# Transcriptome coordination analysis identifies RASSF1 as an ATF4 target

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**Abstract**

Evaluation of gene co-regulation emerges as a powerful approach to revealing regulatory associations between genes and predicting biological function, especially in genetically diverse samples. We have applied this strategy to identify transcripts that are co-regulated with unfolded protein response (UPR) genes in cultured fibroblasts from outbred deer mice. Our analyses showed that RASSF1-associated transcriptome, a tumor suppressor involved in cell cycle regulation and not linked to UPR before, is highly correlated with the transcriptome of several UPR-related genes such as BiP/GRP78, DNAJB9, GRP94, ATF4, DNAJC3 and CHOP/DDIT3. Conversely, gene ontology analyses for genes co-regulated with RASSF1 predicted an involvement, unreported for this gene before, in UPR-associated apoptosis. Bioinformatic analyses indicated the presence of ATF4 binding sites in RASSF1 promoter, which by chromatin immunoprecipitation studies, were shown to be operational. Reporter assays showed that RASSF1 promoter is responsive to ATF4, while ablation of RASSF1 mitigated expression of the ATF4 effector BBC3 and abrogated apoptosis that were triggered by tunicamycin. Collectively these results implicate the role of RASSF1 in the regulation of ER stress-associated apoptosis downstream of ATF4. They also illustrate the power of gene coordination analysis in predicting biological functions and unveiling regulatory associations between genes.

## Introduction

Differential analysis of gene expression is a powerful and extensively used strategy for pointing to regulatory relationships between genes (1,2). Nevertheless, its applicability is highly limited when genetically diverse specimens are being analyzed because they result in high variation in gene expression. Thus, highly variable, albeit biologically significant transcripts are being overlooked because they do not pass the stringency thresholds of differential expression. Conversely, even subtle changes in expression levels may point to transcripts with minimal involvement in specific processes when variation is narrow (3-5).

To overcome the limitations of conventional differential expression analysis we focused on the analysis of patterns of gene co-expression in genetically diverse specimens.

By concentrating on the co-regulation of genes associated with the unfolded protein response (UPR) in specimens from outbred deer mice (Peromyscus), we showed that despite the variation in the levels of expression of individual genes, a striking correlation is maintained in their levels in samples from different individuals (6). This correlation extends to the correlation of the UPR genes with the whole transcriptome and exhibits different profiles when endoplasmic reticulum (ER) stress is induced and pathology is inflicted (7,8). Beyond the UPR, this approach was also shown to be especially meaningful when the pattern of gene coordination was evaluated, at the whole transcriptome level, in outbred genetically diverse specimens. For example, in people suffering from frailty syndrome, this approach readily manifested the involvement of the immune system (9). In brain samples of different species of deer mice, it pointed to a loss of smell at aging and identified transcriptomic coordination differences that accompany the development of histological changes consistent with neurodegeneration (10). In analyses of liver

68 samples from deer mice receiving high fat diet, this strategy demonstrated the engagement of
69 immune system, prior to the development of histologically detectable inflammation (11).
70 In the present study, we sought to exploit this analysis towards the discovery of specific
71 transcripts that may play unrecognized roles as yet in specific processes. We specifically
72 hypothesized that in genetically diverse specimens, transcripts with causal involvement in certain
73 biochemical pathways should exhibit high coordination, with various genes known to be
74 involved in these pathways. Furthermore, it is plausible that this coordination extends beyond the
75 expression of individual genes, to the whole transcriptome, and can be reflected to how tightly
76 each and every gene is co-expressed between the interrogated transcript and known gene targets
77 of the pathway in question.
78 The UPR was selected for this analysis because it represents a central homeostatic
79 response at which different biochemical pathways converge during stress of the ER (12,13).
80 Furthermore, it is associated with considerable changes in gene expression profiles that vary
81 among individuals (6,14,15). Our analyses pointed to RASSF1 that exhibited high coordination
82 with multiple UPR genes. RASSF1 is a tumor suppressor that has an established role in cell
83 cycle regulation and apoptosis, but no links to UPR reported so far (16-18). A combination of in
84 silico predictions that were based on coordination studies and gene ontology analyses, combined
85 with validation experiments in vitro, identified RASSF1 as a UPR target, operating in a manner
86 according to which during ER stress the UPR-related transcription factor ATF4 activates
87 RASSF1 transcription by interacting directly with its promoter. In turn, RASSF1 induces cell
88 cycle arrest and apoptosis. The results, besides implicating causally the response of RASSF1 to
89 ER stress, also illustrate how gene coordination analysis can be applied to genetically diverse
90 specimens and reveal novel associations between genes and specific biological processes.

## Results

**Whole transcriptome coordination between RASSF1 and UPR target genes**. Earlier observations showed that UPR-associated genes exhibit coordinated expression, not only between their individual expression levels, but also when the correlation of each with the whole transcriptome was evaluated and compared to that of other UPR genes, in pairwise comparisons (8). Thus, we hypothesized that genes that have causative involvement in the UPR will also show highly coordinated expression, at the whole transcriptome level, with that of established UPR target genes. To test this hypothesis, we initially calculated the Pearson's correlation coefficient of the expression of a panel of UPR genes with the whole transcriptome (Supplementary Table 1). The analysis was performed in primary fibroblasts isolated from different, outbred deer mouse individuals, that were cultured in the presence or absence of tunicamycin. The gene that exhibited the highest correlation with BiP/GRP78/HSPA5, the major UPR regulator (19,20), was RASSF1 that also exhibited high correlation with various UPR targets as well (Fig. 1A). To explore if the coordination identified is conserved across experimental and biological systems, we also performed the same analysis in RNA-Seq data of human liver specimens (7) (Supplementary Table 2) and found similar relationships between RASSF1 and UPR target genes although the correlations are not as tight as those in primary fibroblasts (Fig. 1B). RASSF1 (Ras association domain-containing protein 1) encodes for a Ras effector protein that has been studied primarily in the context of tumorigenesis (19-22). It is a tumor suppressor gene and its expression is lost in human cancers by mechanisms that usually involve aberrant DNA methylation. No evidence to our knowledge exists linking RASSF1 with the UPR yet. As shown in Fig. 1, an astonishing degree of coordination was unveiled with all UPR targets examined, implying a potential role of RASSF1 in the regulation of the UPR. Interestingly, association was

only reduced with CHOP (wider plot in Fig. 1A, lower right, as compared to other combinations) which is consistent with the fact that CHOP is also regulated by alternative to UPR mechanisms (23).

**In silico analysis of RASSF1 function**. In order to further explore the function of RASSF1, we calculated Pearson's correlation between RASSF1 and the whole transcriptome and subjected the top 1129 genes ($p < 0.05$ Pearson's) to Gene Ontology analysis for biological function prediction. As shown in Table 1 and Supplementary Table 3, this analysis showed a striking association with processes relevant to ER stress response, especially in relation to PERK signaling that represents one of the 3 major branches of the UPR, along with IRE1 and ATF6 (24,25). In conformity with these discoveries, coordination analysis showed tight association between the transcriptomes of RASSF1 and both BBC3 and GADD45A, two pro-apoptotic genes primarily induced through the PERK-eIF2α branch of the UPR (26-29) while the correlation between the transcriptomes of RASSF1 and RCAN1 was less tight, aligning with the fact that RCAN1 is an ATF6-dependent, pro-survival regulator during ER stress (30-32) (Suppl. Fig. 1). Other biological processes predicted by this analysis were related to signal transduction and are consistent with known functions of RASSF1 (Table 1).

**Regulation of RASSF1 by ATF4**. The fact that our results, so far, were based on RNA expression data, in combination with the prediction that RASSF1 is associated with the response to ER stress, prompted us to test if RASSF1 harbors consensus ER stress responsive elements within its promoter. Thus, a ~1kb region in the 5'-UTR of RASSF1 was identified and subjected to bioinformatic analysis for prediction of transcription factor binding sites. This analysis readily

identified an ATF4 binding site that was located between -210 and -203 positions from the transcription start site (TSS) of RASSF1 (Fig. 2A) and the sequence 5'-TCAGCAAA-3' was similar to canonical CARE sequence 5′-TGATGxAAx-3′ (33). ATF4 is an established UPR target downstream of PERK (34,35).

Subsequently we tested if the promoter of RASSF1 is responsive to ER stress. Thus, a luciferase-based reporter construct was constructed bearing the RASSF1 promoter (-931 to +38) and its activity was evaluated following co-transfection of human embryonic kidney 293 cells (HEK293) with human wild type or mutant ATF4 expression plasmids. As shown in Fig. 2B, the activation of luciferase activity in RASSF1 promoter reporter was significantly higher by the wild type ATF4. Chromatin immunoprecipitation studies were also performed and confirmed that ATF4, physically interacts with RASSF1 promoter (Fig. 2C).

**Induction of RASSF1 by tunicamycin (Tun) and thapsigargin (Thap).** The aforementioned results predict that RASSF1 is a UPR target gene. To test this hypothesis, we exposed HEK293 and human fetal foreskin fibroblasts (HFFF2) to tunicamycin and thapsigargin, the established UPR activators (36) and monitored the levels of RASSF1. As shown in Fig. 3, the levels of both RASSF1 and of its splice variants RASSF1A and RASSF1C increased significantly in the tunicamycin treated cells, among which the level of RASSF1A expression increased more than 4-fold compared to about 2-fold increase of RASSF1C. Similarly, ATF4 levels and of its downstream target BBC3 (37,38), were induced by tunicamycin. The significant induction of RASSF1 and ATF4 were also seen in HFFF2 treated with either tunicamycin (Fig. 4A) or thapsigargin (Fig. 4B). In addition, the integrated stress response inhibitor (ISRIB), an

established inhibitor of the PERK branch of UPR, significantly reduced the induction of RASSF1 and ATF4 in HEK293 by either tunicamycin (Fig. 5A) or thapsigargin (Fig. 5B).

When, however, the expression of RASSF1 was inhibited by shRNA (Fig. 6A), the tunicamycin-induced activation of BBC3, but not of ATF4, was abrogated (Fig. 6B). This is consistent with the notion that RASSF1 is downstream of ATF4 but upstream of BBC3, during tunicamycin-induced ER stress. Consistent with these findings coordination analysis between RASSF1 and each of BBC3 or CCNA2-associated transcriptomes showed coordination with the former but not with the latter (Suppl. Fig. 1).

**RASSF1-induced cell cycle arrest and apoptosis during ER stress**. In order to functionally evaluate the integration of RASSF1 into UPR signaling, we evaluated the consequences of RASSF1 inhibition in tunicamycin-induced cell cycle arrest. As shown in Suppl. Fig. 2, exposure of HEK293 cells to tunicamycin induced G1 cell cycle arrest and reduced the fraction of cells in G2/M phase of cell cycle. However, shRNA-mediated RASSF1 inhibition reduced the fraction of cells in G1 and increased the fraction of cells in G2/M phase, but these effects were not seen in cells treated with tunicamycin. In line with these findings were the effects of RASSF1 inhibition in apoptosis. Tunicamycin exposure significantly induced TUNEL-positivity in HEK293 cells but this effect was abolished when RASSF1 was inhibited (Fig. 7). Thus, RASSF1 is required for the effects of tunicamycin on cell apoptosis.

## Discussion

In the present study we applied a novel in silico approach based on analysis of RNA-Seq data to identify UPR-associated genes. Our analysis identified the tumor suppressor gene

184 RASSF1 that is involved in Ras signaling, as a UPR target gene. The premise of our analysis is
185 that coordination analysis of gene expression can be applied to genetically diverse specimens and
186 reveal regulatory relationships between genes. We tested our hypothesis by assessing transcripts
187 of which the transcriptome exhibits co-regulation with the transcriptome of UPR target genes in
188 outbred deer mouse specimens. Our analyses indicated that RASSF1 is highly co-expressed with
189 the major UPR chaperone BiP/GRP78. Furthermore, this co-regulation is extended beyond the
190 individual levels of expression, to the whole transcriptome, when the correlation of each and
191 every gene was evaluated in comparison, between RASSF1 and BiP/GRP78. This association
192 was readily detectable when additional UPR genes were interrogated, and was present in both
193 deer mouse fibroblasts and human liver specimens, albeit the fact that in the former was more
194 pronounced, likely because it was assessed in cells as opposed to whole tissue samples. The only
195 exception was recorded with Ddit3/CHOP that exhibited more relaxed coordination at the
196 transcriptome level with RASSF1 and is consistent with the fact that Ddit3/CHOP is also
197 regulated by alternative to UPR pathways.
198 Strong evidence regarding the functional integration of RASSF1 to UPR signaling was
199 obtained after subjecting the RASSF1-correlated transcriptome to GO analyses, which showed
200 high enrichment for ER stress-associated biological processes. Among those, involvement with
201 UPR-associated cell death was predicted, especially in relation to PERK signaling. In silico
202 analysis for transcription binding sites to RASSF1 promoter pointed to the presence of ATF4
203 binding sites which is an established transducer of PERK signaling. These predictions were all
204 subsequently confirmed by a combination of promoter reporter assays and chromatin IP studies
205 that indeed demonstrated that ATF4 activates and physically interacts with the RASSF1
206 promoter. Functional studies regarding the implications of RASSF1 into UPR signaling

suggested that RASSF1 is required for cell cycle arrest and ER stress-induced apoptosis, in response to tunicamycin exposure.

RASSF1 is an established tumor suppressor that induces cell cycle regulation and apoptosis and is inactivated in various cancers by hypermethylation or mutations. Nevertheless, no connection with the UPR was established for RASSF1 before. The present findings suggest that ATF4 activation downstream of PERK during ER stress activates, at the transcriptional level, RASSF1 which in turn induces cell cycle arrest and stimulates apoptosis. The proposed integration of RASSF1 into UPR signaling suggests that RASSF1 activation may contribute to UPR-associated pathologies at which excessive cell death is recorded. Conversely, in the context of anticancer therapy these findings imply that UPR activation may be beneficial in cancers that are RASSF1-dependent. Furthermore, DAXX was recently found as a new type of protein-folding enabler (39) and it also plays a critical role in the p53-mediated RASSF1A inactivation (40). And RASSF1A associates with DAXX and MDM2 in the nucleus, promoting MDM2 self-ubiquitination by the disruption of MDM2-DAXX-HAUSP complex (41). These results may indicate the involvement of RASSF1 in the protein folding network.

RASSF1A and RASSF1C are two well-studied RASSF1 isoforms. RASSF1A reduces cell proliferation and stimulates apoptosis while RASSF1C functions as an oncogene and shows the opposite activities (42,43). The remarkably higher RASSF1A induction by tunicamycin in HEK293 cells, in combination with the abrogation of tunicamycin-induced apoptosis in cells subjected to shRNA-mediated RASSF1 inhibition suggest that it is RASSF1A that mainly mediates apoptosis when UPR is induced. That during RASSF1 knock-down, G2/M arrest was only induced in the absence of tunicamycin, is likely indicative for the fact that during ER stress, arrested cells have already been sensitized towards apoptosis. Therefore, no considerable

changes are recorded in their G2/M fraction during stress, nevertheless, apoptosis was significant alleviated during tunicamycin treatment, when RASSF1 expression was compromised. BH3-only sensor BBC3/PUMA, a PERK/ eIF2α dependent pro-apoptotic gene (26,27) can be activated by RASSF1A (16,37) and plays an important role in the ER-stress induced apoptosis (44). However, it is worth noting that other BH3-only proteins, such as Bid and Bim can activate apoptotic signaling independent of BBC3/PUMA during ER stress (32,45,46).

Besides the significance of attributing RASSF1 UPR-associated functionality, the present study illustrates how coordination analysis of gene expression may reveal causative associations between genes and biochemical pathways. In addition, GO analyses based on the enrichment of co-regulated, as opposed to differentially expressed genes, may predict with high accuracy biological functions that can be validated experimentally. This highly versatile strategy is particularly applicable to the analysis of transcriptomic data from genetically diverse specimens, such as human samples, at which the observed variation in gene expression levels limits the statistical significance of conventional differential expression analyses and restricts their informative value. By focusing on the degree of transcriptomic coordination, as opposed to the magnitude of differential expression, it is plausible to unveil associations that would remain unnoticed by conventional approaches.

## Methods and Materials

### In silico analysis of RASSF1 transcript

The RNA-Seq data used here have been published (8, 47) and deposited in GEO (Accession numbers: GSE129534 and GSE130970). The flowchart of the process and analysis was described previously (8). Briefly, The Pearson's correlation coefficients were calculated between the whole

transcriptome as obtained by the RNA-Seq analysis and the transcripts indicated. Subsequently, the coordination between the UPR-associated transcripts and RASSF1 was calculated as the correlation of their Pearson's R values. For the Gene Ontology Enrichment analysis, the transcripts were sorted according to the R values of the whole transcriptome versus RASSF1 and the identification of associated biological processes was performed using the gene ontology online platform (48,49) at which the list of genes exhibiting $p < 0.05$ (Pearson's). The putative transcription factor binding sites of RASSF1 promoter were analyzed using MatInspector (50).

**Cell culture**

HFFF2 (Sigma) and HEK293FT (Life Technologies) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.292 mg/ml L-glutamine (HyClone). Cells were maintained at 37 °C in a humidified environment with 5% $CO_2$ and 95% air. For ER stress induction, cells were split into six-well plates, at 300,000 cells/well, and cultured for 24 h. Then cells were treated with either tunicamycin (5 µg/ml, Sigma) or thapsigargin (3 µM, Sigma) with or without the addition of ISRIB (500 µM, Sigma) for 5 h, immediately followed by RNA extraction.

**RASSF1 luciferase reporter constructs**

The genomic DNA was extracted from HFFF2 cells using DNeasy Blood & Tissue Kit (Qiagen) according to the supplied protocol. The RASSF1 promoter region (-930 to +38 relative to the transcription initiation site) was amplified by PCR using 100 ng genomic DNA, Q5 High-Fidelity DNA polymerase (New England BioLabs), and the primers 1 (forward) (5'-GCTGGAGCGAGAAAACAGAG) and 2 (reverse) (5'-CAATGGAAACCTGGGTGCAG). The

PCR product size was 969 base pairs. Following PCR, the generated fragment was subcloned into a pCR-Blunt II-TOPO vector (Invitrogen). Then the target fragment, co-digested by KpnI and EcoRV (New England BioLabs), was subcloned into the KpnI and EcoRV sites of pBV-Luc vector (51) (a gift from Bert Vogelstein; Addgene plasmid # 16539; http://n2t.net/addgene:16539; RRID: Addgene_16539), carrying a firefly luciferase coding sequence under control of a minimal promoter. All constructs were confirmed by sequencing.

**Luciferase assay**

HEK293FT cells were co-transfected with the RASSF1 luciferase reporter plasmid, and pRK-ATF4 expression plasmid (52) (a gift from Yihong Ye; Addgene plasmid # 26114; http://n2t.net/addgene:26114; RRID: Addgene_26114) or pRK-ATF4 ΔC (1-275) expression plasmid (52) (a gift from Yihong Ye, Addgene plasmid # 26118; http://n2t.net/addgene:26118; RRID: Addgene_26118) using Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Luciferase activity in cell lysates was measured using luciferase assay system (Promega). Luciferase activity was normalized by the amount of the total protein.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed using the ChIP kit (Abcam, ab500) according to the supplied protocol. Briefly, HEK293FT cells were exposed to 5 μg/mL tunicamycin (Sigma) for 5 h, cross-linked with 1.1% formaldehyde (Thermo Scientific, Cat# 28906) for 10 min at room temperature, and quenched in 0.125M glycine. The cells were then incubated with lysis buffer and sonicated to produce 200-500 base pair DNA fragments. DNA fragments were immunoprecipitated from the cell lysates using anti-ATF4 antibody (Abcam, ab184909) or rabbit IgG (Abcam, ab171870) and

299 immunoprecipitates were recovered by addition of DNA purifying slurry. After reverse
300 crosslinking and washing, purified DNA was quantified by SYBR Green real-time PCR (Bio-Rad)
301 using specific primers (Table 2). The samples added rabbit IgG was used as a control. Data were
302 expressed as the percentage of input.
303
304 **Establishment of RASSF1 knockdown cells**
305 The hRASSF1-RNAi lentiviral vector pLV-EGFP-Puro shRNA and lentiviral carrying
306 scrambled shRNA were constructed by VectorBuilder (US). The RNAi target sequence against
307 RASSF1 is AGACAGAAGTCTCCTCAATTT and the scrambled shRNA was served as a control.
308 The vector packaging and harvesting were performed by transfection of HEK293FT cells using
309 PEI transfection reagent (Polysciences). Briefly, HEK293FT cells were co-transfected with 1.5 μg
310 of pMD2.G, 4.5 μg of psPAX2 and 6 μg of RASSF1 or control shRNA and cultured for 48 h.
311 Supernatant containing lentiviral vectors was collected and filtered, and then mixed 1:1 volume
312 with complete culture media and added to cells. 8 μg/ml of polybrene was also added to the virus
313 to increase transduction efficiency. Cells were selected with 2 μg/ml of puromycin and the
314 knockdown efficiency was confirmed by western blot.
315
316 **Western blots**
317 Whole cell lysates were obtained from RASSF1 and control shRNA transfected
318 HEK293FT cells treated with tunicamycin (5 μg/mL) for 5 h. The cells were harvested with RIPA
319 lysis buffer (Thermo Fisher). Lysates were sonicated for 30 seconds, and the protein concentration
320 was measured by DC protein assay (Bio-Rad). Protein samples (30 μg each) were separated by 4-
321 12% PAGE Gel (GenScript) and then transferred onto PVDF membranes (Millipore). Membranes

were blocked with 5% non-fat milk for 60 minutes at room temperature and incubated overnight at 4°C with recombinant anti-RASSF1 rabbit monoclonal antibody (1:500, Abcam, ab126764) or anti-α-Tubulin monoclonal mouse antibody (1:5,000, Sigma, T9026). After washing, membranes were incubated for 1 hour at room temperature with horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (1:10,000; Abcam) or goat anti-mouse IgG secondary antibody (1:10,000; ThermoFisher) at room temperature. The immobilized proteins were detected using the enhanced chemiluminescence reagent plus (PerkinElmer). Images were obtained with ChemiDoc™ Touch Imaging System (Bio-Rad) and analyzed with Image Lab.

**RNA extraction, cDNA synthesis and qPCR**

RNA was extracted with a Qiagen RNeasy Plus Mini kit as per manufacturer's recommendations (Qiagen). Complementary DNA (cDNA) synthesis was conducted using an iScript cDNA synthesis kit (Bio-Rad) according to the supplied protocol. Quantitative PCR (qPCR) was performed on a T100 thermocycler (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad). Specific oligonucleotide primers for target gene sequences are listed in Table 2. Arbitrary units of target mRNA were normalized to the level of GAPDH expression.

**Cell cycle analysis**

RASSF1 and control shRNA transfected HEK293FT cells were treated with tunicamycin (5 μg/mL) for 24 h, and then fixed in 70% ethanol overnight at 4°C. Cells were washed once with PBS and labeled with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS/0.1% Triton X-100 solution for 30 min at room temperature. Cell cycle phases were analyzed with BD LSR II flow cytometer (BD Biosciences, Franklin, NJ).

## Cell apoptosis assay

The apoptotic cells were detected using the In Situ Cell Death Detection Kit, Fluorescein (Roche) according to the supplied protocol. Briefly, cells were treated with tunicamycin (5 μg/mL) for 24 h, then washed with PBS and fixed with freshly prepared 2% paraformaldehyde for 1 hour at room temperature. The cells were permeabilized with 0.1% Triton X-100 solution for 2 min on ice, and then labeled with TUNEL reaction mixture for 1 hour at 37°C in a humidified atmosphere in the dark. The cells were resuspended in FBS and smeared over a coverslip. The number of the apoptotic cells was counted with a fluorescence confocal microscope (Carl Zeiss LSM 700) and analyzed with ImageJ.

## Statistical analysis

Statistical analyses were performed using Prism software (version 9.2.0; GraphPad Software). The data were expressed as mean±s.e.m, unless specified otherwise. Results were analyzed using unpaired two-tailed *t*-test, one-way ANOVA followed by Tukey's multiple comparisons test or Pearson's correlation as indicated. $P<0.05$ was considered significant.

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## Conflict of interest

The authors declare no conflict of interest.

## Author Contributions

YZ and HK conceived and designed the project, YZ, TD, XD, VS and CL acquired the data, YZ and HK analyzed and interpreted the data, HK and EB supervised the project and provided resources; HK and YZ wrote the original draft. All authors discussed the results and contributed to the final manuscript.

## References


1. Crow M, Lim N, Ballouz S, Pavlidis P, Gillis J. Predictability of human differential gene expression. Proc Natl Acad Sci U S A. 2019 Mar 26;116(13):6491-6500. doi: 10.1073/pnas.1802973116. Epub 2019 Mar 7. PMID: 30846554; PMCID: PMC6442595.
2. Rodriguez-Esteban, R., Jiang, X. Differential gene expression in disease: a comparison between high-throughput studies and the literature. BMC Med Genomics 10, 59 (2017). https://doi.org/10.1186/s12920-017-0293-y
3. Uygun S, Peng C, Lehti-Shiu MD, Last RL, Shiu SH. Utility and Limitations of Using Gene Expression Data to Identify Functional Associations. PLoS Comput Biol. 2016;12(12):e1005244. Published 2016 Dec 9. doi:10.1371/journal.pcbi.1005244
4. Hirsch CD, Springer NM, Hirsch CN. Genomic limitations to RNA sequencing expression profiling. Plant J. 2015 Nov;84(3):491-503. doi: 10.1111/tpj.13014. Epub 2015 Oct 6. PMID: 26331235.
5. Rapaport, F., Khanin, R., Liang, Y. et al. Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. Genome Biol 14, 3158 (2013). https://doi.org/10.1186/gb-2013-14-9-r95
6. Havighorst A, Zhang Y, Farmaki E, Kaza V, Chatzistamou I, Kiaris H. Differential regulation of the unfolded protein response in outbred deer mice and susceptibility to metabolic disease. Dis Model Mech. 2019 Feb 27;12(2). pii: dmm037242. doi: 10.1242/dmm.037242.
7. Zhang Y, Chatzistamou I, Kiaris H. Coordination of the unfolded protein response during hepatic steatosis identifies CHOP as a specific regulator of hepatocyte ballooning. Cell Stress Chaperones. 2020 Jun 23. doi: 10.1007/s12192-020-01132-x

8. Zhang Y, Lucius MD, Altomare D, Havighorst A, Farmaki E, Chatzistamou I, Shtutman M, Kiaris H. Coordination Analysis of Gene Expression Points to the Relative Impact of Different Regulators During Endoplasmic Reticulum Stress. DNA Cell Biol. 2019 Sep;38(9):969-981. doi: 10.1089/dna.2019.491.
9. Zhang Y, Chatzistamou I, Kiaris H. Identification of frailty-associated genes by coordination analysis of gene expression. Aging (Albany NY). 2020; https://doi.org/10.18632/aging.102875
10. Soltanmohammadi E, Farmaki E, Zhang Y, Naderi A, Kaza V, Chatzistamou I, Kiaris H. Coordination in the unfolded protein response during aging in outbred deer mice. Experimental Gerontology. 2020 Dec 5;144:111191. doi: 10.1016/j.exger.2020.111191
11. Zhang Y, Chatzistamou I, Kiaris H. Transcriptomic coordination at hepatic steatosis indicates robust immune cell engagement prior to inflammation. BMC Genomics. 2021. https://doi.org/10.1186/s12864-021-07784-y
12. Hetz, C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* **13,** 89–102 (2012). https://doi.org/10.1038/nrm3270
13. Bhattarai KR, Chaudhary M, Kim HR, Chae HJ. Endoplasmic Reticulum (ER) Stress Response Failure in Diseases. Trends Cell Biol. 2020 Sep;30(9):672-675. doi: 10.1016/j.tcb.2020.05.004. Epub 2020 Jun 16. PMID: 32561138.
14. Dombroski BA, Nayak RR, Ewens KG, Ankener W, Cheung VG, Spielman RS. Gene expression and genetic variation in response to endoplasmic reticulum stress in human cells. Am J Hum Genet. 2010 May 14;86(5):719-29. doi: 10.1016/j.ajhg.2010.03.017. Epub 2010 Apr 15. PMID: 20398888; PMCID: PMC2869002.

15. Chow CY, Wang X, Riccardi D, Wolfner MF, Clark AG. The genetic architecture of the genome-wide transcriptional response to ER stress in the mouse. PLoS Genet. 2015 Feb 4;11(2):e1004924. doi: 10.1371/journal.pgen.1004924. PMID: 25651210; PMCID: PMC4412289.
16. Dubois F, Bergot E, Zalcman G, Levallet G. RASSF1A, puppeteer of cellular homeostasis, fights tumorigenesis, and metastasis-an updated review. Cell Death Dis. 2019 Dec 5;10(12):928. doi: 10.1038/s41419-019-2169-x. PMID: 31804463; PMCID: PMC6895193.
17. Malpeli G, Innamorati G, Decimo I, Bencivenga M, Nwabo Kamdje AH, Perris R, Bassi C. Methylation Dynamics of *RASSF1A* and Its Impact on Cancer. Cancers (Basel). 2019 Jul 9;11(7):959. doi: 10.3390/cancers11070959. PMID: 31323949; PMCID: PMC6678546.
18. Iwasa H, Hossain S, Hata Y. Tumor suppressor C-RASSF proteins. Cell Mol Life Sci. 2018 May;75(10):1773-1787. doi: 10.1007/s00018-018-2756-5. Epub 2018 Jan 20. PMID: 29353317.
19. Elfiky AA, Baghdady AM, Ali SA, Ahmed MI. GRP78 targeting: Hitting two birds with a stone. Life Sci. 2020 Nov 1;260:118317. doi: 10.1016/j.lfs.2020.118317. Epub 2020 Aug 22. PMID: 32841659; PMCID: PMC7442953.
20. Pobre KFR, Poet GJ, Hendershot LM. The endoplasmic reticulum (ER) chaperone BiP is a master regulator of ER functions: Getting by with a little help from ERdj friends. J Biol Chem. 2019 Feb 8;294(6):2098-2108. doi: 10.1074/jbc.REV118.002804. Epub 2018 Dec 18. PMID: 30563838; PMCID: PMC6369273.

21. Hu H, Zhou C, Li B, Chen Y, Dai J, Mao Y, Huang T, Yu H, Chen M, Zhao J, Duan S. Diagnostic value of RASSF1A hypermethylation in colorectal cancer: a meta-analysis. Pathol Res Pract. 2018 Oct;214(10):1572-1578. doi: 10.1016/j.prp.2018.07.031. Epub 2018 Jul 27. PMID: 30082160.
22. Schmidt ML, Hobbing KR, Donninger H, Clark GJ. RASSF1A Deficiency Enhances RAS-Driven Lung Tumorigenesis. Cancer Res. 2018 May 15;78(10):2614-2623. doi: 10.1158/0008-5472.CAN-17-2466. Epub 2018 May 7. PMID: 29735543; PMCID: PMC5955812.
23. Jauhiainen A, Thomsen C, Strömbom L, Grundevik P, Andersson C, Danielsson A, Andersson MK, Nerman O, Rörkvist L, Ståhlberg A, Åman P. Distinct cytoplasmic and nuclear functions of the stress induced protein DDIT3/CHOP/GADD153. PLoS One. 2012;7(4):e33208. doi: 10.1371/journal.pone.0033208
24. Guo J, Ren R, Sun K, He J, Shao J. PERK signaling pathway in bone metabolism: Friend or foe? Cell Prolif. 2021 Apr;54(4):e13011. doi: 10.1111/cpr.13011. Epub 2021 Feb 21. PMID: 33615575; PMCID: PMC8016635.
25. Read A, Schröder M. The Unfolded Protein Response: An Overview. Biology (Basel). 2021 Apr 29;10(5):384. doi: 10.3390/biology10050384. PMID: 33946669; PMCID: PMC8146082.
26. Cunha DA, Igoillo-Esteve M, Gurzov EN, Germano CM, Naamane N, Marhfour I, Fukaya M, Vanderwinden JM, Gysemans C, Mathieu C, Marselli L, Marchetti P, Harding HP, Ron D, Eizirik DL, Cnop M. Death protein 5 and p53-upregulated modulator of apoptosis mediate the endoplasmic reticulum stress-mitochondrial dialog triggering

lipotoxic rodent and human β-cell apoptosis. Diabetes. 2012 Nov;61(11):2763-75. doi: 10.2337/db12-0123. Epub 2012 Jul 6. PMID: 22773666; PMCID: PMC3478544.

27. Yang Y, Li C, Xiang X, Dai Z, Chang J, Zhang M, Cai H, Zhang H, Zhang M, Guo Y, Wu Z. Ursolic acid prevents endoplasmic reticulum stress-mediated apoptosis induced by heat stress in mouse cardiac myocytes. J Mol Cell Cardiol. 2014 Feb;67:103-11. doi: 10.1016/j.yjmcc.2013.12.018. Epub 2014 Jan 3. PMID: 24389342.

28. Lee D, Hokinson D, Park S, Elvira R, Kusuma F, Lee JM, Yun M, Lee SG, Han J. ER Stress Induces Cell Cycle Arrest at the G2/M Phase Through eIF2α Phosphorylation and GADD45α. Int J Mol Sci. 2019 Dec 13;20(24):6309. doi: 10.3390/ijms20246309. PMID: 31847234; PMCID: PMC6940793.

29. Kapoor A, Chen CG, Iozzo RV. Endorepellin evokes an angiostatic stress signaling cascade in endothelial cells. J Biol Chem. 2020 May 8;295(19):6344-6356. doi: 10.1074/jbc.RA120.012525. Epub 2020 Mar 23. PMID: 32205445; PMCID: PMC7212646.

30. Belmont PJ, Tadimalla A, Chen WJ, Martindale JJ, Thuerauf DJ, Marcinko M, Gude N, Sussman MA, Glembotski CC. Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. J Biol Chem. 2008 May 16;283(20):14012-21. doi: 10.1074/jbc.M709776200. Epub 2008 Mar 3. PMID: 18319259; PMCID: PMC2376224.

31. Bartoszewski R, Gebert M, Janaszak-Jasiecka A, Cabaj A, Króliczewski J, Bartoszewska S, Sobolewska A, Crossman DK, Ochocka R, Kamysz W, Kalinowski L, Dąbrowski M, Collawn JF. Genome-wide mRNA profiling identifies RCAN1 and GADD45A as regulators of the transitional switch from survival to apoptosis during ER stress. FEBS J.

2020 Jul;287(14):2923-2947. doi: 10.1111/febs.15195. Epub 2020 Jan 10. PMID: 31880863.

32. Gebert M, Sobolewska A, Bartoszewska S, Cabaj A, Crossman DK, Króliczewski J, Madanecki P, Dąbrowski M, Collawn JF, Bartoszewski R. Genome-wide mRNA profiling identifies X-box-binding protein 1 (XBP1) as an IRE1 and PUMA repressor. Cell Mol Life Sci. 2021 Nov;78(21-22):7061-7080. doi: 10.1007/s00018-021-03952-1. Epub 2021 Oct 12. PMID: 34636989; PMCID: PMC8558229.
33. Shan J, Zhang F, Sharkey J, Tang TA, Örd T, Kilberg MS. The C/ebp-Atf response element (CARE) location reveals two distinct Atf4-dependent, elongation-mediated mechanisms for transcriptional induction of aminoacyl-tRNA synthetase genes in response to amino acid limitation. Nucleic Acids Res. 2016 Nov 16;44(20):9719-9732. doi: 10.1093/nar/gkw667. Epub 2016 Jul 28. PMID: 27471030; PMCID: PMC5175342.
34. Fusakio ME, Willy JA, Wang Y, et al. Transcription factor ATF4 directs basal and stress-induced gene expression in the unfolded protein response and cholesterol metabolism in the liver. *Mol Biol Cell*. 2016;27(9):1536-1551. doi:10.1091/mbc.E16-01-0039
35. Zhang K, Wang M, Li Y, Li C, Tang S, Qu X, Feng N, Wu Y. The PERK-EIF2α-ATF4 signaling branch regulates osteoblast differentiation and proliferation by PTH. Am J Physiol Endocrinol Metab. 2019 Apr 1;316(4):E590-E604. doi: 10.1152/ajpendo.00371.2018. Epub 2019 Jan 22. PMID: 30668150.
36. Wu, J., Chen, S., Liu, H. *et al.* Tunicamycin specifically aggravates ER stress and overcomes chemoresistance in multidrug-resistant gastric cancer cells by inhibiting N-glycosylation. *J Exp Clin Cancer Res* **37,** 272 (2018). https://doi.org/10.1186/s13046-018-0935-8

37. Matallanas D, Romano D, Yee K, Meissl K, Kucerova L, Piazzolla D, Baccarini M, Vass JK, Kolch W, O'neill E. RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. Mol Cell. 2007 Sep 21;27(6):962-75. doi: 10.1016/j.molcel.2007.08.008. PMID: 17889669; PMCID: PMC2821687.

38. Qing G, Li B, Vu A, Skuli N, Walton ZE, Liu X, Mayes PA, Wise DR, Thompson CB, Maris JM, Hogarty MD, Simon MC. ATF4 regulates MYC-mediated neuroblastoma cell death upon glutamine deprivation. Cancer Cell. 2012 Nov 13;22(5):631-44. doi: 10.1016/j.ccr.2012.09.021. PMID: 23153536;

39. Huang L, Agrawal T, Zhu G, Yu S, Tao L, Lin J, Marmorstein R, Shorter J, Yang X. DAXX represents a new type of protein-folding enabler. Nature. 2021 Sep;597(7874):132-137. doi: 10.1038/s41586-021-03824-5. Epub 2021 Aug 18. PMID: 34408321.

40. Zhang H, He J, Li J, Tian D, Gu L, Zhou M. Methylation of RASSF1A gene promoter is regulated by p53 and DAXX. FASEB J. 2013 Jan;27(1):232-42. doi: 10.1096/fj.12-215491. Epub 2012 Oct 4. PMID: 23038753; PMCID: PMC3528318.

41. Song MS, Song SJ, Kim SY, Oh HJ, Lim DS. The tumour suppressor RASSF1A promotes MDM2 self-ubiquitination by disrupting the MDM2-DAXX-HAUSP complex. EMBO J. 2008 Jul 9;27(13):1863-74. doi: 10.1038/emboj.2008.115. Epub 2008 Jun 19. PMID: 18566590; PMCID: PMC2486425.

42. Dubois F, Bergot E, Levallet G. Cancer and RASSF1A/RASSF1C, the Two Faces of Janus. Trends Cancer. 2019 Nov;5(11):662-665. doi: 10.1016/j.trecan.2019.10.001. Epub 2019 Oct 28. PMID: 31735284.

43. Estrabaud E, Lassot I, Blot G, Le Rouzic E, Tanchou V, Quemeneur E, Daviet L, Margottin-Goguet F, Benarous R. RASSF1C, an isoform of the tumor suppressor RASSF1A, promotes the accumulation of beta-catenin by interacting with betaTrCP. Cancer Res. 2007 Feb 1;67(3):1054-61. doi: 10.1158/0008-5472.CAN-06-2530. PMID: 17283138.

44. Gurzov EN, Germano CM, Cunha DA, Ortis F, Vanderwinden JM, Marchetti P, Zhang L, Eizirik DL. p53 up-regulated modulator of apoptosis (PUMA) activation contributes to pancreatic beta-cell apoptosis induced by proinflammatory cytokines and endoplasmic reticulum stress. J Biol Chem. 2010 Jun 25;285(26):19910-20. doi: 10.1074/jbc.M110.122374. Epub 2010 Apr 26. PMID: 20421300; PMCID: PMC2888402.

45. Verfaillie T, Rubio N, Garg AD, Bultynck G, Rizzuto R, Decuypere JP, Piette J, Linehan C, Gupta S, Samali A, Agostinis P. PERK is required at the ER-mitochondrial contact sites to convey apoptosis after ROS-based ER stress. Cell Death Differ. 2012 Nov;19(11):1880-91. doi: 10.1038/cdd.2012.74. Epub 2012 Jun 15. PMID: 22705852; PMCID: PMC3469056.

46. Lomonosova E, Chinnadurai G. BH3-only proteins in apoptosis and beyond: an overview. Oncogene. 2008 Dec;27 Suppl 1(Suppl 1):S2-19. doi: 10.1038/onc.2009.39. PMID: 19641503; PMCID: PMC2928556.

47. Hoang SA, Oseini A, Feaver RE, Cole BK, Asgharpour A, Vincent R, Siddiqui M, Lawson MJ, Day NC, Taylor JM, Wamhoff BR, Mirshahi F, Contos MJ, Idowu M, Sanyal AJ. Gene Expression Predicts Histological Severity and Reveals Distinct Molecular Profiles of Nonalcoholic Fatty Liver Disease. Sci Rep. 2019 Aug

29;9(1):12541. doi: 10.1038/s41598-019-48746-5. PMID: 31467298; PMCID: PMC6715650.

48. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics. 2009 Feb 3;10:48. doi: 10.1186/1471-2105-10-48. PMID: 19192299; PMCID: PMC2644678.
49. Eden E, Lipson D, Yogev S, Yakhini Z. Discovering motifs in ranked lists of DNA sequences. PLoS Comput Biol. 2007 Mar 23;3(3):e39. doi: 10.1371/journal.pcbi.0030039. PMID: 17381235; PMCID: PMC1829477.
50. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics. 2005 Jul 1;21(13):2933-42. doi: 10.1093/bioinformatics/bti473. Epub 2005 Apr 28. PMID: 15860560.
51. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell. 1999 Oct 29;99(3):335-45. doi: 10.1016/s0092-8674(00)81664-5. PMID: 10555149; PMCID: PMC3779681.
52. Wang Q, Mora-Jensen H, Weniger MA, Perez-Galan P, Wolford C, Hai T, Ron D, Chen W, Trenkle W, Wiestner A, Ye Y. ERAD inhibitors integrate ER stress with an epigenetic mechanism to activate BH3-only protein NOXA in cancer cells. Proc Natl Acad Sci U S A. 2009 Feb 17;106(7):2200-5. doi: 10.1073/pnas.0807611106. Epub 2009 Jan 22. PMID: 19164757; PMCID: PMC2629785.

**Table 1. Gene ontology enrichment analysis for transcripts that exhibited positively correlated expression (P < 0.05, Pearson's) with RASSF1**

| GO term | Description | P-value | FDR q-value |
|---|---|---|---|
| GO:0034976 | Response to endoplasmic reticulum stress | 1.03E-06 | 6.40E-03 |
| GO:2001233 | Regulation of apoptotic signaling pathway | 1.47E-05 | 4.58E-02 |
| GO:1901565 | Organonitrogen compound catabolic process | 3.89E-05 | 8.07E-02 |
| GO:0033554 | Cellular response to stress | 5.57E-05 | 8.68E-02 |
| GO:2001235 | Positive regulation of apoptotic signaling pathway | 8.40E-05 | 1.05E-01 |
| GO:0006986 | Response to unfolded protein | 1.08E-04 | 1.12E-01 |
| GO:0035966 | Response to topologically incorrect protein | 1.08E-04 | 9.61E-02 |
| GO:0006915 | Apoptotic process | 1.22E-04 | 9.53E-02 |
| GO:0030163 | Protein catabolic process | 1.26E-04 | 8.73E-02 |
| GO:0012501 | Programmed cell death | 1.77E-04 | 1.10E-01 |
| GO:0060548 | Negative regulation of cell death | 1.77E-04 | 1.00E-01 |
| GO:1903912 | Negative regulation of endoplasmic reticulum stress-induced eif2 alpha phosphorylation | 2.19E-04 | 1.14E-01 |
| GO:0008219 | Cell death | 3.09E-04 | 1.48E-01 |
| GO:0051246 | Regulation of protein metabolic process | 4.32E-04 | 1.92E-01 |
| GO:0032270 | Positive regulation of cellular protein metabolic process | 5.01E-04 | 2.08E-01 |

584 **Table 2. Oligonucleotide Primers for RT-qPCR**

| Name | Forward (5′ – 3′) | Reverse (5′ – 3′) | Product (bp) |
|---|---|---|---|
| ***ATF4*** | CCCCAGACGGTGAACCCAAT | CTGGAGTGGAGGACAGGACC | 121 |
| ***BBC3*** | ACGACCTCAACGCACAGTAC | CTGGGTAAGGGCAGGAGTC | 112 |
| ***CCNA2*** | AGCATGTCACCGTTCCTCCT | CCAGGGCATCTTCACGCTC | 132 |
| ***GAPDH*** | AGAAGGTGGTGAAGCAGGCG | AAGGTGGAGGAGTGGGTGTC | 109 |
| ***RASSF1*** | TGCCCAGATCAACAGCAACC | CTGCAAGGAGGGTGGCTTCT | 130 |
| ***RASSF1A*** | TTCACCTGCCACTACCGCTG | GTCTCCCACTCCACAGGCTC | 122 |
| ***RASSF1C*** | AATGACCTGGAGCAGCACGA | GTCTCCCACTCCACAGGCTC | 103 |
| ***RASSF1*** (ChIP) | GATCTCCCTCCTCCTCACCC | CCTGGTCCGGTTTGCTGAA | 94 |

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587

588 **Figure legends**

589 **Fig. 1.** Whole transcriptome coordination analysis shows positive correlations between RASSF1
590 and UPR target genes. A. Scatterplots showing the R (Pearson's) values for the whole
591 transcriptomes of RASSF1 and each of UPR target genes HSPA5/BiP, DNAJB9, HSP90B1,
592 ATF4, DNAJC3 and DDIT3 in primary fibroblasts of deer mice with or without tunicamycin
593 treatment (n = 6). The data are shown in Suppl. Table 1 and the methods can be found in our
594 previous publication (8). B. Scatterplots showing the R (Pearson's) values for the whole
595 transcriptomes of RASSF1 and each of UPR target genes HSPA5/BiP, DNAJB9, HSP90B1,
596 ATF4, DNAJC3 and DDIT3 in human liver specimens (n = 6). The data are shown in Suppl. Table
597 2 and the methods can be found in our previous publication (7).

598 **Fig. 2**. ATF4 occupies the RASSF1 promoter and regulates its expression. **A.** Schema of the
599 RASSF1 promoter with the localization of putative ATF4 binding site. **B.** Luciferase activity in
600 HEK293FT cells co-transfected with pRASSF1-Luc and pRK-ATF4 or pRK-ATF4 ΔC (1-275) (n
601 = 2 biological replicates). The results were expressed as relative luciferase activity normalized
602 with the total protein concentration. P value was calculated with unpaired two-tailed *t*-test. **C.**
603 Soluble chromatin from HEK293FT cells was precipitated with anti-ATF4 antibody or rabbit IgG
604 (n = 3 biological replicates). The final DNA samples were amplified by qPCR with primers for the
605 RASSF1 promoter listed in Table 2. The results were expressed as the percentage to the input
606 DNA. P value was calculated with unpaired two-tailed *t*-test.. * $P < 0.05$, ** $P < 0.01$.

607 **Fig. 3.** ER stress induced by tunicamycin (Tun) in HEK293FT cells upregulates RASSF1,
608 RASSF1A, RASSF1C, ATF4 and BBC3 expression. HEK293FT cells were treated with
609 tunicamycin (5 μg/mL) and the relative gene expression was detected by RT-qPCR using primers
610 listed in Table 2 and normalized with GAPDH expression (n = 3 biological replicates). Tun –

611 tunicamycin treatment. Ctrl – control. P values were calculated with unpaired two-tailed *t*-test. *
612 $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns non-significant.

613 **Fig. 4.** ER stress induced by **A.** tunicamycin (Tun) or **B.** thapsigargin (Thap) in HFFF2 cells
614 upregulates RASSF1 and ATF4 expression. HFFF2 cells were treated with tunicamycin (5 μg/mL)
615 or thapsigargin (3 μM), and the relative gene expression was detected by RT-qPCR using primers
616 listed in Table 2 and normalized with GAPDH expression (n = 3 biological replicates). Tun –
617 tunicamycin treatment. Thap – thapsigargin treatment. Ctrl – control. P values were calculated
618 with unpaired two-tailed t-test. * $P < 0.05$, *** $P < 0.001$.

619 **Fig. 5.** ER stress induced by **A.** tunicamycin (Tun) or **B.** thapsigargin (Thap) in HEK293FT cells
620 upregulates RASSF1 and ATF4 expression, and the effects were reduced by ISRIB addition.
621 HEK293FT cells were treated by tunicamycin (5 μg/mL) or thapsigargin (3 μM) with or without
622 ISRIB (500 μM) addition, and the relative gene expression was detected by RT-qPCR using
623 primers listed in Table 2 and normalized with GAPDH expression (n = 3 biological replicates).
624 Tun – tunicamycin treatment. Thap – thapsigargin treatment. Ctrl – control. P values were
625 calculated with one-way ANOVA followed by Tukey's multiple comparisons test. * $P < 0.05$, **
626 $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

627 **Fig. 6.** RASSF1 knockdown by ShRNA modulates RASSF1 and its related genes expression in
628 cells under ER stress conditions. **A.** The relative expression of RASSF1 protein in HEK293FT
629 cells transfected with RASSF1or control shRNA and treated with tunicamycin (5 μg/mL), detected
630 with western blotting and normalized with α-Tubulin levels (representative images of n = 3). **B.**
631 The relative expressions of RASSF1, ATF4 and BBC3 mRNA in HEK293FT cells transfected
632 with RASSF1 or control shRNA and treated with tunicamycin, detected with RT-qPCR and
633 normalized with GAPDH expression (n = 3 biological replicates). Control shRNA Ctrl – cells

transfected with scrambled shRNA and without tunicamycin treatment, RASSF1 shRNA Ctrl – cells transfected with hRASSF1-shRAN and without tunicamycin treatment, Control shRNA Tun – cells transfected with scrambled shRNA and treated with tunicamycin, RASSF1 shRNA Tun – cells transfected with hRASSF1-shRAN and treated with tunicamycin. P values were calculated with one-way ANOVA followed by Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Fig. 7**. RASSF1 knockdown by ShRNA reduces the cell apoptosis under ER stress conditions. HEK293FT cells were transfected with hRASSF1 or control shRNA and treated with tunicamycin (5 μg/mL). The ratios of TUNEL positive to EGFP positive cells were detected with the In Situ Cell Death Detection Kit and analyzed under a fluorescence microscope (representative images of n = 10). Scale bars: 30 μm. Control shRNA Ctrl – cells transfected with scrambled shRNA and without tunicamycin treatment, RASSF1 shRNA Ctrl – cells transfected with hRASSF1-shRAN and without tunicamycin treatment, Control shRNA Tun – cells transfected with scrambled shRNA and treated with tunicamycin, RASSF1 shRNA Tun – cells transfected with hRASSF1-shRAN and treated with tunicamycin. P values were calculated with one-way ANOVA followed by Tukey's multiple comparisons test. ** $P < 0.01$, **** $P < 0.0001$, ns non-significant.

**Supplementary Figure 1.** Whole transcriptome coordination analysis between RASSF1 and each of BBC3, GADD45A, RCAN1 and CCNA2-associated transcriptomes. The transcriptome of RASSF1 showed tight association with those of BBC3 and GADD45A, two PERK-eIF2α dependent pro-apoptotic genes, but the correlation was reduced with the transcriptome of RCAN1, an ATF6-dependent pro-survival regulator. In line with the RNA expression data of Fig. 3, the RASSF1-associated transcriptome was coordinated with the BBC3-associated transcriptome but not with the CCNA2-associated transcriptome. Data were obtained from deer mouse fibroblasts

RNA-Seq data for BBC3, GADD45A and RCAN1 (8), and human liver RNA-Seq data for BBC3, GADD45A, RCAN1 and CCNA2 (47) (CCNA2 was not detected in the deer mouse data).

**Supplementary Figure 2.** RASSF1 knockdown by ShRNA alters the cell population distribution in different stages of cell cycle under ER stress conditions. HEK293FT cells were transfected with hRASSF1- or control shRNA and treated with tunicamycin (5 μg/mL). The cell populations in different stages of cell cycle were measured with flow cytometry (n = 2 biological replicates). Control shRNA Ctrl – cells transfected with scrambled shRNA and without tunicamycin treatment, RASSF1 shRNA Ctrl – cells transfected with hRASSF1-shRAN and without tunicamycin treatment, Control shRNA Tun – cells transfected with scrambled shRNA and treated with tunicamycin, RASSF1 shRNA Tun – cells transfected with hRASSF1-shRAN and treated with tunicamycin. P values were calculated with one-way ANOVA followed by Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns non-significant.

**Supplementary Table 1.** The calculation of Pearson's R values for whole transcriptome coordination between RASSF1 and UPR target genes HSPA5/BiP, DNAJB9, HSP90B1, ATF4, DNAJC3 and DDIT3 in primary fibroblasts of deer mice.

**Supplementary Table 2.** The calculation of Pearson's R values for whole transcriptome coordination between RASSF1 and UPR target genes HSPA5/BiP, DNAJB9, HSP90B1, ATF4, DNAJC3 and DDIT3 in human liver specimens.

679 **Supplementary Table 3.** Gene ontology enrichment analysis for transcripts that exhibited
680 significantly ($P < 0.05$, Pearson's) positive, or positive and negative correlated expression to
681 RASSF1 in *Mus musculus* or *Homo sapiens* genome.
682