Oncogenic JAK1 and JAK2-activating mutations resistant to ATP-competitive inhibitors

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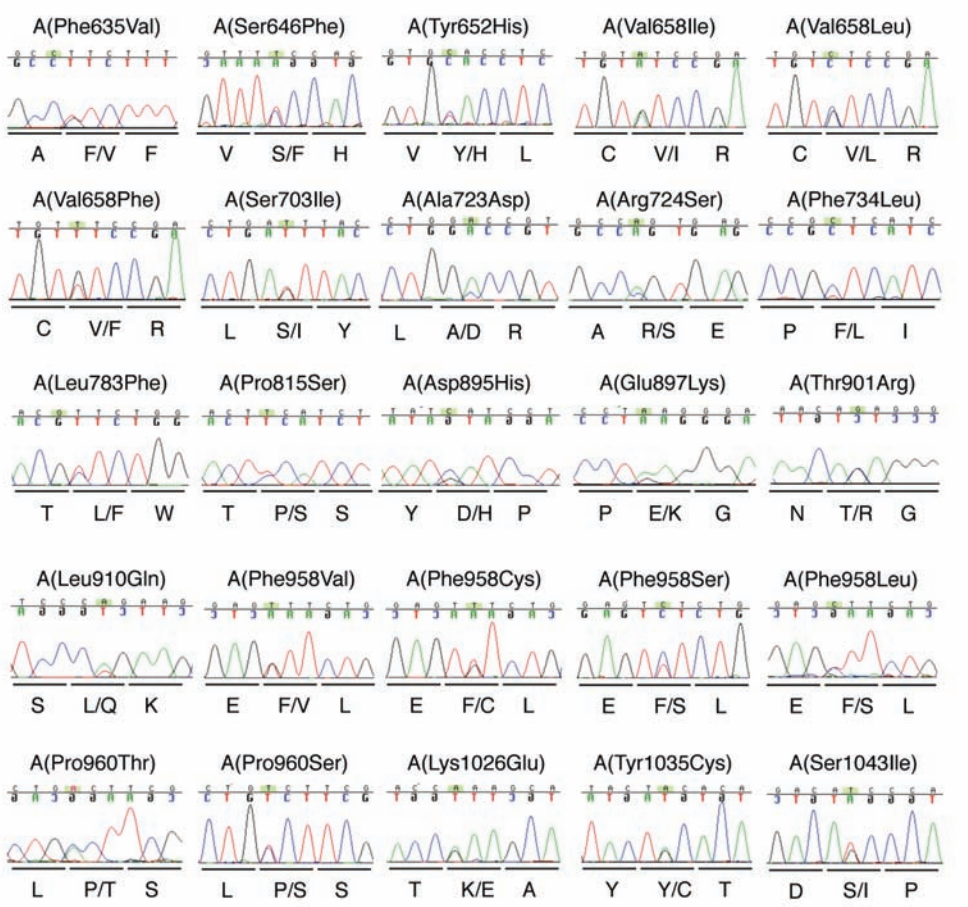
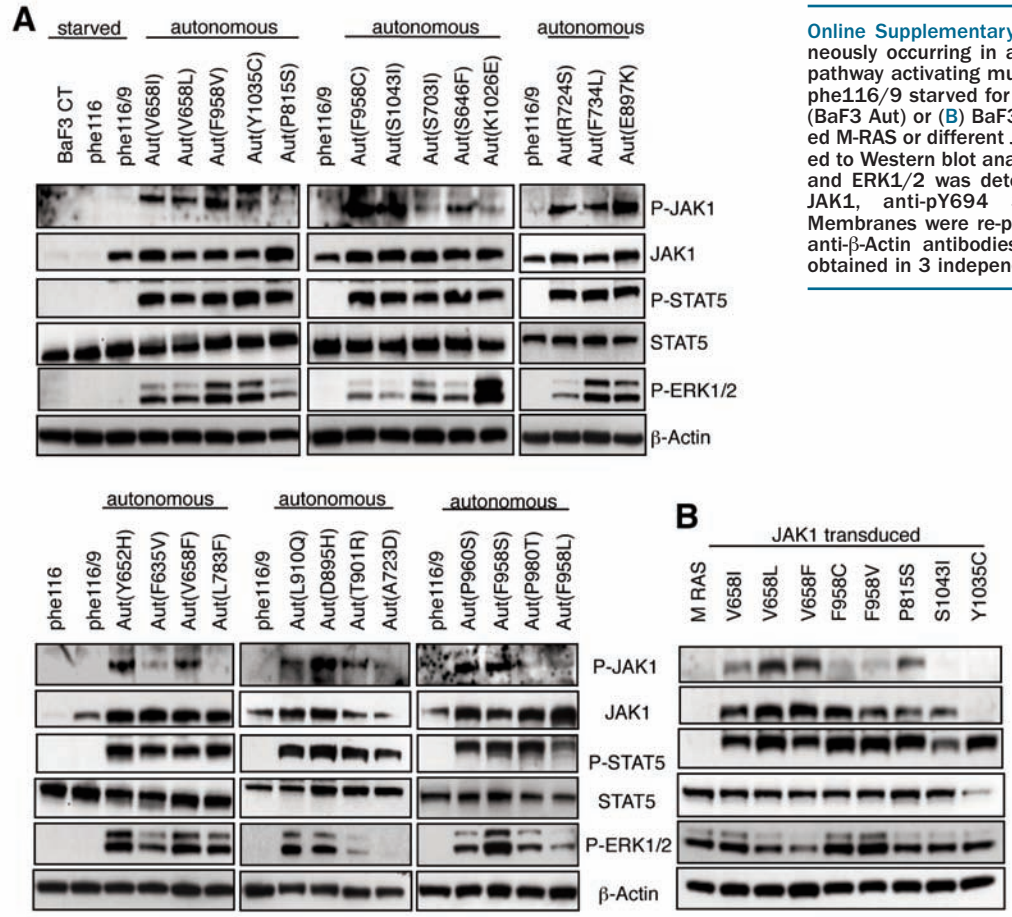
Online Supplementary Table S1. ***De novo*** **mutations in JAK1 identified** ***in vitro*** **in autonomous clones. List of mutates residues in JAK1 with their nucleotide and amino acid change, localization within the JAK1 structure in parallel to their effect on activation of JAK1, STAT5 and ERK1/2 proteins, on BaF3 cells transformation to IL-3-independent proliferation, occurence in ALL patients (9-11, 23) and resistance to ATP-competite JAK inhibitors.**

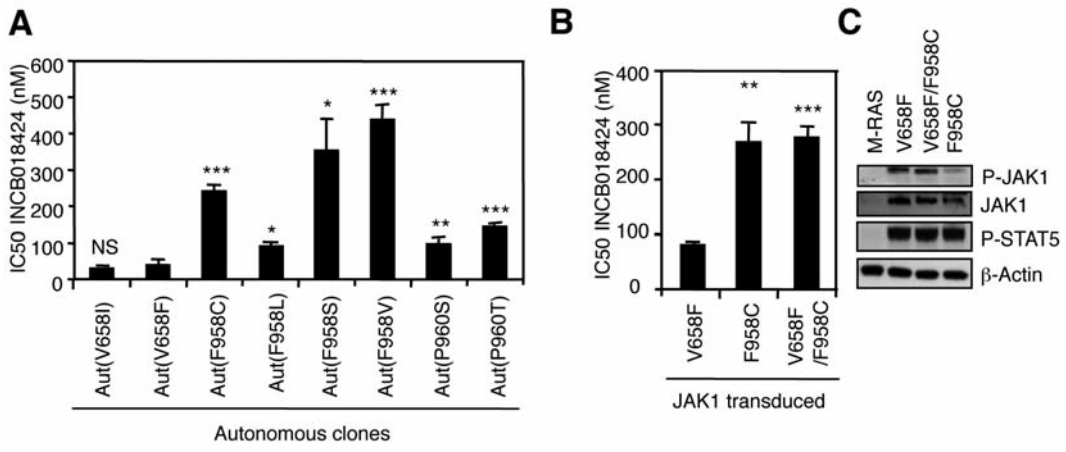
JAK1 MUTATION	NUCLEOTIDE CHANGE	LOCALIZATION	CONSTITUTIVE P-JAK1	SIGNALING P-STAT5	SIGNALING P-ERK1/2	TRANSFORMING POTENTIAL IN BAF3 CELLS	PRESENT IN PATIENT	JAK INHIBITOR RESISTANT
F635V	TTC->GTC	pseudoKD	+	+	+	+	-	-
S646F	TCC->TTC	pseudoKD	+	+	+	+	+	-
Y652H	TAC->CAC	pseudoKD	++	+	++	+	+	-
V658I	GTC->ATC	pseudoKD	++	+	+	+	-	-
V658L	GTC->CTC	pseudoKD	++	+	+	+	-	-
V658F	GTC->TTC	pseudoKD	++	+	++	+	+	-
S703I	AGT->ATT	pseudoKD	+	+	+	+	-	-
A723D	GCC->GAC	pseudoKD	+/-	+	-	not tested	-	-
R724S	CGT->AGT	pseudoKD	++	+	+	+	+	-
F734L	TTC->CTC	pseudoKD	++	+	++	not tested	-	-
L783F	CTC->TTC	pseudoKD	+/-	+	++	+	+	-
P815S	CCA->TCA	pseudoKD	+	+	+	+	-	-
D895H	GAT->CAT	KD	++	+	+	not tested	-	-
E897K	CGT->AGT	KD	++	+	++	not tested	-	-
T901R	ACA->AGA	KD	+	+	+	not tested	-	-
L910Q	CTG->CAG	KD	+	+	+/-	not tested	-	-
F958V	TTT->GTT	KD	++	+	++	+	-	+
F958C	TTT->TGT	KD	++	+	+	+	-	+
F958L	TTT->CTT	KD	+	+	+	not tested	-	+
F958S	TTT->TCT	KD	++	+	++	not tested	-	+
P960T	CCT->ACT	KD	+	+	+	+	-	+
P960S	CCT->TCT	KD	++	+	+	not tested	-	+
K1026E	AAA->GAA	KD	+	+	++	+	-	-
Y1035C	TAC->TGC	KD	+	+	++	+	-	-
S1043I	AGC->ATC	KD	+	+	+/-	+	-	-



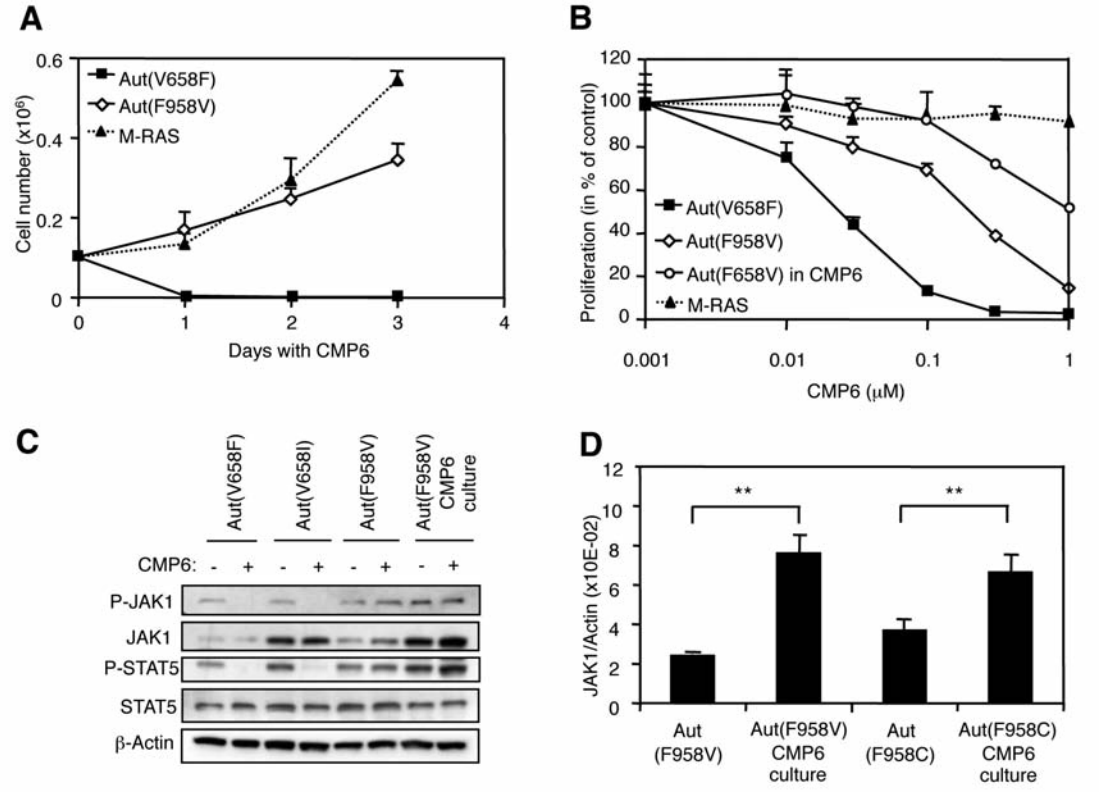
Online Supplementary Figure S1. *De novo* JAK1 mutations occur frequently during the selection of BaF3 autonomous clones. Autonomous BaF3 clones (BaF3 AUt) were selected from IL-9 responding BaF3 phe116/9 cells. Direct sequencing of JAK1 RT-PCR product from BaF3 Aut clones was performed showing *de novo* point mutations in JAK1 in autonomous cells. The JAK1 sequence of one representative autonomous clone is shown for each of the 25 detected JAK1 mutations from a total of 139 clones heterozygous for a single JAK1 mutation.



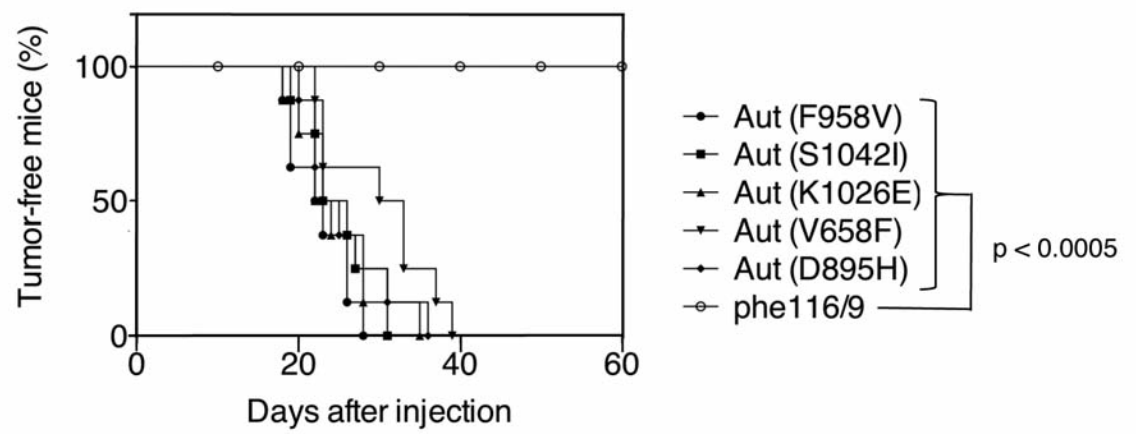
Online Supplementary Figure S2. JAK1 mutations spontaneously occurring in autonomous BaF3 cells are JAK-STAT pathway activating mutations. (A) 10^6 BaF3 phe116, BaF3 phe116/9 starved for 4 h and autonomous BaF3 cell lines (BaF3 Aut) or (B) BaF3 cells stably transduced with activated M-RAS or different JAK1 mutants were lyzed and subjected to Western blot analysis. Phosphorylation of JAK1, STAT5 and ERK1/2 was detected using specific anti-pY1034/35 JAK1, anti-pY694 STAT5, anti-pP42/44 antibodies. Membranes were re-probed with anti-JAK1, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments.



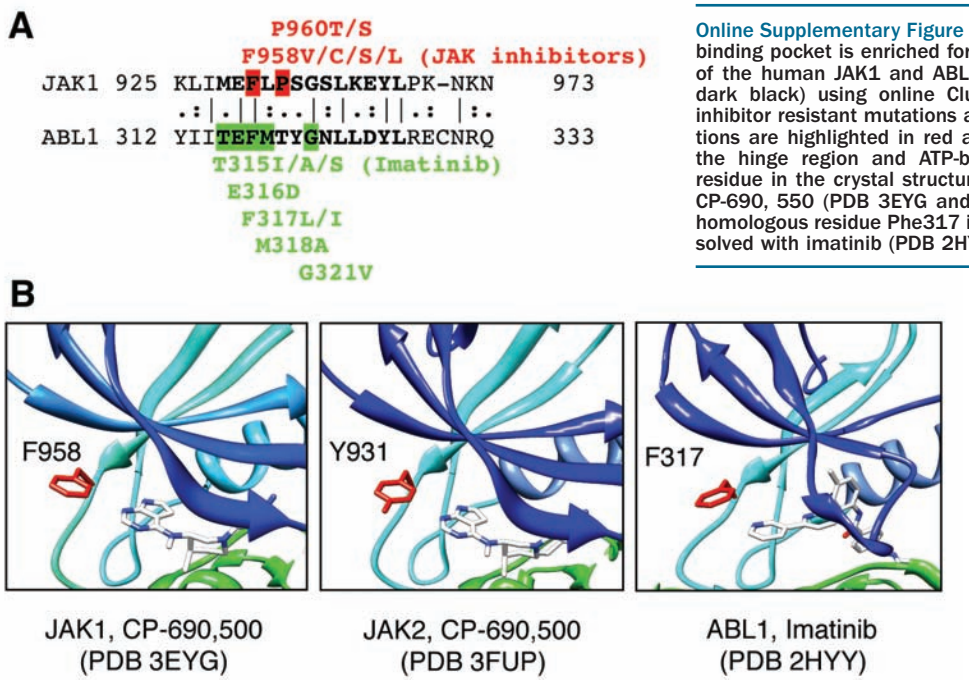
Online Supplementary Figure S3. Mutations targeting JAK1 Phe958 and Pro960 confer JAK inhibitor resistance. IC50 for INCB018424 inhibitor calculated based on dose-dependent proliferation curves of (A) BaF3 Aut(V658F), Aut(V658I), Aut(F958C), Aut(F958L), Aut(F958S), Aut(F958V), Aut(P960T) and Aut(P960S) or (B) BaF3 cells stably transduced by V658F, F958C and V658F/F958C double JAK1 mutant. Results are mean ± SEM of at least 3 independent experiments with 2 different autonomous clones for each JAK1 mutation (*P<0.05; **P<0.01; ***P<0.001). Similar results were observed with CMP6 inhibitor. (C) BaF3 cells stably transduced with activated M-RAS or JAK1 V658I and F958V mutants were lyzed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT5 was detected using specific anti-JAK1 Y1034/35 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK1 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments.



Online Supplementary Figure S4. Long-term culture of F958V-positive BaF3 cells with JAK inhibitor leads to increased expression of JAK1 that correlates with increased resistance to JAK inhibitor. (A) BaF3 Aut(V658F), Aut(F958V) and BaF3 stably transfected with activated M-RAS were seeded in 6-well plates at a concentration of 100,000 cells/mL in culture medium in the presence of 1 μM CMP6 inhibitor. Living cells were counted at 24 h intervals. Results are mean ± SD of triplicate cultures. Similar results were obtained in 3 independent experiments. (B) 10,000 BaF3 Aut(F958V) cells that had been cultured for two weeks with 1 μM CMP6, or BaF3 Aut(V658F), Aut(F958V) or stably transfected with activated M-RAS, that were never cultured with the inhibitor, were seeded in 96-well plates and treated with increasing concentrations of CMP6. After 48 h, tritiated thymidine was added to the cells for 4 h and thymidine incorporation was measured. Results are mean ± SD of triplicate cultures. Similar results were obtained in 3 independent experiments with 2 different autonomous clones for F958V and F958C JAK1 mutation. (C) 10^6 BaF3 Aut(V658I), Aut(V658F), Aut(F958V) never cultured with the inhibitor and BaF3 Aut(F958V) cells cultured for two weeks with 1 μM CMP6 were treated for 30 min with 500 nM CMP6 inhibitor or with 0.1 % DMSO as a control (- condition), lyzed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT5 was detected using specific anti-pY1034/35 JAK1 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK1, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments with 2 different autonomous clones for F958V and F958C JAK1 mutation. (D) 10^6 BaF3 Aut(F958V), Aut(F958C) never cultured with the inhibitor and BaF3 Aut(F958V) or Aut(F958C) cells cultured for two weeks with 1 μM CMP6 were lyzed and used for total RNA extraction. Quantitative PCR was performed using primers for murine JAK1 and murine Actin as a normalization reference. Results are mean ± SD of biological triplicates (**P<0.01). Similar results were obtained in 3 independent experiments with 2 different autonomous clones for F958V and F958C JAK1 mutation.



Online Supplementary Figure S5. *In vivo* tumorigenic activity of autonomous BaF3 clones. 2x10^6 BaF3 autonomous or phe116/9 cells were injected i.p. with immunodeficient 9-11 weeks old Rag2-/- mice. Three to four weeks after cell transfer, Rag2-/- mice injected with JAK1 mutation-positive BaF3 clones developed tumors. Mice injected with parental, JAK1 mutation-negative phe116/9 cells stayed tumor-free. No significant difference in rate of tumor development was observed between clones with strong (K1026E) or faint (S1043I) ERK1/2 activation and between mutations affecting kinase or pseudokinase domain (Gehan-Breslow-Wilcoxon test).



Online Supplementary Figure S6. Hinge region at the periphery of the ATP-binding pocket is enriched for inhibitor resistant mutations. (A) Alignment of the human JAK1 and ABL protein sequences around hinge region (in dark black) using online ClustalW2 software. The JAK1-activating JAK inhibitor resistant mutations and currently known imatinib resistant mutations are highlighted in red and yellow, respectively. (B) Magnification of the hinge region and ATP-binding pocket around the Phe958/Tyr931 residue in the crystal structure of JAK1/JAK2 kinase domain solved with CP-690, 550 (PDB 3EYG and PDB 3FUP, left and middle panel) and the homologous residue Phe317 in the crystal structure of ABL kinase domain solved with imatinib (PDB 2HYY, right panel).