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The p53-inducible long noncoding RNA TRINGS protects cancer cells from necrosis under glucose starvation

Muhammad Riaz Khan[1,†], Shaoxun Xiang[1,†], Zhiyin Song[2,*] & Mian Wu[1,3,**]

Abstract

The tumor suppressor p53 is activated in response to cellular stress to prevent malignant transformation. However, several recent studies have shown that p53 can play protective roles in tumor cell survival under adversity. Whether p53-regulated long noncoding RNAs are involved in this process remains to be fully understood. Here, we show that under glucose starvation condition, p53 directly upregulates a novel lncRNA named TRINGS (Tp53-regulated inhibitor of necrosis under glucose starvation) in human tumor cells. TRINGS binds to STRAP and inhibits STRAP–GSK3β–NF-κB necrotic signaling to protect tumor cells from cell death. Interestingly, TRINGS appears to respond to glucose starvation specifically, as it is not activated by serum, serine, or glutamine deprivation. Collectively, our findings reveal that p53-induced lncRNA TRINGS controls the necrotic pathway and contributes to the survival of cancer cells harboring wild-type p53 under glucose stress.

Keywords glucose starvation; necroptosis; p53; STRAP; TRINGS
Subject Categories Autophagy & Cell Death; Cancer; Signal Transduction
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Introduction

Tp53 (also known as p53) is a well-known tumor suppressor gene, and ~50% of the tumors harbor mutations in p53 gene (Caron de Fromentel & Soussi, 1992; Chao *et al*, 2003). As a transcription factor, p53 recognizes its target genes by directly binding to p53 response element (p53RE) located upstream or even downstream of the transcription start site (Menendez *et al*, 2009). p53 not only transcribes messenger RNAs, but also noncoding RNAs including microRNAs and long noncoding RNAs (lncRNA; Huarte *et al*, 2010).

The lncRNAs are transcripts without potential open reading frame (Dinger *et al*, 2008; Khalil *et al*, 2009). They play multifaceted roles in regulating gene expression, monitoring mRNA or protein stability and acting as miRNA decoys (Wang & Chang, 2011). Most lncRNAs are expressed in a tissue-specific manner and their expression is correlated with biological pathways and associated with human diseases (Guttman *et al*, 2009; Sánchez & Huarte, 2013). A growing number of studies demonstrate that several lncRNAs are functional components of p53-regulated pathways and play critical roles in tumor suppression (Huarte *et al*, 2010; Hung *et al*, 2011; Chen *et al*, 2016), and therefore, the identification of p53-regulated long noncoding RNAs and the mechanistic investigation of their functions are important for p53-regulated cancer studies.

Due to insufficiencies of tumor vasculature, cancer cells are often exposed to a hostile tumor microenvironment including low nutrients and oxygen. Therefore, cancer cells need to adapt themselves to this harsh microenvironment for survival. Upon stimuli of diverse stresses, cancer cells proceed into cell death by at least three different ways including programmed cell death (apoptosis), autophagic cell death, and necrosis (Edinger & Thompson, 2004; Green *et al*, 2014; Vanden Berghe *et al*, 2014). Apoptosis is a cell suicide by activating an intracellular death program; it can be characterized by cleavage of caspases or PARP-1 (Indran *et al*, 2011). Autophagic cell death is an autophagy-dependent non-apoptotic form of cell death, and it can be suppressed by inhibition of various autophagy pathways (Tsujimoto & Shimizu, 2005). On the other hand, necrosis is originally viewed as an unprogrammed cell death and occurs upon external force including infection, body injury, or poison, and it also causes inflammation (Kono & Rock, 2008). Accumulating evidences suggest that necrosis can be programmed and regulated by signal pathways, and programmed or regulated necrosis is termed necroptosis (Fu *et al*, 2013; Thapa *et al*, 2013); HMGB1 and LDH are released from necrotic cells as markers of necrosis (Scaffidi *et al*, 2002; Chan *et al*, 2013).

GSK3β (glycogen synthase kinase 3β) is a serine–threonine kinase that belongs to the glycogen synthase kinase subfamily

1 CAS Key Laboratory of Innate Immunity and Chronic Disease, CAS Center for Excellence in Cell and Molecular Biology, Innovation Center for Cell Signaling Network, School of Life Sciences, University of Science & Technology of China, Hefei, Anhui, China
2 Hubei Key Laboratory of Cell Homeostasis, College of Life Sciences, Wuhan University, Wuhan, Hubei, China
3 Translational Research Institute, School of Medicine, Henan Provincial People's Hospital, Henan University, Zhengzhou, China
*Corresponding author. Tel: +86 027 68752235; E-mail: songzy@whu.edu.cn
**Corresponding author. Tel: +86 551 63606264; E-mail: wumian@ustc.edu.cn
†These authors contributed equally to this work

(Kashikar *et al*, 2011) and is involved in regulating numerous pathways including cell death and survival. GSK3β was reported to be inactive through its phosphorylation at serine 9 in response to various stimuli (Doble & Woodgett, 2003). Besides, GSK3β still plays conflicting roles in cell death pathways (Jacobs *et al*, 2012). STRAP, which localizes both in cytoplasm and nucleus, was originally identified as TGF-β interacting protein (Datta & Moses, 2000; Kashikar *et al*, 2011). STRAP was shown to regulate GSK3β function by directly interacting with GSK3β (Kashikar *et al*, 2011). The exact role of STRAP and GSK3β in cell death regulation is still unknown.

p53 is activated in response to external environmental or physiological changes (stresses) such as DNA damage, hypoxia, or nutrient starvation (Hu *et al*, 2012; Maddocks *et al*, 2013). On activation, p53 was shown to mediate important cellular processes such as cell death, cell-cycle arrest, senescence, and metabolic reprogramming (Yee & Vousden, 2005; Okoshi *et al*, 2008; Riley *et al*, 2008; Green & Kroemer, 2009). Upon various stress conditions, p53 acts as a key killer and promotes apoptosis by activating pro-apoptotic genes such as *bax*, *puma*, *noxa,* or *p53AIP1* (Riley *et al*, 2008). In addition, p53 was shown to trigger necrosis by opening the mitochondrial permeability transition pore (Vaseva *et al*, 2012). Interestingly, p53 was recently reported to protect hypoxia-induced cell death by directly inhibiting BNIP3 expression (Feng *et al*, 2011). Moreover, p53 was also shown to promote pro-survival autophagy under condition of nutrient stress (Feng *et al*, 2007), suggesting that besides its pro-apoptotic role, p53 possesses anti-death function under some specific circumstances. Approximately half of the tumors still harbor wild-type (WT) p53, and whether and how the WT p53 in cancer cells respond to tumor microenvironments for survival is not well understood. Our previous study has demonstrated a protective role of WT p53 in melanoma cells by upregulating miR-149* (Jin *et al*, 2011). Particularly, whether p53 is able to trigger a signal pathway to prevent necrosis in tumor cells via lncRNA upon nutritional stress has never been reported. In this study, we report a newly identified p53-regulated long noncoding RNA which protects cancer cells from necrotic cell death upon glucose starvation; we therefore name it as TRINGS (Tp53-regulated inhibitor of necrosis under glucose starvation). lncRNA TRINGS interacts with STRAP and blocks necrotic cell death via inhibiting STRAP–GSK3β–NF-κB signal pathway. Thus, our findings provide a new pathway for the survival of cancer cells harboring WT p53 under glucose stress.

Results

p53 upregulates lncRNA TRINGS expression

To identify new long noncoding RNAs involved in the regulation of p53 function, microarray analysis was performed using H1299 cells carrying a p53 tet-on system. After initial screening, levels of four lncRNAs (lncRNA-4, lncRNA-6, lncRNA-8, and lncRNA-9) were shown to be significantly elevated upon doxycycline treatment (Appendix Fig S1A and Table EV1). The four lncRNAs were further quantified through qRT–PCR analysis under glucose starvation condition and only lncRNA-9 (lncRNARP11-115D19.1, ENST00000507916, named as TRINGS in this manuscript) among others was found to be markedly upregulated (Appendix Fig S1B). The nucleotide sequence of TRINGS is shown in Appendix Fig S1C, lncRNA TRINGS is a mature transcript consisting of three exons, and its size is ~600 nts long. The full length of TRINGS was further verified by Northern blotting (Fig 1A). In addition, TRINGS is poorly conserved among species examined (Appendix Fig S1D). Furthermore, doxycycline treatment induced remarkable increase in the level of TRINGS and lncRNA DINO (a positive control; Schmitt *et al*, 2016) in H1299 cells carrying a p53 tet-on system (Fig 1B). TRINGS was therefore chosen for further investigation. We firstly examined the TRINGS expression profiling in various cell lines including HAFF, HepG2, U2OS, U87, H1299 (p53 tet-on), SMCC7721, Mel-CV, and HCT116, all of which contain wild-type p53. Levels of TRINGS were exhibited particularly high in p53 highly expressed cell lines such as HCT116 and U2OS as compared to other cell lines (Fig 1C). Interestingly, the expression of TRINGS does not entirely reflect the p53 status similar to the bona fide p53 target gene p21 (Fig 1C), which may be explained by two possible reasons: (i) besides p53, some other unknown factor(s) may also affect(s) TRINGS expression, and (ii) TRINGS expression may be cell-type dependent. Of note, level of TRINGS in normal HAFF cells is near to the ground (Fig 1C). We also explored the subcellular distribution of TRINGS and found that it resides mainly in the cytoplasm by lncRNA fluorescence *in situ* hybridization (FISH; Fig 1D). To further verify whether TRINGS is regulated by p53, HCT116 cells containing wild-type (WT) p53 were treated with doxorubicin (Dox, a DNA-damaging agent) and Dox-induced p53 led to an increase in TRINGS expression (Fig 1E). Conversely, TRINGS expression was significantly reduced when p53 was depleted by short hairpin RNA (shRNA;

Figure 1. TRINGS is upregulated by p53.

A Total RNA from U2OS cells treated with or without doxorubicin was subjected to Northern blot analysis to determine the molecular size of lncRNA TRINGS. 28S and 18S RNA were used as loading controls.

B H1299 cells with doxycycline-inducible expression of wild-type p53 (H1299 Tet-On p53) were incubated with doxycycline (1 μg/ml) for the indicated periods of time. Cell lysates were analyzed by Western blotting with indicated antibodies; total RNA was analyzed by real-time RT–PCR analysis for lncRNA TRINGS and lncRNA DINO. Data shown are mean ± SD (n = 3).

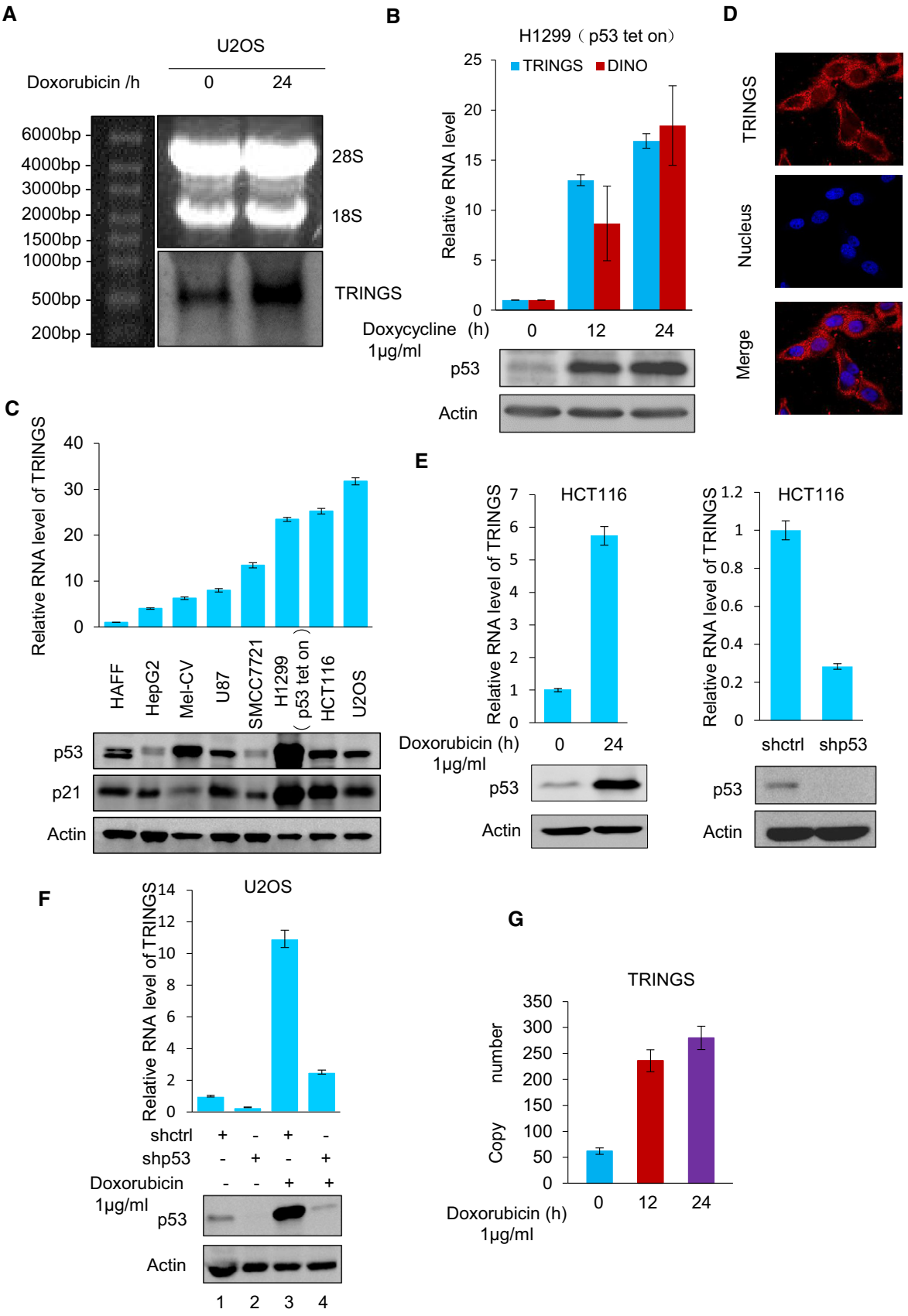
C Levels of TRINGS from different cell lines including HAFF, HepG2, Mel-CV, U87, SMCC-7721, HCT116, and U2OS were compared by real-time RT–PCR. Cell lysates were analyzed by Western blotting with indicated antibodies. Data shown are mean ± SD (n = 3).

D RNA FISH was performed with Alexa Fluor® 488-labeled probe (red) recognizing TRINGS in U2OS cells. The nucleus was stained by Hoechst (blue). The representative fluorescence images taken by confocal microscopy are shown.

E HCT116 cells were incubated with doxorubicin to induce expression of p53. After 24 h, cell lysates and total RNA were subjected to Western blot and real-time RT–PCR analyses, respectively. Data shown are mean ± SD (n = 3). HCT116 cells were infected with lentiviruses expressing control shRNA or p53 shRNA. 48 h after infection, total RNA and cell lysates were analyzed by real-time RT–PCR and Western blot, respectively. Data shown are mean ± SD (n = 3).

F U2OS cells were infected with lentiviruses encoding a shRNA against p53 or an irrelevant control shRNA. Cells were then treated with or without doxorubicin for 24 h. Total RNA and cell lysates were analyzed by real-time RT–PCR and Western blotting, respectively. Data shown are mean ± SD (n = 3).

G The copy number of TRINGS in U2OS cells treated with doxorubicin at the indicated time was determined. Data shown are mean ± SD (n = 3).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Doxycycline (h)
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Figure 1.

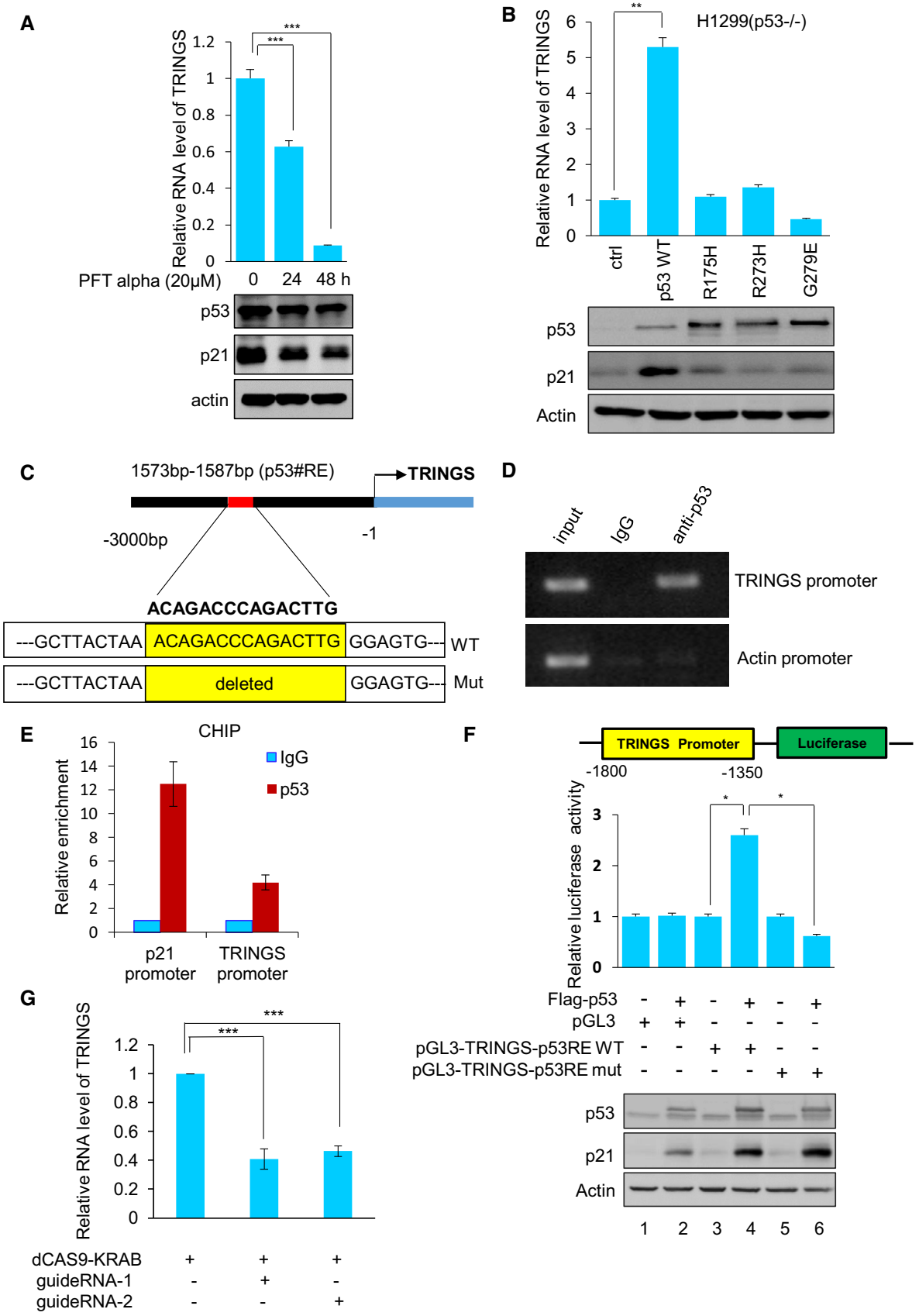

Figure 2.

Figure 2. TRINGS is a direct target of p53.

A U2OS cells were treated with PFT-α for the indicated periods of time, and cell lysates were then analyzed by Western blotting with anti-p53, anti-p21, and anti-Actin antibodies. Total RNA was analyzed by real-time RT–PCR analyses. Data shown are mean ± SD (n = 3; ***P < 0.001).

B H1299 cells were transfected with the plasmids encoding wild-type p53 or indicated mutant forms of p53. 24 h after transfection, total RNA were then analyzed by real-time RT–PCR analyses. The data are represented as mean ± SD (n = 3). Cell lysates were analyzed by Western blotting with anti-p53, anti-p21, and anti-Actin antibodies (**P < 0.01).

C Schematic illustration of putative p53 binding site in *TRINGS* gene promoter. pGL3-based wild-type and mutant reporter constructs used for luciferase assays are also shown. "mut" indicates the promoter region (−1,573 to −1,587 bp) with deletion of the functional p53 binding site.

D U2OS cells lysates were analyzed by ChIP assay with anti-p53 antibody or an isotype-matched IgG. ChIP products were amplified by PCR with the indicated pairs of primers (Appendix Table S1).

E U2OS cells were harvested, and cell lysates were subjected to ChIP analysis. The promoter fragments for p21 and TRINGS (−1,800 to −1,350 bp in the promoter) were amplified by quantitative real-time PCR. Data are shown as the mean ± SD (n = 3).

F U2OS cells were co-transfected with either Flag-p53 or control vector plus the TRINGS promoter (−1,800 to −1,350 bp) reporter constructs and Renilla luciferase plasmid. 24 h after transfection, reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity (mean ± SD; n = 3; *P < 0.05).

G U2OS cells were infected with dCas9-KRAB and indicated guide RNA lentiviruses. After induction with doxycycline (1 μg/ml) for 48 h, total RNA was analyzed by real-time RT–PCR analysis for TRINGS expression. Data shown are mean ± SD (n = 3; ***P < 0.001).

Data information: (A, B, F, G) Student's t-test.

Fig 1E). Moreover, in human osteosarcoma U2OS cells, p53 knockdown led to a reduction whereas Dox-induced p53 to an increase in TRINGS expression (Fig 1F). Again, the elevation of TRINGS induced by Dox treatment was diminished when p53 was suppressed (Fig 1F, lane 4), which excluded the effect of Dox alone on TRINGS expression. Moreover, we have determined TRINGS copy numbers per cell and found that each U2OS cell contains more than 60 TRINGS molecules. Noticeably, TRINGS molecules will increase fivefold after doxorubicin treatment for 24 h (Fig 1G). It was concluded that TRINGS is indeed positively regulated by p53.

TRINGS is a direct target of p53

p53 is widely known as a transcription factor that regulates many stress response genes including noncoding RNA genes (Huarte *et al*, 2010). To examine whether the p53 transcriptionally upregulates TRINGS, pifithrin-alpha (PFT-α), an inhibitor of p53-dependent transcription was used; TRINGS expression was reduced by 90% after PFT-α treatment for 48 h (Fig 2A), indicating that TRINGS expression is dependent on p53 transcriptional activity. Next, we examined whether the tumor-associated p53 mutants, which are reported to lose p53 transcriptional activity (Dearth *et al*, 2007), are able to activate the TRINGS expression. Unlike the WT p53, all three mutant p53 (p53-R175H, p53-R273H, and p53-G279E) failed to upregulate p21 and TRINGS in H1299 cells (Fig 2B). Since TRINGS expression is dependent on p53 transcriptional activity, we further explored the p53 binding site within *TRINGS* promoter region. We analyzed the *TRINGS* gene sequence for potential p53 response elements (p53RE) using JASPAR profile database (Sandelin *et al*, 2004). A consensus binding site (ACAGACCCAGACTTG) was identified at 1.5 kb upstream of *TRINGS* transcription start site (Fig 2C). Chromatin immunoprecipitation (ChIP) assay was performed and a band representing chromatin fragment corresponding to this p53RE was clearly detected (Fig 2D), despite the fact that the binding affinity between p53 and TRINGS promoter is weaker than that between p53 and p21 promoter (Fig 2E). We also evaluated whether this putative p53 binding site confers p53-dependent transcriptional activity; DNA fragments containing wild-type or mutant p53 binding site were inserted into the promoter region of a firefly luciferase reporter plasmid, and the luciferase assay was performed. The luciferase expression from the reporter containing WT-p53RE, but not mutant-p53RE, was indeed induced by ectopic expression of p53 (Fig 2F, lane 4 vs. lane 6). Furthermore, we used dCas9-KRAB, guided by gRNAs targeting p53 binding site of TRINGS promoter region to impede p53 binding and TRINGS expression was successfully downregulated upon dCas9-KRAB transduction (Fig 2G). In addition, we analyzed TRINGS expression in various tumor cells containing wild-type p53 or mutated p53 from TCGA datasets. TRINGS expression is clearly decreased in all p53 mutated tumor samples (Appendix Fig S2A). Taken together, our results demonstrate that p53 physically interacts with p53RE in the promoter region of gene *TRINGS* to induce its expression.

TRINGS protects cancer cells from necrosis induced by glucose starvation

p53 has previously been reported to be activated under nutritional stresses (Hu *et al*, 2012; Maddocks *et al*, 2013), and the roles of p53 under stresses are not totally clear. Particularly, the functions of p53-regulated long noncoding RNA(s) under nutritional stresses are still unknown. Glucose starvation and FBS, serine, and glutamine deprivation were used to test for the response of TRINGS to these metabolic stresses. Among all stresses examined, only low glucose treatment is able to elicit TRINGS expression in p53-positive cells but not in p53 null cells such as H1299 (Fig 3A and Appendix Fig S2B and E). Moreover, glucose starvation-induced upregulation of TRINGS was found in cancer cell lines expressing WT p53 such as U2OS, U87 (human primary glioblastoma cells), Mel-CV (human melanoma cells), and SMCC7721 (human liver cancer cells; Fig 3A and Appendix Fig S3A–C) but not in some normal cells such as HAFF (human adult foreskin fibroblasts), IMR90 (Human fetal lung fibroblasts), or MCF10A (human breast epithelial cells; Fig 3B and Appendix Fig S3D and E). Compared with cancer cells, p53 is minimally activated in these normal cells upon glucose starvation (Fig 3B and Appendix Fig S3D and E), indicating that upregulation of TRINGS induced by glucose starvation correlates with p53 activation. Expectedly, the elevation of TRINGS in U2OS, U87, Mel-CV, and SMCC7721 cells are diminished under glucose starvation when p53 is depleted (Fig 3A and Appendix Fig S3A–C), indicating the important role of p53 in upregulating TRINGS. Additionally, we showed that glucose starvation induced cell death in U2OS but not in HAFF cells (Fig 3C), and p53 knockdown led to increased cell

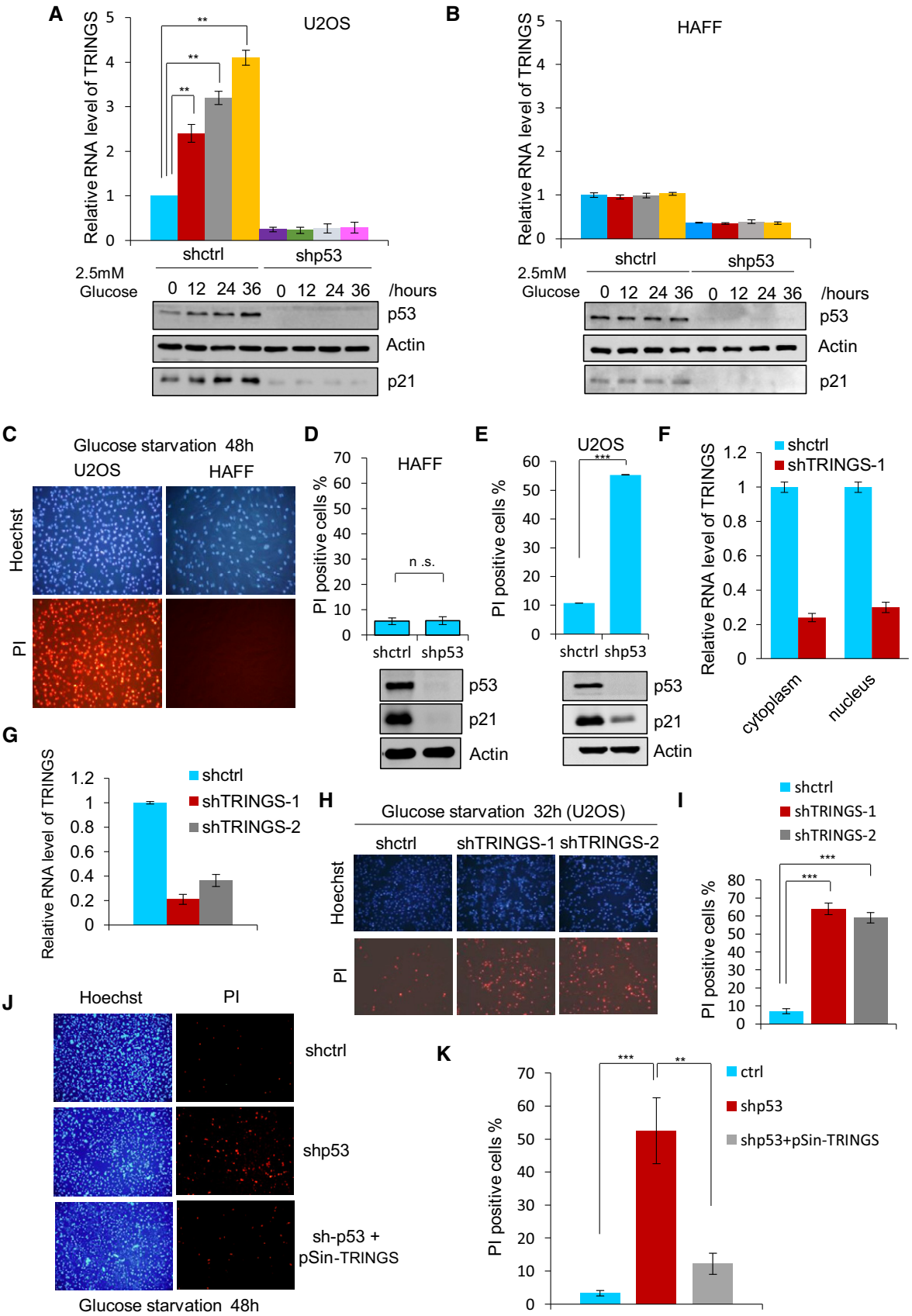

Figure 3.

Figure 3. TRINGS promotes cell survival under glucose starvation condition.

A, B U2OS (A) or HAFF (B) cells were infected with control shRNA or p53 shRNA, and 48 h after infection, cells were cultured in 2.5 mM glucose medium for indicated time. Levels of TRINGS of U2OS (A) or HAFF (B) were analyzed using real-time RT–PCR assay. Data shown are mean ± SD (n = 3) and ** indicates $P < 0.01$. Cell lysates were also analyzed by Western blotting with anti-p53, anti-p21, and anti-Actin antibodies.

C Fluorescence microscopy images of U2OS and HAFF cells cultured in 2.5 mM glucose starvation medium for 48 h and followed by double staining with PI and Hoechst 33342.

D, E HAFF (D) or U2OS (E) cells treated with lentivirus encoding control shRNA or p53 shRNA (shp53) were cultured in 2.5 mM glucose medium for 72 and 48 h respectively. Cells were then stained with PI and Hoechst 33342. The extent of cell death was expressed as the percentage of PI-positive cells to Hoechst 33342-positive cells. Bars represent means ± SD of three independent experiments, and *** indicates $P < 0.001$. Cell lysates were analyzed by Western blotting with indicated antibodies.

F The knockdown efficiency of TRINGS in the cytosol or nucleus was detected by real-time RT–PCR. Data shown are mean ± SD (n = 3).

G–I Lentiviral infected U2OS cells containing control shRNA (shctrl), shTRINGS-1, or shTRINGS-2 were cultured in 2.5 mM glucose medium for 36 h. Cells were then stained with PI and Hoechst 33342 and visualized by fluorescence microscope (H); PI and Hoechst 33342 double staining cells were considered as necrotic cells, and PI staining cells were counted under microscope from 300 Hoechst 33342 staining cells. The percentage of necrotic cells in both TRINGS and control knockdown cells was plotted. Bars represent means ± SD of three independent experiments, and *** indicates $P < 0.001$ (I); the efficiency of TRINGS knockdown was analyzed by real-time RT–PCR assay. Data shown are mean ± SD (n = 3) (G).

J, K Lentiviral infected U2OS cells containing control shRNA (shctrl), shp53 or shp53 plus pSin-TRINGS were cultured in 2.5 mM glucose medium for 48 h. Cells were then stained with PI and Hoechst 33342 and visualized by fluorescence microscope (J). PI staining cells were counted under microscope from 300 Hoechst 33342 staining cells. The percentage of PI-positive cells in both TRINGS and control knockdown cells was then quantified. Bars represent means ± SD of three independent experiments, and ** indicates $P < 0.01$, *** indicates $P < 0.001$ (K).

Data information: (A, E, I, K) Student's t-test.

death in U2OS but not in HAFF cells under glucose starvation (Fig 3D and E), indicating the protective function of p53 for cell survival occurs predominately in cancer cells, and whether p53 can protect other type of cells from glucose starvation-mediated cell death through TRINGS awaits further investigation. Consistently, TRINGS knockdown, which resulted in remarkable decrease in both cytoplasm and nucleus fractions (Fig 3F and G), also led to increased cell death as evidenced by more positive propidium iodide (PI) staining upon low glucose treatment in four examined cancer cells (Fig 3H and I, and Appendix Fig S4A–C). Moreover, overexpression of TRINGS was shown to rescue p53 knockdown-induced cell death under glucose starvation (Fig 3J and K). TRINGS knockdown specificity was also verified, and silencing of TRINGS showed no effect on three identified p53-inducible lncRNAs (Appendix Fig S3F). These results suggest that TRINGS is a survival factor in cancer cells.

To clarify the type of cell death induced by TRINGS knockdown, we first checked whether apoptotic cell death is involved. The cleavage of PARP and caspase-3 was not observed in the cell death induced by TRINGS knockdown or glucose starvation as was seen in Dox-treated cells (Fig 4A, lane 2). Moreover, the cell death was not inhibited by the treatment of pan caspases inhibitor Z-VAD-FMK (Fig 4B). These results demonstrate that cell death induced by glucose starvation is not apoptosis. TRINGS depletion-caused cell death does not appear to be classical necroptosis, ferroptosis, or autophagic cell death, since none of Nec1 (necrostatin-1, necroptosis inhibitor), DFO (iron chelator deferoxamine, ferroptosis inhibitor), or CQ (Chloroquine, autophagy inhibitor) was able to prohibit the cell death (Fig 4C). Finally, to check whether TRINGS depletion-induced cell death type is necrosis, we examined the ATP depletion and HMGB1/lactate dehydrogenase (LDH) release. Upon

Figure 4. Glucose starvation causes necrotic cell death in U2OS.

A U2OS cells were treated with DMSO (control) or Doxorubicin (Doxo) for 16 h; U2OS cells expressing control shRNA, TRINGS shRNA-1, shRNA-2 were cultured in 25 mM glucose (mock) or 2.5 mM glucose medium (glucose starvation) for 32 h. Cell lysates were then analyzed by Western blot analysis using anti-PARP, anti-Caspase3, or anti-Actin antibodies.

B U2OS cells expressing control shRNA or TRINGS shRNA-1 were cultured in 2.5 mM glucose medium with or without apoptosis inhibitor Z-VAD (20 μM) for 32 h, and cells were then stained with PI and Hoechst 33342. PI staining cells were then counted from 300 Hoechst 33342 staining cells; bars represent means ± SD of three independent experiments.

C Control and TRINGS knockdown U2OS cells were treated with DMSO (mock), Nec1, DFO, or CQ respectively, and cells were then stained with PI and Hoechst 33342. PI staining cells were counted in 300 Hoechst 33342 staining cells; bars represent means ± SD of three independent experiments.

D Control and TRINGS knockdown U2OS cells were cultured in 2.5 mM glucose starvation medium for 32 h. Cells were then harvested, and the intracellular levels of ATP were determined by ATP Assay Kit (Beyotime) according to the manufacturer's protocol (***$P < 0.001$; error bars are ±SE, n = 3).

E Control and TRINGS knockdown (shTRINGS-1 and shTRINGS-2) U2OS cells were incubated with 2.5 mM glucose starvation medium for 32 h. Both culture medium and cell lysates were then analyzed by Western blotting using anti-HMGB1 antibody. HMGB1-Ex indicates extracellular HMGB1 and HMGB1-In indicates intracellular HMGB1.

F Control and TRINGS knockdown (shTRINGS-1 and shTRINGS-2) U2OS cells were incubated with 2.5 mM glucose starvation medium for 32 h. Lactate dehydrogenase (LDH) release was then used to test natural cytotoxicity of treated cells by LDH Cytotoxicity Assay Kit (Promega) according to the manufacturer's protocol (***$P < 0.001$; error bars are ±SE, n = 3).

G Necrosis of U2OS cells under glucose starvation conditions was observed under transmission electron microscope at the micrometer scale. The left are U2OS cells without glucose starvation.

H Control and RIP1 knockdown (shRIP1-1, shRIP1-2, or shRIP1-3) U2OS cells were incubated with 2.5 mM glucose starvation medium for 48 h. Cells were then stained with PI and Hoechst 33342. PI staining cells were counted in 300 Hoechst 33342 staining cells. Bars represent means ± SD of three independent experiments. Cell lysates were analyzed by Western blotting using anti-RIP1 and anti-Actin antibodies. n.s. indicates no significance.

Data information: (B–D, F, H) Student's t-test.

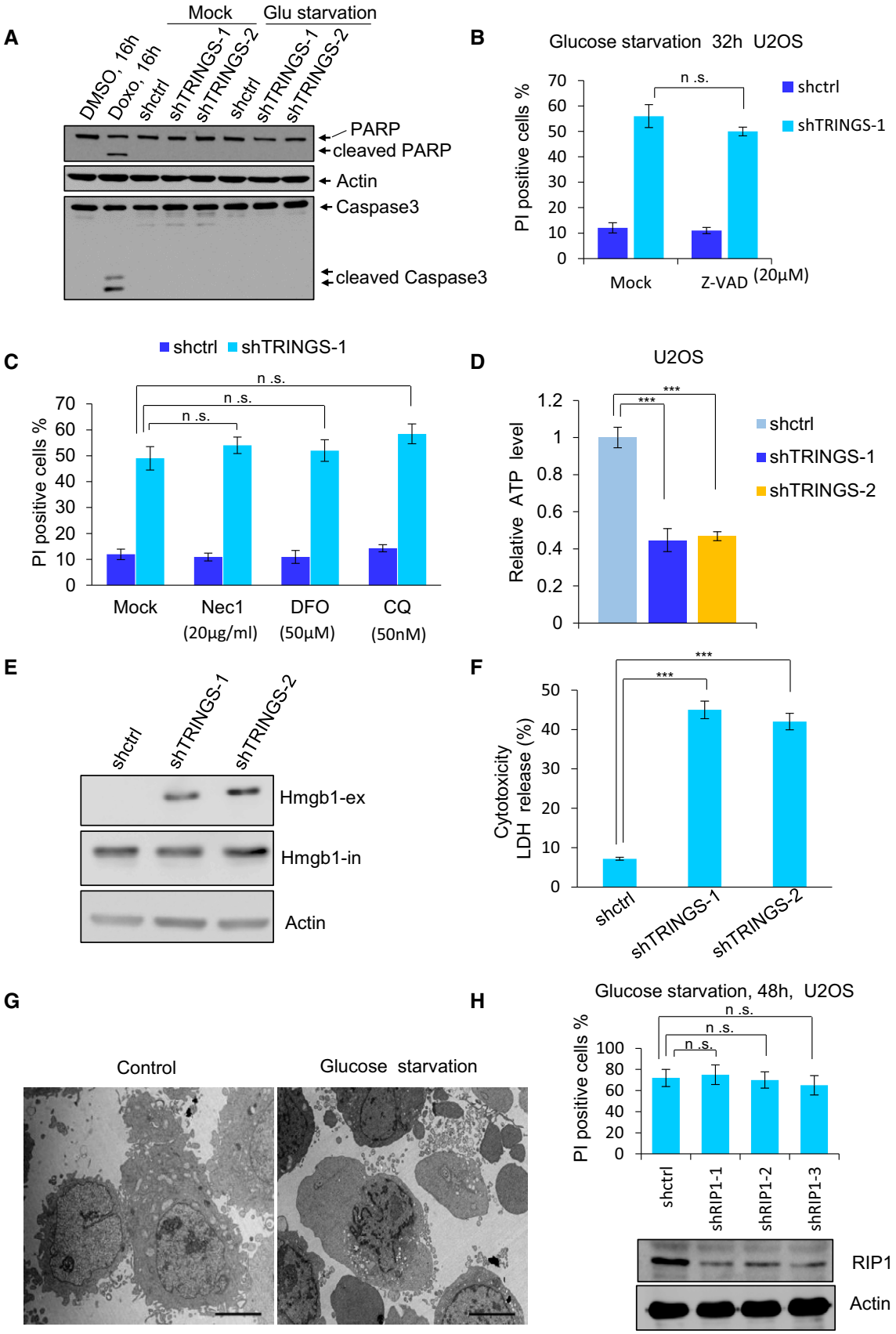

Figure 4.

glucose starvation, TRINGS knockdown led to a significant decrease in intracellular ATP level (Fig 4D), and remarkable increase in HMGB1 and LDH release (Fig 4E and F), indicating that the TRINGS depletion caused necrotic cell death under glucose deprivation. We further determined glucose starvation induced necrotic cell death by transmission electron microscopy (TEM). Membrane destruction and loss of electron density in the cytosol characterized as necrotic phenotype were observed (Fig 4G), further suggesting that TRINGS knockdown-induced cell death belongs to necrosis. RIP1 is a crucial mediator of programmed necrosis (Cho *et al*, 2009). We additionally demonstrated that TRINGS knockdown-induced necrosis is RIP1 independent, since cell death was not affected when RIP1 was depleted (Fig 4H). Moreover, U2OS cells express little if any RIP3 (He *et al*, 2009), thus, the necrosis induced by TRINGS knockdown cannot be RIP1/RIP3/MLKL-mediated necroptosis. To further confirm the effect of TRINGS on necrosis, H_2O_2 with high concentration (1 mM) was used as necrosis inducer (Saito *et al*, 2006). TRINGS was observed to be induced in response to H_2O_2 treatment (Appendix Fig S4D). TRINGS knockdown U2OS cells exhibited remarkable necrosis by treating with H_2O_2 (Appendix Fig S4E and F), suggesting TRINGS also inhibits H_2O_2-induced necrotic cell death. Taken together, these results suggest that TRINGS plays a protective role in cancer cells against necrosis induced by glucose starvation or H_2O_2.

To test whether TRINGS affects cell proliferation or tumor growth *in vivo*, cell proliferation assay was measured by counting cell numbers and anchorage-independent growth in soft agar was performed. Control and TRINGS knockdown cells were assessed individually under normal growth condition. Reduced cell growth (Appendix Fig S5A) and decreased cell colony formation in soft agar (Appendix Fig S5B) were observed in TRINGS knockdown cells. In addition, control or TRINGS knockdown U2OS cells, which were pools rather than clones, were injected subcutaneously into nude mice for investigation of tumor growth *in vivo*. Importantly, TRINGS knockdown resulted in marked retardation of tumor growth *in vivo* (Appendix Fig S5C). These data suggest that TRINGS knockdown inhibits cell proliferation both *in vitro* and *in vivo*.

TRINGS physically interacts with STRAP

Since knockdown of TRINGS led to increased cell death under glucose starvation, we then focused on the investigation of the mechanism behind the role of TRINGS in protection against necrosis. A biotin-labeled RNA pull-down assay followed by mass spectrometric analysis was performed to identify the proteins that might interact with TRINGS (Fig 5A and Dataset EV1). One of these proteins, named STRAP, had caught our attention, since it has been reported to be involved in regulation of cell death (Seong *et al*, 2014). To further confirm the interaction between TRINGS and STRAP, a biotin pull-down assay was performed and TRINGS was observed to interact with STRAP (Fig 5B). In addition, ectopically expressed Flag-STRAP was shown to co-precipitate with TRINGS approximately 4.3-fold more than Flag alone (Fig 5C). These results suggest that STRAP represents a TRINGS binding protein. We also compared the STRAP–TRINGS interactions in both stressed (glucose starvation) and unstressed cells by IP experiment. More STRAP–TRINGS interactions were detected in stressed than in unstressed cells (Fig 5D). Therefore, glucose starvation results in increased

Figure 5. TRINGS interacts with STRAP.

A U2OS cells lysates were incubated with *in vitro*-synthesized biotin-labeled antisense and control sense DNA probes against TRINGS, and biotin pull-down assay was performed. Precipitated complexes from biotin pull-down assay were separated by SDS–PAGE and visualized by Coomassie brilliant blue staining. Gel-separated proteins were analyzed by MALDI mass spectrometry. Putative RNA-binding proteins were indicated.

B U2OS cells lysates were incubated with *in vitro*-synthesized biotin-labeled antisense and control sense DNA probes against TRINGS, and biotin pull-down assay was performed. The pull-downed complexes were analyzed by Western blot analysis using anti-p53, anti-STRAP, anti-CIP2A, and anti-Actin antibodies; the same complexes were also used for semi-quantitative PCR analysis to ensure the presence of TRINGS.

C Lysates from U2OS cells expressing Flag or Flag-STRAP were used for co-immunoprecipitation (co-IP) using anti-Flag resin. Eluted products were used for examining TRINGS and STRAP by real-time RT–PCR and Western blot analysis respectively (***$P < 0.001$; error bars are ±SE, $n = 3$).

D Lysates from U2OS cells under normal condition or glucose starvation (2.5 mM, 32 h) were used for co-immunoprecipitation (co-IP) using IgG or anti-STRAP coupled resin. Eluted products were used for examining TRINGS and STRAP by real-time RT–PCR and Western blotting analysis respectively. The intensity of STRAP bands was quantified by densitometry analysis (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; error bars are ±SE, $n = 3$).

E Western blot analysis of STRAP in control and TRINGS knockdown U2OS cells. Actin was served as protein loading control. The intensity of STRAP bands was quantified by densitometry analysis.

F Total RNA from control or TRINGS knockdown U2OS cells was analyzed by real-time RT–PCR to determine the STRAP mRNA levels and validate the knockdown efficiency of TRINGS as well. Data shown are mean ± SD ($n = 3$).

G U2OS cells were infected with dCas9-KRAB and indicated guide RNA lentiviruses. After induction with doxycycline (1 μg/ml) for 48 h, lysates was analyzed by Western blotting.

H Control or TRINGS knockdown U2OS cells were treated with 50 μg/ml of cycloheximide (CHX) for indicated times. Subsequently, cell lysates were analyzed by Western blotting with anti-STRAP and anti-Actin antibodies.

I Control or TRINGS overexpression U2OS cells were treated with 50 μg/ml of cycloheximide (CHX) for indicated times. Subsequently, cell lysates were analyzed by Western blotting with anti-STRAP and anti-Actin antibodies.

J U2OS cells were treated with 50 μg/ml of cycloheximide (CHX) for indicated times. Furthermore, cells were treated with vehicle or MG132 (10 μM) for 4 h before harvesting the samples for protein. Subsequently, cell lysates were analyzed by Western blotting with anti-STRAP and anti-Actin antibodies.

K U2OS cells were transfected with Flag-STRAP and HA-Ub plasmids as indicated in the figure. Cells were treated with MG132 for hours before cell lysis. Ectopically expressed Flag-STRAP was immunoprecipitated with anti-Flag antibody followed by Western blotting with anti-Flag and anti-HA antibodies.

L U2OS cells were transfected with Flag-STRAP, HA-Ub, and pSin-TRINGS in combination as indicated. Cells were treated with MG132 for hours before cell lysis. Ectopically expressed Flag-STRAP was immunoprecipitated with anti-Flag antibody followed by Western blotting with anti-Flag and anti-HA antibodies.

M Control or TRINGS knockdown U2OS cells were transfected with Flag-STRAP, HA-Ub in combination as indicated. Cells were treated with MG132 for hours before cell lysis. Ectopically expressed Flag-STRAP was immunoprecipitated with anti-Flag antibody followed by Western blotting with anti-Flag and anti-HA antibodies.

Data information: (C, D) Student's *t*-test.

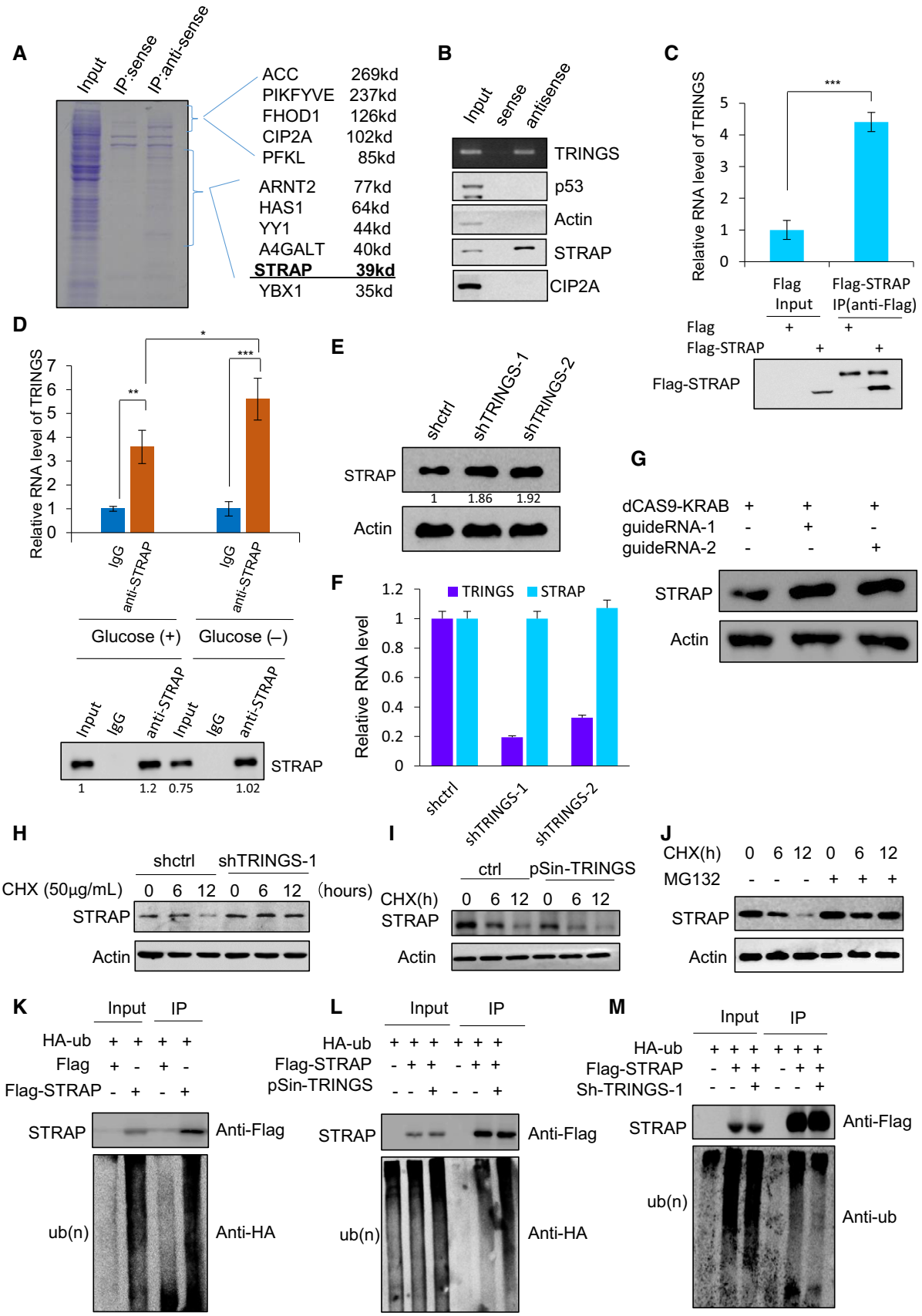

Figure 5.

interaction between STRAP and TRINGS. Additionally, we assessed the TRINGS binding domain in STRAP by co-IP and real-time RT–PCR, and the N-terminal and WD1-7 domains of STRAP are required for TRINGS binding (Appendix Fig S5D). We also examined the subcellular localization of STRAP and found that STRAP is localized largely in the cytoplasm (Appendix Fig S6A). We further explored whether the binding of TRINGS to STRAP affects the protein stability. Knockdown of TRINGS led to an increase in STRAP protein but not the mRNA level (Fig 5E and F). In addition, dCas9-KRAB-mediated TRINGS knockdown also caused an increase in STRAP (Fig 5G). These data indicate TRINGS may reduce STRAP stability through their interaction. To confirm this hypothesis, both control and TRINGS knockdown cells were treated with cycloheximide (CHX) to study the stability of STRAP protein. After CHX treatment for 6 or 12 h, STRAP protein is decreased in control cells but not in shTRINGS cells (Fig 5H), suggesting that STRAP turnover is altered and it became more stable in shTRINGS cells. Reversely, TRINGS overexpression promoted degradation of STRAP (Fig 5I). To further investigate whether the degradation of STRAP is through ubiquitin proteasome-dependent pathway, we treated U2OS cells with proteasome inhibitor MG132 and showed that this treatment did inhibit the degradation of STRAP (Fig 5J). In addition, we performed an ubiquitination assay for STRAP and found the ubiquitylated STRAP was much increased upon TRINGS overexpression (Fig 5K and L) and was clearly decreased when TRINGS was knocked down (Fig 5M). These results suggest that TRINGS-mediated STRAP destabilization is through ubiquitin proteasome pathway.

Combined, these data suggest that TRINGS destabilizes STRAP. The detailed mechanism underlying this is still unclear. Moreover, STRAP remained unchanged upon p53 knockdown or overexpression under normal condition (Appendix Fig S6B–D). However, under low glucose treatment, p53 knockdown led to increased protein level of STRAP (Appendix Fig S6E). Thus, in response to glucose starvation, p53 knockdown stabilizes STRAP through down-regulation of TRINGS.

TRINGS-promoted cell survival under glucose starvation is mediated via STRAP–GSK3β–NF-κB axis

STRAP is reported to regulate various biological processes by interacting with cellular proteins such as p53 and GSK3β (Datta *et al*, 1998; Seong *et al*, 2014). Since knockdown of TRINGS results in upregulation of STRAP (Fig 5E), we then explored the role of STRAP in TRINGS-regulated cell survival. As expected, cell death induced by TRINGS depletion under glucose starvation is markedly rescued by STRAP knockdown (Fig 6A); in addition, shTRINGS-induced necrosis under H_2O_2 treatment was rescued by further STRAP knockdown (Appendix Fig S4F). These data suggest that STRAP is required for necrotic cell death induced by TRINGS depletion under glucose starvation.

STRAP is required for GSK3β function in recruiting its downstream substrates (Kashikar *et al*, 2011). We firstly confirmed that STRAP interacts with GSK3β by co-IP assay (Fig 6B). Biotin pull-down assay also showed TRINGS interacts with STRAP but not GSK3β (Fig 6C). Moreover, the increased interaction between STRAP and GSK3β was detected when TRINGS was silenced (Fig 6B and Appendix Fig S6F, upper panel, lane 6 vs. lane 8), indicating the TRINGS and GSK3β compete to interact with STRAP. We then performed co-IP assay with anti-GSK3β antibody and found that glucose starvation decreases the interaction between STRAP and GSK3β (Appendix Fig S6G), implying that glucose starvation-induced TRINGS impairs the interaction between STRAP and GSK3β. In addition, we performed co-IP assay in Flag-GSK3β overexpressed and control cells using anti-STRAP antibody, and TRINGS

Figure 6. TRINGS promotes cell survival through STRAP–GSK3β–NF-κB signaling.

A U2OS cells infected with control shRNA (shctrl), TRINGS shRNA (shTRINGS), TRINGS shRNA plus STRAP shRNA (shTRINGS plus shSTRAP), or STRAP shRNA (shSTRAP) were incubated with 2.5 mM glucose starvation medium for 32 h. Cells were then stained with PI and Hoechst 33342 and visualized by fluorescence microscope. PI-positive cells were counted from 300 Hoechst 33342-positive cells. Bars represent means ± SD of three independent experiments, ** indicates $P < 0.01$. Cell lysates were analyzed by Western blotting using anti-STRAP antibody.

B Control or TRINGS knockdown U2OS cells lysates were used for co-immunoprecipitation assay with anti-STRAP coupled resin. The input and co-immunoprecipitates products were analyzed by Western blotting using anti-STRAP or anti-GSK3β antibodies. The intensity of STRAP-binding GSK3β bands was quantified by densitometry analysis.

C Lysates of U2OS cells were incubated with *in vitro*-synthesized biotin-labeled sense or antisense DNA probes against TRINGS, and then, biotin pull-down assay was performed. The precipitates were then analyzed by RT–PCR using primers against TRINGS and by Western blotting using anti-STRAP, anti-GSK3β, or anti-RelA antibodies.

D Control shRNA and STRAP shRNA knockdown U2OS cells were cultured in DMEM medium (high glucose) or 2.5 mM glucose starvation medium for 32 h. Cell lysates were then analyzed by Western blot analysis using anti-STRAP, anti-p-Gsk3β (ser 9), anti-GSK3β, and anti-Actin antibodies. The p-Gsk3β (ser 9) bands were quantified by densitometry analysis.

E U2OS cells expressing control shRNA, TRINGS shRNA, or TRINGS shRNA plus STRAP shRNA were cultured in DMEM medium (high glucose) or 2.5 mM glucose starvation medium for 32 h. Cell lysates were then analyzed by Western blotting using anti-p-Gsk3β (ser 9), anti-GSK3β, anti-STRAP, or anti-Actin antibodies. The p-Gsk3β (ser 9) bands were quantified by densitometry analysis.

F Control shRNA (shctrl) or TRINGS shRNA (shTRINGS) U2OS cells were incubated with 2.5 mM glucose starvation medium with or without 3 μM CHIR99021 (inhibitor of GSK3β activity) for 32 h. Cells were then stained with PI and Hoechst 33342 and visualized by fluorescence microscope. PI-positive cells were counted in 300 Hoechst 33342-positive cells and plotted. Bars represent means ± SD of three independent experiments (** indicates $P < 0.01$).

G Control and TRINGS knockdown U2OS cells were incubated with 2.5 mM glucose starvation medium for 32 h. Cell lysates were then analyzed by Western blot analysis using anti-NF-κB-p50, anti-RelA, and anti-Actin antibodies.

H Cell lysates from control and TRINGS knockdown U2OS cells were analyzed by Western blot analysis using anti-IKBα and anti-Actin antibodies.

I U2OS cells expressing shctrl, shTRINGS or shGSK3β, shTRINGS plus shGSK3β were co-transfected with pGL-NF-κB reporter construct and Renilla luciferase plasmid, respectively. 12 h after transfection, the cells were incubated with 2.5 mM glucose starvation medium. Reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity (mean ± SD). Cell lysates were analyzed by Western blotting using anti-GSK3β and anti-Actin antibodies (* indicates $P < 0.05$, ** indicates $P < 0.01$).

Data information: (A, F, I) Student's *t*-test.

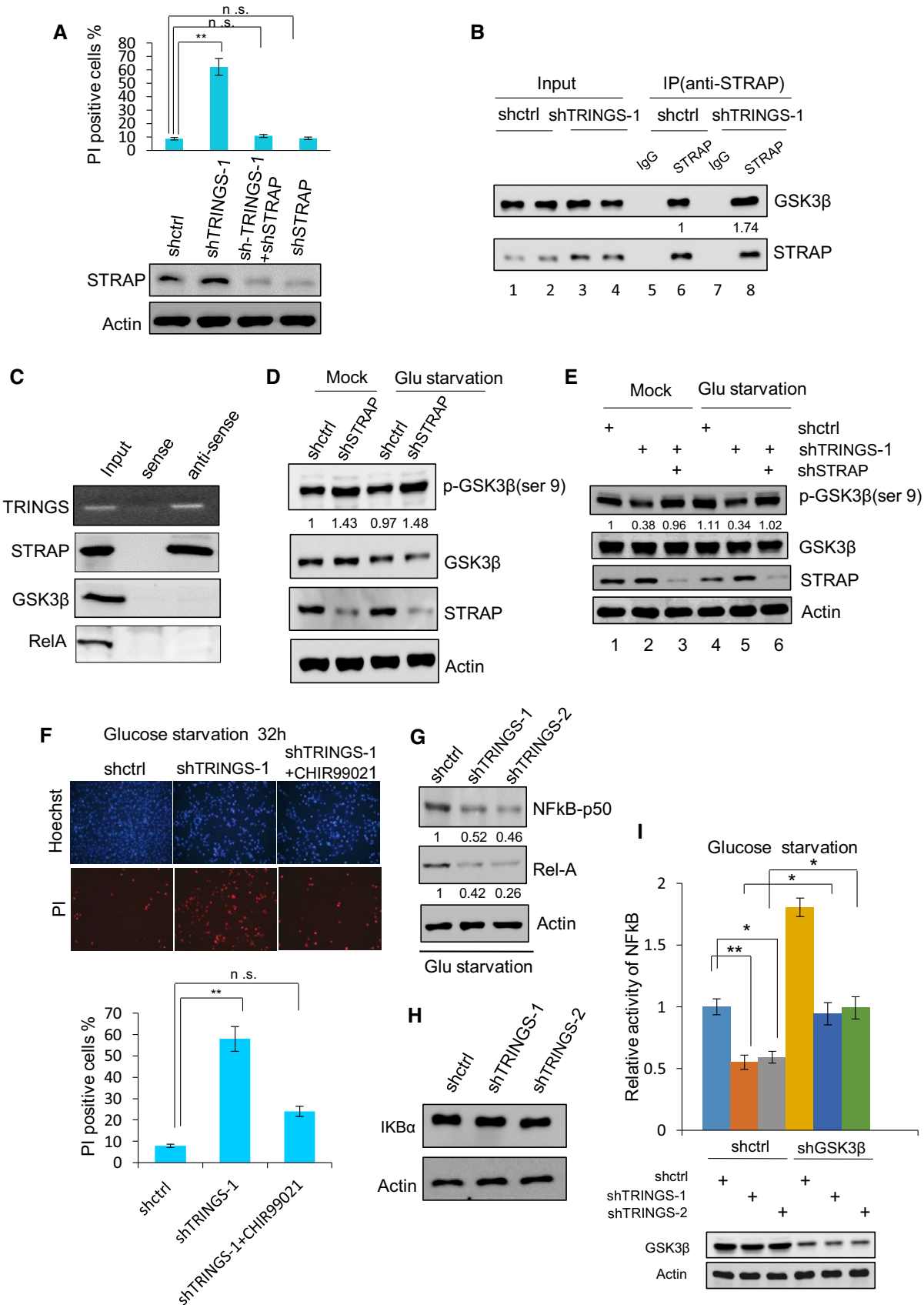

Figure 6.

co-precipitated by STRAP was significantly reduced in GSK3β-overexpressed cells than in control cells (Appendix Fig S6H), suggesting that increased dosage of GSK3β inhibits STRAP-associated TRINGS. Interestingly, knockdown of STRAP caused increase in GSK3β phosphorylation at serine 9 which inhibits GSK3β activity (Fig 6D). These results suggest that the interaction between TRINGS and STRAP may impair GSK3β activity. We therefore examined the effect of TRINGS on GSK3β phosphorylation. TRINGS knockdown remarkably reduced GSK3β phosphorylation at serine 9 (Fig 6E, top panel, compare lane 1 vs. 2, and lane 4 vs. 5), and the reduction was inhibited by further knockdown of STRAP (Fig 6E, compare lane 2 vs. 3, and lane 5 vs. 6), indicating that TRINGS knockdown-induced cell death is through STRAP–GSK3β pathway. We then investigated whether the inhibition of GSK3β activity would block this cell death. Indeed, TRINGS depletion-induced cell death was notably restored by inhibition of GSK3β by treatment of inhibitor of CHIR99021 or CHIR98014 (Fig 6F and Appendix Fig S4G), suggesting that GSK3β is truly the downstream mediator of TRINGS–STRAP–GSK3β death signaling.

The mechanism of GSK3β underlying the regulation of necrosis induced by glucose starvation still remains obscure. It has been reported that the GSK3 activation leads to inhibition of NF-κB activity (Sanchez *et al*, 2003; Bachelder *et al*, 2005), which in turn induces necrosis (Mauro *et al*, 2011). We therefore examined the effect of TRINGS on NF-κB activity. TRINGS knockdown resulted in downregulation of NF-κB subunits p50 and RelA (NF-κB p65) protein levels under glucose starvation (Fig 6G), but TRINGS knockdown showed no effect on stability of IKBα which is an inhibitor of NF-κB (Fig 6H), indicating TRINGS-mediated necrosis may not be through IKBα. Furthermore, NF-κB luciferase activity was clearly decreased by TRINGS depletion and partly restored when GSK3β was depleted (Fig 6I). In addition, TRINGS failed to interact with RelA (Fig 6C), indicating that TRINGS is unlikely to affect NF-κB activity directly. These results suggest that TRINGS regulates NF-κB activity through STRAP–GSK3β cascade. Taken together, TRINGS prevents cancer cells from necrosis under glucose starvation through inhibiting STRAP–GSK3β–NF-κB signaling pathway. Thus, shTRINGS-induced necrosis is programmed and is a non-canonical necroptosis.

Discussion

In this study, we found that p53 transcriptionally upregulates lncRNA TRINGS under glucose starvation condition. Furthermore, loss of TRINGS remarkably promotes glucose starvation-induced necrosis of several cancer cells. In addition, TRINGS was found to interact with STRAP and protect cancer cells from necrosis by inhibiting a new STRAP–GSK3β–NF-κB necrotic signaling pathway. Thus, the role of TRINGS in defending cancer cells against necrosis provides novel insight into an unexpected role of p53 for cancer cell survival. Liu *et al* (2013) reported that lncRNA loc285194 is induced by p53 and inhibits tumor cell growth. Here, we identified an lncRNA TRINGS, which was positively regulated by p53. Interestingly, by Western blotting analysis, we found that expression of TRINGS in different cell lines does not entirely reflect the p53 level. This observation is not too surprising, since p21 (WAF1/CIP1), a p53 tightly regulated target gene, also failed to exhibit a perfect corresponding relationship with p53 level (Fig 1C). Mizuta *et al* (2013) had reported that this lncRNA gene is positively regulated by YY1, despite the underlying mechanism is not clear. We did knock down YY1 and found it showed no effect on cell death under glucose starvation conditions (data not shown). But one thing is for sure, TRINGS expression will not be induced in p53 null cells upon glucose starvation. In addition, the binding affinity between p53 and TRINGS promoter is observed to be weaker than that between p53 and p21 promoter (Fig 2E). The Cancer Genome Atlas (TCGA) is a coordinated and comprehensive effort to improve our understanding of the molecular basis of cancer. TCGA data provide valuable insights into the underlying genetic and genomic basis of cancer. By analyzing eight types of tumor samples from TCGA, we found that tumor samples harboring wild-type p53 display higher level of TRINGS than tumor samples with mutant p53 (Appendix Fig S2A), further confirming the conclusion that the expression of TRINGS is positively regulated by wild-type p53. Of note, Ashouri *et al* recently identified a lncRNA RP11-115D19.1 (we named it as TRINGS), which is negatively associated with p53 mutation, and exhibited reduced transcription in p53 null cells as compared to p53 wild-type cells. In addition, expression for this lncRNA (TRINGS) was similarly seen to be reduced in p53-mutated tumor samples (Ashouri *et al*, 2016).

It is interesting to note that although p53 can be activated in response to diverse nutritional stresses such as FBS deprivation, serine deprivation, glutamine deprivation (Maddocks *et al*, 2013), and glucose starvation, yet only glucose starvation-induced p53 is able to upregulate TRINGS (Fig 3A and Appendix Fig S2B–E). Moreover, although p53-activated TRINGS expression responds to nutritional, oxidative, or genotoxic stress, yet only glucose- and oxidative-stress-induced upregulated TRINGS will affect cell death. As doxorubicin-upregulated TRINGS has no effect on apoptosis induced by this genotoxic drug, and similarly, the pharmacological p53 activator Nutlin3-induced TRINGS was unable to affect cell viability (Appendix Fig S7A and B). These results suggest that TRINGS is necessary but not sufficient to protect the cell survival. Moreover, TRINGS protects against necrosis and does not protect against apoptosis (Fig 4A and B), RIP1/RIP3/MLKL-mediated necroptosis, ferroptosis, or autophagic cell death (Fig 4C and H) upon glucose stress. The underlying mechanism of this phenomenon is still unclear. However, this may suggest that p53-mediated upregulation of TRINGS specifically participates in a new programmed necrosis pathway. Thus, TRINGS acts as a mediator linking p53 and necrosis. In addition, TRINGS binds to STRAP and inhibits STRAP–GSK3β–NF-κB necrotic signaling (Fig 7). These findings provide a signaling pathway for p53 in regulating necrosis which involves a long noncoding RNA.

Metabolic pathway reprogramming is a hallmark of cancer cell growth, and our findings support the notion that cancer cells may alter their metabolism in order to maintain cellular proliferation and survival in unfavorable conditions. As described by Otto Warburg, the energy in cancer cells is preferentially generated by glycolytic pathways (Warburg *et al*, 1924; Warburg, 1956). Here, we showed that besides its tumor suppressor function, p53 also helps cancer cells to survive glucose starvation by upregulating a long noncoding RNA TRINGS.

p53 is frequently mutated in many types of cancer, and mutant p53 protein not only exhibits loss of WT p53 function but also

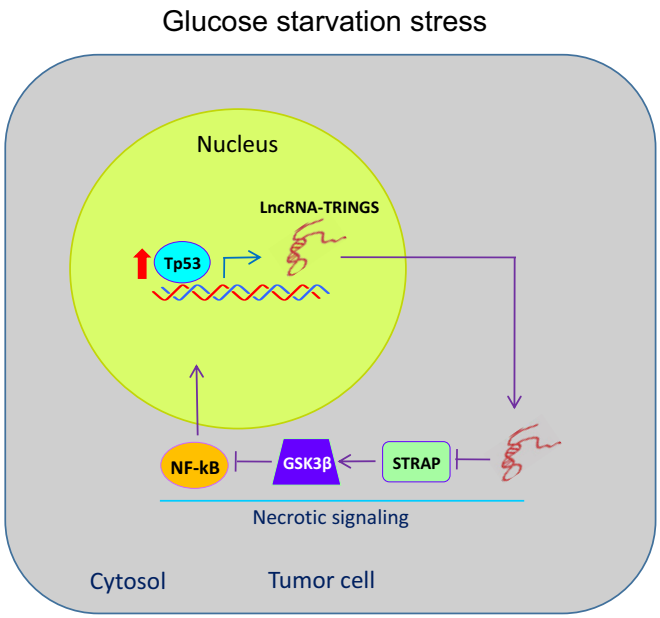

Figure 7. The mode of lncRNA TRINGS in protecting cancer cells from necrosis induced by glucose starvation.

TRINGS is activated by p53 upon glucose starvation. Upregulated TRINGS competes with GSK3β for binding to STRAP, and thus diminishes the interaction between STARP and GSK3β, and eventually TRINGS activates NF-κB to protect cancer cells against necrosis.

often gains new oncogenic activities favoring cancer development (Caron de Fromentel & Soussi, 1992; Chao *et al*, 2003). However, about half of cancer cells still harbor WT p53 and some tumor cells even show high levels of p53 expression which appears to be in contradiction with the definition of tumor suppressor. Whether WT p53 in the remaining 50% tumor cells is tumor-preventing or tumor-provoking is as yet an unanswered question. Recently, some researchers found that p53 acts as an anti-apoptotic factor under certain circumstances (Feng *et al*, 2011). However, there is still no report on p53-dependent lncRNA(s) in protecting cancer cells against necrosis. Here, we found a long noncoding RNA TRINGS that is induced in response to p53 activation by glucose starvation. It competes with GSK3β for binding to STRAP, leading to increased phosphorylation of GSK-3β (Ser9) with still unknown mechanism. Increased phospho-GSK3β (Ser9; less active form) stabilizes NF-κB and thus protects cancer cells from necrosis (Fig 7). Importantly, we have uncovered an intricate mechanism by which WT p53 protects some tumor cells from necrosis via regulating a long noncoding RNA, which then elicits a novel NF-κB signaling. This knowledge will expand our understanding of the non-canonical pathway involved in p53-mediated tumorigenesis.

Materials and Methods

Reagents and antibodies

The following reagents and antibodies used in this study were purchased from the indicated sources: CHIR99021 (Millipore), CHIR98014 (Selleck), Doxorubicin (Sigma), Doxycycline (Sigma), Cycloheximide (Sigma), Propidium iodide (Sigma), Nutlin-3 (Selleckchem), PFT-α (Sigma), ATP Assay Kit (Beyotime), LDH Release Assay Kit (Promega), DMEM without Glucose or Glutamine (Gibco), Z-VAD-FMK (Santa Cruz), Chloroquine/CQ (Sigma), Deferoxamine/DFO (Sigma), Necrostatin-1/Nec-1 (Sigma), anti-Actin (Cell Signaling), anti-STRAP (Proteintech), anti-CIP2a (Santa Cruz), anti-p53 (Santa Cruz), anti-p21(Sigma), anti-PARP (Santa Cruz), anti-Caspase3 (Cell Signaling), anti-GSK3β (Santa Cruz), anti-p-GSK3β (Cell Signaling), anti-Flag (Sigma), anti-HMGB1 (Proteintech), anti-RelA (Proteintech), anti-NF-κB-p50 (Proteintech), anti-IKBα (Proteintech), anti-RIP1 (Boster), HRP-conjugated secondary antibodies against mouse and rabbit IgG (Promega). The primers and oligo probes used in this study are listed in Appendix Table S1.

Cell culture and transfection

H1299, 293T, HCT116, U2OS, HAFF (human adult foreskin fibroblast), MEL-CV, SMCC-7721, U87, Mel-CV, HepG2, and IMR90 cell lines (human lung normal fibroblast cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; with glucose concentration of 25 mM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% sodium pyruvate at 37°C with 5% CO_2. For glucose starvation conditions, DMEM (without glucose, GIBCO 11966025) was supplemented with pure glucose (Gibco A2494001) to make final concentration of 2.5 mM. Lipofectamine 2000 and Opti-MEM I (Invitrogen) were used for transient transfection according to the manufacturer's protocol. MCF10A cells (human mammary normal epithelial cells) were cultured in DMEM/F12 medium containing 5% horse serum, 20 μg/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml CholeraToxin, and 10 μg/ml insulin. Preparations of DMEM medium deprived of FBS, serine, or glutamine were performed according to previously published methods (Maddocks *et al*, 2013).

RNA interference and TRINGS overexpression

To generate lentiviruses expressing shRNAs, 293T cells grown on a 6-cm dish were co-transfected with pLKO.1-shRNA (2 μg), pREV (2 μg), pGag/Pol/PRE (2 μg), and pVSVG (1 μg). 48 h after transfection, the supernatant was collected. U2OS cells were incubated with the medium containing lentivirus particles supplemented with 8 μg/ml polybrene (Sigma). After 12 h, lentivirus-containing medium was replaced by fresh medium. The knockdown efficiency was evaluated by Western Blotting or real-time RT–PCR analysis. For overexpression of TRINGS, full-length TRINGS (602 bp) was cloned into pSin vector and the resultant pSin-TRINGS was transfected into 293T cells for lentivirus production, and U2OS cells were then infected with lentivirus encoding TRINGS for TRINGS overexpression.

Real-time RT–PCR

Total RNA was isolated by TRIzol reagent (Invitrogen). 1 μg of RNA was used to synthesize cDNA using the first-strand cDNA Synthesis System (Marligen Biosciences). Real-time PCR was performed using SYBR Green real-time PCR analysis (Takara) with the specific primers (Appendix Table S1). PCR results,

recorded as cycle threshold (Ct), were normalized against an internal control (β-actin).

Western blot analysis and co-immunoprecipitation

Western blot analysis was performed as we have described previously (Zhang *et al*, 2014). For co-immunoprecipitation, cells were lysed in IP lysis buffer (0.5% NP-40, 150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM EDTA, and 1.5 mM $MgCl_2$) supplemented with protease inhibitor cocktail for half an hour on ice. Cell lysates were incubated with protein A/G-sepharose beads coated with the indicated antibodies for 4 h at 4°C, and then, the IP products were washed three times by IP lysis buffer; after that, the eluted products were boiled at 95°C for 10 min, and the protein extracts were analyzed on SDS–PAGE followed by Western blot analysis.

Luciferase assay

To determine the effect of p53 on TRINGS promoter, U2OS cells were transfected with control plasmid or Flag-p53 together with the pGL3-based construct containing TRINGS promoter plus Renilla luciferase plasmid. 24 h later, the reporter activity was measured by using a luciferase assay kit (Promega) and plotted after normalizing with respect to Renilla luciferase activity (mean ± SD).

Biotin pull-down assay

All processes were performed in the RNase-free conditions. For antisense oligomer affinity pull-down assay, sense (negative control) and antisense biotin-labeled DNA oligomers corresponding to human TRINGS (1 μg) were incubated with streptavidin-coupled Dynabeads (Invitrogen). One hour after incubation, the Dynabeads were washed and subsequently incubated with lysates from 2×10^7 U2OS cells for further 4 h to isolate the RNA–protein complex. The retrieved proteins were subjected to SDS–PAGE. The gel was then stained using Coomassie Brilliant Blue G-250 Dye according to the manufacturer's instructions. After Coomassie staining, specific bands were cut and sent to Core Facility of Molecular Biology (Institute of Biochemistry and Cell Biology, Shanghai, CAS) for mass spectrometry (MS) analysis by Thermo-Finnigan LTQ LC/MS-MS. Identified proteins by MS are listed in Dataset EV1.

ChIP assay

U2OS cells were crosslinked with 1% formaldehyde for 10 min. Cells were then harvested and lysed by RIPA buffer, and the lysates were sheared by sonication to produce fragments of 300–1,000 base pairs (bp) in length. Lysates were centrifuged for 10 min using a refrigerated ultracentrifuge at 12,000 *g* at 4°C, and the supernatant was collected in a clean tube and the pellet was then discarded. ChIP assay was performed by using anti-STRAP antibody and the Pierce Agarose ChIP kit (Thermo Scientific, USA) according to the manufacturer's instructions. Anti-Rabbit immunoglobulin G was also used as a negative control. The bound DNA fragments were subjected to real-time PCR using the specific primers (Appendix Table S1).

Ubiquitination assay

U2OS cells were cultured in 10-cm dish plate and transfected with relevant plasmids. Four hours prior to cell harvesting for ubiquitination assay, MG132 was added at the concentration of 10 μM. Ubiquitination assay was performed according to previous report (Choo & Zhang, 2009) with little modifications. Simply, cells were washed with cold PBS three times. 200 μl of lysis buffer (RIPA buffer, Beyotime P0013B) was added to the cells, and with the help of cell scrapper, cells were collected into 1.5-ml Eppendorf tube. Immediately, cells were heated for 10 min at 95°C, followed by sonication. Then, cell lysate was diluted with 800 μl of dilution buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton) and incubated at 4°C rotator for 30 min. After incubation, lysate was centrifuged at 20,000 *g* for 20 min at 4°C. Supernatant was transferred into Eppendorf tubes containing M2 beads and incubated at 4°C rotator for 4 h. Beads were centrifuged at 1,000 *g* for 1 min and washed five times with washing buffer (10 mM Tris–HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1% NP-40), followed by Western blot analysis.

dCas9-KRAB experiment

293T cells were cultured in 6-cm dish for overnight, followed by transfection with pHAGE TRE dCas9-KRAB (Addgene-50917) plasmid and packaging plasmids psPAX2, pMD2.G at the ratio of 3:2:1. Similarly, guide RNAs cloned in pLKO.1-puro U6 sgRNA vector (Addgene-50920) were packaged with pREV, pGag/Pol/PRE, and pVSVG plasmids at the ratio of 2:2:2:1. Viruses were harvested at 48 h of post-transfection. U2OS cells were incubated with the medium containing lentivirus particles supplemented with 8 μg/ml polybrene (Sigma). After 12 h, lentivirus-containing medium was replaced by fresh medium. Subsequently, cells were selected with G418 (1 mg/ml) and puromycin (5 μg/ml), followed by induction with doxycycline for 48 h. The knockdown efficiency was evaluated by real-time RT–PCR analysis.

TCGA data

TRINGS expression analysis in tumors with wild-type p53 and mutant p53 was analyzed from the TCGA datasets thorough online TANRIC database tool (Li *et al*, 2015).

Northern blotting

Equal amounts of total RNAs collected from U2OS cells treated with or without doxorubicin were re-solved on 1.5% agarose gels, and Northern blot was carried out as described previously (Zhang *et al*, 2013). Digoxigenin-labeled antisense TRINGS antisense probe was made using T7 RNA polymerases by IVT with the DIG Northern Starter Kit (Roche).

RNA *in situ* hybridization

RNA FISH was carried out as described previously (Yin *et al*, 2012). Hybridization was carried out using *in vitro*-transcribed antisense probe labeled with Alexa Fluor® 488 (ULYSIS® Nucleic Acid Labeling Kits, U21650). The nuclei were counterstained with

Hoechst. The fluorescence images were taken by confocal microscopy.

Cytosolic/nuclear fractionation

1×10^7 U2OS cells were incubated with hypotonic buffer (25 mM Tris–HCl, PH 7.4, 1 mM $MgCl_2$, 5 mM KCl) on ice for 5 min. An equal volume of hypotonic buffer containing 1% NP-40 was then added, and each sample was left on ice for another 5 min. After centrifugation at 5,000 *g* for 15 min, the supernatant was collected as the cytosolic fraction. The pellets were resuspended in nucleus resuspension buffer (20 mM HEPES, PH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and incubated at 4°C for 30 min. Nuclear fraction was collected after removing insoluble debris by centrifugation at 12,000 *g* for 15 min.

Quantitation of TRINGS expression levels

The exact copy numbers of TRINGS transcripts per cell in U2OS cells treated with doxorubicin at the indicated time were quantified by using quantitative real-time RT–PCR assay. In this assay, serially diluted TRINGS-PCR products were used as templates to formulate standard curves. 5×10^5 U2OS cells treated with doxorubicin for 24 h were collected, and total RNA was isolated by TRIzol reagent. The RNA sample was subjected to qRT–PCR analysis, and then, the exact copies of lncRNA TRINGS per cell were calculated accordingly.

Electron microscopy

Transmission electron microscopy analysis was performed as we have described previously (Li *et al*, 2016). U2OS cells were fixed in 3% glutaraldehyde/1% paraformaldehyde in 0.1 M MOPS buffer for 12 h at 4°C. The cells were then embedded in Spurr's resin at room temperature for 4 h and polymerized at 60°C for 2 days. The blocks were cut into micrometer sections with a diamond knife, picked up on 200-mesh grids, stained, and observed according to the standard electron microscopy procedures.

Microarray analysis

To investigate the functions of p53-regulated long noncoding RNAs in tumorigenesis, microarray analysis was performed by Kang Chen Biotech Co., Ltd (Shanghai, China). H1299 cells carrying a p53 tet-on system were treated with or without 1 μM doxycycline for 24 h, and cells were then collected in TRIzol reagent and sent to company for lncRNA expression profiling using Arraystar Human LncRNA Microarray V3.0. Approximately 30,586 lncRNAs can be detected using this third-generation lncRNA microarray. The arrays were scanned by the Agilent Scanner G2505C, and the acquired array images were analyzed by the Agilent Feature Extraction software (version 11.0.1.1). Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). Differentially expressed lncRNAs were identified through fold change filtering. The full dataset has been submitted to GEO repository (GSE95186).

LncRNAs screening

To identify new long noncoding RNAs involved in the function of p53, microarray analysis was performed using H1299 cells carrying a p53 tet-on system. The long noncoding RNAs responsive positively to doxycycline treatment were chose from the microarray data and were further confirmed by qRT–PCR analysis. All long noncoding RNAs selected are with a length less than 2,000 bp.

RNA-IP

1×10^7 cells were lysed in hypotonic buffer supplemented with RNase A inhibitor and DNase I before centrifugation. Cell lysates were precleared with protein A/G beads (Pierce) before they were incubated with protein A/G beads coated with the indicated antibodies at 4°C for 3 h. After extensive washing, the bead-bound immunocomplexes were eluted using elution buffer (50 mM Tris, pH 8.0, 1% SDS, and 10 mM EDTA) at 65°C for 10 min. To isolate protein-associated RNAs from the eluted immunocomplexes, samples were treated with proteinase K, and RNAs were extracted by phenol/chloroform. Purified RNAs were then subjected to RT–PCR analysis.

Colony formation assay

Control or TRINGS knockdown U2OS cells were plated at a density of 2,000 cells per well on a six-well plate in soft agar medium. After 2 weeks, the cells were then fixed with 10% cold methanol for 5 min and stained with 0.005% (m/v) crystal violet for 30 min at room temperature. After extensive wash, the colonies were photographed.

Xenograft mouse model

U2OS cells expressing control shRNA or TRINGS shRNA-1 (2×10^6) were subcutaneously injected into the dorsal flank of 4-week-old male athymic nude mice (Shanghai SLAC Laboratory Animal Co. Ltd.; $n = 7$ mice per group). After 2 weeks, mice were sacrificed, representative photographs of xenografts were taken and tumors were excised. Studies on animals were conducted with approval from the Animal Research Ethics Committee of the University of Science and Technology of China.

Reproducibility

All the data were repeated at least three times. The Western blot and ChIP analyses were representatives of three independent experiments.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel software and GraphPad Prism to assess differences between experimental groups. Densitometry was performed using ImageJ software. Statistical significance was analyzed by Student's *t*-test and expressed as a *P* value. *P* values lower than 0.05 were considered to be statistically significant. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$,

respectively. Moreover, we adjusted P values where multiple comparisons were performed (Benjamini & Hochberg, 1995).

Expanded View for this article is available online.

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Author contributions
MRK and SX designed research and performed experiments. ZS analyzed the data and wrote the manuscript.MW supervised the study and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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