SUPPLEMENTARY APPENDIX

Oncogenic fusion protein BCR-FGFR1 requires the breakpoint cluster region-mediated oligomerization and chaperonin Hsp90 for activation

Malalage N. Peiris,[1] April N. Meyer,[1] Katelyn N. Nelson,[1] Ezra W. Bisom-Rapp,[1] and Daniel J. Donoghue[1,2]

[1]Department of Chemistry and Biochemistry, University of California San Diego and [2]Moores Cancer Center, University of California San Diego, La Jolla, CA, USA

 doi:10.3324/haematol.2019.220871

Received: March 1, 2019.

Accepted: August 14, 2019.

Pre-published: August 22, 2019.

Correspondence: *DANIEL J. DONOGHUE* - ddonoghue@ucsd.edu

Supplemental Information

Oncogenic fusion protein BCR-FGFR1 requires BCR-mediated oligomerization and chaperonin Hsp90 for activation

Malalage N. Peiris [1], April N. Meyer [2], Katelyn N. Nelson [3], Ezra W. Bisom-Rapp [4], and Daniel J. Donoghue [5,6]

SUPPLEMENTAL METHODS

DNA Constructs

All DNA constructs were confirmed by DNA sequencing. The following plasmids in pcDNA3 vectors were used for transient transfection in HEK293T cells: pNP13, BCR-FGFR1; pNP14, BCR-FGFR1-K656E; pNP12, BCR-FGFR1-K514A; pNP15, FGFR1-K514A; pMW170, FGFR1; and pMW171 FGFR1-K656E. The following plasmids in pLXSN vectors were used for transfection in NIH3T3 cells and 32D cells: pNP23, FGFR1-K514A; pNP17, BCR-FGFR1-K514A; pNP20, FGFR1; pNP16, BCR-FGFR1; pNP21, FGFR1-K656E; pNP18, BCR-FGFR1-K656E; pKN34, FGFR3-TACC3; pNP26, BCR-Y177F-FGFR1; pNP41, BCR-Y436F-FGFR1; pNP43, BCR-Y55F-FGFR1; pNP44, BCR-S122A-FGFR1; pNP45A, BCR-Y246F-FGFR1; pNP46B, BCR-S459A-FGFR1; pNP47A, BCR-Y554F-FGFR1; pNP48A, BCR-T359A-S367A-S369A-S377A-FGFR1; pNP35, BCR-E34R-FGFR1; pNP37B, BCR-E52R-FGFR1; pNP40, BCR-R55E-FGFR1; pNP36A, BCR-E34R-E52R-FGFR1; pNP52, BCR-E46R-FGFR1; pNP53, BCR-R53E-FGFR1; pNP49A, BCR-R53E-R55E-FGFR1; pNP54, BCR-E34R-E46R-FGFR1;

pNP57, BCR-E46R-E52R-FGFR1; pNP58, BCR-E52R-R53E-FGFR1; pNP50A, BCR-E34R-E46R-E52R-FGFR1.

Antibodies and Reagents

Antibodies were obtained from the following sources: Flg (C-15), Hsp90 (F-8), STAT3 (C-20), STAT5 (C-17) from Santa Cruz Biotechnology (Dallas, TX, USA); phosphotyrosine (4G10) from MilliporeSigma (Burlington, MA, USA); FGFR1 (D8E4), phospho-STAT3 (Tyr705; D3A7), phospho-STAT5 (Tyr694), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) from Cell Signaling (Danvers, MA, USA); GAPDH (GT239) from GeneTex (Irvine, CA, USA); mouse FGFR1 (0AAB11171) from Aviva Systems Biology (San Diego, CA, USA); horseradish peroxidase (HRP) anti-mouse, HRP anti-rabbit, and Enhanced chemiluminence (ECL and Prime-ECL) reagents were from GE Healthcare (Little Chalfont, UK). MG132, aFGF, and recombinant mouse IL-3 were obtained from R&D Systems (Minneapolis, MN, USA). Heparin and Pierce protein A/G magnetic beads were from MilliporeSigma (Burlington, MA, USA), Geneticin (G418) was from Gibco (Waltham, MA, USA), and Lipofectamine 2000 Reagent was from Invitrogen (Carlsbad, CA, USA). Ganetespib (STA-9090) and BGJ398 (Infigratinib) were from Selleck Chemicals (Houston,TX, USA).

Cell Culture

HEK293T cells were maintained in 10% Fetal Bovine Serum (FBS) in DMEM media with 1% penicillin/streptomycin in 10% CO_2, 37 °C. NIH3T3 cells were maintained in 10% Bovine Calf Serum (CS) in DMEM media with 1% penicillin/streptomycin 10% CO_2, 37 °C.

32D clone 3 (ATCC CRL-11346) cells were maintained in RPMI 1640 media with 10% FBS, 1% penicillin/streptomycin and 5 ng/mL mouse IL-3 in 5% CO_2, 37 °C.

Cell Transfection, Immunoprecipitation, Immunoblot Analysis

HEK293T cells were transfected with 3 μg of the pcDNA3 plasmid constructs using calcium phosphate transfection as described (1). Approximately 24 h after transfection, cells were starved with no FBS for 18 h. Cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation assay buffer [RIPA; 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% TritionX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, and 10 μg/mL aprotinin]. Lowry assay was used to measure total protein concentration. For immunoprecipitation, FGFR1 antibody (D8E4) was added to lysates for overnight incubation at 4°C with rocking, immune complexes were bound to Protein A-Sepharose (MilliporeSigma, Burlington, MA, USA) and washed extensively with RIPA buffer. Samples were separated by 10% or 12.5% SDS-PAGE and transferred to Immobilon-P membranes (MilliporeSigma, Burlington, MA, USA). Immunoblotting was performed as described (2). NIH3T3 focus assays were performed as described (3). Number of foci were, normalized by transfection efficiency, and quantitated relative to a positive control +/-SEM.

Ganetespib and BGJ398 Experiments

Experiments involving Ganetespib were performed using HEK293T cells transiently transfected as described. Approximately 24 h after transfection, cells were starved with no FBS for 18 h. Ganetespib was added to a final concentration of 200 nM 14 h into the starvation period. Cells were then collected and lysed as described for immunoblotting and immunoprecipitation

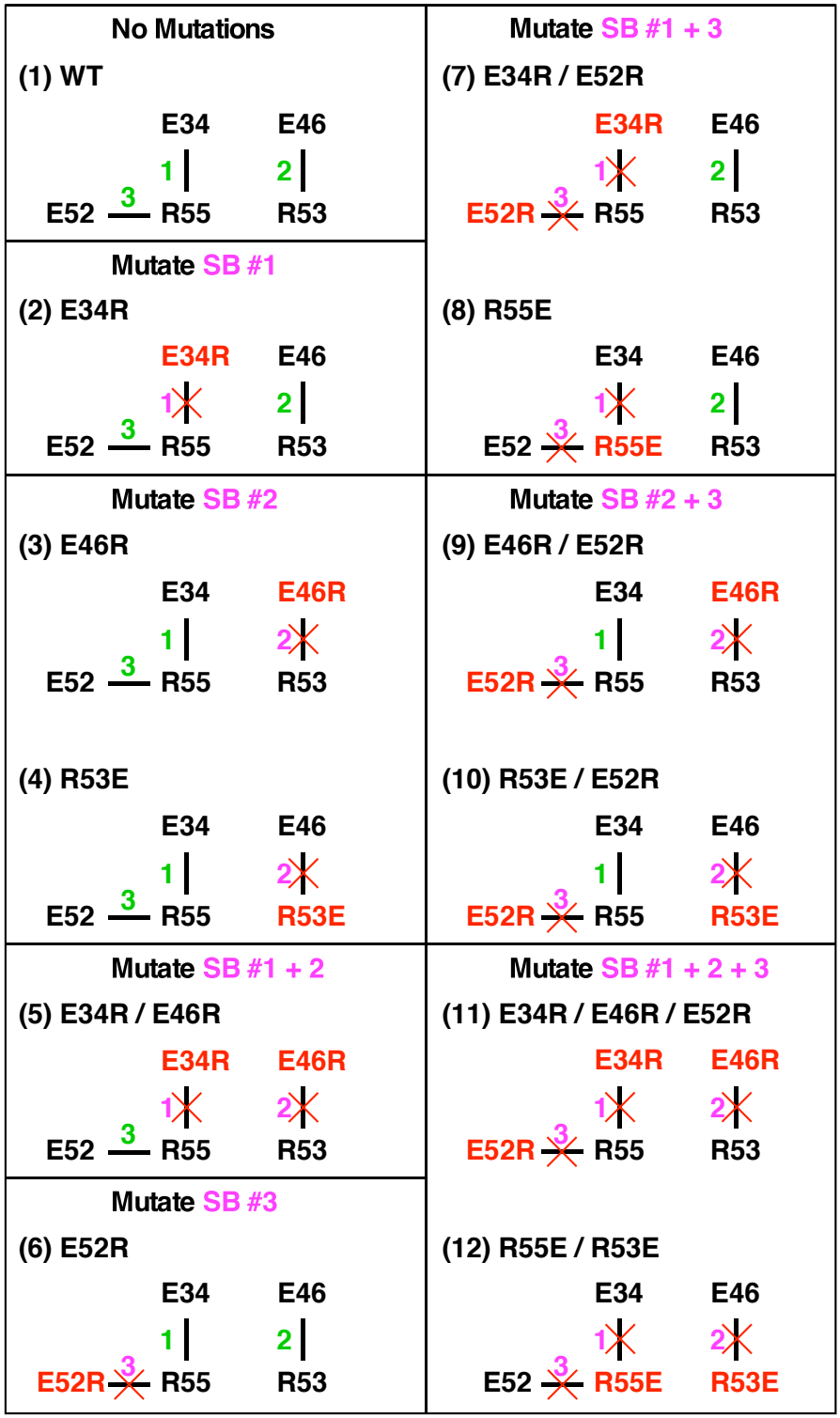
analyses. For experiments involving Ganetespib performed in NIH3T3 cell focus assays, cells were transfected and split out for focus assay as described. NIH3T3 cells expressing either BCR-FGFR1 or FGFR1 derivatives were dosed with either 0, 10, 20, 23, 26, or 30 nM Ganetespib 24 h following the split out onto 10 cm plates. These cells were re-fed with drug in 2.5% CS DMEM media every 3-4 days, after which they were fixed and scored for transfection efficiency as described. NIH3T3 cells were transfected and split as described for combination experiments involving both Ganetespib and FGFR inhibitor BGJ398. NIH3T3 cells expressing either BCR-FGFR1 or FGFR1 derivatives were dosed with no drug, or 15 nM Ganetespib and 0, 2.5, 5.0, or 10 nM BGJ398. These cells were re-fed with drug every 3-4 days, and then fixed and scored for transfection efficiency as described. The transient transfection assays in HEK293T cells required an increased concentration of Ganetespib due to the shorter drug incubation time of 4 h, opposed to 14 days for NIH3T3 cells. The amount of drug was initially titrated for each assay in order to avoid toxicity to the various cell lines.

Mass Spectrometry Sample Preparation

HEK293T cells were transfected and treated as described (4). A minimum of two replicates per sample were prepared and analyzed. Clarified lysates were immunoprecipitated with FGFR1 antisera (Aviva OAAB11171) overnight at 4°C with rocking. Immune complexes were collected with Pierce protein A/G magnetic beads as per manufacturer's directions. Following immunoprecipitation, proteins were digested directly on-beads using Trypsin/Lys-C mix, and then subjected to liquid chromatography/mass spectroscopy (LC-MS/MS) as described (4). Phosphopeptides were enriched prior to LC-MS/MS using IMAC (Immobilized Metal

Affinity Chromatography) columns (Thermo Scientific, Waltham, MA, USA). For proteomics analysis, spectral data were analyzed by MaxQuant label free quantitation (LFQ) (5).

Supplemental Figure S1. Diagram showing mutated Salt Bridges #1 + 2 + 3



REFERENCES FOR SUPPLEMETAL INFORMATION

1. Gallo LH, Meyer AN, Motamedchaboki K, et al. Novel Lys63-linked ubiquitination of IKKbeta induces STAT3 signaling. Cell Cycle. 2014;13(24):3964-3976.

2. Meyer AN, McAndrew CW, Donoghue DJ. Nordihydroguaiaretic acid inhibits an activated fibroblast growth factor receptor 3 mutant and blocks downstream signaling in multiple myeloma cells. Cancer Res. 2008;68(18):7362-7370.

3. Nelson KN, Meyer AN, Wang CG, Donoghue DJ. Oncogenic driver FGFR3-TACC3 is dependent on membrane trafficking and ERK signaling. Oncotarget. 2018;9(76):34306-34319.

4. Nelson KN, Meyer AN, Siari A, et al. Oncogenic Gene Fusion FGFR3-TACC3 Is Regulated by Tyrosine Phosphorylation. Mol Cancer Res. 2016;14(5):458-469.

5. Cox J, Hein MY, Luber CA, et al. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol Cell Proteomics. 2014;13(9):2513-2526.