Identification of a Specific Translational Machinery via TCTP–EF1A2 Interaction Regulating NF1-associated Tumor Growth by Affinity Purification and Data-independent Mass Spectrometry Acquisition (AP-DIA)

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In Brief
Translationally controlled tumor protein (TCTP) is a novel biological target for neurofibromatosis type 1 (NF1)-associated tumors. Here, we identified specific TCTP-interacting proteins by affinity purification and data-independent mass spectrometry acquisition (AP-DIA/SWATH). TCTP mainly interacts with elongation factors in NF1-tumor cells. Interestingly, TCTP directly binds to EF1A2 and activates EF1A2-dependent translation machinery. Inhibiting the TCTP–EF1A2 interaction significantly caused dramatic suppression of growth in NF1-tumor cells. Our findings demonstrate that a translation machinery via TCTP–EF1A2 could represent a therapeutic target of NF1-tumors.

Highlights
- AP-DIA/SWATH analysis to identify TCTP-interacting proteins in NF1 tumor cells.
- A highly specific TCTP–EF1A2 interaction but rather than TCTP–EF1A1 interaction.
- TCTP–EF1A2 interaction mediating formation of EF1A2-elongation factor complex.
- TCTP–EF1A2 dependent translation machinery regulating NF1 tumor cell growth.
Identification of a Specific Translational Machinery via TCTP-EF1A2 Interaction Regulating NF1-associated Tumor Growth by Affinity Purification and Data-independent Mass Spectrometry Acquisition (AP-DIA)*

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Neurofibromatosis type 1 (NF1) is an autosomal dominant disease that predisposes individuals to developing benign neurofibromas and malignant peripheral nerve sheath tumors (MPNST). The mechanism of NF1-tumorigenesis or the curatives have not been established. Using unique transcriptome and proteome integration method, iPeach (1), we previously identified translationally controlled tumor protein (TCTP) as a novel biological target for NF1-associated tumors (2). Here, we identified specific TCTP-interacting proteins by sequential affinity purification and data-independent mass spectrometry acquisition (AP-DIA/SWATH) to investigate the role of TCTP in NF1-associated tumors. TCTP mainly interacts with proteins related to protein synthesis and especially to elongation factor complex components, including EF1A2, EF1B, EF1D, EF1G, and valyl-tRNA synthetase (VARS), in NF1-deficient malignant tumor cells. Interestingly, TCTP preferentially binds to EF1A2 (normally found only in neural and skeletal-muscle cells and several cancer cells), rather than EF1A1 despite the high homologies (98%) in their sequences. The docking simulation and further validations to study the interaction between TCTP and EF1A2 revealed that TCTP directly binds with EF1A2 via the contact areas of EF1A2 dimerization. Using unique and common sequences between EF1A2 and EF1A1 in AP-DIA/SWATH, we quantitatively validated the interaction of EF1A2 and TCTP/other elongation factors and found that TCTP coordinates the translational machinery of elongation factors via the association with EF1A2. These data suggest that TCTP activates EF1A2-dependent translation by mediating complex formation with other elongation factors. Inhibiting the TCTP-EF1A2 interaction with EF1A2 siRNAs or a TCTP inhibitor, artesunate, significantly down-regulated the factors related to protein translation and caused dramatic suppression of growth/translation in NF1-associated tumors. Our findings demonstrate that a specific protein translation machinery related to the TCTP-EF1A2 interaction is functionally implicated in the tumorigenesis and progression of NF1-associated tumors and could represent a therapeutic target. Molecular & Cellular Proteomics 18: 245–262, 2019. DOI: 10.1074/mcp.RA118.001014.

Neurofibromatosis type 1 (NF1) is an autosomal-dominant heritable disorder with an estimated prevalence of one in 3,500 persons (3). Its hallmarks include the development of benign tumors in the peripheral nervous system and an increased risk of malignant transformation, such as malignant peripheral nerve sheath tumors (MPNST). The highly variable NF1 phenotypes affect several organ systems, including the bones, skin, irises, and central and peripheral nervous systems, manifesting in the latter as neurofibromas, gliomas, and learning disabilities.

NF1 is located on chromosome 17q11.2 and encodes a protein of 2,818 amino acids called neurofibromin (4). Because the majority of NF1 mutations found in NF1 patients prevent the expression of intact protein, functional disruption of neurofibromin is potentially relevant in some of the various abnormalities (5). Despite the high frequency of and the predispositions caused by NF1 mutations, neither a specific molecular mechanism nor a biomarker or therapeutic target directly related to NF1 pathogenesis has been identified. The radical curing of phenotypes, such as NF1-associated tumors, thus presents a considerable challenge.

Previously, we demonstrated a novel role for neurofibromin as a Ras regulator via its GTPase-activated protein (GAP)-related domain (NF1-GRD) (6) by using neural model cells and rat hippocampal neuronal cells. We also showed that the...
function of neurofibromin with collapsing response mediator protein (CRMP)-2 is essential for neuronal cell differentiation (7). In these studies, down-regulation/inhibition of neurofibromin activity using NF1-specific siRNA (siNF1) caused a unique neurite retraction in NGF-stimulated PC12 cells via CRMP-2 phosphorylation due to activation of the Ras-MAPK-cyclin-dependent kinase (CDK)5/glycogen synthase kinase (GSK)/Rho-kinase (7). These results indicated that neurofibromin-deficient neural cells are useful for the study of NF1-related molecular pathology.

Recently, we conducted quantitative transcriptomic and proteomic analyses of neurofibromin-deficient PC12 cells (1) using a newly established integration tool termed iPEACH (1)/MANGO (8). We uniquely identified translationally controlled tumor protein (TCTP) and its related signaling as antiapoptotic factors that are regulated in response to NGF stimulation in PC12 cells (8). Interestingly, TCTP expression was up-regulated in NF1-deficient neural model cells, such as PC12 cells and human-derived Schwann cells (1, 2) in response to growth factor stimulation via MAPK and PI3K-AKT signaling. Importantly, specific TCTP up-regulation was also found in NF1-associated tumors, such as neurofibromas and MPNST, correlating with their malignancy (2). Moreover, in NF1-deficient MPNST cells, TCTP protein expression, but not mRNA expression, was down-regulated by NF1-GRD or MAPK/PI3K inhibitors; this down-regulation was correlated with suppression of mTOR signaling. Inhibition of mTOR signaling by rapamycin also down-regulated TCTP protein expression, while knockdown or overexpression of TCTP suppressed or activated mTOR signaling, respectively (2). These phenomena were also observed in tumor cells derived from an NF1-associated neurofibroma, suggesting that a positive feedback loop between TCTP and mTOR contributes to NF1-associated tumor formation. Down-regulation of TCTP with siRNA or the specific inhibitory drug artesunate dramatically decreased NF1-deficient cell viability and cell dimensions, demonstrating that TCTP can serve not only as a diagnostic biomarker but also as a therapeutic target in NF1-associated tumors (2).

TCTP was originally discovered in Ehrlich ascites tumor cells (9, 10) and was identified as a histamine-releasing factor (11). Highly conserved across species, it regulates cytoskeletal proteins such as tubulin (12, 13) and inhibits apoptosis by interacting with the Bcl2 protein family (14–17). Although known to be a multifunctional protein (18), as corroborated by our findings, TCTP is primarily considered as a positive regulator of cellular growth and proliferation (19, 20). However, the precise molecular mechanism of TCTP function, especially in malignant neural cells, is not well understood.

Here, we identified TCTP-interacting proteins by affinity purification and data-independent mass spectrometry acquisition (AP-DIA/SWATH) which is a powerful method to identify specific interacting proteins (21, 22), to investigate in detail the specific function of TCTP during NF1-associated tumorogenesis. The TCTP-interacting proteins predominantly included molecules related to the protein translation machinery, especially such protein elongation factors as EF1A, EF1B, EF1D, and EF1G. Several studies have reported the interaction of TCTP with EF1A1 components (18, 19). Cans et al. demonstrated that TCTP functions as a guanine nucleotide dissociation inhibitor of EF1A1 by yeast two hybrid analysis using U937 cell cDNA (19). The biophysical and structural studies by Wu et al. also suggested the interaction of TCTP and EF1D (23). Interestingly, our quantitative AP-DIA/SWATH analyses revealed that TCTP preferentially binds to EF1A2 rather than EF1A1, despite the high similarity between their amino acid sequences. Although EF1A2 peptide was observed in large scale in the Bioplex TCTP interactome database using HEK293T cells recently (24), further confirmation has not been performed. Several validations and docking simulations to study the interaction between TCTP and EF1A2 revealed that TCTP coordinates the translational machinery of elongation factors via the association with EF1A2. This interaction was dissociated under the treatment of the TCTP inhibitor artemesunate, which is known as a specific drug for Plasmodium TCTP in the treatment of malaria. Upon artesunate treatment, we observed decrease in the TCTP–EF1A2 interaction that coincided with the down-regulation of protein synthesis followed by the cellular damages. Our findings demonstrate that the specific interaction between TCTP and EF1A2 contributes to NF1-associated tumor growth and may be a specific therapeutic target for NF1-associated tumors.

EXPERIMENTAL PROCEDURES

Cell Culture, Preparation of Cell Lysate, and Evaluation of Cell Growth—Hela, sNF96.2, and sNF02.2 cells were cultured under 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. HSCs were purchased from ScienCell Research Laboratories (San Diego, CA), and cultured according to manufacturer recommendations. For preparation of cell lysate, cells were washed with PBS twice and solubilized with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, and 1% (v/v) protease inhibitor mixture (Sigma) for 15 min at 4 °C on a shaker. Lysates were centrifuged at 20,400 g for 20 min at 4 °C, and the protein concentration of the supernatants was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Cell growth was assayed with a Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan), following the manufacturer’s instructions.

Experimental Design and Statistical Rationale—To identify TCTP-, EF1A1-, and EF1A2-binding proteins, in sNF96.2 MPNST cells, AP-DIA/SWATH experiments were performed in three biological replicates. The cells transfected with FLAG-expression plasmid alone (FLAG alone) were used as a control set. To identify differentially expressed proteins in EF1A1- and EF1A2-deficient cells, DIA/SWATH

1 The abbreviations used are: TCTP, translationally controlled tumor protein; NF1, neurofibromatosis type 1; siRNA, short interfering RNA; MPNST, malignant peripheral nerve sheath tumor; HSCs, human Schwann cells; AP-DIA/SWATH, affinity purification and data-independent mass spectrometry acquisition; VARS, valyl-tRNA synthase.
experiments were performed in three biological replicates. The cells transfected with negative control siRNA were used as a control set. All values are expressed as the mean and S.D., and significant differences between groups were assessed by Student’s t test.

Construction of Expression Plasmids—To construct the c-terminal FLAG-tagged TCTP expressing plasmids, PCR was performed with Takara LA Taq® DNA polymerase (Takara Bio, Inc., Kusatsu, Japan) using TCTP cDNA obtained in our previous study (2) as the template for PCR. The forward and reverse primers were designed to ligate TCTP cDNA with expression plasmid at EcoRI and BglII site as follows: 5’-GAATTCATGATTATCTACCGGGACCTC-3’ (TCTP-forward) and 5’-AGATTCATCATTTCCTACTTGAACATC-3’ (TCTP-reverse), respectively. The PCR reaction was performed using an Applied Biosystems 9700 thermalcycler with the following thermal cycling parameters: 94 °C for 1 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min, and the final extension was performed at 72 °C for 10 min. The cDNA was cloned into pCMV-(DYKDDDDK)-C vector (Takara Bio, Inc.), and the constructed expression plasmid was used for the transfection of cultured cells. To obtain EF1A1 and EF1A2 cDNA, total RNA was extracted from normal HSCs using Qiagen RNeasy® mini kit, and reverse transcription of first strand cDNA was performed with SuperScript II reverse transcriptase (Invitrogen) using the total RNA as a template. The forward and reverse primers for EF1A1 and EF1A2 were designed to ligate EF1A1 cDNA with expression plasmid at EcoRI and BglII site as follows: 5’-GAATACTGAGGGAGAACATG-3’ (EF1A1-forward) and 5’-GAAGATGTGTCTTGCCTTGGG-3’ (EF1A1-reverse), and 5’-GAATACTGAGGGAGAACATG-3’ (EF1A1-forward) and 5’-GAAGATGTGTCTTGCCTTGGG-3’ (EF1A1-reverse), respectively. The PCR reaction was performed using an Applied Biosystems 9700 thermalcycler, with the following thermal cycling parameters: 55 °C for 30 min, 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min, and the final extension was performed at 72 °C for 10 min. The PCR product was cloned into the pGEM-T easy vector (Clontech) and sequenced. The cDNA was cloned into pCMV-(DYKDDDDK)-C vector and pCMV-Myc-C vector (Takara Bio, Inc.), and these constructed expression plasmids were used for the transfection of cultured cells.

To construct the plasmids expressing EF1A2 deletion mutant, PCR were performed with Takara LA Taq® DNA polymerase (Takara Bio, Inc.). The forward and reverse primers for EF1A2 deletion mutants were designed to ligate these cDNAs with expression plasmid at EcoRI and BglII site as follows: 5’-GAATACTGAGGGAGAACATG-3’ (EF1A2-forward), and 5’-GAAGATGTGTCTTGCCTTGGG-3’ (EF1A2-reverse), respectively. The PCR reaction was performed using an Applied Biosystems 9700 thermalcycler, with the following thermal cycling parameters: 94 °C for 1 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min, and the final extension was performed at 72 °C for 10 min. The PCR product was cloned into the pGEM-T easy vector (Clontech) and sequenced. The cDNA was cloned into pCMV-(DYKDDDDK)-C vector and pCMV-Myc-C vector (Takara Bio, Inc.), and these constructed expression plasmids were used for the transfection of cultured cells.

To construct the plasmids expressing chimera EF1A1/EF1A2 proteins, PCR were performed with Takara LA Taq® DNA polymerase (Takara Bio, Inc.). The forward and reverse primers for chimera EF1A1 and EF1A2 domain at Ncol as follows: 5’-GAATACTGAGGGAGAACATG-3’ (EF1A1-D1-forward), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D1-reverse), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D2-forward), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D3-forward), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D2-reverse), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D3-reverse). Translation Machinery via TCTP–EF1A2 in NF1 Tumors

AAAGACTCATAC-T3’ (EF1A1-D1-forward), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D1-reverse), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D2-forward), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D3-forward), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D2-reverse), 5’-GAACATGGAGCGACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D3-reverse). The PCR reaction was performed using an Applied Biosystems 9700 thermalcycler with the following thermal cycling parameters: 94 °C for 1 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min, and the final extension was performed at 72 °C for 10 min. The PCR product was cloned into the pGEM-T easy vector (Clontech) and sequenced. Each EF1A1 and EF1A2 domain was ligated at Ncol, and cloned into pCMV-(DYKDDDDK)-C vector. Because the cDNAs encoding chimera EF1A1/EF1A2 proteins contain additional amino acid tryptophan at the Ncol site, the inverse PCR was performed to exclude the three nucleotides encode the tryptophan at Ncol site. The primers for inverse PCR were designed as follows: 5’TGGCAGAGTGGTTGT-AGGA-3’ (EF1A1-D1-reverse), 5’-GACTGACAAGCCCCCTTGGCGCTC-3’ (EF1A1-D2-forward), 5’-GTTGGGCTTTTTCGTTCAGT-3’ (EF1A1-D2-reverse), 5’-ATGGGACACCATGCTGTCCTAC-3’ (EF1A1-D3-forward), 5’-TTGGCCGCTGGGGGGCAAGA-3’ (EF1A1-D2-reverse), 5’-ACGGAACACCCCTGGCCCT-3’ (EF1A1-D2-forward), 5’-TTGGCCGCTGGGGGGCAAGA-3’ (EF1A1-D2-reverse), 5’-ACGGAACACCCCTGGCCCT-3’ (EF1A1-D3-forward). The reverse PCR reaction was performed using an Applied Biosystems 9700 thermalcycler with the following thermal cycling parameters: 94 °C for 1 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min, and the final extension was performed at 72 °C for 10 min. The nucleotide exclusion was confirmed by sequencing.

Transfection of Cultured Cells with Expression Plasmids and siRNAs—Transfection of Hela, sNF96.2, and sNF02.2 cells and HSCs with siRNA or expression vectors was performed using the Neon Transfection System (Invitrogen/Life Technologies) according to the manufacturer’s protocol. The transfection efficiency of FLAG-tagged expressing plasmids were assumed to be more than 90% by the immunocytochemical analyses using FLAG antibody as shown in Figs. S2 and S5. The cells transfected with the expression plasmids were cultured for 24 h and harvested to be used for FLAG-immunoprecipitation. Target sequences for TCTP, EF1A1, and EF1A2 siRNA were designed as follows: 21-oligonucleotide siRNA duplexes was designed as recommended elsewhere (7, 8) and synthesized by Gene Link (Japan) to target the TCTP, EF1A1, and EF1A2 sequences. To prepare the sample for mass spectrometry, 100 μM glycine HCl (PH 2.0) were added to the washed resin beads, and the supernatant was applied to pH 7.4. For the Western blotting, SDS-PAGE sample buffer was added to the washed resin beads, and the supernatant was applied to SDS-PAGE. To prepare the sample for mass spectrometry, 100 μM glycine HCl (PH 2.0) were added to the washed resin beads, and the supernatant was applied to SDS-PAGE. To prepare the sample for mass spectrometry, 100 μM glycine HCl (PH 2.0) were added to the washed resin beads, and the supernatant was applied to SDS-PAGE. To prepare the sample for mass spectrometry, 100 μM glycine HCl (PH 2.0) were added to the washed resin beads, and the supernatant was applied to SDS-PAGE.translation machinery via TCTP–EF1A2 in NF1 Tumors
The supernatant was obtained and neutralized with 10 μl of 10×TBS (0.5 mM Tris HCl, 1.5 mM NaCl, pH 7.4). These samples were further treated to analyze, or frozen at −70 °C until use. All amount of the FLAG-IP product was subjected to LCMS and DIA/SWATH analysis.

Sample Preparation for Mass Spectrometric Analysis—To identify and quantify the binding proteins, the obtained FLAG-IP samples were used for LC-MS and DIA/SWATH analyses. To adjust pH at 8.5 for the digestion using trypsin/lysyl endopeptidase (LysC), 1 μl Tris solution was added to the sample. For cysteine reduction, 1.5 μg of DTT in 50 mM Tris-HCl (pH 8.5) were added to the samples that were incubated at 56 °C for 1 h. Following cysteine alkylation, 3.75 μg of iodoacetamide in 50 mM Tris-HCl (pH 8.5) were added, and the samples were incubated at 24 °C for 45 min. For the peptide digestion, 150 ng of Trypsin/Lys-C Mix (Promega) in the solution containing 50 mM Tris-HCl (pH 8.5) were added, and the samples were incubated at 37 °C overnight. To stop the reaction, trifluoroacetic acid was added to the sample at final concentration of 1%. The samples were desalted using ZipTip C18 pipette tips (Millipore) or styrenedivinylbenzene (SDB)-StageTip (25), and dissolved with 0.1% TFA in 2% acetonitrile for LC-MS and DIA/SWATH analyses.

To quantify the total proteins, each cell lysate containing 50 μg of total proteins was precipitated using sing a 2-D Clean-Up kit (GE Healthcare), and the precipitant was dissolved in 10 μl of 6 M urea. Twenty μl of 50 mM Tris-HCl (pH 8.5) were added to the samples. For cysteine reduction, 1 μl of 150 μM DTT in 50 μl Tris-HCl (pH 8.5) was added to the samples that were incubated at 37 °C for 1 h. Following cysteine alkylation, 1 μl of 450 mM iodoacetamide in 50 mM Tris-HCl (pH 8.5) was added, and the samples were incubated at 24 °C for 45 min. For the peptide digestion, 1 μg of Trypsin (Promega) in the solution containing 50 mM Tris-HCl (pH 8.5) was added, and the samples were incubated at 37 °C overnight. To stop the reaction, trifluoroacetic acid was added to the sample at final concentration of 1%. The samples were desalted using styrenedivinylbenzene (SDB)-StageTip (25) and dissolved with 0.1% TFA in 2% acetonitrile for LC-MS and DIA/SWATH analyses.

LC-MS and DIA/SWATH Analysis—To identify TCTP-binding proteins, LC separation of samples were performed using the Dionex Ultimate 3000 RSLC nano instrument (Thermo Scientific, Waltham, MA). Samples were loaded onto a 300-μm internal diameter × 5-mm length reverse phase (RP) C18 trap column (Thermo Scientific Dionex) at 25 μl/min and washed for 10 min before switching the trap column in line with the analytical column, which was a 75-μm internal diameter × 150-mm length ChromXP RP column (AB SCIEX) packed with 3-μm C18 beads with 120-Å pores. The flow rate used for separation on the RP column equilibrated with solvent A (0.1% formic acid in water) was 300 nl/min with a 120-min linear gradient of solvent B (0.1% formic acid in acetonitrile) from 5% to 40%. MS/MS spectra of the sample were performed using a Triple TOF 5600 mass spectrometer (AB SCIEX). Analyst TF 1.7.1 software (AB SCIEX) was used for data acquisition with the scan cycles set to perform a 25-msec MS scan followed by 30 MS/MS scans of the most abundant peaks for 20 ms each. Data acquisition was performed with an exclusion of 15 s for previous target ions. For data-independent acquisition, the precursor ions were sequentially isolated using a wide quadrupole 100 windows with variable m/z overlapping by 1 m/z and covering the precursor mass range of 400–1,250 m/z. The m/z ranges of 100 variable windows are shown in supplemental Table S13. The product ion spectra were collected from 100 to 1,500 m/z at 25 ms/scan. The total duty cycle was 2.6 s, including 100 product ion scans and 1 survey scan for precursor ions. The collision energy for each window was determined based on the appropriate collision energy for a charge 2+ ion with a spread of 5 eV. The Paragon™ algorithm (26) of ProteinPilot Version 5.0 (AB SCIEX) and the SwissProt human database (release2011_07) with contaminant proteins (total 20,416 entries) was used for the protein identification. Identified proteins were grouped by the Paragon algorithm to minimize redundancy. The six user-defined options included (i) cysteine alkylation, iodoacetamide; (ii) digestion, trypsin digestion; (iii) special factors, none; (iv) species, none; (v) identification focus, biological modifications; and (vi) search effort, thorough identification search.

DIA data were processed by Peakview ver 1.2. The group file obtained from ProteinPilot was used for the library of MS/MS spectra. The modified peptides and shared peptides in the protein identification were excluded for the quantification. The parameters in Peakview software were set as follow; number of proteins for quantification: all proteins in the search using ProteinPilot, peptide identification confidence: 95%, number of transition: 5, extracted ion chromatogram (XIC) extraction window: 5 min, XIC width: 0.05 Da.

For identification of EF1A1- and EF1A2-binding proteins, quantification of EF1A2-binding proteins and expression profiling in TCTP-deficient MPNST cells, and identification of differentially expressed proteins in EF1A1- and EF1A2-deficient cells, LC separation of samples were performed using the Eksigent ekspert™ nanoLC 415 instrument (AB SCIEX). Samples were loaded onto a 200-μm internal diameter × 0.5-mm length ChromXP-C18CL RP trap column (AB SCIEX) packed with 3-μm C18 beads with 120-Å pores at 2 μl/min, and washed for 10 min before switching the trap column in line with the analytical column, which was a 75-μm internal diameter × 150-mm length ChromXP RP column (AB SCIEX) packed with 3-μm C18 beads with 120-Å pores. The flow rate used for separation on the RP column equilibrated with solvent A (0.1% formic acid in a water) was 300 nl/min with a 120-min linear gradient of solvent B (0.1% formic acid in acetonitrile) from 5% to 32%. Mass spectrometric analyses of the sample were performed using a Triple TOF 6600 mass spectrometer (AB SCIEX). Analyst TF 1.7.1 software (AB SCIEX) was used for data acquisition with the scan cycles set to perform a 25-msec MS scan followed by 30 MS/MS scans of the most abundant peaks for 20 ms each. Data acquisition was performed with an exclusion of 15 s for previous target ions. For data-independent acquisition, the precursor ions were sequentially isolated using a wide quadrupole 100 windows with variable m/z overlapping by 1 m/z and covering the precursor mass range of 400–1,250 m/z. The m/z ranges of 100 variable windows are shown in supplemental Table S13. The product ion spectra were collected from 100 to 1,500 m/z at 25 ms/scan. The total duty cycle was 2.6 s, including 100 product ion scans and 1 survey scan for precursor ions. The collision energy for each window was determined based on the appropriate collision energy for a charge 2+ ion with a spread of 5 eV.
RESULTS

Identification of Specific TCTP-interacting Proteins in MPNST Cells by AP-DIA/SWATH Analysis—To investigate the specific function of TCTP in NF1-associated tumors, TCTP-interacting proteins were identified by quantitative AP-DIA/SWATH analysis. A plasmid-expressed FLAG-tagged version of TCTP was constructed to purify TCTP-interacting proteins using beads conjugated to a specific antibody for the FLAG tag. The constructs were transfected into shNF96.2 NF1-associated MPNST cells, and cell lysates were used for purification. The purified interacting proteins were identified by two independent strategies based on mass spectrometry, namely, a direct in-solution LC-shotgun analysis using a DIA/SWATH method and a gel-based (SDS-PAGE) analysis (Fig. 1A and Fig. S1A). The gel-based identification revealed that TCTP predominantly interacts with proteins related to the protein translation machinery, such as elongation factors and the ribosomal protein P0 (Fig. S1B and supplemental Table S1). To identify TCTP-interacting proteins in more detail, the samples were subjected to direct in-solution digestion using trypsin and lysyl endopeptidase and analyzed by a quantitative DIA/SWATH method. In total, 3,113 peptides and 345 proteins were identified with 95% confidence by data-dependent acquisition (DDA) (supplemental Table S2).

Using these data to generate the reference spectra for DIA/SWATH analysis, 337 proteins except for TCTP were quantified (supplemental Table S3). The quantitative data were normalized using the DIA/SWATH intensities of trypsin signals as well as total ions and were filtered at a significance level with a p value <0.05 and a fold change >4 to obtain/extract 113 TCTP-interacting proteins with high confidence (Fig. 1B and supplemental Table S4). These proteins included novel TCTP-binding partner candidates, such as EF1B, EF1G, EF1A2, VARS, and the 60S acidic ribosomal stalk protein P1, as well as known proteins, such as EF1A1, EF1D, tubulin, and actin (18). To understand the functional relationships among these 113 proteins, network analysis was performed using String. As shown in Fig. 1C, the network of identified TCTP-binding proteins were significantly assembled into nine groups of functional relationships, such as translation, stress response/chaperones, RNA binding, cytoskeleton, nucleosome/transcription, signal transduction, metabolic enzymes, and proteasome, among others (Fig. 1C). A statistical analysis with GO based on the “Biological Process” criterion revealed that this network primarily contains proteins related to translation (p = 9.94 × 10−21), translational elongation (p = 2.10 × 10−20), peptide metabolic process (p = 7.98 × 10−20), and viral process (p = 1.13 × 10−20) (Fig. 1D and supplemental Table S4).

These results strongly suggest that TCTP has important roles in protein translation in NF1-associated tumors.

To validate the localizations of TCTP-FLAG, we examined them by immunocytochemical analyses using anti-FLAG and -TCTP antibody. The TCTP-FLAG localizes entirely in cells,
which is slightly different from endogenous TCTP which localized mainly nuclear and perinuclear (Fig. S2). Although there are some differences in their localizations, our data include some interactors already reported, such as EF1D (23) and EF1A1 (19) indicating that TCTP functions on elongation machinery are maintained.

To validate our AP-DIA/SWATH data, the list of TCTP-binding proteins was compared with two supplementary tables of TCTP-binding proteins, one identified by the yeast two-hybrid system (18) and the other by gel-based MS identification coupled with affinity purification using anti-TCTP in HeLa cells (29). The TCTP-binding proteins identified by AP-DIA/SWATH analysis in our study were categorized into three groups according to confidence in the interactions with TCTP: high (p value <0.05 and fold change >4; 113 proteins), medium (p value <0.05 and fold change >2 or fold change >4 and p value >0.05; 108 proteins), and low (fold change <2 or fold change >2 and <=4 and p value >0.05; 116 proteins) (supplemental Table S3). Compared with the TCTP-binding proteins identified by the yeast two-hybrid system (18) or the previous gel-based analysis with HeLa cells (29), only 8 proteins (4%) or 45 proteins (20%), respectively, overlapped with the proteins identified with high and medium confidence in our study (Figs. S3A and S3B). These data suggest that our quantitative AP-DIA/SWATH method identifies novel candidates of TCTP-binding proteins.

Validation of the Interaction Between TCTP and EF1A1/EF1A2; TCTP Predominantly Interacts with EF1A2 Rather than EF1A1—Among the TCTP-interacting proteins, we focused on the interaction between translation elongation factors and TCTP because our data showed that all components of the translation elongation factor complex predominantly interacted with TCTP. Our AP-DIA/SWATH data in particular showed that TCTP preferentially interacts with EF1A2 rather than with EF1A1 (EF1A2 ratio, 81.1; EF1A1 ratio, 19.0, as shown in Fig. 1B) despite the high homology (98%) of their sequences (Fig. S4). As the TCTP–EF1A2 interaction was not observed in previous studies using U937 (18) and HeLa cells (29), we reasoned that this interaction might be relatively specific to NF1-associated tumors. To confirm the difference between the strength of the EF1A1 and EF1A2 interactions with TCTP, plasmids expressing Myc-tagged elongation factors EF1A1 and EF1A2 and FLAG-tagged TCTP were transfected into HeLa cells. In these cells, we could not observe any TCTP–EF1A2 interaction by mass spectrometric analysis (data not shown), in accordance with previous results (29). Therefore, we used HeLa cells to analyze the interaction between TCTP and exogenously expressed EF1A2 protein. The samples were purified using anti-FLAG antibody-conjugated beads. The interactions between TCTP and elongation factor EF1A1/EF1A2 were confirmed by Western blotting, which showed that TCTP preferentially interacts with EF1A2 over EF1A1, in accord with our AP-DIA/SWATH data (Fig. 2A). To further validate these interactions, plasmids expressing FLAG-tagged elongation factor EF1A1/EF1A2 were constructed and transfected into MPNST cells. The samples were purified using anti-FLAG antibody-conjugated beads and subjected to Western blotting. The FLAG-EF1A2–TCTP interaction was strongly observed; however, an EF1A1–TCTP interaction was not clearly observed (Fig. 2B). These data support our AP-DIA data that TCTP preferentially interacts with EF1A2 rather than EF1A1.

We reasoned that TCTP might predominantly interact with EF1A2 due to a different cellular localization of EF1A1, and so we used immunocytochemistry to examine the cellular distribution of FLAG-tagged EF1A1 and EF1A2. However, the proteins were both observed in the cytoplasmic/peri-nuclear area, and both seemed to co-localize with endogenous TCTP (Fig. S5), suggesting that the difference between EF1A1 and EF1A2 with respect to TCTP interaction is not due to different localization patterns. In addition, the observed localization patterns of exogenous EF1A1 and EF1A2 in our study were consistent with a previous report showing their endogenous localization (30). To gain further insight into the interaction difference, we attempted to identify the domain in EF1A2 that mediates binding to TCTP. Plasmids expressing six FLAG-tagged deletion mutants were constructed according to the three domains of EF1A2, namely the GTPase domain, the M domain, and the C-terminal domain. However, none of the deletion mutants showed binding activity to TCTP (Fig. 2C), indicating that TCTP specifically recognizes the overall structure of EF1A2. Next, plasmids expressing chimeric fusion proteins of EF1A2 and EF1A1 were constructed to determine the domains of EF1A2 that mediate its binding to TCTP. Only one chimeric protein, which contained domain I-II of EF1A2 and domain III of EF1A1, bound to TCTP (Fig. 2D). These data indicate that TCTP recognizes the conformations of all three domains of EF1A2 and that the main EF1A2 domains respon-

Fig. 1. Identification of TCTP-binding proteins in NF1-associated MPNST cells. (A) Workflow for the identification of TCTP-binding proteins by AP-DIA/SWATH MS. A plasmid expressing TCTP-FLAG was transfected into sNF96.2 MPNST cells. The samples obtained from cells transfected with a FLAG alone plasmid were used as a control. (B) The identified TCTP-binding proteins by AP-DIA MS. The X-axis indicates the log_{2}(fold change of TCTP/FLAG alone), and the Y-axis indicates the log_{10}(p value of FLAG alone versus TCTP). At a significance level of p < 0.05 (shown by the red dotted line) and fold change >4 (shown by the blue dotted line) compared with the FLAG alone-IP fraction, 113 proteins were identified as highly reliable candidate TCTP-interacting proteins. The proteins highlighted with red letters show that the elongation factor complex consisted of EF1A1, EF1A2, EF1B, EF1D, and EF1G. (C) Network analysis of 113 TCTP-interacting proteins by STRING. These proteins include the molecules related to protein translation highlighted with the red square. (D) Functional enrichment analysis in the network of TCTP-interacting proteins by the GO term “biological processes.” The top five significantly enriched GO groups are listed in rank order of their p values. The complete list of enriched GO terms is given in supplemental Table S4.
sible for binding to TCTP are domains I and II. These results demonstrate that TCTP specifically interacts with EF1A2 rather than with EF1A1.

**TCTP Inhibits Dimer Formation by EF1A2**—To discover the effect of TCTP binding on EF1A2, the binding status between TCTP and EF1A2 was predicted by docking simulations. In all ten docking structures with the highest scores, TCTP bound to the groove formed by the three domains of EF1A2 (Fig. 3A). Interestingly, the docking model also revealed that the areas of EF1A2 in contact with TCTP overlap with the EF1A2 dimer interface, based on a comparison of the model with the crystal structure (31) (Fig. 3B). This result suggests that TCTP binds to the EF1A2 dimerization region and may inhibit EF1A2 dimer formation. To test this prediction, FLAG-tagged and Myc-tagged EF1A2 were co-transfected into HeLa cells, and the relationship between EF1A2 dimer formation and the TCTP–EF1A2 interaction was examined. Surprisingly, the EF1A2–EF1A2 interaction showed the highest affinity relative to those of EF1A1–EF1A1 or EF1A1–EF1A2 (Fig. 3C). In addition, the EF1A2–EF1A2 interaction seemed to interfere with the TCTP–

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**Fig. 2. Validation of the interaction between TCTP and EF1A1/EF1A2 and the determination of the EF1A2 domains responsible for its binding to TCTP.** (A) Validation of the interactions between FLAG-tagged TCTP and Myc-tagged EF1A1/EF1A2 in HeLa cells by affinity purification using anti-FLAG antibody and Western blotting. (B) Validation of the interactions between FLAG-tagged EF1A1/EF1A2 and endogenous TCTP in MPNST cells by affinity purification using anti-FLAG antibody and Western blotting. (C, D) Identification of the domains responsible for the binding of EF1A2 and TCTP. (C) Evaluation of the interaction between each FLAG-tagged deletion mutant and endogenous TCTP in HeLa cells by affinity purification using anti-FLAG antibody and Western blotting. (D) Evaluation of the interaction between each chimeric protein containing EF1A1 and EF1A2 domains and endogenous TCTP in HeLa cells by affinity purification using anti-FLAG antibody and Western blotting.
EF1A2 interaction (Figs. 3C and 3D), suggesting that the interaction site of TCTP–EF1A2 competes with that of EF1A2–EF1A2. To further validate our hypothesis, a plasmid expressing native (nontagged) TCTP was co-transfected with plasmids expressing FLAG-tagged and Myc-tagged EF1A2 into HeLa cells, and the influence of TCTP on the EF1A2–EF1A2 interaction was examined. TCTP overexpression resulted in an increase of TCTP–EF1A2 interaction and effectively reduced the EF1A2–EF1A2 interaction (Figs. 3E and 3F), indicating that TCTP inhibits the EF1A2–EF1A2 interaction. Collectively, we conclude that TCTP binding changes dimeric EF1A2 to its monomeric form, which is assumed to be functionally active as a translation elongation factor (31).

**TCTP Regulates the Formation and Activity of the Elongation Factor Complex with EF1A2**—To address the functional differences between EF1A1 and EF1A2, we identified EF1A1- and EF1A2-interacting proteins by AP-DIA/SWATH analysis. Plasmids expressing FLAG-tagged EF1A1 and EF1A2 were constructed to purify interacting proteins using beads conjugated to a specific FLAG-tag antibody. The constructs were transfected into sNF96.2 NF1-associated MPNST cells, and interacting proteins were purified using anti-FLAG antibody and subjected to DIA/SWATH analysis after digestion with trypsin and lysyl endopeptidase (Fig. 4A). First, the quantitative data for the seven common peptides between EF1A1 and EF1A2 were used to normalize for the amount of bait protein. The factors for normalization of bait isoform amount (FNBIA, z) were calculated using the quantitative data of total intensity area value for the seven common peptides between EF1A1 and EF1A2 (supplemental Table S5) as follows:

$$z = \frac{y}{x_{EF1A1}}$$

where $y = \text{total intensity area value of SWATH quantitative data of the seven common peptides in EF1A1-IP experiment}$, and $x_{EF1A1} = \text{total intensity area value of SWATH quantitative data of the seven common peptides in each EF1A1- or EF1A2-FLAG-IP experiment}$ (FNBIA$_{EF1A1-1}$ = 1, FNBIA$_{EF1A1-2}$ = 1.09, FNBIA$_{EF1A1-3}$ = 1.10, FNBIA$_{EF1A2-1}$ = 0.74, 0.74, 0.74).
FIG. 4. TCTP positively regulates the interaction between EF1A2 and elongation factors. (A–F). Quantification of TCTP and elongation factors bound to EF1A1- and EF1A2-FLAG by AP-DIA/SWATH MS analyses. (A) Workflow for the AP-DIA/SWATH MS. (B, C) The identified EF1A1- and EF1A2-binding proteins by AP-DIA/SWATH MS. The X-axis indicates the log2(fold change of EF1A1/FLAG alone and EF1A2/FLAG alone), and the Y-axis indicates the -log10(p value of FLAG alone versus TCTP). Proteins at a significance level of p/H110210.05 and fold change >10 compared with the FLAG alone-IP fraction were annotated with their abbreviations. The proteins highlighted with red letters show that the elongation factor complex consisted of EF1A1, EF1A2, EF1B, EF1D, EF1G, and VARS/SYVC. (D–H) The amount of (D) TCTP, (E) EF1B, (F) EF1D, (G) EF1G, and (H) VARS bound to EF1A1- and EF1A2-FLAG, as quantified by AP-DIA/SWATH MS. *p < 0.01 versus AP-DIA/SWATH
MPNST cells and quantified the EF1A2-interacting proteins by TCTP siRNA and an EF1A2-FLAG-expressing plasmid into EF1G, and VARS. To test this hypothesis, we co-transfected the normalization by FNBIA, the data using FNBIA was compared using with the data using trypsin signal adjustment factors, as shown in Fig. S6. The total DIA ion intensity of proteins in EF1A2 fraction is nearly two times higher than that in EF1A1 without any normalization factor (Fig. S6A). Despite the normalization of trypsin signal, total DIA ion intensity of proteins in EF1A2 fraction remains to be almost two times higher than that in EF1A1 (Fig. S6C). After the normalization by FNBIA, total DIA ion intensity of proteins in EF1A2 fraction becomes almost same level as EF1A1 (Fig. S6E).

To identify significant interactors of EF1A1 and EF1A2, the EF1A1 and EF1A2 AP-DIA/SWATH data were compared with FLAG alone data as a control (Figs. 4A–4C). The components of elongation factor complex, such as EF1B, EF1D, EF1G, and VARS, dominantly were included in both data, whereas one of the most confident EF1A2-interacting proteins was TCTP, which was not included in the EF1A1 data, in accord with Western blot analysis (Fig. 2B). The comparison of the amount of interacting proteins between EF1A1 and EF1A2 shows that these proteins have the higher affinities with EF1A2 than those with EF1A1 (Fig. S6F, Figs 4D–4H, and supplemental Table S6). Therefore, we hypothesized that TCTP contributes to the binding activity of EF1A2 to other elongation factors that form the EF1 elongation factor complex, such as EF1B, EF1D, EF1G, and VARS. To test this hypothesis, we co-transfected TCTP siRNA and an EF1A2-FLAG-expressing plasmid into MPNST cells and quantified the EF1A2-interacting proteins by AP-DIA/SWATH analysis (Fig. 4I). Although the TCTP knockdown efficiency was 79% compared with the control, the quantity of elongation factors bound to EF1A2, especially EF1B (25%) and VARS (20%), was significantly decreased by TCTP knockdown in MPNST cells (Figs. 4J–4O and supplemental Table S7). To evaluate which EF1A2-interacting proteins were significantly reduced by TCTP knockdown with siRNA, the amounts of these proteins were quantified as the ratios of the TCTP-siRNA/control-siRNA data. The 30 proteins of high-confident EF1A2-interactors (p value <0.05 and fold change >4; 206 proteins, listed in supplemental Table S7), including elongation factors such as EF1B and EF1G, significantly reduced by TCTP-knockdown (Fig. S7). We confirmed that TCTP knockdown did not influence the expression of these elongation factors by quantifying their protein levels in the cell lysates using DIA/SWATH analysis (Fig. S8 and supplemental Table S8). These data indicate that TCTP regulates the formation of the EF1A2-elongation factor complex.

Protein Synthesis Associated with the Growth of MPNST Cells Largely Depends on EF1A2–TCTP Function—To evaluate the differences in the biological functions of EF1A1 and EF1A2 in NF1-associated MPNST cells, we constructed specific siRNAs for EF1A1 and EF1A2 to knock down their expression. These siRNAs were transfected into two MPNST cell lines, sNF96.2 and sNF02.2, and into normal HSCs; decreased expression levels were confirmed by RT-PCR (Figs. S9A and S9B). Interestingly, knockdown of EF1A2 caused significant inhibition of MPNST cell growth, which was not observed for the EF1A1 knockdown; however, knockdown of EF1A2 did not inhibit HSC growth at all (Figs. 5A–5C), indicating that EF1A2 function is essential for MPNST cell growth. To further evaluate the function of EF1A2 in MPNST cells, the protein expression statuses in EF1A1- and EF1A2-deficient MPNST cells and in HSCs were profiled by DIA analysis (Fig. 5D). We confirmed EF1A1 and EF1A2 down-regulation in MPNST cells and in HSCs by DIA/SWATH analysis (Figs. S9C–9F). In total, 2,557 and 2,612 proteins, including 2,168 overlapping proteins, were quantified in MPNST cells and in HSCs, respectively (Fig. 5E and supplemental Tables S9 and S10). Among these quantified proteins, differentially expressed proteins were selected based on a fold-change criterion (more than 1.2 or less than 0.83) and on the p value of Student’s t test (less than 0.05). In MPNST cells, 264 and 374 proteins were up- and down-regulated by EF1A2 knockdown, respectively (Figs. 5F and 5G and supplemental Table S9). Of the 374 proteins, 234 proteins were specifically down-regulated by EF1A2 knockdown but not by EF1A1 knockdown in MPNST cells (Fig. 5G and supplemental Tables S9 and S10). To characterize the functions of these 234 proteins, we performed GO enrichment analysis. These proteins include molecules related to “translation (p = 5.8 × 10−10),” “cell cycle (p = 3.3 × 10−4),” “mRNA metabolic process (p = 1.7 × 10−10),” “protein transport (p = 7.5 × 10−7),” “viral process (p = 7.9 × 10−7),” “protein folding (p = 2.0 × 10−6),” and “nuclear export (p = 2.4 × 10−5).” We also compared the data from the GO enrichment term “translation” among the differentially expressed proteins in MPNST cells and HSCs treated with EF1A1 or EF1A2 siRNAs (Fig. 5H and supplemental Table S11). The data showed that both the full set of 374 down-
regulated proteins and the nonoverlapping set of 234 down-regulated proteins in MPNST cells treated with EF1A2 siRNA predominantly contained translation-related molecules, while the 191 down-regulated proteins in MPNST cells treated with EF1A1 siRNA and the 159 down-regulated proteins in HSCs treated with EF1A2 siRNA did not predominantly include them (Fig. 5/ and supplemental Table S11). These data suggest that the functions of EF1A2 in NF1-associated tumors are different from those of EF1A1 in NF1-associated tumors and/or of EF1A2 in normal HSCs and that EF1A2 predominantly regulates tumor cell translation and growth in NF1-associated tumors. To validate this idea, the protein synthesis activity in EF1A1- and EF1A2-deficient MPNST cells was measured using AHA, a surrogate of L-methionine. In EF1A2-deficient MPNST cells, AHA-containing de novo polypeptides were significantly decreased, while EF1A1 knockdown did not elicit

**Fig. 5.** EF1A2 mediated-translation induces growth in NF1-associated MPNST cells. (A–C) Evaluation of the effect of EF1A1 and EF1A2 knockdown on the growth of (A) sNF96.2 and (B) sNF02.2 MPNST cells and on (C) HSCs using a CCK-8 assay. The growth of these cells was evaluated 48 h after transfection with EF1A1 and EF1A2 siRNA. *p < 0.01 versus control siRNA (Student’s t test). (D) Workflow for the quantification of proteins expressed in sNF96.2 MPNST cells and in HSCs treated with EF1A1 and EF1A2 siRNA. (E) Venn diagram of the quantified proteins in MPNST cells and HSCs, including 2,168 overlapping proteins. (F, G) Venn diagram of (F) up-regulated and (G) down-regulated proteins by EF1A1 and EF1A2 knockdown in MPNST cells and HSCs. (H) GO enrichment analysis of 234 down-regulated, nonoverlapping proteins in MPNST cells treated with EF1A2 siRNA. The representative enriched GO terms are listed. The complete list of enriched GO terms is given in supplemental Table S11. (I) Comparison of GO “translation” enrichment between sets of differentially expressed proteins. (J) Evaluation of the quantity of de novo polypeptides using AHA to analyze translational activity in MPNST cells treated with EF1A1- and EF1A2-siRNA.
any decrease (Fig. 5J). These results indicate that the protein synthesis machinery in MPNST cells depends on EF1A2 rather than EF1A1.

To evaluate the difference of TCTP function on EF1 complex in MPNST cells and normal HSCs, the amounts of these elongation factors bound to TCTP were quantified in each cell. Plasmids expressing FLAG-tagged TCTP were transfected into sNF96.2 MPNST cells and normal HSCs, and interacting proteins were purified using anti-FLAG antibody and subjected to quantitative MS analysis (Fig. S10A and supplemental Table S12). The amount of TCTP was used to normalize the amount of elongation factors bound to TCTP (average TCTP ion intensities: HSC = 1.86 × 10^5; MPNST = 8.74 × 10^5). The amount of EF1A2 bound to TCTP in normal HSCs was hardly detected compared with MPNST cells (Fig. S10B) beside the detectable expression level of EF1A2 in normal HSCs analyzed by DIA/SWATH MS (Fig. S9F). In contrast, the amounts of other elongation factors bound to TCTP were detectable in both cells (Figs. S10C–S10F). Especially, the EF1A1 amount in normal HSCs was higher than that in MPSNT (Fig. S10C). These results show that the TCTP interaction with EF1A1 and/or EF1A2 seems to determine its function on EF1 complex in each cell. Collectively, our findings suggest that the TCTP interaction with EF1A2 prominently found in MPNST cells (Fig. S10B) is essential to exert EF1A2-dependent translation, positively regulating the growth in NF1-associated MPNST cells but not in HSCs (Fig. 5).

Artesunate Inhibits the TCTP–EF1A2 Interaction and Down-regulates Protein Synthesis in MPNST Cells—To inhibit the effect of TCTP on EF1A2, we used the artemisinin derivative artemesunate, which has been reported to bind to TCTP (32–34). To learn whether artemesunate inhibits the TCTP–EF1A2 interaction, the binding of TCTP to EF1A2 was evaluated in the presence of artemesunate and hemin, the activator of artemisinin and its derivatives (Fig. 6A). TCTP binding to EF1A2 was significantly inhibited in the presence of artemesunate and hemin (Fig. 6A). We also evaluated the binding of TCTP to EF1A2 in MPNST cells expressing EF1A2-FLAG treated with artemesunate (Fig. 6B). TCTP binding to EF1A2 was significantly inhibited in the presence of artemesunate in vivo (Fig. 6B). Next, the protein synthesis activity of MPNST cells treated with artemesunate was measured using AHA to evaluate whether artemesunate treatment would significantly affect the protein translation machinery. A dose-dependent inhibition of protein synthesis (Fig. 6C), followed by a decrease in cellular dimensions and induction of cell damage (2), was observed following artemesunate treatment in MPNST cells. These results suggest that the functional disruption of TCTP–EF1A2 by artemesunate leads to the inhibition of protein synthesis activity followed by NF1-associated MPNST cell damage (2). However, further study will be needed to understand whether these effects are involved in another iron-regulated process mediated by artemesunate besides its effect on TCTP–EF1A2 binding.

DISCUSSION

In our previous study, we demonstrated that TCTP knock-down/overexpression causes the defect/promotion of NF1-deficient cell growth (2). To investigate the role of TCTP function in NF1-associated tumors, in this study, we identified TCTP-interacting proteins with a sequential interactome approach using quantitative AP-DIA/SWATH. We demonstrate that TCTP significantly interacts with proteins related to protein translation and stress responses, suggesting that TCTP has essential roles in the protein translation machinery and in stress response/defense during NF1-associated tumorogenesis. Among these proteins, translation elongation factors showed the most significant interactions with TCTP. Our results clearly indicate that TCTP forms a specific complex with translation elongation factors via its interaction with EF1A2, which was identified as an important functional translation factor in this study. Our data also uncover a specific TCTP function as a positive regulator of the EF1A2-mediated translation elongation machinery, especially in NF1-associated malignant tumors.

We schematically summarized our findings about the effect of TCTP on EF1A2 in NF1-associated tumors by comparing the elongation machinery of TCTP–EF1A2 with the known canonical elongation cycles in eukaryotic cells (Fig. 7A). EF1A is a G-protein that is responsible for the delivery of aminocyl tRNAs to the ribosome. EF1A-GTP is hydrolyzed during the entrance of the tRNA into the A site of the ribosome, and EF1A-GDP is released from the ribosome. The GTP exchange factor complex for EF1A, composed of EF1B, EF1D, and EF1G, induces the exchange of GDP for GTP bound to EF1A. The EF1A dimer is thought to be an inactive form of the translation machinery, as it has been reported that the EF1A dimer has functions outside the ribosome (35–37). Our data demonstrate for the first time that TCTP inhibits EF1A2 dimer formation and facilitates the interaction of EF1A2 with other elongation factors. Thus, in addition to the "canonical" translation elongation machinery, TCTP functions on EF1A2 to regulate growth-related translation in NF1-associated tumor cells (Fig. 7B). Inhibitors of TCTP–EF1A2 binding, such as artemesunate, down-regulate the specific translational machinery in tumor cells; such inhibitors could serve as therapeutic agents against NF1-related tumor formation (Fig. 7C).

We previously demonstrated that TCTP is an NGF-inducible protein that regulates neurite outgrowth with antiapoptotic functions in differentiating PC12 cells (8) and that TCTP up-regulation in neurofibromin-deficient NF1-model PC12 cells (1) and Schwann cells activates cell growth or inhibits neural differentiation. These effects cause an increase in the dimensions of cells related to malignant NF1 tumors (2). Interestingly, EF1B and EF1G, which were identified as TCTP-binding proteins in this study, were up-regulated in neurofibromin-deficient PC12 cells after NGF stimulation in our previous proteomic study (1). Because major components of the trans-
Translation Machinery via TCTP–EF1A2 in NF1 Tumors

Fig. 6. The effect of artesunate on the TCTP–EF1A2 interaction and on translational activity in MPNST cells. (A) Lysates from MPNST cells expressing EF1A2-FLAG were prepared and treated with 500 μg/ml artesunate and 50 μM hemin at room temperature for 24 h. Cellular EF1A2-FLAG was purified by anti-FLAG antibody, and the bound TCTP was analyzed by Western blotting using anti-TCTP antibody. (B) MPNST cells expressing EF1A2-FLAG were treated with 20 μg/ml artesunate for 24 h. The lysates from these cells were prepared, and cellular EF1A2-FLAG was purified by anti-FLAG antibody, and the bound TCTP was analyzed by Western blotting using anti-TCTP antibody. (C) MPNST cells were treated with 5, 10, and 20 μg/ml artesunate for 24 h. The amount of de novo polypeptides in these cells was evaluated using AHA to analyze cellular translational activity.

The translational machinery are frequently up-regulated in human cancers, including neurofibromatosis-associated tumors (38), it may be reasonable to assume that the up-regulation and interaction of both TCTP and elongation factors contributes to the hyperactivation of protein synthesis in NF1-associated tumors.

Our quantitative AP-DIA/SWATH analysis using NF1-deficient MPNST cells significantly identified a specific interaction...
Fig. 7. A specific translation elongation machinery mediated by the effect of TCTP on EF1A2. (A) “Canonical” translation elongation machinery in eukaryotic cells. Eukaryotic elongation factor 1A (EF1A) delivers the aminoacylated tRNA to the A-site of the ribosome. GTP is hydrolyzed during the entrance of the tRNA into the A-site of the ribosome, and eEF1A-GDP is released from the ribosome. The GTP exchange factor for EF1A is a complex with three subunits, namely, EF1B, EF1G, and EF1D, which induces the exchange of the GDP for GTP. The EF1A dimer is thought to be an inactive form of the translation machinery. (B) Elongation machinery mediated by the effect of TCTP on EF1A2 and observed in NF1-associated tumor cells. Our results demonstrate that TCTP inhibits EF1A2 dimer formation and recruits elongation factors to EF1A2 to activate growth-related translational machinery in NF1-associated tumor cells. (C) Our data showed that artemisin inhibits the interaction of TCTP-EF1A2 and its GEF complex, causing the inactivation of EF1A2-induced translation function in NF1-associated tumor cells.
between TCTP and EF1A2. EF1A exists as two separately encoded tissue-specific isoforms, EF1A1 and EF1A2. These two isoforms are 92% identical and 98% similar to each other at the amino acid level. Although functional differences between EF1A1 and EF1A2 have not been fully clarified, their expression patterns in tissues are quite different. EF1A1 is almost ubiquitously expressed, whereas EF1A2 is confined to nerve tissues and skeletal muscle. Hence, the EF1A2–TCTP interaction could be identified in our study thanks to the use of neural cells. On the other hand, only a TCTP–EF1A1 interaction was observed in previous studies using U937 (18) and HeLa cells (29), as EF1A2 expression might have been weak or absent in these cells. It is very difficult to use an antibody-based approach to detect the EF1A2 protein because almost all commercially available EF1A2 antibodies recognize both EF1A1 and EF1A2. As antibodies that specifically recognize EF1A2 but not EF1A1 have not yet become available, other methods, such as a targeted proteomics by mass spectrometry, multiple/selected reaction monitoring or DIA/SWATH, must be used to quantitatively identify EF1A2. In this study, we identified and used a unique peptide that was present in the EF1A2 sequence but not in the EF1A1 sequence in the AP-DIA/SWATH analysis to establish an easy method for the quantification of EF1A2. Therefore, we identified preferential interaction between TCTP–EF1A2 over TCTP–EF1A1 in NF1-associated tumors by an MS-based approach.

Our observations indicate that both EF1A1 and EF1A2 interact with other elongation factors such as EF1B, EF1D, and EF1G, but the interaction of EF1A2 with TCTP is much higher than that of EF1A1. If TCTP–EF1A2 interaction is mainly mediated by another protein such as EF1B, EF1A1 should considerably interact with TCTP indirectly. However, we did not observe considerable level of TCTP–EF1A1 interaction compared with the TCTP–EF1A2 interaction. The specific TCTP–EF1A2 interaction is thought to facilitate the guanine nucleotide exchange reaction by recruiting its GEF complex, as TCTP interacts with other elongation factors and regulates the formation of the EF1A2-elongation factor complex. On the other hand, previous reports demonstrated that TCTP functions as a guanine nucleotide dissociation inhibitor of EF1A1 (19). We conclude that the binding modes of TCTP to EF1A1 and EF1A2 are quite different, as the TCTP–EF1A2 interaction, which not only needs domains I + II but also the overall structure, is highly specific compared with the TCTP–EF1A1 interaction. We therefore posit that TCTP has different effects on EF1A1 and EF1A2. As shown in previous studies (19, 23), TCTP is thought to have important functions as a positive regulator of the elongation machinery, though the underlying mechanism has not yet been fully clarified. Further investigation is needed to uncover the precise functions of TCTP as an elongation regulator.

Recently, EF1A2 was thought to be an oncogene, as it has been reported that its mRNA expression or EF1A1/2 antibody reactions are up-regulated in several kinds of cancers, including ovarian cancers (39), breast cancers (40, 41), hepatocellular carcinoma (42), and lung cancers (43, 44). On the other hand, previous reports have demonstrated that TCTP expression is also up-regulated in several cancers, such as breast cancers and hepatocellular carcinomas (45). However, the functional interactions between EF1A2 and TCTP in those cancers have not yet been studied. Our previous work showed that TCTP expression is significantly up-regulated under NF1 deficiency and is correlated with the malignancy of NF1-associated tumors (2). As neural cells selectively express EF1A2, the translation machinery mediated by EF1A2 may tend to be induced in neural tumor cell growth or dimensional expansion. TCTP up-regulation in NF1-associated tumors should positively regulate EF1A2 function and be correlated with malignancy. We hypothesize here that TCTP activates translation elongation via its interaction with EF1A2 in NF1-associated tumors. Our findings indicate a substantial functional effect of the TCTP protein on EF1A2 in the genesis and progression of NF1 tumors. They also suggest that agents targeting the TCTP–EF1A2 interaction, such as artesunate, could be developed as an effective new therapeutic approach for NF1-associated tumors, which are inexcisable even after multiple surgical resections.

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DATA AVAILABILITY
All MS raw data were stored in jPOSTrepo (46) (https://repository.jpostdb.org/). jPOSTIDs/PXIDs for the projects containing these data were JPST000107/PXD007578 (for TCTP-binding proteins identified by AP-DIA/SWATH), JPST000310/PXD007607 (for TCTP-binding proteins identified by gel-based proteome analysis), JPST000307/PXD007604 (for EF1A1, and EF1A2-binding proteins identified by AP-DIA/SWATH), JPST000309/PXD007605 (for EF1A2-binding proteins in TCTP-deficient MPNST cells identified by AP-DIA/SWATH), JPST000311/PXD007606 (for the expression profiling of EF1A2-binding proteins in TCTP-deficient MPNST cells analyzed by DIA/SWATH), and JPST000308/PXD007603 (for the identification of differentially
expressed proteins in EF1A1- and EF1A2-deficient MPNST cells and HSCs analyzed by DIA/SWATH.

The authors declare no competing financial interests.

This article contains supplemental material Tables S1–S13 and Figs. S1–S10.

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Author contributions: D.K. and N.A. designed research; D.K., T.T., and N.A. performed research; D.K., T.T., H.O., and N.A. analyzed data; D.K. and N.A. wrote the paper; N.A. contributed new reagents/analytic tools; K.S. performed structural predictions; and M.N., M.-K., S.O., and N.A. performed the analyses by mass spectrometry.

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