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Transducin beta-like 2 is a potential driver gene that adapts to endoplasmic reticulum stress to promote tumor growth of lung adenocarcinoma

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Running head: TBL2 is a driver gene in lung adenocarcinoma

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Disclosure

There is no conflict of interest.

Synopsis

Transducin beta-like 2 (TBL2) is overexpressed in lung adenocarcinoma (LUAD) cells. High *TBL2* mRNA expression is an independent prognostic factor in LUAD. TBL2 promotes tumor growth under ER stress *in vitro*. TBL2 may be a novel driver gene of LUAD.

Abstract

Background Endoplasmic reticulum (ER) stress has a close relation with cancer progression. Blocking the adaptive pathway of ER stress could be an anti-cancer strategy. Here, we identified an ER stress-related gene, Transducin beta-like 2 (TBL2), an ER-localized type I transmembrane protein, on increased chromosome 7q as a candidate driver gene of lung adenocarcinoma (LUAD). **Methods** The association between *TBL2* mRNA expression and prognostic outcomes and clinicopathological factors was analyzed using The Cancer Genome Atlas (TCGA) datasets of LUAD and lung squamous cell carcinoma (LUSC). Localization of TBL2 in tumor tissues was observed by immunohistochemical staining. Gene set enrichment analysis (GSEA) was conducted using TCGA dataset. In vitro cell proliferation assays were performed using TBL2 knockdown LUAD cells, LUSC cells, and LUAD cells overexpressing TBL2. Apoptosis and ATF4 expression (ER stress marker) were evaluated by western blotting. **Results** TBL2 was overexpressed in LUAD and LUSC cells. Multivariate analysis indicated high *TBL2* mRNA expression was an independent poor prognostic factor of LUAD. GSEA revealed high TBL2 expression was positively correlated to the ER stress response in LUAD. TBL2 knockdown attenuated LUAD cell proliferation under ER stress. TBL2 inhibited apoptosis in LUAD cells under ER stress. TBL2 knockdown reduced ATF4 expression under ER stress. **Conclusions** TBL2 may be a novel driver gene that facilitates cell proliferation, possibly by upregulating ATF4 expression followed by adaptation to ER stress, and is a poor prognostic biomarker of LUAD.

1. Methods

2.1 Public datasets

RNA sequencing, DNA copy number, and clinical data for 503 LUAD and 495 LUSC patients were obtained from the Broad Institute Firehose in TCGA

(https://gdac.broadinstitute.org/runs/stddata__2016_01_28/data/LUAD/20160128/,
https://gdac.broadinstitute.org/runs/stddata__2016_01_28/data/LUSC/20160128/).

Subject gene alterations with mutations were found in the Catalogue of Somatic Mutations in Cancer (COSMIC) dataset (<https://cancer.sanger.ac.uk/cosmic>) (accessed 17 September 2022).

2.2 Clinical samples for immunohistochemistry

Formalin-fixed, paraffin-embedded sections of tumor and normal tissue were obtained from 10 cases each of LUAD and LUSC patients who underwent surgical resection at Kyushu University. All protocols used in this study were approved by the local ethics review board of Kyushu University (#2020-613).

2.3 Selection of candidate driver genes

We extracted candidate driver genes from 7311 genes on chromosome 7 in TCGA using the following criteria: Overexpressed (>1.5 -fold) in tumor tissues compared with normal tissues, DNA copy number positively correlated to the mRNA expression level (cutoff for correlation coefficient was 0.4), and the high mRNA expression group was significantly associated with a poor prognosis ($P < .05$).

2.4 Cell lines and culture

Human LUAD cell line A427, A549 and LUSC cell line H226 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LUAD cell line PC9 was obtained from the the RIKEN Cell Bank (Tokyo, Japan). LUAD cell line RERF-LC-Ad1 was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). LUSC cell line Qg56 was obtained from IBL (Gunma, Japan). Cell lines were

cultured in Roswell Park Memorial Institute 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and streptomycin 100 U/ml at 37 °C with 5% CO₂. To induce ER stress conditions, cells were incubated in 6 hours under hypoxia and low nutrition using glucose-free Roswell Park Memorial Institute 1640 medium (Wako) supplemented with 10% fetal bovine serum and Anaero Pack Kenki for Cell (Mitsubishi Gas Chemical, Tokyo, Japan). ER stress was determined by measuring the expression level of ER stress marker ATF4 by western blot analysis.

2.5 RNA extraction and reverse transcription-quantitative polymerase chain reaction

RNA was extracted from cell lines using ISOGEN-II (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's instructions. Reverse transcription-quantitative polymerase chain reaction was performed as reported previously¹⁷. Polymerase chain reaction was performed using the following oligonucleotide primers.

TBL2: 5'-GAAGAAGTCTTGTCAACTAAA-3' (sense) and
5'TTAGATCATGACATAAGGGA-3' (antisense)

GAPDH: 5'-AGCCACATCGCTCAGAC-3' (sense) and 5'-
GCCCAATACGACCAAATCC-3' (antisense)

mRNA expression was normalized to *GAPDH* mRNA expression as an internal control in each sample.

2.6 Immunohistochemical analysis

Immunohistochemistry of TBL2 in LUAD and LUSC tissue samples was performed as reported previously²⁰. A rabbit polyclonal anti-TBL2 antibody (12488-1-AP; ProteinTech, Chicago, IL) diluted 1:200 was used as the primary antibody. Histological evaluation of tumors was performed by an experienced pathologist (T.T.).

2.7 Knockdown by small interfering RNAs

TBL2-specific siRNAs (#s25572 and #s226084; Thermo Fisher Scientific, Waltham, MA, USA) and negative control siRNA (Thermo Fisher) were used. Transfection of

LUAD cells and LUSC with siRNAs was performed using Lipofectamine RNAiMAX (Thermo Fisher) in accordance with the manufacturer's instructions.

2.8 Transient overexpression of TBL2 in lung cancer cell lines

A TBL2 plasmid (NM_012453; Origene, Rockville, MD, USA) was transfected into A427 cells using a jetPRIME kit (Polyplus Transfection, USA). Control A427 cells were transfected with an empty vector (pCMV6-Entry Vector; Origene). Twenty-four hours after transfection, proliferating clones were collected and cultured. TBL2 expression was confirmed by western blotting. A427 cells were selected in medium containing 600 µg/ml G418 (Sigma, St. Louis, MO, USA).

2.9 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl 2H-Tetrazolium Bromide (MTT) Assay

MTT cell proliferation assays were conducted using a Cell Proliferation Kit I (Roche Applied Science, Penzberg, Germany), following the manufacturer's instructions, as described previously ²¹.

2.10 Colony Formation Assay

Cells were seeded at 1000 cells/well in triplicate in 6-well plates. After 10 days, cell colonies were stained using a Differential Quick Stain Kit (Sysmex, Kobe, Japan) in accordance with the manufacturer's instructions. Visible colonies were photographed using the Fusion Solo S imaging system. Colony counts were determined by ImageJ software (version 1.80, NIH, Bethesda, MD, USA).

2.11 Protein extraction and Western blot analysis

Cells were cultured at 37 °C in a CO₂ incubator with RPMI 1640 medium. After changing the medium to antibiotic-free RPMI 1640 medium supplemented with 5% fetal bovine serum, we incubated the cells for 24 hours at 37 °C in a CO₂ incubator with transfection mixtures containing the siRNA or TBL2 plasmid. The medium was then replaced with glucose-free medium, and the cells were incubated under the hypoxic conditions for another 6 h at 37 °C in a CO₂ incubator. After 6 hours, the medium was

replaced with fresh glucose-containing RPMI 1640 medium. Control cells were given equal refreshment but were incubated in glucose-containing RPMI 1640 medium at 37 °C in a CO₂ incubator after transfection. Then, the cells were harvested for protein extraction for Western blot analysis. Western blot analysis was performed as reported previously²². Blots were analyzed using a FUSION SOLO S (VILBER) to obtain optimal images to quantify protein bands. The primary rabbit polyclonal antibody against TBL2 at a 1:1000 dilution, a rabbit polyclonal antibody against ATF4 (ab23760; abcam, Cambridge, UK) at a dilution of 1:1000, and primary mouse polyclonal antibody against β -actin (sc47778; Santa Cruz Biotechnology, Sanata Cruz, CA, USA) at a dilution of 1:10,000 were used in experiments. Each protein was normalized to β -actin. An Apoptosis Western Blot Cocktail (ab136812; abcam, Cambridge, UK) was used in accordance with the manufacturer's protocol.

2.12 Gene set Enrichment analysis

Associations between TBL2 mRNA expression and previously defined gene sets were analyzed by gene set enrichment analysis (GSEA) using TBL2 expression profiles in a TCGA dataset²³. GSEA analysis method was described on the website (<https://www.gsea-msigdb.org/gsea/index.jsp>). Biologically defined gene sets were obtained from Molecular Signatures Database v5.2 (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>).

2.13 Statistical analysis

Patient data from TCGA dataset are expressed as the mean $\pm$ standard deviation and were divided into high and low *TBL2* mRNA expression groups. The cutoff was determined by the median. Associations between variables were tested by the Mann–Whitney U-test, Student's t-test, or Fisher's exact test. Overall survival (OS) curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate analyses were performed using Cox proportional hazards models to identify independent variables that predicted OS. Statistical analyses were

performed using JMP Pro 16 software (SAS Institute) and R v3.6.3 (The R Foundation for Statistical Computing). Statistical significance was set at $P < .05$.

2. Results

3.1 TBL2 is a candidate driver gene on chromosome 7q in lung adenocarcinoma

To identify novel potential driver genes in LUAD, bioinformatics analysis was performed using TCGA dataset. As shown in Figure 1a, the DNA copy number was increased in chromosome arms 7p and 7q in LUAD and LUSC. Using TCGA dataset, we found 11 genes on chromosome 7 that met the three screening parameters described in the methods (Table S1). Among them, we focused on TBL2, an ER stress-related gene, as a candidate driver gene (Figure 1b).

TBL2 is an ER-localized type I transmembrane protein located on chromosome 7q.11.23. The TBL2 gene is a genetic locus affecting the lipid concentration, leading to hypertriglyceridemia and increasing the risk of coronary artery disease ²⁴. Additionally, TBL2 plays an important regulatory role in expression of activating transcription factor 4 (ATF4) ^{25 26}. ATF4 activates the transcription of various genes to adapt to a stress environment ²⁷.

A TBL2 DNA copy number in LUAD and LUSC datasets was positively correlated to *TBL2* mRNA expression ($R = .57$, $P < .01$ and $R = .62$, $P < .01$, respectively) (Figure 1c). The increased TBL2 DNA copy number ($\log_2$ copy number ratio $> .10$) was found in 386/512 (75.4%) of LUAD tissues and 402/498 (80.7%) of LUSC tissues in TCGA dataset (Figure 1d). In genetic mutation analysis, only 1.42% of TBL2 gene alterations with mutations were found in the COSMIC dataset (Figure 1e).

Next, *TBL2* mRNA expression was observed in LUAD and LUSC. In TCGA datasets, *TBL2* mRNA expression was significantly upregulated in tumor tissues compared with normal tissues in both LUAD and LUSC patients (Figure 1f). Immunohistochemical analysis showed significant staining of TBL2 in tumor cells and weak staining in normal alveolar cells in both LUAD and LUSC patients (Figure 1g, 1h). The results were the same for all 10 cases of each histology. In magnified images, TBL2 immunostaining was mainly localized in the cytoplasm of LUAD cells (Figure

1g). These results suggest that *TBL2* is overexpressed in tumor cells, possibly because of amplified chromosome 7q, providing evidence that it is a candidate driver gene in LUAD and LUSC.

3.2 Prognostic significance of *TBL2* mRNA expression in LUAD and LUSC patients in TCGA dataset

We evaluated overall survival (OS) in accordance with *TBL2* mRNA expression in LUAD and LUSC patients. Interestingly, the OS of patients with high *TBL2* mRNA expression was significantly lower than that of patients in the low *TBL2* mRNA expression group for LUAD, but not LUSC (Figure 2a).

In univariate analyses of LUAD, *TBL2* mRNA expression, pathological T, pathological N, and pathological stage were significantly associated with OS (Table 1). Next, we performed multivariate analysis with the pathological stage and *TBL2* mRNA expression. The results showed that high *TBL2* expression was an independent prognostic factor for a poor outcome (HR = 1.43, 95% CI = 1.06–1.95, $P < .019$) (Table 1).

Next, we performed subgroup analysis of OS in accordance with the pathological tumor stage of LUAD. As shown in Figure 2b, the high *TBL2* mRNA expression group had lower OS than the low expression group in pathological stage III.

These findings suggest that high *TBL2* mRNA expression is a poor prognostic biomarker of LUAD, especially in advanced tumor stages.

The prognostic significance of mRNA expression of ER-resident sensor proteins EIF2AK3, ERN1, and ATF6, which are ER stress-related genes, was examined using the LUAD dataset in TCGA²⁸. However, there was no difference in the expression of any of the genes between tumor and normal tissues, and no significant correlation between the expression level and prognosis (Figure 2c, 2d), providing evidence that *TBL2* is a critical gene among ER stress-related genes in LUAD.

3.3 Clinicopathological significance of *TBL2* mRNA expression in LUAD patients

Because high *TBL2* mRNA expression was a poor prognostic factor only in LUAD, we performed clinicopathological analysis of *TBL2* in LUAD. We examined the association between *TBL2* mRNA expression and clinicopathological factors in LUAD patients from TCGA dataset, but there was no significant association between *TBL2* mRNA expression and clinicopathological features (Table 2).

3.4 *TBL2* expression is positively correlated to ER stress response-related pathway

GSEA was performed on TCGA dataset to investigate the biological effect of *TBL2* mRNA expression in LUAD. We found a positive correlation between the gene sets involved in the “ER stress response-related pathway” (Figure 3a). On the basis of GSEA results, we investigated whether *TBL2* regulates tumor cell proliferation and apoptosis under ER stress in LUAD.

3.5 *TBL2* promotes LUAD cell proliferation under ER stress

We performed MTT and colony formation assays of LUAD cell lines with or without ER stress to clarify the relationship between *TBL2* expression and proliferation. We induced ER stress by hypoxia and low glucose which is the physiological condition in the tumor microenvironment^{25,29}. A427, an LUAD cell line, was used for proliferation analysis because of its high expression level of *TBL2* (Figure S1). MTT and colony formation assays showed that *TBL2* knockdown inhibited cell proliferation under ER stress, but not in the absence of ER stress (Figure 3b, c). Moreover, *TBL2* overexpression significantly increased cell proliferation under ER stress (Figure 3d, e). Of note, *TBL2* knockdown reduced ATF4 expression under ER stress³⁰ (Figure 3b). These data suggest that *TBL2* increases the proliferative ability of LUAD cells under ER stress.

3.6 *TBL2* knockdown in LUSC cells does not suppress proliferation under ER stress

We performed knockdown experiments in the LUSC cell line Qg56 to clarify the relationship between *TBL2* expression and cell proliferation in LUSC. Interestingly, cell proliferation was not suppressed by *TBL2* knockdown under ER stress (Figure 3f).

Furthermore, ATF4 was not induced by ER stress. These data suggest that LUSC may be resistant to ER stress, because TBL2 knockdown did not affect the proliferative ability of LUSC cells (Figure 3f).

3.7 TBL2 knockdown induces apoptosis of LUAD cells under ER stress

We examined whether TBL2 knockdown induced apoptosis in A427 cells. TBL2 knockdown suppressed expression of ATF4 and cleaved poly (ADP-ribose) polymerase (PARP), and increased cleaved caspase 3, an apoptosis marker, compared with control cells under ER stress, but not without ER stress (Figure 4a). These results indicated that TBL2 knockdown in LUAD cells induced apoptosis by decreasing ATF4 expression under ER stress.

3. Discussion

We found that TBL2, an ER stress-related gene, may be a novel driver gene on amplified chromosome 7 and a prognostic biomarker of LUAD. To our knowledge, this is the first study to explore the clinical and biological significance of TBL2 in LUAD.

The ER stress response plays a role in regulating cellular homeostasis. When the ER stress response fails to function because of prolonged or severe stress, cells initiate apoptosis and the DNA damage response⁷. Cancer cells are usually exposed to many stressful conditions, such as hypoxia and nutrient starvation, which results in continued ER stress^{31 32 33}. Adaptation to stress is necessary for cancer cell survival and proliferation. In fact, high expression of ER stress-related genes GPR37, XBP1, and ERGIC3 is associated with a poor prognosis of LUAD^{34 35 36}. ERGIC3 knockdown suppresses lung cancer cell growth³⁶. Additionally, inhibition of ER stress enhances the apoptotic effect of cisplatin, a cytotoxic anti-cancer drug, in LUAD³⁷. We also found that knockdown of the ER stress regulator TBL2 promoted apoptosis, and decreased the proliferative ability of LUAD cells, providing additional evidence that the ER stress response is critical for cancer progression. Thus, the ER stress response is a promising therapeutic target for malignancy.

Clinically, ubiquitous amplification and overexpression of TBL2 were found in LUAD. Furthermore, high TBL2 expression was an independent poor prognostic factor. These findings suggest that TBL2 is a therapeutic target to overcome tumor heterogeneity and a prognostic biomarker of LUAD.

We also found that TBL2 knockdown reduced ATF4 expression of LUAD cells. ATF4 is upregulated in cancer cells and contributes to cell survival in the stressful tumor microenvironment^{38 39 40}. ATF4 binds to target genes, such as CHOP and ATF3, to regulate their expression, inducing adaptive responses to stress and induction of cell apoptosis^{41 42 43}. Depletion of ATF4 reduces cell viability under stress conditions, such as glucose deprivation and hypoxia, in solid tumors^{44 29}. Moreover, ATF4 promotes the growth, invasion, and metastasis of LC, breast cancer, and glioma cells^{38 39 40}. Jiang et

al. showed that ATF4 contributes to LC progression by promoting Wnt/ β -catenin signaling in LUAD³⁸. TBL2 regulates ATF4 expression by controlling translation through binding to *ATF4* mRNA via the WD40 domain^{25 26}. These findings suggest that TBL2 facilitates cell growth by resistance against apoptosis, possibly through ATF4 expression under ER stress in LUAD (Figure 4b).

Interestingly, TBL2 expression was not associated with prognosis and did not affect LUSC cell proliferation *in vitro*, although TBL2 was overexpressed in LUSC like LUAD. A significant correlation between the expression level of other representative ER stress-related genes, such as GRP78 and the spliced form of X-box-binding protein 1, and prognosis was also observed in LUAD, but not in LUSC^{34 45}. These findings may be due to differences in the ER stress response between LUAD and LUSC. Further research is needed to clarify this issue.

In conclusion, we identified a novel driver gene, TBL2, on amplified chromosome 7 that facilitates tumor growth by upregulating ATF4 expression, followed by adaptation to ER stress in LUAD. Furthermore, we found that TBL2 may be a prognostic biomarker and therapeutic target of LUAD.

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Figure Legends

Fig. 1 *TBL2* is a candidate driver gene of lung adenocarcinoma.

a, DNA copy number variations in chromosomal arms of 518 lung adenocarcinoma (LUAD) and 501 lung squamous cell carcinoma (LUSC) tissues using data from TCGA.

b, Schematic diagram of the strategy to select candidate driver genes of LUAD.

c, Correlation between the DNA copy number and mRNA expression in LUAD and LUSC datasets from TCGA. *R* is the Pearson correlation coefficient.

d, Percentage of *TBL2* DNA copy number gain in LUAD and LUSC in the TCGA datasets.

e, Frequency of *TBL2* mutations in LUAD in the COSMIC dataset.

f, *TBL2* mRNA expression in tumor and normal lung tissues in LUAD and LUSC datasets from TCGA.

g, Immunohistochemical staining of *TBL2* in LUAD and normal tissues. N, normal tissue; T, tumor tissue. Original magnifications: $\times 50$, $\times 200$, and $\times 400$.

h, Immunohistochemical staining of *TBL2* in LUSC and normal tissues. N, normal tissue; T, tumor tissue. Original magnifications: $\times 50$ and $\times 200$.

Fig. 2 *TBL2* expression predicts a poor prognosis of LUAD.

a, Kaplan–Meier overall survival curves of LUAD and LUSC patients in accordance with *TBL2* mRNA expression in LUAD ($n=503$, $P=.030$) and LUSC ($n=495$, $P=.683$) datasets from TCGA.

b, Kaplan–Meier overall survival curves of LUAD patients in accordance with *TBL2* mRNA expression at pathological stage I ($n=262$, $P=.843$), stage II ($n=119$, $P=.338$), and stage III ($n=89$, $P=.010$) in TCGA LUAD dataset.

c, *EIF2AK3*, *ERN1*, and *ATF6* mRNA expression in tumor and normal lung tissues in LUAD datasets from TCGA.

d, Kaplan–Meier overall survival curves of LUAD patients in accordance with *EIF2AK3* (n=502, $P=.250$), *ERN1* (n=502, $P=.093$), and *ATF6* (n=502, $P=.561$) mRNA expression in LUAD datasets from TCGA.

Fig. 3 TBL2 expression is associated with cell proliferation under ER stress in lung adenocarcinoma.

a, Gene set enrichment analysis (GSEA) of TCGA dataset. FDR, false discovery rate; NES, normalized enrichment score.

b, Left: TBL2 and ATF4 protein expression in TBL2 knockdown LUAD cells with or without ER stress analyzed by western blotting. Right: MTT assays of TBL2 knockdown LUAD cells with or without ER stress. *** $P<.005$.

c, Colony formation assays of TBL2 knockdown LUAD cells with or without ER stress.

d, Left: TBL2 protein expression in TBL2-overexpressing LUAD cells with or without ER stress analyzed by western blotting. Right: MTT assays of TBL2-overexpressing LUAD cells with or without ER stress. *** $P<.005$.

e, Colony formation assay of TBL2-overexpressing LUAD cells with or without ER stress.

f, Western blot analysis of TBL2 and ATF4 protein expression in TBL2 knockdown LUSC cells with or without ER stress, and MTT assays of TBL2 knockdown Qg56 cells with or without ER stress.

Fig. 4 TBL2 expression is associated with apoptosis of LUAD cells under ER stress.

a, Western blot analysis of TBL2, ATF4, cleaved PARP, procaspase 3, and cleaved caspase

b, Schema indicating how TBL2 promotes apoptosis and tumor proliferation

Table 1. Univariate and multivariate analyses of clinicopathological factors for overall survival in the LUAD dataset from TCGA

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
Age (≥ 65 yrs / < 65 yrs)	1.11 (0.82-1.51)	.506		
Sex (male/female)	1.04 (0.77-1.41)	.785		
Smoking(no/yes)	1.09 (0.79-1.49)	.611		
Pathological T(T1-2/T3-4)	0.43 (0.29-0.63)	$<.001$		
Pathological N(N0/N1-3)	0.41 (0.30-0.55)	$<.001$		
Pathological stage (III-IV/I-II)	2.03 (1.27-3.23)	.003	2.10(1.31-3.35)	.002
<i>TBL2</i> mRNA expression(high/low)	1.41 (1.04-1.90)	.026	1.43 (1.06-1.95)	.019

Abbreviations: CI, confidential interval; LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas; HR, hazard ratio.

Table 2. Correlation between *TBL2* mRNA expression in tumor tissues and clinicopathological factors in the LUAD dataset from TCGA

Factor		<i>TBL2</i> high (n=252)	<i>TBL2</i> low (n=251)	<i>P</i> value
Age mean±SD		64.9±9.6	65.8±10.0	
	65yrs ≥	123	125	.54
	65yrs <	122	111	
Gender				
	Male	114	118	.68
	Female	138	133	
Smoking				
	Former or Current	172	170	.89
	Never	80	81	
Pathological T				
	T1-2	213	224	.32
	T3-4	38	25	
Pathological N				
	N0	155	171	.35
	N1-3	93	73	
Pathological stage				
	Stage I-II	229	231	.85
	Stage III-IV	18	17	
Abbreviations: LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas				

Figure. 1

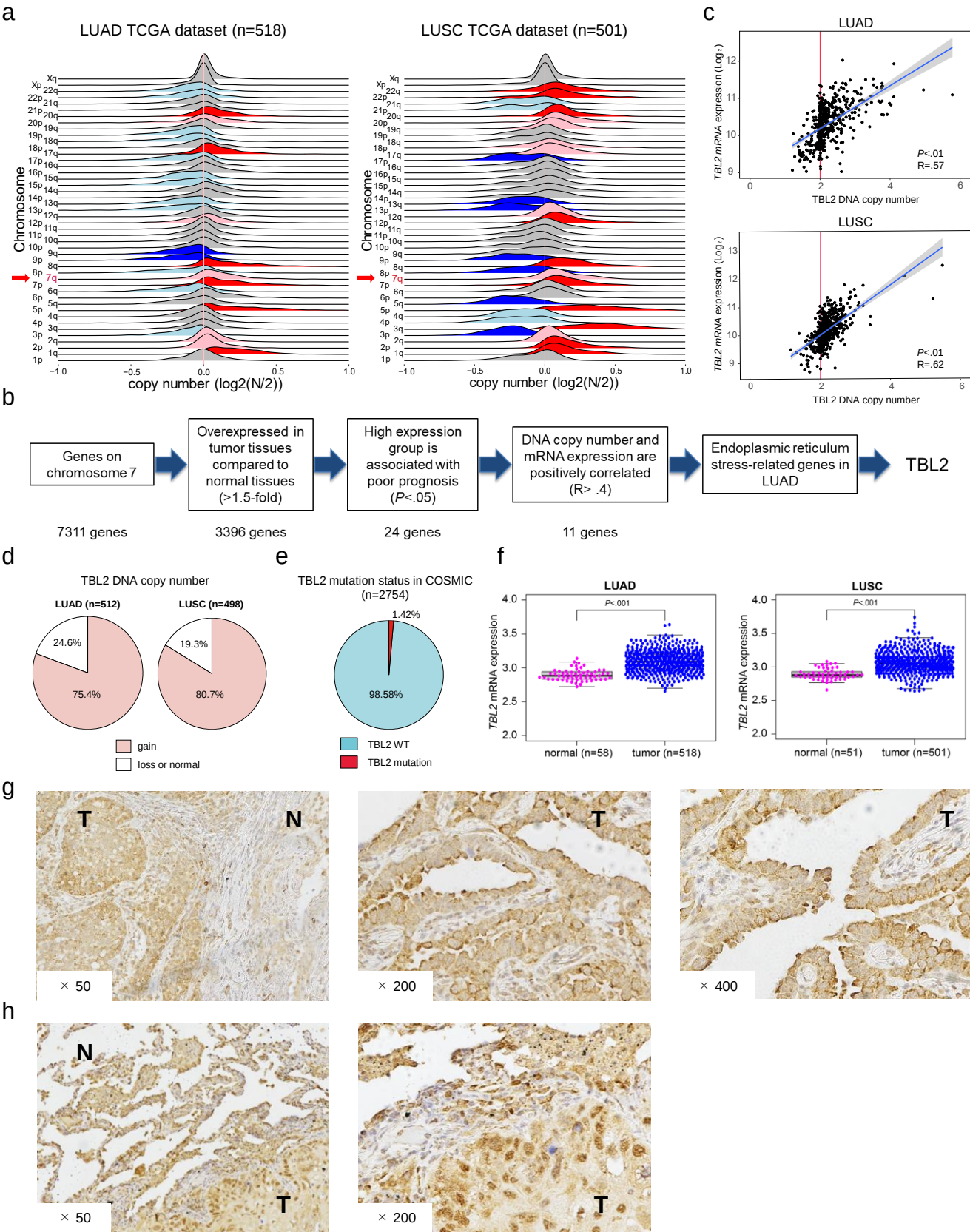


Figure. 2

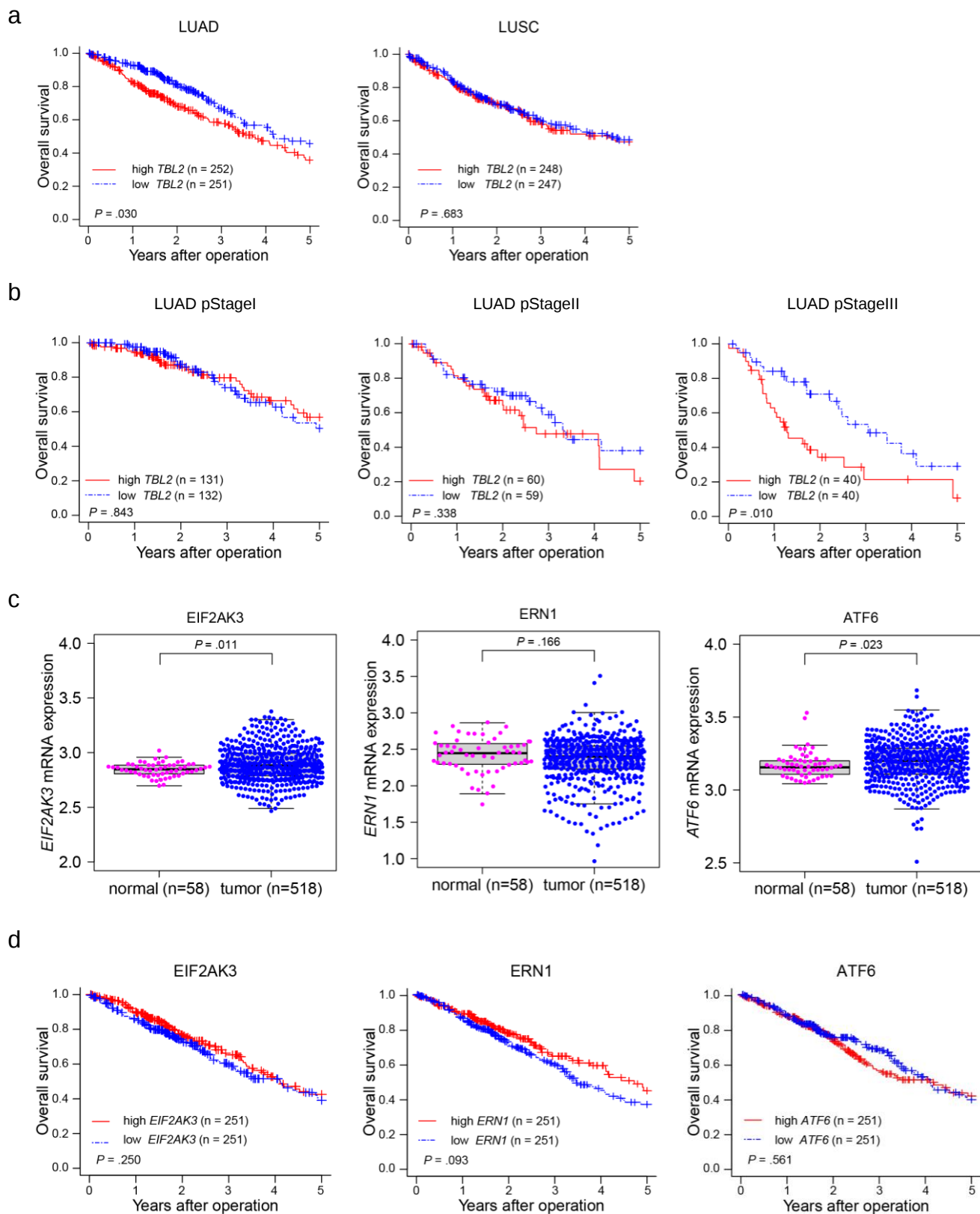
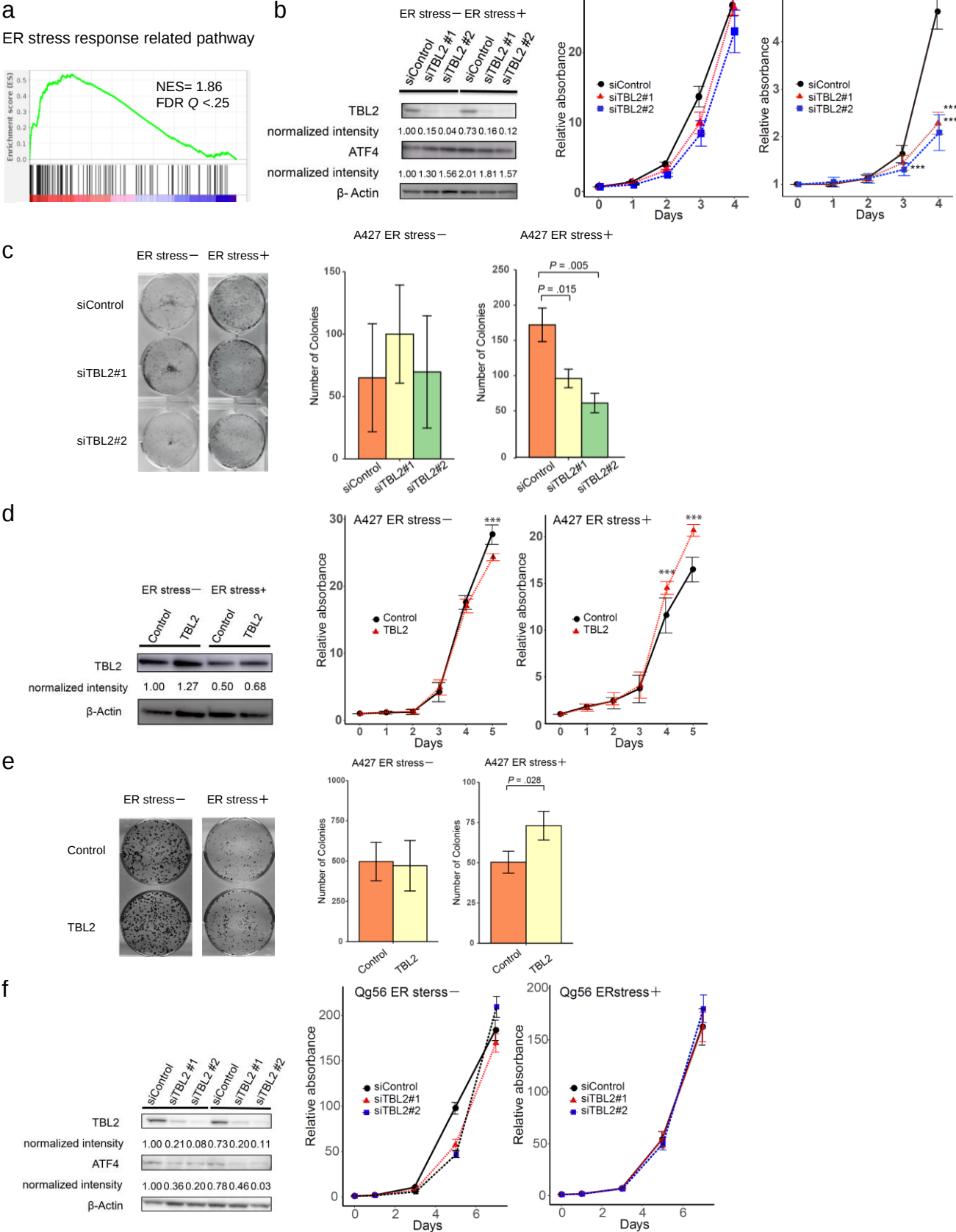


Figure. 3



a

