Supplemental Materials and Methods

Vectors and constructs. Reporter constructs for the MET promoter, MET promoter with both ETS sites mutated (METp, ΔE1ΔE2), and a vector with a minimum promoter (for the RET gene, cRetpm-Luc), as well as expression constructs for ETS1, PAX3, and DN-ETS (pcDNA3-ETS1-DN-IRES-GFP) were previously constructed as described (1). An expression construct for MET, pT3-EF1aH c-Met was a gift from Xin Chen (Addgene plasmid # 86498, RRID:Addgene_86498) (2). Primers containing four common ETS binding domains (3) were cloned 5' of the minimum promoter of cRetpm-Luc to create an ETS reporter vector (EBD-cRetpm-Luc). The ETS binding domain oligonucleotide (5'-TCG AGA CCG GAA GTA CCG CAG AGG AAG TGC CCC ACC GGA TGT ACC GCC CCG GAA GTA CCC-3') was inserted between the XhoI and NcoI sites of the pGL2 backbone. HA tag containing wild-type ETS1 and ETS1 Δ4 were cloned into pcDNA3 using Gibson Assembly Cloning Kit (New England Biolabs) following the manufacturer's instructions. Primers used for creating alanine mutations are 5'- GCA GTG GAC CAA TCG CGC TAT GGC AG-3' for Q336A, 5'-CTG AGC CGT GGC CTA CGC GCC TAT TAC GAC AAA AA-3' for Y395A, and 5'-TAC GCC GCA AAC ATC ATC CAC AAG ACA GCG-3' for mutation of the last four epitopes K399A and D398A. All vectors constructed were verified through DNA sequencing.

Luciferase Reporter Assays. Cells were transfected with either Lipofectamine 2000 (Invitrogen)(293T cells) or Trans-IT 2020 transfection reagent (Mirus)(A375 and MEL624 cells) according to manufacturer's protocols. Cells were transfected with promoter reporter constructs with a luciferase expression cassette and a vector expressing an independent reporter normalization control (either renilla or beta-galactosidase), with or without the addition of expression vectors. For HGF studies, 30 ng/ml of HGF was added to the growth media 7-10 minutes prior to cell collection and analysis. For YK-4-279 studies, 1 μM of drug was added to the media. For calculation of fold induction, arbitrary light units were measured in each sample, values were normalized against renilla or beta-

galactosidase activity, and divided by the readings for reporter constructs alone. Each experiment was conducted at a minimum in triplicate.

Data Mining. The UCSC Cancer Genomics Browser was used to assess the gene expression of PAX3 and ETS family members in human TCGA Skin Cutaneous Melanoma samples (n=473), using IlluminaHiSeq RNASeq, normalized to all TCGA cohorts (16 cancer subtypes in total) and Log2(x+1) transformed. The data demonstrated a melanoma expression pattern depicted as a proportions plot with the red indicating higher expression and the blue lower expression.

Reverse-transcriptase PCR. RNA was isolated from cultured cells using Trizol (Invitrogen). Complementary DNA (cDNA) was created using the BioRad iScript cDNA synthesis (BioRad) according to the manufacturer's instructions. Samples without the addition of reverse transcriptase were used as a control against genomic DNA contamination. PCR for the ETS family transcription factors used primers previously described (4). MCF10a and MCF7 cell RNA were used as positive controls.

MTT/MTS Assays. Cells were seeded in 96 well plates at a density of 5,000 (MTT) or 10,000 (MTS) cells/well. Cells were dosed with YK-4-279 or DMSO alone with fresh drug added every 24 hours. At end points, either MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or CellTiter 96® AQueous One Solution containing MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added directly to each well. After an incubation at 37°C, colorimetric absorbance was read at 570 nM (MTT) or 490 nM (MTS) with the minimum of duplicate wells and experiments repeated in triplicate.

Invasion Assay. The Corning Matrigel Basement Membrane Matrix (Cat# 356230 Lot#7107329) was diluted to 200-300ug/ml in sterile 0.01M Tris (pH8.0) 0.7% NaCl and 100ul of the diluted mixture was pipetted into each falcon cell culture insert (Corning, 8uM, cat#353097). After solidification of the matrix, A375 and SKMEL28 cells were seeded into the wells at 50% confluency. Six hours post cell

seeding media with 0.05% DMSO or 2uM YK-4-279 (diluted in 50% DMSO) was added, with media supplemented with 5% FBS within the insert and 10% in the lower well. At 16 hours post drug addition, a cotton swab was used to remove cells on the top of the insert, and cells were fixed and stained following the Kwik-Diff protocol (Fisher, 9990700). The membrane from the insert was then excised with a blade and mounted on a glass coverslip for cell visualization. Five images were taken from each condition and total cell number in each field was counted using the imageJ cell counter application. The numbers from each image were summed up to get an approximate “total” cell number per well. Experiments were performed in triplicate.

Zymogram. Cells treated with YK-4-279 (0.0, 0.1, 0.5, 1.0, 2.0 uM) for 7 days, and the media was collected then concentrated to 20x using centrifugal filters (Amicon® Ultra-2 mL Centrifugal Filters for DNA and Protein Purification and Concentration, Millipore, UFC200324). Cells were lysed with urea buffer and total protein concentration was quantified following the Pierce 660nm Protein Assay Reagent (Pierce 660nM, Fisher, 22660) with ionic detergent compatibility reagent protocol (Ionic Detergent Compatibility Reagent for Pierce™ 660nm Protein Assay Reagent, Fisher, 22663). The media was normalized using the total protein concentrations from the cells. 5x non-reducing sample buffer was added to the samples then loaded onto the novex 10% zymogram plus gel (Novex™ 10% Zymogram Plus (Gelatin) Protein Gels, 1.0 mm, 10-well, Fisher, ZY00100BOX). Gels were incubated in 1x zymogram renaturing buffer (Novex Zymogram Renaturing Buffer, Fisher, LC2670). The buffer was then decanted and 1x zymogram developing buffer (Novex Zymogram developing buffer, Fisher, LC2671) was added to the gel. After development, the buffer was decanted and Coomassie blue stain (Bio-Safe™ Coomassie Stain, Biorad, 1610786) was added for 1 hour and then destained (Coomassie Brilliant Blue R-250 Destaining Solution, biorad, 1610438). Bands were visualized on the BioRad chemidoc.

Proximity Ligation Assay (PLA). Cells or tissue were fixed with PFA, fixation quenching with 50 mM NH_4Cl, permeablized with a 0.25% TritonX-100 solution, and blocked with Duolink Blocking solution (Duo-link In Situ Red Starter Kit Mouse/Rabbit, Sigma, Duo92101). Samples were incubated with primary antibodies: PAX3 (1:100) (Abcam, Ab180754), ETS1(1:50) (Santa Cruz Cat#sc-55581, RRID:AB_831289) and/or ETV5 (1:200)(R&D Systems, Cat# MAB7107, RRID:AB_10997135). The samples were incubated with secondary antibody conjugated with PLA probes (PLUS and MINUS, Duo92101), treated with ligase, and PLA signal was amplified with the addition of polymerase (Duo92008). The slides were mounted with coverslips with minimal volume of duolink in situ mounting medium with DAPI. Five representative images were captured from each group. For each image, puncta within the nucleus were counted then divided by the number of cells per image to calculate nuclear puncta per cell. For mouse tumor sections, a sequential cut of the same tissue was stained with anti-PAX3 and F594 anti-mouse IgG secondary antibody as a control to normalize the PAX3 positive tumor cells for PLA analysis. PAX3 positive tumor cells from each mouse were counted as a method for normalizing puncta counts, and nuclear puncta numbers/PAX3 expressing nuclei per cell were calculated.

Co-Immunoprecipitation analysis. 293-T cells were transfected with plasmid constructs expressing human PAX3, ETS1 WT, ETS1 Δ4 or empty vector pcDNA3 for 24 hours. Cells were lysed with M-PER mammalian protein extraction buffer (Thermo Scientific) and centrifuged, with the protein collected in the supernatants. Protein extraction was diluted and incubated with control IgG, anti-PAX3 or HA-tag antibody (0.5-1ug) in 0.5 ml M-PER buffer containing a 1x protease inhibitor cocktail (Thermo Scientific) for 30 min at 4 °C, then with protein A/G magnetic beads (EMD Millipore) for an additional 30 min. The resulting immunocomplex were washed five times in PBS-T buffer, resuspended in 2 X SDS sample buffer, and then resolved on 4-15 % SDS–polyacrylamide gel electrophoresis gels and evaluated by standard western blot analysis with indicated antibodies.

MET or ETS1 rescue from YK-4-279 cell loss. For each cell line (A375, MEL624, SKMEL28) cells were divided into three groups transfected with expression constructs (empty vector (mock), ETS1, MET) and treated with either 2 μM YK-4-279 or DMSO. After 48 hours, all cells are counted on an Incucyte FLR live cell analysis system (Essen Bioscience/Sartorius). For each group, DMSO treated cell numbers are set at 100%, and percent survival is calculated as the difference in cell numbers between DMSO and YK-4-279 treated sets. To determine if there was a significant partial or full rescue, percent cell survival numbers for ETS1 and MET were compared to mock transfected and analyzed with one tailed student t tests with mock groups set as controls, and ANOVA analysis of all three groups. For each experiment, at least 400 cells per group was counted, and the experiments were performed minimally in triplicate.

Antibody array for phosphorylated receptor tyrosine kinases (RTKs). Cell lysates from two melanoma cell lines, MEL624 and A375, were assayed for RTK phosphorylation with an array (Human Phospho-RTK Array Kit (R&D Systems) following manufacturer's protocols. Briefly, Cells were solubilized in 250 μL of lysis buffer 17 with addition of 1x Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) and incubated at 4^{o}C on a rocker for 30 minutes. Lysates were centrifuged at 14,000g for five minutes and supernatant collected. Array membranes wee blocked with a BSA solution (RTK Array Buffer 1) and developed with a horseradish peroxidase-conjugated phosphor-tyrosine detection antibody (1:5000). Array membranes were imaged with a ChemiDoc Imager System (Bio-Rad, Hercules, CA). Relative intensities of the averaged signal from each pair of duplicated spots were determined after normalization with averaged signal from the loading controls and quantified using ImageJ.

References for supplemental methods

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