

XIAP RING domain mediates miR-4295 expression and subsequently inhibiting p63 α protein translation and promoting transformation of bladder epithelial cells

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Keywords: bladder cancer transformation, XIAP, RING domain, p63 α

Received: March 02, 2016

Accepted: June 13, 2016

Published: July 18, 2016

ABSTRACT

The X-linked inhibitor of apoptosis protein (XIAP) contains three N-terminal BIR domains that mediate anti-apoptosis and one C-terminal RING finger domain whose function(s) are not fully defined. Here we show that the RING domain of XIAP strongly inhibits the expression of p63 α , a known tumor suppressor. XIAP knockdown in urothelial cells or RING deletion in knockin mice markedly upregulates p63 α expression. This RING-mediated p63 α downregulation is critical for the malignant transformation of normal urothelial cells following EGF treatment. We further show that the RING domain promotes Sp1-mediated transcription of miR-4295 which targets the 3'UTR of p63 α mRNA and consequently inhibits p63 α translation. Our results reveal a previously unknown function of the RING of XIAP in promoting miR-4295 transcription, thereby reducing p63 α translation and enhancing urothelial transformation. Our data offer novel insights into the multifunctional effects of the XIAP RING domain on urothelial tumorigenesis and the potential for targeting this frequently overexpressed protein as a therapeutic alternative.

INTRODUCTION

The X-linked inhibitor of apoptosis protein (XIAP) is a member of the IAP family and a potent inhibitor of apoptosis [1, 2]. It has been reported that XIAP is upregulated in acute and chronic leukemia [3, 4], prostate cancer [5], breast cancer [6–8], and many other cancers [9–11], thus indicating an association between XIAP overexpression and cancer development [12, 13]. Our recent studies reveal several non-apoptosis-related functions of XIAP, including upregulation of Cyclin D1, which promotes bladder cancer cell growth [14] and promotion of F-actin formation and colon cancer cell invasion [15] *via* inhibition of SUMOylation of RhoGDI α (Rho GDP-dissociation inhibitor 1) at lys-138 [16]. Other investigators have reported the association of XIAP

overexpression with cancer progression, chemoresistance and poor prognosis in cancer patients [3, 9, 11, 17].

XIAP consists of four major structural domains, including three repeats of the baculovirus IAP repeat (BIR) domain at its NH₂ terminus and a RING finger domain near its COOH terminus [18]. The BIR domains inhibit caspase 3, 7 and 9, thereby antagonizing apoptosis, while the RING domain exerts E3 ubiquitin ligase activity, enabling IAPs to ubiquitinate themselves, caspase-3, and caspase-7 *via* the proteasome [19–21]. More recently, we found that the BIR domains of XIAP can bind directly to E2F1 (E2F transcription factor 1) and increases its transactivation [22]. In contrast, the biological function and molecular mechanisms underlying the RING domain of XIAP are not well understood. We have demonstrated that the RING domain participates in the inhibition of

RhoGDI α SUMOylation at lys-138, in turn suppressing F-actin formation and human colon cancer invasion [16]. In the current study, we show a novel function and mechanism of the action of the RING domain in the downregulation of tumor suppressor p63 α protein expression where XIAP promotes the malignant transformation of urothelial cells.

The p63 protein is a member of the p53 family of transcription factors that has been shown to be important in the development of epithelial tissues. It has been shown that p63-deficient mice have several developmental defects, such as the lack of limbs, teeth and mammary glands [23]. p63 gene encodes two major isoforms by alternative promoters: TAp63 and Δ Np63, with different transcription abilities [24]. TAp63 contains a transactivation domain (TAD) and can initiate transcription of p53-regulated genes, such as p21, bax, mdm2, and other unique targets [25], whereas Δ Np63 lacks the transactivation domain (TAD) [24]. It has been reported that loss of p63 results in spontaneous tumor formation, although the mechanism underlying the tumorigenesis is not yet fully understood [26]. The p63 α is the longest TA transcript variant of p63, and has been characterized as a tumor suppressor responsible for preventing cancer development [27–31]. However, most of the existing studies focused on p63 α -regulated downstream effectors and much less is known about the upstream regulators of p63 α . It was this lack of knowledge regarding the upstream regulators of p63 α that motivated us to carry out the present studies. Our explorations led us to discover that XIAP could inhibit p63 α protein translation *via* its RING domain-initiated miR-4295 expression.

RESULTS

XIAP inhibited p63 α protein expression specifically via its RING domain in bladder epithelial cells both *in vitro* and *in vivo*

XIAP is a well-known anti-apoptosis protein with three repeat BIR domains in the N terminal and one RING domain in the C terminal as shown in Figure 1A. Our most recent studies show that RING domain of XIAP is crucial for XIAP upregulation of Cyclin D1 expression [14], while BIR domains specifically mediate E2F1 transactivation and Cyclin E expression in human colon cancer HCT116 cells [22]. We have also demonstrated that XIAP inhibits RhoGDI α SUMOylation through the RING domain using the same experimental system [16]. To evaluate the potential role of XIAP in human bladder carcinogenesis, we transfected shRNA specific targeting human XIAP into immortalized normal human bladder epithelial UROtsa cells and established the stable transfectant, UROtsa(shXIAP). As shown in Figure 1B, knockdown of XIAP resulted in profound upregulation of p63 α protein expression, while it exhibited a reduction of Cyclin D1 expression, but no effect on RhoGDI α expression. These

results reveal a novel function of XIAP in the suppression of p63 α expression in addition to its positive regulatory effect on Cyclin D1 expression in human bladder epithelial cells. To identify the specific functional domain that are responsible for XIAP suppression of p63 α , we stably transfected HA- Δ BIR and HA- Δ RING expression constructs into UROtsa cells, respectively, to establish two stable transfectants, UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING). As shown in Figure 1B, ectopic expression of HA- Δ BIR consistently inhibited p63 α protein expression in UROtsa with upregulation of Cyclin D1 expression, whereas overexpression of HA- Δ RING did not show these biological effects in comparison to vector control transfectant, UROtsa(Vector) under same experimental conditions (Figure 1C). The inhibitory effect of XIAP RING domain on p63 α expression was greatly supported by the results obtained from XIAP Δ RING domain knockin mice, showing that p63 α expression in the bladder epithelial cells collected from XIAP Δ RING knockin mice exhibited much higher than that observed in XIAP-WT mice (Figure 1D). This finding of p63 α upregulation in XIAP Δ RING knockin mice was extended to be tested *in vivo* bladder tissues from both types of mice with immunohistochemistry (IHC) staining (Figure 1E & 1F). Taken together, our results clearly demonstrate that RING domain of XIAP provides an inhibitory effect on p63 α protein expression in bladder epithelial cells both *in vitro* and *in vivo*.

p63 α upregulation was crucial for XIAP RING-mediated malignant transformation of human bladder epithelial cells

Epidermal growth factor (EGF) is a well-known tumor promoter that has been widely used to induce cell transformation [32–35]. Epidermal growth factor receptor (EGFR) has been reported to be highly expressed in Bladder cancers [36]. UROtsa is a normal bladder epithelial cell line [37]. Upon exposure to bladder carcinogens, such as arsenate or cadmium, UROtsa is transformed to a malignant status, and expresses a high level of epidermal growth factor (EGF) [38]. Thus, it is thought that EGF and its receptor play an important role in bladder cancer development. To evaluate the potential role of the XIAP RING domain in malignant transformation of UROtsa cells, we exposed UROtsa cells to EGF and malignant transformative capability was evaluated by testing their anchorage-independent growth ability in soft agar. The results showed that EGF treatment did induced UROtsa cell growth in soft agar, whereas knockdown of XIAP led to almost complete abolishment of this EGF-induced malignant transformation (Figure 2A & 2B). We next tested whether the specific structural domain of XIAP was responsible for malignant transformation following EGF treatment. As shown in Figure 2C-2D, ectopic expression of HA-

Δ BIR domain alone resulted in significantly increased UROtsa cell growth in soft agar, and remarkably promoted EGF-induced malignant transformation in comparison to those in UROtsa(Vector) transfectants, revealing that RING domain of XIAP exhibited strong promotion effects on both the basal level and the EGF-induced level of UROtsa cell transformation. Very interestingly, overexpression of HA- Δ RING of XIAP did not show any increase in either basal level or EGF-induced level

of UROtsa cell growth in soft agar. Our results revealed that the RING domain, but not the BIR domain, mediates XIAP promoting UROtsa cell anchorage-independent growth accompanied with inhibition of p63 α protein expression. It was very surprising to note that knockdown of XIAP or overexpression of HA- Δ BIR domain did not show any observable effect on UROtsa cell monolayer growth although both show regulatory effects on Cyclin D1 expression in UROtsa cells (Figure 2E & 2F). It was

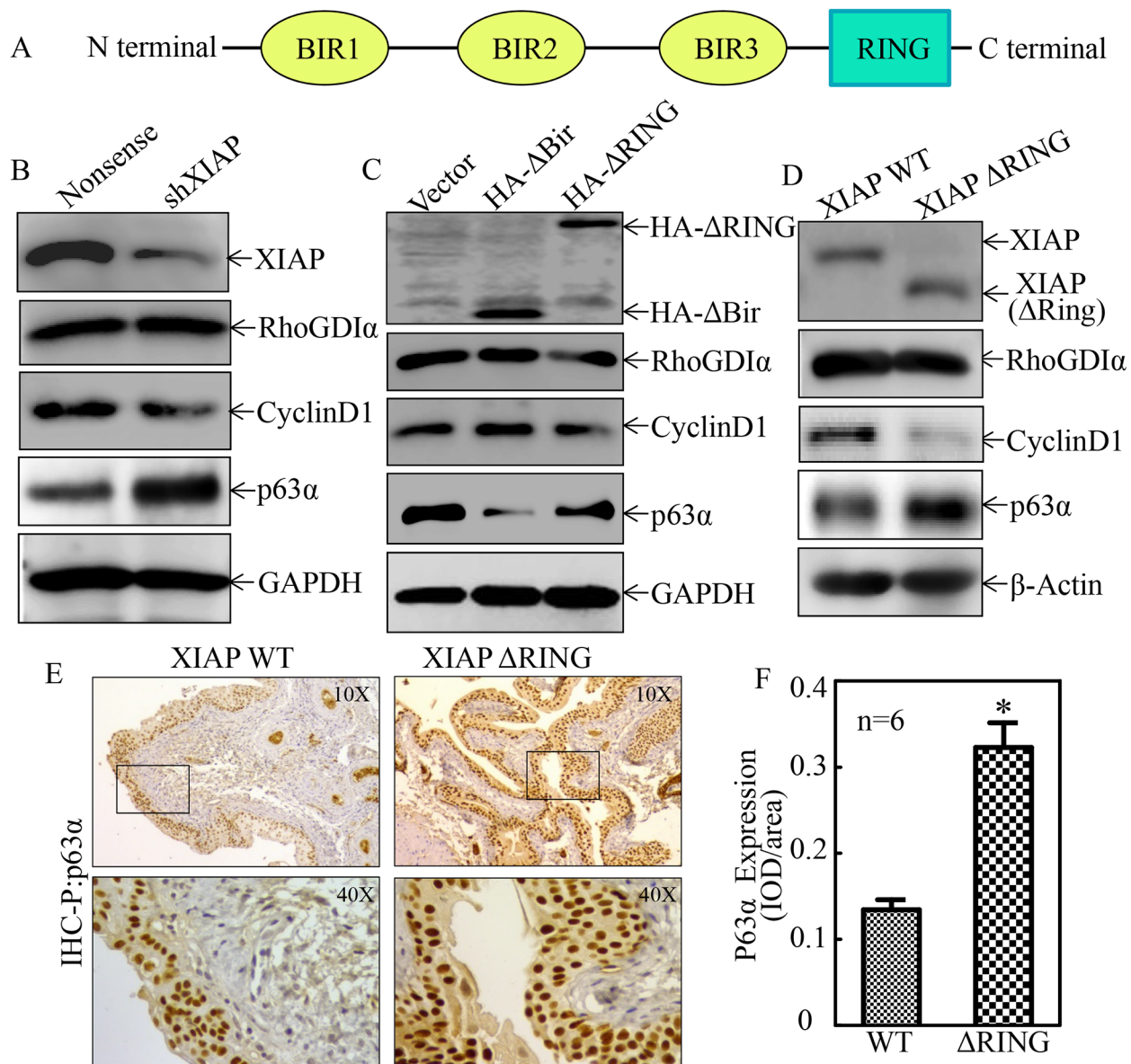


Figure 1: XIAP inhibited p63 α expression *in vitro* and *in vivo* through its RING domain. A. Schematic representation of XIAP protein and identified function of each domain; B. and C. The indicated cell extracts were subjected to Western blot for determination of expression of XIAP, RhoGDI α , CyclinD1 and p63 α . GAPDH was used as protein loading controls; D. Protein extracts of mouse primary bladder epithelial cells collected from either WT-XIAP mice or XIAP- Δ RING knockin mice were subjected to Western blot for determination of expression of XIAP, RhoGDI α , CyclinD1 and p63 α . β -Actin was used as protein loading controls; E. and F. IHC-P was carried out to evaluate p63 α expression in mouse bladder epithelial cells obtained from WT-XIAP mice and XIAP Δ RING mice. The optical density was analyzed as described in “materials and methods”. The symbol (*) indicates a significant increase in comparison to that of WT-XIAP mice ($P < 0.05$).

even more surprising to observe that introduction of the HA- Δ RING domain completely blocked EGF-induced cell transformation of UROtsa cells and profoundly inhibited monolayer growth of UROtsa cells accompanied by slight reduction of Cyclin D1 expression (Figures 1B, 2C, 2D & 2F). These results suggest that Cyclin D1 might not be a major player in RING domain-regulated cell transformation in EGF-treated UROtsa cells. We anticipate that XIAP RING domain-inhibited p63 protein expression might be associated with XIAP promotion of EGF-induced

malignant transformation of UROtsa cells. To test this notion, shRNA specific targeting p63 α (shp63 α) was stably transfected into UROtsa cells, and the stable transfectant UROtsa(shp63 α) and its vector control transfectant UROtsa(Nonsense) were established and identified as shown in Figure 3A. The knockdown of p63 α led to an increase in EGF-induced malignant transformation of UROtsa cells as compared with that observed in UROtsa(Nonsense) (Figure 3B & 3C), suggesting that p63 α does provide an inhibitory effect on malignant

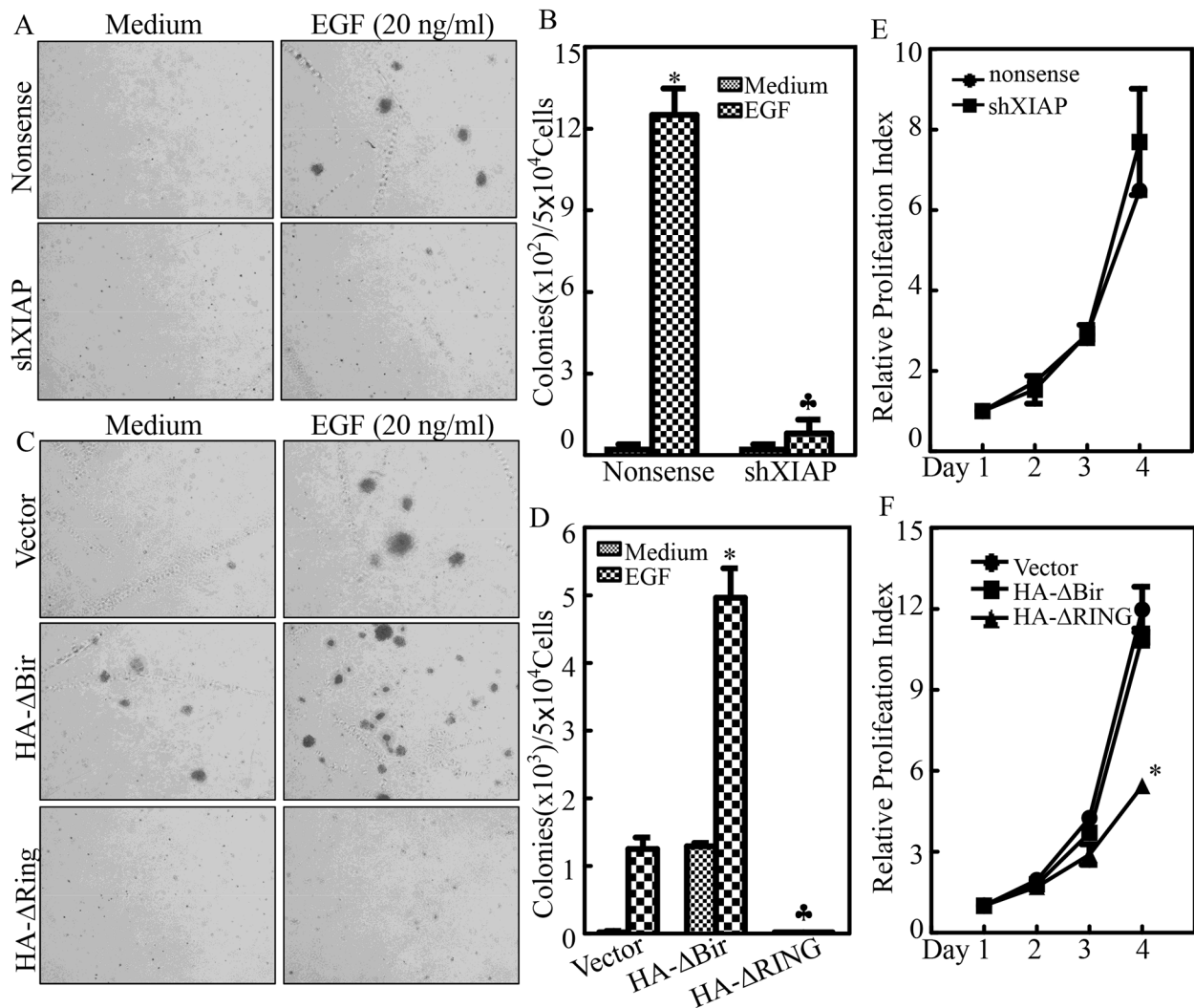


Figure 2: XIAP RING domain upregulated EGF induced bladder cell transformation. A. and B. UROtsa(shXIAP) and UROtsa(Nonsense) cells were subjected to anchorage-independent assay in the presence or absence of EGF as indicated using the protocol described in the section of “Materials and Methods”. Representative images of colonies of UROtsa(shXIAP) and UROtsa(Nonsense) cells were captured under microscopy following 3 week incubation period; (A) the number of colonies was counted under microscopy and the results were presented as colonies per 50,000 cells from three independent experiments. The asterisk (*) indicates a significant increase in comparison to medium control and the symbol (♣) indicates a significant inhibition as compared with UROtsa(Nonsense) cells ($p < 0.05$) (B). C. and D. UROtsa(Vector), UROtsa(HA- Δ BIR) or UROtsa(HA- Δ RING) cells were subjected to anchorage-independent assay in the presence or absence of EGF as indicated. Representative images of colonies of indicated cells were captured under microscopy following 3 week incubation period (C); the number of colonies was counted under microscopy and the results were presented as colonies per 50,000 cells from three independent experiments. The asterisk (*) indicates a significant increase as compared with UROtsa(Vector) cells, while the asterisk (♣) indicates a significant decrease as compared with UROtsa(Vector) cells ($p < 0.05$) (D). E. and F. ATP assay to determine proliferation of cells was performed as indicated. The asterisk (*) indicates a significant inhibition as compared with UROtsa(Vector) cells.

transformation induced by EGF in UROtsa cells. This notion was greatly supported by the results observed in p63 α overexpressed UROtsa cells. The results showed that ectopic expression of p63 α -FLAG significantly reversed HA- Δ BIR-mediated promotion of transformation in UROtsa(HA- Δ BIR/p63 α -FLAG) cells following EGF treatment in comparison to that in UROtsa(HA- Δ BIR/Vector) under the same experimental conditions (Figure 3D-3F). It was noted that overexpression of p63 α -FLAG did not show any effect on expression of either endogenous XIAP or exogenous HA- Δ BIR, excluding the possibility of XIAP as p63 α downstream effector. Taken together, these results demonstrate that p63 α downregulation plays

a key role in XIAP RING domain promoting malignant transformation of UROtsa cells induced by EGF treatment.

RING domain of XIAP inhibited p63 α protein translation in human bladder UROtsa cells

Our above results indicate that the XIAP RING domain inhibits p63 α protein expression, which plays a critical role in RING domain promoting bladder cell malignant transformation. To elucidate the molecular mechanisms underlying the RING domain regulation of p63 α expression, we first compared the mRNA levels of p63 α in UROtsa(shXIAP) and UROtsa(HA- Δ BIR), with

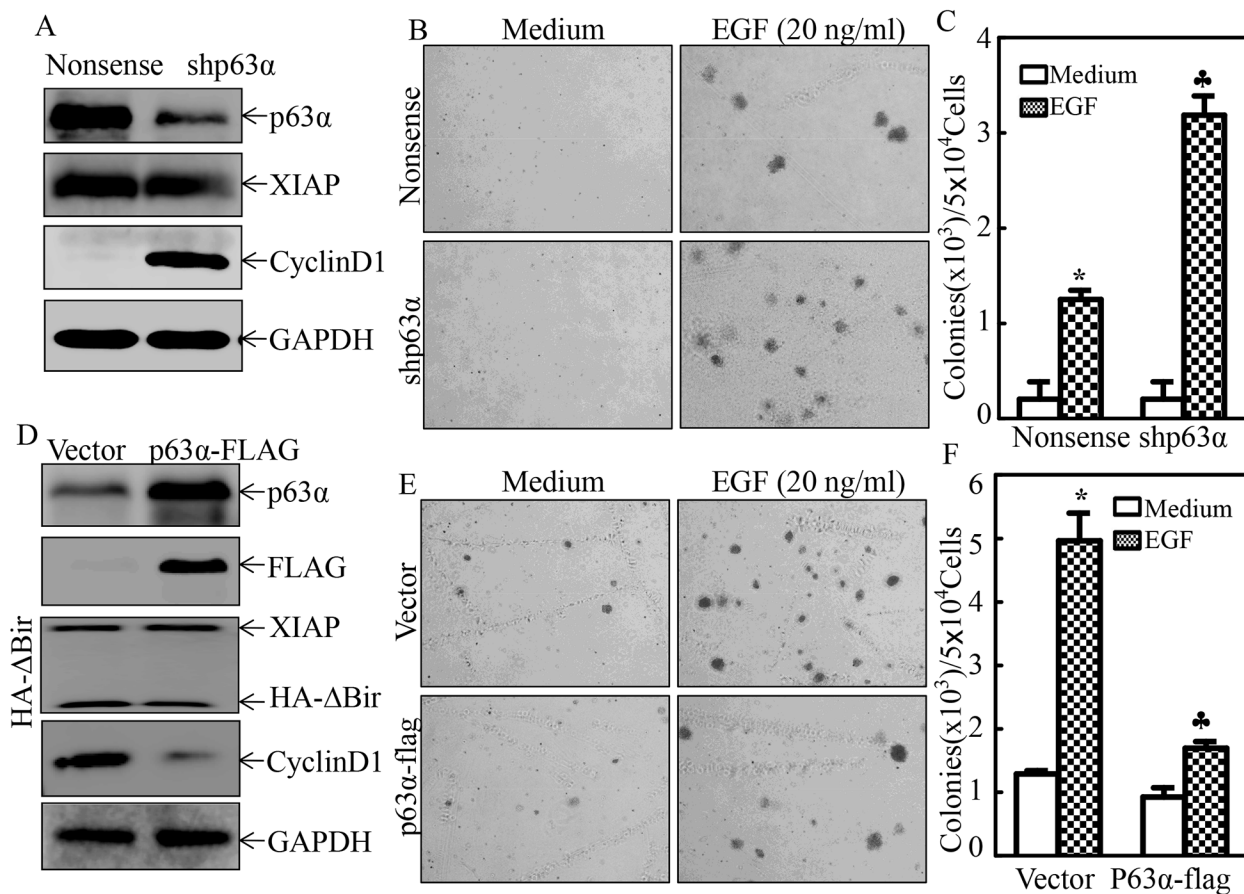


Figure 3: p63 α inhibited EGF-induced malignant transformation of human bladder epithelial cells. **A.** The cell extracts of UROtsa(Nonsense) and UROtsa(shp63 α) transfectants were subjected to Western blot as indicated for identification of knockdown efficiency of shRNA targeting p63 α . **B.** and **C.** UROtsa(Nonsense) and UROtsa(shp63 α) transfectants were subjected to anchorage- independent growth in the presence or absence of EGF as indicated. Representative images of the colonies of indicated cells were captured under microscopy following 3 week incubation period (**B**); the number of colonies was counted under microscopy and the results were presented as colonies per 50,000 cells from three independent experiments. The asterisk (*) indicates a significant increase as compared with medium control, and the symbol (\clubsuit) indicates a significant increase in comparison to UROtsa(Nonsense) cells ($p < 0.05$) (**C**). **D.** Western blot was used to identify the stable overexpression level of p63 α -Flag in UROtsa(HA- Δ BIR) cells in comparison to UROtsa(HA- Δ BIR/Vector) control cells; **E.** and **F.** UROtsa(HA- Δ BIR/Vector) and UROtsa(HA- Δ BIR/p63 α -FLAG) cells were subjected to anchorage-independent growth in the presence or absence of EGF as indicated. Representative images of the colonies of indicated cells were captured under microscopy following 3 week incubation period (**E**); the number of colonies was counted under microscopy and the results were presented as colonies per 50,000 cells from three independent experiments. The asterisk (*) indicates a significant increase as compared with the medium control, and the symbol (\clubsuit) indicates a significant inhibition in comparison to UROtsa(HA- Δ BIR/Vector) cells ($p < 0.05$) (**F**).

their vector control transfectants, UROtsa(Nonsense), and UROtsa(HA-ΔBIR), respectively. As shown in Figure 4A, either knockdown of XIAP or overexpression of HA-ΔBIR did not show any observable alteration of p63α mRNA expression, excluding the possibility of XIAP regulation of p63α transcription and mRNA stability in UROtsa cells. Our confidence in this finding was strengthened by the results which showed the comparable p63α mRNA expression levels between mouse bladder epithelial cells from XIAPΔRING knockin mice and WT mice (Figure 1A). Thus, we next tested to see if XIAP and its RING domain could modulate p63α protein degradation. In presence of a new protein synthesis inhibitor Cycloheximide (CHX), p63α degradation rates were evaluated between UROtsa(shXIAP) and

UROtsa(Nonsense). The results showed that p63α protein degradation in UROtsa(shXIAP) cells was even greater than degradation in UROtsa(Nonsense) cells (Figure 4B). Consistent with this finding, overexpression of HA-ΔBIR profoundly delayed endogenous p63α protein degradation in UROtsa cells (Figure 4C), while overexpression of HA-ΔBIR in UMUC3 did not affect exogenous p63α-FLAG protein expression (Figure 4B). Collectively, our results exclude the possibility that XIAP and its RING domain regulate p63α at the protein degradation level. Therefore, we next tested XIAP regulation of p63α at the protein translation level. To this end, short term protein synthesis pulse-labeling assay was performed to compare p63α protein translational rates between UROtsa(Nonsense) and UROtsa(shXIAP) cells. As shown in Figure 4E, the

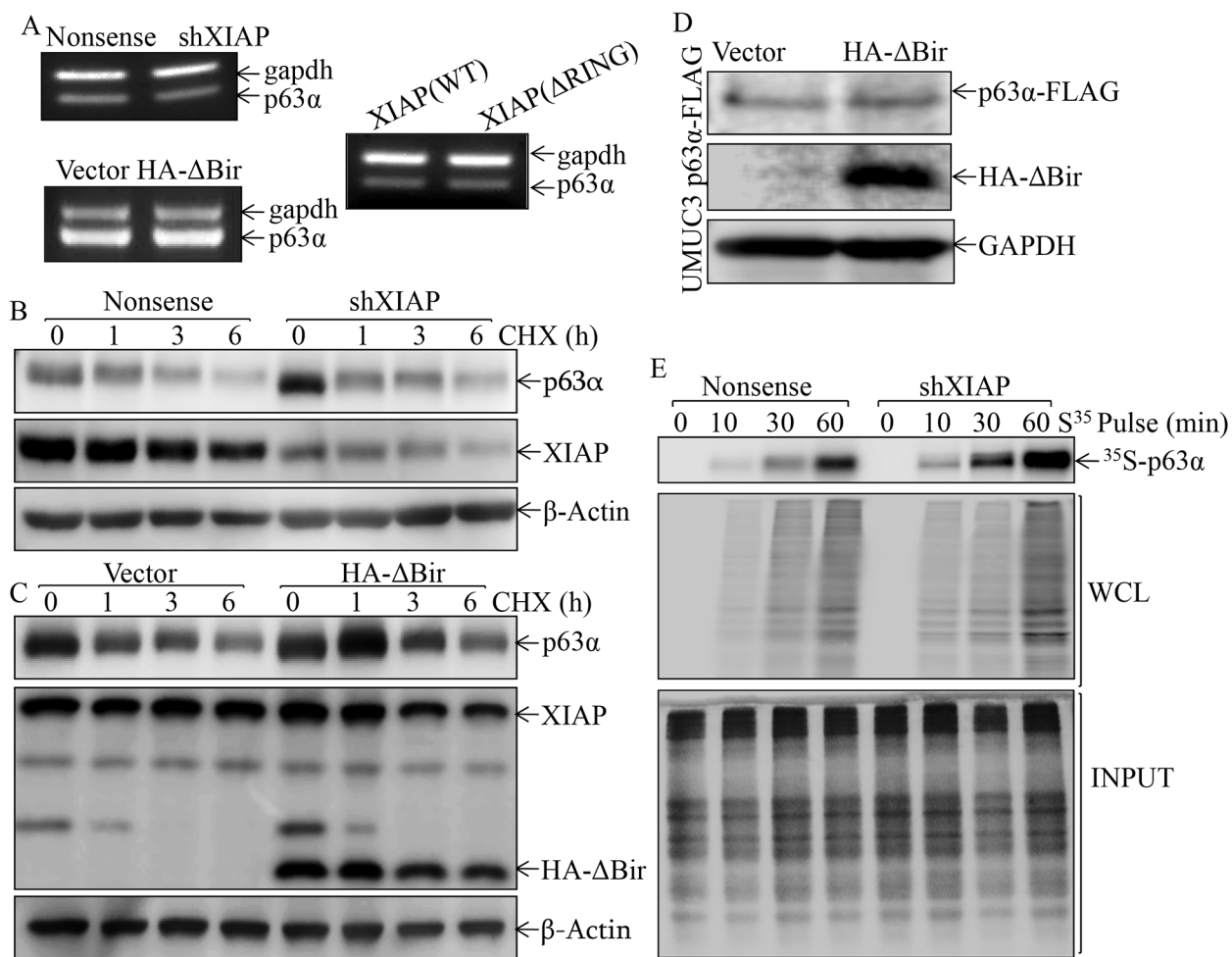


Figure 4: XIAP RING domain inhibited p63α protein translation in human bladder epithelial cells. **A.** Total RNA was extracted from UROtsa transfectants or mouse primary bladder epithelial cells as indicated, then subjected to RT-PCR for evaluation of p63α mRNA expression. GAPDH was used as a loading control; **B.** and **C.** UROtsa(Nonsense) vs. UROtsa(shXIAP) cells (**B.**) or UROtsa(Vector) vs. UROtsa(HA-ΔBIR) cells (**C.**), were treated with 50 μg/ml cycloheximide (CHX) for the indicated times. The cell extracts were then subjected to Western Blot analyses of p63α protein degradation rates among the indicated cells. β-Actin was used as protein loading control. **D.** The cell extracts obtained from UMUC3(p63α-Flag/Vector) and UMUC3(p63α-Flag/HA-ΔBIR) cells were subjected to Western Blot to evaluate the effect of ectopic HA-ΔBIR expression on p63α-FLAG expression. **E.** Newly synthesized p63α protein in UROtsa(Nonsense) and UROtsa(shXIAP) cells was monitored by pulse assay using ³⁵S-labeled methionine/cysteine as described in the section of “Materials and Methods”, WCL stands for whole cell lysate. Coomassie blue staining was used for protein loading control.

incorporation rate of ^{35}S -methionine/cysteine into newly synthesized p63 α protein was gradually increased along with the incubation time periods in UROtsa(shXIAP) cells in comparison to those in UROtsa(Nonsense) cells, demonstrating that XIAP plays a crucial role in p63 α protein translation in human bladder epithelial cells.

miR-4295 expression mediated RING domain inhibition of p63 α protein translation

The 3-terminal untranslated region of mRNA, named 3'UTR has been reported to be involved in the regulation of its mRNA translatability [39]. To

determine whether 3'UTR of p63 α mRNA is involved in XIAP regulating p63 α protein translation, we constructed p63 α 3'UTR luciferase reporter, which was further stably transfected into two paired transfectants, UROtsa(Nonsense) vs. UROtsa(shXIAP) cells, or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING) cells. As shown in Figure 5A, knockdown of XIAP significantly promoted p63 α 3'UTR activity, whereas ectopic expression of HA- Δ BIR, but not HA- Δ RING, dramatically inhibited p63 α 3'UTR activity (Figure 5B), suggesting that the RING domain, but not the BIR domain, inhibits p63 α protein translation by targeting 3'UTR of p63 α mRNA. The microRNA, a

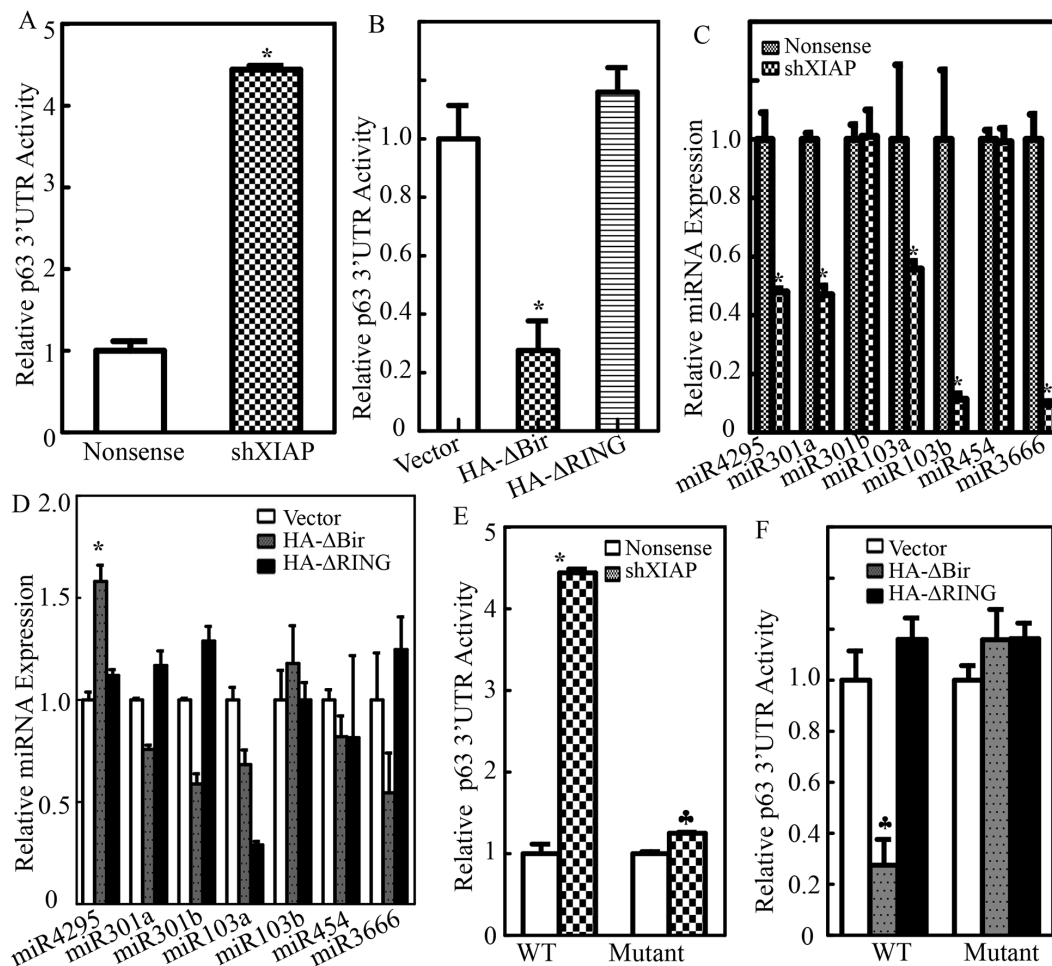


Figure 5: XIAP RING domain initiated miR-4295 expression and in turn inhibited p63 α mRNA 3'UTR activation. **A.** and **B.** Wild-type p63 α 3'UTR luciferase reporters were co-transfected together with pRL-TK into UROtsa(Nonsense) vs. UROtsa(shXIAP) cells (**A**), or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING) cells (**B**), respectively. Twenty-four hours post transfection, the transfectants were extracted to evaluate the luciferase activity. TK was used as internal control. The results were presented as p63 α 3'UTR activity relative to control vector transfectant, and each bar indicates a mean \pm SD from three independent experiments. The symbol (*) indicates a significant difference ($P < 0.05$); **C.** and **D.** The expression levels of miR-4295, miR-301a, miR-301b, miR-103a, miR-103b, miR-454 and miR-3666 were evaluated by real-time PCR as indicated. The results were normalized to U6; **E.** and **F.** UROtsa(Nonsense) vs. UROtsa(shXIAP) cells (**E**), or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING) cells (**F**), were transfected with either p63 α 3'UTR luciferase reporter or mutant of p63 α 3'UTR luciferase reporter that has an miR-4295 binding site mutation, Twenty-four hours post transfection, the transfectants were extracted to evaluate the luciferase activity. TK was used as internal control. The results were presented as p63 α 3'UTR activity relative to control vector transfectant. Each bar indicates a mean \pm SD from three independent experiments. The symbol (*) shows a significant increase and the symbol (♣) indicates a significant inhibition ($P < 0.05$).

Table 1: The potential miRNAs binding sites in the p63 α mRNA 3'UTR region

p63 α :	5' ..(406) UGAAAGAAAAUUGAGUUGCACUU (429)...
hsa- miR-4295:	3' UCCUUUUGUAACGUGAC
p63 α :	5' ..(406) UGAAAGAAAAUUGAGUUGCACUU (429)...
hsa-miR-301a:	3' CGAAACUUAUGUAACGUGAC
p63 α :	5' ... (406) UGAAAGAAAAUUGAGUUGCACUU (429)...
hsa- miR-301b:	3' CGAAACUGUUAUAGUAACGUGAC
p63 α :	5' ... (406) UGAAAGAAAAUUGAGUUGCACUU (429)...
hsa- miR-103a:	3' UACGGGAAAAUUGUAACGUGAC
p63 α :	5' ... (406) UGAAAGAAAAUUGAGUUGCACUU (429)...
hsa- miR-103b:	3' UACGGGAAAAUUGUAACGUGAC
p63 α :	5' ... (406) UGAAAGAAAAUUGAGUUGCACUU (429)...
hsa- miR-454:	3' UGGGAUAUCGUUAUACGUGAU
p63 α :	5' ... (406) UGAAAGAAAAUUGAGUUGCACUU (429)...
hsa- miR-3666:	3' AGCCGUGAUGUGAACGUGAC

class of ~22-nucleotide noncoding small RNAs, has been reported to be able to bind to the 3'UTR of target mRNA and inhibits its protein translation. Therefore, we used online Targetscan 6.2 to analyze the potential miRNAs that could bind to 3'UTR of p63 α mRNA. The result was shown in Table 1, and there were multiple potential miRNA binding sites, such as miR-4295, miR-301a, miR-301b, miR-103a, miR-103b, miR-454 and miR-3666, in 3'UTR of p63 α mRNA. We next evaluated and compared these miRNAs expression levels in two paired transfectants, UROtsa(Nonsense) vs. UROtsa(shXIAP) cells, or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING) cells. The results obtained from the quantitative real-time PCR assay showed that only miR-4295 expression was consistent with its anticipated regulation of p63 α mRNA 3'UTR activity in two paired transfectants, UROtsa(Nonsense) vs. UROtsa(shXIAP) cells, or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING) cells, showing that miR-4295 expression was profoundly inhibited in UROtsa(shXIAP) cells and was significantly upregulated in UROtsa(HA- Δ BIR) cells (Figure 5C & 5D). To provide direct evidence demonstrating the crucial role of miR-4295 binding site in p63 α mRNA 3'UTR activation, we did point mutation of miR-4295 binding site in p63 α 3'UTR luciferase reporter and both the WT-p63 α 3'UTR luciferase reporter and its mutant were then transfected into two paired transfectants, UROtsa(Nonsense) vs. UROtsa(shXIAP) cells or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and

UROtsa(HA- Δ RING) cells, respectively. The results showed that point mutation of miR-4295 binding site in p63 α 3'UTR-luciferase reporter completely abolished the promotion of p63 α 3'UTR activity by XIAP knockdown and also reversed the inhibition of p63 α 3'UTR activity upon HA- Δ BIR overexpression (Figure 5E & 5F). These results demonstrate that miR-4295 expression mediates the RING domain inhibition of p63 α protein translation by directly binding to and targeting p63 α mRNA 3'UTR in UROtsa cells.

To evaluate the biological effect of miR-4295 on p63 α mRNA 3'UTR activation, protein expression and malignant cell transformation of human bladder epithelial cells, we constructed a miR-4295 overexpression plasmid, and the construct was then stably transfected into UROtsa cells together with p63 α mRNA 3'UTR luciferase reporter. The overexpression of miR-4295 in UROtsa cells was identified by quantitative real-time PCR, showing over 12-fold expression of miR-4295 in comparison to vector control transfectant (Figure 6A). Ectopic expression of miR-4295 resulted in a significant inhibition of p63 α 3'UTR activity (Figure 6B); this inhibition of p63 α 3'UTR activity by miR-4295 was not observed in the transfectant of p63 α 3'UTR luciferase reporter with miR-4295 binding site mutant (Figure 6C), revealing that miR-4295 inhibition of p63 α 3'UTR activity depends on miR-4295 binding site of p63 α 3'UTR luciferase reporter. Consistently, overexpression of miR-4295 impaired

p63 α protein expression (Figure 6D) accompanied with the promoting of EGF-induced malignant transformation of human epithelial UROtsa cells (Figure 6E & 6F). Collectively, our results demonstrate that RING domain of XIAP is able to initiate miR-4295 expression, which subsequently binds to p63 α mRNA 3'UTR, and inhibits p63 α protein translation, in turn promoting EGF-induced malignant transformation of UROtsa cells.

RING domain of XIAP promoted miR-4295 transcription by upregulating Sp1 protein expression and transcriptional activation

To explore the mechanisms underlying RING domain regulation of miR-4295 expression, the host gene of miR-4295 was identified by searching NCBI. The results showed that the precursor of miR-4295 was encoded by

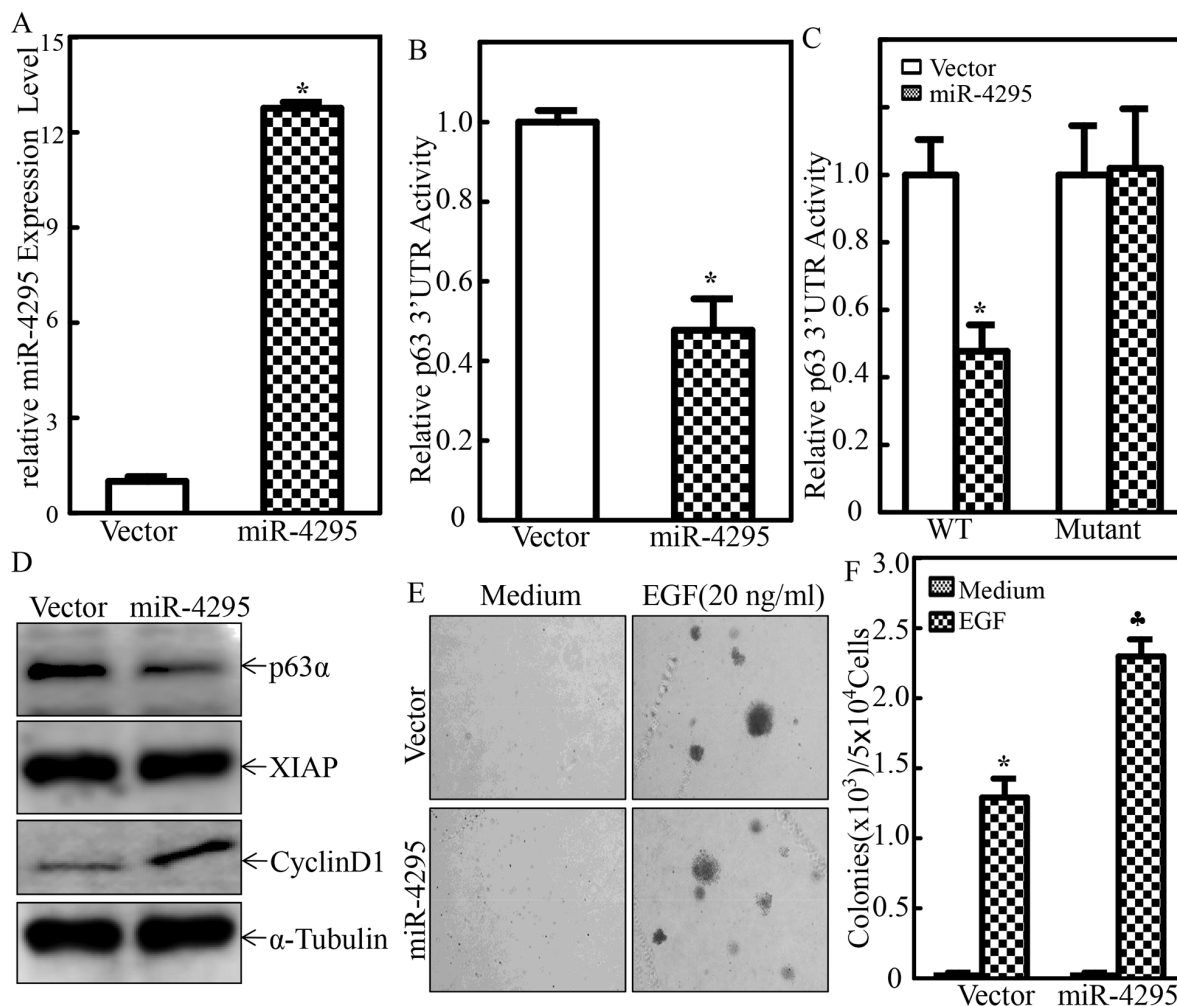


Figure 6: miR-4295 was able to directly bind to p63 α 3'UTR resulting in blockage of p63 α protein translation and subsequently promoting EGF-induced malignant transformation of human bladder epithelial cells. **A.** Real-time PCR was used to identify the expression level of miR-4295 in UROtsa cells; **B.** p63 α 3'UTR luciferase reporters were co-transfected together with pRL-TK into UROtsa(Vector) and UROtsa(miR-4295) cells, respectively. Twenty-four hours post transfection, the transfectants were extracted for measurement of the luciferase activity, while TK was used as internal control. The results were presented as p63 α 3'UTR activity relative to vector control transfectant, and each bar indicates mean \pm SD from three independent experiments. The symbol (*) shows a significant difference ($P < 0.05$); **C.** UROtsa(Vector) and UROtsa(miR-4295) were transfected with either p63 α 3'UTR luciferase reporter or mutant of p63 α 3'UTR luciferase reporter that has miR-4295 binding site mutation, Twenty-four hours post transfection, the transfectants were extracted to evaluate the luciferase activity, with TK used as an internal control. The results were presented as p63 α 3'UTR activity relative to control vector transfectant. Each bar indicates mean \pm SD from three independent experiments. The symbol (*) shows a significant difference ($P < 0.05$); **D.** The cell extracts obtained from UROtsa(Vector) and UROtsa(miR-4295) cells were subjected to Western blot to determine expression of p63 α and XIAP, while α -Tubulin was used as a protein loading control; **E.** and **F.** UROtsa(Vector) and UROtsa(miR-4295) transfectants were subjected to anchorage-independent growth in the presence or absence of EGF as indicated. Representative images of the colonies of indicated cells were captured under microscopy following 3 week incubation period (**E**); the number of colonies was counted under microscopy and the results were presented as colonies per 50,000 cells from three independent experiments. The asterisk (*) indicates a significant increase as compared with the medium control, and the symbol (♣) indicates a significant inhibition in comparison to UROtsa(Vector) cells ($p < 0.05$) (**F**).

the intron region of vesicle transport through interaction with t-SNAREs homolog 1A (VTI1A) gene. Thus, we first evaluated the effect of XIAP and its RING domain on VTI1A expression. As shown in Figure 7A, knockdown of XIAP impaired VTI1A expression, while overexpression of HA- Δ BIR, but not HA- Δ RING, exhibited an increase VTI1A expression at both protein and mRNA levels (Figure 7A & 7B). To test the possibility that XIAP and its RING regulate VTI1A transcription, the VTI1A promoter-driven luciferase reporter was constructed and transfected into two paired transfectants, UROtsa(Nonsense) vs. UROtsa(shXIAP) cells, or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) cells, or UROtsa(HA- Δ RING) cells, respectively.

The results indicated that VTI1A promoter-driven luciferase reporter transcription activity was significantly downregulated in UROtsa(shXIAP) cells and upregulated in UROtsa(HA- Δ BIR) cells, demonstrating that XIAP and its RING domain is essential for VTI1A transcription level (Figure 7C and 7D).

miRNA transcription is modulated by RNA Pol II-associated transcription factors and epigenetic regulators [40]. To explore the transcription factor that might be involved in transcriptional regulation of VTI1A and miR-4295, the VTI1A promoter-driven

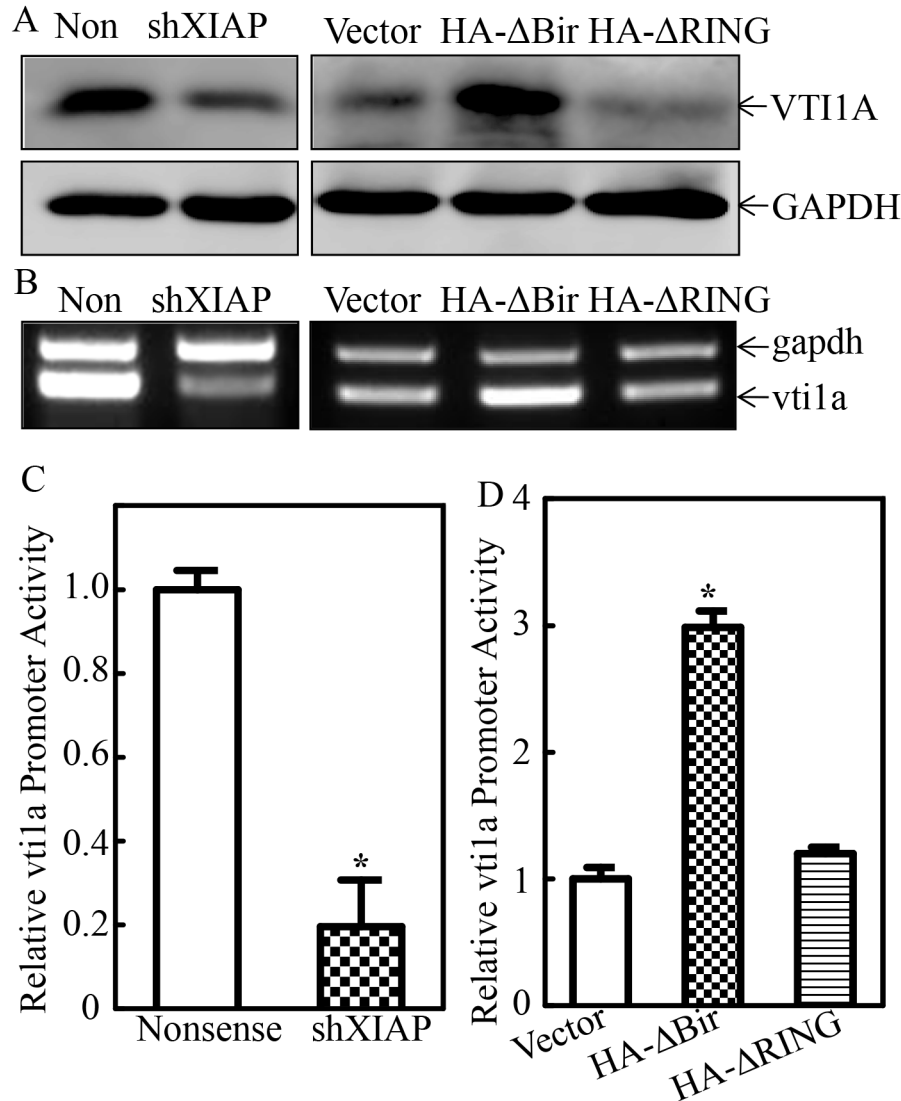


Figure 7: XIAP RING domain promoted miR-4295 host gene VTI1A transcription in human bladder epithelial cells. A. and B. The cell extracts were subjected to Western Blot to determine expression levels of VTI1A protein (A) and mRNA (B) in UROtsa(Nonsense) vs. UROtsa(shXIAP) cells (A); or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING) cells as indicated. C. and D. VTI1A promoter-driven luciferase reporter was transfected together with TK reporter into UROtsa(Nonsense) vs. UROtsa(shXIAP) cells (C), or UROtsa(Vector) vs. UROtsa(HA- Δ BIR) and UROtsa(HA- Δ RING) cells (D). Twenty-four hours post transfection, the transfectants were extracted to evaluate the luciferase activity, with TK used as internal control. The results were presented as luciferase activity relative to vector control transfectant. Each bar indicates mean \pm SD from three independent experiments. The symbol (*) shows a significant difference ($P < 0.05$).

luciferase reporter was bioinformatically analyzed. As shown in Figure 8A, the promoter contains the binding sites of multiple transcription factors, including c-Jun, E2F1, Sp1, STAT3 (Signal transducer and activator of transcription 3), STAT1 (Signal transducer and activator of transcription 1) and STAT5 (Signal transducer and activator of transcription 5). To identify the specific transcription factors implicated in XIAP and its RING domain promotion of VTI1A transcription, we evaluated the expression of these transcription factors in two paired transfectants, UROtsa(Nonsense) vs. UROtsa(shXIAP) cells, and UROtsa(Vector) vs. UROtsa(HA-ΔBIR) cells. Comparison of the results obtained from these two paired cell transfectants, revealed that only alteration of Sp1 and c-Jun/c-Jun Ser63 was consistent with XIAP and

its RING domain regulation of VTI1A and miR-4295 (Figure 8B). The results obtained from investigating AP-1-dependent transcription activity in two paired transfectants, UROtsa(Nonsense) vs. UROtsa(shXIAP) cells, and UROtsa(Vector) vs. UROtsa(HA-ΔBIR) cells, showed that AP-1 transactivation was inhibited in both UROtsa(shXIAP) and UROtsa(HA-ΔBIR) cells, which was not consistent with their effects on the transcriptional regulation of VTI1A and miR-4295, in turn further excluding involvement of AP-1/c-Jun in transcriptional regulation of VTI1A and miR-4295 (Figure 8C). We noted with great interest that Sp1-dependent transcription activity was downregulated in UROtsa(shXIAP), while it was upregulated in UROtsa(HA-ΔBIR) cells, consistently supporting the Sp1 transcriptional regulation of VTI1A and

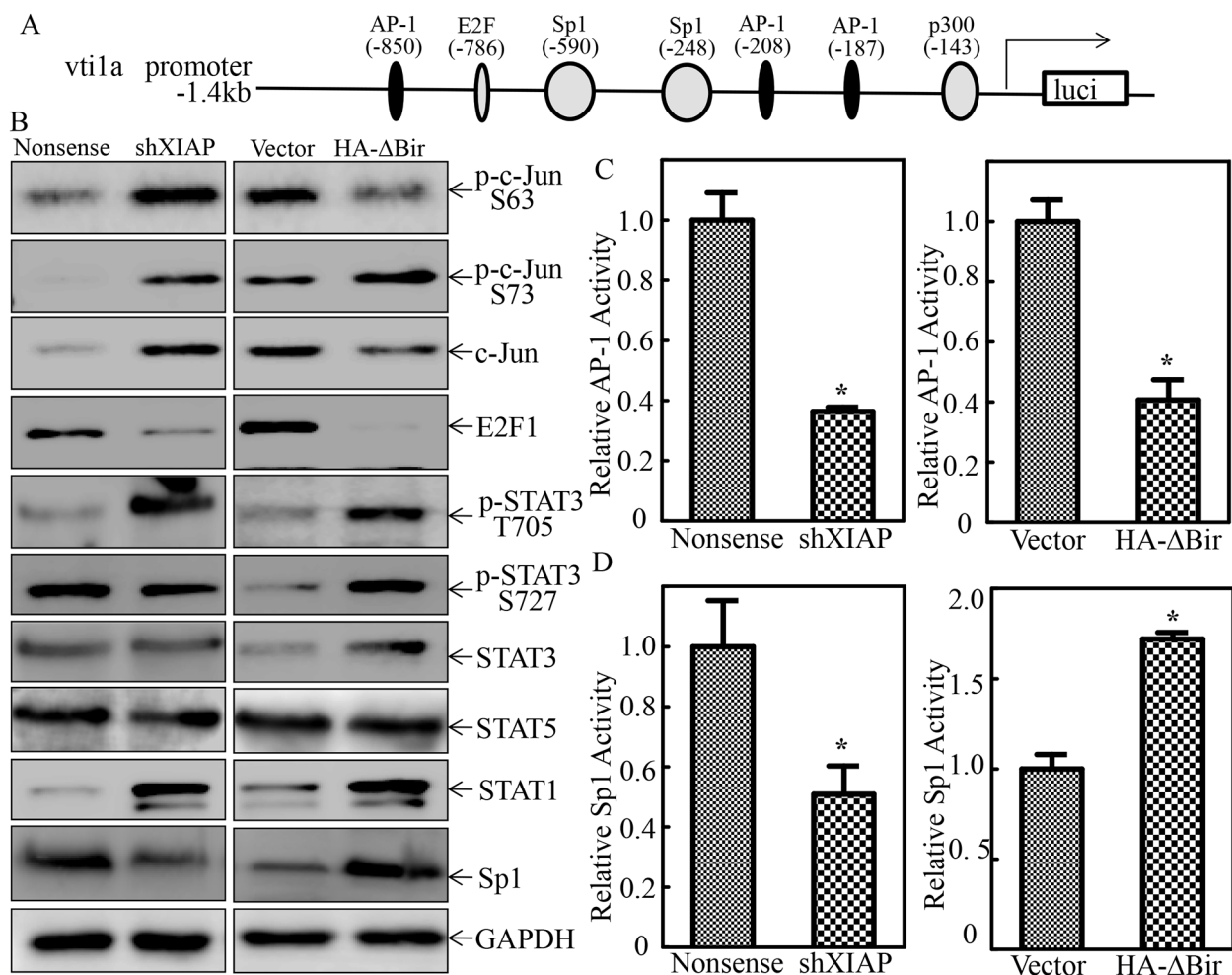


Figure 8: XIAP RING domain was crucial for Sp1 protein expression and transactivation in human bladder epithelial cells. **A.** The potential transcription factors binding sites of VTI1A promoter. **B.** The cell extracts obtained from UROtsa(Nonsense), UROtsa(shXIAP), UROtsa(Vector), and UROtsa(HA-ΔBIR) cells were subjected to Western Blot for determination of expression of p-c-Jun S63, p-c-Jun S73, c-Jun, E2F1, p-STAT3 705, p-STAT3 727, STAT3, STAT5, STAT1 and Sp1 as indicated. **C.** and **D.** UROtsa(Nonsense), UROtsa(shXIAP), UROtsa(Vector), and UROtsa(HA-ΔBIR) cells were co-transfected with AP-1 luciferase reporter or Sp1 luciferase reporter, together with pRL-TK. Twenty-four hours post transfection, the transfectants were extracted to evaluate the luciferase activity, with normalization to TK. The results were presented as luciferase activity relative to vector control transfectant. Each bar indicates mean±SD from three independent experiments. The symbol (*) shows a significant difference (P<0.05).

miR-4295 (Figure 8D). To test this notion, shRNA specific targeting Sp1 was stably transfected into UROtsa cells. The stable transfectants were established and identified as shown in Figure 9A. The knockdown of Sp1 dramatically inhibited VTI1A protein expression accompanied by p63 α induction (Figure 9A). Consistent with the protein expression, VTI1A promoter-driven luciferase activity

was downregulated in Sp1 knockdown transfectant, UROtsa(shSp1) (Figure 9B) and EGF-induced malignant transformation of UROtsa cells was almost completely abolished (Figure 9C & 9D). To further determine whether RING-induced cell transformation was specifically mediated by Sp1, The stable transfectant UROtsa(HA- Δ BIR/shSp1) was established by co-transfecting shRNA

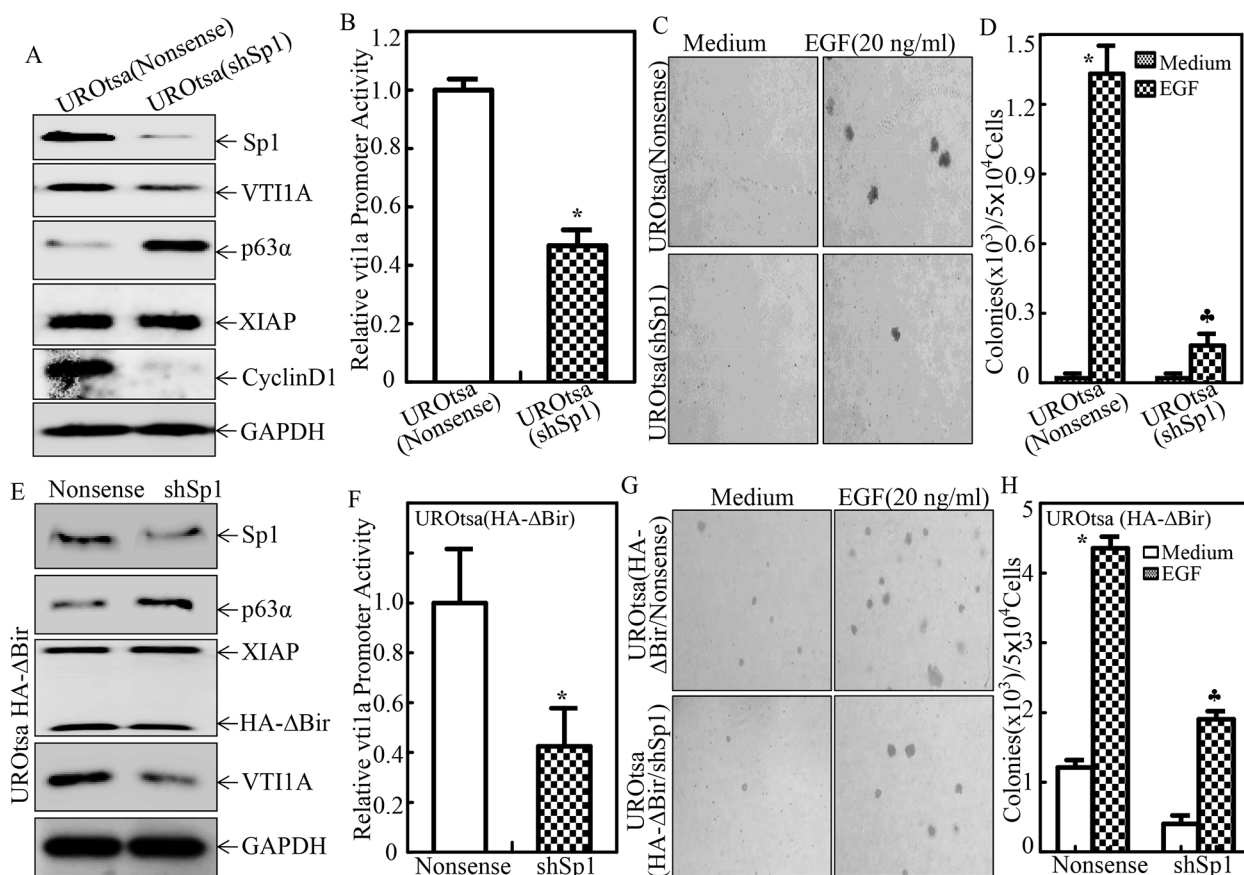


Figure 9: Sp1 was crucial for VTI1A transcription, p63 α expression and EGF-induced malignant transformation of human bladder epithelial cells. **A.** The cell extracts obtained from UROtsa(Nonsense), and UROtsa(shSp1) were subjected to Western Blot for determination of expression of protein expression as indicated; **B.** UROtsa(Nonsense) and UROtsa(shSp1) were co-transfected with VTI1A promoter-driven luciferase reporter and pRL-TK. Twenty-four hours post transfection, the transfectants were extracted to evaluate the luciferase activity, with normalization to TK. The results were presented as luciferase activity relative to nonsense control transfectant. Each bar indicates mean \pm SD from three independent experiments. The symbol (*) shows a significant decrease as compared to UROtsa(Nonsense) cells ($P < 0.05$); **C.** and **D.** UROtsa(Nonsense) and UROtsa(shSp1) were subjected to anchorage-independent growth in the presence or absence of EGF as indicated. Representative images of the colonies of indicated cells were captured under microscopy following 3 week incubation period (**C**); the number of colonies was counted under microscopy and the results were presented as colonies per 50,000 cells from three independent experiments. The asterisk (*) indicates a significant decrease as compared with the medium control, and the symbol (\clubsuit) indicates a significant inhibition in comparison to UROtsa(Nonsense) cells ($p < 0.05$) (**D**). **E.** The cell extracts obtained from UROtsa(HA- Δ BIR/Nonsense), and UROtsa(HA- Δ BIR/shSp1) were subjected to Western Blot to determine expression of protein expression as indicated; **F.** UROtsa(HA- Δ BIR/Nonsense) and UROtsa(HA- Δ BIR/shSp1) were co-transfected with vti1a promoter-driven luciferase reporter and pRL-TK. Twenty-four hours post transfection, the transfectants were extracted to evaluate the luciferase activity, with normalization to TK. The results were presented as luciferase activity relative to nonsense control transfectant. Each bar indicates mean \pm SD from three independent experiments. The symbol (*) shows a significant decrease as compared to UROtsa(HA- Δ BIR/Nonsense) cells ($P < 0.05$); **G.** and **H.** UROtsa(HA- Δ BIR/Nonsense) and UROtsa(HA- Δ BIR/shSp1) were subjected to anchorage-independent growth in presence or absence of EGF as indicated. Representative images of colonies of indicated cells were captured under microscopy following 3 week incubation period (**G**); the number of colonies was counted under microscopy and the results were presented as colonies per 50,000 cells from three independent experiments. The asterisk (*) indicates a significant decrease as compared with the medium control, and the symbol (\clubsuit) indicates a significant inhibition in comparison to UROtsa(HA- Δ BIR/Nonsense) cells ($p < 0.05$).

specifically targeting Sp1 together with vt1a promoter-driven luciferase reporter into UROtsa(HA- Δ BIR) cells as shown in Figure 9E. As anticipated, the deficiency of Sp1 in UROtsa(HA- Δ BIR) cells resulted in attenuation of vt1a promoter transcriptional activity caused by HA- Δ BIR overexpression (Figure 9F), which consequently profoundly inhibited EGF-induced cell transformation (Figure 9G & 9H). Collectively, our results demonstrate that XIAP and its RING domain can initiate Sp1-dependent transcription of VTI1A/miR-4295 expression, which subsequently inhibits p63 α translation by targeting its mRNA 3'-UTR, in turn promoting malignant transformation of human bladder epithelial cells.

DISCUSSION

Biochemical and structural studies indicate that XIAP is a protein with three BIR domains and a RING-finger domain [18]. XIAP has been reported to be upregulated in many cancers, including acute and chronic leukemia [3, 4], prostate cancer [5], breast cancer [6–8], and many other cancers [9–11]. Our most recent studies have also shown that XIAP expression is overexpressed in human bladder cancers, revealing a crucial association between XIAP and human bladder cancer development (Data not shown). However, the molecular mechanisms and specific XIAP functional domain(s) responsible for XIAP regulation of human bladder carcinogenesis has not been explored. In this study, we show that XIAP and its RING domain specifically provide an inhibitory effect on p63 α protein expression both *in vitro* human bladder epithelial UROtsa cells and *in vivo* normal mouse bladder epithelial cells obtained from XIAP Δ RING knockin mice. Further studies reveal that RING of XIAP is the structural functional domain responsible for this inhibition by initiating Sp1-mediated transcription of miR-4295, which is able to bind to 3'-UTR of p63 α mRNA and blocks p63 α protein translation. Moreover, our studies demonstrate that the miR-4295 induction/p63 α downregulation by RING domain of XIAP plays a disposable role in malignant transformation of human bladder epithelial cells following EGF treatment. These findings help us to understand the association and mechanisms of XIAP overexpression with bladder cancer development, they also enable us to explore the utilization of RING domain as a potential therapeutic target for treatment of bladder cancer patients.

Although endogenous TAp63 proteins are barely detectable in normal somatic cells, their alterations are still reported in various types of cancer tissues [41]. In comparison with well-characterized p63 α downstream targets and mechanisms involved in its tumor suppressive effects, the upstream regulators for control of the p63 expression have been much less thoroughly explored, although p63 levels are known to be regulated by protein phosphorylation/ubiquitin-dependent proteasome degradation and miRNAs [42]. p63 protein stability is

regulated by the ubiquitin–proteasome system [43]. For example, p53-induced E3 ubiquitin ligase Pirh2 can directly bind to p63 and cause its poly ubiquitination and degradation in keratinocytes [44]. Another E3 ubiquitin ligase Ring1B has also been shown to target p63 [45]. To the best of our knowledge, there are only a few reports showing miRNAs involvement in the regulation of p63 protein expression [46]. miR-203 can repress p63 expression in supra-basal epithelial cells, contributing to definition of the border between progenitors and differentiated epithelial cells [47]. miR-302 has also been reported to suppress p63 expression in germ cells [48], while iASPP protein inhibits the expression of miR-574-3p and miR-720, which in turn inhibits p63 expression [49, 50]. In our current studies, we discovered that XIAP and its RING domain were able to inhibit p63 α protein expression in bladder epithelial cells *in vitro* human cell culture model and *in vivo* mouse bladder tissues. By excluding the possibility of XIAP/RING regulation of p63 α transcription and mRNA stability, as well as protein degradation, we were able to identify the regulation of p63 α protein expression by the XIAP/RING domain and identify that it occurred at translation level by probing new p63 α protein synthesis using [³⁵S] methionine pulse assays. We further found that 3'UTR of p63 α mRNA was targeted by miR-4295, which was upregulated by XIAP/RING domain. These new discoveries provide us with significant insight into understanding the upstream regulators for control of the p63 expression in the regulation of human bladder epithelial carcinogenesis.

Micro-RNAs (miRNAs) are non-coding RNAs involved in the post-transcriptional regulation of gene expression in cellular organisms by affecting mRNAs stability and translation [51]. The miRNA expression is tightly regulated at multiple levels, including at the levels of miRNA transcription, maturing, modification by RNA editing, RNA methylation, uridylation and adenylation, argonaute loading, and RNA decay [40]. Although miR-4295 could be found in the NCBI Gene database, nothing is known about its transcriptional promoter, upstream regulators/downstream effector(s). In humans, the majority of miRNAs are encoded by either introns or exons of transcripts [52]. Our studies here indicated that VTI1A is the host gene of miR-4295 and that precursor mRNA of miR-4295 is located in the intron region of VTI1A. We also found that the RING domain, but not the BIR domain, of XIAP, was able to regulate transcription of VTI1A/miR-4295, while transcription factor Sp1 expression/transactivation was regulated by the RING domain of XIAP, and was required for XIAP/RING-mediated miR-4295 expression. The matured miRNA is incorporated into a RNA-induced silencing complex (RISC), which recognizes target mRNAs through imperfect base pairing with the miRNA and consequently results in either translational inhibition or alteration of the targeted mRNA stability [51]. In this study, we found that miR-4295 as

an oncogenic miRNA could target tumor suppressor gene p63 α protein expression and promotes malignant transformation of human bladder epithelial UROtsa cells. These studies demonstrate novel information related to the host gene, transcriptional promoter, and upstream regulators/downstream effector(s) of miR-4295, which provide a link between XIAP overexpression and bladder epithelial carcinogenesis.

In summary, the current studies discover that XIAP/RING domain initiates Sp1-mediated miR-4295 transcription, which directly targets the 3'UTR of p63 α mRNA and inhibits p63 α protein translation, and further in turn promoting transformation of human bladder epithelial cells. The complete elucidation of the XIAP RING domain in the initiating of Sp1 expression/transactivation and new effector(s) of miR-4295 is next goals of undergoing projects in our group and we will test this regulation pathway in other kind of tumors in the future. Our novel findings of XIAP RING domain in regulation of Sp1/miR4259/p63 α cascade as well as identifying the crucial role of this cascade in malignant transformation of human bladder epithelial cell, may help us to utilize miR-4295 as biomarkers for the diagnosis and/or targets for the prevention and therapy of human bladder cancers.

MATERIALS AND METHODS

Cell lines, plasmids, and antibodies

UROtsa cell line was normal human bladder epithelial cells kindly provided by Dr. Scott H. Garrent (University of North Dakota, Grand Forks, ND), and was cultured in RPMI1640 with 10% FBS [37]; UMUC3 was a high grade human bladder cancer cell line described in our previous publications [53, 54]. It was cultured in DMEM with 10% FBS (ATLANTA, Flowery Branch, GA). The shRNA that specifically targets human XIAP, Sp1 and p63 α was purchased from Open Biosystems (GE, Pittsburgh, PA). The p63 α -Flag expression plasmid was bought from Addgene (Cambridge, MA). HA- Δ BIR and HA- Δ RING expression plasmids were described in our previously studies [15, 16, 22]. AP-1- and Sp1-dependent luciferase reporters were described in our previous papers [55–57]. The expression plasmid containing hsa-miR-4295 was cloned into pmR-ZsGreen1 Vector using BglIII and KpnI restriction endonuclease using primers: forward 5'-GGA AGA TCT AGG ATC ACA GTT AAC TCA GAA T-3', reverse 5'-CGG GGT ACC GCA CAA TCC AAA ACA AGA A-3'. The plasmid containing luciferase reporter under control of human VTI1A gene promoter was constructed into PGL3-BASIC vector using the primers: forward 5'-CCG CTC GAG CTA GGA GAA TCC AGT GTC-3', reverse 5'-CCC AAG CTT TGC TTC CGG AGG GAG GTT T-3'. The plasmid containing luciferase reporter under control of human WT p63 α mRNA 3'UTR were constructed into pMIR-report vector using the primers:

forward 5'-TAA CTC GAG GCC TCA CCA TGT GAG CTC TTC CTA-3', reverse 5'-GGC TCG ACT AGT ACC AAC ACT GAG AAA AGA ATA-3'. The primers for cloning mutant of p63 α mRNA 3'UTR luciferase reporter are: forward 5'-AAA TGA AAG AAA ATT GAG ACT TAT TGA CCA TTT TTT AA-3', reverse 5'-TTA AAA AAT GGT CAA TAA GTC TCA ATT TTC TTT CAT TT-3'. The specific antibodies against p63 α were bought from Genetex (Invine, CA). Anti-XIAP antibody was purchased from BD (Franklin Lakes, NJ). Antibodies specific against HA, p-STAT3 Ser727, p-STAT3 Tyr705, STAT3, STAT5, and GAPDH, were purchased from Cell Signaling (Beverly, MA). Antibodies specific for VTI1A, Sp1, E2F1, β -Actin and α -Tubulin, were from Santa Cruz (Dallas, TX).

Cell transfection

Cell transfections were performed with PolyJet™ DNA *in Vitro* Transfection Reagent (SignaGen Laboratories, Rockville, MD) according to the manufacturer's instructions. For stable transfection, UROtsa cells were subjected to selection with hygromycin B (30 μ g/mL), G418 (300 μ g/mL) or puromycin (0.5 μ g/mL) depending on the different antibiotic resistance plasmids transfected. The cells surviving from the antibiotics selection were pooled as mass stable transfectants. UMUC3 overexpressing p63 α -FLAG were selected by hygromycin (300 μ g/mL).

Western blot analysis

Whole cell extracts were prepared with the cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, and 1 mM Na₃VO₄) as described in our previous studies [57, 58]. Protein extracts were subjected to Western Blot with the indicated primary antibodies, and probed with the AP-conjugated secondary antibody together with the enhanced chemifluorescence system as described in a previous reports [57, 58]. The images were acquired by scanning with the PhosphorImager Typhoon FLA 7000 (GE, Pittsburgh, PA).

Immunohistochemistry paraffin (IHC-P) of mouse bladder specimens

Mouse bladder tissues obtained from the sacrificed mice were formalin-fixed and paraffin-embedded. The antibodies specific against p63 α (Genetex, CA) were used for immunohistochemical (IHC) staining as described in our previous publications [57, 59]. The resulting immunostaining images were captured using the AxioVision Rel.4.6 with computerized image analysis system (Carl Zeiss, Oberkochen, Germany). Protein expression levels were analyzed by calculating the integrated optical density per stained area (IOD/area) using Image-Pro Plus version 6.0 (Media Cybernetics, MD).

Luciferase reporter assay

p63 α mRNA 3'UTR luciferase reporter, VT11A promoter-driven luciferase reporter, Sp1-dependent transcriptional activation luciferase reporter or AP-1-dependent transcriptional activation luciferase reporter and pRL-TK, were transiently transfected into UROtsa(shXIAP), UROtsa (HA- Δ BIR), UROtsa (HA- Δ RING) and their related control vector transfectants. Twenty-four hours after the transfection, luciferase activity was determined using the luciferase assay system kit purchased from Promega (Madison, WI,) as described in our previous studies [60, 61]. The results were normalized by internal TK signal. The results are expressed as mean \pm SD error from triplicate experiments.

RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Grand Island, NY,) as described in the manufacturer's instructions and our previous studies [62, 63]. 5 μ g total RNA was used for first-strand cDNA synthesis with oligdT primer by SuperScriptTM III First-Strand Synthesis system (Invitrogen, Grand Island, NY) and described in our previous studies [64]. Specific primers (Invitrogen, Grand Island, NY,) used for PCR amplification were: *human p63 α* , forward 5'-aga cat gaa tgg act cag cc-3', reverse 5'-ctc aat ctg ata gat ggt ggt-3'; *human vti1a*, forward 5'-aga act gct tga aca gat gga ttt g-3', reverse 5'-agg agc tea ttc cgt act tgc tca-3'; and *human gapdh*, forward 5'-gat gat ctt gag gct gtt gtc-3', reverse 5'-cag ggc tgc ttt taa ctc tg-3'.

Quantitative RT-PCR

The cells used for total RNA extraction using miRNeasy Mini Kit (QIAGEN, Valencia, CA). 1 μ g total RNA was used for reverse transcription. Analysis of miR-4295, miR-301a, miR-301b, miR-130a, miR-130b, miR-454, and miR-3666 expression were carried out using the miScript PCR system (QIAGEN, Valencia, CA) by 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA). The primer for miR-4295 assay was purchased from QIAGEN (Valencia, CA), and U6 (Invitrogen, Grand Island, NY) was used as internal control. The initial activation was performed at 95°C for 15 min, followed by 40 cycles (denaturation at 95°C for 15s, annealing at 55°C for 30s and extension at 72°C for 30s). The data were analyzed as described in the previous publication [58].

[³⁵S] methionine pulse assays

UROtsa(Nonsense) and UROtsa(shXIAP) were incubated with methionine-cysteine-free DMEM (Gibco-BRL, Grand Island, NY) containing 2% dialyzed fetal calf serum (Gibco-BRL, Grand Island, NY) in presence

of MG132 (50 μ M) for 1 hours. The cells were then incubated with 2% fetal bovine serum methionine-cysteine-free DMEM containing ³⁵S-labeled methionine/cysteine (250 mCi per dish, Trans ³⁵S-label; Perkin Elmer, Boston, MA) for the indicated time periods. The cells were extracted with lysis buffer (Cell Signaling, Beverly, MA) containing complete protein inhibitor mixture (Roche, Branchburg, NJ) on ice for 10 mins. Total lysate of 500 mg was incubated with anti-p63 α antibody-conjugated agarose beads (R&D Systems, Minneapolis, MN) at 4°C overnight. The immunoprecipitated samples were washed with the cell lysis buffer five times, heated at 100°C for 5 min and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The ³⁵S-labeled p63 α protein was detected with the PhosphorImager Typhoon FLA 7000 (GE, Pittsburgh, PA).

Anchorage-independent growth assay

The anchorage-independent growth ability of human bladder epithelial UROtsa and its transfectants following EGF treatment was determined in soft agar as described in our previous studies [56, 65]. Briefly, 3 ml of 0.5% agar in basal modified Eagle's medium supplemented with 10% FBS with or without EGF, respectively, was layered onto each well of 6-well tissue culture plates. One ml of UROtsa cells or their transfectants (5 \times 10⁴) was mixed with 0.5% agar BMEM (1:3) supplemented with 10% FBS with or without EGF, then layered on top of the 0.5% agar layer in each well of 6-well tissue culture plates. Plates were incubated at 37°C in 5% CO₂ for 2–3 weeks. The colonies with more than 32 cells were scored and are presented as colonies/10⁴ cells.

Statistical analysis

The student's t-test was used to determine the significance between treated and untreated group. The results are expressed as mean \pm SD from at least three independent experiments. P<0.05 was considered to be a significant difference between compared groups.

Abbreviations

CHX, Cycloheximide; EGF, Epidermal growth factor; EGFR, Epidermal growth factor Receptor; E2F1, E2F Transcription Factor 1; miRNA, microRNA; RhoGDI α , Rho GDP-dissociation inhibitor 1; Sp1, Specificity Protein 1; STAT1, Signal transducer and activator of transcription 1; STAT3, Signal transducer and activator of transcription 3; STAT5, Signal transducer and activator of transcription 5; UTR, Untranslated region; VT11A, Vesicle Transport Through Interaction With T-SNAREs homolog 1A; XIAP, X-linked inhibitor of apoptosis protein.

ACKNOWLEDGMENTS

We thank Dr. Scott H. Garrent from University of North Dakota, Grand Forks, ND, for providing us with UROtsa cells. This work was partially supported by the grants of NIH/NCI CA165980, CA177665, CA112557, and NIH/NIEHS ES000260, as well as Key Project of Science and Technology Innovation Team of Zhejiang Province (2013TD10) and the Xinmiao Talent Program of Zhejiang Province (2015R413066).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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