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# P300 participates in ionizing radiation-mediated activation of cathepsin L by mutant p53

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**List of nonstandard abbreviations:**

CTSL: cathepsin L

Egr-1: early growth response factor-1

IR: ionizing radiation

## Abstract

Our previous studies have shown that cathepsin L (CTSL) is involved in the ability of tumors to resist ionizing radiation (IR), but the specific mechanisms responsible for this remain unknown. We report here that mutant p53 (mut-p53) is involved in IR-induced transcription of CTSL. We found that irradiation caused activation of CTSL in mut-p53 cell lines whereas there was almost no activation in p53 wild-type (wt-p53) cell lines. Additionally, luciferase reporter gene assay results demonstrated that IR induced the p53 binding region on the CTSL promoter. We further demonstrated that the expression of p300 and Egr-1 was up-regulated in mut-p53 cell lines after IR treatment. Accordingly, the expression of Ac-H3, Ac-H4, AcH3K9 was up-regulated after IR treatment in mut-p53 cell lines, while HDAC4 and HDAC6 were reciprocally decreased. Moreover, knockdown of either Egr-1 or p300 abolished the binding of mut-p53 to the promoter of CTSL. ChIP assay results showed that the IR-activated transcription of CTSL was dependent on p300. To further delineate the clinical relevance of interactions between Egr-1/p300, mut-p53 and CTSL, we accessed primary tumor samples to evaluate the relationships between mut-p53, CTSL and Egr-1 /p300 ex vivo. The results support the notion that mut-p53 is correlated with CTSL transcription involving the Egr-1/p300 pathway. Taken together, the results of our study revealed that p300 is an important target in the process of IR induced transcription of CTSL, which confirms that CTSL participates in mut-p53 gain of function.

**Keywords:** cathepsin L, mutant p53, p300, ionizing radiation.

## Significance Statement

Transcriptional activation of cathepsin L by ionizing radiation required the involvement of mutated p53 and Egr-1/p300. Interference with Egr-1 or p300 could inhibit the expression of cathepsin L induced by ionizing radiation. The transcriptional activation of cathepsin L by p300 may be mediated by p53 binding sites on the cathepsin L promoter.

## Introduction

Ionizing radiation (IR), which is one of the most important treatments for therapy of malignant tumors, has been in clinical use for almost a century. DNA of tumor cells is the major target of IR treatment. Nonetheless, a number of studies have indicated that under certain conditions, tumor cells not only acquire radiation resistance but also exhibit greater invasiveness (Lee *et al*., 2017). However, the mechanisms responsible for the induction of radiation resistance in tumors and the biological effects that increase invasiveness are still unclear.

It has been known for many years that mutation or deletion of the p53 gene is very common in cancer, with more than 50% of malignant tumors having this phenotype. Mutant p53 (mut-p53) not only fails to function in the same manner as wild-type p53 (wt-p53) but also acquires a range of new functions (gain-of-function mutations). This contributes to increased tumor invasiveness, metastasis and resistance to conventional anticancer treatments in mut-p53 tumors. Previous research indicated that cathepsin L (CTSL) plays a key role in IR-induced radiation resistance and

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invasiveness of tumor cells (Fei *et al*., 2018). We also reported that IR could activate transcription of CTSL in mut-p53 tumor cells (Wang *et al*., 2019). CTSL can be activated by various different growth factors and even can be regarded as a tumor-related gene, or oncogene (Urbanelli *et al*., 2010). However, the transcriptional mechanisms controlling CTSL expression remain largely unknown, and there have been very few studies on relationships between mut-p53 and CTSL so far (Navab *et al*., 2008).

What is known, is that the transcription factor CREB and early growth response factor (Egr-1) are likely involved in transcriptional regulation of CTSL (Sriraman *et al*., 2004). Katara et al. reported that there are two p53 binding sites on the CTSL promoter. Their data showed that only wt-p53 could activate CTSL transcription by directly binding to the promoter of CTSL or indirectly inducing C/EBPα, the key transcription factor of CTSL (Katara *et al*., 2010). Our laboratory has investigated the effect of IR on CTSL expression and intriguingly found mut-p53 participated in this regulatory relationship. We recently reported that CTSL is highly expressed in wt-p53 cells when quiescent, but is expressed only at low levels in mut-p53 cells or in p53 null cells, which is consistent with the work of Katara et al. (Wang *et al*., 2016). However, our further studies found that IR significantly induced CTSL expression in mut-p53 cells.

The combination of the activity of mut-p53 with downstream transcription factor genes such as Egr-1, and the upregulation of its expression is also an important factor in mut-p53 GOF (Sauer *et al*., 2010). Notably, Egr-1 is not only induced by IR but also has a close relationship with mut-p53 (Orgad *et al*., 2005). It has been reported that Egr-1 can either promote or inhibit the proliferation of cells by activation of the transcription of its downstream genes (Peng *et al*., 2017). Our recent work has documented that mut-p53 up-regulates transcription of CTSL by activating

Egr-1 in lung cancer cells. Normally, the IR-induced apoptosis activation function of Egr-1 is mediated directly by wt-p53 (Zagurovskaya *et al*., 2009). Nevertheless, some studies showed that excessive activation of Egr-1 is an important activity of mutant p53 GOF (de Belle *et al*., 1999)**.** Consequently, we hypothesized that mut-p53 mediated activation of CTSL by IR may be correlated with the excessive activation of Egr-1.

An important large protein molecule of the family of histone acetyltransferases (HATs), p300, mediates transcription by interacting with specific sequence activators and plays a key role in DNA impair, cell proliferation and apoptosis (Magni *et al*., 2019). It was observed that p300, as an important acetyltransferase for wt-p53, could also form a large transcription-activating complex by interacting with mut-p53 and other transcription factors, activators or protein factors. These may either up- or down-regulate downstream genes, acting as a bridge between transcription factor and transcriptional protein complex (Barlev *et al*., 2001). Silverman et al. discovered that there is a binding site for Egr-1 on the promoter of p300, and Egr-1 could thus activate the transcription of this HAT. In addition, p300 was also found to inhibit the expression of C/EBPα in the liver (Breaux *et al*., 2015). Therefore, there might be a relationship between p300 and CTSL.

The purpose of the present study was to clarify the significance of mut-p53 in the transcriptional activation of CTSL induced by IR, and to explore whether p300 is an important target in the CTSL transcription process induced by IR.

## Materials and methods

### *Cell lines and culture*

Three pairs of tumor cell lines (human glioma cell lines U251 and U87, colon cancer cell lines HT-29 and RKO, breast cancer cell lines MDA-MB-468 and MCF-7) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U251, U87, HT-29 and RKO cells were cultured in high glucose DMEM medium (HyClone, Los Angeles, USA) containing 10% fetal bovine serum (Gibco, United States, California). MCF-7 cells were cultured in DMEM medium (HyClone, Los Angeles, USA) containing 10% fetal bovine serum (Gibco, United States, California). The above five cells were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. MDA-MB-468 cells were cultured in Leibovitz's L-15 medium (HyClone, Los Angeles, USA) containing 10% fetal bovine serum (Gibco, United States, California) and cultured in a $CO_2$ free incubator at 37°C.

### *Clinical tissue samples*

Colon cancer tissues (n=10) and breast cancer tissues (n=7) were collected from patients in the Affiliated Hospital of Jiangsu University (Zhenjiang, China). Glioma tissues (n=6) were taken from the Suzhou Kowloon Hospital (Suzhou, China). None of the patients had received chemical prevention treatment before surgery. All samples were informed to the patients and given their informed consent before being obtained. At the same time, it is approved by the Ethics Committee of Affiliated Hospital of Jiangsu University and Suzhou Kowloon Hospital.

### *IR condition and antibodies*

The cells were irradiated with Rad Source biological X-ray irradiance (RS-2000 Pro, Rad source, Inc) under the condition of vertical X-ray irradiation, dose rate of 1.2 Gy/min, and total dose of 10 Gy. The antibodies to Ac-H3 (1:1000), H3 (1:1000), Ac-H4 (1:1000), H4 (1:1000), HDAC4 (1:1000), HDAC6 (1:1000), AcH3K9 (1:1000) and Flag (1:1000) were purchased from Cell Signaling Technology (Massachusetts, USA). P300 (1:5000) was obtained from Bethyl Laboratories (Montgomery, TX, USA). Egr-1 (1:1000) and CTSL (1:1000) were purchased from

Abcam (Abcam, Massachusetts, UK). β-actin (1:1000) was obtained from MultiSciences (Lianke) Biotech (MultiSciences, Hangzhou, China).

### *Comet assays*

The slices were immersed in 1% normal melting point agarose (SolarBio, Beijing, China)/phosphate buffered saline (PBS) for pretreatment, and then dried into thin films. The appropriate number of cells were treated as needed, resuspended in PBS, and then quickly mixed with 0.8% low melting point agarose/PBS. A cover glass was added on each slide, and the slides were placed and cured according to the experimental requirements. The slides were immersed in alkaline lysis buffer (freshly prepared) at 4 °C for 1 h and kept away from light. Then, the slides were placed in an electrophoretic buffer (freshly prepared) at 4 °C for 20 min. The samples were electrophoresed at 25 V and 300 mA at 4 °C for 30 min. After electrophoresis, the slides were neutralized in Tris (0.4 mol/L, pH 7.5) for 5 min and then fixed with methanol for 10 min. Finally, the slides were stained with GelRed (Biotium, California, America), observed and photographed under the fluorescence microscope.

### *Apoptosis assay*

Cell apoptosis was detected by the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Beyotime, Jiangsu, China). The cells were cultured and treated as required in 6-well plates (NEST Biotechnology Co. LTD. Wuxi, China). 24 h after treatment, cells were collected and resuspended with 500 uL binding buffer, and then incubated with 5 μL Annexin V-FITC and 10 μL PI at room temperature for 15 min in the dark. At the end, apoptosis was analyzed by LSRII flow cytometry and FACSDiva software.

### *siRNA and plasmid transfection*

siRNA for Egr-1 and p300 were purchased from GenePharma (GenePharma, Shanghai, China). Mutant p53 type and wild p53 type plasmids were purchased from Suzhou Golden Wisdom Biological Technology Co., Ltd. Cells were cultured in 6-well plates for 24 h and transfected with either siRNA or plasmids using lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

### *Western blot assay*

The cells were cultured in 6-well plates (NEST Biotechnology Co. LTD. Wuxi, China) and then treated as required. The protein samples were then collected by lysis and centrifuge, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulocellulose membrane (NC) in Bio-Rad (Hercules, CA, USA). The membranes were then incubated with primary antibodies at 4℃ overnight, followed by incubation with secondary antibodies at room temperature for 1 hour in the dark. The blots were detected by Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA) and Image J software was used to quantify the data.

### *Luciferase reporter assay*

The luciferase reporter gene of p53 binding region (SBE) in the CTSL promoter was constructed by using pGL4 enhancer carrier from Suzhou Golden Wisdom Biological Technology Co., Ltd. Cells were cultured with or without silencing of Egr-1/p300 expression were transfected with pGL4-SBE-Luciferase plasmid using lipofectamine 2000. After 48 hours, the cells were collected in the special lysate, added 50 uL beta-gal substrate into 10 uL protein sample at 37 ℃ in the dark, and added 10 uL Luciferase substrates into another 10 uL protein sample, using the Luciferase Reporter Assay System and Iuminometer to detect the luciferase activity before 30 min.

### *Chromatin Immunoprecipitation (CHIP) assay*

Cells were treated as indicated and CHIP assay was conducted according to the manufacturer's protocol (Millipore, USA). DNA was extracted by using the TIANGEN kit (TIANGEN, Beijing, China) and Polymerase Chain Reaction (PCR) was used to test the performance of CHIP. The gene of p53 was amplified by touch-down PCR. The sequences for the primers used for the ChIP assay were as follows: p53 forward primer 5'-CATGC CCGGG GCACC AGCTC-3'. p53 reverse primer 5'-TTCGC CTGAC TCTGC TTCTA-3'. The primers of p53 gene were synthetized by Shanghai Abm Co., Ltd. The amplification products were electrophoresed in 2.0% agarose gel and detected by Bio-Rad GelDoc XR System.

### *Immunohistochemical staining*

The Vectastain ABC kit (Vector) was used for immunostaining according to the instructions. In brief, the endogenous peroxidase activity of the slides was blocked with 3% hydrogen peroxide solution after deparaffinized, rehydrated, and treated with a citric acid solution for antigen repair. The slides were incubated in blocking solution (PBS, 3% bovine serum albumin), then incubated with primary antibody, followed by counterstained with hematoxylin for nuclear staining. The negative control slides, without primary antibodies, did not exhibit nonspecific staining and the slides were examined blind by two researchers.

### *Statistical analysis*

Data analysis was performed using GraphPad Prism 5 software. All experiments were independently repeated for at least 3 times. The experimental results were expressed as the mean ± SD and Student's t-test. $P < 0.05$ were considered statistically significant.

## Results

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### *The effect of IR on CTSL expression in tumor cells of different p53 status*

Preliminary colony formation assays showed that the survival rate was significantly different after IR in different cell lines. For example, U87, RKO and MCF-7 cells were obviously sensitive to IR. However, U251, HT29 and MDA-MB-468 cells showed a low sensitivity to IR and a strong survival rate (Fig. 1a). Correspondingly, comet assay showed that U87, RKO and MCF-7 cells produced long comet tails after IR treatment, suggesting that DNA was damaged to varying degrees (Fig. 1b). Annexin V-FITC/PI apoptosis assay also showed that the apoptosis was not significantly increased after IR stimulation in U251, HT29 and MDA-MB-468 cells (Fig. 1c). Further study found that the differences in response to IR may be related to the status of p53 gene among these 3 pairs of tumor cell lines.

In addition, our previous studies have shown that CTSL is involved in the ability of tumors to resist IR (Yang *et al*., 2015; Wang *et al*., 2019; Hashimoto *et al*., 2006), therefore, we continued to investigate the correlation between p53 gene status and CTSL expression after IR stimulation. These cell lines are described in the Materials and Methods section. They were exposed to 10 Gy of X-rays. As is shown by Western blotting, levels of CTSL expression in mut-p53 cell lines were increased by IR, relative to wt-p53 cell lines (Fig. 2a). To further confirm the effect of IR on the regulation of the expression of CTSL in the mut-p53 cell lines U251, HT-29 and MDA-MB-468, we have generated a luciferase reporter vector containing a p53 binding site on the CTSL promoter and have transfected this vector into the cell lines. As shown in Fig. 2b, the activity of the luciferase reporter in U251, HT-29 and MDA-MB-468 cells was increased by IR, while this was not the case in wt-p53 cells. These results suggest that the IR-activated transcription of CTSL was mediated by increasing the activity of the p53 binding site on the CTSL promoter. Interestingly, we found that mut-p53 was selectively recruited to the relevant promoter region after IR treatment. This finding further confirmed the regulatory effect of mut-p53 for IR-induced expression of CTSL. Additionally, based on the sequence of the p53 binding regions on the CTSL promoter as reported in the literature, chromatin immunoprecipitation (ChIP) primers were designed and constructed. By means of this ChIP experiment (Fig. 2c), we found that the mut-p53 cells markedly increased CTSL expression after IR via promoter binding. This further documented the regulatory role of mut-p53 on CTSL expression after IR.

### *Egr-1 and p300 are involved in the IR-induced expression of CTSL*

It has been reported that Egr-1 expression is increased in tumor cells transfected with mut-p53. Additionally, Egr-1 was significantly increased by IR in p53 knockout mice (Zhang *et al*., 2001; Rousselet *et al*., 2004). P300 is not only a downstream protein of Egr-1, but is also closely related to C/EBPα (Wang *et al*., 2019), which is a key regulatory factor of CTSL (Katara *et al*., 2010; Muller *et al*., 2009). To investigate this issue, we investigated Egr-1 and p300 expression in 6 cell lines. Egr-1 and p300 were both markedly increased by IR in mut-p53 cell lines compared with wt-p53 cell lines (Fig. 3a, 3b).

Next, we investigated whether Egr-1 knockdown had any influence on IR-regulated p300 expression by transfecting Egr-1 siRNA into U251, HT-29 and MDA-MB-468 cells. After knocking down Egr-1, p300 expression was found to be significantly inhibited. More importantly, the IR-induced expression of CTSL was also abolished by knocking down Egr-1, compared with levels following IR of unmanipulated cells (Fig. 3c). Next, we determined that p300 knockdown also prevented the activation of CTSL transcription by IR (Fig. 3d). Correspondingly, p300 knockdown accompanied with IR caused serious DNA damage and reduced the survival rate in U251, HT-29 and MDA-MB-468 cells (Fig. 3g, 3h). Furthermore, we assessed luciferase reporter activity of p53 binding sites on the CTSL promoter in Egr-1 and p300-siRNA-transfected cells. As shown in Fig. 3e, 3f, the luciferase reporter activity in Egr-1/p300-siRNA-transfected cells did not change after IR. This indicated knockdown of both Egr-1 and p300 abolished the binding of mut-p53 to the promoter of CTSL.

### *IR enhances the acetylation level of mut-p53 cells and mediates CTSL expression*

Previous studies demonstrated that p300, which is a broadly functional transcription co-factor, participates in the activation or inhibition of the transcription and expression of many genes by interacting with mut-p53 (Barlev *et al*., 2001; Silverman *et al*., 1998). As an important HAT, p300 catalyzes acetylation at multiple sites of lysine in histones. In our experiments, we found that IR induced H3 and H4 acetylation in mut-p53 cell lines (Fig. 4a). Using H1299 cells, we also found that IR significantly increased the acetylation level of H3 and H4 in p53 hot spots (p53-175, p53-248, and p53-273) relative to wt-p53 expressing H1299 cells, which is consistent with the results in endogenous mut-p53 cell lines (Fig. 4b).

Histone deacetylases (HDACs) modify histone tails through deacetylating amino-terminal lysine residues. This not only results in chromatin remodeling observed in numerous diseases, especially in tumorigenesis, but also counteracts the activity of HATs (Choudhary *et al*., 2009). After treating cells with IR, we also found that the expression level of HDAC4 and HDAC6 in mut-p53 cell lines decreased compared with the decrease in wt-p53 cell lines (Fig. 4a). In addition, the expression of AcH3K9 was also assessed, and interestingly, we found that AcH3K9 in mut-p53 cell lines was notably induced by IR compared with wt-p53 cell lines. These results further indicate that IR improves the acetylation level of histones.

### ***P300 activates transcription by directly acting on the CTSL promoter***

To further study the mechanism by which p300 is involved in IR-activated transcription of CTSL, we first conducted an immunoprecipitation experiment (IP) to determine whether p300 bound to the CTSL promoter and subsequently activated transcription by forming a complex with mut-p53. However, no binding of p300 and p53 was found in any of the 6 cell lines before or after IR, indicating that p300 did not combine with p53 to form a complex to activate transcription of CTSL. We further conducted ChIP assays on p300, and found that it bound to the p53-binding site on the CTSL promoter after IR only in mut-p53 cell lines (Fig. 5a). These results indicate that p300 can bind directly to the CTSL promoter and thus activate its transcription. Next, we conducted ChIP assays on p300-siRNA-transfected U251, HT-29 and MDA-MB-468 cells and interestingly found that the IR-induced binding of mut-p53 to the CTSL promoter was abolished by knocking down p300 (Fig. 5b). Taken together, the above evidence points to a role for p300 as a positive regulator of CTSL transcription after IR in p53-mutant cell lines.

### *CTSL, Egr-1 and p300 are positively correlated with mutant p53 status in tissues ex vivo*

Our laboratory has explored and reported on the relationship between CTSL expression level and glioma grade, and concluded that the strong expression of CTSL is not only a positive biomarker for these tumors but also participates in the invasion and migration of the tumor cells via various different mechanisms (Xiong *et al*., 2017; Fei *et al*., 2018). To further complement the study, we have now examined CTSL expression characteristics in clinical tumor tissues from other cancers. To this end, we have collected 10 colon tumor samples and 7 breast tumor samples as well as the corresponding paired normal tissue. We also collected clinical glioma samples harboring wild-type p53 (n=3) and mutant p53 (n=3).

By Western blotting assays, we found that CTSL is highly expressed in tumor tissue relative to the paired normal tissue in breast tumor samples (Fig. 6a). Additionally, Egr-1 was expressed more strongly in 50% of the breast tumor samples relative to the paired normal tissue. This result suggests that the level of CTSL expression is positively correlated with Egr-1 in colon cancer tissue. Furthermore, we sequenced the p53 gene in colon cancer samples to detect mutations and found that sample No. 7 and No. 8 harbored the p53-273 mutation, while the remaining samples possessed wild-type p53 or other non-hot spot mut-p53. To explore the relationship between

CTSL, Egr-1 and p300 expression, we conducted immunohistochemical staining assays on mut-p53-273 and wt-p53 colon tumor tissue. As shown in Fig. 6b, CTSL, Egr-1 and p300 were all highly expressed in p53-273 mutated tissue relative to wt-p53 tissue. Interestingly, we found that CTSL located to the nucleus in mut-p53 tissue, which was consistent with our previous findings in lung cancer tissues. Additionally, the expression levels of CTSL, Egr-1 and p300 were all higher in breast tumor tissues than in paired normal tissue. Interestingly, CTSL, Egr-1, and p300 were more highly expressed in p53 mutant samples than wild-type p53 also in glioma samples.

## Discussion

Based on the results of this study, we propose a new mechanism by which p300 is involved in the regulation of CTSL in mut-p53 cells. We mainly focus on findings that the IR-induced expression of CTSL is part of the mut-p53 GOF and reveal that p300 is an important target in the process of IR induced transcription of CTSL.

To verify the relationship between mut-p53 and IR-induced expression of CTSL, we collected 3 pairs of tumor cell lines and quantified CTSL expression levels in these cells after IR. The results showed that CTSL was increased in mut-p53 cell lines whereas there it was almost unaltered in p53 wild-type (wt-p53) cell lines (Fig. 2). ChIP assays on tumor cells confirmed that mut-p53 activated transcription of CTSL by directly binding to the promoter of CTSL after IR. It was reported that p53 mutations fall into two main categories: DNA contact mutations and conformational mutations (Duan *et al*., 2019; Monteith *et al*., 2016). Although the p53 status of all 3 cell lines in our study (U251, HT-29 and MDA-MB-468) is DNA contact mutation, the mut-p53 of these cells was changed by IR to enable its binding to the CTSL promoter. This finding suggested to us that there might be some transcriptional co-factors assisting the binding capacity of mut-p53 to the CTSL promoter.

In previous studies, we found a direct binding relationship between the transcription factor Egr-1 and the CTSL promoter after IR. The results of the present study further confirm that the expression of Egr-1 significantly increased after IR treatment in mut-p53 cells (Fig. 3). Moreover, inhibition of Egr-1 efficiently abolishes the up-regulation of CTSL by IR. Egr-1 is an important participant in the ability of wt-p53 to function in apoptosis, and could be induced by IR to inhibit the proliferation and growth of tumor cells by affecting the DNA binding activity of downstream genes (Li *et al*., 2014; Peng *et al*., 2017; Liu *et al*., 2001). There would therefore be no clinical significance in inhibiting CTSL expression by Egr-1 knockdown directly. In the present study, we report that p300, a multifunctional acetyltransferase, plays an important role as a novel participant in the regulation of CTSL expression by mut-p53. It was previously reported that p300 is not only regulated by Egr-1 but also positively correlates with CEBP/α, which is a known regulatory factor of CTSL (Barlev *et al*., 2001; Breaux *et al*., 2015; Wang *et al*., 2015). Taken together, these data indicate that p300 is an important factor which must be taken into account when investigating the mechanisms regulating Cathepsin L expression.

Di Agostino *et al*. confirmed that during DNA damage, the mut-p53/NF-Y complex had opposite effects on the transcription level compared with the wt-p53/NF-Y complex. Mut-p53 recruited p300 and caused abnormal transcription and cell cycle disorders of NF-Y target genes, while wt-p53 recruited HDAC1 and had an inhibitory effect on NF-Y target genes (Agostino *et al*., 2006). However, it is not clear in which opposing manner they recruit p300 or HDAC1, respectively. The mechanisms of these differential effects are thus still unclear. Our data showed that p300 was increased in mut-p53 cell lines after IR, and that inhibition of p300 significantly decreased the mut-p53 mediated up-regulation of CTSL. The results of this experiment also led to the basic conclusion that mut-p53 cells could mediate transcriptional activation of CTSL by recruiting p300 after IR. In our experiments, we found that IR treatment increased the acetylation levels of H3, H4 and H3K9, but decreased histone deacetylase levels of HDAC4 and HDAC6 in mut-p53 cells. In addition, we established cell lines with different p53 states by using H1299 cells to verify the effect of IR on the acetylation of H3 and H4. The results showed that IR significantly increased the acetylation levels of H3 and H4 in mut-p53 cells (p53-175, p53-248 and p53-273), which is consistent with the results from endogenous mutant p53 cell lines. These results indicate that IR increases the acetylation level of histones in mut-p53 cell lines. Therefore, as a functional acetylated protein closely related to p53, p300 is recruited by mut-p53 under the influence of IR.

To further explore the mechanism by which p300 participates in the IR-regulated expression of CTSL, we conducted ChIP assays on cells of different p53 status exposed or not exposed to IR. Interestingly, the results showed that p300 exhibited enhanced binding to p53 binding regions on the CTSL promoter after IR in mut-p53 cell lines. These results suggest that p300 activates transcription by directly acting on the CTSL promoter and that the binding region for p300 on the CTSL promoter has a high degree of homology with p53 (Fig. 5). When p300 was silenced in p53 mutant cell lines, ChIP analysis revealed that the IR-induced enhanced binding of p53 to CTSL was eliminated, further emphasizing an important role of p300 in mediating transcriptional activation of CTSL in mut-p53 cells. Therefore, although p53 produced binding mutations in U251, HT-29 and MDA-MB-468 cells, the mut-p53 regained the ability to bind DNA with the help of Egr-1 and p300. More importantly, this study found that p300 regulated CTSL by binding to the p53 binding region after IR.

Moreover, our study further showed that the expression level of CTSL was higher in clinical colon and breast cancer tissues than in counterpart paired normal tissues. We also found that the expression levels of Egr-1 and p300 were positively correlated with CTSL in these cancer tissues (Fig. 6). Our previous work on lung cancer tissue found that the CTSL expression level was significantly higher in tumor tissue than in paired normal tissue, especially in p53-273 mutated tissue (Wang *et al*., 2019; Zhang *et al*., 2015). In the present study, we conducted p53 gene sequencing on 10 colorectal tumor tissues and 6 glioma tissues. Interestingly, in glioma, CTSL, Egr-1, and p300 were more strongly expressed in p53 mutant samples than wild-type p53 samples. However, due to the limited source of clinical samples, this pilot study requires validation.

In conclusion, in the present study, we found that the regulatory mechanism controlling CTSL expression may be associated with mutations in the p53 gene, revealing that mut-p53 may regulate the expression of CTSL through interactions with Egr-1/p300 and suggesting that CTSL may be a possible participant of mut-p53 GOF.

## Conflict of interest

The authors declare no competing interests.

## Author contributions

Participated in research design and contributed new reagents or analytic tools: Lin and Liang

Conducted experiments: Xiong, Zhu, Liu, Zhao, Shen and Zuo

Performed data analysis and wrote or contributed to the writing of the manuscript: Zhu and Xiong

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## Footnotes

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**Figure legends**

**Fig.1 The effect of IR in tumor cells of different p53 status.** These 3 pairs of tumor cell lines were treated with different doses of IR, then cell survival was detected by colony formation experiment (a), DNA damage was detected by comet assay (b), and cell apoptosis was detected by Annexin V-FITC/PI apoptosis assay (c). ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control).

**Fig.2 The effect of IR on CTSL expression in tumor cells of different p53 status.** (a) Western blot of CTSL of whole cell extracts from U251, U87, HT-29, RKO, MCF-7 and MDA-MB-468 cells with or without IR treatment. β-actin was used as an internal control. (b) Luciferase reporter gene activity of p53 binding site on CTSL promoter in U251, U87, HT-29, RKO, MCF-7 and MDA-MB-468 cells with or without IR treatment. ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control). (c) ChIP analysis of p53 binding site on CTSL promoter using p53 antibody in U251, U87, HT-29, RKO, MCF-7 and MDA-MB-468 cells with or without IR treatment.

**Fig. 3 Egr-1 and p300 are involved in the IR-induced expression of CTSL.** (a, b) Western blot of Egr-1 and p300 of whole cell extracts from U251, U87, HT-29, RKO, MCF-7 and MDA-MB-468 cells. β-actin was used as an internal control. (c, d) Western blot of Egr-1 and p300 of whole cell extracts from si-Egr-1/p300 RNA treated U251, HT-29 and MDA-MB-468 cells with or without IR. β-actin was used as an internal control. (e, f) Luciferase reporter gene activity of p53 binding site on CTSL promoter in si-Egr-1/p300 RNA treated U251, HT-29 and MDA-MB-468 cells with or without IR. (g) Colony formation experiment. (h) Comet assay. ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control).

**Fig. 4 IR enhances the acetylation level of mut-p53 cells and mediates CTSL expression.** (a) Western blot of Ac-H3, H3, Ac-H4, H4, HDAC4, HDAC6 and AcH3K9 of whole cell extracts from U251, U87, HT-29, RKO, MCF-7 and MDA-MB-468 cells. β-actin was used as an internal control. (b) Western blot of Ac-H3, H3, Ac-H4 and H4 of whole cell extracts from H1299 (null of p53) and H1299 cell lines established with different p53 status plasmids. β-actin was used as an internal control.

**Fig. 5 P300 activates transcription by directly acting on the CTSL promoter.** (a) ChIP analysis of p300 binding site on CTSL promoter using p300 antibody in U251, U87, HT-29, RKO, MCF-7 and MDA-MB-468 cells with or without IR treatment. (b) ChIP analysis of p53 binding site on CTSL promoter using p53 antibody in p300 knockdown U251, HT-29 and MDA-MB-468 cells with or without IR treatment.

**Fig. 6 CTSL, Egr-1 and p300 are positively correlated with mutant p53 status in tissues ex vivo.** (a) Western blot of CTSL, Egr-1 and p300 in clinical breast cancer and glioma tissues. β-actin was used as an internal control. (b) Immunohistochemistry staining of CTSL, Egr-1 and p300 in wt-p53/ mut-p53 clinical colon cancer and normal samples (arrows indicate the histological features of the tissues).

## Tables

Table 1. The sequence of siRNA for transfection cells

| siRNA | Base sequence | |
|---|---|---|
| Negative control (NC) | sense | 5'-UUCUCCGAACGUGUCACGUTT-3' |
| | anti-sense | 5'-ACGUGACACGUUCGGAGAATT-3' |
| si-Egr-1$^{698}$ | sense | 5'-CCAACAGUGGCAACACCUUTT-3' |
| | anti-sense | 5'-AAGGUGUUGCCACUGUUGGTT-3' |
| si-Egr-1$^{1508}$ | sense | 5'-GGCAUACCAAGAUCCACUUTT-3' |
| | anti-sense | 5'-AUUGUUGCUGUAUUUCUGGTT-3' |
| si-Egr-1$^{1819}$ | sense | 5'-GCUGUCACCAACUCCUUCATT-3' |
| | anti-sense | 5'-UGAAGGAGUUGGUGACAGCTT-3' |
| si-p300$^{728}$ | sense | 5'-GUCCUGGAUUAGGUUUGAUTT-3' |
| | anti-sense | 5'-AUCAAACCUAAUCCAGGACTT-3' |
| si-p300$^{1807}$ | sense | 5'-GGACUACCCUAUCAAGUAATT-3' |
| | antisense | 5'-UUACUUGAUAGGGUAGUCCTT-3' |

| siRNA | Base sequence | |
|---|---|---|
| si-p300$^{3797}$ | sense | 5’-CAUCACGGGUAUACAAAUATT-3’ |
| | antisense | 5’-UAUUUGUAUACCCGUGAUGTT-3’ |

figure 1

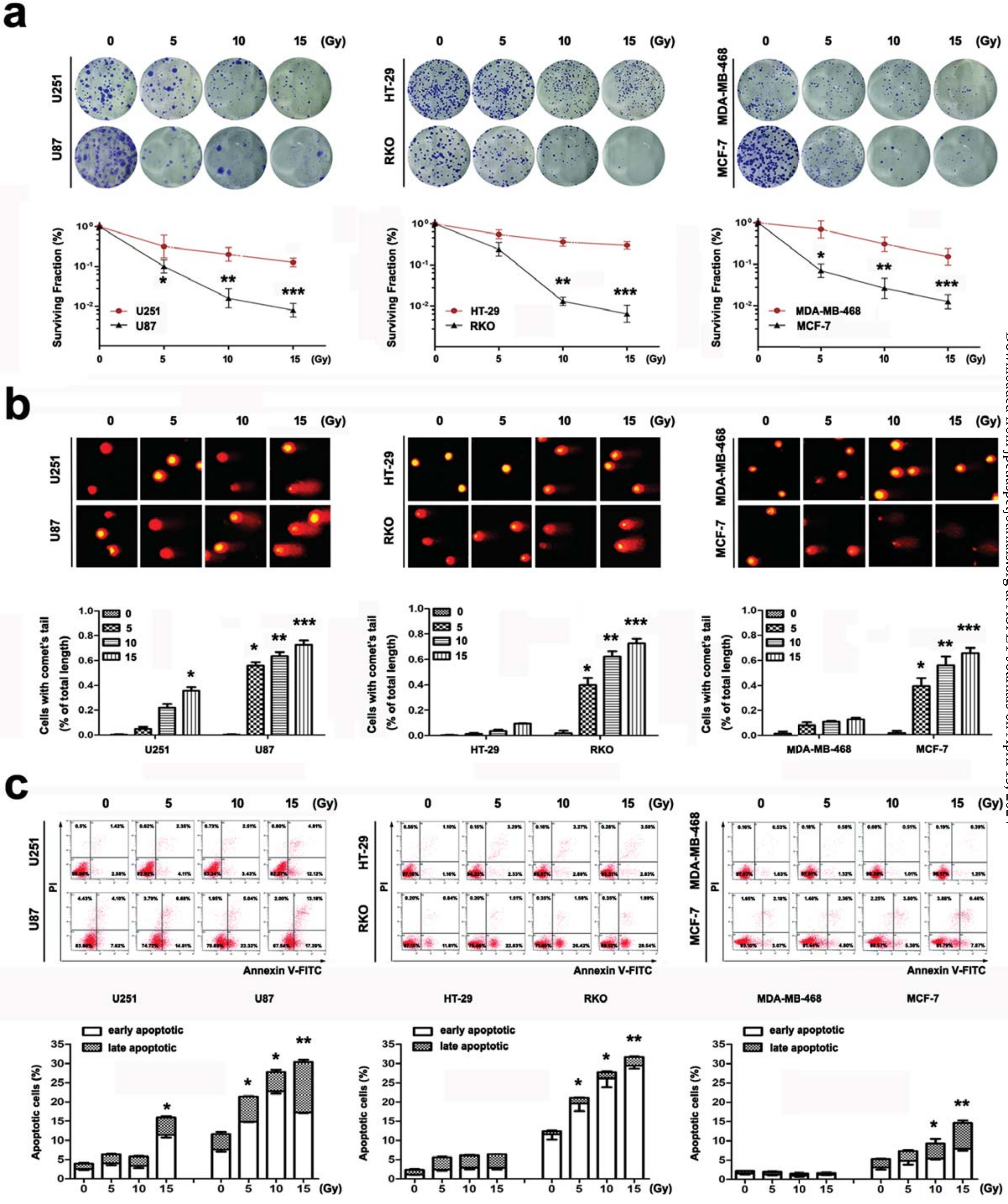
a
0
5
10
15
(Gy)
U251
U87
HT-29
RKO
MDA-MB-468
MCF-7
Surviving Fraction (%)
10⁰
10⁻¹
10⁻²
*
**
***
0
5
10
15
(Gy)
b
Cells with comet's tail (% of total length)
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
c
PI
Annexin V-FITC
early apoptotic
late apoptotic
Apoptotic cells (%)
0
5
10
15
20
25
30
35

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figure 2

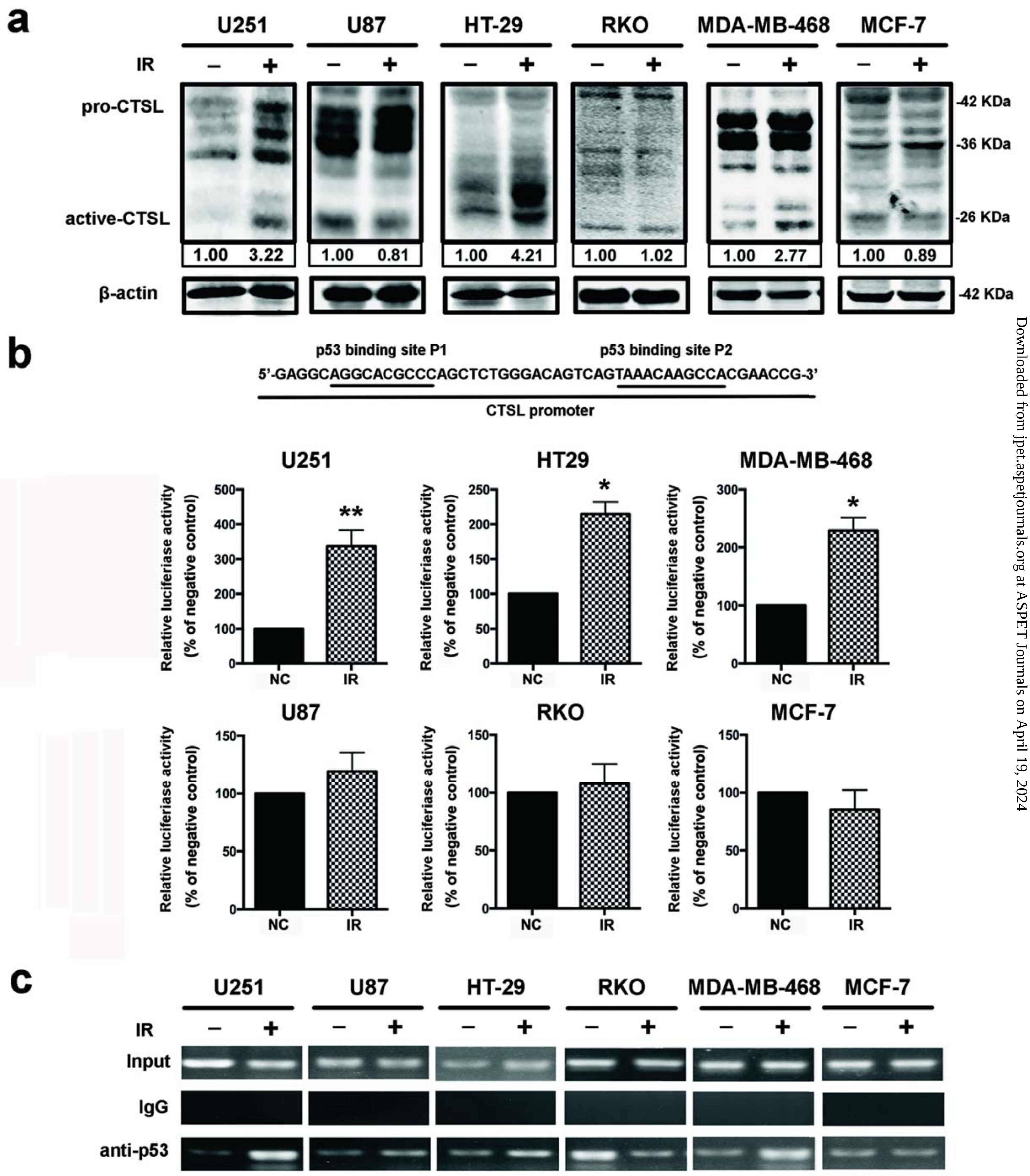

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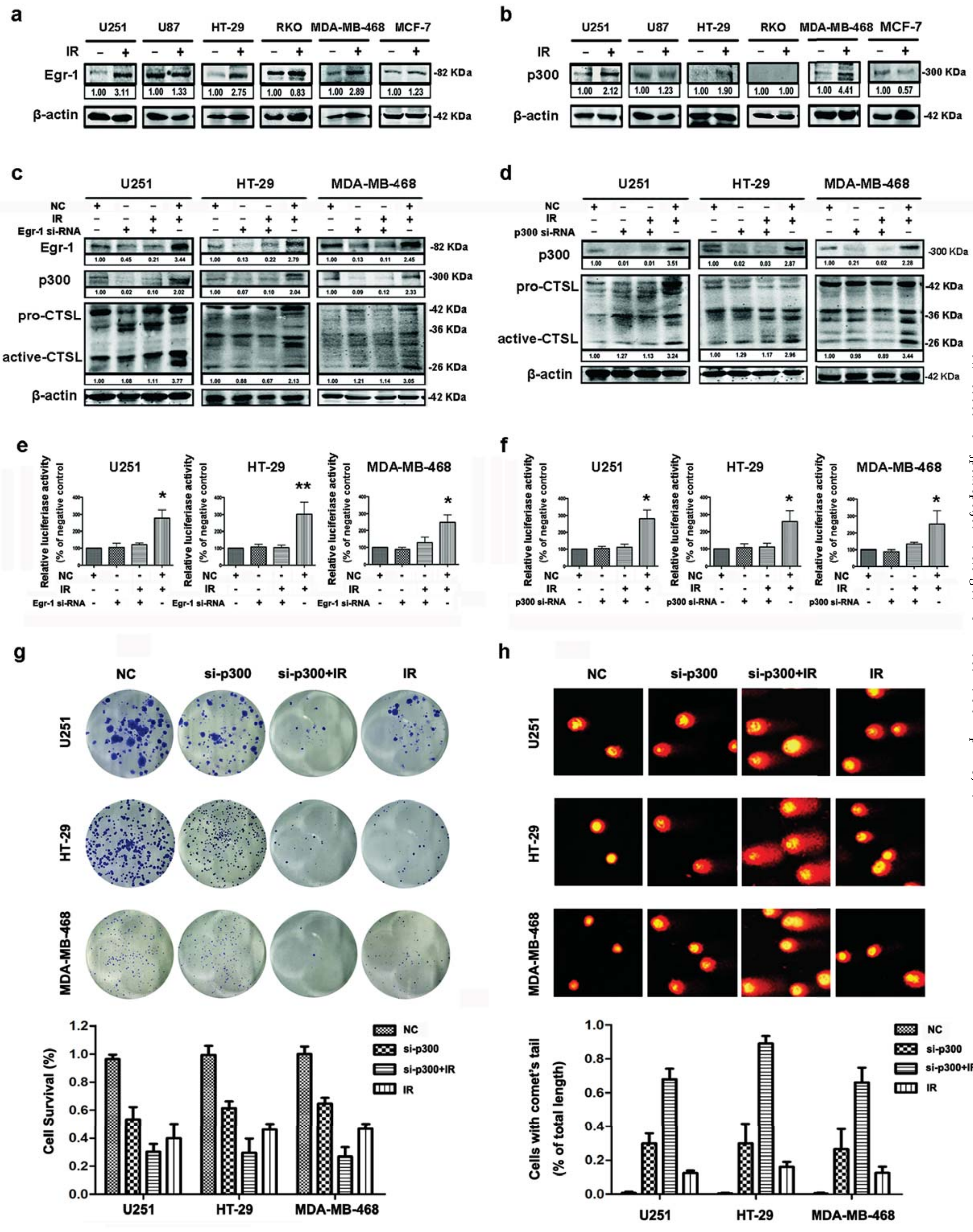
figure 3



JPET Fast Forward. Published on July 12, 2021 as DOI: 10.1124/jpet.121.000639
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**figure 4**

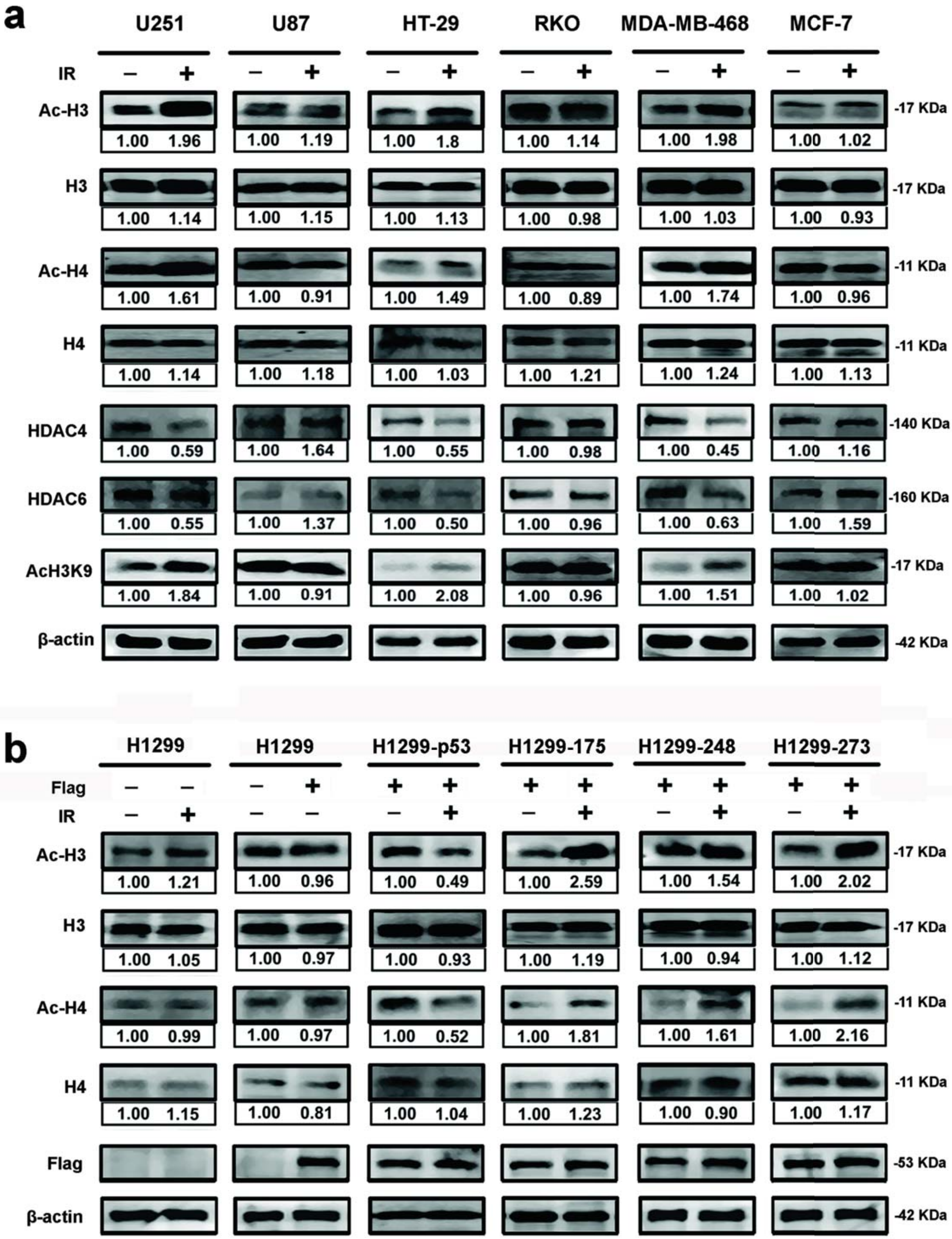
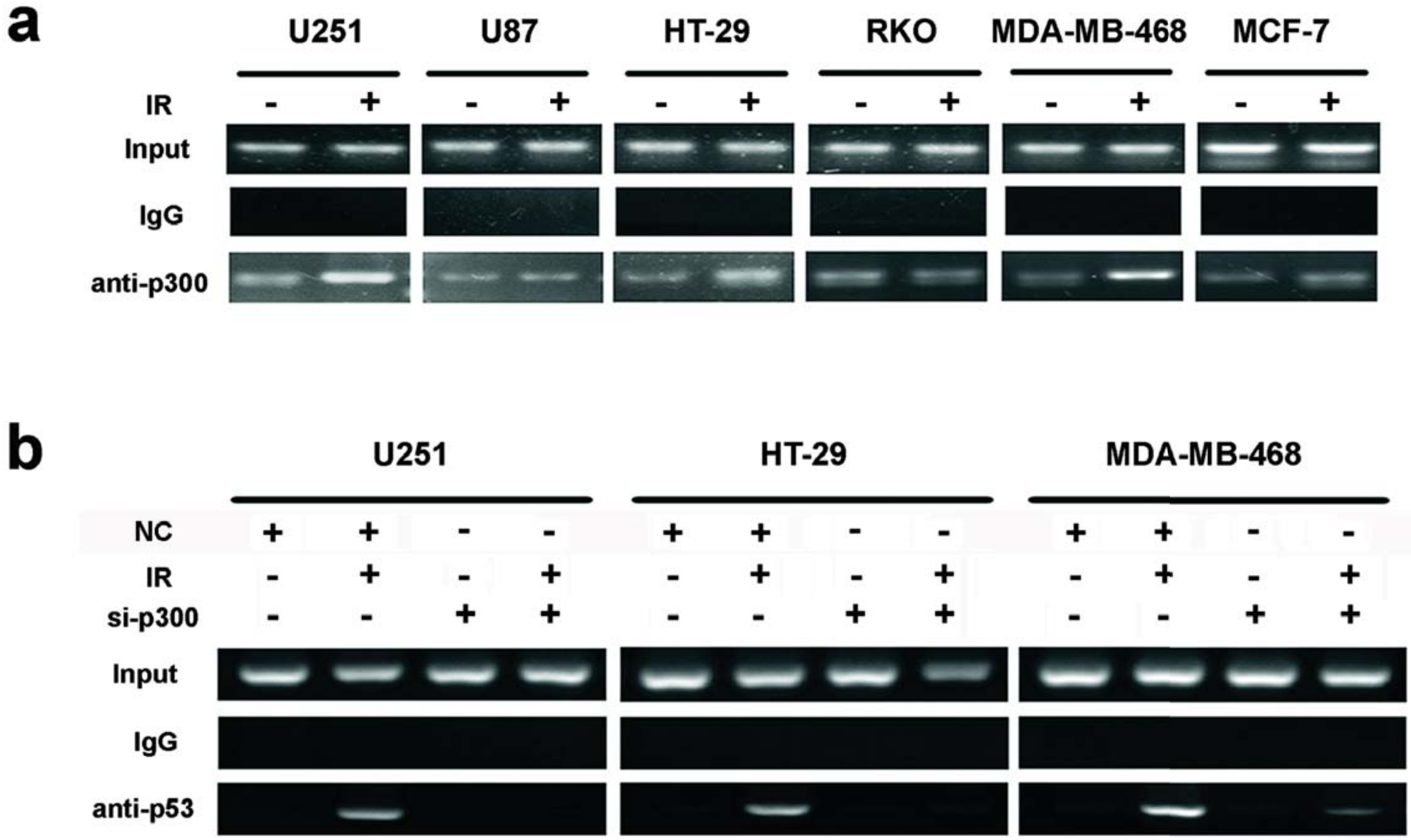


figure 5


a
U251
U87
HT-29
RKO
MDA-MB-468
MCF-7
IR
- + - + - + - + - + - +
Input
IgG
anti-p300
b
U251
HT-29
MDA-MB-468
NC
+ + - - + + - - + + - -
IR
- + - + - + - + - + - +
si-p300
- - + + - - + + - - + +
Input
IgG
anti-p53

JPET Fast Forward. Published on July 12, 2021 as DOI: 10.1124/jpet.121.000639
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figure 6