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NEDD8-conjugating enzyme E2 UBE2F confers radiation resistance by protecting lung cancer cells from apoptosis

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Lung cancer, which is exacerbated by environmental pollution and tobacco use, has become the most common cause of cancer-related deaths worldwide, with a five-year overall survival rate of only 19% (Siegel et al., 2020; Yang et al., 2020; Yu and Li, 2020). Nearly 85% of lung cancers are non-small cell lung cancers, of which lung adenocarcinoma is the most common subtype accounting for 50% of non-small cell lung cancer cases. At present, radiotherapy is the primary therapeutic modality for lung cancer at different stages, with significant prolongation of survival time (Hirsch et al., 2017; Bai et al., 2019; Shi et al., 2020). Irradiation can generate reactive oxygen species (ROS) through the radiolysis reaction of water and oxygen, cause DNA damage and oxidative stress, and subsequently result in cancer cell death (Kim et al., 2019). Nevertheless, radioresistance seriously hinders the success of treatment for lung cancer, owing to local recurrence and distant metastasis (Huang et al., 2021). Compared with small cell lung cancer, non-small cell lung cancer shows more tolerance to radiotherapy. Therefore, it is of great importance to decipher key mechanisms of radioresistance and identify effective molecular radiosensitizers to improve patient survival.

Neddylation is a process that conjugates neuronal precursor cell-expressed developmentally down-regulated protein 8 (NEDD8), a ubiquitin-like molecule, to a lysine residue of the substrate protein via a three-step enzymatic cascade involving a single E1, two E2s and a few E3s (Zhou LS et al., 2018; Zhou and Jia, 2020). NEDD8-activating enzyme E1 is a heterodimer of NEDD8-activating enzyme E1 subunit 1 (NAE1) and ubiquitin-like modifier-activating enzyme 3 (UBA3) (Bohnsack and Haas, 2003). Ubiquitin-conjugating enzyme E2 M (UBE2M) and UBE2F are two independent E2s with distinct biological functions (Huang et al., 2009). So far, the best characterized physiological substrates of neddylation are the cullin subunits of cullin-RING ligases (CRLs); the cullin-RING family is the largest family of E3 ubiquitin ligases. Upon activation by cullin neddylation, CRLs promote ubiquitylation and degradation of various substrates which participate in many important biological processes, including tumorigenesis and metastasis (Zhou et al., 2019a, 2019b). Intensive studies revealed that global protein neddylation and catalytic neddylation enzymes (e.g., NEDD8 E1, NAE1/UBA3; NEDD8 E2, UBE2M/UBE2F) are significantly overactivated in multiple human cancers (Li et al., 2014; Hua et al., 2015; Jia et al., 2019). Moreover, elevation of the neddylation pathway is associated with poor overall patient survival, indicating that overactivated neddylation pathway status might be an oncogenic event (Li et al., 2014; Hua et al., 2015; Jia et al., 2019). Other studies reported that neddylation inhibition caused accumulation of a variety of key CRL substrates, resulting in cell apoptosis and senescence and thus suppressing tumor development (Zhou LS et al., 2018; Zhou and Jia, 2020). Together, these findings highlight the

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neddylation pathway as a potential target for anti-cancer therapy.

UBE2F, one of the NEDD8-conjugating E2 enzymes, specifically pairs with RING-box protein 2 (RBX2) to promote cullin 5 (CUL5) neddylation and activation. Recent studies showed that UBE2F/RBX2/CUL5 tri-complex activated CRL5 and consequently led to polyubiquitination and proteasome-mediated degradation of pro-apoptotic protein NOXA (Zhou WH et al., 2017, 2018). Inhibition of UBE2F causes NOXA accumulation and thus induces apoptosis, indicating that UBE2F is an anti-apoptotic protein (Zhou et al., 2017). One of the most important reasons for radioresistance is the suppression of apoptotic pathways. Thus, as a regulatory protein of apoptosis, the role of UBE2F in radiosensitivity of lung cancer cells deserves further study.

As mentioned above, radiotherapy using ionizing radiation induces cancer cell death mainly by generating ROS. High levels of ROS increase oxidative stress to induce DNA damage and apoptosis. To decipher the role of UBE2F in regulating sensitivity to radiotherapy, we first investigated effects of irradiation or hydrogen peroxide (H_2O_2) treatment on UBE2F expression. Western blot analysis demonstrated that in non-small cell lung adenocarcinoma cell line A549, the expression of UBE2F was remarkably increased at 48 h after irradiation in a dose-dependent manner (0, 2, 4, and 6 Gy) (Fig. 1a). Interestingly, the enhanced protein level of UBE2F induced by irradiation was completely blocked by treatment of cells with *N*-acetyl-L-cysteine (NAC), a classical ROS scavenger (Fig. 1b), indicating that irradiation-induced up-regulation of UBE2F might be associated with the increase in ROS levels. To test this hypothesis, we treated cells with different concentrations of H_2O_2, the major source of ROS, and found that H_2O_2 treatment led to an obvious increase of UBE2F expression in a dose-dependent manner (0, 12.5, 25.0, 50.0, 100.0, and 200.0 μmol/L) for 48 h (Fig. 1c). Moreover, treatment of cells with NAC also restored UBE2F levels (Fig. 1d). We observed similar results in H1299, another human non-small cell lung cancer cell line (Fig. S1). These results suggest that oxidative stress can increase UBE2F expression, which might be a way for cancer cells to enhance antioxidant defense and thus lead to radiation resistance.

By activating CRL5 and promoting the degradation of pro-apoptotic protein NOXA, UBE2F exerts its anti-apoptotic function, and its deletion inhibits lung cancer cell growth by inducing apoptosis (Zhou WH et al., 2017; Zhou LS et al., 2020). Previous studies reported that suppression of NOXA expression protects cells from oxidative stress-induced cell death (Eno et al., 2012). We thus investigated the functional importance of UBE2F in antioxidant defense and radiosensitivity. First, we generated stable A549 cells with

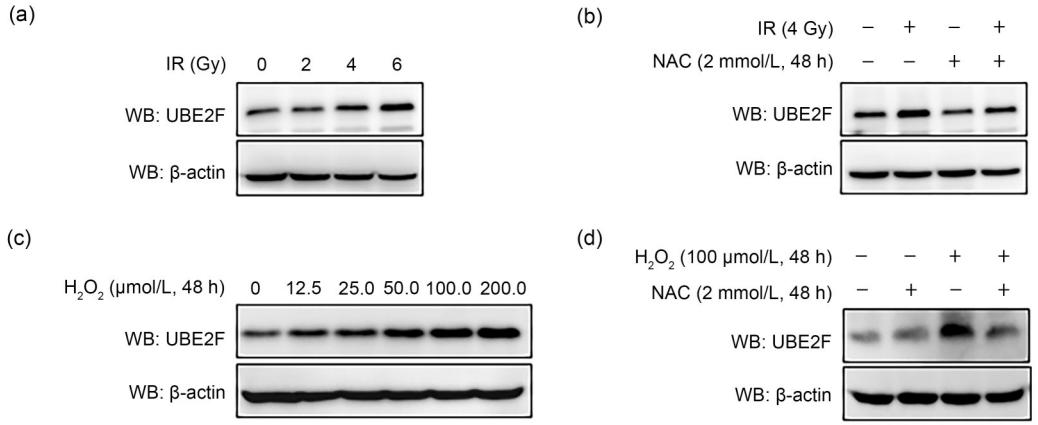


Fig. 1 Up-regulation of ubiquitin-conjugating enzyme E2 F (UBE2F) expression by irradiation (IR) or other stimulus-induced oxidative stress. (a) IR treatment increased UBE2F protein levels. A549 cells were seeded in a 60-mm dish, followed by IR at different doses up to 6 Gy. After IR, cells were cultured for 48 h. (b) Reactive oxygen species (ROS) scavenger *N*-acetyl-L-cysteine (NAC) completely blocked IR-induced up-regulation of UBE2F expression. A549 cells were treated with IR (4 Gy) alone and/or NAC (2 mmol/L) for 48 h. (c) H_2O_2 treatment increased UBE2F protein levels. A549 cells were seeded in a 60-mm dish, followed by H_2O_2 treatment at different doses up to 200 μmol/L for 48 h. (d) ROS scavenger NAC completely blocked H_2O_2-induced up-regulation of UBE2F expression. A549 cells were treated with H_2O_2 (100 μmol/L) and/or NAC (2 mmol/L) for 48 h. Protein levels of UBE2F were determined by western blot (WB) and normalized against β-actin.

UBE2F knockout (KO) using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system to evaluate the effects of UBE2F down-regulation on cellular sensitivity to irradiation and oxidative stress. UBE2F KO cells display lower levels of CUL5 neddylation, in line with higher levels of NOXA expression (Fig. S2). Compared with wild-type (WT) control cells, UBE2F KO cells exhibited a higher incidence of cell death upon high-dose (4 Gy) irradiation, with trypan blue staining identifying the dead and living cells (Fig. 2a). Similarly, UBE2F KO cells appeared to be more sensitive to a high concentration (100 μmol/L) of H_2O_2 (Fig. 2c). In accord with this, the protein levels of NOXA were enhanced in UBE2F KO cells, regardless of the irradiation or H_2O_2 treatment (Figs. 2b and 2d). Since NOXA is a well-known substrate of UBE2F-CRL5 complex, these results support a crucial role for UBE2F in regulating lung cancer cell sensitivity to irradiation or other stimulus-induced oxidative stress via modulating apoptosis.

Next, we performed xenograft experiments using UBE2F WT and KO lung cancer cells, and evaluated the effect of UBE2F on radio-sensitivity. When tumor size reached about 100 mm^3, mice were treated by radiotherapy. We found that tumor volumes in nude mice injected with UBE2F KO cells were significantly smaller than those in mice injected with WT cells (tumor size: 33.89 mm^3 vs. 11.23 mm^3 vs. 14.02 mm^3 for WT vs. UBE2F KO1 vs. UBE2F KO2; P<0.001; Figs. 3a and 3b). The tumor weights in nude mice injected with UBE2F KO cells were also significantly heavier than those in mice injected with WT cells at 7 d after radiotherapy (tumor weight: 11.20 mg vs. 5.81 mg vs. 6.50 mg for WT vs. UBE2F KO1 vs. UBE2F KO2; P<0.05; Fig. 3c). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining demonstrated that after radiotherapy, the apoptosis levels were higher in UBE2F KO tumor cells than in WT cells (Fig. 3d). Collectively, these data further support the notion that inhibition of UBE2F sensitizes lung cancer cells to radiotherapy in vivo by promoting apoptosis.

The discovery that UBE2F was a potential target for improving the sensitivity of lung cancer cells to

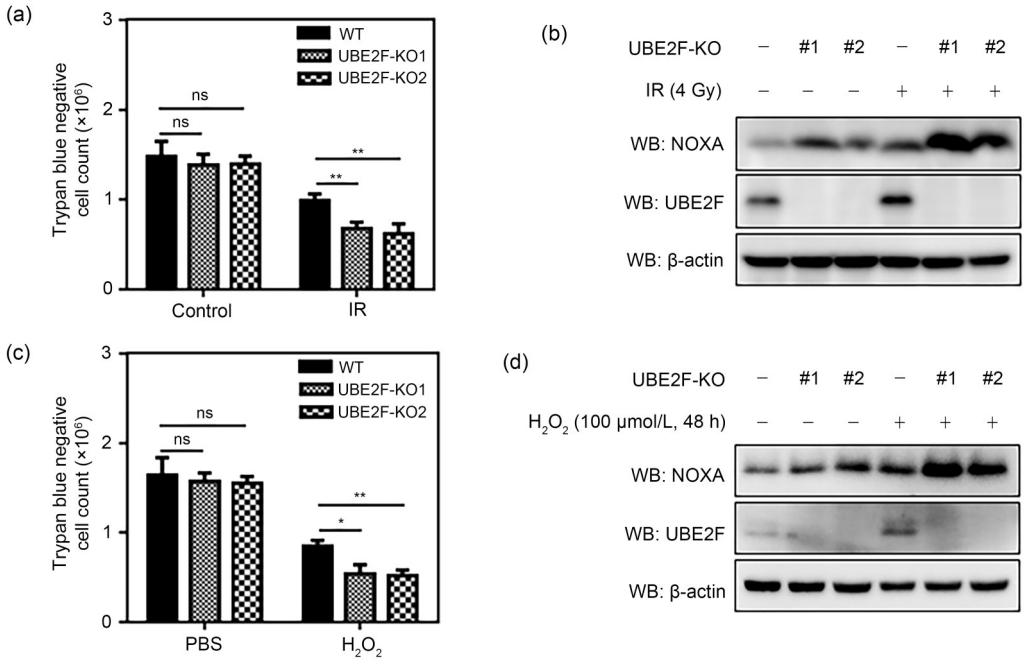


Fig. 2 Sensitization of lung cancer cells to irradiation (IR) by inhibition of ubiquitin-conjugating enzyme E2 F (UBE2F) caused by accumulation of pro-apoptotic protein NOXA. UBE2F knockout (KO) cells exhibited a higher incidence of cell death upon IR (4 Gy) (a) and H_2O_2 (100 μmol/L) for 48 h (c). Cell viability was determined by counting the remaining adherent cells using Nexcelom. Values are shown as average with standard deviation (SD), n=3. $^*P$<0.05, $^{**}P$<0.01, for the indicated comparison; ns: not significant. NOXA expression levels in A549 wild-type (WT) and UBE2F KO cells treated with IR (4 Gy) (b) and H_2O_2 (100 μmol/L) for 48 h (d) are determined by western blot (WB) and normalized against β-actin. PBS: phosphate-buffered saline; NOXA: proapoptotic BH3-only protein.

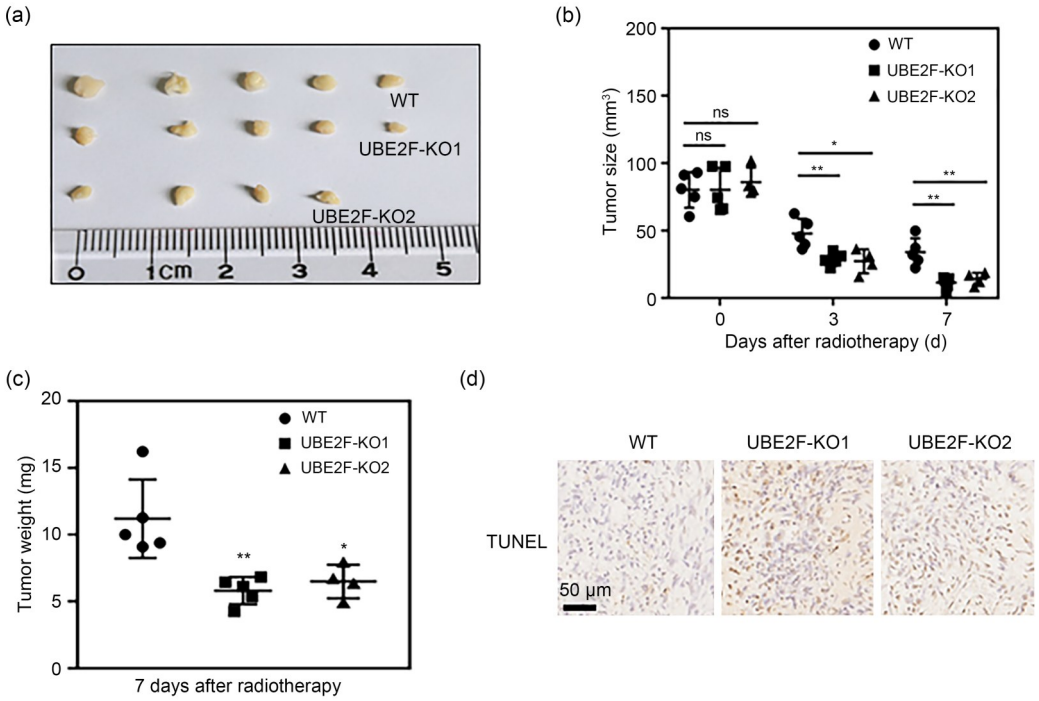


Fig. 3 Effects of ubiquitin-conjugating enzyme E2 F (UBE2F) inhibition on lung cancer cells in vivo. (a–c) UBE2F protected tumors from irradiation-induced growth inhibition: mouse tumor tissue was collected and photographed (a); tumor xenografts were measured for size (b) and weight (c). Values are shown as average with standard deviation (SD), n=4 or n=5. $^{*}P$<0.05, $^{**}P$<0.01, for the indicated comparison; ns: not significant. (d) UBE2F was protective against irradiation-induced apoptosis in vivo. Apoptosis was determined using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. WT: wild-type; KO: knockout.

radiotherapy led us to examine UBE2F expression in clinical samples of human lung adenocarcinoma. Analysis of TCGA RNA-Seq showed that UBE2F expression was broadly increased in lung adenocarcinoma tissue (n=535) compared to adjacent normal tissue (n=59) (P<0.0001; Fig. 4a). Receiver operating characteristic (ROC) curve analysis suggested that UBE2F had a good diagnostic value in lung adenocarcinoma (area under curve (AUC)=0.809) (Fig. 4b). Moreover, Kaplan-Meier analysis revealed that in lung adenocarcinoma patients, high UBE2F messenger RNA (mRNA) levels conferred poorer overall survival than low levels (P=0.048, hazard ratio (HR)=1.34; Fig. 4c). These findings reaffirm that UBE2F is a promising target for radiotherapy sensitivity.

Radioresistance is a major obstacle to the effective treatment of cancer, particularly in non-small cell lung cancer, resulting in poor patient survival rates. The current study uncovers a novel mechanism: neddylation of UBE2F helps lung cancer cells to escape irradiation-induced cell apoptosis and consequently result in radioresistance. Excessive ROS is a common mechanism of radiation-induced cell death, through increasing oxidative stress that induces DNA damage and apoptosis (Cook et al., 2004). Cancer cells tend to obtain radioresistance mainly by inactivating apoptosis signaling pathways through down-regulation of pro-apoptotic proteins or up-regulation of antiapoptotic proteins. Therefore, finding a way to reactivate the apoptotic signaling of cancer cells has become a new direction in anticancer therapy. Our results reveal that irradiation or other stimulus-induced oxidative stress causes an increase in UBE2F expression, leading to degradation of ROS-induced NOXA and consequently inducing apoptotic resistance to radiotherapy. This model is further supported by the fact that deletion of UBE2F promotes irradiation- or H_2O_2-induced apoptosis. Mechanistically, UBE2F silencing-mediated radiosensitization could be attributable to the inhibition of proapoptotic NOXA degradation which is induced by irradiation-generated ROS (Fig. 4d). In combination with previous findings that UBE2F, when overexpressed, promotes tumor growth by ubiquitylating and degrading NOXA through activating CRL5, UBE2F seems to be a

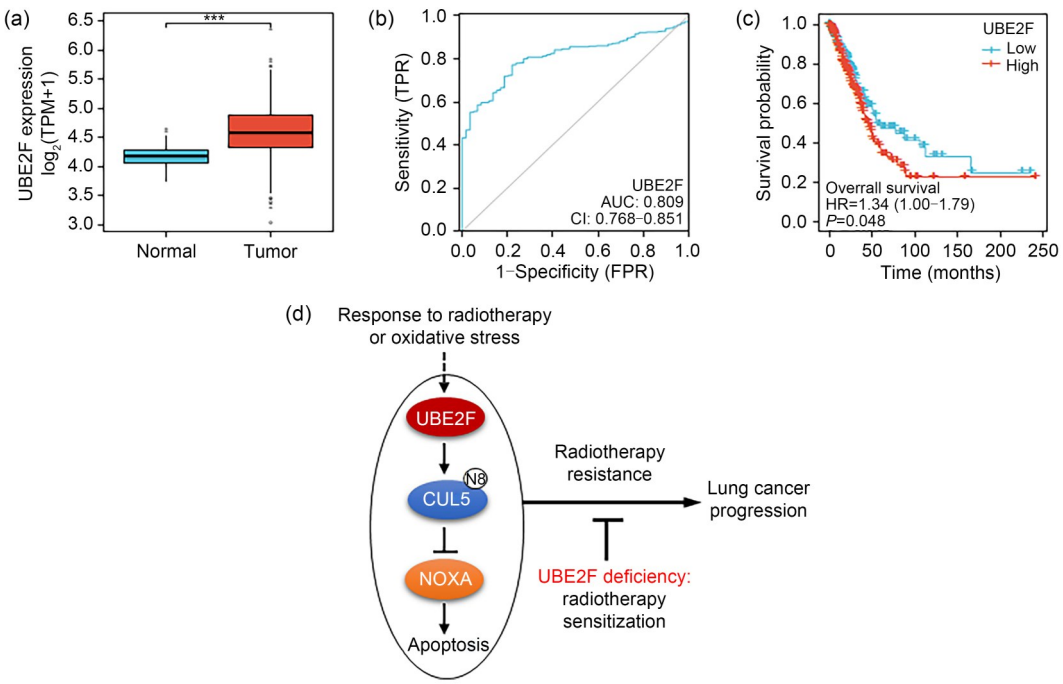


Fig. 4 Ubiquitin-conjugating enzyme E2 F (UBE2F) overexpressed in human lung adenocarcinoma. (a) TCGA database analysis showed that UBE2F expression was broadly increased in lung adenocarcinoma tissue (*n*=535) compared to adjacent normal tissue (*n*=59). (b) Receiver operating characteristic (ROC) curve analysis suggested that UBE2F had a good diagnostic value in lung adenocarcinoma. (c) Kaplan-Meier analysis showed that lung adenocarcinoma patients with high UBE2F messenger RNA (mRNA) levels had poorer overall survival rates than those with low levels. (d) A working model illustrates the role of UBE2F in radiation sensitivity. Irradiation caused an increase of UBE2F expression, leading to degradation of reactive oxygen species (ROS)-induced NOXA and inducing apoptotic resistance to radiotherapy. Meanwhile, knockout of UBE2F sensitized lung cancer cells to radiotherapy. TPM: transcripts per million; TPR: true positive rate; FPR: false positive rate; AUC: area under curve; CI: confidence interval; HR: hazard ratio; N8: neuronal precursor cell-expressed developmentally down-regulated protein 8; CUL5: cullin 5; NOXA: proapoptotic BH3-only protein.

promising anticancer target as well as a radiosensitized target.

UBE2F specifically pairs with RBX2 to promote CUL5 neddylation and activation. The role of RBX2 in cellular radioresistance has been confirmed, based on two pieces of evidence. First, RBX2 is an antioxidant protein that scavenges ROS (Tan et al., 2006). Second, it is an E3 ubiquitin ligase that promotes ubiquitination and degradation of pro-caspase 3, inhibitor of nuclear factor-κBα (IκBα), and NOXA, thus mediating adaptive radioresistance. Consistently, UBE2F, as a neddylation E2, decreases levels of ROS-induced NOXA, indicating the antioxidant protective role of UBE2F. However, whether the radiosensitivity of UBE2F-KO lung cancer cells is partly due to inhibition of ROS scavenging ability still requires further investigation. Our data suggest that oxidative stress-caused UBE2F up-regulation can be blocked by the antioxidant NAC, suggesting that UBE2F can also be induced by oxidative stress, similarly to RBX2 (Sun and Li, 2013). Additionally, it is potentially interesting to further investigate how elevated UBE2F levels are induced by ROS. Does this occur at the transcription level or post-translation level?

In summary, our study demonstrates that inactivation of NEDD8-conjugating enzyme UBE2F serves as a potent radiosensitizing strategy in lung cancer cells by triggering NOXA-mediated apoptosis. These findings lay the foundation for future application of UBE2F inhibitors as a novel radiosensitizer in lung cancer.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Lisha ZHOU, Changsheng DONG, and Yingying ZHU conceived and designed the experiments. Lisha ZHOU and Changsheng DONG performed the experiments and wrote the paper. Lisha ZHOU and Yingying ZHU revised and finalized the paper. Zhuoming XU and Xinran WANG assisted in carrying out the research. Luyi ZHANG, Siyuan CHEN, and Jiahao CHEN assisted in analyzing the data. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Lisha ZHOU, Changsheng DONG, Zhuoming XU, Xinran WANG, Luyi ZHANG, Siyuan CHEN, Jiahao CHEN, and Yingying ZHU declare that they have no conflict of interest.

Animal studies were performed in accordance with animal protocol procedures approved by the Institutional Animal Care and Use Committee of Taizhou University, China (TZYXY2019-503).

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Supplementary information

Materials and methods; Figs. S1 and S2