TRAF6 Activates Fibroblasts to Cancer-Associated Fibroblasts through FGF19 in Tumor Microenvironment to Benefit the Malignant Phenotype of Melanoma Cells

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Cancer-associated fibroblasts (CAFs) are an important component of the tumor microenvironment and mediate tumor progression in various cancers. A previous study demonstrated that TRAF6 promotes the malignant phenotype of melanoma cells. However, the role of TRAF6 in melanoma CAFs remains unclear. In this study, we found that TRAF6 was significantly upregulated in CAFs adjacent to melanoma cells. Functional assays showed that TRAF6 promoted fibroblast proliferation and migration as well as MMP and α-SMA expression. Moreover, the expression of TRAF6 in fibroblasts promoted the malignant phenotype of melanoma cells in vitro and in vivo. Meanwhile, the intervention of TRAF6 expression in melanoma cells affected the activation of CAFs. We found that FGF19 was a key cytokine regulated by TRAF6 through NF-κB1 using luciferase assay and chromatin immunoprecipitation in melanoma cells. Because plasma FGF19 levels are elevated in patients with melanoma, it may significantly induce fibroblast activation in vitro and in vivo. Taken together, our results support that TRAF6 is a key molecule that mediates the interaction between melanoma cells and stromal fibroblasts, suggesting that TRAF6 is a potentially promising target in melanoma therapy.

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INTRODUCTION

Although significant advances have been made in elucidating the pathogenesis of cancers, cancer recurrence, metastasis, and the tumor microenvironment (TME) are three obstacles that remain unsolved for effective treatment in clinical practice (Kalluri, 2016). The TME is composed of immune cells, activated fibroblasts, and extracellular matrix (ECM) components (Rønnov-Jessen et al., 1996). Activated fibroblasts, also named cancer-associated fibroblasts (CAFs), are a key component of the TME (Ostman and Augsten, 2009). CAFs mainly originate from resident fibroblasts but can also originate from bone marrow–derived mesenchymal cells through epithelial-mesenchymal transition (Nwani et al., 2016). Accumulating evidence has shown that crosstalk occurs between CAFs and cancer cells. Fibroblasts have been cocultured with cancer cells, leading to the acquisition of malignant features that enable cell survival in the TME and promote cancer progression (Öhlund et al., 2014; Yan et al., 2019). CAFs exhibit aggressive characteristics, including enhanced proliferation and migration, and secrete GFs such as basic fibroblast GF(FGF), epidermal GF, and HGF (Hanahan and Coussens, 2012; Saadi et al., 2010). More importantly, CAFs produce matrix-remodeling enzymes such as matrix metalloproteinases (MMPs) (MMP-1, -2, -9, etc.), which degrade the ECM and promote the invasion and metastasis of tumor cells (Moro et al., 2014). In addition, tumor cells or specific stimuli in the TME, such as hypoxia, oxidative stress, and GFs, facilitate the reprogramming of normal fibroblasts to CAFs. For example, GFs secreted by tumor cells, such as FGF, TGFβ, and PDGF, bind to surface receptors on fibroblasts, which activates fibroblasts to become CAFs with enhanced secretion, proliferation, and migration, and secrete GFs such as basic fibroblast GF(FGF), epidermal GF, and HGF (Hanahan and Coussens, 2012; Saadi et al., 2010). More importantly, CAFs produce matrix-remodeling enzymes such as matrix metalloproteinases (MMPs) (MMP-1, -2, -9, etc.), which degrade the ECM and promote the invasion and metastasis of tumor cells (Moro et al., 2014). In addition, tumor cells or specific stimuli in the TME, such as hypoxia, oxidative stress, and GFs, facilitate the reprogramming of normal fibroblasts to CAFs. For example, GFs secreted by tumor cells, such as FGF, TGFβ, and PDGF, bind to surface receptors on fibroblasts, which activates fibroblasts to become CAFs with enhanced secretion, proliferation, invasiveness, and metastasis (Chen and Song, 2019; Long et al., 2019).

Melanoma originates from melanocytes and is one of the most lethal cancers (Gansler et al., 2010). Genetic factors and environmental stimulants contribute to the development of melanoma. Melanoma cells have been documented to affect their microenvironment through direct interactions...
with stromal cells or the ECM, and by secreting GFs and cytokines. Melanoma cells establish a specific microenvironment that benefits tumor-cell migration, invasion, and survival (Fischer et al., 2018).

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a member of the TNF receptor–associated factor family. Structurally, the N-terminus of TRAF6 contains a RING domain with E3 ligase activity, which ubiquitinates target proteins. The C-terminus contains a TRAF domain, which mediates interactions with other proteins. Evidence has shown that TRAF6 plays important roles in immunity, inflammation, bone metabolism, and development (Walsh et al., 2015). In addition, recent studies showed that TRAF6 is an oncogene and found that the membrane localization and oncogenic activation of protein kinase B require the E3 ligase activity of TRAF6 for protein kinase B K63–linked ubiquitination (Starczynowski et al., 2011; Zhang et al., 2018). Our previous study demonstrated that TRAF6 is highly expressed in melanoma and promotes the proliferation, invasion, and metastasis of melanoma cells by regulating the expression of MMP-9 (Luo et al., 2014). We discovered that TRAF6 is overexpressed in the fibroblasts of melanoma. However, the role of TRAF6 in CAFs and melanoma TME remains unknown.

In this study, we showed that overexpression of TRAF6 significantly promoted fibroblast proliferation and migration as well as MMP and α-SMA expression. Activated fibroblasts induced by TRAF6 overexpression enhanced melanoma cell migration, invasion, and metastasis. Furthermore, knockdown of TRAF6 in melanoma cells inhibited FGF19 expression, which is a key GF for CAF activation, suggesting that TRAF6 is a key molecule regulating crosstalk between melanoma cells and CAFs.

RESULTS

TRAF6 promotes fibroblast activation

Our previous study showed that TRAF6 was highly expressed in melanoma tissues. We noticed that TRAF6 was highly expressed in the area rich in CAFs. To confirm the finding, we studied the localization of TRAF6 in melanoma tissues by immunofluorescence staining. We used α-SMA as a marker of CAFs and S100A4 as a control for stromal cells. As expected, TRAF6 was highly expressed in CAFs, which are α-SMA-positive cells. The average fluorescence intensity of TRAF6 was related to the expression of α-SMA (Figure 1a). To investigate the role of TRAF6 in fibroblasts, we isolated the foreskin fibroblasts from healthy donors and found that ectopic TRAF6 expression significantly promoted fibroblast proliferation (Figure 1b) and migration (Figure 1c–e). Fibroblast activation is closely linked with the expression of MMPs such as MMP-1, -2, and -9. Therefore, we examined the expression of MMPs and other CAFs biomarkers in TRAF6-overexpressing fibroblast cells. As shown in Figure 1f, overexpression of TRAF6 increased the expression of MMP-2, MMP-9, vimentin, PD transcript, desmin, and α-SMA, supporting that TRAF6 may activate fibroblasts. Moreover, we found that overexpression of TRAF6 had similar effects in BJ cells (Supplementary Figure S1). To validate the role of TRAF6 in fibroblasts activation, we generated BJ cells with stable knockdown of TRAF6 using two independent targeting sequences. As expected, inhibition of TRAF6 expression dramatically attenuated BJ cell proliferation (Supplementary Figure S2a), migration, and invasion (Supplementary Figure S2b–d). Furthermore, knockdown of TRAF6 decreased the expression of CAF biomarkers such as MMPs in BJ cells (Supplementary Figure S2e).

Conditioned medium derived from TRAF6-deficient and/or -overexpressing fibroblasts regulates the malignant phenotype of melanoma cells

Given that CAFs are key components of the TME, we hypothesized that conditioned medium (CM) derived from fibroblasts with TRAF6 intervention might influence the biological functions of melanoma cells. Compared with CM from control, CM from TRAF6-overexpressing BJ cells significantly enhanced melanoma cell growth (Figure 2a). Next, scratch assays and transwell assays were performed to examine the effect of CM on melanoma cell migration and invasion. The migration and invasion abilities of melanoma cells treated with CM derived from TRAF6-overexpressing BJ cells were significantly increased compared with those of melanoma cells treated with CM from control BJ cells (Figure 2b and c) (Supplementary Figure S3a). To validate the effect of fibroblast TRAF6 expression on melanoma cells, we collected CM from BJ cells with TRAF6 knockdown to culture melanoma cells with the CM. Consistent with the previous results, CM from BJ cells with TRAF6 knockdown significantly reduced melanoma cell growth, migration, and invasion (Supplementary Figure S4).

Moreover, we generated a xenograft mouse model by mixing BJ cells and melanoma cells (1.5:1) to study the effect of TRAF6 on melanoma cell growth in vivo. Similar to the in vitro results, overexpression of TRAF6 in BJ cells significantly promoted the growth of xenografted melanoma cells in nude mice (Figure 2d) (Supplementary Figure S3b). We examined Ki67 expression in paraffin-embedded mouse tumor tissue. As shown in Supplementary Figure S3c, the Ki67-positive rate was significantly increased in the ectopic TRAF6 expression groups. As expected, knockdown of TRAF6 in BJ cells significantly inhibited the growth of the xenografted melanoma mouse model (Supplementary Figure S5), supporting that expression levels of TRAF6 in fibroblasts regulate melanoma cell growth in vitro and in vivo.

Expression of TRAF6 in melanoma cells plays a critical role in fibroblast activation

Normal fibroblasts maintain microenvironment homeostasis by inhibiting the proliferation of adjacent cells (Kim et al., 2013), whereas CAFs increase tumor-cell growth and invasion by inducing growth factor expression, angiogenesis, and ECM remodeling (Komohara and Takeya, 2017). Evidence has demonstrated that tumor cells produce a series of growth factors or cytokines to activate fibroblasts and generate CAFs (Sharon et al., 2015). Thus, we investigated whether TRAF6 intervention in melanoma cells could regulate fibroblast activation. BJ cells were treated with CM derived from melanoma cells expressing short hairpin (sh)-TRAF6 or sh-scrambled (Figure 3a). Cell viability was significantly reduced after treatment with CM from TRAF6-knockdown melanoma cells (Figure 3b). Consistent with this finding, CM from TRAF6-knockdown melanoma cells attenuated fibroblast migration compared with that of
Figure 1. TRAF6 is highly expressed in melanoma CAFs and promotes fibroblast growth and migration in vitro. (a) TRAF6 is overexpressed in melanoma tissues. The expression of TRAF6 and α-SMA in clinical samples were tested by immunofluorescence and in conjunction with tyramide signal amplification as described in Supplementary Materials and Methods. The representative images of α-SMA (orange), TRAF6 (purple), S100A4 (green), and DAPI (blue) in melanoma tissues are marked in white arrow. Bar = 100 μm. The correlation of average fluorescence intensity between α-SMA and TRAF6 are shown in the lower right corner. (b) Overexpression of TRAF6 promotes primary fibroblast growth in vitro. TRAF6-overexpressing fibroblasts showed an increased growth rate. Stable overexpression of TRAF6 in primary human foreskin fibroblast cells was generated by lentiviral infection. Cells were seeded into 96-well plates, and cell viability was examined by Cell Counting Kit-8 as described in Supplementary Materials and Methods. Data from multiple experiments are expressed as mean ± SD. Significant differences were evaluated using two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (c–e) Overexpression of TRAF6 promotes fibroblast migration and invasion. The migration assay was performed as described in Supplementary Materials and Methods. Representative images were taken.
fibroblasts cultured with control CM (Figure 3c). Moreover, the number of invaded cells was significantly decreased in the group treated with CM from TRAF6-knockdown melanoma cells (Figure 3d). As expected, biomarkers of fibroblast activation, including MMP-9, MMP-2, vimentin, PDPN, desmin, and α-SMA, were significantly down-regulated in fibroblasts cultured with CM derived from TRAF6-knockdown melanoma cells (Figure 3e), suggesting that the expression levels of TRAF6 in melanoma cells influence fibroblast activation.

Effect of TRAF6 on the gene expression profile in melanoma cells
To investigate the effects of TRAF6 on melanoma cell gene expression profiles, we analyzed global transcriptome alterations in melanoma cells after the suppression of TRAF6 expression. The RNA-sequencing results showed 2,075 genes overlapping between cells transfected with sh-TRAF6#1 and sh-TRAF6#4 (Figure 4a). Then, we examined the differentially expressed genes by Kyoto Encyclopedia of Genes and Genomes analysis and found that the differentially expressed genes are involved in multiple pathways, such as the MAPK and phosphatidylinositol 3-kinase-protein kinase B pathways (Figure 4b), and protein-protein interaction network with growth factors, including FGF13, nerve growth factor, and FGF19 (Figure 4c). Quantitative real-time reverse transcriptase-PCR was performed to verify differentially expressed key genes (Figure 4d) (Supplementary Figure S6 and S7), which showed that the expression of FGF13 and FGF19 decreased 2- to 3-fold when TRAF6 was knocked down in Sk-Mel-5 and Sk-Mel-28 cells. There are six subfamilies of FGFs: five paracrine subfamilies and one endocrine subfamily (Belov and Mohammadi, 2013). FGF19 belongs to the FGF19 subfamily (FGF19, FGF21, and FGF23), which is an endocrine subfamily that acts through endocrine secretion to drive interorgan crosstalk (Fukumoto, 2008). In addition, FGF19 has been found to be overexpressed in a subgroup of hepatocellular carcinoma, breast cancer, prostate cancer, and lung squamous carcinomas (Chen et al., 2020; Nagamatsu et al., 2015; Zhang et al., 2017; Zhao et al., 2018), whereas FGF13 is typically not classified into any of the six FGF subfamilies, and it is not a secreted factor (Turner and Grose, 2010). Therefore, we focused on the study of the FGF19 function.

TRAF6 enhances the activation of fibroblasts through FGF19
Our previous results demonstrated that knockdown of TRAF6 inhibited FGF19 expression. Consistent with the previous finding, the production of FGF19 in the medium was decreased after inhibition of TRAF6 in melanoma cells (Figure 5a). To study the TRAF6 regulation of FGF19 expression at the mRNA level, we analyzed the FGF19 promoter and generated the pGL3-FGF19-luc plasmid as shown in the upper panel of Figure 5b. Luciferase reporter assays showed that suppression of TRAF6 expression significantly reduced the FGF19 reporter luciferase activity (Figure 5b, lower panel). Then, we analyzed the FGF19 promoter in PROMO (http://alggen.lsi.upc.es/) and identified several NF-κb1 recognition sequences (Figure 5d, upper panel). Consistent with previous results (Huang et al., 2015; Min et al., 2018), ectopic TRAF6 significantly increased NF-κB1 luciferase activity (Figure 5c, upper panel), whereas knockdown of TRAF6 significantly suppressed the nuclear accumulation of NF-κB1 (Figure 5c, lower panel). To further validate that TRAF6 regulates FGF19 expression through NF-κB1, chromatin immunoprecipitation assays were performed to examine the effect of TRAF6 on NF-κB1 binding to the FGF19 promoter. As shown in Figure 5d, knockdown of TRAF6 reduced the association of NF-κB1 with the FGF19 promoter in melanoma cells.

To study the role of FGF19 in melanoma, we tested the production of FGF19 in the plasma of patients with melanoma by ELISA, which showed that FGF19 was significantly elevated in patients with melanoma compared with age- and gender-matched healthy controls (Figure 6a). The clinical characteristics are included in Supplementary Materials and Methods. In addition, we found that the concentration of FGF19 varied from 10 to 80 pg/ml in patients with melanoma. Meanwhile, age (P = 0.543), gender (P = 0.287), and presence of metastasis (P = 0.292) had no significant effect on FGF19 production in patients with melanoma. To study the effect of FGF19 on fibroblasts, we treated BJ cells with FGF19 at 25 pg/ml and 50 pg/ml, which are similar to the concentration measured in the medium from melanoma cells. FGF19 increased cell viability in a dose-dependent manner (Supplementary Figure S8a). Moreover, FGF19 promoted BJ cell migration and invasion (Supplementary Figure S8b and c). As a consequence, biomarkers of CAFs were significantly upregulated after FGF19 treatment (Figure S8d). However, FGF19 could not affect TRAF6 expression (Supplementary Figure S8d). To validate the effects of FGF19 on cell growth in vivo, a melanoma xenograft mouse model was generated by mixing vehicle or FGF19-pretreated BJ cells with melanoma cells (1:5:1). Consistent with the in vitro data, FGF19 treatment significantly increased melanoma cell growth in nude mice (Figure 6b–d), supporting that FGF19 is a key factor for fibroblast activation.

DISCUSSION
Tumor progression depends not only on cancer cells but also on complex TME comprising multiple cell populations that
Figure 2. CM derived from TRAF6-overexpressing fibroblasts regulates the proliferation, migration, and invasion of melanoma cells. (a) CM derived from TRAF6-overexpressing BJ cells promotes the growth of melanoma cells. SK-Mel-5 (left panel) and SK-Mel-28 (right panel) cells were treated with CM derived from BJ cells as described in Supplementary Materials and Methods, and cell viability was tested by CCK-8 assays as described in the Supplementary Materials and Methods. Data from multiple experiments are expressed as the mean ± SD. Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (b, c) CM derived from TRAF6-overexpressing BJ cells promotes melanoma cell migration and invasion. The scratch assay was performed as described in Supplementary Materials and Methods. The bar chart graphs of SK-Mel-5 (left panel) and SK-Mel-28 (right panel) shown are from three independent experiments. **P < 0.01, ***P < 0.001. (d) CM derived from TRAF6-overexpressing BJ cells promotes melanoma cell invasion. The bar chart graph of tumor volume shown are from three independent experiments. **P < 0.01, ***P < 0.001.
form architectural and functional interdependencies (Hui and Chen, 2015). Recent studies have highlighted the significance of CAFs in cancer development, especially in epithelial-derived cancers such as breast, gastric, lung, ovarian, and prostate carcinoma (Olumi et al., 1999; Orimo et al., 2005; Vicent et al., 2012; Yang et al., 2006).

CAFs have been documented to mediate the invasion, metastasis, and therapeutic resistance of melanoma. CAFs secrete a Wnt antagonist, frizzled-related protein 2, which activates a multistep signaling cascade in melanoma cells involving in angiogenesis, metastasis, and chemotherapeutic resistance in melanoma (Kaur et al., 2016). Growth factors such as nerve growth factor 1 activate extracellular signal–regulated kinase 1 and 2 or induce survival signals associated with the ECM in melanoma cells, which in turn results in the progression of melanoma (Hirata et al., 2015). Melanoma cells produce tumor-cell–derived factors such as extracellular MMP inducers, which activate fibroblasts to favor tumor-cell invasion or survival in the TME (van den Oord et al., 1997). In primary melanoma and lymph node metastatic melanoma, MMPs (such as MMP-2 and MMP-9) are mainly located in melanoma cells adjacent to the ECM or in connective tissue compartments where melanoma cells have accumulated (Kurschat et al., 2002).

We found that ectopic TRAF6 expression significantly enhanced fibroblast activation. Moreover, melanoma malignant phenotype was affected by TRAF6 expression in fibroblasts, suggesting that CAFs may influence the progression of melanoma through TRAF6. Although the epithelial-mesenchymal transition has a critical role in tumor progression, CM derived from the intervention of TRAF6 expression fibroblasts did not influence epithelial-mesenchymal transition–related biomarkers expression, including E-cadherin, N-cadherin, and vimentin (data not shown), suggesting that TRAF6 in fibroblast cells may affect the biological behavior of melanoma cells through epithelial-mesenchymal transition–independent manner.

Growth factors or cytokines were well-known to involve in the activation of CAFs. For example, TGF-β enhanced the aggressiveness of ovarian cancer cells by activating CAFs (Yeung et al., 2013). BRAFV600E melanoma cells secreted high levels of cytokines such as IL-1β, IL-6, and IL-8, which facilitated the activation of stromal fibroblasts (Whipple and Brinckerhoff, 2014). Through RNA sequencing, we found that growth factors or cytokines such as FGF19, CX3CL1, and transferrin were downregulated in TRAF6 knockdown melanoma cells. CX3CL1 exerted both protumor and antitumor effects (Guo et al., 2003; Marchesi et al., 2008; Sciumé et al., 2010; Zeng et al., 2005). Therefore, the therapeutic role of CX3CL1 in clinical treatment for tumors was controversial. Meanwhile, transferrin, which transports iron to all proliferating cells in the body, has also been shown to exert both protumor or antitumor effects (Beutler et al., 2000). Iron-bound transferrin inhibits tumor-cell growth in ovarian cancer cells and hepatocellular carcinoma (Fassl et al., 2003; Lesnikov et al., 2004, 2001), whereas it induces apoptosis in prostate cancer cells through association with IGFBP-3 (Weinzimer et al., 2001).

Next, we validated that the abundance of FGF19 was highly elevated in the plasma of patients with melanoma and following FGF19 treatment, promoted the activation of fibroblasts, indicating that FGF19 plays a critical role in CAF activation in melanoma. FGF19 is a member of the hormone-like FGF family, and its expression in CAFs has been positively correlated with the clinical relevance of hepatocellular carcinoma (Zou et al., 2018). FGF19 is also overexpressed in various solid tumors such as hepatocellular carcinoma and head and neck squamous cell carcinoma and is associated with FGFR4-mediated activation of downstream signaling pathways such as the GSK3β-Nrf2 and MAPK/ extracellular signal–regulated kinase 1/2 signaling pathways to maintain tumor-cell survival (Gao et al., 2019; Teng et al., 2017).

Therapies targeting FGF19 and/or FGFR4 by sorafenib induce tumor-cell apoptosis (Gao et al., 2017). In addition, evidence showed that FGF19 activated both FGFR1 and FGFR4 to a comparable extent in the presence of β-Klotho (Lan et al., 2017; Yang et al., 2012). FGFR1 inhibitor or genetic silencing by small interfering RNA significantly decreased the expression of α-SMA and type 1 collagen and reduced cell viability (Lou et al., 2018) and administration of FGF receptor inhibitor (PD173074) attenuated the activation of fibroblasts (MacKenzie et al., 2015). Those studies demonstrated that FGF19 could increase α-SMA, activate fibroblasts, and lead to the activation of CAFs.

In conclusion, our study demonstrates that TRAF6 promotes the activation of stromal fibroblasts into CAFs. Mechanistically, TRAF6 regulates the secretion of FGF19 in melanoma cells through the NF-kB1 pathway to promote fibroblasts activation into CAFs and enhance the malignant phenotype of melanoma cells. Therefore, TRAF6/FGF19 axis plays a critical role in the crosstalk between melanoma cells and CAFs. Targeting the TRAF6/FGF19 axis may be a novel strategy for melanoma treatment.

**MATERIALS AND METHODS**

**Cell lines and cell culture**

The human malignant melanoma cell lines Sk-Mel-5 and Sk-Mel-28, the human fibroblast cell line BJ (ATCC cell line HTB-70, HTB-72, CRL-2522), and HEK293T cells (maintained in our laboratory) were used in this study. Cells were grown in DMEM (Biological Industries, Beit HaEmek, Israel) supplemented with 10% fetal bovine serum independent experiments. Data are presented as the mean ± SD (n = 3). Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (a) Transwell assays were performed as described in Supplementary Materials and Methods. Bar = 100 μm. The number of invasive cells per field was calculated, and (c) data are presented as the mean ± SD of each group (n = 4) right panel. The significant difference between cells was evaluated by Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001. (d) TRAF6-overexpressing BJ cells promote melanoma cell growth in vivo. The melanoma (1 × 10⁶ cells) and B) cells (1.5 × 10⁶ cells) were mixed in 100 μL and xenografted into the right flank of nude mice as described in Supplementary Materials and Methods. (d), Representative xenografted tumors from melanoma mouse models at 3 weeks after implantation left panel. Bar = 1 cm. (d) Tumor growth was measured three times a week (right panel). The results are shown as the mean tumor volume ± SD. Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. CCK-8, cell counting kit-8; CM, conditioned medium; OD, optical density.
Figure 3. CM derived from TRAF6-knockdown melanoma cells inhibits the proliferation and migration of fibroblasts. (a) Knockdown of TRAF6 in melanoma cells. Whole-cell lysate of SK-Mel-5 (left panel) and SK-Mel-28 (right panel) infected by lentiviral particles (sh-Mock, sh-TRAF6#1, and sh-TRAF6#4) were extracted and subjected to immunoblot analysis using antibodies to TRAF6 as described in Supplementary Materials and Methods. GAPDH was used as control.
Protein preparation and immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer, and protein concentrations were determined by a BCA protein assay kit (PP1001, BioTeck, Beijing, China). Nuclear protein was extracted by NE-PER nuclear and cytoplasmic extraction reagents (78835, Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. A total of 30–50 μg of protein or 10 μg of nuclear protein was separated by SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes (Millipore, Burlington, MA). Additional experiments were carried out as previously described (Luo et al.).

**Protein digestion and absorption**

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**Mismatch repair**

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**Mismatch repair**

Figure 4. RNA-sequence analyses of the effect of TRAF6 on gene expression profile in SK-Mel-5 cells. (a) The effect of TRAF6 on gene expression profile. Stable knockdown of TRAF6 was generated in SK-Mel-5 cell lines by two independent shRNAs. RNA sequencing was performed as described in the Supplementary Materials and Methods, and differential expression genes were analyzed using DESeq2. (b) The top 20 positively enriched KEGG pathways are shown in the bubble chart. The x-axis is the enrichment score, and the y-axis is the enriched pathways. (c) FGFR1 is a nodes protein in the PPI network of TRAF6-knockdown melanoma cells. The PPI network was analyzed using STRING online (https://string-db.org/). (d) FGFR1 is decreased in TRAF6-deficient melanoma cells. Total RNA was extracted from Sk-Mel-5 and Sk-Mel-28 cells, and qRT-PCR was then performed as described in Supplementary Materials and Methods. The data from multiple experiments are expressed as the mean ± SD (n = 3). Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; qRT-PCR, quantitative real-time reverse transcriptase-PCR; shRNA, short hairpin RNA.
**Figure 5. TRAF6 regulates FGF19 expression through NF-κB1.** (a) The knockdown of TRAF6 reduces secretory production of FGF19 in melanoma cells. CM of melanoma cells was collected and subjected to FGF19 ELISA as described in Supplementary Materials and Methods. The data from multiple experiments are represented as the mean ± SD (n = 3). Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (b) Suppression of TRAF6 attenuates FGF19 luciferase activity in melanoma cells. (b) Schematic diagram of pGL3-FGF19-luc plasmid construction (upper panel). The pGL3-FGF19-luc and Renilla luciferase genes (200 ng) were transfected into TRAF6-knockdown melanoma cells. After 24 hours of transfection, Firefly luciferase activity was examined by normalizing against Renilla luciferase activity as described in the Supplementary Materials and Methods (lower panel). The data from multiple experiments are expressed as the mean ± SD (n = 4). Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (c) NF-κB1 luciferase activity is regulated by TRAF6 in melanoma cells. TRAF6 was transfected with pGL3-NF-κB1 promoter-luciferase reporter and the Renilla luciferase gene, and the NF-κB1 luciferase activity was assessed by luciferase assay as described in the Supplementary Materials and Methods (upper panel). The data from multiple experiments are expressed as the mean ± SD (n = 4). Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. Nuclear protein was extracted from TRAF6-knockdown melanoma cells and subjected to immunoblot analysis using antibodies to NF-κB1 as described in Supplementary Materials and Methods. Lamin A/C was used as a control (lower panel). (d) TRAF6 benefits NF-κB1 associated with FGF19 promoter. (d) Schematic diagram of FGF19 promoter and PROMO predicted several binding sites of NF-κB1 (upper panel). ChIP assay was performed to examine the NF-κB1 recognition of the FGF19 promoter as described in the Supplementary Materials and Methods (lower panel). The data from multiple experiments are expressed as the mean ± SD (n = 3). Significant differences were evaluated using Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001. The data of primers 3 and 4 were not shown as they did not work. ChIP, chromatin immunoprecipitation; CM, conditioned medium.
et al., 2014). Immunoreactions were detected by an imaging system (Bio-Rad, Hercules, CA).

**Antibodies and reagents**

Chemical reagents, including Tris, sodium chloride, and SDS for molecular biology and buffer preparation, were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and other supplements were purchased from Life Technologies (Rockville, MD).

The primary antibodies used were rabbit polyclonal anti-TRAF6 (1:1,000, 8028s, Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-MMP-9 (1:1,000, ab76003, Abcam, Cambridge, UK), rabbit polyclonal anti-MMP-2 (1:500, AP13693C, Abgent, San Diego, CA), rabbit monoclonal anti-α-SMA (1:1,000, 19245s, Cell Signaling Technology), rabbit monoclonal anti-vimentin (1:1,000, 5741s, Cell Signaling Technology), rabbit polyclonal anti-desmin (1:500, 16520-1-AP, Proteintech, Wuhan, China), and rabbit polyclonal anti-SMA (1:1,000, 19245s, Cell Signaling Technology).

**Figure 6. FGF19 promotes tumorigenesis of melanoma.** (a) Plasma FGF19 is elevated in patients with melanoma. The plasma of patients with melanoma (n = 12) and healthy volunteers (n = 12) was collected and tested by ELISA as described in Supplementary Materials and Methods. Significant differences were evaluated using Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001. (b–d) FGF19 promotes tumorigenesis of melanoma in vivo. SK-Mel-5 (1 × 10^6 cells) and FGF19 pretreated (25 pg/ml and 50 pg/ml) BJ cells (1.5 × 10^6 cells) were mixed in 100 μl and xenografted into the right flank of the nude mouse as described in Materials and Methods. (b) Representative xenografted tumors (upper panel) and tumor weight (lower panel) of mouse models were shown at three weeks after implantation. Bar = 1 cm. Significant differences were evaluated using two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (c) Tumor growth of xenografted mouse model was measured three times a week as described in Supplementary Materials and Methods. The results are shown as the mean tumor volume ± SD. Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (d) Immunohistochemistry staining of Ki67 (1:350) in xenografted melanoma mouse models as described in Supplementary Materials and Methods. (d) Representative images were taken (left panel) and bar chart graphs of ki67 positive rate. (right panel) Bar = 100 μm. Data are presented as the mean ± SD (n = 4). Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001.
anti-PDPN (1:500, D162603, BBI, Shanghai, China), and mouse monoclonal anti-GAPDH (1:3,000, 60004-1-Ig, Proteintech).

Xenograft tumor model

Xenograft tumor models were previously established by our laboratory (Chen et al., 2006), and the animal study was approved by the Ethics Committee of Xiangya Hospital, Central South University, Hunan, China. BJ cells transduced with lentiviral particles or pre-treated with FGF19 and SK-Mel-5 cells were collected and washed with PBS buffer. Then, 1 x 10^6 SK-Mel-5 cells mixed with 1.5 x 10^6 sh-Mock or sh-TRAF6 BJ cells were resuspended in cold serum-free DMEM and injected subcutaneously into the right flank of 4- to 5-week-old athymic BALB/c female nude mice (Shanghai Laboratory Animal Center, Shanghai, China). Tumors were measured three times a week using calipers, and tumor volumes were calculated using the following formula: length x width x height x 0.52. Tumor tissues were harvested and fixed in 10% buffered formalin, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with H&E or subjected to immunohistochemical analysis.

Ethics approval

The animal protocol was approved by the Ethics Committee of Xiangya Hospital, Central South University.

Statistical analysis methods

Statistical results are presented as the mean ± SD and were analyzed by the Student’s t-test or one-way ANOVA to evaluate the statistical differences. A P-value of <0.05 was considered statistically significant.

Detailed materials and methods are included in the Supplementary materials (Supplementary Materials and Methods) owing to space limitations.

Data availability statement

Data sets related to this article can be found at [https://dataview.ncbi.nlm.nih.gov/object/PRJNA602707?reviewer=4q16ck35vvs] and hosted at (NCBI).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: XC, CP; Data Curation: YG, WZ, YX, LT; Formal Analysis: YG, ZX, ZW, YX; Funding Acquisition: YG, ZX, WZ, JZ, XC, CP; Investigation: YG, ZX, NL; Methodology: LC, ZW; Project Administration: JS, CP; CP; Resources: JS, XC, CP; Software: WZ, LT; Supervision: XC, CP; Validation: XZ, JZ, LT; Visualization: YG, ZX; Writing - Original Draft Preparation: YG, ZX; Writing - Review and Editing: YX, XX, CP

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.03.950.

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Whipple CA, Brinckerhoff CE. BRAF(V600E) melanoma cells secrete factors that activate stromal fibroblasts and enhance tumourigenicity. Br J Cancer 2014;111:1625–33.


SUPPLEMENTARY MATERIALS AND METHODS

Cell proliferation assays

Cell viability was assessed using a cell counting kit-8 assay (Bimake, Houston, TX) according to the manufacturer’s instructions. Cells were seeded into 96-well plates at 3,000 cells per well and cultured for 24, 48, 72, or 96 hours. Then, 10 μl of cell counting kit-8 solution was added to each well, and the 96-well plate was incubated for 2 hours at 37 °C and 5% carbon dioxide. The fluorescence of each plate was measured using a spectrophotometer at an emission wavelength of 450 nm (Beckman, Brea, CA). Six replicates per sample were analyzed.

Generation of conditioned media

SK-Mel-5, SK-Mel-28, and BJ cells were cultured to ~60% confluence on 10 cm culture dishes. The medium was replaced with fresh culture medium when the cells had reached the desired confluence, and culture was continued for 48 hours. Then, the medium was collected and filtered through a 0.22 μm polyethersulfone filter to remove cells and cellular debris. The medium was aliquoted and stored at −80 °C. For the proliferation, scratch, and transwell assays, the conditioned medium was thawed immediately before being added to fresh culture medium (1:1) in 96-well, 6-well, or 24-well plates.

Plasmid and lentiviral vector construction

Lentivirus plasmids containing pLKO.1, pSPAX2, and pMD2G and TRAF6 short hairpin RNAs were purchased from Thermo Scientific (Waltham, MA). Plasmids pCDNA4.0 and TRAF6-Flag were purchased from Vigene Biosciences (Rockville, MD). The pLVX-TRAF6-puro (TRAF6-overexpressing lentivirus plasmids) were constructed in our laboratory. We amplified the full-length cDNA encoding TRAF6 by PCR with the primers (Forward: 5′CCGAATTCATGAGTCATCAAATCTTG3′; Reverse: 5′GGCACTAGTCTATTTGTCATCGTCATCCTTGTACCC CTGCATCAGTACTTC3′). Subsequently, the purified TRAF6 PCR products were ligated to pLVX-IRESPuro Vector (Takara, Shanghai, China), and plasmids were transformed with different plasmids using TurboFect Transfection Reagent (Thermo Scientific). The reagent and DNA were diluted in DMEM and incubated for 20 minutes. The mixture was added to cells growing in the plates for 36 to 48 hours to facilitate transfection. To establish stable TRAF6 knockdown and overexpression cells, pLKO.1-shTRAF6 or pLKO.1-sh-Mock and pLVX-TRAF6-Puro or pLVX-IRESPuro plasmids were cotransfected with packaging plasmids (pSPAX2 and PMD2G) into 293T cells. The supernatant fractions containing lentiviral particles were collected separately at 48 and 72 hours, and SK-MEL-5, SK-MEL-28, and BJ cells were infected with lentiviral particles in medium supplemented with 10 μg/ml polybrene. At 16 hours after infection, the medium was replaced with fresh medium containing a suitable concentration of puromycin. The appropriate experiments were performed with these cells until all control cells (uninfected) were dead (usually 36–48 hours) in the puromycin-containing medium.

Transwell invasion assay

For the invasion assay, a transwell experiment was performed with 8-μm-pore chambers inserted into 24-well plates (Corning, NY). Matrigel (BD Biosciences, Franklin Lakes, NJ) was diluted (1:7) in serum-free DMEM and was then added to each chamber and allowed to solidify completely. Transfected cells were obtained, resuspended in serum-free medium at a concentration of 4 × 10^5/100 μl, and seeded in the upper chambers, whereas 350 μl of DMEM containing 30% fetal bovine serum or different controlled medium derived from melanoma cells or BJ cells was placed into the bottom chamber as a chemotactic factor. After 24 or 48 hours, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Nonmotile or noninvaded cells on the top surface of the filter were removed, whereas motile or invaded cells on the bottom surface were stained with crystal violet. ImageJ software was used to quantify the invaded and migrated cells. Three fields per well were counted with an inverted microscope system (Ti-S, Nikon, Tokyo, Japan).

Scratch assay

Cells in complete medium were seeded in a 6-well plate at a density of 1 × 10^5 cells per well, and a straight line was scratched on the cell monolayer with a 200 μl pipette tip. Then, cells were washed with PBS three times to remove debris. Finally, cells were cultured in a controlled medium derived from SK-Mel-5, SK-Mel-28, or BJ cells and imaged at 24 hours and 48 hours.

Immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded tumor sections were baked at 65 °C, deparaffinized in turpentine, rehydrated through a series of graded alcohols, and immersed in hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed by heat treatment in a pressure cooker in citrate buffer (pH 6.0) for 3 minutes. Primary antibodies are as follows: Ki67 (1:400, ab1667, Abcam, Cambridge, UK), α-SMA (1:250, D261431, Sangon, Shanghai, China), and TRAF6 (1:250, D261103, Sangon, Shanghai, China), and TRAF6 (1:250, D221362, Sangon, Shanghai, China). Sections for immunohistochemistry staining were blocked for nonspecific binding by incubation in normal goat serum at room temperature. Subsequently, slides were incubated overnight with a primary antibody against Ki67 at 4 °C. The next day, sections were incubated for 20 minutes with a biotin-conjugated secondary antibody and then with peroxidase-conjugated streptavidin for an additional 30 minutes. Next, 3,3′-diaminobenzidine tetrahydrochloride was used to visualize the reaction, and slides were then counterstained with hematoxylin. Alternatively, for immunofluorescence, sections were dual-labeled using secondary antibodies conjugated to tyramide signal amplification (TSA, Opal-520 for S100A4, -570 for α-SMA, 690 for TRAF6, PerkinElmer, Waltham, MA). Fluorescence imagery was obtained on the PerkinElmer Vectra Multispectral imaging platform. The images were quantified for average fluorescence intensity using National Institutes of Health ImageJ analysis software (α-SMA, TRAF6) and for percent positive cells using ImageJ analysis software (Ki-67). The Ki67-positive rate was calculated by the percentage of nuclei with positive
staining in tumor cells. Five regions (bottom right, bottom left, top left, top right, and middle left) of each sample and four tissue samples in each group were randomly chosen for an average Ki67 positive rate.

**RNA sequencing**

The cDNA library construction, library purification, and transcriptome sequencing were implemented according to the Wuhan Huada Sequencing Company’s instructions (www.genomics.org.cn, BGI, Shenzhen, China).

**Quantitative real-time PCR analysis**

Total RNA was extracted from cells infected with sh-Mock, sh-TRAF6#1, or sh-TRAF6#4 with Trizol reagent. Total RNA (3 μg) was used as the template for the reverse transcription reaction (SuperScript III First-Strand Synthesis System for Reverse Transcription PCR, Invitrogen, Waltham, MA). All PCR primers used in this study are as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>FGF19</td>
<td>5’ATGGCTCAAATGGTACCGATC3’</td>
<td>3’CTGTGGCGAGATGCAACC3’</td>
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<tr>
<td>TRAF6</td>
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<td>3’TGGACGAGAcAAGACGAGAC3’</td>
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<tr>
<td>FGR3</td>
<td>5’GAGGAAACACGTGAGAAGATCG3’</td>
<td>3’GTTTCTGTCTTCCTTTGAGT3’</td>
</tr>
<tr>
<td>TNFβ</td>
<td>5’AACACACCTGAAGCTCCTCCTC3’</td>
<td>3’GCGAGACGAGACGAGAC3’</td>
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<tr>
<td>FGF13</td>
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<tr>
<td>EBRB4</td>
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<td>3’CTGGGACGAAATGCTGGTTA3’</td>
</tr>
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<td>3’TACccccGAATGGCTGGTTA3’</td>
</tr>
<tr>
<td>FOS</td>
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<td>3’TGGGACAGAGAAGACGAGAC3’</td>
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<tr>
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<td>3’GCTCtCCtACCGACATG3’</td>
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<tr>
<td>VEGFD</td>
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<tr>
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<td>3’AAGCTGATGCGGAGGCTGG3’</td>
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</tbody>
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**ELISA assays**

ELISA was used to detect the levels of FGF19 in conditioned media. FGF19 ELISA kit (INS4060108, Inselisa, Wuhan, China) was used. All operations were performed in strict accordance with the manufacturer’s instructions.

**Luciferase reporter gene assays**

pGL3 was the luciferase reporter vector (E1751, Promega, Madison, WI). pRLE was the vector with kanamycin selection and CMV promoter and C-terminal FLAG and His tags (pREDER, P100001, Vigene Biosciences). pRLTK was the renilla luciferase control reporter vector (P100001, Promega). HEK293T cells were transfected with TRAF6, PGL3-NF-kB, and pRLE. TRAF6-knockdown melanoma cells were transfected with PGL3-FGF19 (constructed in our lab) and pRLE plasmids. After 24 hours of transfection, the firefly and renilla luciferase activity in the cell lysates was analyzed with a dual luciferase assay kit (Promega), following the protocol. For each transfection, the luciferase activity of four replicates was averaged.

**Chromatin immunoprecipitation**

Melanoma cells (10^7) infected with sh-Mock and sh-TRAF6 were collected and chromatin immunoprecipitation was performed according to the protocol provided by EZ ChIP KIT (17-371RF, Millipore, Burlington, MA). Soluble lysates were rotated with 5 μl antibody (NF-kB1, 13586s, Cell Signaling Technology, Danvers, MA) overnight at 4 °C with protease inhibitors. FGF19 promoter regions were amplified by PCR (50 cycles) using the following primer pairs:

**Primer 1:** Forward: 5’TCCCTTGGCTCAGAAGCTT3’

Reverse: 5’TCCCTTGGCTCAGAAGCTT3’

**Primer 2:** Forward: 5’AGAGTTAAACCAAGGGCC3’

Reverse: 5’TCCCTGACTCCATGAGCT3’

**Primer 3:** Forward: 5’TTGAGAAGGGCCAGCTT3’

Reverse: 5’CTGACAGGGCCAGCTT3’

**Primer 4:** Forward: 5’AAGCCAGGGGCGAGCTT3’

Reverse: 5’CTGACAGGGGCGAGCTT3’

**Clinical characteristics of patients with melanoma and healthy volunteers**

Healthy controls (n = 12) or patients with melanoma (n = 12) were recruited at the Xiangya Hospital, Central South University, Hunan, China, in accordance with the Declaration of Helsinki. All sample donors provided signed consent forms, which were approved by the Xiangya Hospital Committee of Ethics. We collected 5 ml of whole blood from each subject into EDTA tubes. Samples were centrifuged at 3,000g for 20 minutes at 4 °C within 1 hour. After centrifugation, plasma samples were collected and immediately stored at −80 °C until use. The detail clinical characteristics are presented in Supplementary Table S1.
Supplementary Figure S1.
**Overexpression of TRAF6 promotes fibroblast growth in vivo.**
(a) Overexpression of TRAF6 promotes BJ cell growth in vitro. TRAF6-overexpressing BJ cells showed an increasing growth rate. Stable overexpression of TRAF6 in human fibroblast (BJ) cells was generated by lentiviral infection. Cells were seeded into 96-well plates, and cell viability was examined by CCK-8 kit as described in Supplementary Materials and Methods. Data from multiple experiments are expressed as the mean ± S.D. Significant differences were evaluated using two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. (b–e) Overexpression of TRAF6 promotes BJ cell migration and invasion. The wound healing assay was performed as described in the Supplementary Materials and Methods. (b) Representative images were taken at indicated time points and (c) bar chart graphs shown are from three independent experiments. Bar = 100 μm. Data are presented as the mean ± SD (n = 3). Significant differences were evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. An asterisk (*) indicates a significant difference (P < 0.05, one-way ANOVA). For transwell assay, the same number of cells (4 × 10^4) were seeded into the upper layer of a chamber. (d) The cells that migrated across the membrane were stained with crystal violet as described in Supplementary Materials and Methods. Bar = 100 μm. (e) Data represent the mean ± SD of each group (n = 4). Significant differences were evaluated using Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001. (f) TRAF6 promotes the acquisition of CAF properties by BJ cells. Whole-cell lysate of BJ cells overexpressed with TRAF6 were extracted and subjected to immunoblot analysis using antibodies to TRAF6, MMP-9, Vimentin, Desmin, PDPN, and α-SMA as described in Supplementary Materials and Methods. GAPDH was used as control. CCK-8, cell counting kit-8; H, hour; hr, hour; OD, optical density.
**Supplementary Figure S2.** Knockdown of TRAF6 inhibits fibroblast growth in vivo. (a) Knockdown of TRAF6 blocks BJ cells proliferation. BJ cells were infected with sh-TRAF6#1, sh-TRAF6#4, or control (sh-Mock), and then cell viability was evaluated by CCK-8 at the indicated time points as described in Supplementary Materials and Methods. Data from multiple experiments are expressed as the mean ± SD. An asterisk (*) indicates a significant difference (\( P < 0.05 \), two-way ANOVA). (b–e) Knockdown of TRAF6 attenuates BJ cells migration and invasion. Wound healing assays of BJ cells infected with lentiviral sh-TRAF6#1, sh-TRAF6#4, or control construct (sh-Mock). (b) Phase-contrast images at \( \times 40 \) magnification were obtained at indicated time points as described in Supplementary Materials and Methods. (c) Bar chart graphs shown are from three independent experiments of wound healing assay. Data are presented as the mean ± SD (n = 3). Significant differences were evaluated using two-way ANOVA, and an asterisk (*) indicates a significant difference (\( P < 0.05 \)). (d) For transwell assay, the same number of cells (4 × 10^4) were seeded into the upper layer of a chamber. The cells that migrated across the membrane were stained with crystal violet and imaged as described in Supplementary Materials and Methods (left panel). Bar = 100 μm. Bar chart data represent the mean ± SD of each group (n = 4). Significant differences were evaluated using one-way ANOVA, and an asterisk (*) indicates a significant difference (\( P < 0.05 \)) (right panel). (e) Biomarkers of CAFs are downregulated in TRAF6-deficient BJ cells. Western blot analysis of CAFs biomarkers in BJ cells infected with sh-TRAF6#1, sh-TRAF6#4, and sh-Mock was generated as described in Supplementary Materials and Methods. GAPDH was used as control. CCK-8, cell counting kit-8; hr, hour; OD, optical density.
Supplementary Figure S3. TRAF6-overexpressing BJ cells promote melanoma cells growth and migration in vitro and in vivo. (a) CM derived from TRAF6-overexpressing BJ cells promotes melanoma cells migration ability. Wound healing assay of SK-Mel-5 (left panel) and SK-Mel-28 (right panel) cells treated with CM from TRAF6-overexpressing BJ cells was generated as described in Supplementary Materials and Methods. Representative images were taken at indicated time points. Bar = 100 μm. (b, c) TRAF6-overexpressing BJ cells promote tumorigenesis of melanoma cells in vivo. (b) A photograph of xenografted tumors in mice xenografted by mixing SK-Mel-5 with BJ cells, which were infected with TRAF6 or Mock as described in Supplementary Materials and Methods (left panel). Bar = 1 cm. Body weight of xenografted mouse were measured three times a week. Data are presented as the mean ± SD (right panel). Significant differences were evaluated using two-way ANOVA. (c) Immunohistochemistry staining of Ki67 (1:350) in xenografted melanoma mouse model tissues as described in Supplementary Materials and Methods. (d) Representative images were taken (left panel) and bar chart graphs of Ki67 positive rate (%) (right panel). Bar = 100 μm. Data are presented as the mean ± SD (n = 4). Significant differences were evaluated using Students’ t-test, and an asterisk (*) indicates a significant difference (p < 0.05). CM, conditioned medium; hr, hour.
Supplementary Figure S4. CM derived from TRAF6-knockdown fibroblasts attenuates the proliferation and migration of melanoma cells in vitro. (a) CM derived from TRAF6-knockdown BJ cells blocks the proliferation of melanoma cells. Melanoma cells were treated with CM derived from BJ cells infected with sh-TRAF6#1, sh-TRAF6#4, or control (sh-Mock), and then cell viability was evaluated by CCK-8 at the indicated time points as described in Supplementary Materials and Methods. Data from multiple experiments are expressed as the mean ± S.D. An asterisk (*) indicates a significant difference (P < 0.05, two-way ANOVA). (b–d) CM derived from TRAF6-knockdown BJ cells reduces the migration and invasion of melanoma cells. (b) Wound healing assay of SK-Mel-5 (left panel) and SK-Mel-28 (right panel) cells treated with CM from TRAF6-knockdown BJ cells was generated as described in Supplementary Materials and Methods. Representative images
Supplementary Figure S5. TRAF6-knockdown BJ cells attenuate the tumorigenic ability of melanoma cells in vivo. (a–e) TRAF6-knockdown BJ cells inhibit tumorigenesis of melanoma cells. (a) SK-Mel-5 (1 x 106 cells) and BJ cells infected with lentiviral sh-TRAF6#1, sh-TRAF6#4, or sh-Mock (1.5 x 106 cells) were mixed in 100 µl and xenografted into right flank of nude mouse as described in Supplementary Materials and Methods. Representative xenografted tumors of mouse models at four weeks after implantation. Bar = 1 cm. (b) Tumor volume and (c) body weight were measured three times a week as described in Supplementary Materials and Methods. Data are presented as the mean ± SD. Significant differences were evaluated using two-way ANOVA, and an asterisk (*) indicates a significant difference (P < 0.05). (d) Immunohistochemistry staining of Ki67 (1:350) in xenografted melanoma mouse model tissues as described in Supplementary Materials and Methods. Representative images were taken (left panel) and bar chart graphs of Ki67 positive rate (%) (right panel). Bar = 100 µm. Data are presented as the mean ± SD (n = 4). Significant differences were evaluated using one-way ANOVA, and an asterisk (*) indicates a significant difference (P < 0.05).

were taken at indicated time points. Bar = 100 µm. (c) Bar chart graphs shown for SK-Mel-5 (upper panel) and SK-Mel-28 (lower panel) are from three independent experiments of wound healing assay. Data are presented as the mean ± SD (n = 3). Significant differences were evaluated using two-way ANOVA, and an asterisk (*) indicates a significant difference (P < 0.05). Bar charts of wound width. Data are presented as the mean ± SD (n = 3). For transwell assay, the same number of cells (4 x 10^4) were seeded into the upper layer of a chamber and CM derived from sh-Mock, sh-TRAF6#1, or sh-TRAF6#4 BJ cells was placed into the bottom chamber. (d) The cells that migrated across the membrane were stained with crystal violet and imaged as described in Supplementary Materials and Methods (upper panel). Bar = 100 µm. Bar chart data represent the mean ± SD of each group (n = 4) (lower panel). Significant differences were evaluated using one-way ANOVA, and an asterisk (*) indicates a significant difference (P < 0.05). CCK-8, cell counting kit_8; CM, conditioned medium; hr, hour; OD, optical density.
Supplementary Figure S6. qRT-PCR analyses of the effect of TRAF6 on the gene expression profile in SK-MEL-5 Cells. RNA was extracted from SK-Mel-5 cells infected by lentiviral sh-Mock, sh-TRAF6#1, or sh-TRAF6#4. qRT-PCR was then performed with different primers (FGFR3, TNFB, FGF13, ERBB4, TF, FOS, PDGFRB, VEGFD, ERBB3, HSPA6, FZD1, SMAD6, CX3CL1, MAP2K6, and SDC2) as described in the Supplementary Materials and Methods. The data from multiple experiments (n = 3) are expressed as the mean ± S.D. Significant differences were evaluated using one-way ANOVA, and an asterisk (*) indicates a significant difference (*P < 0.05, **P < 0.01, ***P < 0.001).
Supplementary Figure S7. qRT-PCR analyses of the effect of TRAF6 on the gene expression profile in SK-MEL-28 cells. RNA was extracted from SK-Mel-28 cells infected by lentiviral sh-Mock, sh-TRAF6#1, or sh-TRAF6#4. qRT-PCR was then performed with different primers (FGFR3, TNFB, FGF13, ERBB4, TF, FOS, PDGFRB, VEGFD, ERBB3, HSPA6, FZD1, SMAD6, CX3CL1 MAP2K6 and SDC2) as described in the Supplementary Materials and Methods. The data from multiple experiments (n = 3) are expressed as the mean ± S.D. Significant differences were evaluated using one-way ANOVA, and an asterisk (*) indicates a significant difference (*P < 0.05, **P < 0.01, ***P < 0.001).
Supplementary Figure S8. FGF19 facilitates BJ cells transfer to CAFs. (a) FGF19 promotes the growth of BJ cells in vitro. BJ cells treated with FGF19 at 25 pg/ml and 50 pg/ml. Cells were seeded into 96-well plates, and cell viability was examined by CCK-8 kit as described in Materials and Methods. Data from multiple experiments are expressed as the means ± SD (n = 3). The asterisk (*) indicates a significant difference (P < 0.05, two-way ANOVA). (b, c) FGF19 promotes the migration and invasion of BJ cells in vitro. Scratch wound healing assays of BJ cells pretreated with FGF19 at 25 pg/ml and 50 pg/ml were generated as described in the Supplementary Materials and Methods. (b) Representative images were taken every 24 hours to 48 hours (left panel), and bar chart graphs shown are from three independent experiments (right panel). Bar = 100 μm. For transwell assay, the same number of BJ cells (4 × 10^4) pretreated with FGF19 were seeded into the upper layer of a chamber for 24 hours. The cells that migrated across the membrane were stained with crystal violet as described in Supplementary Materials and Methods. (c) Data represent the means ± SD (n = 4) of each group. The asterisk (*) indicates a significant difference between cells evaluated using two-way ANOVA (P < 0.05). (d) Whole protein of BJ cells pretreated with FGF19 were extracted and subjected to immunoblot analysis using antibodies to MMP-2, MMP-9, vimentin, desmin, PDPN, and α-SMA as described in Supplementary Materials and Methods. GAPDH was used as control. CCK-8, cell counting kit-8; H, hour; hr, hour; OD, optical density.
Supplementary Table S1. Detailed Clinical Characteristics of Patients with Melanoma and Healthy Volunteers

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Abbreviations: N/A, not applicable.

\(^\dagger\) P-value was calculated by unpaired t-test (age) and chi-square (gender).