp53* mutant mice. The regressive and recurrent tumor tissues were collected from mice at 3 weeks post castration, with the guidance of BLI as previously reported.[2] (**b**) Quantification analysis of protein levels for Skp2, Ezh2, H3K27me3 and Ki67 in prostate tumors from (**a**). (**c**) IHC staining on SKP2 and EZH2 in human PCa tissue microarray (TMA). Scale bars represent 50 μm (inset 100 μm) in (**a** and **c**). (**d**) Statistical analysis of the correlation

between SKP2 and EZH2 levels in PCa TMA. The percentages of EZH2 levels were calculated for each level of SKP2 protein in 35 cases of human PCa specimens. SKP2 and EZH2 levels were graded as 0, 1, 2 and 3 by intensity scores. EZH2 grades are color-coded, and numbers in parenthesis represent sample sizes. The statistical significance was determined by Chi-Square test (Supplementary Table S2). (**e**) A working model of SKP2, EZH2 and TRAF6 network on the epigenetic regulation of H3K27me3 in PCa. SKP2 stabilizes EZH2 by a sequestration of TRAF6-mediated ubiquitination for EZH2 degradation, and SKP2 deficiency results in a reduction of EZH2 and H3K27me3 to suppress PCa progression.