TRIB1 supports prostate tumorigenesis and tumor-propagating cell survival by regulation of endoplasmic reticulum chaperone expression

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Abstract

Endocrine therapy is the standard treatment for advanced prostate cancer, however, relapse occurs in most patients with few treatment options available after recurrence. To overcome this therapeutic hurdle, the identification of new molecular targets is a critical issue. The capability to proliferate in three-dimensional (3D) conditions is a characteristic property of cancer cells. Therefore, factors that regulate 3D growth are considered rational targets for cancer therapy. Here, we applied a functional genomic approach to the 3D spheroid cell culture model and identified TRIB1, a member of the Trib family of serine/threonine kinase-like proteins, as an essential factor for prostate cancer cell growth and survival. RNAi-mediated silencing of TRIB1 suppressed prostate cancer cell growth selectively under the 3D conditions. This effect was rescued by ectopic expression of an RNAi-resistant TRIB1 exogene. Gene signature-based analysis revealed that TRIB1 was related to endoplasmic reticulum (ER) pathways in prostate cancer and was required for expression of the ER chaperone GRP78, which is critical for prostate tumorigenesis. Of note, GRP78 was expressed preferentially in a subpopulation of prostate cancer cells that possess tumor-propagating potential, and these tumor-propagating cells were highly sensitive to TRIB1 and GRP78 depletion. In a xenograft model of human prostate cancer, TRIB1 depletion strongly inhibited tumor formation. Supporting these observations, we documented frequent overexpression of TRIB1 in clinical specimens of prostate cancer. Overall, our results indicated that the TRIB1-ER chaperone axis drives prostate tumorigenesis and the survival of the tumor-propagating cells.
Introduction

Prostate cancer is the most common cancer in men in the western world. The number of prostate cancer patients has also been increasing in Asian countries. Androgen receptor signaling plays a central role in the proliferation of prostate cancer, and androgen ablation therapy is a standard therapy for advanced prostate cancer. In most patients, however, relapse eventually occurs, and there are few treatment options available after recurrence (1). To improve the therapeutic outcome, the identification of new therapeutic targets for drug development is critically important.

Molecular pathways required for cancer-specific growth conditions are rational targets for cancer treatment. Three-dimensional (3D) growth is one of the characteristic properties of cancer cells (2-5). Multicellular spheroid culture is a well-characterized 3D culture system that mimics the in vivo tumor state. In ovarian cancer, there was a similarity in the gene expression of cells cultured in spheroids to that of tumor xenografts (6). Additionally, spheroid formation provides the cancer cells with tissue-like features that are commonly found in prostate cancer (7). These data indicate that spheroid culture is a suitable model system to examine the survival pathways of cancer cells under conditions that are close to the intact tumor state.

A tumor often contains a subpopulation of cells, called tumor-propagating cells or cancer stem cells, which self-renew and are essentially involved in tumor progression (8). In prostate cancer, it has been reported that the tumor-propagating cells exist in the bulk tumor, and these cells could be responsible for the resistance to endocrine therapy and chemotherapy (9-12). Self-renewal ability of the tumor-propagating cells is usually assessed under spheroid (sphere) culture conditions, which evaluates infinitive growth potential under anchorage-independent conditions (13). These observations suggest that
tumor growth under 3D conditions could mainly be mediated by the highly tumorigenic subpopulation of a tumor.

The Trib family of proteins encodes serine/threonine kinase-like proteins that act as adaptor proteins in several cell signaling pathways (14). Among the Trib members, TRIB1 and TRIB2 have been identified as myeloid oncogenes (15,16). TRIB1 mediates leukemogenesis through the mitogen-activated protein kinase (MAPK)/ERK kinase1 (MEK1) and CCAAT/enhancer-binding proteins (C/EBP)-α transcription factors. However, its roles in solid tumor are still unclear.

Tumor microenvironment stresses cause the accumulation of unfolded proteins and induce the unfolded protein response (UPR) in the endoplasmic reticulum (ER) (17). To maintain cellular homeostasis, cancer cells require increased activities of the ER chaperone proteins (18). GRP78 is a major ER chaperone playing a central role in the UPR. In prostate cancer, GRP78 is upregulated and its expression correlates with tumor recurrence (19). Moreover, the functional involvement of GRP78 in prostate tumorigenesis was shown in a mouse model (20).

Here, utilizing a functional genomic approach, we identified TRIB1 as a critical factor for prostate cancer cell survival under 3D conditions. We further demonstrated that TRIB1 regulates GRP78 expression, which is critical for the maintenance of the prostate tumor-propagating cells.
Materials and Methods

Cell lines and patient-derived cells

Human prostate cancer PC3, 22Rv1 and LNCaP cells and human leukemia U937 cells were obtained and cultured as described previously (21,22) or in Supplementary Materials and Methods. The cell lines were last authenticated by short tandem repeat (STR) analysis on December 25, 2013 (PC3, 22Rv1, U937) or on April 2, 2014 (LNCaP). For spheroid culture, the cells were cultured in EZ-BindShut cell culture plates (IWAKI, Chiba, Japan) in PrEBM (Takara, Kyoto, Japan) medium supplemented with 2% B27 (Invitrogen, Carlsbad, CA), 4 μg/ml insulin (Sigma, St. Louis, MO), 20 μg/ml epidermal growth factor (EGF, Invitrogen) and 20 μg/ml basic fibroblast growth factor (bFGF, Invitrogen). To analyze the differences between the conventional two-dimensional (2D) culture and the spheroid culture, we used the spheroid culture medium in both conditions. The primary cancer samples were resected from patients after informed consent was given, and all procedures were approved by the ethics committee of the Cancer Institute Hospital.

Cell proliferation assay and soft agar assay

Cell proliferation was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). For soft agar assay, the cells were mixed with 0.4% top agar (Noble agar, BD, Tokyo, Japan) and were layered over 0.6% bottom agar, in triplicate, in 6-well plates. The cells were allowed to grow for 3 weeks, and the number of colonies was counted using the publicly available Image J software. Relative colony numbers per control sample (%) were calculated.
**Microarray analysis and data deposit**

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). Microarray analysis was done as described previously (23) with the GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA). Data analysis and Gene Ontology (GO) analysis were performed using the GeneSpring GX software (Agilent Technologies, Santa Clara, CA). The gene expression data have been deposited in the Gene Expression Omnibus (GEO) database under the accession number GSM1288491-1288495. The data will be released on December 1, 2014.

**Pooled RNAi screening**

The SBI kinome shRNA library, consisting of 10,454 shRNAs targeting 919 kinases and kinase-related proteins in pSIHI-H1-Puro lentivirus vector, was purchased from Funakoshi (Tokyo, Japan). Viruses were prepared as described in the manufacturer’s protocol. PC3 cells maintained in normal adherent culture or spheroid culture for 3 weeks were transduced with the shRNA virus library at a multiplicity of infection (MOI) of 0.5 for 12 h. At 24 h after virus infection, half of the cells were harvested as initial controls for the analysis; these samples were harvested in duplicate. The virus-transduced cells were selected with 1 μg/ml puromycin-containing medium for 3 days. After selection, the cells were further cultured for 2 weeks in conventional 2D conditions or in spheroid conditions, and the cells were then harvested for further analysis. We took quadruplicate samples for each culture condition. To estimate the amount of shRNAs that were maintained in the harvested cells, we isolated the RNA, synthesized cDNA, and amplified biotin-labeled siRNA targets as described in the manufacturer’s protocol. Purified, biotinylated siRNA targets were hybridized onto the GeneChip Human Genome
U133 Plus 2.0 Array. Analysis of the signal intensity for each siRNA target was performed using GeneNet software (SBI).

Plasmids, transfection, and viral infection

Information on the plasmids is described in Supplementary Materials and Methods. Reporter assay was done as described previously (21). In brief, cells were transfected with the firefly luciferase vector with GRP78 promoter (pGRP78pro160-Luc) and the Renilla luciferase vector phRL-CMV (Promega). Relative activity of firefly luciferase to Renilla luciferase was determined using the Dual-Glo Luciferase Assay System (Promega). Retroviruses and lentiviruses were produced, and infection was carried out as previously described (23,24). At 48 h after virus infection, the cells were treated with 1 μg/ml puromycin or with 400 μg/ml G418 for selection.

Quantitative reverse transcription PCR (qRT-PCR)

cDNAs from 39 prostate cancer tissues and 8 normal prostate tissues (Prostate tissue scan, HRPT102) were obtained from Origene Technologies Inc. (Rockville, MA). cDNA was also synthesized from total RNA, using the Superscript III first-strand synthesis Super Mix RT-for-PCR (Invitrogen). Quantitative PCR was carried out with the Power SYBR Green reagent (Applied Biosystems, Beverly, MA) and the ABI Prism 7000 system (Applied Biosystems). The primers for qRT-PCR are described in Supplementary Materials and Methods.

Western blot analysis

For the C/EBP-α, C/EBP-β and lamin A/C proteins, nuclear extracts were prepared. For
all other proteins, whole cell lysates were prepared. Cell lysates were prepared and western blot analysis was performed as previously described (21) with the following primary antibodies: rabbit anti-TRIB1 and rabbit anti-C/EBPβ (Abcam, Cambridge, UK), mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO), mouse anti-KDEL (Enzo Life Sciences, NY), rabbit anti-ERK and mouse anti-lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-ERK (Thr202/Tyr204), rabbit anti-C/EBPα, rabbit anti-Akt and rabbit anti-phospho-Akt (Ser473) (Cell Signaling Technology, Beverly, MA).

**Immunohistochemistry**

Tissue microarrays containing prostate cancer tissues and corresponding normal tissues were obtained from ISU ABXIS (Seoul, Korea). After deparaffinization and heat-induced epitope retrieval in Tris-EDTA buffer (pH 9.0) at 60°C overnight, the sections were incubated with goat polyclonal anti-TRIB1 (LifeSpan BioSciences, Inc., WA) or with mouse anti-KDEL (Enzo Life Sciences), which mainly detects GRP78 among the ER chaperones in prostate cancer, at 4°C overnight. The Polink-2 Plus HRP Goat with DAB kit (Golden Bridge International, WA) or the Envision kit (HRP labeled for anti-mouse antibody) (Dako, Denmark) was used for detection. The staining intensity was scored semiquantitatively (weak, moderate and strong).

**Immunofluorescence staining**

The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and were permeabilized with 0.03% Triton X-100 in PBS. The fixed cells were blocked in PBS containing 1% bovine serum albumin and 0.03% Triton X-100 and incubated with
the rabbit anti-TRIB1 (Thermo Scientific, Rockford, IL) and mouse anti-calnexin (BD) antibodies. These primary antibodies were detected using the Alexa 488-conjugated anti-rabbit immunoglobulin (IgG) and Alexa 594-conjugated anti-mouse IgG, respectively. The DNA was stained with 0.2 μg/ml 4’,6-diamidino-2-phenylindole (DAPI).

Cell sorting and flow cytometry

Single-cell suspensions were incubated with FITC-conjugated anti-CD151 (Santa Cruz Biotechnology), PE-conjugated anti-CD166 (BD) and Alexa 647-conjugated TRA-1-60 (BD) antibodies for 30 minutes. The cells were washed 3 times in PBS containing 25 mM HEPES (pH 7.0), 1 mM EDTA and 0.5% fetal bovine serum (FBS) and were analyzed using a FACSCalibur flow cytometer (BD). For cell sorting, cells were stained with FITC-conjugated anti-CD151 and PE-conjugated TRA1-60 antibodies (BD). Cell sorting was carried out using a FACSria cell sorter (BD).

Animal experiments

All animal procedures were performed in the animal experiment room of the Japanese Foundation for Cancer Research (JFCR) according to protocols approved by the JFCR Animal Care and Use Committee. PC3 cells were resuspended in Matrigel (BD) and Hanks' balanced salt solution (Life Technologies, Grand Island, NY) in a 1:1 ratio and were subcutaneously implanted into the flanks of 6-week-old CAnN.Cg-Foxn1nu/CrlCrlj nude mice (Charles River Laboratories Japan, Inc., Kanagawa, Japan). The length (L) and width (W) of the tumor mass were measured, and the tumor volume (TV) was calculated using the equation: TV = (L × W²)/2.
Results

Gene expression profiling and RNAi screening of prostate cancer spheroids

We first characterized multicellular spheroids derived from prostate cancer PC3 cells (Figure 1A) by gene expression profiling. Gene ontology (GO) analyses revealed that growth in sphere culture upregulated the expression of genes that reflect the solid tumor microenvironment, such as the genes involved in the response to low oxygen levels/hypoxia, the unfolded protein response (UPR)/endoplasmic reticulum (ER)-related genes, and inflammatory response genes (Supplementary Table 1A). Cell cycle-related genes were downregulated in the spheroids, indicating the suppressed growth state that is observed in solid tumors (Supplementary Table 1B). These data indicate that the prostate cancer spheroids mimic the tumor-like 3D conditions.

To identify functional survival factors in the 3D and in vivo conditions, we screened short hairpin RNAs (shRNAs) that were selectively toxic to the spheroid cells (Figure 1B). The RNAi screening identified 60 candidate genes whose knockdown resulted in a selective anti-proliferative effect in the prostate cancer spheroids but not in the conventional, 2D cultured cells (Supplementary Table 2). The genes included Rho-associated coiled-coil containing protein kinase 1 (ROCK1) and myosin light chain kinase (MLCK). These genes are involved in the 3D growth of cancer cells (25,26), indicating that our screening system worked expectedly. We further examined expression of the genes in clinical prostate cancer specimens using public gene expression databases, focusing on the genes that were overexpressed in prostate cancer. At the same time, we confirmed the spheroid-selective toxicity using 2 additional, validated shRNAs for each gene (Figure 1B). Finally, as described below, we found that TRIB1 is overexpressed in prostate cancer and is required for prostate cancer cell growth under 3D conditions.
TRIB1 overexpression in prostate cancer

As shown in Figure 2A, TRIB1 was frequently overexpressed at the mRNA level in the prostate cancer tissues. TRIB1 protein expression was also elevated in the prostate cancer samples compared to the adjacent normal tissues (Figure 2B and Supplementary Table 3). The Oncomine database analysis further confirmed that TRIB1 is overexpressed in prostate cancer compared with normal prostate tissue and other types of cancer (Supplementary Figure 1A and B). TRIB1 gene is located on chromosome 8q24, the region frequently amplified in prostate cancer (27,28). In Taylor’s genomic profiling data (29), the TRIB1 gene copy number was increased together with other genes on the chromosome 8q24 in a subset of prostate cancer specimens (Supplementary Figure 1C). cBioPortal database analysis further revealed that frequency of the TRIB1 gene amplification in prostate cancer specimens was 4-15% (Supplementary Figure 1D). The TRIB1 gene amplification correlated with the mRNA expression (Supplementary Figure 1E), while TRIB1 was also overexpressed in prostate cancer specimens without gene amplification (Supplementary Figure 1D, Taylor’s data). It was reported that the genes on chromosome 8q24 were overexpressed in PC3 cells (30). Consistently, TRIB1 protein was highly expressed in PC3 cells (Supplementary Figure 2A). In addition to PC3 cells, TRIB1 was also expressed in prostate cancer 22Rv1 and LNCaP cells but was not clearly expressed in the normal prostate epithelial PrEC cells (Supplementary Figure 2A). These data indicate that TRIB1 expression is closely related to prostate cancer.

TRIB1 supports 3D prostate cancer cell proliferation and survival

When we knocked down TRIB1 in PC3 cells (Figure 3A), a morphological change was
markedly observed (Supplementary Figure 2B) and cell proliferation was suppressed preferentially under spheroid culture conditions (Figure 3B). Ectopic expression of TRIB1 reversed the decrease of TRIB1 caused by shRNA, which targeted the 3'-untranslated region of the TRIB1 gene, and rescued spheroid growth (Figure 3C). TRIB1 overexpression itself marginally stimulated spheroid cell proliferation (Figure 3C). TRIB1 knockdown also suppressed the spheroid growth in the 22Rv1 and the LNCaP cells (Figure 3D and Supplementary Figure 3A). To evaluate the involvement of TRIB1 in anchorage-independent 3D growth, we further examined colony formation in soft agar. In PC3 cells, TRIB1 knockdown strongly reduced the colony number (Figure 3E), while TRIB1 overexpression significantly enhanced colony formation (Supplementary Figure 3B and C). These results indicate that TRIB1 enhances the 3D growth of prostate cancer cells.

TRIB1 regulates ERK phosphorylation through its carboxy-terminal MEK1-binding motif (ILLHPWF), while its carboxy-terminal DQXVP motif is required for interaction with the E3 ubiquitin ligase COP1 and C/EBPα degradation (14). Because the MEK-ERK activation and the C/EBPα degradation are critical for TRIB1-mediated leukemogenesis (14), we examined whether ERK activation or C/EBPα regulation were involved in the downstream signaling of TRIB1 in prostate cancer. Unexpectedly, TRIB1 knockdown did not affect ERK phosphorylation in PC3 cells (Supplementary Figure 4A). Similarly, TRIB1 knockdown did not alter the levels of C/EBPα or C/EBPβ (Supplementary Figure 4B). These data indicate that the ERK pathway and the C/EBP proteins could not be involved in the TRIB1-mediated proliferation and survival of prostate cancer cells.
TRIB1-dependent regulation of ER chaperone expression is critical for 3D prostate cancer cell proliferation and survival

To further address the downstream pathways of TRIB1 in prostate cancer, we analyzed the TRIB1-dependent gene expression signature. GO analysis revealed a close molecular link between TRIB1 expression and the ER-related pathways (Table 1). Among the Trib family proteins, TRIB3 is involved in the ER stress response (31), whereas the contribution of TRIB1 to the ER stress response is still unclear. We found that, in PC3 cells, TRIB1 was partially localized in the ER, as TRIB1 co-stained with calnexin, an ER marker (Supplementary Figure 5A). Moreover, TRIB1 knockdown strongly reduced the mRNA expression levels of multiple ER chaperones and related genes (Figure 4A). These data indicated that TRIB1 regulates the ER chaperone expressions in prostate cancer cells.

GRP78 is a central ER chaperone and is involved in the ER stress response and survival under the tumor microenvironment (17,18). In PC3 cells, GRP78 was predominantly expressed compared to GRP94 and other ER chaperones (Figure 4B, left). Moreover, GRP78 expression was markedly elevated in the spheroid cells (Figure 4B, right) and GRP78 knockdown efficiently suppressed spheroid growth (Supplementary Figure 5B and C). These data indicate that GRP78 is critical for survival particularly under the 3D conditions, where the unfolded protein response is highly induced (Supplementary Table 1A). In prostate cancer cells, TRIB1 knockdown strongly attenuated the GRP78 upregulation under the spheroid culture conditions (Figure 4C, D) and overexpression of an shRNA-resistant TRIB1 rescued the GRP78 downregulation caused by the TRIB1 knockdown (Supplementary Figure 5D). These results indicate that TRIB1 regulates the GRP78-mediated survival pathways particularly under the 3D
conditions. To clarify the regulation of GRP78 expression by TRIB1, we examined the GRP78 promoter activity. The GRP78 promoter activity was increased under the 3D spheroid conditions and TRIB1 knockdown suppressed the activity (Figure 4E). Moreover, in prostate cancer tissues, the ER chaperone expression roughly correlated with the TRIB1 expression (Supplementary Table 4). Within a prostate cancer tissue, we observed heterogeneity in TRIB1 expression and the cells with higher TRIB1 level tended to express higher ER chaperone proteins (Supplementary Figure 6).

GRP78 regulates Akt activation, which is essential for prostate tumorigenesis and survival under the spheroid conditions or in the ER stress-induced cells (13,20,32). Consistent with the previous reports, GRP78 knockdown suppressed the Akt phosphorylation (Figure 4F), indicating that the Akt activation is dependent on GRP78. TRIB1 depletion attenuated the Akt phosphorylation particularly under the 3D conditions (Figure 4F). These data suggest that TRIB1 could support 3D cell survival by modulating the GRP78-Akt axis.

Prostate tumor-propagating cells show elevated GRP78 expression and depend on the TRIB1-GRP78 axis

In cancer cell growth under 3D conditions, a subpopulation of cells, known as tumor-propagating cells or cancer stem cells, plays a critical role. Previous studies indicated that prostate cancer cells contain tumor propagating-cells (9-13), which are triple positive for CD151, TRA1-60, and CD166 (11). As previously reported, PC3 cells contained a small subpopulation of these triple positive cells (0.92%; Figure 5A). To confirm the tumorigenic potential of the cells, we sorted the marker-positive and -negative cells. In our PC3 cells, as well as in the patient-derived primary prostate cancer
cells, we found that nearly all of the cells were CD166-positive (Figure 5A and data not shown). Therefore, we sorted the CD151+/TRA1-60+ and the CD151-/TRA1-60- cells for further evaluation. When we subcutaneously transplanted the marker-positive and -negative cells into immunodeficient mice, we observed the preferential tumor formation of the marker-positive cells (Figure 5B). Moreover, the marker-positive cells grew better under spheroid conditions than the marker-negative cells (Figure 5C). These data indicate that prostate cancer cells contain a highly tumorigenic subpopulation. Of note, the CD151+/TRA1-60+ cells also existed in the patient-derived, primary cultured prostate cancer cells (Supplementary Figure 7).

We found that GRP78 was preferentially expressed in the marker-positive subpopulation (Figure 5D). To determine whether the TRIB1-GRP78 axis is involved in the maintenance of the prostate tumor-propagating cells, we compared the sensitivity of the two populations to TRIB1 or GRP78 inhibition. As shown in Figure 5C, under spheroid culture conditions, both TRIB1 and GRP78 knockdown significantly suppressed the cell proliferation of the marker-positive and the marker-negative cells. This effect was more prominent in the marker-positive tumor-propagating cells. These results indicated that the tumor-propagating subpopulation depends on the TRIB1-ER chaperone pathway.

**TRIB1 is required for prostate tumorigenesis**

In order to evaluate the functional significance of TRIB1 during tumor formation in vivo, we utilized a tumor xenograft model. When we overexpressed TRIB1 in PC3 cells, a significant enhancement of in vivo tumor formation was observed (Figure 6A). Conversely, TRIB1 knockdown in the PC3 cells severely suppressed their tumorigenicity (Figure 6B, C). These observations indicate that TRIB1 may be a critical factor for tumor
propagation in vivo.
Discussion

Regulation of ER chaperone expression and 3D cancer cell survival by TRIB1 in prostate cancer

In this study, we showed that TRIB1 regulates the ER chaperone expression, which is essential for prostate cancer cell survival. TRIB1 particularly modulated the GRP78-Akt axis. On the other hand, the action of GRP78 and TRIB1 was not completely equal, since TRIB1 knockdown was less toxic under the 2D conditions, whereas GRP78 knockdown showed growth inhibition even under the 2D conditions. TRIB1 regulated not only GRP78 expression but also various other ER chaperone expressions (Figure 4A). Considering these data, GRP78 would explain a part of the TRIB1 action but the suppression of other ER chaperone expressions could also be critical for the 3D-selective action of TRIB1 depletion.

TRIB1 mediates leukemogenesis through MEK-ERK pathway and C/EBP regulation (14-16). In contrast, TRIB1 depletion did not affect ERK phosphorylation or C/EBP protein levels in the prostate cancer cells (Supplementary Figure 4). These observations suggest that the mechanism of TRIB1-dependent malignant transformation could be different in leukemia and prostate cancer.

GRP78 expression is frequently elevated in prostate cancer (19). Our results indicated that TRIB1 is a regulator of GRP78 expression in prostate cancer. In the mammalian ER, three ER associated proteins act as ER stress sensors: inositol-requiring transmembrane kinase 1 (IRE1), double-stranded RNA-activated protein kinase-like eukaryotic initiation factor 2α kinase (PERK), and activating transcription factor-6 (ATF6) (17). These proteins are responsible for the expression of the ER chaperones, particularly upon accumulation of unfolded proteins in the cells. Because TRIB1 was critical for the
expression of multiple ER chaperones, TRIB1 might directly regulate these ER stress sensor proteins. Among the Trib family members, TRIB3 is involved in the ER stress response downstream of ATF4 and C/EBP homologous protein (CHOP) (31). However, in contrast with TRIB1 and TRIB2, TRIB3 is not a leukemogenic factor (16). Thus, it would be important to clarify the differences in the roles of the Trib family members in the regulation of the ER stress pathway and their effects on tumorigenic activity.

**GRP78 and TRIB1 maintain the prostate tumor-propagating cells**

We demonstrated that GRP78 and its regulator, TRIB1, were important for the survival of the prostate tumor-propagating cells. Loss of the tumor suppressor PTEN and concomitant Akt activation are critical for prostate tumor progression (33,34). It was reported that Akt was preferentially activated in prostate tumor-propagating cells (13). Moreover, GRP78 promotes tumorigenesis through Akt activation in PTEN-deficient prostate cells (20). From these observations, we speculate that TRIB1 may promote the survival of the tumor-propagating cells through GRP78-dependent Akt activation.

GO analysis revealed that TRIB1 knockdown modulated the expression of genes related to anatomical structure morphogenesis and the developmental process (Table 1). We also observed a dramatic morphological change in the TRIB1-depleted cells (Supplementary Figure 2B). These observations suggest that TRIB1 could maintain the prostate cancer cells in an immature state, and TRIB1 loss could trigger a type of differentiation. Further studies will clarify the mechanisms of how TRIB1 and GRP78 mediate the maintenance of the prostate tumor-propagating cells.

**TRIB1 and GRP78 as therapeutic targets of prostate cancer**
TRIB1 gene is amplified in a subset of prostate cancer specimens (Supplementary Figure 1). Moreover, TRIB1 is involved in prostate cancer cell survival. Recently, it was reported that the TRIB1 expression was also regulated by a microRNA (miR-224) in prostate cancer and the TRIB1 upregulation was significantly associated with poor biochemical recurrence-free survival of patients with prostate cancer (35). These data suggest that TRIB1 could be a rational therapeutic target in prostate cancer. When considering TRIB1 as a therapeutic target, the identification of its physiological roles is important to estimate the potential side effects of TRIB1 inhibition in the human body. TRIB1 has been reported to play roles in some physiological processes, such as the differentiation of tissue-resident M2-like macrophages and Toll-like receptor-mediated gene expression (36,37). Further studies will clarify the roles of TRIB1 in normal human tissues.

We showed that the TRIB1-ER chaperone axis is a critical survival pathway for prostate tumor-propagating cells. Recently, several compounds that interfere with GRP78 or the related ER stress response pathways were developed, and some of these compounds are now in clinical trials (38-40). Of note, one of these agents, metformin, was recently shown to be an effective suppressor of tumor-propagating or cancer stem cells (41,42). It was also reported that GRP78 is present on the cell surface of prostate cancer cells, but not on normal cells (43). These findings suggest that GRP78 on cell surface could be a potential target of the prostate tumor-propagating cells. Additionally, it is important to search for druggable TRIB1 regulators in prostate cancer cells.
Authors' Contributions

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GeneChip analysis was performed on PC3 cells infected with lentivirus expressing control shRNA (shGFP) or shRNAs to TRIB1 (#1 or #2). We applied the arbitrary cutoffs of >1.5-fold up- or down-regulation or downregulation and extracted a gene set that were commonly upregulated (484 probe sets) or downregulated (309 probe sets) in the TRIB1 shRNA-expressed cells as compared with control shRNA-expressed cells. GO analysis was further done on the signature gene set (793 probe sets). The GO terms whose P-values were less than 0.05 were indicated. Bold letters indicate the endoplasmic reticulum-related categories.
Figure Legends

Figure 1. Functional genomic screening for the identification of prostate cancer survival factors under 3D conditions.
A, Morphologies of PC3 cells under normal (2D) and spheroid (3D) culture conditions.
B, Functional genomic strategy to identify candidate survival factors of prostate cancer cells under 3D conditions.

Figure 2. Elevated expression of TRIB1 in clinical prostate cancer tissues.
A, TRIB1 expression was examined by qRT-PCR. GAPDH expression was examined as a loading control.
B, TRIB1 expression in prostate cancer and adjacent normal prostate tissues was examined by immunohistochemistry.

Figure 3. Functional involvement of TRIB1 in prostate cancer cell proliferation and survival under 3D conditions.
A, PC3 cells were infected with lentiviruses expressing control shRNA (shGFP) or TRIB1 shRNAs (#1 or #2). TRIB1 protein levels were evaluated using western blot analysis.
B and D, PC3, 22Rv1 and LNCaP cells were infected with lentiviruses expressing the indicated shRNAs. The cells were seeded in normal conditions (2D) or in spheroid conditions (spheroid) and were cultured for 6 days. Cell proliferation was evaluated as described in Materials and Methods.
C, PC3 cells transduced with control virus (PC3/Mock) or with TRIB1 retrovirus (PC3/TRIB1) were infected with lentiviruses expressing control shRNA (shGFP) or
shRNA targeting the 3’ untranslated region (3’UTR) of the TRIB1 gene (#3). TRIB1 expression was examined by western blot (left panel). Cell proliferation was evaluated as in the right panel.
E, PC3 cells were infected with viruses as in A. Anchorage-independent growth was evaluated using a soft agar assay.
Error bars show standard deviations. Statistical evaluations were performed using Student’s t-test. **, $P < 0.01$; *, $P < 0.05$.

**Figure 4.** TRIB1 regulates GRP78 expression, which is critical for prostate cancer 3D survival.

A, Total RNA was prepared from PC3 cells infected with lentiviruses expressing control shRNA (shGFP) or shRNAs (#1 or #2) against TRIB1. The expression levels of the ER chaperones and related proteins were analyzed using qRT-PCR. β-actin expression was also analyzed as a control.
B-D, The expression levels of TRIB1 and the ER chaperones were examined in PC3, 22Rv1 and LNCaP cells infected with lentiviruses expressing control shRNA (shGFP) or TRIB1 shRNAs and cultured in normal conditions (2D) or in spheroid conditions (spheroid) for 6 days.
E, PC3 cells cultured in normal conditions (2D) or in spheroid conditions (spheroid) for 6 days were transiently transfected with the reporter plasmids, and the GRP78 promoter activity was determined.
F, PC3 cells were infected with lentiviruses expressing control shRNA (shGFP), TRIB1 shRNAs (#1), or GRP78 shRNAs (#1). After infection, the cells were cultured in normal conditions (2D) or in spheroid conditions (spheroid) for 6 days, and Akt phosphorylation
was determined by western blot analysis. Akt and actin expressions were also analyzed. Error bars show standard deviations. Statistical evaluations were performed using Student’s t-test. **, P <0.01; *, P <0.05.

**Figure 5. The TRIB1-GRP78 axis plays a preferential role in the maintenance of the prostate tumor-propagating cells.**

A, Flow cytometry analysis of the CD166⁺ or the CD151⁺/TRA1-60⁺ subpopulations of PC3 cells.

B-D, CD151⁺/TRA1-60⁺ cells, as well as CD151⁺/TRA1-60⁻ cells, were sorted from PC3 cells. In B, 500 cells from each sample were subcutaneously implanted into nude mice. Forty days after implantation, the length (L) and width (W) of the tumor mass were measured, and the tumor volume (TV) was calculated using the equation: TV = (L × W²)/2. In C, GRP78 expression was examined using an anti-KDEL antibody. In C, the sorted cells were infected with lentiviruses expressing control shRNA (shGFP), TRIB1 shRNAs (#1), or GRP78 shRNAs (#1). After infection, the cells were seeded in spheroid culture conditions and were cultured for 4 days. Cell proliferation was evaluated as described in Materials and Methods. Error bars show standard deviations. Statistical evaluations were performed using Student’s t-test. *, P <0.05.

**Figure 6. TRIB1 inhibition represses prostate tumor growth in vivo.**

A, PC3 cells were infected with control retrovirus (Mock) or virus expressing TRIB1 (TRIB1). Two thousand cells for each sample were subcutaneously implanted into nude mice. Thirty days after implantation, the length (L) and width (W) of the tumor mass were measured, and the tumor volume (TV) was calculated using the equation: TV = (L ×
B and C, PC3 cells were infected with lentiviruses expressing control shRNA (shGFP) or TRIB1 shRNAs (#1 or #2). One thousand cells for each sample were subcutaneously implanted into nude mice. Sixty days after implantation, the tumor sizes were measured as in A. Photographs of the tumors are shown in C.

Statistical evaluations were performed using Student’s \( t \)-test. *, \( P < 0.05 \).
A

Adherent culture (2D conditions)  Spheroid culture (3D conditions)

B

RNAi screening on prostate cancer 3D model (Selectively toxic to the PC3 spheroid cells)

Elevated expression in clinical prostate cancer

Validation of selective toxicity to the spheroid cells using additional 2 independent shRNAs (PC3, 22Rv1 and LNCaP cells)

Prostate cancer-selective survival factor in 3D conditions (Identification of TRIB1)
Relative TRIB1 mRNA level (GAPDH mRNA level)

A

Normal Prostate

Prostate Cancer

B

Normal Prostate

Prostate Cancer
Mashima et al. Figure 3

A

PC3

<table>
<thead>
<tr>
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<tr>
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B

PC3

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C

PC3/Mock  PC3/TRIB1

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D

22Rv1  LNCaP

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E

Colony Number (%)

Soft agar growth (PC3)
**A**

Tumor volume (L x W x W/2, mm$^3$)

Mock (N=6)  TRIB1 (N=6)

**B**

Tumor volume (L x W x W/2, mm$^3$)

shGFP (N=12)  shTRIB1-#1 (N=6)  shTRIB1-#2 (N=6)

**C**

shGFP (7/12)  
shTRIB1-#1 (0/6)  
shTRIB1-#2 (0/6)

Mashima et al. Figure 6
TRIB1 supports prostate tumorigenesis and tumor-propagating cell survival by regulation of endoplasmic reticulum chaperone expression

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