TFE3 fusions activate MET signaling by transcriptional upregulation, defining another class of tumors as candidates for therapeutic MET inhibition

Masumi Tsuda, Ian J. Davis, Pedram Argani, Neerav Shukla, Gael G. McGill, Makoto Nagai, Tsuyoshi Saito, Marick Laé, David E. Fisher, and Marc Ladanyi

Supplementary methods

Cell culture. 293T, Cos-7, HeLa, A673, and HS-SY-II cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). FUJI cells, RH30, and MCF-7 were maintained in RPMI 1640 medium containing 10% FBS, and MEM non-essential amino acids and 1 mM sodium pyruvate were added to the medium for MCF-7 cells. FU-UR-1 renal carcinoma cells were maintained in DMEM/F12 medium supplemented with 15% FBS.

RNA extraction and cDNA microarray analysis. cDNA microarray hybridization was performed according to the Affymetrix expression analysis technical manual (Affymetrix, Santa Clara, CA). Total RNA was extracted in Trizol reagent and purified using the RNEasy kit (Qiagen, Valencia, CA). cRNA was prepared using biotinylated uridine triphosphate and cytosine triphosphate, and then incubated at 94°C for 35 min in a fragmentation buffer (200 mM Tris-acetate pH 8.1, 500 mM KOAc, 150 mM MgOAc) to produce a distribution of RNA fragment sizes from 35 to 200 bp. cRNA quality was verified on a test array (TEST3 chip) and then hybridized to HG_U133A oligonucleotide arrays (Affymetrix), which contain 22215 probe sets for approximately 18500 sequence verified transcripts.

Compounds. PD98059 (MEK1 inhibitor), LY294002 (PI3 kinase inhibitor), and Rapamycin (FRAP/mTOR inhibitor) were purchased from Cell Signaling, and used at the concentrations described in the Results section.

Adhesion assay. At 72 hours after transfection of *MET*- or control-siRNA into FU-UR1 cells, 4 x 10^4 cells were suspended in 0.1 ml of DMEM containing 0.02% BSA. The cells were plated on 96-well plates coated with collagen IV, fibronectin, or laminin/fibronectin, and incubated for 1 hour at 37°C with or without 50 ng/ml HGF. The bound cells in each well were lysed, stained with 0.2% crystal violet, and quantified by spectrophotometer at O.D. A590 nm.

Wound healing assay. This assay involves creating a "wound" by scraping a line in a cell monolayer, and then examining the effects of a given treatment on directional cell migration over time. Briefly, 72 hours after transfection of MET- or control-siRNA to FU-UR1 cells, cells were scraped-off /wounded using a yellow tip, and incubated with or without 50 ng/ml HGF. After another 16 or 24 hours, the distances of cell movement were measured.

Invasion assay. For this assay, the Bio Coat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ) was used and 40000 cells were seeded in the upper chambers (24-well chambers) in 0.5 ml of complete DEME/F12 medium, and the lower chambers contained complete medium with or without 50 ng/ml HGF as a chemo-attractant. After 22 hours, the non-invading cells on the upper surface of the filters were removed by wiping with a cotton swab. Cell at the bottom side of the membranes were fixed 3.7 % formaldehyde in PBS, and stained with 0.2 % cresyl violet. The number of cells invading through the matrigel was counted under microscope.

Supplementary Figure Legends

Supplementary Figure 1. FU-UR1 cells were treated with 1 μM PHA665752, 50 μM PD98059, or 50 μM LY294002 for 3 hours followed by treatment of with 50 ng/ml HGF for 30 min or no treatment. The phosphorylation status of MET and of its downstream proteins was examined by immunoblotting using Abs as indicated at the right of the panels.

Supplementary Figure 2. Effects of MET, ERK, PI3K, or FRAP/mTOR selective inhibitors on cell viability. **A.** Cell lines were treated with the ERK inhibitor PD98059, the PI3K inhibitor LY294002, and rapamycin, a FRAP/mTOR inhibitor, at the indicated doses for 4 days. Live cells were counted under the microscope, and the numbers normalized to that without inhibitor (indicated as 0 μM) and described as a proportion. **B.** The cell growth of FU-UR1 cells treated with 0.5 μM of the MET selective inhibitor PHA665752 and/or 10 nM rapamycin was examined every other day over 8 days.

Supplementary Figure 3. Effects of MET depletion on cell adhesion, scattering, motility, and matrigel invasion in ASPL-TFE3-positive cells. A. Effects of depletion of MET on HGF-dependent adhesion in FU-UR1 cells. FU-UR1 cells were transfected with or without MET siRNA or non-specific siRNA as control. After 72 hours, 4×10^4 cells were plated on 96-well plates coated with collagen IV, fibronectin, or laminin/fibronectin, and incubated for 1 hour at 37°C with or without 50 ng/ml HGF. The adherent cells in each well were stained with 0.2% crystal violet, lysed with DMSO,

and quantified by spectrophotometer at O.D. A590 nm. Data indicate means ± SD of values from more than three independent experiments. **B.** Analysis of cell motility and scattering by wound healing assay. FU-UR1 cells were transfected with MET siRNA or non-specific siRNA as control. After 72 hours, the confluent cells were scraped-off/wounded using a tip, and further incubated with or without 50 ng/ml HGF for additional 24 hours. The pictures were taken at 0, 16, and 24 hours after wound formation and the distances of cell movement were measured. Top: Representative images at 0 or 16 hours after wound formation in the presence of 50 ng/ml HGF are displayed. Bottom: The graphs indicate the averages of the cell movement distances in the absence of HGF (left panel) or in its presence (right panel) obtained in three independent experiments; bars, ± SD. Statistical analyses were performed by Student's *t* test. *, value of MET knock-down cells was significantly different from MOCK cells at 16 hrs after wound formation, at $P < 0.0001$. MOCK, cells treated with transfection reagent; Con. si, cells transfected with non-specific siRNA; MET-si, MET knock-down cells. **C.** Analysis of matrigel invasion in MET knock-down FU-UR1 cells. FU-UR1 cells were transfected with MET siRNA or non-specific siRNA as control; 72 hours after transfection, 4×10^3 cells were seeded in the upper chamber of a matrigel invasion chamber (Bio Coat), and further incubated for 22 hours with or without 50 ng/ml HGF in the lower chamber. The numbers of invading cells were counted under the microscope. Data indicate means ± SD of values from three independent experiments.

Supplementary Figure 4. Exogenous ASPL-TFE3 is phosphorylated on Serine residues in 293T cells and in FU-UR-1 cells. Following immunoprecipitation with an antibody to

the MYC tag, immunoblotting with anti-phosphoserine antibody shows that both full-length TFE3 and ASPL-TFE3 (both forms) are phosphorylated on Serine residues. The weaker results in the FU-UR-1 cell line may reflect lower expression of the exogenous constructs than in 293T cells.