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## Role of intracellular tyrosines in activating KIT induced myeloproliferative disease

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### Abstract

Gain-of-function mutations in KIT receptor in humans are associated with gastrointestinal stromal tumors (GIST), systemic mastocytosis (SM), and acute myelogenous leukemia (AML). The intracellular signals that contribute to oncogenic KIT induced myeloproliferative disease (MPD) are poorly understood. Here, we show that oncogenic KITD814V induced MPD occurs in the absence of ligand stimulation. The intracellular tyrosine residues are important for KITD814V induced MPD, albeit to varying degrees. Among the seven intracellular tyrosines examined, tyrosine 719 alone plays a unique role in regulating KITD814V induced proliferation and survival *in vitro*, and MPD *in vivo*. Importantly, the extent to which AKT, ERK and Stat5 signaling pathways are activated via the seven intracellular tyrosines in KITD814V impacts the latency of MPD and severity of the disease. Our results identify critical signaling molecules involved in regulating KITD814V induced MPD, which might be useful for developing novel therapeutic targets for hematologic malignancies involving this mutation.

### Keywords

KITD814V; acute myelogenous leukemia (AML); systemic mastocytosis (SM); PI3Kinase; ERK; Stat5

### Introduction

The proto-oncogene KIT belongs to the receptor tyrosine kinase class III family, which includes the macrophage colony stimulating factor receptor (M-CSFR), the platelet-derived growth factor receptor (PDGFR), as well as the F1 cytokine receptor (FLT3) (1). The KIT

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receptor plays a crucial role in cell proliferation, differentiation, survival and migration through activation of diverse signaling pathways (2). Binding of its ligand, stem cell factor (SCF), induces KIT receptor dimerization and autophosphorylation on intracellular tyrosine residues leading to the recruitment and docking of src homology 2 (SH2) containing signaling molecules to the seven critical intracellular tyrosines (3). Although the individual and combined importance of these tyrosines in normal KIT receptor signaling is beginning to emerge; the role of these tyrosines in oncogenic KIT induced myeloproliferative disease (MPD) is poorly understood. Furthermore, it is unclear whether these tyrosines play a unique, redundant or overlapping function in inducing MPD.

Activating mutations in KIT receptor have been shown to be involved in various human diseases including gastrointestinal stromal tumors (GIST), systemic mastocytosis (SM), and acute myelogenous leukemia (AML) (4–7). An activating KIT mutation within the tyrosine kinase domain, such as KITD814V in mice or KITD816V in humans, results in ligand independent tyrosine kinase activity leading to constitutive autophosphorylation and activation of downstream signaling pathways (8, 9). As a result, KITD814V bearing cells demonstrate ligand independent proliferation *in vitro* and MPD *in vivo* (8–12). However, the intracellular mechanisms that contribute to KITD814V induced MPD are not known. In addition, primary hematopoietic stem and progenitors (HSC/Ps) bearing KITD814V show a further increase in proliferation in the presence of KIT ligand, SCF, relative to cells grown in the absence of SCF (11), which suggests that endogenous ligand stimulation may contribute to oncogenic KIT induced transformation *in vivo*. Therefore, it is still unclear whether ligand independent growth observed *in vitro* via KITD814V is sufficient to induce MPD *in vivo* or whether presence of SCF is necessary to drive MPD.

Although KIT mutations within the juxtamembrane domain found in GIST are highly sensitive to inhibition by imatinib (i.e. Gleevec), KIT mutations within tyrosine kinase domain involved in SM and AML, including KITD816V, are resistant to imatinib treatment (13–15). Currently, there are no therapies available for human diseases involving KITD816V mutation. Thus, it is important to identify signaling pathways that are involved in KITD814V induced MPD to develop novel therapeutic targets for diseases involving this mutation. Utilizing biochemical and genetic approaches, we demonstrate that endogenous ligand (i.e. SCF) binding is dispensable for KITD814V induced MPD. Furthermore, the intracellular tyrosine residues are important for KITD814V induced MPD, albeit to varying degrees. Among the seven intracellular tyrosines examined, tyrosine 719 alone plays a unique role in regulating KITD814V induced proliferation *in vitro*, and MPD *in vivo*. Importantly, tyrosine 719 is vital for the activation of PI3Kinase and Stat5 downstream from KITD814V. Our results identify critical signaling molecules downstream from KITD814V, which might be useful for developing therapeutic targets for hematologic malignancies involving oncogenic forms of KIT.

## Materials and Methods

### Chemicals, Mice and Cells

Chemicals, mice and cells used were described in supplementary materials and methods.

### Wild-type (WT) and mutant KIT or CHR receptors

The construction and expression of WT and mutant KIT or CHR receptors was described in supplementary methods.

### Proliferation

Proliferation was assessed by conducting a thymidine incorporation assay as previously described (16) and described in detail in supplementary methods.

### Western blotting

Western blot analysis was performed as previously reported (16).

### Murine bone marrow transplantation

A single intraperitoneal injection of 150 mg/kg of 5-fluorouracil (5-FU) was given to C57BL/6 mice. LDMNCs were collected 72 hours post 5-FU injection and transduced with retrovirus encoding the various KIT or CHR constructs as described previously (16).  $1 \times 10^6$  transduced cells and  $1 \times 10^5$  supporting fresh splenocytes from C57BL/6 mice were intravenously injected through tail vein into lethally irradiated (1100 cGY-split dose) recipient mice. Transplanted mice were monitored for MPD development and survival. Mice were harvested at moribund and bone marrow, spleen, thymus and peripheral blood were harvested for flow cytometric analysis, and histology by hematoxylin and eosin staining.

### Statistics

All graphical data was evaluated by paired Student *t*-test and results were considered significantly different with *p*-value  $<0.05$ . All data are represented as mean values  $\pm$  standard deviations (SD). Survival probability of transplanted mice cohorts were compared using a Kaplan-Meier Survival Analysis in which statistical significance was determined as *p*-values  $<0.05$  by log rank test.

## Results

### Construction of wild-type and mutant chimeric KIT receptors

We and others have previously shown that KITD814V is sufficient to induce ligand independent growth *in vitro* as well as myeloproliferative disease (MPD) *in vivo* (8–11, 17). It is however unclear whether KITD814V induced ligand independent growth observed *in vitro* is sufficient to cause MPD *in vivo* or whether presence of endogenous SCF induced signals are essential for the development of MPD. To determine the contribution of ligand independent growth in KITD814V induced MPD *in vivo*, we generated a chimeric KIT receptor (CHR) in which the extracellular domain of KIT was replaced with the extracellular domain of human macrophage colony stimulating factor receptor (h-MCSFR) to inhibit the endogenous binding of murine SCF, but to maintain the transmembrane and intracellular domains of the murine KIT receptor (18, 19) (Figure S1A). This receptor allows studying the ligand independent functions of KIT receptor *in vivo* as it maintains the intracellular functions of KIT receptor intact without endogenous binding of murine SCF or M-CSF, but is specifically activated by human M-CSF (18, 19). The wild-type chimeric receptor (WT

CHR) is functionally and biochemically similar to the wild-type endogenous KIT receptor as previously reported (18, 19). In addition, we constructed a mutant chimeric receptor (CHRD814V) that contains an oncogenic mutation of aspartic acid to valine at residue 814 of the WT CHR (Figure S1A). Parental and chimeric KIT receptors with or without D814V mutation were cloned into a bicistronic retroviral vector, MIEG3, which expresses EGFP through an internal ribosome entry site as previously described (18, 19).

### Ligand independent growth is sufficient to induce KITD814V induced MPD *in vivo*

We first verified whether our constructed chimeric KIT receptors function similar to their wild-type and KITD814V counterparts. We performed proliferation assay in 32D myeloid cells bearing parental or chimeric KIT receptors with or without D814V mutation by assessing thymidine incorporation. As expected, cells bearing KITD814V or CHRD814V showed similar levels of ligand independent growth (Figure S1B). In contrast, cells bearing WT KIT or WT CHR showed minimal thymidine incorporation in the absence of growth factors (Figure S1B). In addition, cells bearing WT CHR showed increased growth in the presence of human M-CSF, but not murine M-CSF (Figure S1C). These findings demonstrate that chimeric receptors (WT CHR and CHRD814V) function in a manner similar to their parental counterparts with respect to ligand independent growth and specifically respond to human M-CSF, but not murine M-CSF. Therefore, these chimeric receptors could be used to determine the impact of ligand independent growth in KITD814V induced MPD *in vivo*.

To determine the role of ligand independent growth in KITD814V induced MPD *in vivo*, we used a murine transplantation model that we have previously described (17). Low density mononuclear cells (LDMNC) from 5-FU-treated C57BL/6 mice were transduced with WT CHR, WT KIT, KITD814V or CHRD814V, and sorted cells were transplanted into lethally irradiated recipient mice. Transplanted mice were monitored for MPD development and survival. Consistent with our previous results, all mice transplanted with WT CHR or WT KIT bearing cells appeared normal and healthy past 180 days post transplantation (Data not shown). In contrast, all recipient mice transplanted with KITD814V or CHRD814V bearing cells died within 81 days of transplantation (Figure S2A). No significant difference in the survival of mice transplanted with cells bearing KITD814V or CHRD814V was observed suggesting that endogenous SCF mediated stimulation of KITD814V receptor is not necessary for KITD814V induced MPD *in vivo*. Mice transplanted with cells expressing CHRD814V developed a series of fatal diseases, including MPD, as seen by a significant increase in white blood cell counts compared to WT CHR controls (Figure S2B). In addition, mice transplanted with CHRD814V-bearing cells showed splenomegaly (Figure S2C). Figure S2D shows representative pictures of spleen and liver from mice transplanted with cells bearing either WT CHR or CHRD814V.

Histologic analysis of bone marrow (BM), spleen, liver and lungs from mice transplanted with CHRD814V bearing cells showed signs of MPD including infiltration of immature cells in BM and disruption of the normal architecture of red and white pulp in spleen (Figure S2E). Furthermore, flow cytometric analysis of peripheral blood, spleen and BM from mice transplanted with cells bearing CHRD814V showed increase in the presence of Gr-1 and

Mac-1-positive cells compared to mice transplanted with cells bearing WT CHR (Figure S2F). Some mice transplanted with cells bearing CHR814V also demonstrated erythroleukemia, B-cell and T-cell lymphomas in addition to MPD, similar to other published models of oncogene-induced MPD (data not shown) (20). Taken together, these results indicate that KITD814V mutation does not require a direct engagement with its ligand SCF to induce MPD *in vivo*.

### **Intracellular tyrosine residues in KITD814V are essential for ligand independent growth *in vitro***

We next focused our studies to determine the importance of intracellular tyrosine residues in KITD814V induced ligand independent growth. To address this, we constructed a CHR814V mutant receptor (CHR814V-F7) in which all the intracellular tyrosine residues were converted to phenylalanine by site directed mutagenesis as shown in Figure 1A. 32D cells were transduced with WT CHR, CHR814V or CHR814V-F7, sorted to homogeneity based on EGFP expression and used to perform proliferation assay. Consistent with our earlier result (Figure S1B), cells bearing CHR814V, but not WT CHR, showed ligand independent growth (Figure 1B). However, conversion of all the seven intracellular tyrosine residues in CHR814V to phenylalanine (CHR814V-F7) resulted in loss of ligand independent growth (Figure 1B). These results suggest that intracellular tyrosine residues in KITD814V are essential for ligand independent growth.

To identify which of the tyrosine residue plays a critical role in KITD814V induced ligand independent growth *in vitro* and transformation *in vivo*, we generated 7 different single tyrosine add-back mutants at residues 567, 569, 702, 719, 728, 745 and 934 in the KIT intracellular domain using CHR814V-F7 as the template (Figure 2A). These CHR814V mutant receptors were designated CHR814V-Y567, CHR814V-Y569, CHR814V-Y702, CHR814V-Y719, CHR814V-Y728, CHR814V-Y745 and CHR814V-Y934. 32D cells, which lack endogenous KIT receptor, were infected with these mutant CHR814V receptors and sorted to homogeneity. A similar level of expression of these receptors was observed by western blot analysis (Figure 2B). As seen in Figure 2C, 32D cells bearing only the chimeric receptor harboring tyrosine residue at position 719 (CHR814V-Y719) was sufficient to induce ligand independent growth to a level similar to cells bearing the CHR814V receptor. The other tyrosine add-back CHR814V receptors induced modest to no ligand independent proliferation (Figure 2C). Similar results were observed in transduced primary HSC/Ps from *Kit<sup>W-sh/W-sh</sup>* mice lacking endogenous KIT (Data not shown). In addition, cells bearing CHR814V showed significantly increased survival compared to WT CHR bearing cells in the absence of growth factors and loss of intracellular tyrosine residues in CHR814V (CHR814V-F7) abrogated ligand independent survival (Figure S3A). Among all the single tyrosine add-back CHR814V receptors, CHR814V-Y719 was the only receptor whose expression maintained survival at a level similar to that of CHR814V receptor (Figure S3A). There was no significant difference in the cycling status of cells bearing various mutant CHR814V receptors, including CHR814V and CHR814V-Y719, when grown in the absence of growth factors (Figure S3B). These results demonstrate that intracellular tyrosine residues in KITD814V receptor are essential for ligand independent growth. Among these tyrosine residues,

tyrosine at residue 719, which is the binding site for class I<sub>A</sub> PI3Kinase regulatory subunit p85 $\alpha$ , is sufficient to rescue ligand independent proliferation *in vitro* to CHR814V levels.

### **Intracellular tyrosine residues in KITD814V contribute to MPD *in vivo*, albeit to varying extent**

To determine the physiologic role of the intracellular tyrosine residues in KITD814V induced MPD *in vivo*, we transduced primary HSC/Ps from 5-FU-treated C57BL/6 mice with WT CHR, CHR814V or CHR814V with none or single tyrosine add-back mutants. Transduced cells showing similar transduction efficiencies were sorted to homogeneity and transplanted into lethally irradiated recipient mice. Mice were monitored for MPD development and survival. While mice transplanted with cells bearing WT CHR showed no signs of disease and survived throughout the study, mice transplanted with cells bearing CHR814V (red line) succumbed to death within 80 days of transplantation and developed a fatal MPD (Figure S2A and Figure 3A). In addition, mice transplanted with cells bearing CHR814V-F7 (black line), which lack all the seven intracellular tyrosine residues, significantly delayed MPD development and prolonged overall survival (Figure 3A-1). In the CHR814V-F7 group, only 70% of the mice died within 180 days of transplantation and remaining 30% showed no signs of disease and appeared normal. These data suggest that the intracellular tyrosine residues in KITD814V are critical for efficient transformation *in vivo*.

Consistent with *in vitro* proliferation, among all the mice transplanted with cells bearing various CHR814V mutant receptors, only recipient mice expressing CHR814V-Y719 (blue line) showed similar MPD progression and survival as the CHR814V bearing mice (Figure 3A-5). The median time of survival in these two groups was 60 days for CHR814V vs 55 days for CHR814V-Y719. In addition, recipient mice with cells bearing CHR814V-Y567, CHR814V-Y569, CHR814V-Y728, CHR814V-Y745 and CHR814V-Y934 showed a significant delay in MPD development and survival compared to CHR814V bearing mice (Median time of survival 105, 128, 95, 104 and 68 days, respectively, \* $p < 0.05$ ). Restoration of Y702 demonstrated a modest but non-significant delay in the disease onset compared to KITD814V bearing mice (Median time of survival 76 days,  $p = 0.077$ ). Consistent with the survival data, histological analysis of bone marrow, spleen, liver and lungs from the recipient mice transplanted with cells bearing various single tyrosine add-back CHR814V mutants showed variable degree of infiltration of immature cells relative to CHR814V bearing mice or CHR814V-Y719 bearing mice (Figure S2E and Figure 3B). These results suggest that among the seven intracellular tyrosine residues in KITD814V, tyrosine residue at 719 is sufficient to induce fully penetrant MPD *in vivo*. Other tyrosine residues at 567, 569, 702, 719, 728, 745 and 934 do contribute to KITD814V induced MPD, however to a lesser extent, resulting in delayed disease onset and reduced severity.

### **Differential activation of PI3Kinase, Stat5, and ERK MAP kinase in cells bearing various single tyrosine add-back CHR814V mutant receptors**

To assess the biochemical basis for the differential role of single tyrosine add-back CHR814V mutants in ligand independent growth and MPD, activation of AKT, Stat5 and ERK, which are known to be critical for KITD814V induced MPD, was analyzed (11, 17,

21, 22). As seen in Figure 4A, constitutive phosphorylation of AKT, Stat5, and ERK was observed in cells bearing CHR814V, but not in WT CHR bearing cells. Interestingly, loss of seven intracellular tyrosine residues in CHR814V resulted in complete abrogation of the constitutive activation of AKT, Stat5 and ERK (Figure 4A, lane 11). Importantly, among the single tyrosine add-back CHR814V mutants, only restoring tyrosine 719 completely rescued the robust constitutive phosphorylation of AKT, Stat5, and ERK observed in CHR814V expressing cells (Figure 4A, lane 7). Cells bearing CHR814V-Y702 and CHR814V-Y728 showed robust activation of AKT and ERK, but not Stat5 (Figure 4A, lanes 6 and 8, respectively). Furthermore, a modest constitutive activation of AKT and ERK was observed in cells bearing CHR814V-Y569 (Figure 4A, lane 5). In contrast, no activation of AKT, Stat5 and ERK was observed in cells bearing CHR814V-Y567, CHR814V-Y745 or CHR814V-Y934 (Figure 4A, lanes 4, 9 and 10, respectively). These results suggest that differential activation of AKT, Stat5 and ERK in cells bearing various single tyrosine add-back CHR814V mutants likely contributes to variable level of disease progression and survival of transplanted mice.

### **Class I<sub>A</sub> PI3Kinase regulatory subunit p85 $\alpha$ is essential for constitutive activation of Stat5 in KITD814V bearing cells**

The above results showed constitutive activation of both AKT and Stat5 in cells bearing CHR814V and CHR814V-Y719, but not other CHR814V mutant receptors. These results suggest that tyrosine 719 (binding site for p85 $\alpha$  subunit) mediated signaling events might be required for constitutive activation of Stat5 in KITD814V bearing cells. To test this, we infected primary HSC/Ps from WT and *p85 $\alpha$ -/-* mice with WT KIT or KITD814V, sorted the cells and determined constitutive phosphorylation of Stat5 by western blotting. Constitutive phosphorylation of Stat5 was observed in WT cells bearing KITD814V, but not WT KIT bearing cells (Figure 4B). Deficiency of p85 $\alpha$  resulted in significantly reduced KITD814V induced activation of Stat5 (Figure 4B). These results demonstrate that p85 $\alpha$  is essential for constitutive activation of Stat5 in KITD814V bearing cells.

### **PI3Kinase and Stat5 inhibitors suppress KITD814V induced ligand independent growth**

Since cells bearing KITD814V show constitutive activation of AKT, Stat5 and ERK, we examined whether inhibition of PI3Kinase, Stat5 or ERK activation would suppress KITD814V induced ligand independent proliferation. We treated cells bearing WT CHR or CHR814V with the PI3Kinase inhibitor LY294002, Stat5 inhibitor, or MEK inhibitor PD0325901, and assayed proliferation by thymidine incorporation. As seen in Figure 4C, LY294002 treatment completely suppressed the constitutive growth of cells bearing CHR814V. In contrast, Stat5 inhibitor treatment showed only a partial but significant repression in the constitutive growth of cells bearing CHR814V. In addition, MEK inhibitor treatment showed no significant effect on the constitutive growth of cells bearing CHR814V. These results suggest that activation of PI3Kinase and Stat5 plays an essential role in KITD814V induced ligand independent growth. Since Stat5 inhibitor only partially suppressed the constitutive growth of cells bearing CHR814V, PI3Kinase may also regulate the activation of other signaling molecules in addition to Stat5 in cells bearing KITD814V.

### PI3Kinase pathway is essential for KITD814V induced ligand independent proliferation via AKT and mTOR signaling

To further determine the signaling molecules downstream of PI3Kinase that might contribute to the growth of KITD814V bearing cells, we analyzed the involvement of AKT and mTOR. For this, we starved the cells bearing KITD814V in serum- and cytokine-free medium for 6 hours and performed proliferation assays in the presence of indicated amounts of specific PI3Kinase inhibitor (GDC-0941), AKT inhibitor (AKT inhibitor VIII), or mTOR inhibitor (AZD8055) by thymidine incorporation. As seen in Figure 5A, KITD814V induced ligand independent proliferation is significantly suppressed in the presence of the specific inhibitors of PI3Kinase, mTOR and AKT, which suggests that PI3Kinase pathway is essential to KITD814V induced ligand independent proliferation via AKT and mTOR signaling. In contrast, although the MEK inhibitor (PD0325901) did not significantly repress KITD814V induced proliferation even at high doses (Figure 4C), a significant further suppression in ligand independent proliferation in KITD814V bearing cells in the presence of PI3Kinase inhibitor (GDC-0941) and MEK inhibitor (PD0325901) was observed (Figure 5B). These results suggest that while MEK/ERK pathway alone is not critical; it can cooperate with the PI3Kinase pathway to regulate KITD814V induced ligand independent proliferation.

### Discussion

Our studies demonstrate that the direct binding of the ligand SCF to KITD814V receptor is not necessary to induce MPD and intracellular tyrosines are critical to this process. Mice bearing either the parental KITD814V receptor or the chimeric CHR814V receptor (lacking the ability to be activated via its ligand SCF) show similar potency at inducing MPD with similar median survival. Furthermore, no significant difference in disease manifestation was observed between the two transplanted groups. Importantly, intracellular tyrosines show a critical role in CHR814V induced MPD and survival. Loss of seven critical tyrosine residues in CHR814V, which are known to activate PLC- $\gamma$  (728) (23, 24), PI3Kinase [p85 $\alpha$ ; 719] (25), Src family kinases (567 & 569) (26–28), Grb2 (702) (29), Grb7 (934) (29) and Ras-GAP (745) profoundly impaired disease onset and prolonged the survival of transplanted mice. Only 70% mice that were transplanted with cells bearing CHR814V-F7 succumb to death with very long latency, while 30% mice were free of disease and survived for the remaining duration of the study. Using transduced primary HSC/Ps, we have previously demonstrated that KITD814V not only induces ligand independent growth or responds to SCF, but also synergizes with IL-3 and macrophage colony-stimulating factor (M-CSF) receptors to further enhance the growth of these cells *in vitro*. This suggests that KITD814V induced ligand-independent growth and its ability to cooperate with other cytokines might also contribute to activating KIT induced MPD (11). Furthermore, KIT has also been shown to cooperate with granulocyte macrophage colony-stimulating factor (GM-CSF) in a kinase independent manner (30) and to synergize with granulocyte colony-stimulating factor (G-CSF) to induce proliferation (31). Therefore, it is conceivable that CHR814V-F7 is not sufficient to induce robust proliferation without growth factor *in vitro*, but may cooperate with additional cytokine receptors, such as IL-3,



M-CSF and /or G-CSF receptors to induce MPD in the 70% transplanted animals in a significantly delayed and milder manner.

In an effort to assess the role of individual tyrosine mediated signals in KITD814V induced MPD, we employed a unique approach. While single point mutations in important tyrosine residues have been shown to affect KIT function (32, 33), we felt that the approach of interfering with the binding of one or two SH2 binding proteins to CHR814V, when all other SH2 proteins still retain the ability to bind may not allow for complete assessment of the importance of signals emanating from a single pathway, since it is possible that the remaining SH2 consisting proteins may compensate for the loss of a single binding site. We therefore employed a strategy where we could assess the role of an individual tyrosine induced pathway in KITD814V induced MPD. Using a version of the receptor that lacked all 7 tyrosines (i.e. CHR814V-F7) as the template, we restored each of the SH2 binding sites from phenylalanine to tyrosine and tested their ability to induce growth *in vitro* and MPD *in vivo*, along with their potential to activate downstream signaling molecules known to be involved in KITD814V induced MPD, including AKT, ERK and Stat5. We show a unique role for tyrosine 719 in regulating KITD814V induced MPD. We show that restoring this site alone in the CHR814V-F7 receptor is sufficient to completely rescue ligand independent growth *in vitro*, MPD *in vivo* and activation of AKT, ERK and STAT5, similar to the CHR814V receptor. Importantly, while the remaining add-back mutant receptors showed minimal rescue in ligand independent growth *in vitro*; *in vivo* mice bearing these receptors demonstrated delayed MPD onset and prolonged latency compared to CHR814V or CHR814V-Y719 receptor bearing mice. These results suggest that although ligand independent growth is sufficient for KITD814V induced MPD *in vivo*, presence of SCF and other cytokines might further regulate the MPD phenotype.

The variable onset of MPD and latency in mice transplanted with cells bearing various add-back mutant receptors was associated with differential rescue in the activation of AKT, ERK and STAT5. In general, add-back CHR814V mutants that lacked the ability to rescue the activation of AKT, ERK and STAT5, such as CHR814V-F7 receptor bearing mice, demonstrated prolonged latency, while receptors demonstrating rescue in the activation of AKT and ERK, such as the CHR814V-Y702 receptor bearing mice, resulted in MPD, albeit at a slower rate. In contrast, receptors that robustly activated all three signaling molecules (ERK, AKT and Stat5) such as the CHR814V-Y719 and the CHR814V receptor, showed the shortest disease latency. Thus, KITD814V induced MPD *in vivo* is largely dependent on the level of activation of AKT, ERK and STAT5, which to a large extent is regulated by signals emanating from tyrosine 719, suggesting that signals emanating from tyrosine 719 play a dominant role in regulating MPD, while the remaining tyrosine initiated signals contribute to MPD, albeit to a lesser extent. Thus, the impairment in the activation of essential signaling pathways is likely to contribute to a significant delay in the onset of MPD in mice bearing remaining add-back mutants of KITD814V.

While recent studies have suggested that persistent activation of PI3Kinase and Stat5 is frequently observed in hematologic malignancies as well as in solid tumors (34, 35), how exactly PI3Kinase contributes to KITD814V induced MPD and/or Stat5 activation is unclear. We show that tyrosine 719 in KITD814V (binding site for p85 $\alpha$ ) contributes to the

activation Stat5. In cells expressing CHR814V-F7, no binding of p85 $\alpha$  is observed and consistently no constitutive activation of Stat5 is observed (data not shown). Furthermore, deficiency of p85 $\alpha$  in HSC/Ps expressing KITD814V results in complete inhibition of Stat5 activation. Importantly, restoring 719 from phenylalanine to tyrosine in CHR814V-F7 completely rescues Stat5 activation. Thus, binding of p85 $\alpha$  to tyrosine 719 in KITD814V is critical for Stat5 activation. These results suggest that PI3Kinase mediated signaling is essential for constitutive activation of Stat5 in KITD814V bearing cells. While treatment of cells bearing KITD814V with the PI3Kinase, AKT and mTOR inhibitors profoundly suppressed the ligand independent growth *in vitro*, treatment with Stat5 inhibitor only partially repressed the constitutive growth. These results, while consistent with our biochemical findings, suggest that in addition to Stat5, PI3Kinase might cooperate with other signaling molecules including the ERK pathway downstream from KITD814V to induce MPD. Taken together, our studies determine the contribution of SCF to KITD814V induced MPD *in vivo*, and also identify critical tyrosine residues and signaling pathways involved in KITD814V induced MPD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

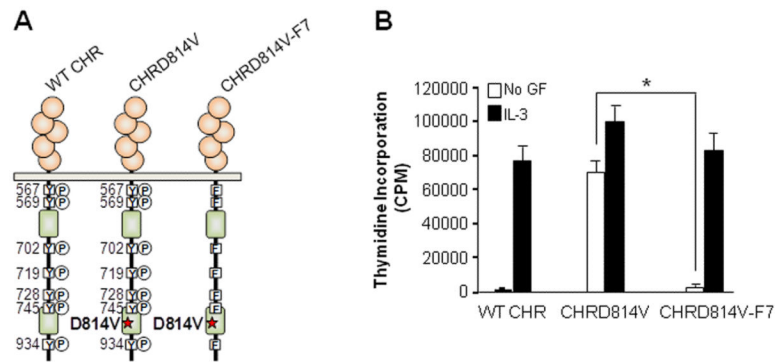
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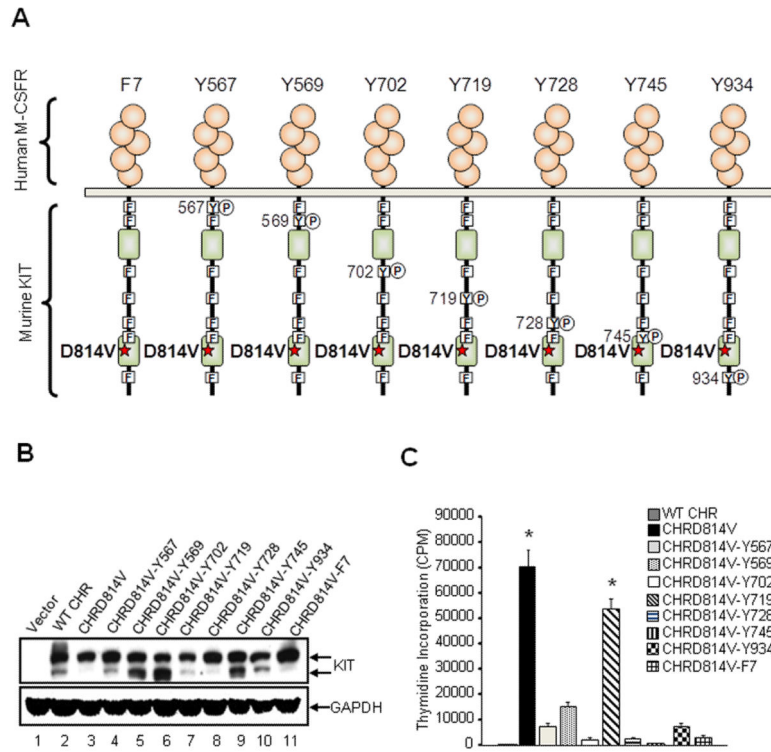
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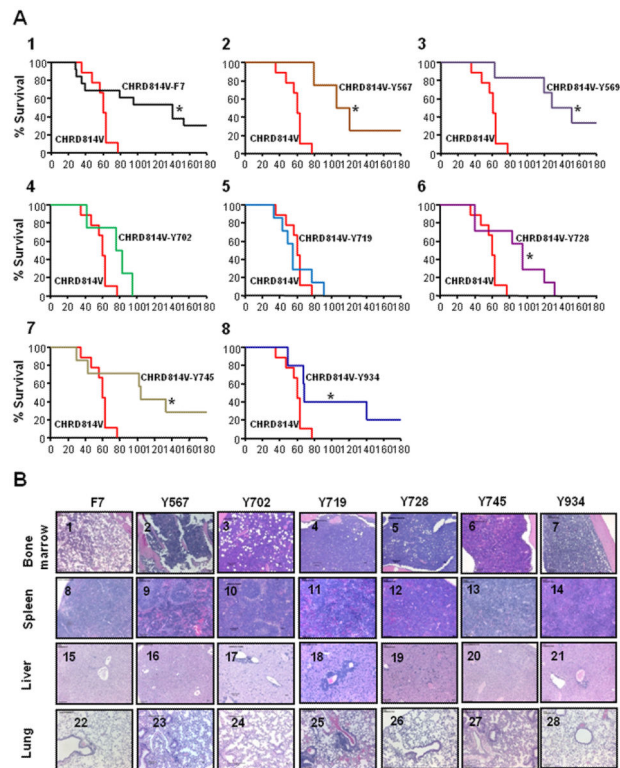


**Figure 1. Intracellular tyrosine residues in KIT receptor are essential for KITD814V induced ligand independent growth *in vitro***

(A) Schematic of WT CHR, CHR D814V and CHR D814V-F7. The D814V mutation in the kinase domain of KIT is indicated in the mutant chimeric receptors. A chimeric receptor (CHR D814V-F7) was constructed in which seven critical tyrosine residues (Y) at indicated positions were changed to phenylalanine (F) by site-directed mutagenesis using CHR D814V as a template. (B) Cells bearing WT CHR, CHR D814V or CHR D814V-F7 receptors were subjected to proliferation assay in the presence or absence of IL-3 by thymidine incorporation. Bars denote the mean thymidine incorporation (CPM  $\pm$  SD) from one of three independent experiments performed in quadruplicate. \* $p < 0.05$ , CHR D814V vs. CHR D814V-F7.

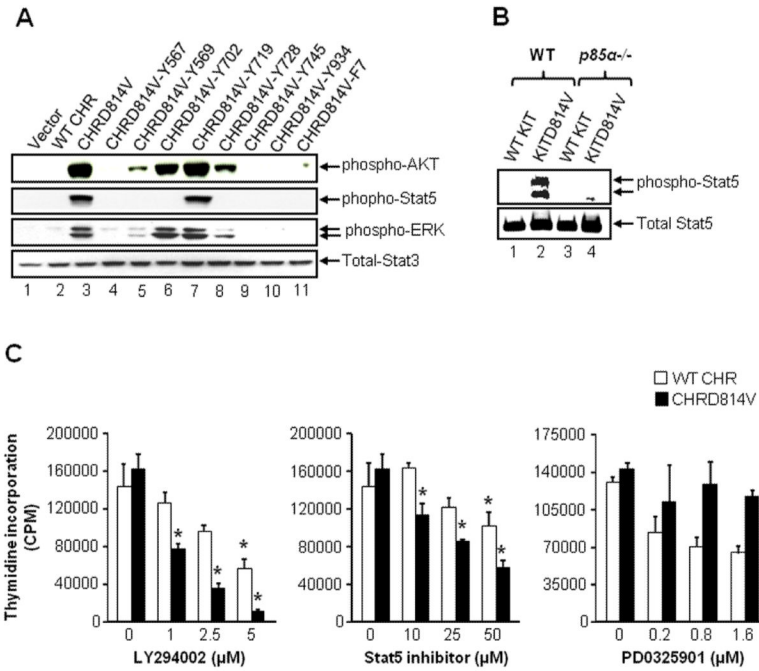


**Figure 2. Differential contribution of intracellular tyrosine residues in KIT receptor to KITD814V induced ligand independent growth in vitro**  
 (A) Schematic of mutant CHRD814V receptors. In the single tyrosine add-back mutant CHRD814V receptors, phenylalanine at indicated positions in the CHRD814V-F7 receptor were restored to tyrosines on an individual basis. (B) Equal amounts of protein lysates from cells bearing MIEG3 vector or indicated chimeric receptors was subjected to western blot analysis using an anti-KIT antibody. Similar results were observed in two additional experiments. (C) 32D cells bearing the indicated chimeric receptors were starved of serum and growth factors for 6 hours and subjected to thymidine incorporation assay in the absence of growth factors. Bars denote the mean thymidine incorporation (CPM± SD) from one of the three experiments performed in replicates of four. \*p<0.05.



**Figure 3. Intracellular tyrosine residues in KIT receptor are essential for KITD814V induced MPD *in vivo***

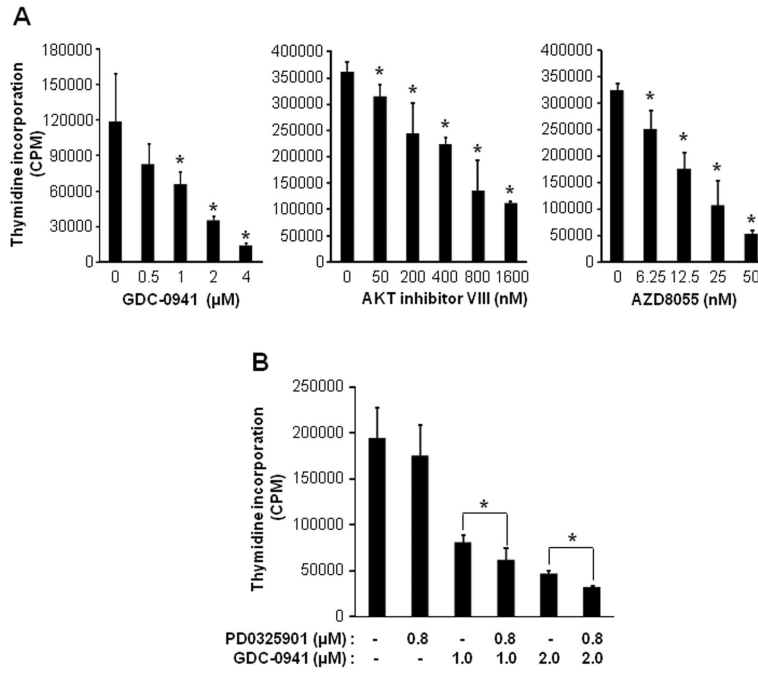
(A) Kaplan-Meier survival analysis of mice transplanted with cells bearing indicated single tyrosine add-back mutant CHRD814V receptors (n=4 to 13 per group). Results show that loss of seven tyrosine residues in CHRD814V significantly delayed MPD development and prolonged overall survival (median survival= 140 days, n=13, \*p<0.05). Restoration of Y719 alone is sufficient to induce MPD *in vivo* (median survival= 55 days, n=7, \*p<0.05). Compared to CHRD814V, restoration of Y567, Y569, Y728, Y745 and Y934 demonstrated a significant delay in disease onset in transplanted mice (median survival= 95–128 days, n=4 to 13, \*p<0.05). There is a modest but non-significant delay in the survival of the recipient mice bearing CHRD814V-Y702 compared to CHRD814V bearing mice (median survival=76 days, n=4, \*p=0.077). (B) Histopathologic analysis of bone marrow, spleen, liver and lung from mice transplanted with cells bearing various single tyrosine add-back mutant CHRD814V receptors. Bone marrow, spleen, liver and lung from mice transplanted with cells bearing various single tyrosine add-back mutant CHRD814V receptors were harvested, fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Shown are representative tissue sections from mice transplanted with cells bearing various single tyrosine add-back mutant CHRD814V. Normal erythroid and myeloid components in bone marrow, spleen, liver and lungs were replaced by sheets of immature tumor cells to various degrees in all the representative animals, but predominately in CHRD814V-Y719 (panel 4) followed by CHRD814V-Y745 (panel 6) and CHRD814V-Y728 (panel 5), respectively (first row).



**Figure 4. Involvement of AKT, ERK and Stat5 signaling in KITD814V induced ligand independent growth**

(A) Cells bearing the indicated chimeric receptors were starved in serum- and growth factor-free medium for 8 hours. Starved cells were lysed and equal amount of protein lysates were subjected to western blot analysis using an anti-phospho-Stat5, phospho-AKT, phospho-ERK, and total Stat3 antibodies as indicated. Similar results were observed in two to three independent experiments. (B) Primary BM derived progenitors expressing WT KIT or KITD814V receptors from WT or *p85α*<sup>-/-</sup> mice were starved for 8 hours from serum and cytokines. Equal amount of protein was subjected to western blot analysis using an anti-phospho-Stat5 and an anti total-Stat5 antibody. Similar results were observed in two independent experiments. (C) Cells bearing WT CHR or CHR814V were starved of serum and growth factors for 6 hours and subjected to proliferation assay in the presence or absence of indicated amounts of PI3Kinase inhibitor (LY294002), Stat5 inhibitor or MEK inhibitor (PD0325901). Assays were performed in the presence of IL-3 (10 ng/mL) for cells expressing WT CHR, whereas for CHR814V bearing cells in the absence of growth factors. Bars denote the mean thymidine incorporation (CPM ± SD) from one of two-three independent experiments performed in quadruplicate. \*p < 0.05.





**Figure 5. PI3Kinase pathway is essential for KITD814V induced ligand independent proliferation via AKT and mTOR signaling**

(A) 32D cells bearing KITD814V receptor were starved in serum- and growth factor-free media for 6 hours and subjected to proliferation assay in the absence of growth factors by thymidine incorporation. The proliferation assay was performed in the presence of individual inhibitors including PI3K inhibitor (GDC-0941), AKT inhibitor (AKT inhibitor VIII), or mTOR inhibitor (AZD8055) at indicated concentrations. Bars denote the mean thymidine incorporation (CPM  $\pm$  SD) from one of two independent experiments performed in quadruplicate. \*p <0.05. (B) 32D cells bearing KITD814V receptor were starved in serum- and growth factor-free media for 6 hours and subjected to proliferation assay in the absence of growth factors by thymidine incorporation. The proliferation assay was performed in the presence of PI3Kinase inhibitor (GDC-0941) and MEK inhibitor (PD0325901) individually or in a combination at indicated concentration. Bars denote the mean thymidine incorporation (CPM  $\pm$  SD) from one of two independent experiments performed in quadruplicate. \*p <0.05.