FLT3 and FLT3-ITD phosphorylate and inactivate the cyclin-dependent kinase inhibitor $p27^{Kip1}$ in acute myeloid leukemia

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Supplementary Data

Supplementary Methods

Reagents

SU5614 and PP2 were purchased from Merck (Darmstadt, Germany), AC220 (Quizartinib) from Selleckchem (Houston, TX, USA) and Dasatinib from Adooq (Irvine, CA, USA). Recombinant human FLT3 ligand was purchased from PeproTech (Rocky Hill, NJ, USA).

Antibodies

The following antibodies were used: polyclonal anti-p27 C19 (sc-528, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HRP mouse anti-p27 (clone 57/Kip1/p27; BD Biosciences, Franklin Lakes, NJ, USA), rabbit anti-FLT3 (06-647; Upstate, Millipore, Billerica, MA, USA), rabbit anti-FLT3 (used for IF) (8F2; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-pY589/591 FLT3 (30D4; Cell Signaling), mouse anti-phospho tyrosine antibody (clone 4G10; Upstate, Millipore), pY-20 (sc-508; Santa Cruz), mouse anti-pY88 p27 (Grimmler M et al. Cell. 2007;128(2):269-280), rabbit-anti pY694 Stat5A/B (C11C5; Cell Signaling), rabbit anti-phospho Src family (Y416; Cell Signaling), mouse anti-pT202/Y204 and pT185/Y187 p44/42-Erk1/2 (#9102; Cell Signaling), mouse anti Skp2 (32-3300, Invitrogen, Carlsbad, CA, USA), rabbit anti-ß-Actin (sc-47778, Santa Cruz), mouse anti-HA tag (clone 12CA5, Abcam, Camebridge, MA, USA), mouse anti-DDDDK (anti-Flag, clone M2, Sigma, St Louis, MO, USA), mouse anti-GAPDH (Sigma), rabbit polyclonal pT187-p27 (Upstate)

Plasmids

p27 plasmids (1,2) and vectors for murine FLT3, murine/human chimeric FLT3-ITD and FLT3-TKD (3-5) have been described previously. C-terminal Flag-tagged FLT3-ITD and FLT3 WT expression vectors were generated from the respective pcDNA3.1 expression vectors by recombination cloning (Gateway technology, Invitrogen, USA). Therefore, FLT3 cDNA was flanked with attB1 and attB2 sites and the sequence DYKDDDDK corresponding to Flag was directly added at the C-terminus in a two-step PCR. Plasmids encoding FLT3 domains were also generated using the Gateway technology. To generate the construct for the cytoplasmic domain of FLT3-ITD and for the tyrosine kinase domain (KD) of FLT3, primers were used that start at nucleotide 1692 and nucleotide 1813 respectively, together with a reverse primer for

the full-length cDNA. For the tyrosine kinase domain 1, the same forward primer was used as for the KD with a reverse primer starting at nucleotide 2238 of FLT3. The delta T-FLAG-pDEST vector (N-terminal addition of Flag) was used to generate plasmids suitable for mammalian expression and the pGEX-SG-pDEST vector (N-terminal GST fusion) for bacterial expression. All vectors were verified by sequencing.

Pull down assays

To characterise the interaction between FLT3 and p27, recombinant purified His-p27, His-p27 C-terminal (AA 97-189) or His-p27 N-terminal (AA 1-96) domains were bound to AffiGel10 (BioRAD) according to manufacturer's instructions. Flag-tagged FLT3 domains were expressed in 293T cells. Cell lysates (lysis buffer: 150 mM NaCl, 20 mM Tris pH 7.5, 0,5% NP-40, phosphatase- and protease inhibitors) were incubated for 2 hours with the indicated AffiGel10-bound p27 protein or -domains. After five washing steps (wash buffer: 1% Triton X-100, 300 mM NaCl, 20 mM Tris pH 7.5) the bound proteins were eluted by boiling in Lämmli buffer (4% SDS, 20% glycerol, 100 mM Dithiothreitol, 100 mM Tris pH 6.8, 0.4% bromphenol blue) and analysed by Western blot.

References

1) Grimmler M, Wang Y, Mund T, Cilensek Z, Keidel EM, Waddell MB, et al. Cdk-inhibitory activity and stability of p27Kip1 are directly regulated by oncogenic tyrosine kinases. Cell. 2007 Jan 26;128(2):269-80.

2) Jakel H, Weinl C, Hengst L. Phosphorylation of p27Kip1 by JAK2 directly links cytokine receptor signaling to cell cycle control. Oncogene. Aug 11;30(32):3502-12.

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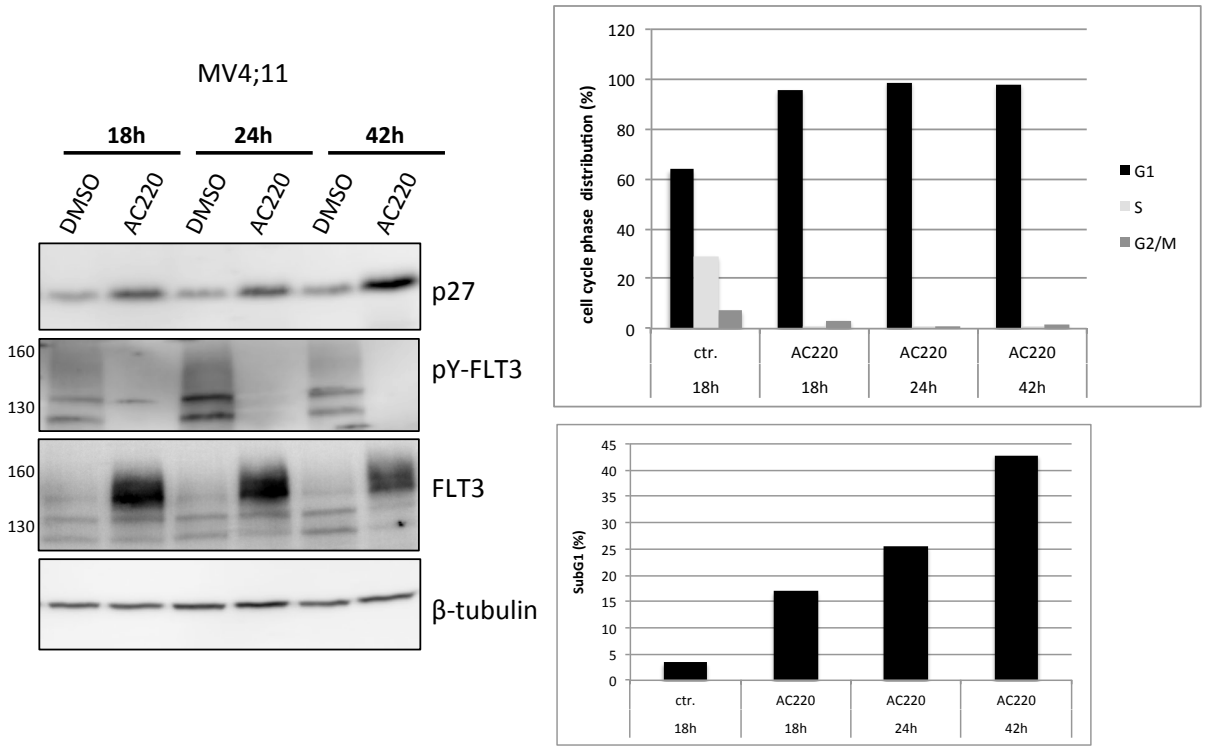
4) Leischner H, Albers C, Grundler R, Razumovskaya E, Spiekermann K, Bohlander S, et al. SRC is a signaling mediator in FLT3-ITD- but not in FLT3-TKD-positive AML. Blood. Apr 26;119(17):4026-33.

5) Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Muller C, et al. Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. Blood. 2000 Dec 1;96(12):3907-14.

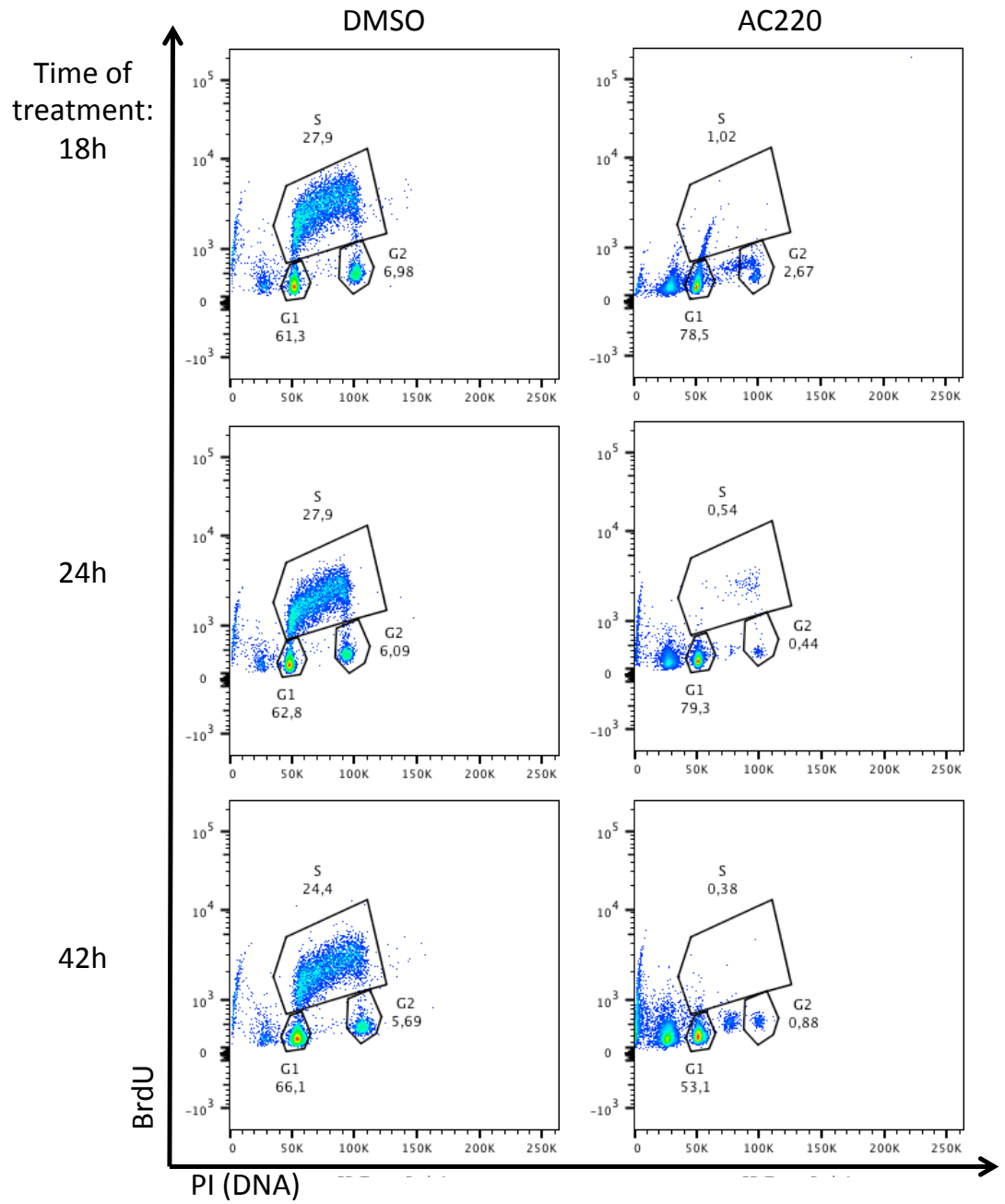
Supplementary Figures

Figure S1



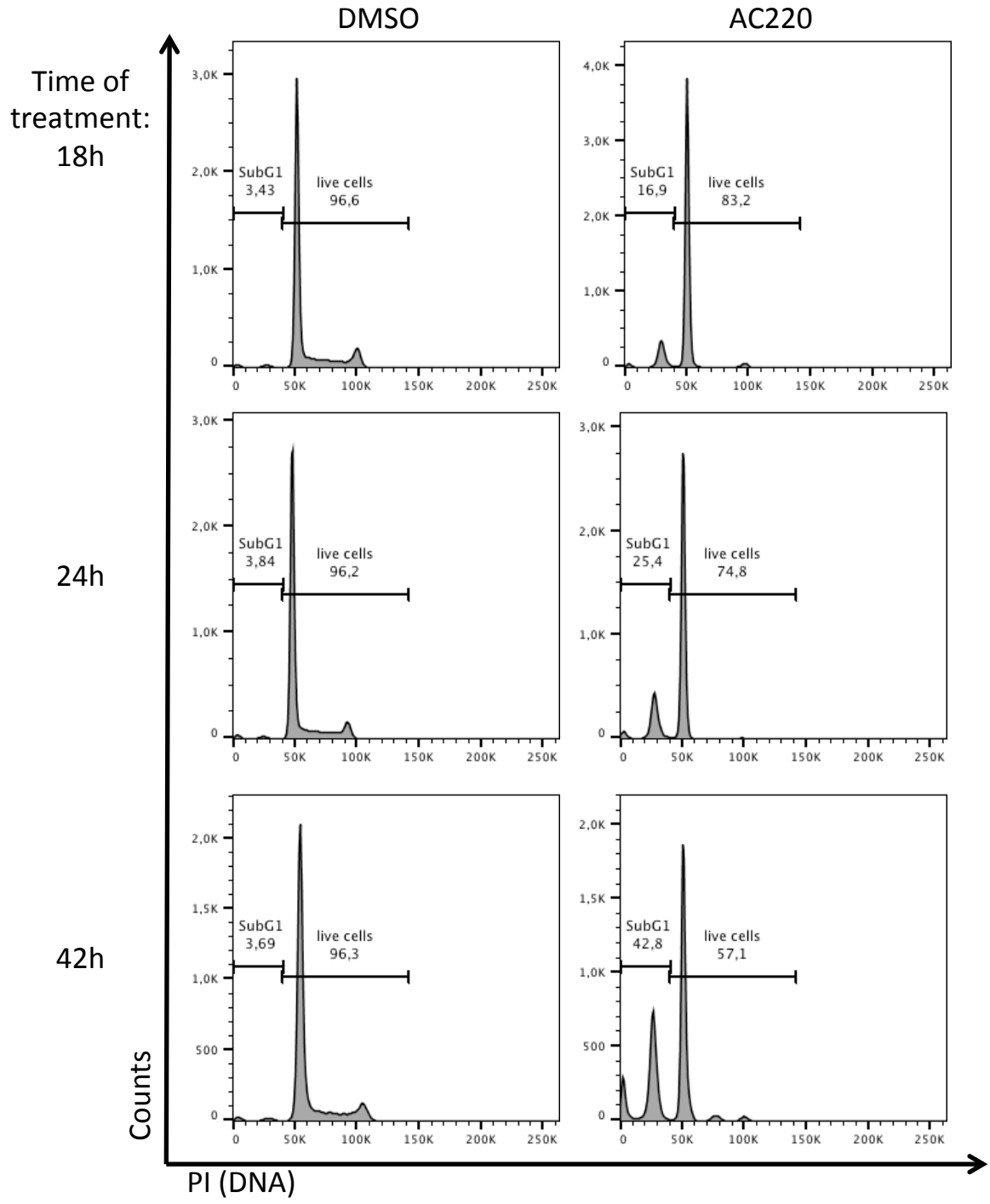
Inhibition of FLT3-ITD with AC220 in MV4;11 cells results in upregulation of p27 levels and cell cycle arrest in G1 phase. MV4;11 cells were incubated with AC220 and vehicle (DMSO) for the times indicated. Western blot analyses with anti-p27, anti-pY-FLT3, anti-FLT3 and anti-tubulin antibodies (left panel) as well as flow cytometry analyses of PI and BrdU stained cells to determine cell cycle phase distribution (see Figure S2 and upper right panel) were performed. Percentage of sub-G1 cells (lower right panel) was determined from a histogram of PI stained cells (see Figure S3).

Figure S2



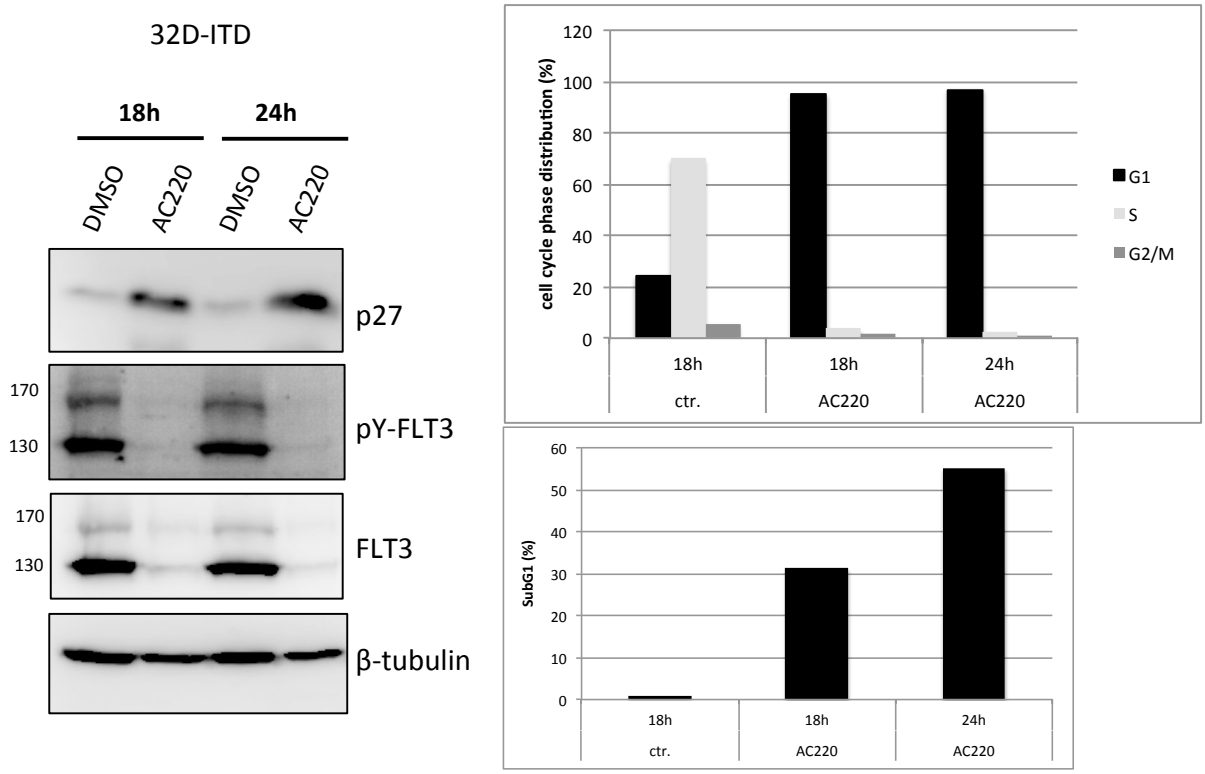
FACS profiles of MV4;11 cells treated with vehicle or AC220. Cells were double stained with BrdU (S-phase) and PI (DNA) and cell cycle phase distribution was analysed using FlowJo software.

Figure S3



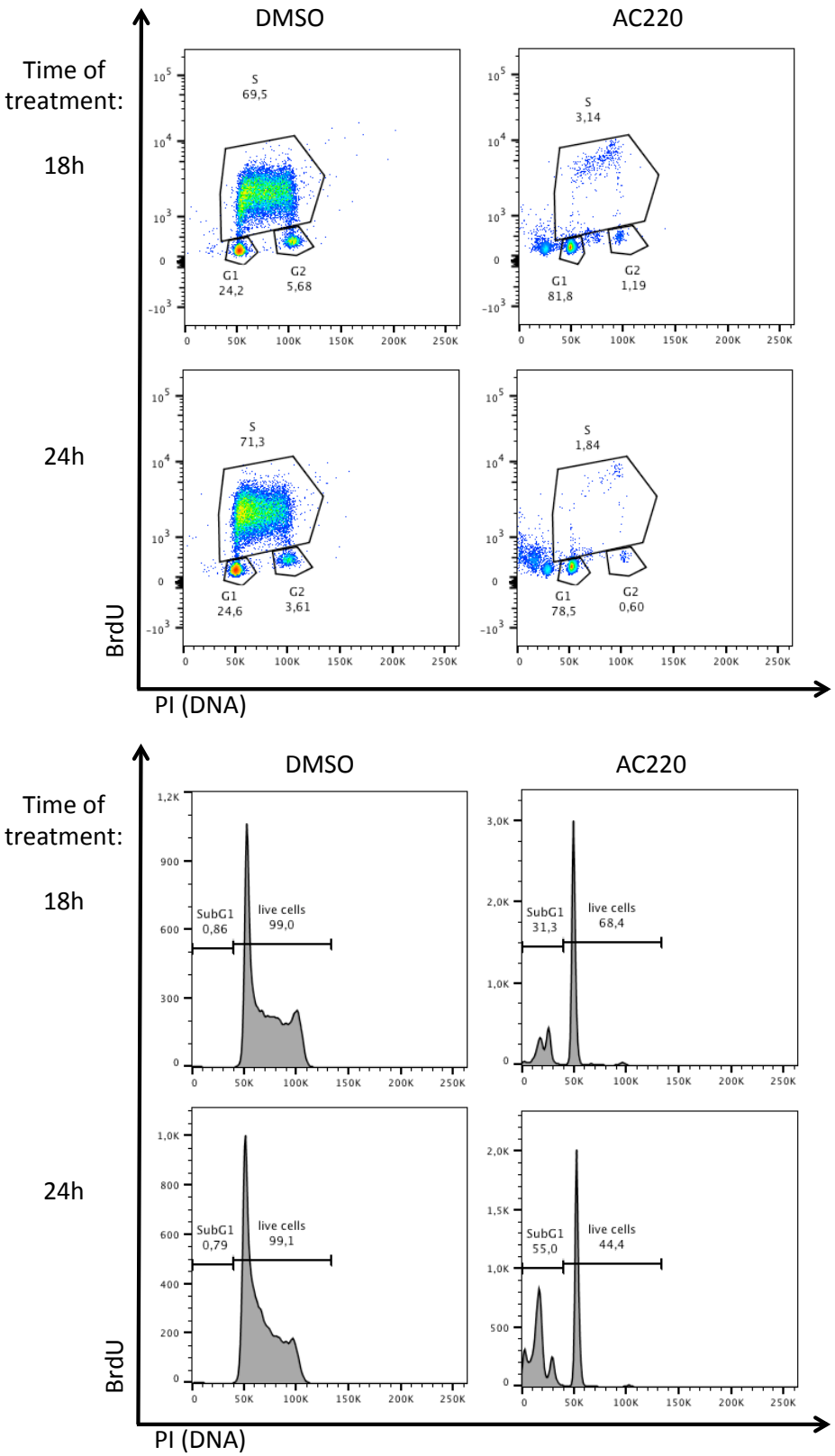
FACS profiles of MV4;11 cells treated with vehicle or AC220. Cells were stained with PI (DNA) and proportion of cells in subG1 was analyzed using FlowJo software.

Figure S4



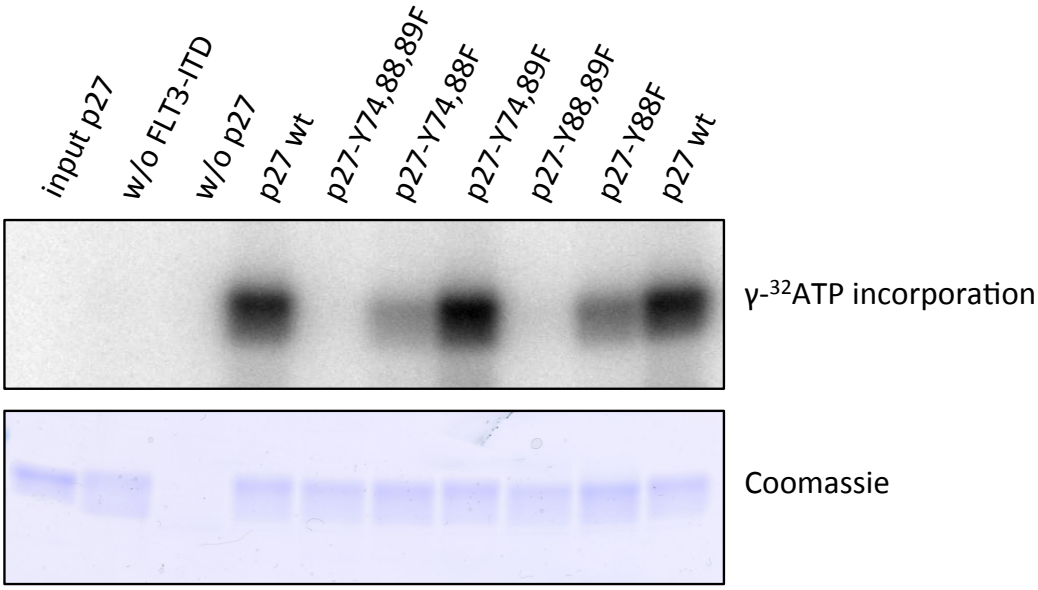
Inhibition of FLT3-ITD with AC220 in 32D-ITD cells results in upregulation of p27 levels and cell cycle arrest in G1 phase. 32D-ITD cells were incubated with AC220 and vehicle (DMSO) for the times indicated. Western blot analyses with anti-p27, anti-pYFLT3, anti-FLT3 and anti-tubulin antibodies (left panel) as well as flow cytometry analyses of PI and BrdU stained cells to determine cell cycle phase distribution (Figure S5 and upper right panel) were performed. Percentage of subG1 cells (lower right panel) was determined from a histogram of PI stained cells (Figure S5 lower panel).

Figure S5



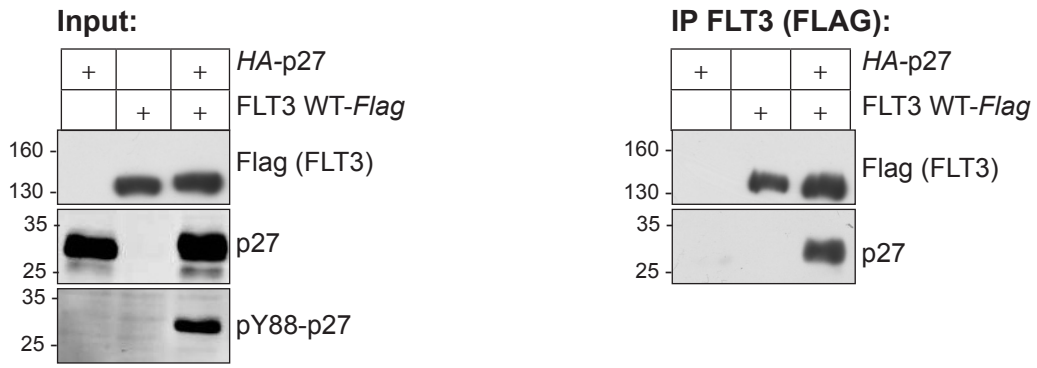
FACS profiles of 32D-ITD cells treated with vehicle or AC220. Upper panel: Cells were double stained with BrdU (S-phase) and PI (DNA) and cycle phase distribution was analysed using FlowJo software. Lower panel: Cells were stained with PI (DNA) and proportion of cells in subG1 was analysed.

Figure S6



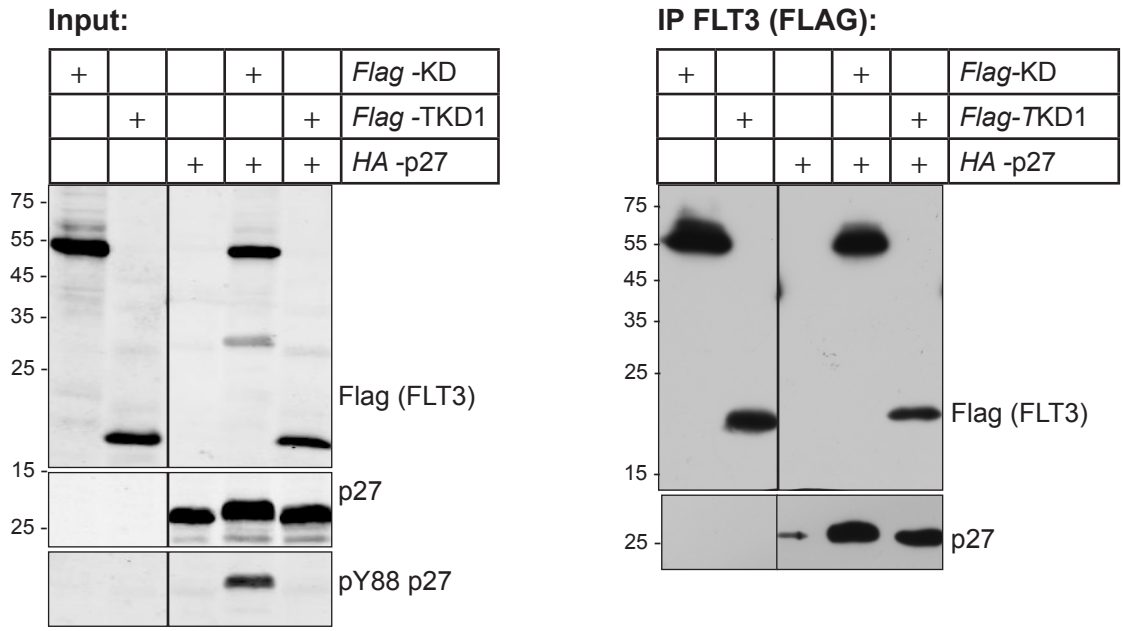
p27 is phosphorylated by recombinant FLT3-ITD kinase domain isolated from insect cells (ProQinase). In vitro kinase assay with wildtype and mutant p27 purified from bacteria reveal that p27 is directly phosphorylated on tyrosine 88 by isolated recombinant FLT3-ITD kinase domain (γ-32-ATP incorporation, upper panel). Coomassie staining of the gel (lower panel) indicates the presence of equal p27 protein levels for each condition.

Figure S7



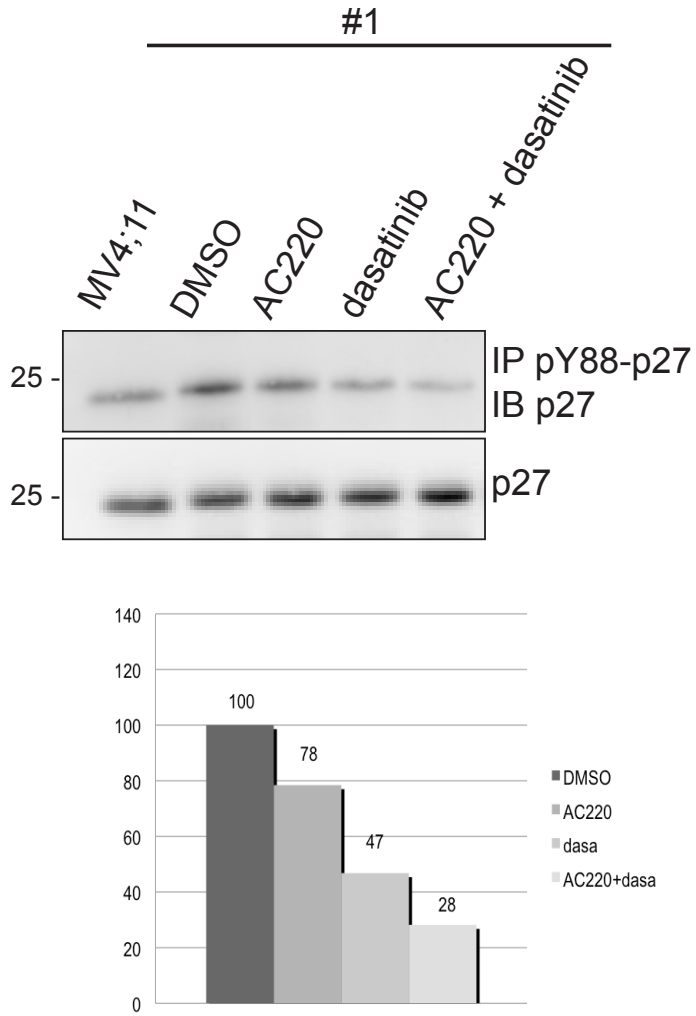
p27 co-precipitates with FLT3 WT. HA-p27 and Flag-FLT3 were over-expressed in 293T cells. Protein expression (Input) was analysed by Western blot. FLT3 was immunoprecipitated with anti-Flag antibodies. Immunoprecipitates were analysed by Western blot using anti-Flag antibodies and co-immunoprecipitates were detected with anti-p27 antibodies (right panel).

Figure S8



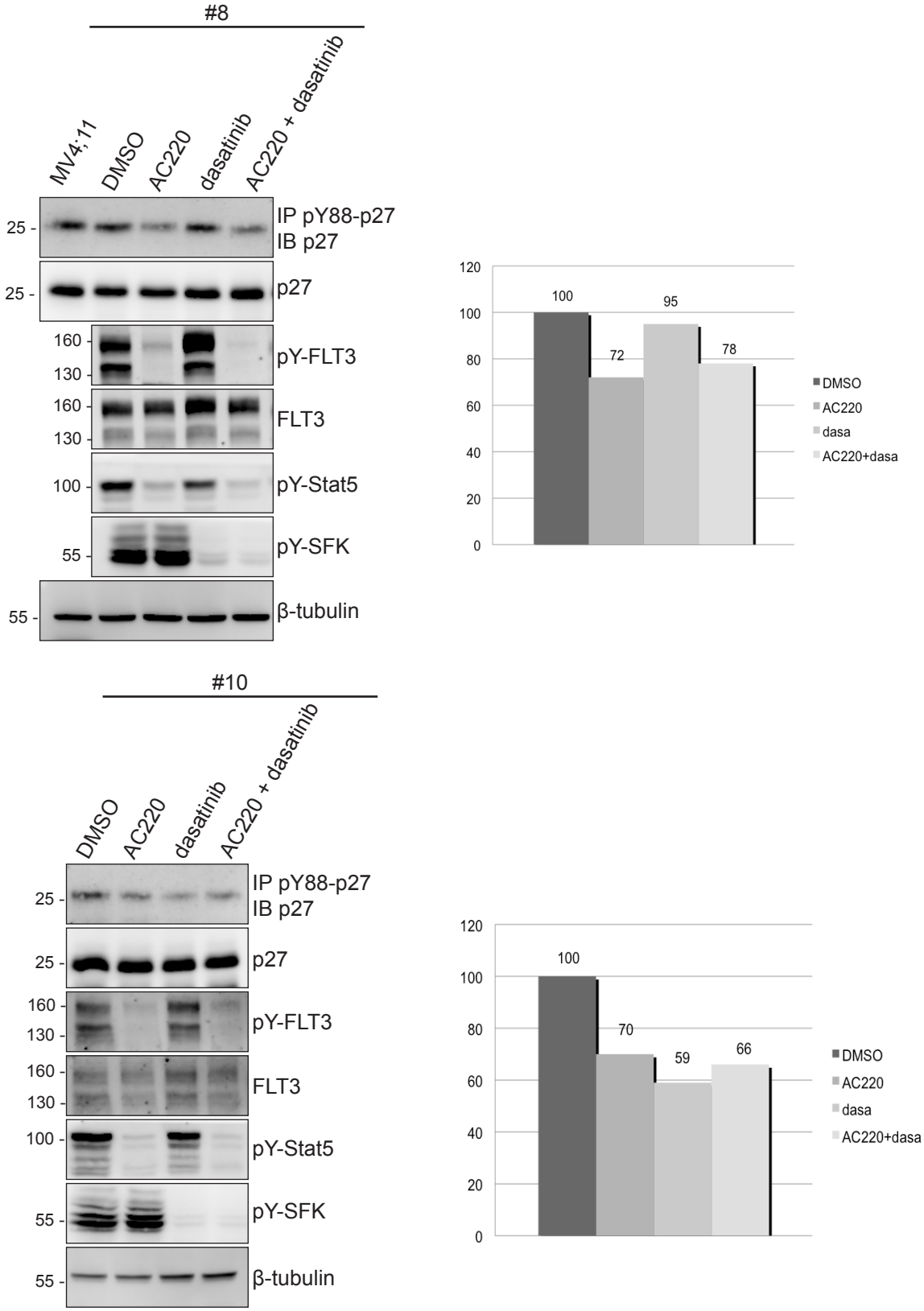
p27 binds to TKD1 of FLT3. HA-p27 and cytoplasmic domains of Flag-FLT3 were over-expressed in 293T cells and analysed by Western blot (left panel). p27 is phosphorylated on Y88 after Flag-FLT3-KD expression (left panel). HA-p27 co-precipitates with Flag-FLT3-KD and Flag-FLT3-TKD1 (right panel).

Figure S9



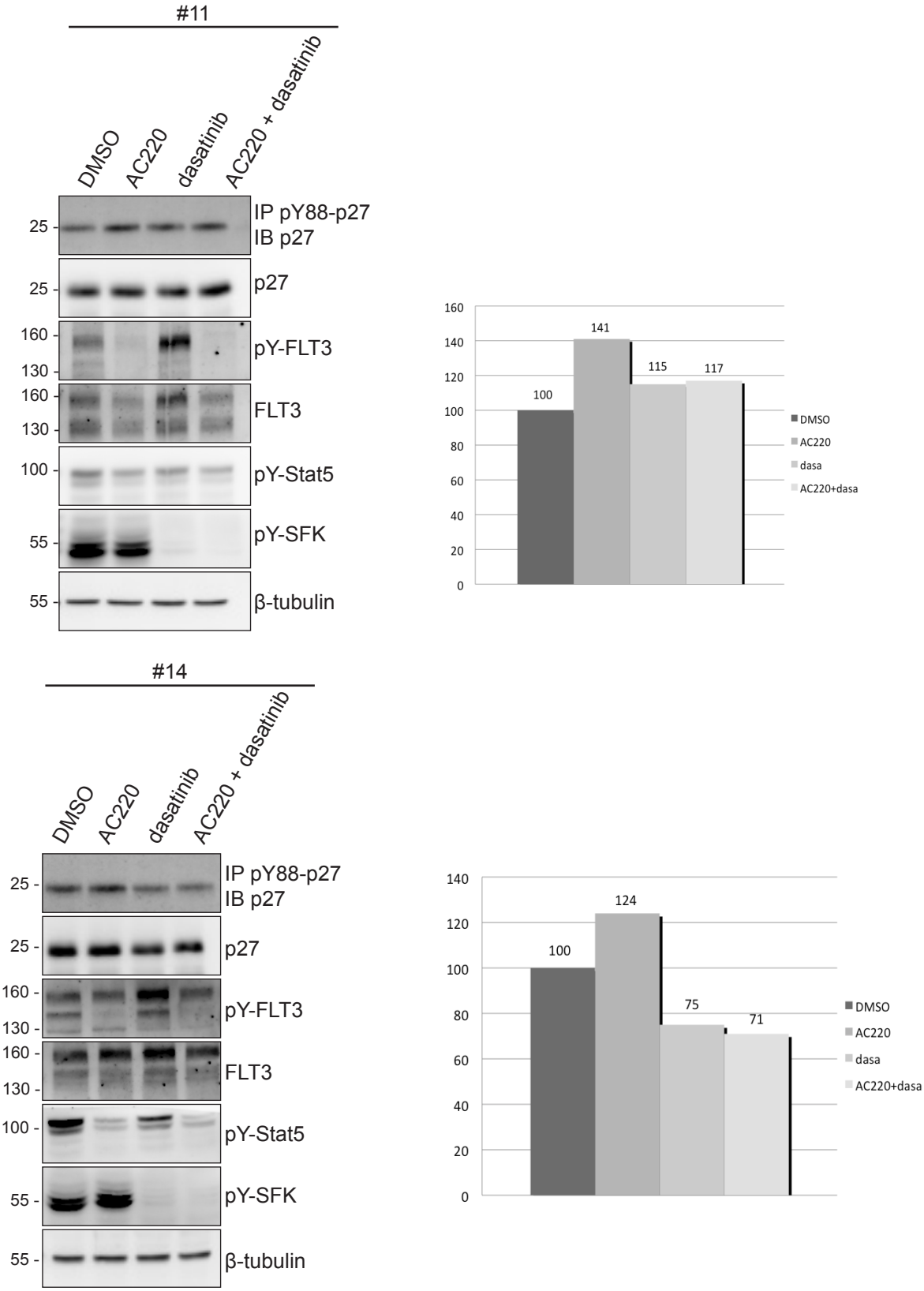
Dasatinib treatment decreases pY88-p27 in patient sample #1 expressing wildtype FLT3. Treatment of patient sample #1 (wildtype FLT3) with AC220, dasatinib and the combination of the two kinase inhibitors. Western blot analyses show that AC220 treatment decreases pY88-p27 levels. Dasatinib and the combination of AC220 with dasatinib decreases pY88-p27 even further. Of note, signals for p27 and pY88-p27 of patient #1 and MV4;11 cells are within a comparable range (same amounts of total protein analysed).

Figure S10



Dasatinib treatment decreases pY88-p27 in one out of two FLT3-ITD positive patients where AC220 treatment decreases pY88-p27 levels. Western blot analyses show that dasatinib treatment does not affect pY88-p27 in one patient (#8) but decreases pY88-p27 in the second patient (#10). Of note, the levels of p27 and pY88-p27 are similar between patient #8 and MV4;11 cells (same amounts of total protein analysed).

Figure S11



Dasatinib treatment decreases pY88-p27 in one out of two FLT3-ITD positive patients where AC220 treatment increases pY88-p27 levels. Western blot analyses show that dasatinib treatment barely affects pY88-p27 in one patient (#11) and decreases pY88-p27 in the second patient (#14).