Ring Finger Protein 180 Suppresses Cell Proliferation and Energy Metabolism of Non-Small Cell Lung Cancer Through Down-Regulating C-myc

Yi Ding
First Affiliated Hospital of Soochow University
Yi Lu
Pudong new area people's hospital
Xinjie Xie
Pudong new area people's hospital
Lei Cao
Pudong new area people's hospital
Shiying Zheng (shiyingzhengsz@163.com)
First Affiliated Hospital of Soochow University

Research article

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Abstract

Background

Non-small cell lung cancer (NSCLC) causes a great number of cancer-related mortality worldwide, but the biomarkers for prognosis of NSCLC are scarce because of their inconsistent efficiency. Proteins containing RING finger domain are the key mediator for ubiquitination, which controls cell cycle and regulates tumor progression. Ring Finger Protein 180 (RNF180) has been reported to suppress gastric cancer, whereas its function in NSCLC is still unclear. In this study, the association between RNF180 expression and NSCLC as well as the effect of RNF180 in cellular proliferation and metabolism of NSCLC were investigated.

Methods

Quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical staining were performed to analyse RNF180 levels. Moreover, RNA interference (RNAi) and lentiviral-mediated vector transfections were performed to silence and overexpress RNF180. Further, Cell Counting Kit-8 (CCK-8) was used to assess its biological function in cell proliferation. A xenograft model was used to examine RNF180 function *in vivo*.

Results

We found that the expression of RNF180 was decreased in NSCLC tissues, and its expression was positively correlated with the survival rate of NSCLC patients. Furthermore, the overexpression of RNF180 in NSCLC cells suppressed their proliferation, glycolytic activities, and mitochondrial respiration *in vitro*, and it restricted the tumorigenicity in mice. In addition, RNF180 knockdown promoted NSCLC cell proliferation and energy metabolism, whereas these promotive effects were counteracted by C-myc inhibitor. The underlying anti-NSCLC mechanism of RNF180 involved in the down-regulation of C-myc through ubiquitin-dependent degradation, and subsequently reduced C-myc downstream: lactate dehydrogenase-A (LDHA) and hexokinase-2 (HK2).

Conclusions

These results firstly indicated the anti-tumor properties of RNF180 and its significant correlation with NSCLC, which endorses RNF180 with the potential as efficient prognostic biomarker for tumor recurrence in NSCLC.

Background

Lung cancer has extremely poor prognosis and high mortality rate which exceeds the combined rate of three of the other most common human cancers, i.e., breast, colorectal, and pancreatic cancers [1]. The 5-year survival rate of lung cancer is no more than 18%, and its death rate within one year after diagnosis and treatment is more than 50% [2]. NSCLC accounts for approximately 85% of all the lung cancer, and its

treatments mainly consist of radiotherapy, chemotherapy, and immunotherapy, based on the different stages of the cancer [1]. However, tumor resistance towards these therapies remains to be the key factor in causing recurrence and further metastasis, leading to a worse prognosis[3, 4]. The advancement of biomarker tests is beneficial in distinguishing the prognosis of NSCLC patients at certain clinical stages [5], thus it is crucial to recognize and develop practical molecular markers for the prophecy of tumor recurrence and progression.

As a matter of fact, the enormous metabolic demands for tumor cell proliferation drives the physiological switching from mitochondrial function to aerobic glycolysis, during which the tumor cells acquire essential components from the generated byproducts, including nucleotides for DNA replication, amino acids for protein synthesis, and lipids for cell membrane formation [6, 7]. The metabolism of cancer cells, influentially different from normal cells, is mainly dependent on glycolysis which is the aerobic process of generating adenosine triphosphate (ATP) from glucose [8, 9]. This hallmark metabolic pathway by cancer cells was first reported by Warburg (1956) and known as the Warburg effect. [10] Whereas, the compensatory aerobic glycolysis in tumor cells is suggested to be independent from the normal mitochondrial oxidative phosphorylation as it has no impact on the basic function of mitochondria [11].

Several key enzymes and transcription factors are involved in regulating cellular metabolism in cancer development, for example, the proto-oncogene *c-myc* [12, 13]. According to previous studies, c-Myc up-regulates the expression of lactate dehydrogenase-A (LDHA) and hexokinase-2 (HK-2) genes [13, 14]. These two genes encode crucial enzymes LDHA and HK-2 in catalyzing glycolysis, the former of which converts pyruvate into lactate, and the latter of which phosphorylates glucose [15, 16].

Proteins containing RING finger domain play a crucial role in mediating the ubiquitination, and they are reported to be active in regulating *protein levels* of *c-myc* [17-19], thus participating in the regulation of tumorigenesis especially in NSCLC and serving as the prospective biomarkers for cancer clinical management [20, 21]. Many RING family members, such as RNF126 and RNF146, have been implicated in the regulation of proliferation and aerobic glycolysis of NSCLC [22, 23]. RNF180, a member of RING family, possesses anti-tumor properties in inhibiting tumor growth in gastric cancer [24], however, whether and how RNF180 function in NSCLC remains largely unidentified.

In this study, we proposed to investigate the correlation between RNF180 and NSCLC prognosis, and examine its effectiveness on NSCLC cellular processes including cell proliferation and metabolic activities. We demonstrate the correlation between RNF180 expression and mortality of NSCLC patients, the suppressive effects of RNF180 on NSCLC cell proliferation, mitochondrial respiration, and glycolytic function, as well as the underlying mechanism which involves the down-regulation of C-myc through the ubiquitin ligase activity of RNF180.

Methods

Human NSCLC tissues samples

A total of 30 pairs of tumorous and adjacent non-tumorous lung tissues were surgically resected from NSCLC patients and used for determining the expression of RNF180. Moreover, 93 NSCLC tissues were used for IHC assay. Patients that received chemotherapy or radiotherapy were excluded from this study. All patients had informed and written consent. This study was approved by the independent ethics committee of The First Affiliated Hospital of Soochow University and was in accordance with the Declaration of Helsinki.

Cell lines and culture

A549, H292, H358, H1975, and PC9 cells purchased from ATCC (Manassas, USA) were cultured in Hyclone DMEM/F12 (SH30023.01B) with 10 % fetal bovine serum 16000-044 (Gibco, USA) supplementation and 100 U/ml penicillin (Solarbio, China). Cells were allowed for adherent growth under 5 % CO_2 at 37°C in a cell incubator.

RNF180 overexpression vectors construction and transfection

The primers for human RNF180 gene (NM_152925.2) were: 5'-CGGAATTCATGAAAAGAAGCAAAGAATTGATAAC-3' (EcoR I) (forward) and 5'-CGGGATCCCTAAAACGGAAAGAAAAAATAGC -3' (BamH I) (reverse). The cDNA fragment encoding RNF180 was inserted into pLVX-Puro (Clontech) between the cloning sites EcoR I and BamH I (underlined). Plasmids pLVX-Puro-RNF180, pMD2G, and psPAX2 (Addgen, USA) were packaged into lentiviruses via 293T cells by Lipofectamine™ 2000 (Invitrogen, USA). At 4-6 h later, the cells were then transferred into the complete medium, followed by harvesting lentiviruses after both 48 and 72 h. The lentiviruses (~ 1.5 μg) with RNF180 expression were then transfected in 293T cells, and the transfected cells were considered as oeRNF180 group. Meanwhile, the cells transfected with lentiviruses with no expression of RNF180 were considered as the Vector group for control.

RNF180 silencing vectors construction and transfection

The system of lentivirus vector was consisted of PLKO.1, pMD2G, and psPAX2. A short hairpin RNAs (shRNAs), whose expression was controlled by U6 promoter, was contained in the PLKO.1 vector. A 20-23 nt gene fragment from the RNF180 cDNA was selected as the siRNA target (siRNA-RNF180). The sequences of siRNA-RNF180 are listed as follows: siRNF180-1 (Site: 219-237): GGAGTATCTTGAGAATCAA; siRNF180-2 (Site: 1211-1229): GCATTAATCAGAGGCTTAA; siRNF180-3 (Site: 1748-1766): GGATGGATTACCTGCACTT.The target siRNA-RNF180 was introduced into the PLKO.1 (pLKO.1-shRNF180), and the accuracy of insertion was analyzed (Shanghai Majorbio Bio-Pharm Technology Co., Ltd, China). The system of lentivirus vector was constructed followed by co-transfection in 293T cells according to the method described above. The control shRNA vector was constructed and

transfected similarly. The NSCLC (H292) cells with RNF180-siRNA-pLKO.1 were marked as the siRNA-RNF180 group, and the cells with the control shRNA vector were used for the control group (siNC).

Xenograft model

Twenty nude mice with 4 to 6 weeks old were purchased (Shanghai Laboratory Animal Company, China), and they were randomly allocated into Vector and oeRNF180 groups. Mice in Vector and oeRNF180 groups were injected with 7×10^5 H358 cells with Vector and oeRNF180 transfection subcutaneously. The mice in each group were reared with independent feeding and regular bedding change. The tumor volume (mm^3) was measured every third days from the 12^{th} to the 33^{th} day and recorded as length × (width^2/2), while at the 33^{th} day, the mice were sacrificed. The tumor tissues were weighted (g) as well. In vivo study was approved by the ethics committee of the The First Affiliated Hospital of Soochow University. All mice were handled according to the Institutional Animal Care and Use Committee (IACUC) guidelines and experiments were conducted following the institute's guidelines for animal experiments.

Cell proliferation assay

The proliferation of NSCLC (H292 and H358) cells was assessed by Cell Counting Kit-8 (CCK-8). Briefly, the control or treated cells in individual well were mixed with 10 ml of CCK-8 reagent and 90 ml of the 0, 24, 48, or 72 h incubated serum-free cultural medium, followed by another 1 h incubation. A microplate reader (Bio-Rad, USA) was used for the measurement of 450 nm optical density.

Immunohistochemistry (IHC) assay

The tumorous or normal lung tissue sections (~ 4-7 μm thick) were incubated with RNF180 antibody (ab127548, Abcam) overnight at 4°C and then with secondary antibodies (D-3004, Long Island Biotech, China) for 30 min at 25°C. DAB (3,3'-diaminobenzidine) substrate (Long Island Biotech, USA) and hematoxylin 714094 (BASO Diagnostic Inc., China) were applied for IHC staining. The Eclipse Ni-E/Ni-U microscope (NIKON, Japan) with DS-Ri2 imaging system (NIKON, Japan) was used to visualize and count RNF180-postive cells.

Western blotting

The total protein level in the supernatant of NSCLC cell lysis was determined by BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific, China). The samples were boiled for 10 min at a temperature of 95°C. An aliquot of 30 mg of protein was separated by SDS-PAGE (10% gel) followed by PVDF membranes transfer, 1 h blocking in 5% skim milk, and incubation with separate antibodies (Abcam, Inc., USA) of 1:1000 diluted antibody Ab127548 (anti-RNF180), 1:500 diluted antibody Ab39688 (anti-C-myc),

1:5000 diluted antibody Ab227198 (anti-HK-2), 1:1000 diluted Ab101562 antibody (anti-LDHA), and 1:2000 diluted antibody against GAPDH (#5174, Cell Signaling Technology) overnight at 4°C. Then the membranes were incubated with horseradish peroxidase secondary antibodies (A0208, A0181 and A0216, Beyotime Biotechnology) at room temperature for 1 h. The ECL plus substrate (GE Healthcare, USA) with LAS-400 image analyzer (FujiFilm Medical Systems, USA) was used for the detection of horseradish peroxidase signal.

Quantitative real-time (qRT)-PCR

The trizol regent (Invitrogen, USA) was used for extraction of the total RNA from cultured cells or tissue samples. The first-strand cDNA synthesis was carried out using RevertAid First Stand cDNA Synthesis Kit (Fermentas, USA). Quantified analysis on mRNA levels of RNF180 and GAPDH was conducted using SYBR Green Master Mixes (Thermo Fisher Scientific, China) on 7300 Real-Time PCR System (Applied Biosystems, USA). GAPDH was used for the control of normalization. The sequences of related primers used are summarized in as follows: RNF180: F 5'-TGACTTTCCTGATGGACCTG-3', R 5' – ATCCCACTCCTGAGTATTTACC-3'; GAPDH: F 5'-GGATTGTCTGGCAGTAGCC-3', R 5' – ATTGTGAAAGGCAGGGAG-3'

Analysis of cellular aerobic glycolysis and mitochondrial respiration

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cell lines H358 and H292 were measured and analyzed by the Seahorse XFe24 Analyzer (Seahorse Bioscience, USA). Approximately 50,000 target cells per well in a 24-well plate (Seahorse Bioscience, USA) with a total of 250 µl culture medium were seeded, followed by overnight incubation with 5% CO_2 at 37°C.

For ECAR assay, the adherent cells were washed with phosphate-buffered saline (PBS) and resuspended in 500 µl XF Base Medium with a pH at 7.4 (Seahorse Bioscience, USA) containing 2 mM L-glutamine (basal conditions) and 25 mM of glucose (the main substrate in aerobic glycolysis), 1 µM of oligomycin (an inhibitor of oxidative phosphorylation), or 50 mM of 2-deoxy-D-glucose (2-DG) (an inhibitor of glycolysis) in real-time conditions.

For OCR assay, the adherent cells were maintained in XF Base Medium containing 1 µM of oligomycin, 1 µM of protonophore trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP), and 0.5 µM of Antimycin A/Rotenone in real-time conditions. The applications of oligomycin (mitochondrial inhibitors) for ATP synthase blocking, FCCP for inner mitochondrial membrane permeability induction, and rotenone & antimycin A for complexes I and III inhibition, respectively, were required for both assays.

Immunofluorescence

The cells were mounted onto microscope slides with 30 min fixation using 4% formaldehyde, followed by 10 min permeabilization using 0.5% Triton X-100 (Solarbio, China). After 30 min blocking using 1% bovine serum albumin (Solarbio, China), the cells were incubated with antibody ab15580 (anti-Ki67) from Abcam overnight at 4°C, and then they were further incubated with a secondary antibody (Beyotime Biotechnology, China) for 30 min in dark at 37°C. 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) from Beyotime Biotechnology was used for cell nuclei staining. ECLIPSE Ni Fluorescent microscope from NIKON was used for visualization.

Co-immunoprecipitation (Co-IP) and in vitro ubiquitination assays

Approximately 100 μg immune complexes in the lysed NSCLC cell supernatant were harvested by Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, USA). IgG sc-2027 from Santa Cruz Biotechnology, anti-RNF180 antibody Orb2721 from Biorbyt, and anti-C-myc antibody ab32072 from Abcam were used in Co-IP assays. Anti-RNF180 and anti-C-myc antibodies (ab127548 and ab39688) from Abcam were used in western-blot. The quantity of total protein was controlled. Anti-ubiquitin antibody ab7780 from Abcam was used to determine the C-myc ubiquitination in the immune-complexes precipitation.

Statistical analyses

Data were displayed as mean ± SEM, which were calculated from 3 parallels in each experiment. Comparison between groups was conducted using One-Way Analysis of variance and Tukey's test as post-hoc, and statistical significance was determined at $P < 0.05$.

Results

The Expression of RNF180 was down-regulated in NSCLC

The overall expression level of RNF180 was found to be significantly lower in NSCLC patients, compared with that in normal individuals (Fig. 1). To be specific, the mRNA level of RNF180 was investigated by qRT-PCR and compared among 30 pairs of tumorous and adjacent non-tumorous lung tissues. We observed a significant ($P < 0.01$) down-regulation of RNF180 transcriptional level in tumorous tissues in comparison with that in normal tissues (Fig. 1A). Through IHC assay, we were able to visually detect the difference in RNF180 expressions between tissues from healthy individual and NSCLC patient. In a total of 93 NSCLC patients, we observed 53 tumorous tissues with high expression of RNF180 and 40 tumorous tissues with low expression of RNF180 (Fig. 1B).

In addition, by analyzing the data obtained from TCGA database (http://ualcan.path.uab.edu/analysis.html), we found a similar expression pattern of RNF180 in patients with Lung Adenocarcinoma (LUAD), the main type of NSCLC (Fig. 1C). Specifically, the transcriptional

level of RNF180 in LUAD patients from a total of 515 primary tumorous tissues, was numerically (P< 0.05) lower than that in healthy individuals from a total of 59 normal tissues (Fig. 1C).

High RNF180 expression was correlated with low mortality rate in NSCLC patients

Based on the Kaplan-Meier method, we found a significantly (P< 0.001) lower survival rate of NSCLC patients with high expression of RNF180 than that in the patients with low expression of RNF180, throughout 60 months (Fig. 1B). Meanwhile, based on the data from TCGA database, we also observed a similar connection between RNF180 expression level and the survival rate in Lung Adenocarcinoma (LUAD) patients (Fig. 1C). The survival probability over 6,000 days was observed significantly higher in 126 LAUD patients with high expression of RNF180, compared with that in 376 LAUD patients with low/medium expression of RNF180 (Fig. 1C).

Differential expressions of RNF180 in NSCLC cell lines and the establishment of its overexpression in H358

The mRNA and protein expression levels of RNF180 in five NSCLC cell lines (A549, H358, H292, H358, and PC9) and human lung bronchial epithelial (HBE) cell line were examined by qRT-PCR and western-blot separately. Compared with HBE cell line, the relative transcriptional levels of RNF180 in A549, H358, H292, H358, and PC9 cell lines were numerically (P < 0.05) lower (Fig. 2A). At the same time, the relative RNF180 protein levels in A549, H358, H292, H358, and PC9 cell lines were relatively lower than that in HBE cell line (Fig. 2A).

Based on both RNF180 mRNA and protein levels, we established the overexpression of RNF180 in H358 cell line. The relative mRNA and protein expression levels of RNF180 in the developed cells (oeRNF180) were analyzed using the same methods described above, and we found that both of them were significantly (P< 0.01) higher in oeRNF180 group when compared with those in either control or Vector cell groups (Fig. 2A).

RNF180 overexpression inhibited the proliferation and metabolism of NSCLC cells

The proliferation of NSCLC cells was assessed by CCK-8 analysis and is shown in Fig. 2B. In comparison with the Vector control cells, the proliferation of oeRNF180 cells was significantly (P< 0.01) lower at 12, 24, and 48 h (Fig. 2B). Moreover, with Ki67 and DAPI immunofluorescence staining, we were able to visualize and compare the NSCLC cells that were undergoing cellular proliferation (Fig. 2G).

The effects of oeRNF180 on glycolytic function and mitochondrial respiration in NSCLC cells were evaluated by extracellular acidification and oxygen consumption rates separately through Seahorse tests, and the overall inhibitory effect is shown in Fig. 2C. In comparison with the Vector control cells, the glycolysis, glycolytic capacity, glycolytic reserve, basal respiration, ATP-linked respiration, maximal respiration, and spare capacity in oeRNF180 cells were all decreased (Fig. 2C).

Furthermore, the protein level of C-myc and the levels of glycolysis related proteins HK-2 and LDHA in the Vector control or oeRNF180 cells were analyzed based on western-blot. We observed that all these three proteins were significantly ($P < 0.01$) suppressed in oeRNF180 cells, compared with those in the control (Fig. 2D).

Additionally, based on TCGA database and gene set enrichment analysis, the correlation between RNF180 gene expression and c-Myc protein expression or cellular glycolysis level was explored separately. The gene expression level of RNF180 was negatively correlated with the protein expression level of c-Myc in NSCLC ($P < 0.01$, NES score = -3.35) (Suppl. Figure 1). Similarly, the gene expression level of RNF180 was also negatively correlated with the level of glycolytic functions ($P < 0.01$, NES score = -2.48) (Suppl. Figure 1).

RNF180 overexpression restricted NSCLC tumorigenicity in mice

In order to investigate the *in vivo* effect of RNF180 overexpression on NSCLC tumorigenicity, Vector control-transfected or oeRNF180-transfected H358 cells were subcutaneously injected into nude mice, with five mice in each group. The mean tumor volumes in oeRNF180-H358 cells-injected mice were observed significantly ($P < 0.01$) lower than those in Vector-H358 cells-injected mice after 21 days (Fig. 2E). To be specific, the mean tumor volumes in mice injected with oeRNF180-H358 cells were decreased at Day 21, 24, 27, 30, and 33, respectively, compared with those in the control. Meanwhile, at Day 33, the mean tumor weight in mice injected with oeRNF180-H358 cells were also significantly ($P < 0.01$) reduced, in comparison with that in the control group (Fig. 2E).

C-myc inhibition counteracted the promoted effects of RNF180 knockdown on NSCLC cell proliferation and metabolism

The RNF180 knockdown in H292 cell line was established and confirmed by qRT-PCR and western-blot (Fig. 3A). In details, the relative mRNA levels of RNF180 in siRNF180-1, 2, and 3 cells were all significantly ($P < 0.01$) repressed compared to those in H292 control and siNC cells; while the relative protein levels of RNF180 in siRNF180-1, 2, and 3 cells were all significantly ($P < 0.01$) repressed as well compared to those in H292 control and siNC cells.

In comparison with the siNC cells, the siRNF180 cells significantly ($P < 0.01$) promoted their proliferation at 12, 24, and 48 h (Fig. 3B). On the contrary, the treatment of C-myc inhibitor 10058-F4 significantly ($P < 0.01$) inhibited the proliferation of siNC cells, and it even significantly ($P < 0.01$) counteracted the siRNF180-promoted cell proliferation at 12, 24, and 48 h (Fig. 3B).

Furthermore, the effects of RNF180 knockdown and C-myc inhibitor on glycolytic function and mitochondrial respiration in NSCLC cells are shown in Fig. 3C. In terms of glycolytic function, the siRNF180 cells stimulated their glycolysis, glycolytic capacity, and glycolytic reserve. In terms of mitochondrial respiration, the siRNF180 cells promoted their basal respiration, ATP-linked respiration, maximal respiration, and spare capacity. However, C-myc inhibitor 10058-F4 restrained the glycolytic

function and mitochondrial respiration in siNC cells, and the 10058-F4 treatment on siRNF180 cells induced the reduction of their extracellular acidification and oxygen consumption rates as well.

In addition, the relative protein levels of C-myc, HK-2, and LDHA in siRNF180 cells were observed significantly ($P < 0.01$) elevated, compared with those in siNC cells (Fig. 3D). Whereas, C-myc inhibitor 10058-F4 significantly ($P < 0.01$) reduced C-myc, HK-2, and LDHA levels in siNC cells. At the same time, the treatment of 10058-F4 significantly ($P < 0.01$) counteracted the stimulation of C-myc, HK-2, and LDHA in siRNF180 cells and decreased their expression levels.

RNF180 enhanced the ubiquitination of C-myc in NSCLC cells

The transcriptional levels of C-myc in RNF180-overexpressed H358 and RNF180-knockdown H292 cells were analyzed by qRT-PCR and compared with that in separate control cells, but no significant difference was detected (Fig. 4A). The mRNA level of C-myc in oeRNF180 cells was only numerically ($P < 0.05$) higher than that that in Vector control cells, While the C-myc mRNA levels in siRNF180-1 and siRNF180-2 cells were only numerically ($P < 0.05$) higher than that in siNC cells as well.

However, through Co-IP, we observed the association between RNF180 and C-myc proteins in H292 cells (Fig. 4B). Furthermore, we also detected the enhanced ubiquitination of C-myc protein in oeRNF180-transfected H358 cells, by immunoprecipitation and immunoblotting, compared with that in Vector control cells (Fig. 4C). Finally, analyzed by western-blot, we found that the treatment of proteasome inhibitor MG132 significantly ($P < 0.01$) increased the relative protein levels of C-myc in NSCLC cells (Fig. 4D). To be specific, MG132 treatment induced the elevation of C-myc in both Vector control and oeRNF180 cells, though the relative C-myc level in MG132 treated oeRNF180 cells was still lower than that in MG132 treated Vector control cells (Fig. 4D).

Discussion

Our study focused on proteins containing RING finger domain, since they are mediators of either E2 ubiquitin-conjugating enzyme- or E3 ubiquitin ligase-dependent ubiquitination, essentially participating in regulating multiple biological processes including cellular apoptosis and carcinogenesis[25, 26]. Based on the expression data of NSCLC patients, recruited in our hospital, we found that RNF180 was significantly reduced in NSCLC (Fig. 1A), and comparing the survival of NSCLC patients with RNF180 ^{high} (n = 53) and RNF180 ^{low} (n = 40) tumor tissues, we revealed that high RNF180 predicted a long overall survival (Fig. 1B), which were further confirmed in an online RNF180 analysis in LUAD (one major subtype of NSCLC) from TCCA database (Fig. 1C). Moreover, a non-significant but numerical correlation was also found between the expression of RNF180 and the survival rate of Lung Squamous Cell Carcinoma (LUSC) patients (**Suppl. Figure 2**). Accordantly, these results demonstrate the potential of RNF180 as a biomarker for NSCLC prognosis, though further univariate survival analysis is warrant for determining its independency as NSCLC prognostic predictor.

RNF180 is a membrane-bound E3 ubiquitin ligase[27], and its function as tumor inhibitor in gastric cancer has been studied previously [28]. Deng et al. (2016) demonstrated that RNF180 acted as a tumor suppressor gene in gastric cancer, and its anti-cancer effects included inhibiting the cell proliferation and repressing tumor growth *in vivo*, which was further confirmed in NSCLC (Fig. 2B, D, F, G).

Besides, the direct and indirect functions of RNF in regulating cellular energy metabolism were documented previously [29, 30]. In this work, our data showed that RNF180 restricted both aerobic glycolysis and mitochondrial respiration (Fig. 2C), and the molecular mechanisms involving the suppression of protein levels C-myc and its subsequent down-streams: HK-2, and LDHA related to energy metabolism (Fig. 2D). All these findings could suggest the inhibited effect of RNF180 in NSCLC energy metabolism.

RNF family were shown to be active in interacting with C-myc proteins [18–20]. Furthermore, we studied whether C-myc was the mechanism, whereby RNF180 regulated NSCLC cell proliferation and energy metabolism. Our data confirmed an inhibited effect of C-myc inhibitor on NSCLC cell proliferation, glycolytic function and mitochondrial respiration (Fig. 3), and the effect of which were significantly weakened by RNF180 knockdown. The ubiquitination-dependent degradation of C-myc in counteracting carcinogenesis and tumor progression has been reported [31, 32], and in this study, we firstly suggested the anti-cancer effect of RNF180 via a down-regulating C-myc through ubiquitination-dependent degradation pathway (Fig. 4).

Conclusion

Our findings demonstrate the inhibitory effects of RNF180 on NSCLC cell proliferation, metabolic activities, and tumorigenicity as well as their potential mechanism. To our knowledge, we are the first to display the anti-tumor function of RNF180 in lung cancer. Most importantly, besides all the biological findings, our study is significant for the clinical management of NSCLC patients as well. The survival analysis illustrate that RNF180 can effectively predict NSCLC patient outcomes after therapy. Considering the significance of RNF180 in both functional and clinical aspects, we believe that RNF180 is a prospective biomarker in guidance of the prognosis of NSCLC.

Abbreviations

NSCLC
Non-small cell lung cancer; RNF180：Ring Finger Protein 180；
qRT-PCR
Quantitative real-time polymerase chain reaction;RNAi： RNA interference；
CCK-8
Cell Counting Kit-8;LDHA：lactate dehydrogenase-A；HK2：hexokinase-2；
IHC
Immunohistochemistry;OCR：oxygen consumption rate；

ECAR
extracellular acidification rate;PBS phosphate-buffered saline
Co-IP
Co-immunoprecipitation;

Declarations

Ethics approval and consent to participate

All human and animal procedures that involved in current study were approved by the independent ethics committee of The First Affiliated Hospital of Soochow University, Suzhou 215006, P. R. China and was in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Fundings

Not applicable

Author contributions

SZ designed this project and wrote the manuscript; YD performed the experiments; YL XX and LC analyzed the data and edited diagrams. All authors have read and approved the manuscript.

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Figures

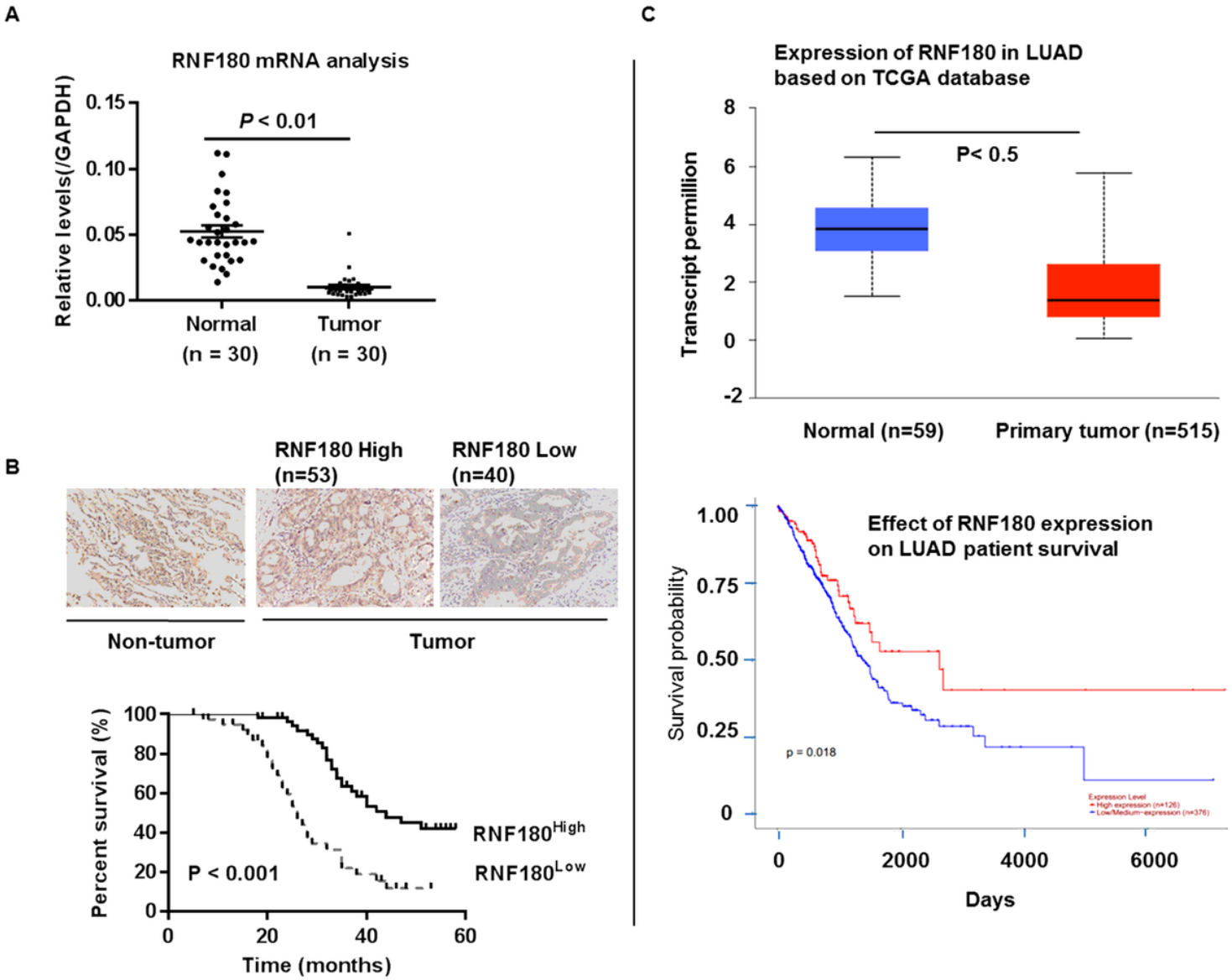

Figure 1

Expression of RNF180 and survival probability in non-small cell lung cancer (NSCLC) patients. A: The mRNA level of RNF180 in 30 pairs of tumorous and adjacent normal tissues was measured by qRT-PCT. B: Expression of RNF180 in tissue microarrays from healthy controls (n = 5) and NSCLC patients (n = 93) was assessed using IHC assay (original magnification at 200×). Overall survival probability of NSCLC patients was analyzed by Kaplan-Meier method and compared between RNF180 high-expressed group (n

= 53) and RNF180 low-expressed group (n = 40). C: Expression of RNF180 in Lung Adenocarcinoma (LUAD) patients (n = 515) and healthy individuals (n = 59) was analyzed based on TCGA database. Survival probability of LUAD patients with high expression of RNF180 (n = 126) and low/medium expression of RNF180 (n = 376) was analyzed and compared by Kaplan-Meier method.

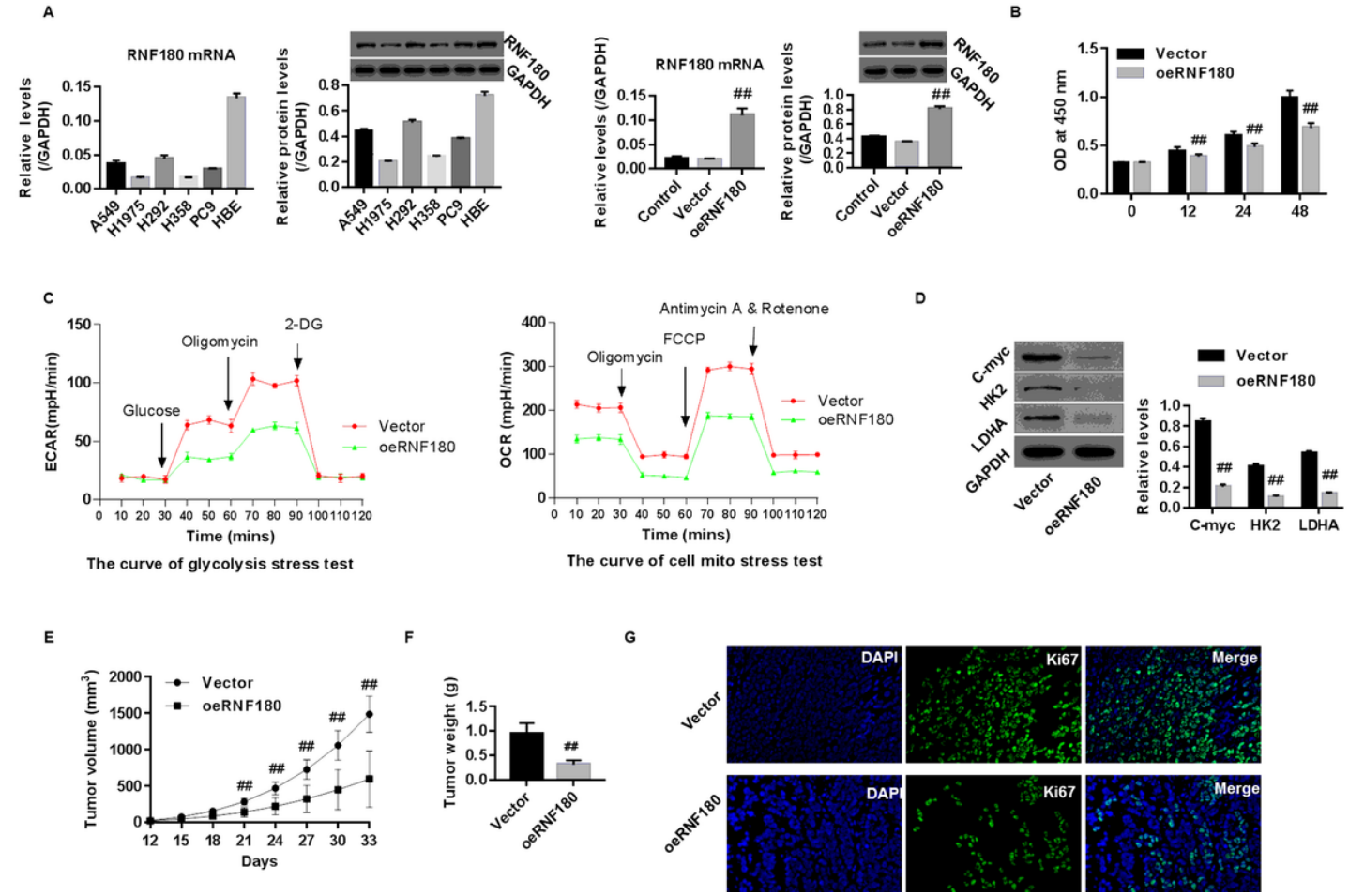

Figure 2

Effects of RNF180 on cell proliferation, metabolism, and tumorigenicity of non-small cell lung cancer (NSCLC). A: The mRNA and protein levels of RNF180 in NSCLC cell lines A549, H358, H292, H358, PC9, and human lung bronchial epithelia cell line HBE were detected by qRT-PCR and western-blot. The established overexpression of RNF180 at both mRNA and protein levels in H358 cell line was also confirmed. Full-length blots/gels are presented in Supplementary Figure 2A B: Cellular proliferation of control (Vector) and oeRNF180-transfected H358 cells (oeRNF180) was analyzed and compared by CCK-8. C: Glycolytic function and mitochondrial respiration in control (Vector) and oeRNF180-transfected H358 cells (oeRNF180) were analyzed by Seahorse tests. D: The protein levels of C-myc, HK-2, and LDHA in control (Vector) and oeRNF180-transfected H358 cells (oeRNF180) were analyzed and compared by western-blot. Full-length blots/gels are presented in Supplementary Figure 2D. E: Tumor volume (mm3) in nude mice from the 12th to the 33rd day after control (Vector) or oeRNF180-transfected H358 cells (oeRNF180) injection (n = 5 in each group). F: Tumor weight (g) in nude mice on the 33rd day after control (Vector) or oeRNF180-transfected H358 cells (oeRNF180) injection (n = 5 in each group). G:

Immunofluorescence staining for DAPI (blue) and Ki67-positive cells (green); ## P < 0.01 vs. Vector; GAPDH as loading control.

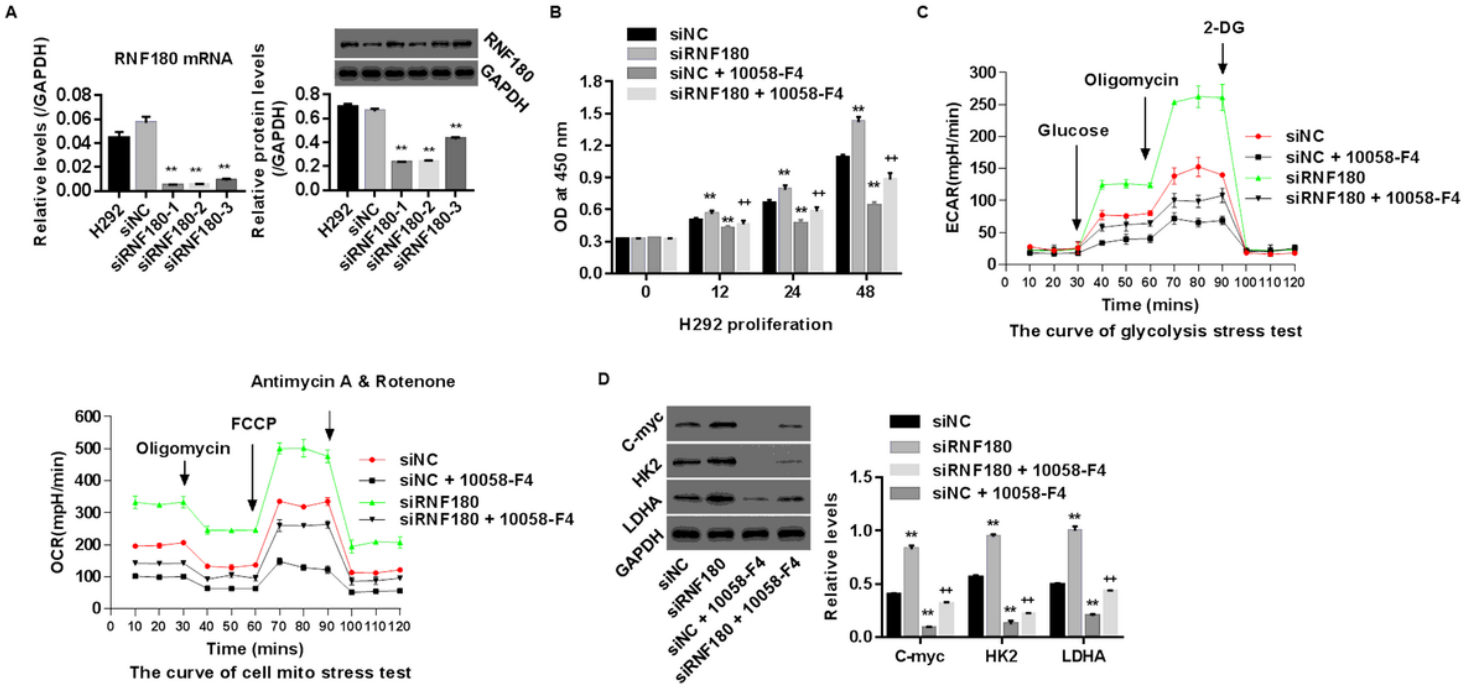

Figure 3

Effects of RNF180 knockdown and C-myc inhibitor on cell proliferation and metabolism of non-small cell lung cancer (NSCLC). A: The established RNF180 knockdown in H358 cell line (siRNF180) was confirmed at both mRNA and protein levels through qRT-PCR and western-blot separately. Full-length blots/gels are presented in Supplementary Figure 3A. B: Cellular proliferation of control (siNC) and siRNF180-1-transfected H292 cells (siRNF180) with or without 100 μmol/l of 10058-F4 treatment was analyzed and compared by CCK-8. C: Glycolytic function and mitochondrial respiration in control (siNC) and siRNF180-1-transfected H292 cells (siRNF180) with or without 100 μmol/l of 10058-F4 treatment were analyzed by Seahorse tests. D: The protein levels of C-myc, HK-2, and LDHA in control (siNC) and siRNF180-1-transfected H292 cells (siRNF180) with or without 100 μmol/l of 10058-F4 treatment were analyzed and compared by western-blot. ** P < 0.01 vs. siNC; ++ P < 0.01 vs. siNC+10058-F4; GAPDH as loading control. Full-length blots/gels are presented in Supplementary Figure 3D.

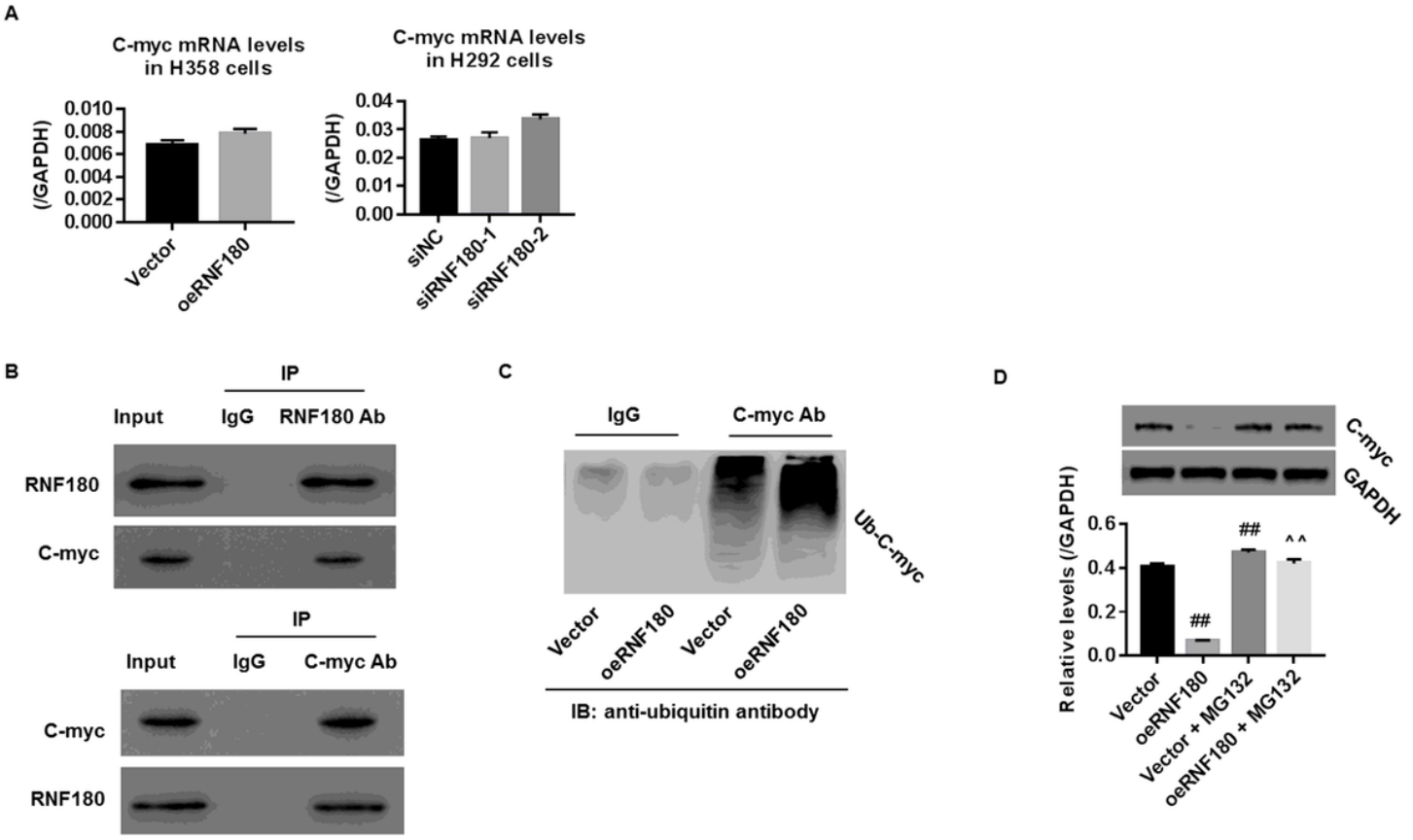

Figure 4

Effects of RNF180 on C-myc ubiquitination in non-small cell lung cancer (NSCLC) cells. A: The mRNA levels of C-myc in RNF180-overexpressed H358 (oeRNF180) and RNF180-knockdown H292 cells (siRNF180) were assessed by qRT-PCR and compared with their own control cells (Vector and siNC) separately. B: Co-immunoprecipitation showed the potential association between RNF180 and C-myc in H292 cells. C: Immunoprecipitation and immunoblotting of C-myc in control (Vector) and oeRNF180-transfected H358 cells (oeRNF180) with C-myc antibody or anti-ubiquitin antibody. D: The protein expression levels of C-myc in control (Vector) and oeRNF180-transfected H358 cells (oeRNF180) with or without 10 μmol/l of MG132 treatment were assessed by western-blot; ## P < 0.01 vs. Vector; ++ P < 0.01 vs. oeRNF180; IgG and GAPDH as loading control. Full-length blots/gels are presented in Supplementary Figure 4D.

Supplementary Files

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