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Syk kinase and Shp2 phosphatase inhibition cooperate to reduce FLT3-ITD-induced STAT5 activation and proliferation of acute myeloid leukemia

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Acute myeloid leukemia (AML) continues to be a difficult-to-treat disease with a high relapse rate, and individuals presenting with FLT3-ITD+ status exhibit a poor prognosis[1]. We demonstrated previously that genetic disruption or pharmacologic inhibition of the protein tyrosine phosphatase, Shp2, reduced FLT3-ITD-induced STAT5 hyperactivation, hyperproliferation, and leukemia-induced mortality *in vivo*[2]. We also demonstrated that mutation of duplicated tyrosine (Y) 599, located in the juxtamembrane domain of FLT3 and known to interact with Shp2[3], to phenylalanine (F) reduced FLT3-ITD-induced STAT5 hyperactivation and hyperproliferation[2]. As Shp2 is a phosphatase, we anticipated that Shp2 cooperates with a STAT5-activating kinase to promote cellular hyperproliferation. Given that Syk has been found to interact with Y599 on FLT3-ITD and phosphorylates inter-kinase domain Y768[4], that Y768 recruits Grb2, Gab2, and p85α[5], and that Shp2 is reported to participate in multi-protein complexes containing Gab2 and p85α[6,7], we hypothesized that

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increased Shp2 recruitment to duplicated Y599 on FLT3-ITD cooperates with Syk to enhance constitutive STAT5 phosphorylation and to promote Y768-dependent Erk and Akt phosphorylation. Consequently, we predicted that mutation of duplicated Y599 or of Y768 to phenylalanine would lead to reduced proliferation *in vitro* and reduced FLT3-ITD-induced myeloproliferative disease (MPD) *in vivo*.

To investigate this hypothesis, we generated 32D cells transfected with modified N51-FLT3-ITD[2,8] constructs bearing mutation of either the duplicated juxtamembrane Y599 to phenylalanine (N51-FLT3-Y599F1/2)[2] or of Y768 to phenylalanine (N51-FLT3-Y768F, Fig. 1A). Cells expressing N51-FLT3-Y599F1/2 or N51-FLT3-Y768F demonstrated significant reduction in proliferation compared to N51-FLT3-expressing cells in the absence and presence of FLT3 Ligand (FL, Fig. 1B), although the N51-FLT3-Y599F1/2 mutation had a stronger effect.

We hypothesized that reduction in hyperpoliferation *in vitro* would prolong latency to onset of MPD *in vivo.* To investigate disease development, transfected 32D cells were transplanted into syngeneic C3H/HeJ hosts, and animals demonstrating ≥0.5% EGFP positive cells in peripheral blood at three weeks post-transplant were followed for overall survival. While mice transplanted with N51-FLT3-Y768F cells failed to show reduced MPD or improved survival, mice transplanted with N51-FLT3-Y599F1/2 cells demonstrated prolonged overall survival compared to N51-FLT3 (Fig. 1C). In spite of the differential effect of the Y599 and Y768 mutations on overall survival, we found similarly reduced Shp2 interaction with both N51-FLT3-Y599F1/2 and N51-FLT3-Y768F compared to N51-FLT3 in immunoprecipitation assays (Fig. 1D, compare lanes 3 and 4 to lane 2). Taken together, these findings suggest duplicated Y599 and Y768 have differential effects on FLT3-ITD-induced MPD, most likely due to varied activation of downstream signaling effectors. Additionally, although duplicated Y599 plays a more prominent role, it is not the only factor contributing to disease, as its mutation slows but does not ablate leukemia progression.

We next examined potentially altered signals emanating from N51-FLT3 bearing mutation of duplicated Y599 or Y768. We subjected each of the 32D cell lines to serum and growth factor deprivation for 3 hours or overnight, followed by examination of STAT5, Akt, and Erk activation. When examining Akt activation, we did not observe increased Akt phosphorylation in the N51-FLT3 compared to WT FLT3 control cells regardless of the starvation conditions (data not shown). As there have been inconsistencies in the literature about hyperactivation of Akt in FLT3-ITD+ AML[9–11], we turned our attention toward STAT5 and Erk. Interestingly, we saw differences in STAT5 and Erk activation depending on the starvation time length. We therefore focused on the effect of duplicated Y599 or Y768 mutation at time points post-starvation where N51-FLT3-expressing cells most consistently demonstrated elevated phospho-STAT5 (3 hours) or phospho-Erk (6 hours to overnight) compared to WT FLT3-expressing cells. Consistent with previous findings[2], we saw that N51-FLT3-expressing cells had higher phospho-STAT5 compared to WT FLT3-expressing cells, and N51-FLT3-Y599F1/2 resulted in lower STAT5 activation compared to N51-FLT3 (Fig. 1E); however, when combining multiple experiments, N51-FLT3-Y599F1/2-induced STAT5 activation was not completely normalized to WT levels (Fig. 1F), consistent with incomplete ablation of N51-FLT3-mediated MPD *in vivo* (Fig. 1C). The N51-FLT3-Y768F

mutation did not reduce STAT5 activation compared to N51-FLT3-expressing cells, again consistent with the *in vivo* findings (Fig. 1E and 1F). As we hypothesized the protein complex at Y599 is critical for the phosphorylation of Y768 and consequent protein complex recruitment to Y768, we predicted that mutation of duplicated Y599 would also lower Erk activation. However, the N51-FLT3-Y599F1/2-expressing cells did not demonstrate lower Erk activation (Fig. 1E and 1G), suggesting that mutation of duplicated Y599 is not adequate for reduction of Erk activation. Interestingly, upon mutation of Y768, we did observe normalization in Erk activation (Fig. 1E and 1G), even though animals transplanted with N51-FLT3-Y768F-expressing cells did not demonstrate improved survival (Fig. 1C). Collectively, these findings indicate that the inter-kinase domain Y768 is needed for FLT3-ITD-induced Erk hyperactivation, but that normalization of Erk activation in the presence of persistent FLT3-ITD-induced STAT5 hyperactivation is insufficient to prevent the onset and progression of FLT3-ITD-induced MPD.

Given that we did not observe a strong differential effect of Akt activation in our various experimental cell types, we focused pharmacologic studies on Shp2 phosphatase and Syk kinase inhibition. Based on our hypothesis that increased Shp2 recruitment to duplicated Y599 cooperates with Syk kinase to promote STAT5 hyperactivation, and our observation that reduced STAT5 activation significantly modulated FLT3-ITD-induced proliferation *in vitro* (Fig. 1B) and MPD *in vivo* (Fig. 1C), we predicted that pharmacologic inhibition of Shp2 phosphatase (using II-B08[12]) would cooperate with pharmacologic inhibition of Syk kinase (using R406) to reduce proliferation of FLT3-ITD-expressing AML cells. Additionally, since STAT5 activation is an aberrant pathway in FLT3-ITD-expressing cells, we hypothesized that FLT3-ITD-expressing cells would be uniquely sensitive to Shp2 and Syk inhibition. To test this hypothesis, we first looked at WT FLT3- or N51-FLT3-expressing 32D cells. Cells expressing WT FLT3 demonstrated minimal response to R406 alone or the combination of R406 plus II-B08 (Fig. 2A). In contrast, proliferation of N51-FLT3-expressing cells was significantly reduced in response to R406 alone compared to the WT FLT3-expressing cells (Fig. 2A). Furthermore, pharmacologic inhibition of Shp2 and Syk cooperated to further reduce proliferation of N51-FLT3-expressing cells compared to R406 alone (Fig. 2A), suggesting that FLT3-ITD-expressing cells are uniquely dependent on Shp2 and Syk activity. This reduction in proliferation was consistent with the effect of drug treatment on STAT5 activation. While II-B08 or R406 alone reduced phospho-STAT5 levels, we saw a significant reduction upon dual Shp2 and Syk inhibition (Fig. 2B and 2C), again displaying cooperativity between Shp2 and Syk in FLT3-ITD-expressing cells.

We next examined the effect of Shp2 and Syk inhibition on primary AML samples. R406 alone substantially reduced proliferation of primary AML cells; however, the addition of II-B08 further significantly reduced proliferation (Fig. 2D, upper panel). Of these AML samples, we next compared the response of FLT3-ITD- (n=4) and FLT3-ITD+ (n=7) samples to these compounds. Similar to that previously published[4], we found that FLT3-ITD- samples demonstrated a more variable and less pronounced response to Syk inhibition alone compared to FLT3-ITD+ samples (Fig. 2D, lower panel). Furthermore, in response to the combination of R406 plus II-B08, FLT3-ITD+ samples proliferated less compared to FLT3-ITD- samples and compared to FLT3-ITD+ samples treated with R406 alone (Fig. 2D, lower panel). From these studies, we also noted a trend that FLT3-ITD+ samples appeared

more sensitive to II-B08 alone. To investigate further, we compiled data from all FLT3-ITD- and FLT3-ITD+ AML samples tested in our lab in response to increasing concentrations of II-B08. These data clearly demonstrate that FLT3-ITD+ samples bear increased sensitivity to II-B08 compared to FLT3-ITD- samples (Fig 2E).

Collectively, these data show that both duplicated Y599 and Y768 positively promote FLT3-ITD-induced MPD, and that signaling from Y599 through STAT5 more strongly promotes aggressiveness of disease. In the context of FLT3-ITD, Syk and Shp2 cooperate to promote STAT5 activation and AML cell proliferation. As Syk has been shown to phosphorylate Y768, we expected that mutation of Y599 would reduce the activation of effectors emanating from Y768, and that mutation of Y768 would phenocopy mutation of duplicated Y599. However, we did not observe normalization of Erk activation upon mutation of duplicated Y599, and mutation of Y768 failed to prolong survival. As Syk has been shown to be recruited to Y589, Y591, and Y597 as well as Y599[4], mutation of just one of the binding sites for Syk may not be sufficient to reduce Syk signaling to the inter-kinase domain of FLT3, and the other juxtamembrane domain tyrosines may compensate to maintain Erk activation. Additionally, Syk has also been shown to phosphorylate inter-kinase domain Y955 as well as Y768; therefore, Syk phosphorylation of Y955 may compensate for mutation of Y768, thus precluding normalized survival in mice transplanted with N51-FLT3-Y768F cells. Taken together, our findings suggest that reduced STAT5 activation overrides reduced Erk activation, likely accounting for the increased sensitivity of FLT3-ITD+ samples to Shp2 inhibition alone or to the combination of Shp2 and Syk inhibition. Overall, our findings indicate a novel signaling relationship between the tyrosine phosphatase, Shp2, and the tyrosine kinase, Syk, in FLT3-ITD+ AML, and provide evidence that targeting this pathway at multiple points may hold therapeutic benefit for treating FLT3-ITD+ AML patients.

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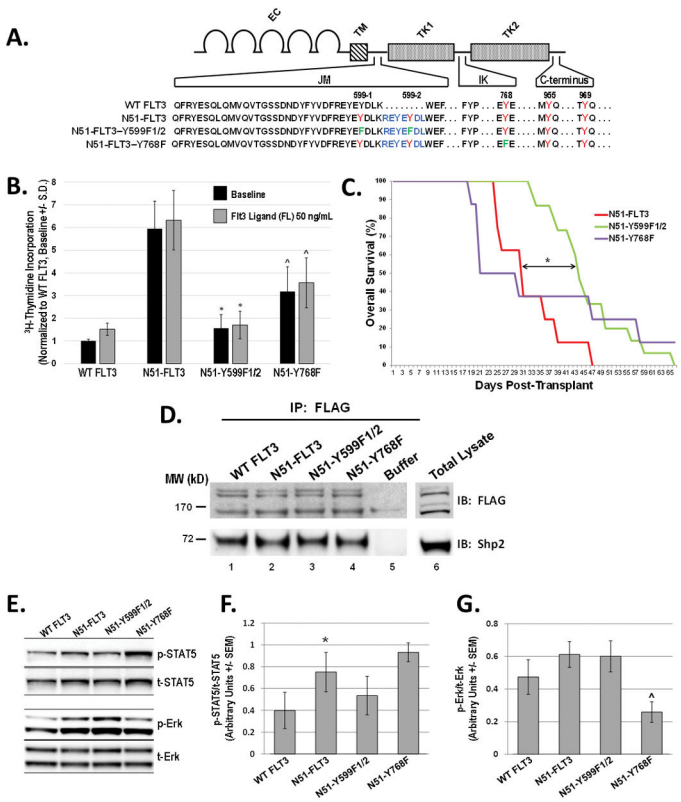

Figure 1.

(A) Schematic diagram of WT FLT3, the internal tandem duplication, N51-FLT3, and mutated constructs used in the current studies, cDNAs were FLAG-tagged and cloned into pMSCV in tandem with enhanced green fluorescent protein (EGFP). **(B)** ^{3}H-thymidine incorporation analyses of 32D cells transfected with WT FLT3, N51-FLT3, N51-FLT3-Y599F1/2, or N51-FLT3-Y768F and sorted to homogeneity based on EGFP expression, proliferation normalized to average WT FLT3-expressing cells at baseline within each independent experiment, data compiled from 4 independent experiments, *n=4, $p<0.05$ comparing N51-FLT3-Y599F1/2 to N51-FLT3 in the absence or presence of FL, ^n=4, $p<0.05$ comparing N51-FLT3-Y768F to N51-FLT3 in the absence or presence of FL, statistics performed by unpaired, two-tailed student's *t* test. **(C)** 3 X 10^{6} 32D cells transfected with respective constructs and sorted to homogeneity based on EGFP expression were transplanted into C3H/HeJ syngeneic recipients, and animals with > 0.5% EGFP positive peripheral blood 3 weeks post-transplant were followed for overall survival, data presented by Kaplan-Meier analysis, n=8 in the N51-FLT3 group, n=15 in the N51-FLT3-Y599F1/2 group, and n=8 in the N51-FLT3-Y768F group, *$p<0.005$ comparing N51-FLT3 to N51-FLT3-Y599F1/2 by log-rank test. **(D)** Total cellular proteins from exponentially growing 32D cells transfected with respective constructs were immunoprecipitated (IP) with anti-FLAG and immunoblotted (IB) with anti-Shp2 or anti-FLAG, experiment performed on two independent occasions. **(E)** Representative immunoblot examining phosphorylation of STAT5 and Erk in WT FLT3-, N51-FLT3-, N51-FLT3-Y599F1/2-, and N51-FLT3-Y768F-expressing 32D cells. **(F)** and **(G)** Densitometry and quantitation of immunoblot analyses

examining phospho-STAT5 levels normalized to total STAT5 (F) and of phospho-Erk normalized to total Erk (G) using ImageJ software (NIH, Bethesda, MD), *n=3, $p<0.05$ comparing p-STAT5/t-STAT5 in N51-FLT3- to WT FLT3-expressing cells (paired, two-tailed student's *t* test) and ^n=5, $p<0.01$ comparing p-Erk/t-Erk in N51-FLT3-Y768F- to N51-FLT3-expressing cells (unpaired, two-tailed student's *t* test).

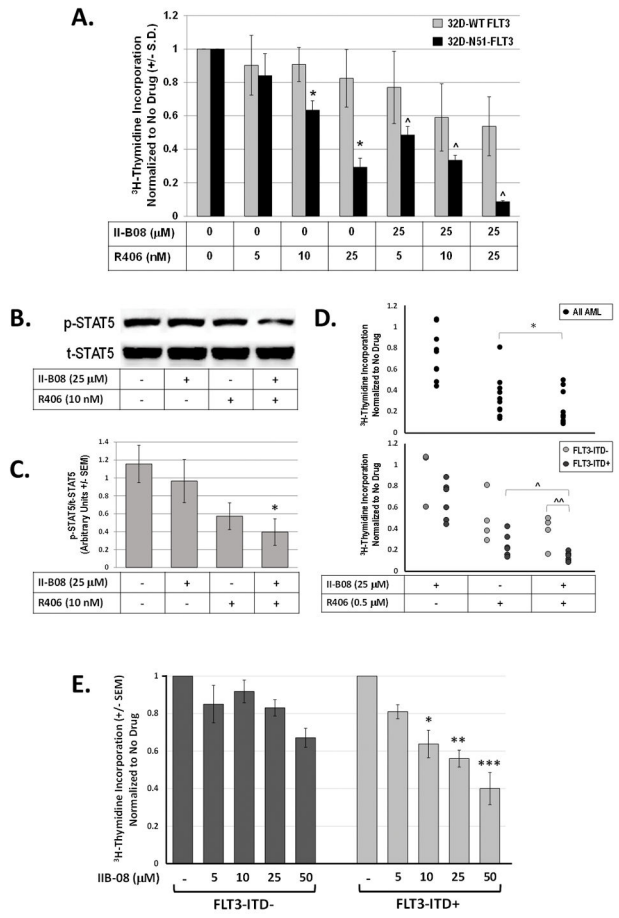

Figure 2.

(A) ^{3}H-thymidine incorporation assay of WT FLT3- and N51-FLT3-expressing 32D cells cultured in FL 50 ng/mL in the presence Syk inhibitor, R406, alone or R406 plus Shp2 inhibitor, II-B08; data presented as % of average proliferation in the absence of drug for each cell type in each independent experiment, n=3, *p<0.05 comparing to N51-FLT3 to WT FLT3 in response to 10 nM and 25 nM R406, ^p<0.05 comparing N51-FLT3 in response to 5 nM, 10 nM, or 25 nM R406 plus 25 μM II-B08 compared to 5 nM, 10 nM, or 25 nM R406 alone, statistics performed using unpaired, two-tailed student's *t* test. **(B)** Representative immunoblot examining phosphorylation of STAT5 in N51-FLT3-expressing 32D cells in the presence of II-B08 and/or R406. **(C)** Densitometry and quantitation of immunoblot analyses comparing p-STAT5/t-STAT5 in N51-FLT3-expressing 32D cells in the presence of II-B08 and/or R406 using ImageJ software (NIH, Bethesda, MD), *n=4, p<0.05 for II-B08 + R406 v. no drug, statistics using unpaired, two-tailed student's *t* test. **(D)** ^{3}H-thymidine incorporation assay of primary AML cells cultured in FL 50 ng/mL in the presence of II-B08 and/or R406, data represented as % of average proliferation in the absence of drug for each independent experiment, *n=11 independent experiments using 11 independent AML samples, p<0.01 for 0.5 μM R406 plus 25 μM II-B08 v. R406 alone (upper panel), statistics performed using paired, two-tailed student's t test; ^n=7 FLT3-ITD+ samples, p<0.05 for 0.5 μM R406 plus 25 μM II-B08 v. R406 alone (lower panel), statistics

performed using paired, two-tailed student's t test; ^^n=4 FLT3-ITD- samples and n=7 FLT3-ITD+ samples, $p<0.05$ comparing FLT3-ITD- v. FLT3-ITD+ in response to 0.5 μM R406 plus 25 μM II-B08 (lower panel), statistics using unpaired, two-tailed student's *t* test. **(E)** ^{3}H-thymidine incorporation assay of primary AML cells cultured in the presence of increasing concentrations of the Shp2 phosphatase inhibitor, II-B08. *n=3 FLT3-ITD+ and 6 FLT3-ITD- AML samples, $p<0.05$ comparing FLT3-ITD+ to FLT3-ITD- at II-B08 10 μM; **n=10 FLT3-ITD+ and n=12 FLT3-ITD- AML samples, $p<0.001$ comparing FLT3-ITD+ to FLT3-ITD- at 25 μM II-B08; ***n=7 FLT3-ITD+ and n=9 FLT3-ITD- AML samples, $p<0.05$ comparing FLT3-ITD+ to FLT3-ITD- at 50 μM, statistics performed using unpaired, two-tailed student's *t* test.