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Mutant p53 enhances leukemia-initiating cell self-renewal to promote leukemia development

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Acute myeloid leukemia (AML) is an aggressive blood cancer with poor prognosis.[1] AML is thought to be initiated and maintained by a rare population of leukemia stem cells (LSCs) or leukemia-initiating cells (LICs) that have acquired the capacity for self-

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AUTHOR CONTRIBUTIONS

SCN and YL Designed the research. SCN, SC, RG, CY, MK, SV, ACF, CW, and CD Performed the research. SCN, SC, and YL Analyzed the data and performed the statistical analysis. GES Performed pathological analysis. HSB, LDM, and RK Provided reagents and constructive advice to the study. SCN, SC, and YL Wrote the manuscript. All authors read, commented on, and approved the manuscript.

CONFLICT OF INTEREST

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renewal and is blocked in their ability to differentiate by the accumulation of a series of mutations and/or epigenetic changes.[2-3] Clinical studies show that LICs are resistant to conventional chemotherapy and/or targeted therapies.[3] Thus, there is an unmet need to elucidate the molecular mechanisms governing LIC self-renewal and develop novel therapeutic approaches that can target LICs and improve leukemia treatment.[3]

The tumor suppressor p53 is a stress response protein that regulates a large number of genes in response to a variety of cellular insults, including oncogene activation, DNA damage and inflammation.[4] These signals activate p53 primarily through post-translational modifications that result in augmented levels of p53 protein and transactivation activity.[4] Activated p53 suppresses cellular transformation mainly by inducing growth arrest, apoptosis, DNA repair and differentiation in damaged cells.[4] Accordingly, p53 function is always compromised in tumor cells, usually as a result of somatic mutations and deletions, which occur in approximately half of all human cancers.[5] The *TP53* gene encodes the tumor suppressor p53. The frequency of *TP53* mutations in AML is approximately 10%. However, in AML with complex karyotype, the frequency of *TP53* mutations and/or deletions is almost 70%.[6] While *TP53* mutations confer drug resistance and poor prognosis in AML, the role of mutant p53 in the initiation and progression of AML is largely unknown.[6-7]

We have been investigating the role of tumor suppressor p53 in normal and malignant hematopoiesis. We found that wild type p53 maintains HSC quiescence and inhibits HSC self-renewal.[8] Codon 248 of p53 is frequently mutated in AML and $p53^{R248W}$ has been shown to be a gain-of function (GOF) mutant in human cancer cells as well as in animal models.[6-7, 9] We recently reported that $p53^{R248W}$ enhances HSC self-renewal in steady state and promotes HSC expansion following genotoxic stresses.[10] Of note, homozygous $p53^{-/-}$ and $p53^{R248W/R248W}$ mice develop lymphoid tumors, including lymphoma and thymoma, but not myeloid malignancies,[9] suggesting that expression of mutant p53 is not sufficient for inducing myeloid leukemia in mice. This has led to a search for potential second hits that cooperate with mutant p53 in the pathogenesis of myeloid malignancies, primarily focused on using mouse models.

While coexisting mutations with *TP53* mutations in AML are limited,[6-7] previous studies indicate that *TP53* mutations co-occur with AML driver mutations in oncogenic signaling molecules such as FMS-like tyrosine kinase receptor-3 (FLT3).[11] Mutations in FLT3 have been identified in myeloid malignancies, including myeloproliferative neoplasms (MPN) and AML.[12] Internal tandem duplications in the juxtamembrane domain (FLT3-ITD) and mutations in the activating loop of FLT3 (FLT3-TKD) are seen in 30 to 35% of AML patients.[12] Both ITD and TKD mutations of FLT3 lead to constitutive activation of the tyrosine kinase, promoting proliferation and survival of leukemic blasts.[12] Given that expression of FLT3-ITD in the hematopoietic compartment results in MPN in mice and that FLT3-ITD impairs HSC self-renewal *in vivo*,[13] we reasoned that mutant p53 might synergize with FLT3-ITD in driving the development of myeloid leukemia through enhancing LIC self-renewal.

To test this hypothesis, we generated $p53^{R248W/+}FLT3^{ITD/+}$ mice and monitored overall survival and tumor development of these mice. We observed that both $FLT3^{ITD/+}$ and

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p53$^{R248W/+}$*FLT3*$^{ITD/+}$ mice have decreased life span compared to *p53*$^{+/+}$ mice (Figure 1a). Some *p53*$^{R248W/+}$ mice develop myelodysplastic syndromes (MDS) with age and other *p53*$^{R248W/+}$ mice developed lymphoma and sarcoma based upon pathological analysis of bone marrow (BM), spleen, liver, and peripheral blood (PB) (S.C. and Y.L., unpublished data). However, the majority of *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice developed MPN as seen in *FLT3*$^{ITD/+}$ mice (Figures 1b, 1c and data not shown), suggesting that FLT3-ITD-induced MPN development does not depend on mutant p53. Histological observation of spleen sections from MPN mice showed disarray of normal splenic architecture with a reduction and almost total absence of the white pulp in some cases and increased red pulp area with increased extramedullary hematopoiesis (Figure 1c). These features appeared in conjunction with hepatosplenomegaly, variable leukocytosis and overproduction of myeloid cells in bone marrow, spleen and peripheral blood (Figure 1c). We noted that bone marrow cellularity decreased as splenomegaly increased, consistent with increased extramedullary hematopoiesis. Notably, approximately 25% of *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice developed chronic myeloid leukemia (CML).[14] Upon necropsy, mice with CML displayed severe splenomegaly, and some also displayed hepatomegaly. Morphological analysis of peripheral blood smears revealed increased myeloid cells with dysplastic features (Figure 1c). Bone marrow cellularity varied from hypocellular to hypercellular among animals. Increased number of myeloid cells (blast to immature myeloid cells) was observed in bone marrow with extensive spread of myeloid elements in spleen and in a few livers (Figure 1c). While *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice showed marked splenomegaly compared to *p53*$^{+/+}$ and *FLT3*$^{ITD/+}$ mice (Figure 1d), this is not likely due to CML development as majority of double-mutant mice developed MPN (Figures 1b and 1c).

Given that patients with homozygous FLT3-ITD mutations have a more severe disease compared to those with heterozygous FLT3-ITD mutations,[11-12] we examined whether mutant p53 cooperates with homozygous FLT3-ITD mutant in leukemia development. We transplanted 3 x 10^6 whole bone marrow cells from *p53*$^{+/+}$, *FLT3*$^{ITD/ITD}$*, or* *p53*$^{R248W/+}$*FLT3*$^{ITD/ITD}$ mice into lethally irradiated recipient mice and measured their overall survival. Both *FLT3*$^{ITD/ITD}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/ITD}$ recipient mice had decreased life spans compared to *p53*$^{+/+}$ recipient mice (Figure S1a). Interestingly, approximately 30% of the *p53*$^{R248W/+}$*FLT3*$^{ITD/ITD}$ transplanted animals developed CML (Figure S1b), similar to that seen in *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ animals (Figure 1b). Rest of the *p53*$^{R248W/+}$*FLT3*$^{ITD/ITD}$ mice developed MPN (Figure S1b).

Given that some *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice develop CML, we next examined the impact of mutant p53 on FLT3-ITD$^+$ hematopoietic stem and progenitor cells (HSPCs) in order to understand the underlying mechanisms. We first analyzed peripheral blood (PB), bone marrow (BM), and spleen of *p53*$^{+/+}$, *p53*$^{R248W/+}$, *FLT3*$^{ITD/+}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice (8 to 12 week-old). PB white blood cell (WBC) counts, BM cellularity, and spleen weight were comparable among the four groups of mice (Figures S1c, S1d, and S1e). We then examined the frequency of hematopoietic stem and progenitor cells in the BM of *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice. While the number of LT-HSCs and ST-HSCs was comparable among these mice, LSKs and MPPs were expanded in the *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice compared with that of the *p53*$^{+/+}$, *p53*$^{R248W/+}$ and *FLT3*$^{ITD/+}$mice (Figure S2a). We also observed increased frequency of myeloid progenitors (Lin$^-$Kit$^+$ cells) in the bone marrow of

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p53$^{R248W/+}$FLT3$^{ITD/+}$ mice (Figure S2b). These findings suggest that the effects of mutant p53 and FLT3-ITD on myeloid progenitor cell expansion appears additive. However, the number of common lymphoid progenitors (CLPs) was comparable among four group of mice (Figure S2c).

FLT3 mutations have been show to enhance the proliferation of hematopoietic stem and progenitor cells.[13] We then examined the cell cycle status of LSKs isolated from *p53$^{+/+}$*, *p53$^{R248W/+}$*, *FLT3$^{ITD/+}$* and *p53$^{R248W/+}$FLT3$^{ITD/+}$* mice. We confirmed that *FLT3$^{ITD/+}$* LSKs shown enhanced proliferation compared with *p53$^{+/+}$* LSKs (Figure S2d). However, mutant p53 did not alter the proliferation of *FLT3$^{ITD/+}$* LSKs (Figure S2d). To determine the impact of mutant p53 on myeloid progenitors, we performed serial replating assays using BM cells from *p53$^{+/+}$*, *p53$^{R248W/+}$*, *FLT3$^{ITD/+}$* and *p53$^{R248W/+}$FLT3$^{ITD/+}$* mice. While the colony formation potential of *p53$^{+/+}$* and *FLT3$^{ITD/+}$* BM cells was comparable in serial replating assays, *p53$^{R248W/+}$FLT3$^{ITD/+}$* BM cells show enhanced replating potential compared to *p53$^{R248W/+}$* and *FLT3$^{ITD/+}$* BM cells (Figure 2a), suggesting that expanded myeloid progenitors in *p53$^{R248W/+}$FLT3$^{ITD/+}$* mice are functional *in vitro*.

To examine the impact of mutant p53 on HSCs *in vivo*, we performed serial competitive bone marrow transplantation assays. We transplanted 5 x 10^5 donor BM cells (*p53$^{+/+}$*, *p53$^{R248W/+}$*, *FLT3$^{ITD/+}$* or *p53$^{R248W/+}$FLT3$^{ITD/+}$*, CD45.2$^+$) into lethally irradiated (11 Gy) F1 recipient mice (CD45.1$^+$CD45.2$^+$) along with 5 x 10^5 competitor BM cells (CD45.1$^+$). Peripheral blood white blood cell counts were comparable among the four groups of mice following transplantation (Figure S2e). While *FLT3$^{ITD/+}$* BM cells showed decreased repopulating ability compared to *p53$^{+/+}$* cells 16 weeks post transplantation, *p53$^{R248W/+}$FLT3$^{ITD/+}$* BM cells displayed enhanced engraftment compared to *FLT3$^{ITD/+}$* BM cells (Figure 2b). We then sacrificed the recipient mice and examined the frequency of donor-derived hematopoietic stem and progenitor cells in their bone marrow. We found increased number of donor-derived LSKs in the BM of recipient mice repopulated with *p53$^{R248W/+}$* BM cells compared to that of the *p53$^{+/+}$* and *FLT3$^{ITD/+}$* BM cells, whereas the frequency of donor-derived LSKs in the BM of recipient mice repopulated with *p53$^{R248W/+}$* and *p53$^{R248W/+}$FLT3$^{ITD/+}$* cells was comparable (Figure S2f). We found increased number of donor-derived GMPs in the BM of recipient mice repopulated with *p53$^{R248W/+}$FLT3$^{ITD/+}$* bone marrow cells compared to that of the *FLT3$^{ITD/+}$* BM cells (Figure S3a). The spleen size was comparable in recipient mice repopulated with four group of BM cells (Figure S3b).

To determine the impact of mutant p53 on the self-renewal potential of FLT3-ITD$^+$ HSCs, we transplanted 3 x 10^6 BM cells isolated from the primary recipient mice repopulated with *p53$^{+/+}$*, *p53$^{R248W/+}$*, *FLT3$^{ITD/+}$* or *p53$^{R248W/+}$FLT3$^{ITD/+}$* cells into lethally irradiated secondary F1 recipients. Sixteen weeks after transplantation, *p53$^{R248W/+}$FLT3$^{ITD/+}$* cells continued to show increased repopulating ability compared to *FLT3$^{ITD/+}$* BM cells (Figure 2c). These findings suggest that mutant p53 may promote leukemic transformation through enhancing LIC self-renewal.

To examine the impact of mutant p53 on oncogenic signaling pathways, we performed western blot analysis on macrophage progenitor cells derived from *p53$^{+/+}$*, *p53$^{R248W/+}$*, *FLT3$^{ITD/+}$* or *p53$^{R248W/+}$FLT3$^{ITD/+}$* bone marrow cells. Consistent with previous studies,

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cells from *FLT3*$^{ITD/+}$ mice had activated FAK, STAT5, and AKT (Figure 2d). Further, expressing FLT3-ITD in a mutant p53 background enhances activated ERK levels but slightly decreases activated FAK and STAT5 levels (Figure 2d). We found increased levels of FLT3 in *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ macrophage progenitor cells (Figure S3c). Thus,expressing FLT3-ITD in a mutant p53 background has no effect on FLT3-ITD-induced activation of signaling pathways. However, ERK inhibitor treatment decreased the replating potential of *p53*$^{R248W/+}$, *FLT3*$^{ITD/+}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ bone marrow cells (Figure S3d). These findings suggest that mutant p53 and FLT3-ITD may function through different signaling pathways in the pathogenesis of hematological malignancies. In the future, we will elucidate the mechanisms by which mutant p53 upregulates FLT3 in HSPCs.

While *TP53* and *FLT3* mutations are rarely co-occur in MPN and AML,[11] the underlying mechanisms are not known. We found that the majority of *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice developed MPN, as seen in *FLT3*$^{ITD/+}$ mice.[13] Further, we discovered that mutant p53 and FLT3-ITD cooperate in CML development in mice. Functionally, mutant p53 synergizes with FLT3-ITD to expand the myeloid progenitor cell pool and enhance the self-renewal potential of LICs. *TP53* mutations are present in both chronic and blast crisis phase of CML,[15] underscoring the importance of mutant p53 in CML pathogenesis. Delineating the role of mutant p53 and FLT3-ITD in LIC self-renewal and pathogenesis of hematological malignancies may facilitate the development of novel therapeutic approaches that can improve leukemia treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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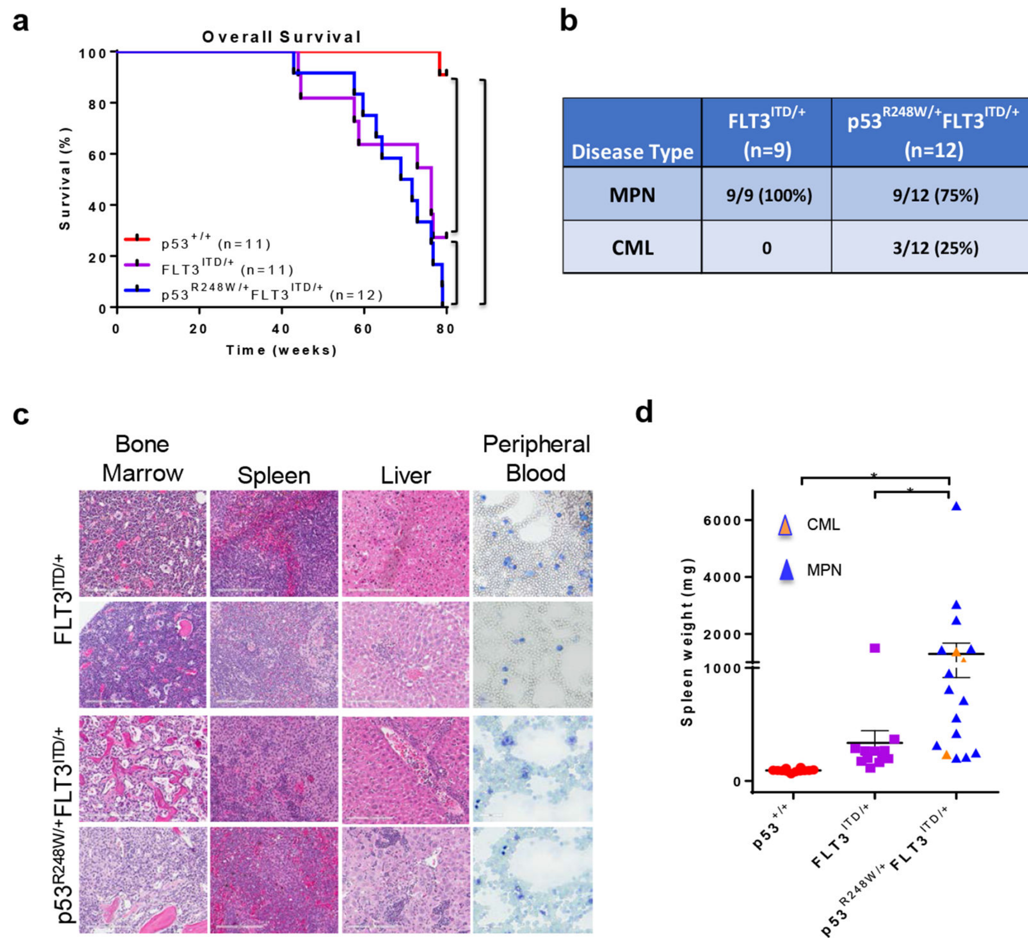

Disease Type	FLT3$^{ITD/+}$ (n=9)	p53$^{R248W/+}$FLT3$^{ITD/+}$ (n=12)
MPN	9/9 (100%)	9/12 (75%)
CML	0	3/12 (25%)

Figure 1.

Mutant p53 cooperates with FLT3-ITD in the pathogenesis of myeloid leukemia.

(**a**) *FLT3$^{ITD/+}$* and *p53$^{R248W/+}$FLT3$^{ITD/+}$* mice show decreased survival compared to *p53$^{+/+}$* mice (n=11, *p53$^{+/+}$*; n=11, *FLT3$^{ITD/+}$*; n=12, *p53$^{R248W/+}$FLT3$^{ITD/+}$*, **$p<0.01$, ****$p<0.0001$). (**b**) Disease spectrums in *FLT3$^{ITD/+}$* and *p53$^{R248W/+}$FLT3$^{ITD/+}$* mice were determined by pathological analysis of bone marrow, spleen, liver, and peripheral blood (n=9, *FLT3$^{ITD/+}$*; n=12, *p53$^{R248W/+}$FLT3$^{ITD/+}$*). (**c**) Representative H&E (20X) images of bone marrow, spleen, liver and peripheral blood smears from *FLT3$^{ITD/+}$* mice with MPN and *p53$^{R248W/+}$FLT3$^{ITD/+}$* mice with CML. (**d**) Spleen weight of *p53$^{+/+}$*, *FLT3$^{ITD/+}$*, and *p53$^{R248W/+}$FLT3$^{ITD/+}$* mice. Mean values (±SEM) are shown (n=12, *p53$^{+/+}$*; n=12, *FLT3$^{ITD/+}$*; n=17, *p53$^{R248W/+}$FLT3$^{ITD/+}$*, *$p<0.05$).

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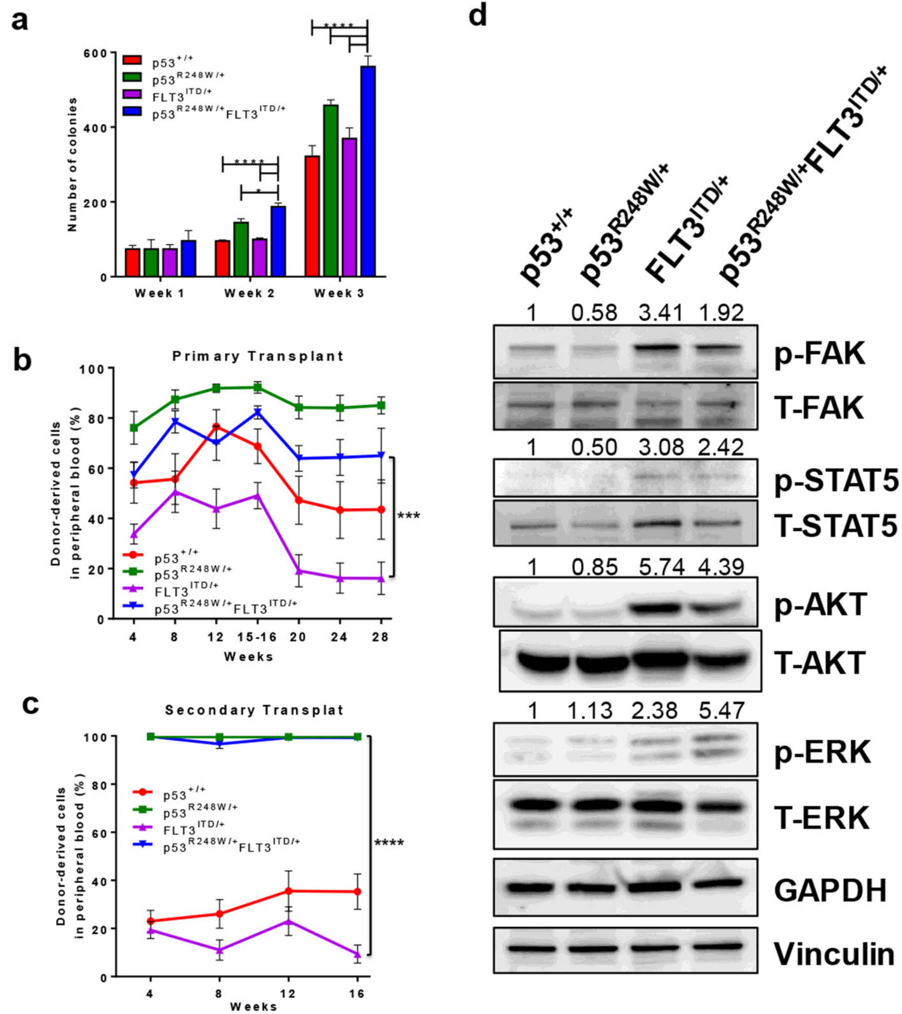

Figure 2.

Mutant p53 enhances the self-renewal potential of FLT3-ITD$^+$ LICs. (**a**) Serial replating assays of bone marrow cells from young *p53*$^{+/+}$, *p53*$^{R248W/+}$, *FLT3*$^{ITD/+}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice. Mean values (±SD) are shown (n=3, *p<0.05, ****p<0.0001). (**b**) p53^{R248W} enhances the repopulating potential of *FLT3*$^{ITD/+}$ hematopoietic cells. Percentage of donor-derived (CD45.2$^+$) cells in the peripheral blood of primary recipient mice post-transplantation, measured at 4-week intervals. Mean values (±SEM) are shown (n=7, ***p<0.001). (**c**) The percentage of donor-derived cells in the peripheral blood of secondary recipient mice. Mean values (±SEM) shown, (n=7, *p53*$^{R248W/+}$ vs *FLT3*$^{ITD/+}$ and *FLT3*$^{ITD/+}$ vs *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$, ****p<0.0001). (**d**) Western blot analysis of activated and total FAK, STAT5, AKT, and ERK protein levels in *p53*$^{+/+}$, *p53*$^{R248W/+}$, *FLT3*$^{ITD/+}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mononuclear cells differentiated into macrophage progenitors. Loading controls GAPDH and Vinculin are also shown. Quantification of phosphorylated proteins was calculated relative to total protein level and is displayed above each respective phospho-protein.