Depalmitoylation Rewires FLT3-ITD Signaling and Exacerbates Leukemia Progression

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Supplemental Methods

Mice and primary human samples

NOD/SCID/IL2rγ^{null} (NSG) mice were originally purchased from Jackson Laboratories, and bred in the pathogen-free facility at the Children's Hospital of Philadelphia (CHOP). Xenograft experiments with NSG mice were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of CHOP. We used both male and female mice (8~12 weeks old) and randomly assigned them for all animal experiments. BM- or peripheral blood-derived mononuclear cells (MNCs) from primary AML patients were obtained from the Stem Cell and Xenograft Core Facility at the University of Pennsylvania. AML cells were cultured in SFEM II (STEMCELL) media supplemented with 10% fetal bovine serum, 10ng/mL IL3, 50ng/mL SCF and 10ng/mL GM-CSF. All cytokines were purchased from PeproTech.

Cell lines

TF-1 cells (ATCC Cat# CRL-2003) were obtained from American Type Culture Collection and cultured in RPMI-1640 media with 10% calf serum and 2ng/mL GM-CSF. MV4;11 (ATCC Cat# HTB-189) and SEMK2 cells were maintained in RPMI media with 10% fetal bovine serum.

Constructs, virus packaging and cell infection

MigR1 (MSCV-ires-GFP) -FLT3-ITD plasmid was kindly provided by Drs. Jian Huang and Stephen Sykes (Temple University, Pennsylvania). The 23 HA-tagged ZDHHCs constructs in the pEF-BOS vector were generously gifted by Dr. Masaki Fukata[1]. LRG vector carrying guide RNA

(gRNA) for CRISPR/Cas9-mediated knockout was generously provided by Dr. Junwei Shi (University of Pennsylvania, Pennsylvania)[2]. CRISPR/Cas9-mediated knockout efficiency was evaluated by Tracking of Indels by DEcompostion (TIDE) analysis[3]. The FLT3-WT, FLT3-D835Y and their respective C563S mutants were obtained by QuickChange mutagenesis. MiR30-based shRNAs to *ZDHHC2* or *ZDHHC6* were cloned into a pCL20-based lentiviral vector (pCL20.MSCV.mir30.PGK.mCherry)[4]. The shRNA sequences are: sh*ZDHHC2*#1: CCAGTATTTCGACATGGAA; sh*ZDHHC2*#2: TGCAGCTATGTTTTCTGTC; sh*ZDHHC6*#1: ACAACTGTTGTGGTTACCA; sh*ZDHHC6*#2: GAGGAACTTTAAACAGGTA; The gRNA sequences are: sg*ZDHHC2*#1: + GGTGCTGTACTGGATCCCGG; sg*ZDHHC2*#2: - CGTAGTAGGACCAGCCGAGC; sg*ZDHHC6*#1: - CCACAACACAGAGTCAATCA; sg*ZDHHC6*#2: - AAAGCCCGGACCGACAAACA.

The lentiviral vector together with the helper plasmids (pMDLg/pRRE, VSVG, pRSV-Rev) were transfected with FuGENE 6 (E2691, Promega) into HEK293T cells (ATCC Cat# CRL-3216). 48 hours later, the virus-containing supernatant was collected by centrifugation and frozen in -80°C for later use. For viral infection, 0.5-1 x10^6 TF-1 or MV4;11 cells were mixed with 1mL viral supernatant plus 10ug/mL polybrene (TR-1003, Sigma-Aldrich) and spin-infected for 90min at room temperature (RT). For the infection of human AML cells, the plate was firstly precoated with RetroNectin (T100B, Takara) overnight at 4ºC, followed by blocking with PBS+2% BSA for 30 min at RT and rinsed with HBSS buffer once. Viral supernatant was then pre-loaded and centrifuged at 2,000 rpm for 30 minutes at 4ºC. After removing the supernatant, AML cells were suspended with LentiBlast (LBPX500, 1:500 A and B) (OZ Biosciences) containing media, added

to viral-coated plates, and centrifuged at 1,500 rpm for 90 min at 37ºC. 48 hours post infection, cells were sorted for subsequent experiments.

Antibodies

The following antibodies were used for western blot: anti-pFLT3 (Y591) (Cell Signaling Technology Cat# 3466, RRID: AB_2263033) , FLT3 (Cell Signaling Technology Cat# 3462, RRID: AB_2107052), pSTAT5 (Y694) (Cell Signaling Technology Cat# 9351, RRID: AB_2315225), pAKT (S473) (Cell Signaling Technology Cat# 4051, RRID: AB_331158), AKT (Cell Signaling Technology Cat# 9272, RRID: AB_329827), pERK1/2 (T202/204) (Cell Signaling Technology Cat# 9106, RRID: AB_331768), ERK1/2 (Cell Signaling Technology Cat# 9102, RRID: AB_330744), Calnexin (Cell Signaling Technology Cat# 2679, RRID: AB_2228381), GAPDH (Cell Signaling Technology Cat# 97166, RRID: AB_2756824), HA (Cell Signaling Technology Cat# 3724, RRID: AB_1549585) were from Cell Signaling; Actin (Santa Cruz Biotechnology Cat# sc-47778 HRP, RRID: AB_2714189) was from Santa Cruz.

Immunoprecipitation (IP) and Western blot (WB)

293T cells were transfected with MigR1-FLT3 variants along with HA-GST (control), HA-ZDHHC2 or ZDHHC6 for 24 hours. Cells were lysed with IP buffer (10mM Tris, pH7.4, 150mM NaCl, 0.5% NP-40, 1mM NaF, 1mM Na_3VO_4, PMSF, protease inhibitor cocktail) for 30 min in cold room. The resultant supernatant was obtained by centrifugation at 13, 000 rpm for 10 min at 4°C, and pre-cleared with protein A/G beads for 30 min. The interacting proteins were captured by HA-EZ Agarose beads (E6779, Sigma-Aldrich) for 2 hours at 4°C, followed by extensively washing with IP buffer. Samples were then subject to WB analysis.

For WB analysis, an SDS-PAGE gel was transferred to a nitrocellulose membrane. The blot was first blocked with 5% BSA in TBS-T (for anti-phospho-protein antibodies) or 5% nonfat milk (for anti-total protein antibodies) for 30min. The membrane was then probed with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies for 1 hour at room temperature after extensively washing. The membrane was developed by KwikQuant imager (Kindle Biosciences, LLC).

Acyl-PEG exchange (APE) assay

Cells were washed with PBS and lysed in TEA lysis buffer (50mM triethanolamine, pH 7.3, 150mM NaCl, 5mM EDTA, 4% SDS, protease inhibitors). 200ug total proteins in 92.5uL lysis buffer were treated with 5uL 200mM neutralized reductive TCEP (pH7.3, #20490, ThermoFisher) for 30 min with nutation at RT to liberate cysteines from disulfide bonds. 2.5uL 1M NEM (E1271, Sigma) was then added for 2 hours to block non-palmitoylated thiol groups of cysteines. NEM was then removed by three repeats of methanol-chloroform-H_2O precipitation. The last precipitation of proteins was reconstituted in 60uL TEA buffer containing 4mM EDTA and 4% SDS, and split into two aliquots. One aliquot was treated with 90uL 0.2% Triton X-100/TEA buffer as the negative control, while the other was treated with 90uL 1M hydroxylamine (HAM, #379921, Sigma) in 0.2% Triton X-100/TEA for 1 hour to remove the palmitate group. Three repeats of methanol-chloroform-H_2O precipitation were done subsequently to remove residual HAM. The dry pellets were then dissolved in 30ul TEA buffer containing 4mM EDTA and 4% SDS and incubated with 60uL of 1.33mM 10kDa methoxypolyethylene glycol maleimide (mPEG-Mal, #712469, Sigma-Aldrich) for 2 hours at RT to allow for the binding of mPEG-Mal to the thiol

group of cysteines before subjecting to the final precipitation of total proteins. The potential palmitoylated proteins were analyzed by Western blot.

Flow cytometry

For surface FLT3 staining, TF-1 cells transduced with retroviruses expressing MigR1-FLT3 variants, MV4;11 or primary AML cells were stained with APC- or PE-conjugated FLT3 antibodies (#313307 or #313305, eBiolegend) for 30 min on ice, followed by analysis on a BD FACS Canto flow cytometer. For total FLT3 staining, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at RT, permeabilized with 0.2% Triton X-100 for 15 min. Subsequently, the cells were blocked with Fc blocker (#550270, BD Biosciences, 1:500), stained with APC- or PE-conjugated FLT3 antibodies for 30 min on ice, and analyzed on a FACS Canto flow cytometer.

Immunofluorescence (IF)

293T cells were grown on coverslips and transfected with different constructs for 24 hours. Cells were washed with PBS and fixed with 4% PFA for 15 min at RT, permeabilized with 0.2% Triton X-100 for 15 min, and then blocked with 5% BSA/PBS. Cells were then stained with anti-FLT3 antibody (MAB812, R&D Systems, 1:100), together with anti-calnexin (#2679, Cell signaling, 1:100) or anti-GM130 (#12480, Cell signaling, 1:3200) as markers for the ER or Golgi complex, respectively, overnight at 4°C. After extensive washing with PBS, AF568-conjugated anti-mouse (A11004, Invitrogen) or AF647 anti-rabbit secondary antibodies (#A21244, Invitrogen) were incubated for 1 hour at RT. To stain the plasma membrane, Alexa Fluor647-conjugated wheat germ agglutinin (WGA) (#W32466, Invitrogen, 1ug/mL) was added to cell culture for 10min in the incubator. Cells were then washed with PBS to remove excess WGA, followed by fixation,

permeabilization and staining with anti- FLT3 antibodies. Coverslips were mounted with DAPI-containing solution on slides (2.5ug/mL, Sigma) and images were acquired on a Zeiss LSM 710 confocal microscope equipped with a 100 x /1.4 numerical aperture oil. The image analysis was performed using Fuji software.

Cytokine-mediated signaling

For signaling sensitivity, TF-1 cells transduced with retroviruses expressing various MigR1-FLT3 variants were starved in 0.5% BSA-containing RPMI-1640 media for 2 hours, and then stimulated with a graded concentration of FLT3L for 10 min. For signaling kinetics, cells were starved, stimulated for indicated time points, and the cell pellets were snap-frozen on dry ice. Samples were subjected to WB analysis with indicated antibodies.

Cell proliferation assay

MTT assays were used to examine the cell growth of cell lines. TF-1 cells expressing various FLT3 variants were cultured in a 96-well plate (10k cells/100uL per well) in a graded concentration of FLT3L for 3 days. A final concentration of 0.5mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; M6494, Invitrogen) was added for 3-4 hours at 37°C, followed by terminating the reaction with stopping buffer. The absorbance was read by a spectrophotometer at 570 nm wavelength.

To examine primary AML cell growth, cells under different growth conditions were counted in the present of trypan blue using a hemacytometer at different days of culture.

Quantitative real-time PCR (qRT-PCR)

Total RNA was purified from cells with the RNeasy Mini Kit (#74106, Qiagen). The cDNAs were synthesized by reverse-transcription reaction using qScript cDNA Supermix (#95047, Quanta Biosciences), and quantitative PCR was performed with SYBR Green Master Mix (A25742, Applied Biosystems) according to manufacturer's instructions in a ViiA 7 real-time PCR system (Applied Biosystems). qPCR primers are: *ZDHHC2*-F: AGTCTAGGTGATGGCTGCTC; *ZDHHC2*-R: TTATGCTGCTCTCCGTCCAA; *ZDHHC*6-F: ATGGGTACGTTCTGTTCGGTT; *ZDHHC6*-R: AAGGGCCAATACCACAACACA; *ZDHHC15*-F: GGTGCCAGTGCTCGTTATTGT; *ZDHHC15*-R: AAGACGTAGGCATAGTAGGACC; *FLT3*-F: TGACAGAAACCCAAGCTGGA; *FLT3*-R: CAAAGCACCCATTCCACGAT; *GAPDH*-F: CCACCCATGGCAAATTCC; *GAPDH*-R: TGGGATTTCCATTGATGACAAG.

Colony-forming cell (CFC) assay For CFC assay with MV4;11 cells, 500 cells under different conditions were plated in triplicates onto methylcellulose culture (Methocult H4230, STEMCELL Technologies) for 7-10 days[5]. Colonies were manually counted and quantified.

For primary patient AMLs, PB- or BM-MNCs were transduced with lentiviruses expressing miR30-based shRNA to *ZDHHC2* or *ZDHHC6* with mCherry as a fluorescent marker. 48 hours later, $mCherry^+$ cells were sorted and plated onto methylcellulose culture (Methocult H4230) (50k/plate) supplemented with 5U/ml EPO (NDC 55513-144-10, EPOGEN), 10ng/ml IL-3, 5ng/ml SCF, 5ng/ml GM-CSF. Colonies were counted after 14 days' culture.

CRISPR/Cas9-mediated knockout and genome editing

For genome editing of the endogenous FLT3-ITD locus in MV4;11 cells, a guide RNA (gRNA) (TACTTGTGACAAATTAGCA) in proximity to C563 codon targeting to the *FLT3* antisense sequence was first introduced into MV4;11/Cas9 stable cell line by lentiviral LRG vector (carrying GFP fluorescent marker) and the targeting efficiency of this gRNA was found to be high using TIDE analysis (87.1% of frameshift). Subsequently, a 180bp synthesized DNA template with the C563S (TGT to AGT) and PAM (GGG to GCG) mutations was electroporated into MV4;11/Cas9 cells using a 4D-nucleofector (Lonza) with the SF cell line solution box (Cat# PBC2-00675, Lonza). GFP^+ cells were sorted into 96-well plates, and single cell clones were cultured and sequenced for heterozygous and homozygous C563S mutations. Since gRNA-mediated knockout of *FLT3-ITD* without the C563S replacement were detrimental to MV4;11 cell growth, the surviving clones had a high probability of successful C563S editing.

Drug treatments and synergy analysis

For cell growth assay with gilteritinib (Cat# S7754, Selleck Chem), TF-1 cells reconstituted with ITD, ITD/C563S, D835Y or D835Y/C563S were cultured in cytokine-free media treated with a graded concentration of gilteritinib as indicated for 3 days, followed by MTT assay. MV4;11 ITD, ITD/C563S Het or ITD/C563S Homo clones were seeded in H4230 methylcellulose semi-solid media with indicated concentrations of gilteritinib for 7~10days for colony growth, and colony numbers were counted manually. Experiments with all cell types with drug treatments were performed in triplicates.

For xenograft assay in NSG mice, MV4;11 cells were pretreated with DMSO, 60uM Palm B (Cat# 178501, Sigma), 50nM gilteritinib or dual drugs for 6 hours, and then transplanted into sublethally irradiated NSG mice for bioluminescence imaging.

For drug synergy analysis, MV4;11 cells or primary AML cells were cultured with combinations of varying concentrations of Palm B and gitleritinib in 96-well plates for 3 days. Cell growth was measured by MTT assay. The drug synergy scoring was calculated by CompuSyn software. CI: Combination index. CI<1: Synergism; CI=1: Additive effect; CI>1: Antagonism.

Reference

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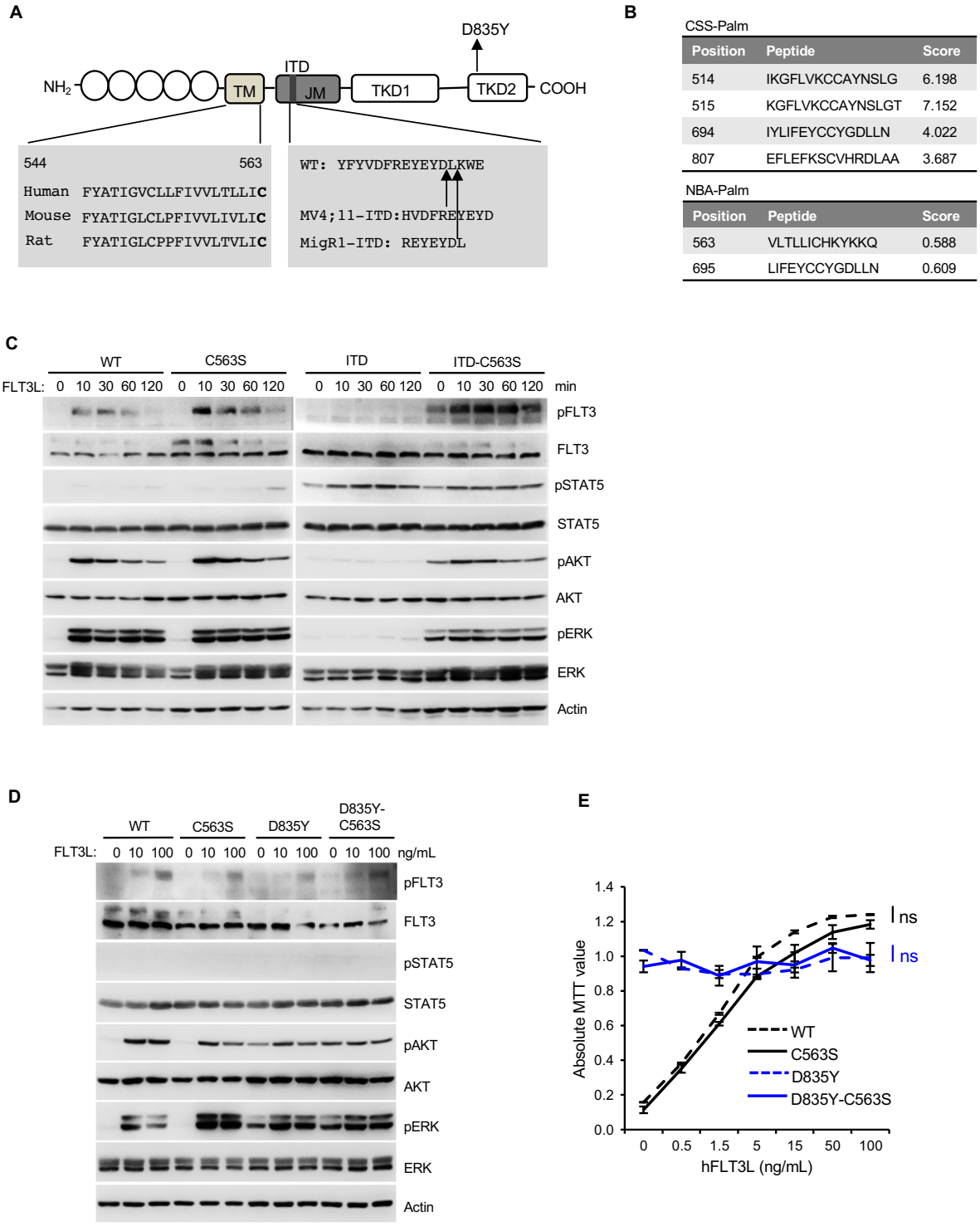
Supplemental Table 1 Clinical and Molecular Characteristics of the AML samples. (see attached Excel file)

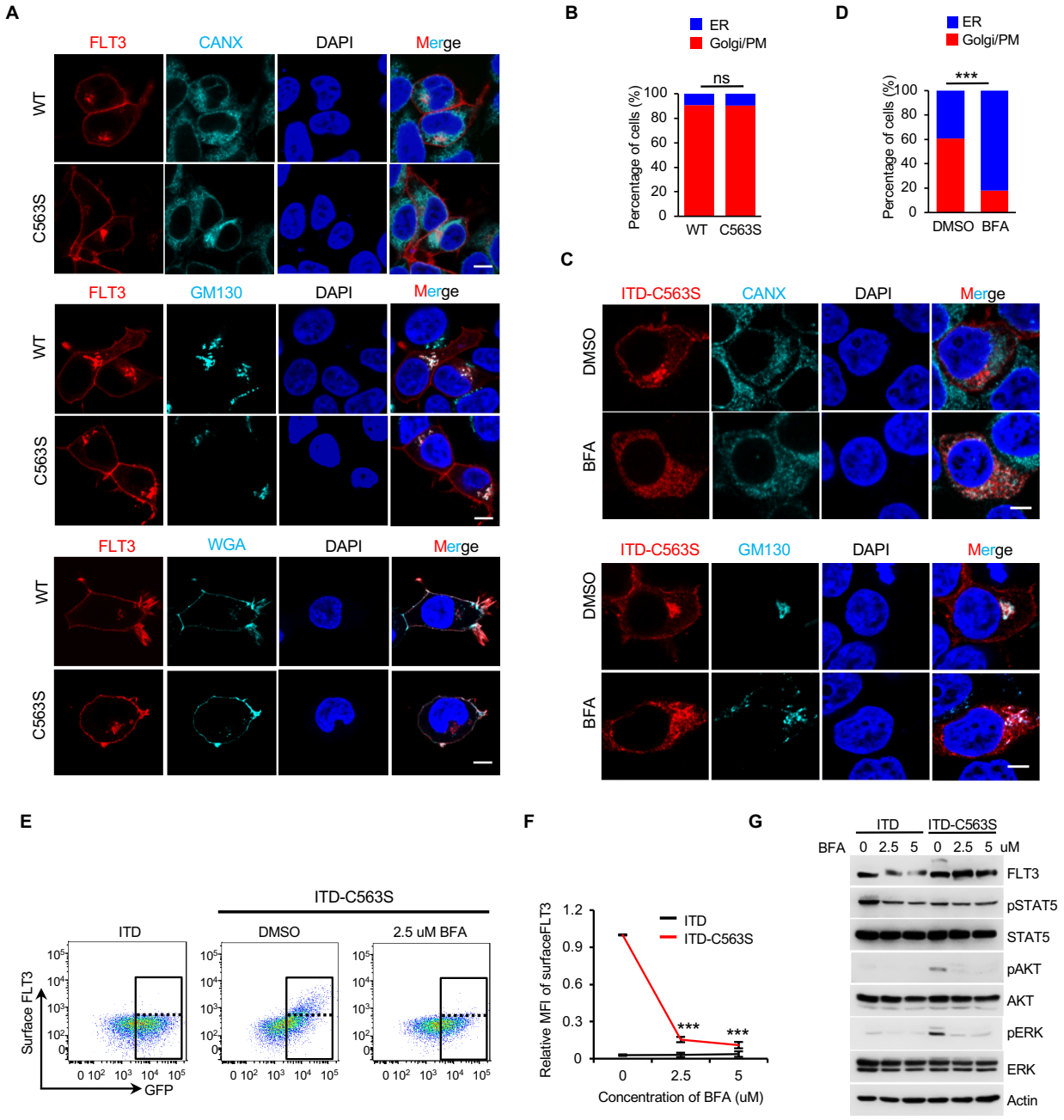
Supplemental Figures

Supplemental Figure 1



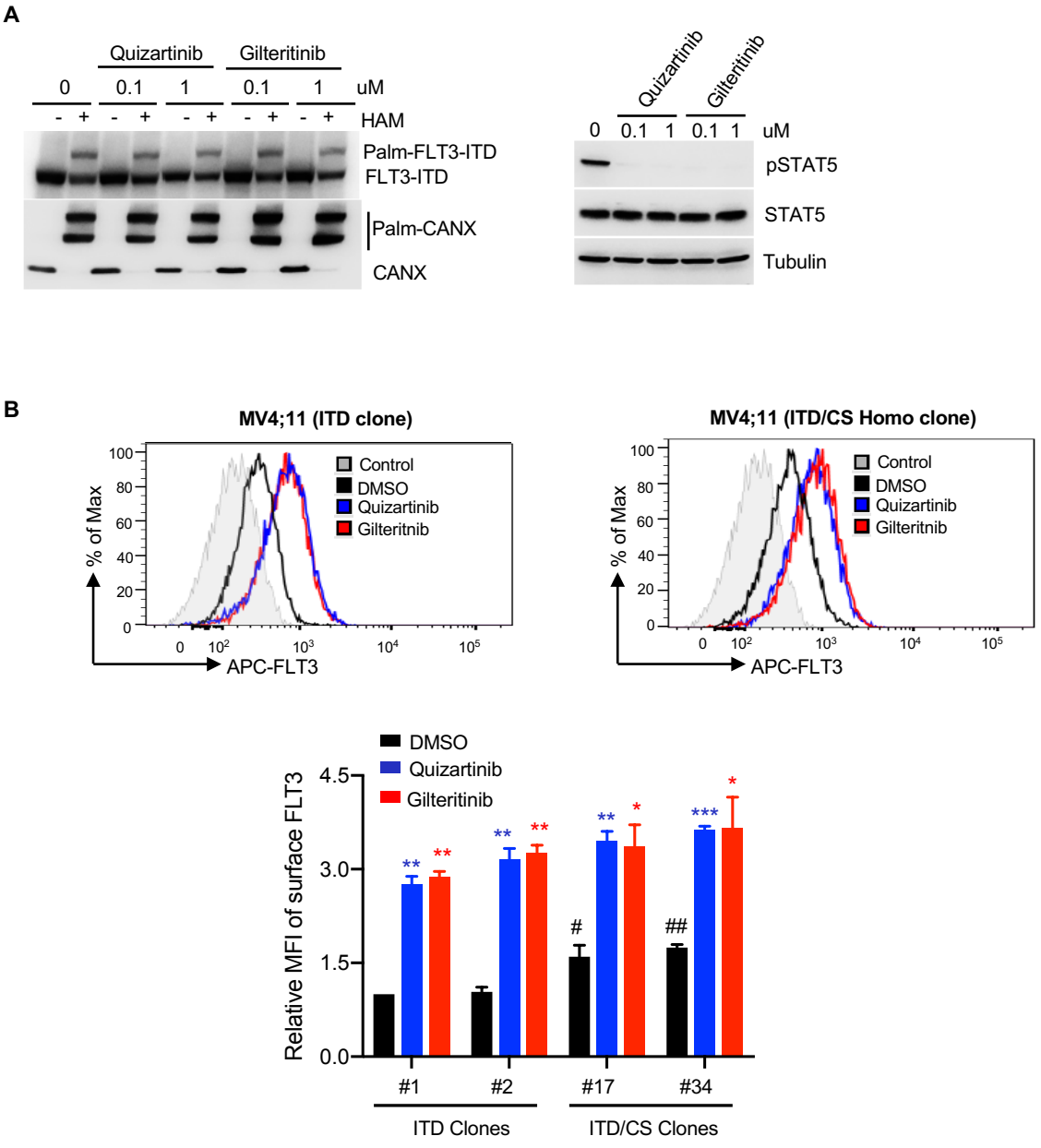
Supplemental Figure 1. Disruption of FLT3-ITD palmitoylation activates PI3K/AKT and RAS/MAPK pathways. (A) Schematic representation of the domain structure of FLT3-WT, FLT3-ITD and FLT3-D835Y mutants. The conserved cysteine^{563} in the TM domain among human, mouse and rat is highlighted in bold. The WT or ITD sequences are shown in the shaded box. The positions of ITD insertion in the endogenous FLT3 locus in MV4;11 cells and in the MigR1-FLT3-ITD construct are labeled by short and long arrows, respectively. TM, transmembrane domain; JM, juxtamembrane domain; TKD, tyrosine kinase domain. (B) The cysteine sites predicted to be palmitoylated in FLT3 with two separate prediction websites are shown. (C) Signaling kinetics in response to FLT3L. TF-1 cells stably expressing indicated constructs were starved and stimulated with 100ng/mL FLT3L for different time points and cell lysates were analyzed by WB. (D) Signaling sensitivity to FLT3L. TF-1 cells stably expressing indicated FLT3 constructs were starved and stimulated with a graded concentration of FLT3L for 10min, followed by WB analysis. (E) TF-1 cells expressing indicated constructs were cultured in a graded concentration of FLT3L in triplicates. Cell growth was quantified after 3 days' culture by MTT assay. Data are represented as mean± SD. ns, not significant, as determined by two-tailed Student's *t*-tests.

Supplemental Figure 2



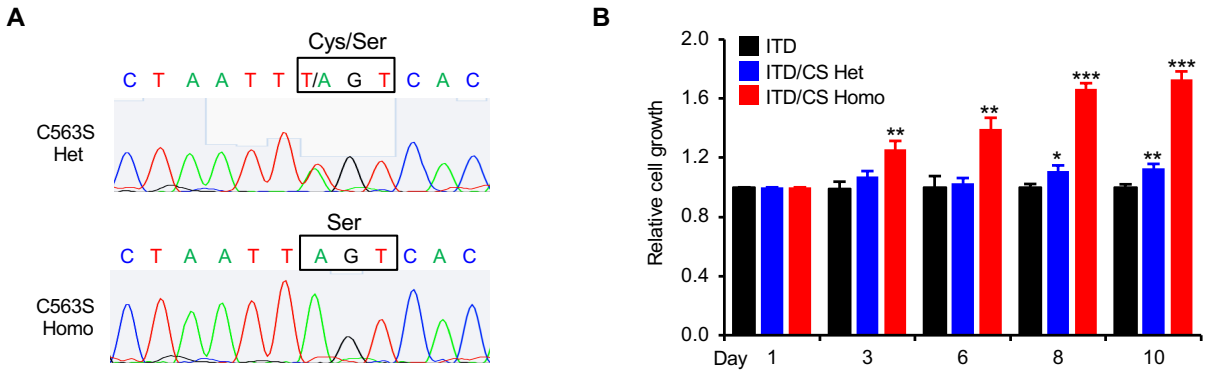
Supplemental Figure 2. (A-B) Disruption of palmitoylation does not affect FLT3-WT subcellular distribution. (A) Representative immunofluorescent confocal images of FLT3 (Red) with the ER marker Calnexin (Cyan, top panel), Golgi marker GM130 (Cyan, middle panel) or AlexaFluor647-conjugated plasma membrane marker wheat germ agglutinin (WGA) (bottom panel) in 293T cells expressing MigR1-FLT3-WT or FLT3-C563S. The nuclei were stained with DAPI (blue). Scale bar, 10um. (B) Quantification of percentages of cells expressing FLT3-WT (n=128) or FLT3-C563S (n=132) with the FLT3 distribution predominantly in the ER or Golgi/PM. ns: not significant, as determined by Fisher's exact test. **(C-G) Blockade of ER-Golgi trafficking prevents ITD-C563S localization to the PM and inactivates PI3K/AKT and RAS/ERK pathways.** (C) Representative immunofluorescent confocal images of 293T cells expressing FLT3-ITD-C563S in the presence of DMSO or BFA for 3 hours. FLT3 (Red) was co-stained with calnexin (ER marker, cyan) or GM130 (Golgi marker, cyan) to show the impact of BFA treatment on FLT3-ITD-C563S localization. The nuclei were stained with DAPI. Scale bar, 10um. (D) Quantification of percentage of cells expressing FLT3-ITD-C563S in the presence of DMSO (n=94) or BFA (n=100) with the FLT3 distribution predominantly in the ER or Golgi/PM as shown in (C). ***: $p<0.001$, Fisher's exact test. (E) Surface FLT3 levels of ITD or ITD-C563S expressing TF-1 cells treated with or without BFA for 3 hours were determined using flow cytometry analysis. (F) MFIs of surface FLT3 levels (mean±SD) as shown in (E) from three independent experiments. ***: $p<0.001$, two-tailed Student's *t*-tests. (G)TF-1 cells expressing FLT3-ITD or FLT3-ITD-C563S were treated with BFA for 3 hours and signaling transduction was examined by WB with indicated antibodies.

Supplemental Figure 3



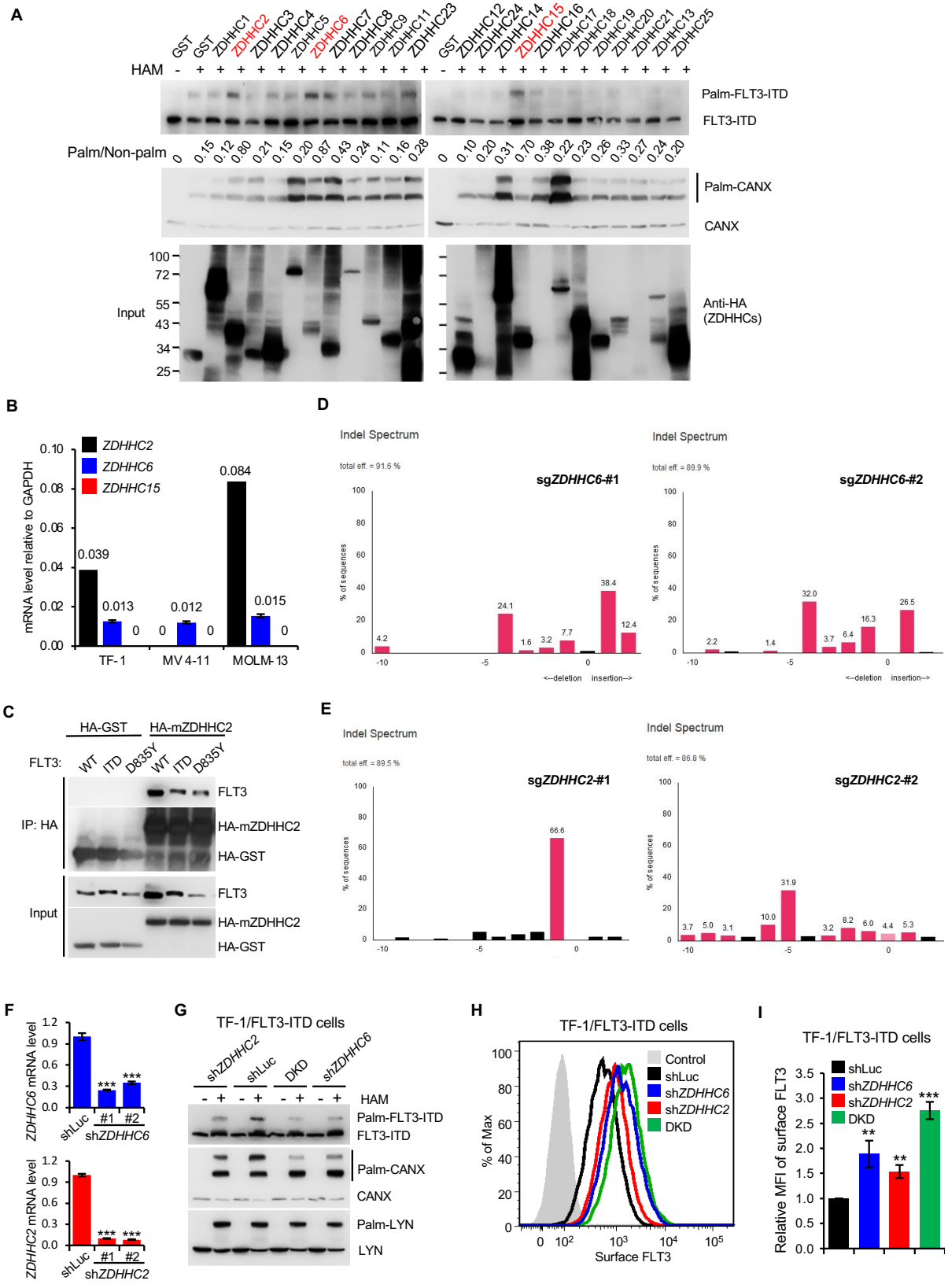
Supplemental Figure 3. TKIs do not affect FLT3 palmitoylation and regulate FLT3-ITD surface expression independent of palmitoylation. (A) FLT3-ITD expressing TF-1 cells were treated with indicated doses of quizartinib or gilteritinib, followed by APE palmitoylation assay of FLT3-ITD. The efficacy of drug treatments was confirmed by pSTAT5 immunoblot. (B) Flow cytometry analysis of surface FLT3 levels in MV4;11 ITD or ITD/CS Homo clones treated with gilteritinib or quizartinib. Quantifications of MFI from three independent experiments are shown. The asterisk signs (*) in (B) denote the comparison of drug treated groups to DMSO, whereas # signs denote the comparison of ITD/CS Homo clones to ITD clones. * or #: $p<0.05$; ** or ##: $p<0.01$; ***: $p<0.001$, as determined by two-tailed Student's *t*-tests.

Supplemental Figure 4



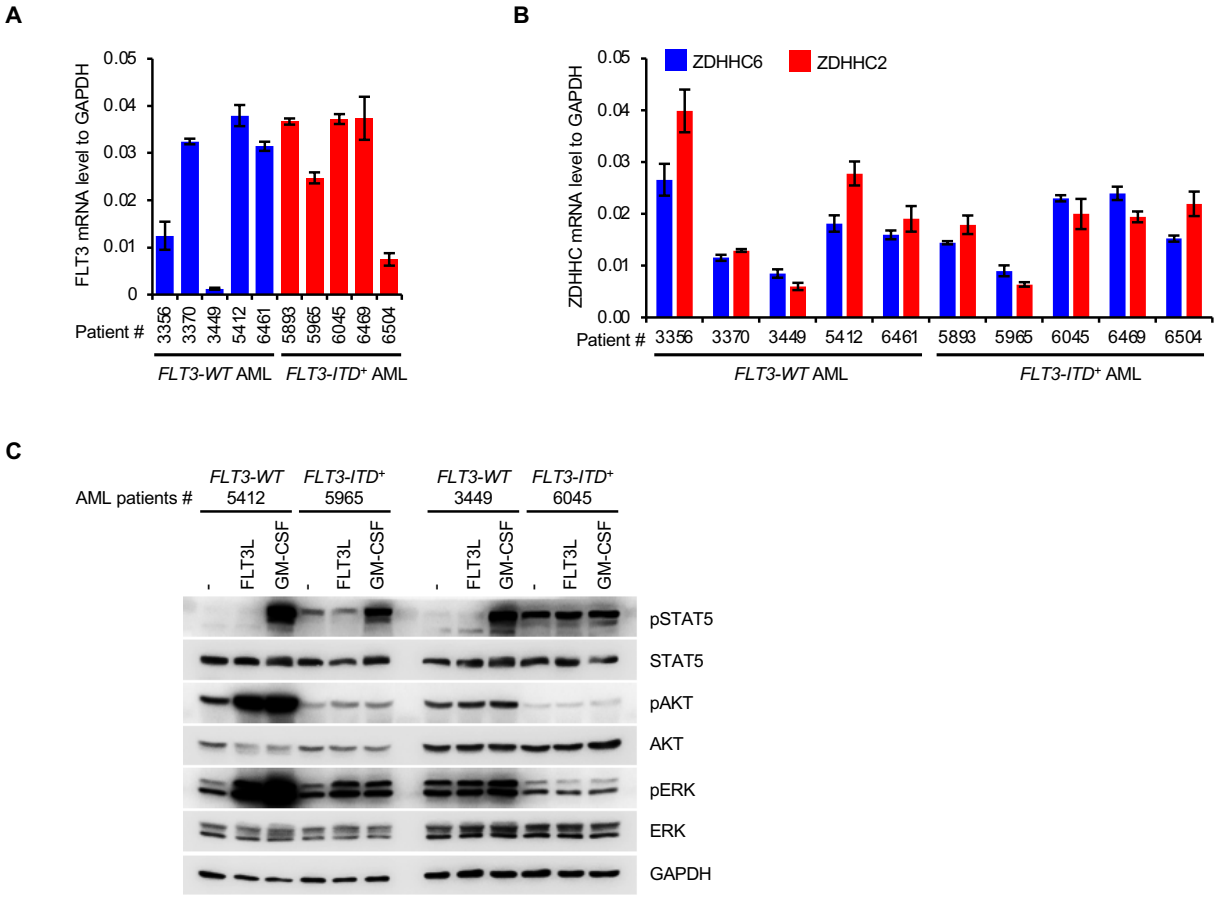
Supplemental Figure 4. Ablation of palmitoylation of endogenous FLT3-ITD in MV4;11 cells enhances cell proliferation. (A) Sequencing trace chromatogram of genomic DNA showing regions of genomic editing by CRISPR/Cas9 in targeted cell clones. Cysteine 563 to serine change is boxed in the heterozygous (Het) and homozygous (Homo) MV4;11 clones. (B) Relative cell growth of ITD, ITD/CS Het and ITD/CS Homo MV4;11 clones. Cell numbers of ITD/CS Het and Homo clones at each time point were normalized to that of ITD clones in triplicates. In all relevant panels, data are shown as mean± SD. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, as determined by two-tailed Student's *t*-tests.

Supplemental Figure 5



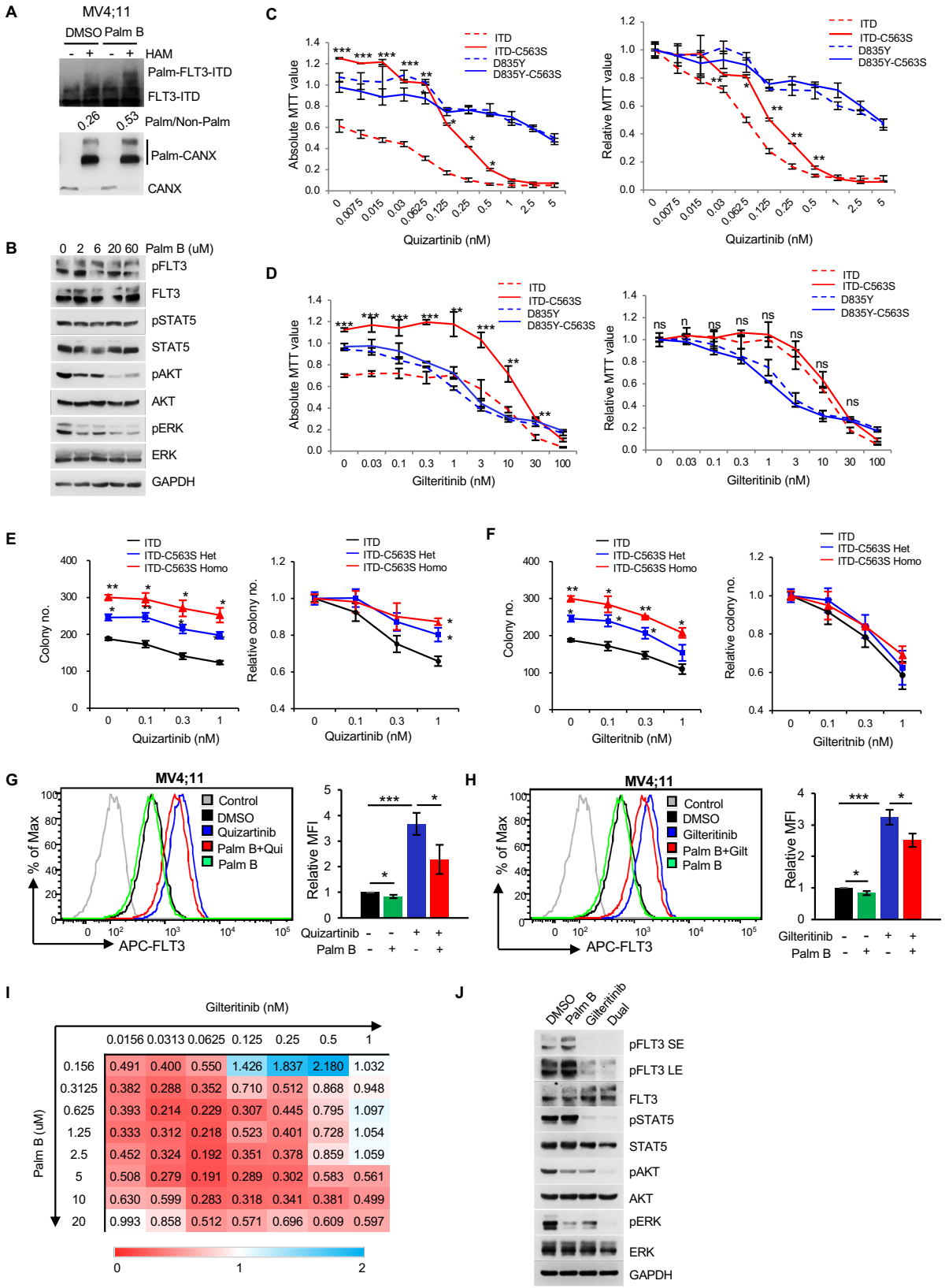
Supplemental Figure 5. ZDHHC6 is the major palmitoyl acyl-transferase (PAT) for FLT3 palmitoylation. (A) The APE assays to screen for ZDHHCs responsible for FLT3 palmitoylation. 293T cells were co-transfected with MigR1-FLT3-ITD and 23 HA-tagged ZDHHCs individually, subsequently cell lysates were subjected to the APE assay. The ratios of palmitoylated FLT3-ITD (top band) relative to non-palmitoylated FLT3-ITD (bottom band) are indicated at the bottom of each lane. (B) mRNA levels of three candidate *ZDHHCs* (2, 6, and 15) were examined by qRT-PCR in three different hematopoietic cell lines in triplicates. Note that *ZDHHC15* showed negligible expression in all three cell lines. (C) Interaction between ZDHHC2 and FLT3. 293T cells transfected with HA-GST (control) or HA-ZDHHC2 along with indicated FLT3 constructs were immunoprecipitated by HA-EZ agarose beads, followed by WB analysis. (D, E) Both *ZDHHC6* and *ZDHHC2* were efficiently deleted by CRISPR/Cas9-mediated genome editing. Tracking of Indels by DEcomposition (TIDE) analysis for the genome editing efficiency using various gRNAs to *ZDHHC6* (D) or *ZDHHC2* (E) in MV4;11 cells. (F) Evaluation of shRNA-mediated knockdown efficiency of *ZDHHC6* (top panel) or *ZDHHC2* (bottom panel) by qRT-PCR in TF-1 cells in triplicates. (G) TF-1/FLT3-ITD cells with single or double depletion of *ZDHHC2* or *ZDHHC6* were analyzed for FLT3-ITD palmitoylation using APE assays. DKD, double knockdown of *ZDHHC2* and *6*. (H) Flow cytometric plots of surface FLT3 expression in TF-1 cells stably expressing FLT3-ITD with single or double depletion of *ZDHHC2* or *ZDHHC6*. (I) Quantification of surface FLT3 level by flow cytometry from four independent experiments. Data in (F) and (I) are represented as mean± SD. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: not significant, as determined by two-tailed Student's *t*-tests.

Supplemental Figure 6



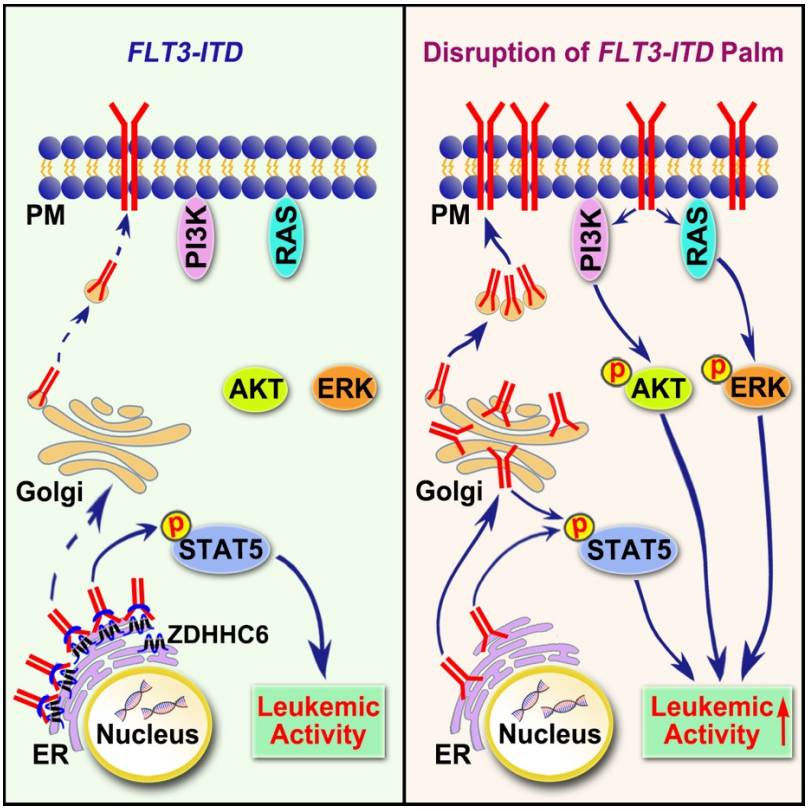
Supplemental Figure 6. Primary *FLT3-ITD*$^+$ but not *FLT3-WT* AMLs show constitutively activated STAT5. (A, B) Primary *FLT3-WT* and *FLT3-ITD*$^+$ AMLs from various AML patients were examined for their mRNA levels of *FLT3* (A) or *ZDHHC2* and *ZDHHC6* (B) by qRT-PCR analysis. Data were performed in triplicates and are represented as mean± SD. (C) Two pairs of *FLT3-WT* or *FLT3-ITD*$^+$ AML patient cells were either left untreated (-) or stimulated with FLT3L or GM-CSF for 10min. Downstream signaling was examined by WB analysis.

Supplemental Figure 7



Supplemental Figure 7. Palm B synergizes with gilteritinib in constraining FLT3-ITD mediated signaling and growth in MV4;11 cells. (A) Examination of endogenous FLT3-ITD palmitoylation level in MV4;11 cells with or without Palm B treatment by the APE assay. (B) WB analysis of FLT3-ITD downstream signaling in MV4;11 cells treated with different doses of Palm B for 6 hours. (C, D) Drug sensitivity of various FLT3 variants. TF-1 cells expressing indicated FLT3 variants were plated in triplicates in cytokine-free media containing a graded concentration of quizartinib (C) or gilteritinib (D) for 3 days. Cell growth was examined using the MTT assay. The left panels show absolute MTT absorbance and the right panels show normalized values to the respective untreated group. (E, F) Colony forming assay of MV4;11 ITD, ITD/CS Het and ITC/CS Homo clones in a graded concentration of quizartinib (E) or gilteritinib (F). The left panels show absolute colony numbers and the right panels show normalized values to the respective untreated group. (G, H) Flow cytometric analysis of surface FLT3-ITD levels in MV4;11 cells treated with Palm B or TKIs singly or combined. TKI: quizartinib (G) or gilteritinib (H). MFIs from three independent experiments are shown. In all relevant panels, data are shown as mean± SD. * or #: $p<0.05$; ** or ##: $p<0.01$; ***: $p<0.001$, as determined by two-tailed Student's *t*-tests. (I) Drug synergy analysis of MV4;11 cells cultured in combinations of varying concentrations of Palm B and gilteritinib. Cell growth in triplicates was measured by MTT assay after 3-day cultures. Drug synergy scores calculated by CompuSyn software are shown. CI: Combination index. CI<1: Synergism; CI=1: Additive effect; CI>1: Antagonism. (J) WB analysis of FLT3-ITD downstream signaling in MV4;11 cells treated with DMSO, Palm B, gilteritinib, or dual drugs for 6 hours.

Supplemental Figure 8



Supplemental Figure 8. A working model. Constitutively active FLT3-ITD mutant proteins are localized in the ER, and this localization is further enhanced by ZDHHC6-mediated palmitoylation. ER-resident FLT3-ITD aberrantly activates cytoplasmic STAT5 that promotes leukemia progression. In circumstances where palmitoylation is inhibited either by palmitoylation inhibitors or by *ZDHHC6* depletion, non-palmitoylated FLT3-ITD is released from the ER and transported through the Golgi to the plasma membrane, where PI3K/AKT and RAS/MAPK pathways are activated. Combinatorial activation of three major downstream signaling pathways exacerbates AML leukemogenesis.