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Discovery of TBC1D7 as a Potential Driver for Melanoma Cell Invasion

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Abstract

Metastasis is the leading cause for mortality in melanoma patients. Here, an unbiased mass spectrometry-based quantitative proteomic method is utilized to assess differential protein expression in a matched pair of primary/metastatic melanoma cell lines (i.e., WM-115/WM-266–4) derived from the same patient. It is found that TBC1D7 is overexpressed in metastatic over primary melanoma cells, and elevated expression of TBC1D7 promotes the invasion of these melanoma cells in vitro, partly through modulating the activities of secreted matrix metalloproteinases 2 and 9. Additionally, interrogation of publicly available data show that higher mRNA expression of TBC1D7 predicts poorer survival in melanoma patients. Together, the results suggest TBC1D7 as a driver for melanoma cell invasion, which is an important element in

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Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest
The authors declare no conflict of interest.

melanoma metastasis. The proteomic data generated from this study may also be useful for exploring the roles of other proteins in melanoma metastasis.

Keywords

mass spectrometry; melanoma metastasis; quantitative proteomics; SILAC; TBC domain-containing proteins; TBC1D7

Melanoma is the deadliest type of skin cancer because its strong tendency to metastasize. We set out to identify new protein players that may drive or suppress melanoma metastasis. To this end, we utilized an unbiased quantitative proteomic approach to examine the differential protein expression in WM-115 and WM-266–4 cells, which are paired primary/metastatic melanoma cells derived from the same patient.[1]

By employing stable isotope labeling by amino acids in cell culture (SILAC) together with off-line 2D LC-MS/MS analysis, we were able to quantify over 7300 proteins (Figure 1A and Table S1, Supporting Information). Among them, 5955 (81%) were quantified in at least two replicates of SILAC-labeling experiments, and 1551 proteins (21%) displayed at least 1.5-fold differences between these two cell lines (Table S2, Supporting Information).

We next conducted pathway analyses for those proteins exhibiting at least 1.5-fold changes using the database for annotation, visualization, and integrated discovery,[2] which revealed enrichments of pathways with known functions in signal transduction, oxidation-reduction process, and positive regulation of GTPases activity (Figure S1, Supporting Information). Our laboratory recently examined the roles of aberrant expression of small GTPase proteins in melanoma metastasis.[3] We are also interested in how regulatory proteins of small GTPases, especially GTPase-activating proteins (GAPs), modulate melanoma metastasis. In this vein, many TBC domain-containing proteins are GAP proteins for small GTPases, and one of the them, TBC1D16, is a driver for melanoma metastasis.[4] Therefore, we placed the emphasis of this study on understanding whether differential expression of other TBC proteins affects melanoma metastasis.

Our proteomic data led to the quantification of 24 TBC domain-containing proteins, which accounts for 55% of this protein family, in WM-115 and WM-266–4 cells (Figure 1B, Tables S3 and S4, Supporting Information). On the grounds that the average relative standard deviation for the quantification results of TBC proteins was 12% (Table S3, Supporting Information), we chose 1.5-fold as a cutoff for identifying up- and downregulated proteins. With this criterion, TBC1D4, TBC1D14, TBC1D16, TBC1D7, and TBC1D10A were upregulated, whereas TBC1D24 was downregulated, in WM-266–4 relative to WM-115 cells. In particular, TBC1D7 was upregulated by 2.5-fold in WM-266–4 over WM-115 cells (Figure S2, Supporting Information shows the MS and MS/MS of a representative tryptic peptide derived from TBC1D7). We also verified the differential expression of TBC1D7 protein by Western blot analysis (Figure 1C).

We next assessed if differential expression of these TBC family proteins is associated with patient survival. Kaplan–Meier survival analyses, based on the gene expression data of TBC

family proteins for the skin cutaneous melanoma (SKCM) patients in the Cancer Genome Atlas (TCGA) database, showed that patients with higher expression levels of *TBC1D16* or *TBC1D7* exhibit poorer overall survival rates (Figure 2A), which is in line with our proteomic results. However, the same survival analyses of TBC1D4, TBC1D14, TBC1D25, TBC1D10A, and TBC1D24 did not reveal correlations between the differential expressions of these genes at mRNA levels, if consistent with differential protein expression as revealed from our proteomic data, and overall survival of melanoma patients. This might be attributed to patient heterogeneity, where WM-115 and WM-266–4 cells were derived from a single patient, or discrepancy between mRNA and protein expression. Notably, TBC1D16 is known to promote melanoma progression.[4] We also carried out distant metastasis-free survival (DMFS) analysis based on data from a cohort of 150 melanoma patients in the GSE65904 dataset. We found that higher levels of expression of TBC1D7 are significantly correlated with lower DMFS of melanoma patients (Figure 2B).

Interrogation of the TCGA data also unveiled significantly higher levels of mRNA expression of *TBC1D7* gene in SKCM and uveal melanoma (UVM) patients compared to other types of cancer (Figure 2C). Likewise, analyses of the publicly available gene expression data for The Cancer Cell Line Encyclopedia (CCLE) and NCI-60 human tumor cell lines showed that the *TBC1D7* mRNA expression levels in melanoma cell lines were upregulated relative to other types of cancer (Figure 2D,E). Together, the above results suggest TBC1D7 as a potential driver for melanoma metastasis.

We next asked if elevated TBC1D7 expression promotes melanoma cell invasion in vitro. Using transwell assays, we observed that siRNA-mediated knockdown of TBC1D7 led to significantly decreased migration and invasion of WM-266–4 cells (Figure 3A and Figure S3A, Supporting Information). Reciprocally, overexpression of TBC1D7 in WM-115 cells results in elevated invasion of these cells, though no increase in migratory capacity was observed (Figure 3B and Figure S4, Supporting Information).

Matrix metalloproteinases 2 (MMP2) and 9 (MMP9) are secreted by cells, and their degradation of basement membranes is crucial for cancer metastasis.[5] Therefore, we next explored if MMP2 and MMP9 play a role in the invasive phenotype of melanoma cells modulated by TBC1D7. Gelatin zymography assay results revealed substantially diminished activities of MMP2 and MMP9 in the conditioned media after siRNA-mediated knockdown of TBC1D7 in WM-266–4 cells compared to treatment with non-targeting control siRNA (Figure 3C). The enzymatic activities of MMP2 and MMP9 were, however, not modulated by ectopic overexpression of TBC1D7 in WM-115 primary melanoma cells (Figure 3D).

We next asked whether the finding can be extended to another pair of melanoma cell lines, that is, the IGR39 and IGR37 cells initiated from a separate patient.[6] Western blot analyses revealed that TBC1D7 was upregulated by 2.5-fold in metastatic (IGR37) relative to primary (IGR39) melanoma cells (Figure 1C). In addition, we observed slight increases in migratory and invasive capabilities upon overexpression of TBC1D7 in IGR39 cells, which is accompanied with elevated activities of secreted MMP2, but not MMP9 (Figure S5A,C, Supporting Information). Intriguingly, knockdown of TBC1D7 in IGR37 cells led to significantly elevated migration and invasion of IGR37 cells, which is associated with

augmented activities of secreted MMP9 after genetic depletion of TBC1D7 in IGR37 cells (Figures S3B and S5B,D, Supporting Information). Hence, both decreased expression of TBC1D7 in IGR37 cells and increased expression of TBC1D7 in IGR39 cells promote melanoma cell invasion. One plausible explanation for the seemingly discrepant findings is that TBC1D7, which forms a complex with tuberous sclerosis complex 1 (TSC1) and TSC2,[7] is a downstream target of protein kinase B (AKT)[8] and AKT pathway is known to be subject to sophisticated feedback regulation.[9] Hence, we speculate that, similar to the findings made for SRPK1,[10] upregulation of TBC1D7 in IGR39 cells and its downregulation in IGR37 cells can both activate AKT, and AKT activation was shown to promote melanoma metastasis in a mouse model.[11] Future studies are needed to test this speculation.

We also performed gel zymography assays using cell lysates, and our results showed that genetic depletion of TBC1D7 in WM-266–4 cells did not alter the enzymatic activity of MMP2 in the cell lysate (Figure S6A, Supporting Information), and similar findings were made for WM-115 cells upon ectopic overexpression of TBC1D7 (Figure S6B, Supporting Information). Additionally, no MMP9 activity was detectable in the lysate of WM-115 or WM-266–4 cells. Because knockdown of TBC1D7 did not alter the MMP2 activity in lysate of WM-266–4 cells, but diminished the MMP2 activity in the secreted proteome, our results suggest that TBC1D7 may promote the transport of MMP2 secretory vesicles.

We further preformed the MTT assay and found that genetic depletion of *TBC1D7* led to drastically diminished proliferation of WM-266–4 cells (Figure S7A, Supporting Information). Along this line, it is worth noting that lung cancer cell growth was shown to be suppressed by siRNA-mediated knockdown of TBC1D7.[12] Overexpression of *TBC1D7* in WM-115 cells attenuated proliferation prior to day 4, though no significant change was found at day 6 (Figure S7B, Supporting Information).

In conclusion, our SILAC-based quantitative proteomic experiment led to the quantification of 7387 proteins, including 55% of known TBC proteins, in WM-115 and WM-266–4 cells. We also discovered that TBC1D7 promotes invasion of this pair of cultured melanoma cells partly through modulating MMP2 and MMP9 activities. However, diminished expression of TBC1D7 in IGR37 cells did not attenuate their invasive capacity or the activity of secreted MMPs, which might be attributed to patient heterogeneity and alternative pathway(s) modulated by AKT. Nevertheless, bioinformatic analyses of clinical data of melanoma patients support that elevated expression of TBC1D7 is significantly associated with poorer overall patient survival and distant metastasis-free survival, suggesting TBC1D7 as a potential driver for melanoma metastasis, at least for a large majority of melanoma patients. In addition, the differentially expressed proteins in primary/metastatic melanoma cells revealed from this study may allow for uncovering other potential modulators of melanoma metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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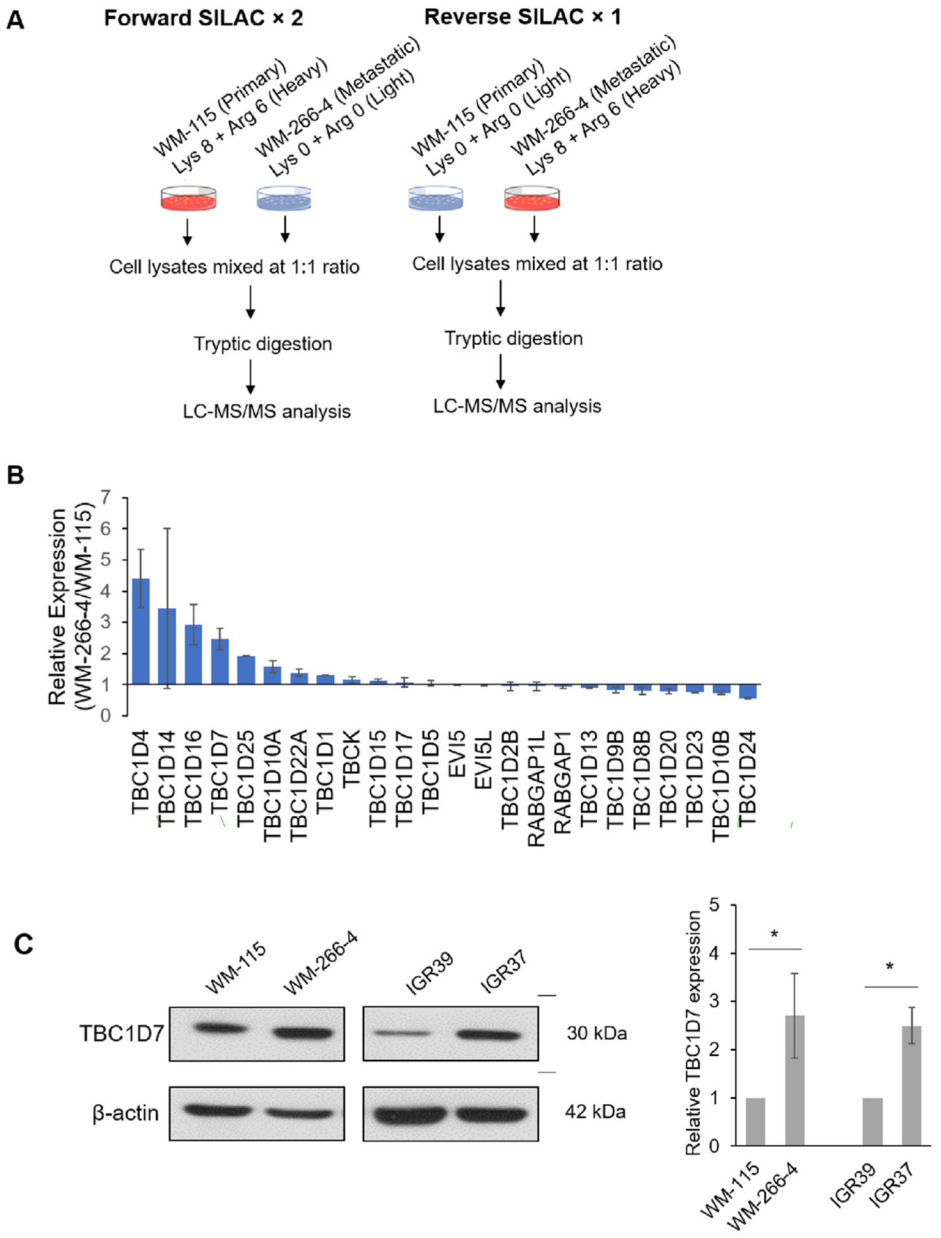

Figure 1.

SILAC-based quantitative proteomic experiment revealed differential expression of TBC domain-containing proteins in WM-115/WM-266–4 paired primary/metastatic melanoma cells. A) A flowchart showing the SILAC-based quantification of the global proteome of WM-115 (primary melanoma) and WM-266–4 (metastatic melanoma) cells. B) A bar graph showing the differential expression of TBC domain-containing proteins in WM-115 and WM-266–4 cells. C) Western blot for monitoring the expression of TBC1D7 protein in the two pairs of primary/metastatic melanoma cell lines. The data represent the mean ± S.D. (n = 3 or 4).

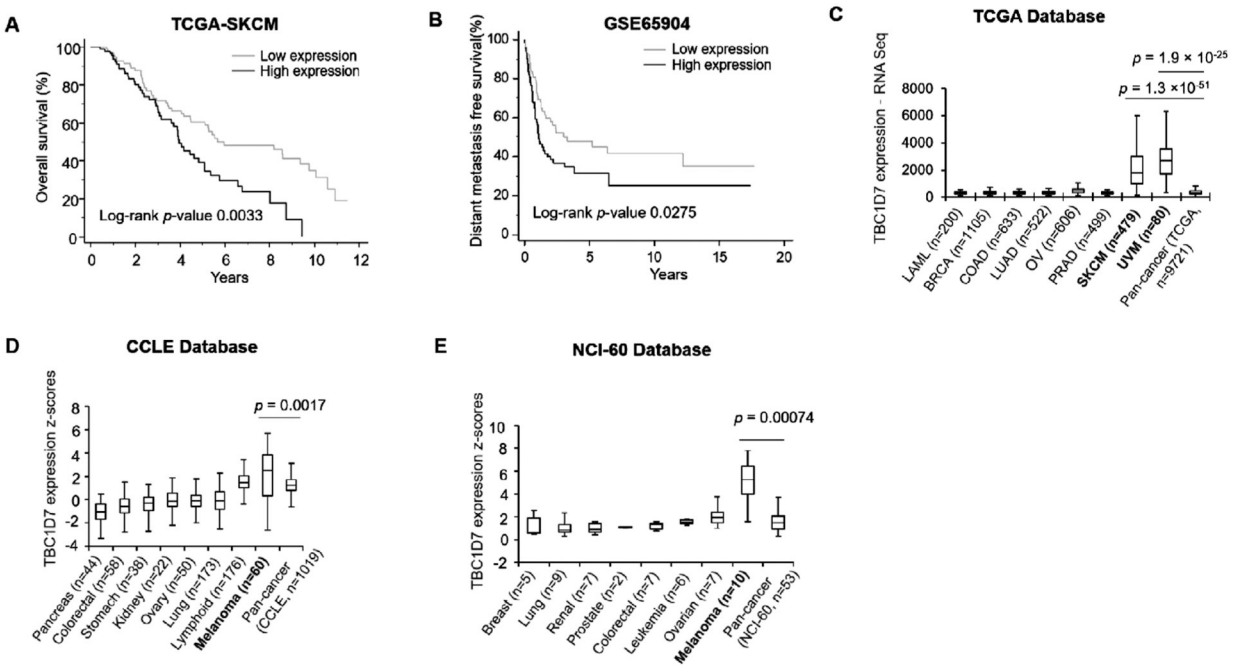

Figure 2.

Bioinformatic analysis suggests that TBC1D7 plays a role in melanoma progression. A) Kaplan–Meier plot for overall survival of skin cutaneous melanoma (SKCM) patients in the TCGA database. High and low expression levels refer to those patients with TBC1D7 expression being among the top and bottom quartiles of the TCGA-SKCM dataset, respectively ($n = 404$). B) Kaplan–Meier plot for the distant metastasis-free survival of melanoma patients in the GSE65904 cohort ($n = 150$). Patients were stratified by the median mRNA expression level of *TBC1D7*. C–E) Box–Whisker plot showed *TBC1D7* mRNA expression in SKCM and UVM patients in the TCGA database (C), in 60 melanoma cell lines in CCLE database (D), and in ten melanoma cell lines from the NCI-60 human tumor cell lines database (E). The horizontal edges of the box and line inside the box represent the top/bottom quartiles and median, respectively. The ends of the whisker denote the highest and lowest values. The survival analyses and p values in (A) and (B) were obtained using MedCalc, and all p values of (C–E) were calculated using the unpaired, two-tailed t-test.

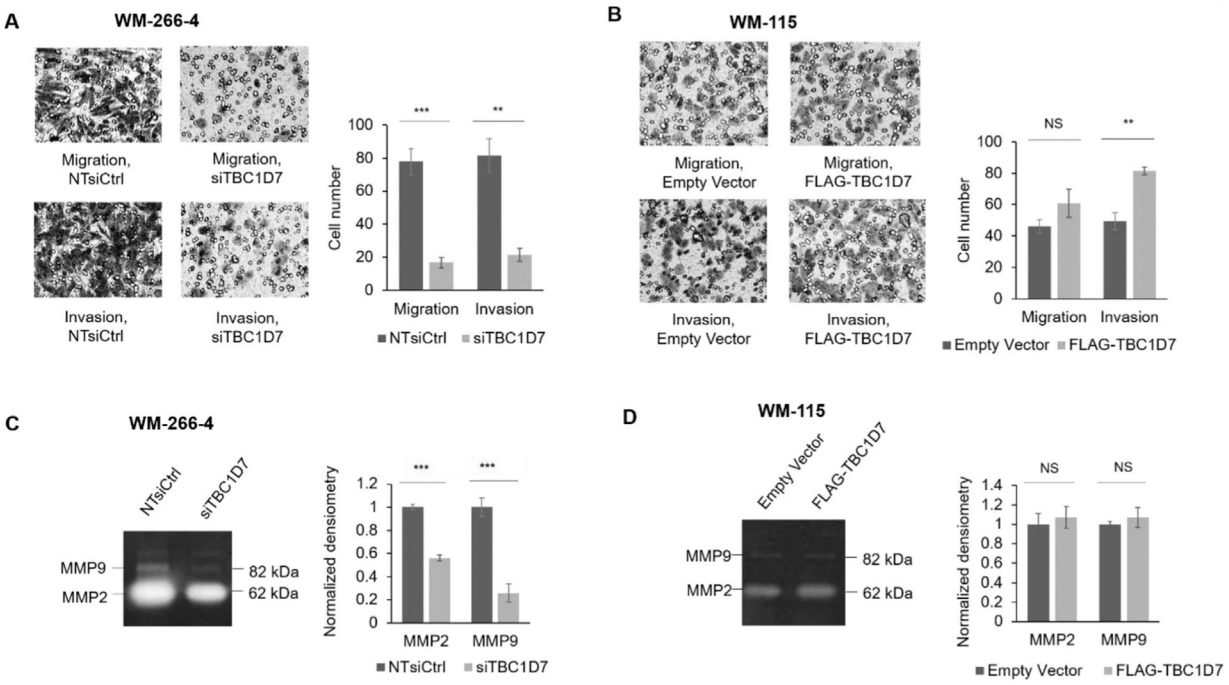

Figure 3.

TBC1D7 enhances melanoma cell invasion in vitro, and it involves the alterations of enzymatic activities of MMP2 and MMP9. A,B) Images and quantification results about the alterations in migration and invasion rates of WM-266–4 cells upon treatment with siTBC1D7 and non-targeting siRNA control (NTsiCtrl) (A), and WM-115 cells with ectopic overexpression of FLAG-TBC1D7 or empty vector control (B). C,D) Gelatin zymography assays and quantification results for the enzymatic activities of MMP2 and MMP9 in WM-115 and WM-266–4 cells upon modulation of TBC1D7 expression levels. The data represent the mean ± S.D. ($n = 3$). All p values were calculated using the unpaired, two-tailed t-test. "NS," $p > 0.05$; "**," $0.001 \leq p < 0.01$; "***," $p < 0.001$.