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RESEARCH ARTICLE

Identification of an E3 ligase-encoding gene *RFWD3* in non-small cell lung cancer

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Abstract **In order to unveil ubiquitin pathway genes (UPGs) that are essential for non-small cell lung cancer (NSCLC) cell proliferation, we recently conducted a siRNA screening experiment to knockdown the expression of 696 UPGs found in the human genome in A549 and H1975 NSCLC cells. We found that silencing of one of the candidates, *RFWD3* that encodes an E3 ubiquitin ligase essential for the repair of DNA interstrand cross-links in response to DNA damage, led to dramatic inhibition of NSCLC cell proliferation with significant *Z*-scores. Knockdown of *RFWD3* suppressed colony forming activity of NSCLC cells. We further evaluated the significance of *RFWD3* in NSCLCs and found that this gene was more elevated in tumor samples than in paired normal lung tissues and was inversely associated with the clinical outcome of patients with NSCLC. Moreover, *RFWD3* expression was significantly higher in smokers than in non-smokers. These results show for the first time that *RFWD3* is required for NSCLC cell proliferation and may have an important role in lung carcinogenesis.**

Keywords *RFWD3*; NSCLC; prognosis; tobacco smoke

Introduction

The ubiquitin E3 ligases catalyze the transfer of ubiquitins from E2 enzymes to substrate proteins for their proteasomal degradation. The stability of many oncoproteins and tumor suppressors is controlled by E3 ligases [1–3], which are divided into the following three major subtypes according to their molecular structure and mechanisms of action: the homologous to E6-associated protein carboxyl terminus (HECT), really interesting new gene (RING), and RING-in-between-RING (RBR) [4]. E3 ligases exhibit higher substrate specificity than E1 and E2; thus, targeting oncogenic E3 ligases is a possible strategy for cancer treatment [5,6].

The E3 ligase RING finger and WD repeat domain 3 (*RFWD3* or RNF201/FLJ10520) was identified as a substrate protein of the Ataxia Telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) in 2007 [7]. *RFWD3* can stabilize p53 in response to DNA damage [8]. *RFWD3* also plays a role in controlling replication checkpoints and responses to DNA damage in cancer cells despite E3 ligase activity [9,10]. Biallelic mutations have been found in *RFWD3* in patients with Fanconi anemia, and these mutations cause this disease [11]. A genome-wide association study demonstrated that *RFWD3* is a susceptibility locus for malignant neoplasms, including multiple myeloma [12] and testicular germ cell tumor [13]. However, the significance of *RFWD3* in cancers, including lung cancer, remains to be elucidated.

To unveil critical ubiquitin pathway genes (UPGs) for non-small cell lung cancer (NSCLC), the 696 UPGs found in human genome were silenced by small interfering RNA (siRNA) in NSCLC cells, and 31 UPGs including *RFWD3* were identified [14]. Here, the significance of *RFWD3* in NSCLC was investigated.

Materials and methods

Patients' samples and immunohistochemistry analysis

This study was approved by the research ethics committees

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of Institute of Zoology, Chinese Academy of Sciences and the Third Affiliated Hospital of Kunming Medical University (Yunnan Tumor Hospital). All lung cancer samples were collected with informed consent. Immunohistochemistry (IHC) assay was performed using an anti-RFWD3 antibody (Proteintech, Chicago, USA) and the formalin-fixed, paraffin-embedded lung cancer tissue specimens (5 μm). Immunoreactivity score (IRS) was calculated as IRS (0–12) = RP (0–4) × SI (0–3), where RP is the percentage of staining-positive cells and SI is the staining intensity.

Cell culture and siRNA library

NSCLC lines A549 and H1975 were obtained from the American Tissue Culture Collection (Manassas, VA, USA). These cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The Human siGENOME SMARTpool siRNA Libraries containing 696 UPGs were purchased from the Thermo Scientific Dharmacon (Lafayette, CO, USA). siRNAs were transfected into the cells according to the manufacturer's instructions, and cell viability was assessed using a CellTiter-Glo Reagent (Promega, Fitchburg, WI, USA) following the manufacturer's instructions. *Z*-scores were calculated as follows: $z = (x - m)/s$, where x is the raw score to be standardized, m is the mean of the plate, and s is the standard deviation of the plate [15,16]. The *Z*-score of a gene reflects its requirement for cell proliferation, and *Z*-score $\leqslant -2$ indicates that the gene is required for cell proliferation and that the silencing of the gene significantly inhibits cell proliferation. *Z*-score $\geqslant 2$ indicates that the gene inhibits lung cancer cell proliferation and that inhibition of the gene significantly promotes cell proliferation [15].

Cell proliferation and colony-forming activity assessment

A549 and H1975 cells were transfected with si*RFWD3* (5′-GUUAAGAUGUUGAGUACUG-3′) for 24 h, and cell proliferation was assayed by a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instruction. Cell viability was estimated by trypan blue dye exclusion. Foci formation was facilitated by seeding A549 or H1975 cells transfected with negative control or *RFWD3*-specific siRNA onto 35 mm plates (300 cells per plate) in triplicate. The cells were stained with Giemsa after 14 days of culturing, and clones containing more than 50 cells were counted.

Online data availability

The cancer microarray database Oncomine [17], including the databases of Hou Lung [18], Selamat Lung [19], Landi Lung [20], Okayama Lung [21], Wachi Lung [22], and Su Lung [23], were explored. The transcriptome data and clinical data of 518 lung adenocarcinomas (LUADs) and 500 lung squamous cell carcinomas (LUSCs) were downloaded from the Cancer Genomics Hub. The Cancer Genome Atlas (TCGA) database was assessed using accession code phs000178. The expression of *RFWD3* in Asian population, including Chinese [24,25] and Japanese [26,27], NSCLCs, was assessed in the Gene Expression Omnibus (GEO) datasets under the accession codes GSE19804, GSE29250, GSE31210, and GSE66759. The Online Survival Analysis Software containing the microarray data of 1928 LUADs and LUSCs [28] was utilized. The correlation of *RFWD3* expression with overall survival (OS) was analyzed by entering *RFWD3* into the database and setting different clinical parameters. The Kaplan–Meier survival plots and log rank *P* values were obtained from the webpage. The baseline demographic characteristics of the patients are available in the reference [28].

Statistical analyses

Statistical analyses were conducted using SPSS 16.0 for Windows (Chicago, IL, USA). Differences between data groups were evaluated for significance using Student's *t*-test of unpaired data, and all statistical tests were two-sided. The survival curve was estimated with the Kaplan–Meier method and log-rank test using the Online Survival Analysis Software [28]. *P* values less than 0.05 were deemed statistically significant in all cases.

Results

Unveiling *RFWD3* in NSCLCs

We conducted a systematic knockdown of UPGs in NSCLC cell lines A549 and H1975 (Fig. 1A) by using the siRNA human siGENOME SMARTpool library obtained from Dharmacon [14,16]. The robust *Z*-scores of the SMARTpools were calculated from duplicate experiments [15]. A total of 31 candidates were identified, and *RFWD3* was required for the proliferation of the two NSCLC cell lines with significant *Z*-scores (Fig. 1B). We verified the results by transfecting si*RFWD3* in A549 and H1975 NSCLC cells. si*RFWD3* significantly inhibited cell proliferation (Fig. 1C) and suppressed the colony-forming activity of the cells (Fig. 1D). These data suggest that *RFWD3* may have important roles in NSCLC.

RFWD3 expression in NSCLCs

The expression level of RFWD3 in NSCLCs was evaluated by performing IHC assays in tumor samples of 22 NSCLCs (Table 1). RFWD3 staining among 14 (64%)

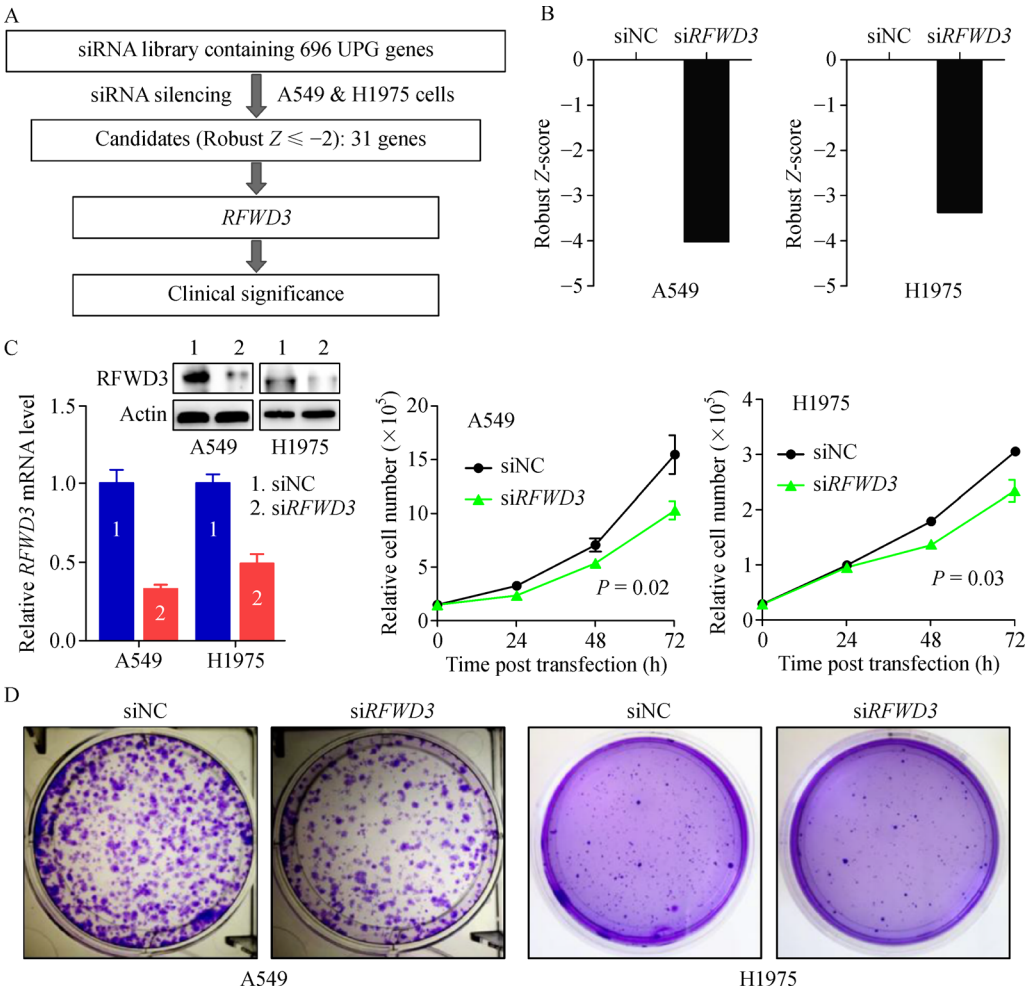

Fig. 1 RFWD3 is required for NSCLC cell proliferation. (A) Schematic of the study workflow. (B) *Z*-scores of si*RFWD3* in A549 and H1975 cells treated with 50 nmol/L indicated siRNAs from the siGenome library (containing 696 UPGs) for 72 h. (C) The cells were transfected with si*RFWD3*, transfection efficacy was tested using real-time RT-PCR and Western blot analysis, and cell proliferation was assessed by CCK-8 analysis. (D) Colony-forming activity of the cells transfected with siNC or si*RFWD3*.

Table 1 Baseline demographic characteristics of 22 patients

Characteristics	Cases, *n*	RFWD3-high, *n* (%)	*P* values*
Total number	22	14 (64)	
Histology			
Adenocarcinoma	17	11 (64.7)	0.519
Squamous cell carcinoma	5	4 (80)	
Smoking			
Smoker	10	9 (90)	0.019
Non-smoker	12	6 (50)	
TNM stage			
I–II	12	8 (66.7)	0.87
III–IV	10	7 (70)	

**P* values were calculated using a two-sicled Fisher's exact test.

patients was strong, whereas RFWD3 staining was weak among 8 (36%) cases (Fig. 2A and 2B). RFWD3 was mainly localized in the cytoplasm of the lung cancer cells (Fig. 2A).

The observations were confirmed by exploring the Oncomine cancer microarray database [17] (www.oncomine.org). In the databases of Hou Lung [18], Selamat Lung [19], Landi Lung [20], Okayama Lung [21], and Wachi Lung [22], the *RFWD3* RNA levels were more elevated in the tumor tissues than in normal lung tissues (Fig. 3A–3E). In the Su Lung database [23], the *RFWD3* expression was slightly higher in the tumor samples than in normal control lung tissues, although the difference was not statistically significant (Fig. 3F, $P = 0.1$).

We explored *RFWD3* RNA levels in the TCGA transcriptome database containing 1018 NSCLC tumor samples and 109 normal lung specimens. A significantly elevated expression of *RFWD3* was found in the NSCLC tumor samples relative to normal lung tissue samples (Fig. 3G). The expression level of *RFWD3* was significantly higher in the tumor tissues than in normal lung samples (Fig. 3H–3I; $P < 0.0001$) in LUADs ($n = 518$) and LUSCs ($n = 500$). In the paired tumor-adjacent samples of TCGA datasets, *RFWD3* was more significantly expressed in the tumor tissues than in their adjacent lung samples (Fig. 3J and 3K). The expression of *RFWD3* in Asian NSCLCs was assessed in GEO datasets, and the results showed that the Chinese and Japanese NSCLCs exhibited higher *RFWD3* in tumor tissues than in normal lung tissues (Fig. 4A and 4B).

RFWD3 is inversely associated with the prognosis of NSCLCs

The Online Survival Analysis Software [28] was utilized to analyze the association between *RFWD3* expression and the lifespan of patients. The OS of NSCLCs with high *RFWD3* expression was significantly shorter than that of NSCLCs with low *RFWD3* expression (Fig. 5A, $P = 1.9 \times 10^{-7}$). NSCLCs with high *RFWD3* exhibited a shorter survival time than those with low levels of *RFWD3* in LUADs and LUSCs (Fig. 5A). In the TCGA datasets, the NSCLC patients with high *RFWD3* had slightly worse prognosis than those with low *RFWD3*, although the difference was not statistically significant (Fig. 5B). Thus, *RFWD3* might play a role in the pathogenesis of NSCLCs.

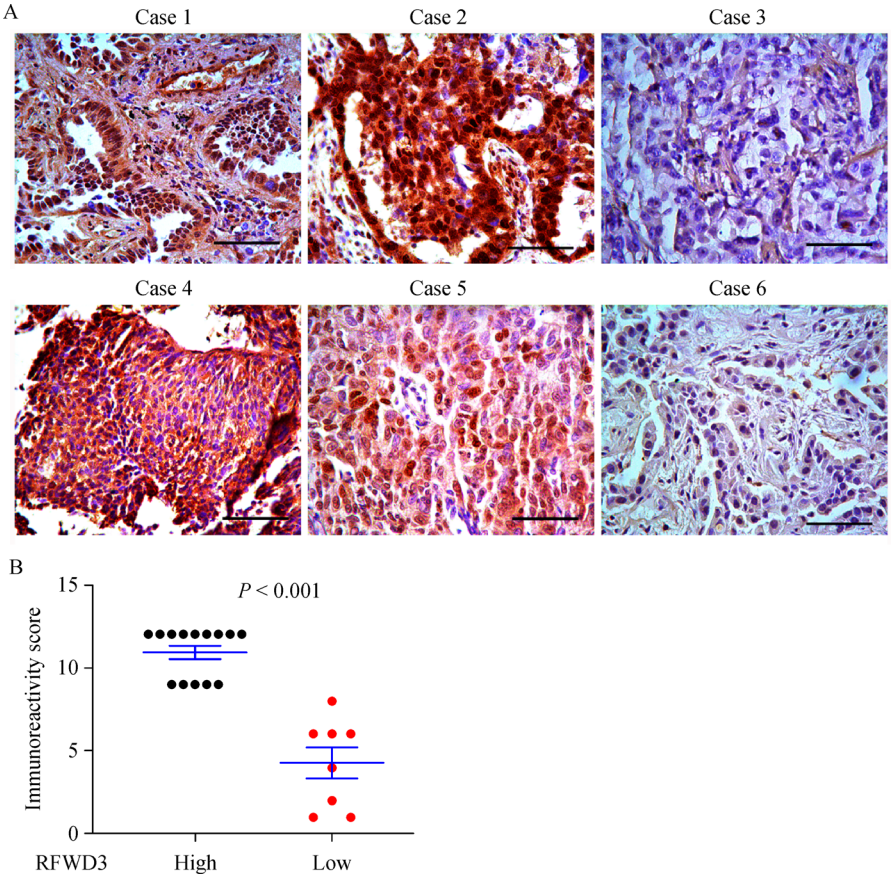

Fig. 2 RFWD3 in NSCLCs. (A) The expression of RFWD3 in tumor samples was detected by immunohistochemistry assay with an anti-RFWD3 antibody. Scale bar, 500 μm. (B) Immunoreactivity of RFWD3 among patients.

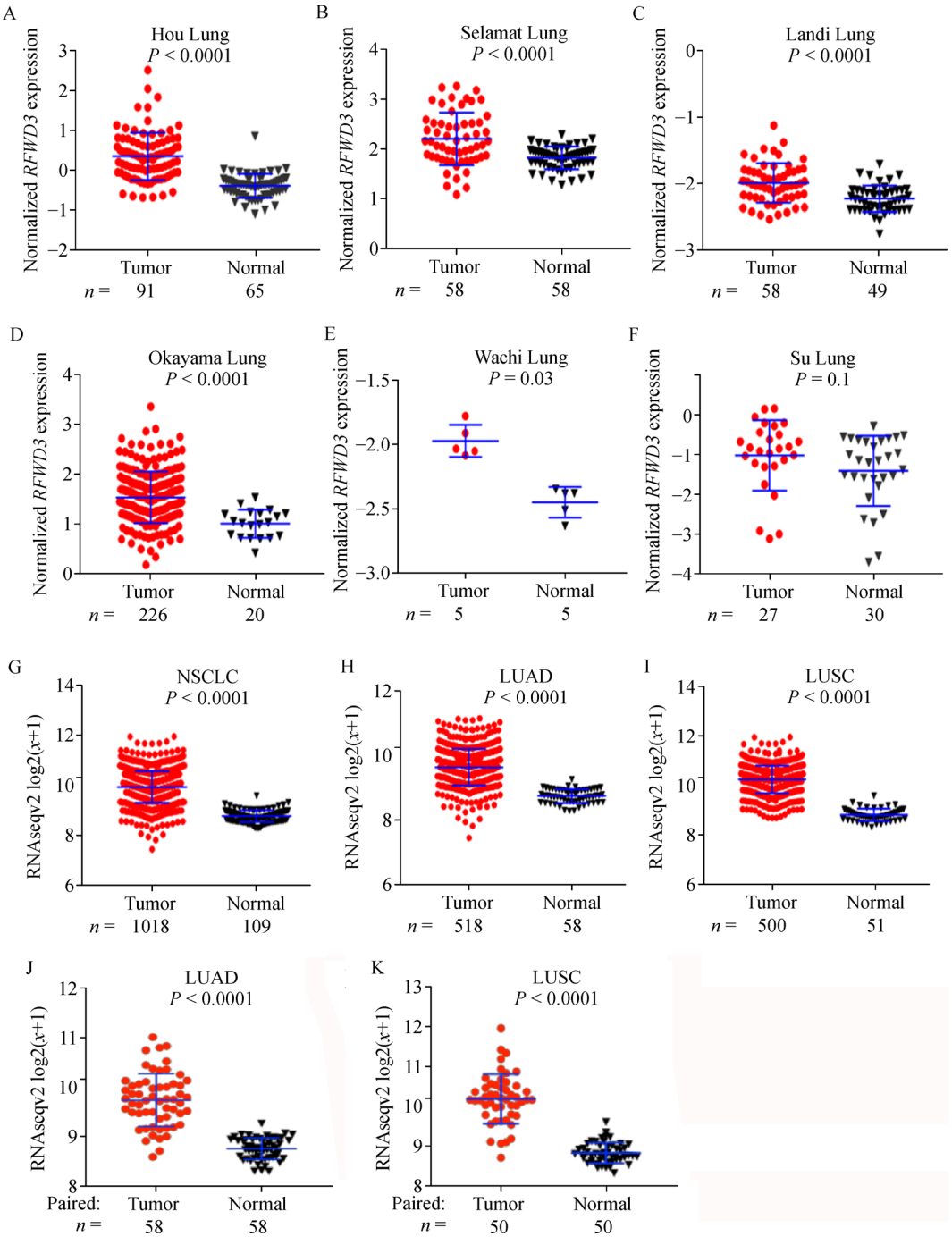

Fig. 3 Expression of *RFWD3* in NSCLCs in Oncomine and TCGA datasets. Expression of *RFWD3* in Oncomine datasets Hou Lung (A), Selamat Lung (B), Landi Lung (C), Okayama Lung (D), Wachi Lung (E), and Su Lung (F); and TCGA NSCLC (G), LUAD (H), and LUSC (I). (J, K) *RFWD3* in tumor samples and adjacent normal lung tissues of TCGA LUAD (J) and LUSC (K).

Association between *RFWD3* expression and tobacco smoke

The association between *RFWD3* expression levels and the tobacco smoking status of patients was explored in the Oncomine and TCGA databases. Data showed that *RFWD3* expression was significantly higher in smoker NSCLCs than in non-smoker NSCLCs in the databases of Lee Lung [29] (Fig. 6A; $P < 0.0001$) and TCGA (Fig. 6B; $P = 0.03$). In the GEO Sweden dataset (GSE29016) [30], smoker LUADs had higher *RFWD3* than their non-smoking counterparts. Thus, tobacco smoke and related

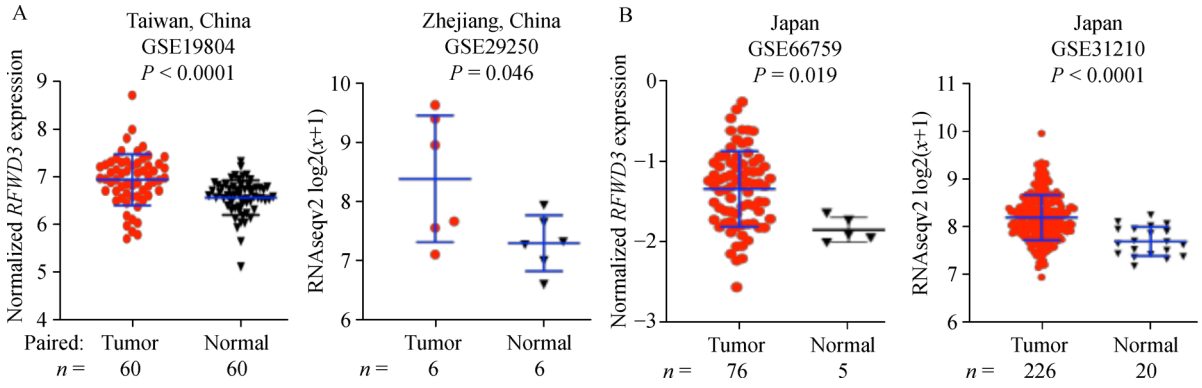

Fig. 4 Expression of *RFWD3* in Asian NSCLCs in GEO datasets. (A) Expression of *RFWD3* in Chinese NSCLCs and (B) in Japanese NSCLCs.

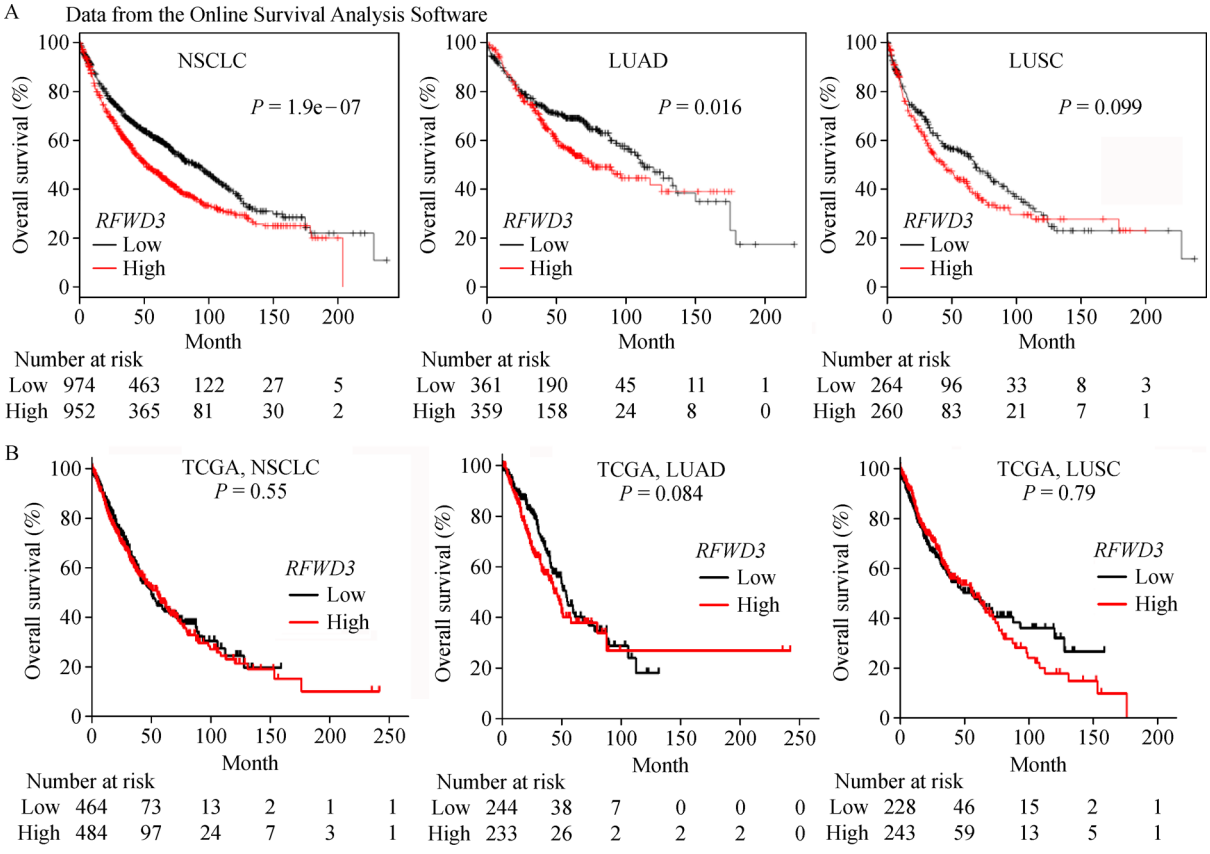

Fig. 5 OS of NSCLCs with high or low levels of *RFWD3* expression. (A) Kaplan–Meier survival curves of NSCLCs, LUADs, and LUSCs of the Online Survival Analysis software. (B) Survival results of TCGA NSCLCs whose tumor-adjacent samples were analyzed for *RFWD3* expression in their transcriptome datasets.

carcinogens may upregulate *RFWD3* and facilitate lung carcinogenesis.

Discussion

Pulmonary homeostasis facilitates normal respiration and is maintained by coping with various circumstances, including airborne particles, pathogens, and other noxious stimuli. The accumulation of misfolded or dysfunctional proteins from critical proteins may cause cell abnormality during division, differentiation, senescence, and death. E3 ligases mediate poly-ubiquitination and the subsequent degradation of proteins in normal and abnormal cells.

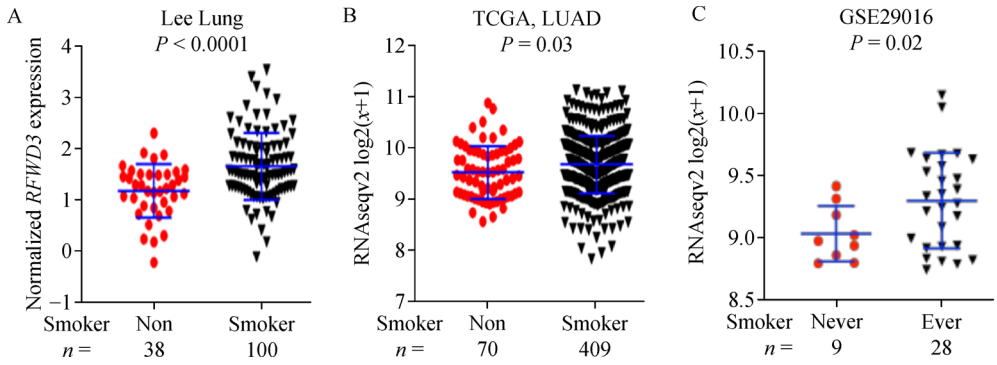

Fig. 6 *RFWD3* expression in smoker and non-smoker NSCLCs in the datasets of Oncomine Lee Lung (A), TCGA (B), and GEO Sweden Lung (C).

Thus, alterations in UPGs may lead to the hyper-activation of oncoproteins and loss of function of tumor suppressors, thereby facilitating cancer initiation and progression [31,32]. Abnormalities in UPGs, such as UBE1L [33], UbcH10 [34], UBE2C [35], Hrad6B [36], c-Cbl [37], SINA [38], Ataxin-3 [39], USP8 [40], USP17 [41], USP37 [42], and Skp1-Cul1-F-box protein complex essential component Skp1 [43], have been reported in lung cancer. Herein, we showed for the first time that *RFWD3* is required for NSCLC cell proliferation because the knock-down of this E3 ligase led to the dramatic inhibition of NSCLC cell proliferation (Fig. 1). We analyzed the expression level of *RFWD3* in NSCLCs in our setting and datasets and found that *RFWD3* was elevated in the tumor samples and inversely associated with the clinical outcome of the patients (Figs. 2–4). These results indicate that *RFWD3* may play an important role in NSCLC, and further research should be performed to unveil how this E3 ligase is involved in lung carcinogenesis.

RFWD3 stabilizes p53 [8] and participates in replication checkpoint [9]. The stabilization of RFWD3 by PCNA at the replication fork is essential for DNA replication [44]. RFWD3-mediated ubiquitination promotes the timely removal of RPA and RAD51 from DNA damage sites to facilitate homologous recombination [45], and the RPA-mediated recruitment of RFWD3 is vital for interstrand crosslink repair and human health [46]. Tobacco smoke and haze (smohaze), which are responsible for > 90% of lung cancer deaths [47–49], result in DNA damage and cause somatic mutations in the genome [50–52]. We found that *RFWD3* was associated with tobacco smoke and that *RFWD3* was more significantly expressed in smokers than in non-smokers (Fig. 6). Thus, RFWD3 may play a role in lung carcinogenesis by perturbing DNA damage repair and/or modifying the expression of oncoproteins/tumor suppressors, thereby affecting the critical pathways involving p53 and other crucial oncoproteins/tumor suppressors and the immune system. These possibilities and the potential of RFWD3 as a therapeutic target for lung cancer should be further investigated in the future.

Acknowledgements

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Compliance with ethics guidelines

Yanfei Zhang, Xinchun Zhao, Yongchun Zhou, Min Wang, and Guangbiao Zhou declare that they have no conflict of interest. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the *Helsinki Declaration* of 1975 as revised in 2000. Additional informed consent was obtained from all patients whose identifying information is included in this article.

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