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Oncogenic KIT mutations in different exons lead to specific changes in melanocyte phospho-proteome

M. Sanlorenzo^{1,2,*}, I. Vujic^{1,3}, C. Posch^{1,3}, J. Ma¹, K. Lin¹, K. Lai¹, J.A. Oses-Prieto⁴, S. Chand⁴, J.L. Rodriguez-Peralto⁵, A. Burlingame⁴, and S. Ortiz-Urda¹

¹University of California San Francisco, Department of Dermatology, Mt. Zion Cancer Research Center, 2340 Sutter Street N461, 94115 San Francisco – USA

²Department of Medical Sciences, Section of Dermatology, University of Turin – Italy

³The Rudolfstiftung Hospital, Academic Teaching Hospital, Medical University Vienna, Department of Dermatology, Juchgasse 25, 1030 Vienna – Austria

⁴University of California San Francisco, School of Pharmacy, Pharmaceutical Chemistry, 600 16th Street, 94158 San Francisco – USA

⁵Hospital Universitario 12 de Octubre, Madrid, Spain

Abstract

Mutations in the proto-oncogene c-KIT (KIT) are found in several cancers, and the site of these mutations differs markedly between cancer types. We used site directed mutagenesis to induce KIT⁵⁵⁹, KIT⁶⁴² and KIT⁸¹⁶ mutations in primary human melanocytes (PHM) and we investigated the impact of each mutation on KIT function. We studied canonical KIT-signaling pathways by immunoblotting, and we used stable isotope labeling by amino acids in cell culture (SILAC) and kinase prediction models to identify kinases differently activated in respective mutants. We validated our results with the analysis of phosphorylation levels of selected substrates for each kinase. We concluded that CK1 ϵ and δ are more active in cell clones harboring KIT⁵⁵⁹ and

*To whom correspondence should be addressed: Martina Sanlorenzo, MD, University of California San Francisco, Department of Dermatology, Mt. Zion Cancer Research Center, 2340 Sutter Street N461, 94115 San Francisco – USA, martina.sanlorenzo@hotmail.it, tel: +1 (415) 732 9965.

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SUPPLEMENTARY MATERIALS:

Supplementary Table 1: p-peptides identified with mass spectrometry (MS)-based quantitative proteomics in non-SCF stimulated cells. The analysis identified 3.655 unique p-peptides.

Supplementary Table 2: p-peptides identified with mass spectrometry (MS)-based quantitative proteomics in SCF stimulated cells. The analysis identified 3.774 unique p-peptides.

Supplementary Table 3: Kinase prediction models identify kinases active in cells with induced KIT mutations. In non-SCF stimulated PHM^{KIT-V559D} and PHM^{KIT-D816H} cells kinases responsible for the phosphorylation of the most peptides belong to the MAPK family. In both clones the most active kinase is p38.

Supplementary Table 4: Kinases prediction in non-SCF stimulated cells. None of the kinases were significantly different comparing non-SCF-stimulated PHM^{KIT-V559D} and PHM^{KIT-D816H} cells.

Supplementary Table 5: The kinases more active in the cells stimulated with SCF. In both PHM^{KIT-V559D} and PHM^{KIT-D816H} the kinases responsible for the phosphorylation of the most peptides belong to the MAPK family and in particular the most active one is p38.

KIT⁶⁴² mutations, whereas PAK4 is more active in clones with KIT⁸¹⁶ mutation. Our findings might help to develop further therapeutic options for tumors with specific KIT mutations in different domains.

Graphical Abstract

Keywords

SILAC; kinases prediction

INTRODUCTION

The proto-oncogene c-KIT (KIT) belongs to the family of growth factor receptors with intrinsic tyrosine kinase activity. KIT is a transmembrane protein and has an extracellular region, a transmembrane domain and an intracellular region that consists of a split kinase and an autoregulatory domain. Stem-cell factor (SCF) is the ligand of KIT, and its binding to the extracellular region induces dimerization of KIT with subsequent autophosphorylation in tyrosine residues which become docking sites for several proteins. This leads to the activation of different downstream pathways: mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinases (PI3K) pathway, and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, the SRC family kinases (SFK) and phospholipase C γ [1–3].

Oncogenic KIT mutations are found in several different cancers. Eighty percent of gastrointestinal stromal tumors (GIST), 26% of testicular seminomas, 17% of acute myeloid leukemia and 5% of melanomas harbor KIT mutations. However, the sites of these KIT mutations differ markedly between cancers. KIT juxtamembrane domain mutations (such as KIT⁵⁵⁹) are predominantly found in GIST, tyrosine kinase II domain mutations (such as KIT⁸¹⁶) in hematological malignancies and tyrosine kinase I domain mutations (such as KIT⁶⁴²) in malignant melanomas [4–7].

We wondered if and how different mutation sites impact KIT function. We sought to understand which pathways are activated by different KIT mutations in order to identify

potential targets for small molecule inhibitors. This is particularly important since in melanoma, the use of KIT inhibitors has yet been disappointing for the treatment of KIT mutant tumors [8].

We used directed mutagenesis to induce KIT⁵⁵⁹, KIT⁶⁴² and KIT⁸¹⁶ mutations in primary human melanocytes (PHM) and we investigated the impact of each mutation on KIT function. We performed the study of canonical KIT-signaling pathways by immunoblotting, and we used stable isotope labeling by amino acids in cell culture (SILAC) and kinase prediction models to identify the kinases differently activated in the respective mutants.

MATERIALS AND METHODS

Cell culture

Primary human melanocytes (PHM) were extracted from left over, human infant foreskin tissue following our established protocol. cKIT plasmids (V559D, D816H, K642E, empty vector) were generated by site directed mutagenesis in a gateway/lenti virus transduction system. Co-expressed GFP was used as a transduction efficacy reporter. SILAC was performed as previously described. The three PHM variants selected for SILAC were empty, V559D and D816H. They propagated for at least 8 doublings in medium containing light (Arg/Lys), medium (Arg6/Lys4) and heavy (Arg10/Lys8) amino acid-isotypes. Two different experimental setting were performed: with and without 5 minutes stimulation with SCF. SILAC encoded cell lysates were mixed, digested with trypsin and enriched for phosphopeptides which is critical because signaling proteins are usually of low abundance and difficult to detect by MS without enrichment. Anti-pTyr IP was used to isolate the pTyr proteome, and the remaining peptides were subjected to chromatographic enrichment of phosphorylated species using a TiO₂ column, followed by extensive fractionation of the phosphopeptide mixture using a C18 reverse phase column at basic pH [9]. Peptides were analyzed by LCMSMS in an Orbitrap Velos, and Mass spectrometry data were analyzed using Protein Prospector (v 5.12.2) as previously described [10].

Immunoblots

All cells used for immunoblots represent biological replicates. Cells were plated in 6 well plates 24h prior to protein extraction. Cells were washed with phosphate buffered saline (PBS) and lysed using radio-immunoprecipitation (RIPA) buffer [150 mM NaCl, 1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS] in 50 mM Tris-HCl (pH 8.0) supplemented with 1× protease and phosphatase inhibitors (78442; Pierce). Protein concentrations were determined using the BCA Protein Assay kit (23235; Pierce). Total protein in 1×Laemmli buffer with 10% 2-mercaptoethanol was separated by SDS/PAGE, transferred for 1 h to a PVDF membrane (IPVH00010; Millipore) by electro blotting with 20% (vol/vol) methanol, and blocked for 1 h in 5% (wt/vol) dry milk/Tris-buffered saline (TBS)/0.1% (vol/vol) Tween-20. Membranes were incubated overnight at 4 °C with primary antibodies following incubation with horseradish peroxidase-conjugated secondary antiserum for 1 h and developed using enhanced chemiluminescence [32106 (Pierce) or 64 – 201BP (Millipore)]. β –Actin protein expression served as a loading control. KIT (3074), P-ERK (4370), ERK (4695), p-AKT (4060), AKT (4691), p-S6 (4857),

S6 (2317), p-MEK (2338), MEK (4694), p-p38 (4511), p38 (9212), p-STAT3 (9145), STAT3 (9139), LIMK1 (3842), p-LIMK1 (3841), β -catenin (9562), p- β -catenin (9564) were obtained from Cell Signaling Technology, β -Actin was purchased from Sigma Aldrich. Image analyses were performed with ImageJ 1.47v (Wayne Rasband, National Institute of Health, USA).

SILAC data analyses

To analyze SILAC results we compared different clones to each other. The SILAC quantification compares a mutated clone (PHM^{KIT-V559D}, PHM^{KIT-D816H}) to the empty vector clone (PHM^{empty}) and provides the log₂ fold change for each phospho-peptide. We calculated the log₂ fold change between PHM^{KIT-V559D} and PHM^{KIT-D816H} to directly compare the two mutant protein species using the formula: $\log_2(PHM^{KIT-V559D}/PHM^{KIT-D816H}) = \log_2(PHM^{KIT-V559D}/PHM^{empty}) - \log_2(PHM^{KIT-D816H}/PHM^{empty})$.

GO terms and pathways analyses

For GO terms only the phosphopeptides that showed a 2× change between different conditions were considered and when multiple peptides mapped to the same protein only highest one was kept. The analysis was run using the functional annotation tool of Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7.

Kinases prediction

For kinase prediction all the ambiguous phosphorylation sites were excluded. The kinases predictions were made in the Group-based Prediction System (iGPS) algorithm with interaction filter (Ver 1.0). The site-specific kinase-substrate prediction was run with a default low threshold and keeping both STRING and experimental protein-protein interaction. Although kinases were predicted to phosphorylate more p-sites, we speculated whether some kinases preferentially modify more or fewer p-sites in one condition compared to the other. To address this problem we used Yates's corrected version of Pearson's chi-squared test with the 2×2 contingency table method [11] (for detail please see Supplementary Methods).

RESULTS

V559D, D816H and K642E mutations in KIT differentially affect downstream signaling

To test how different mutations of KIT change downstream pathway activation in melanocytes, we used site directed mutagenesis to create plasmids bearing the KIT V559D (exon 11, juxtamembrane domain), D816H (exon 17, tyrosine kinase domain II), and K642E (exon 13, tyrosine kinase domain I) mutations (Figure 1A). Plasmids were transduced into pooled primary human melanocytes (PHM) and GFP co-expression used as a transduction efficacy reporter. We confirmed the presence of each KIT mutation in cells by Sanger sequencing.

Figure 1B depicts a schematic of members in the KIT signaling cascade, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) maps and the recent publications about KIT signaling [2,3,12]. To explore mutation effects on these pathways we selected important

members of each pathway and explored their modulation through probing for their phosphorylation status. Clones with different KIT mutations showed differences in pathway activation (Figure 1C). In cells not stimulated with the KIT ligand stem cell factor (SCF), we found a stronger activation of the MAPK, the PI3K/mTOR and the p38-MAPK pathways in PHM^{KIT-V559D} and PHM^{KIT-D816H} compared to the PHM^{empty} and PHM^{KIT-K642E}. Moreover, p-STAT3 was detectable only in PHM^{KIT-D816H}. The SCF-stimulation differently affected each clone: we found higher levels of p-ERK in PHM^{empty} and PHM^{KIT-K642E} compared to the other clones, and detectable levels of p-STAT3 in both PHM^{KIT-D816H} and PHM^{KIT-V559D} (Figure 1C).

Stable isotope labeling by amino acids in cell culture (SILAC) allows the global analysis of the phospho-proteome induced by different cKIT mutations

As we saw differences in pathway activation for different KIT mutants by immunoblotting (Figure 1), we expanded our studies to investigate changes of the phospho-proteome on an omic level. We used SILAC to investigate mass spectrometry based quantitative proteomics of primary human melanocytes (PHM) stably transduced with different KIT mutations. We compared three clones - PHM^{KIT-V559D}, PHM^{KIT-D816H} and PHM^{empty} - each with and without stimulation using SCF. We first enriched for low abundant phosphorylated tyrosine sites (pY) by antibody mediated pull-down [13]. For global phosphoprotein analyses these pY-enriched peptides were pooled with p-serine (pS) and p-threonine (pT) peptides (Figure 2). We identified and quantified 3.655 unique p-peptides in non-SCF-stimulated cells (Supplementary Table 1) and 3.774 in cells stimulated with SCF (Supplementary Table 2).

Next, we compared the phospho-peptide profiles of PHM^{KIT-V559D} and PHM^{KIT-D816H}. The calculated phospho-peptide differences were used for further analyses. For kinases prediction modeling, all ambiguous phospho-sites were removed, and for GO terms prediction only phospho-peptides with biologically relevant changes ($\geq 2\times$ fold difference between conditions) were considered (Figure 3).

SCF stimulation changes the most enriched GO terms in PHM^{KIT-V559D} and in PHM^{KIT-D816H}

GO terms analyses were performed using the functional annotation tool DAVID. In non-stimulated cells the top three GO terms in PHM^{KIT-V559D} and in PHM^{KIT-D816H} were identical: 'intracellular signaling cascade', 'protein transport' and 'protein localization' (Figure 4). Among the phospho-proteins responsible for these GO terms categories were β -catenin in PHM^{KIT-V559D} and AKT1 in PHM^{KIT-D816H}. When we stimulated the cells with the KIT ligand SCF, the predicted GO terms changed and were different for the two clones. In PHM^{KIT-V559D} the top three GO terms were 'protein localization', 'establishment of protein localization' and 'protein transport'; and in PHM^{KIT-D816H} they were 'intracellular signaling cascade', 'phosphate metabolic process' and 'phosphorus metabolic process' (Figure 4). Among the phospho-proteins responsible for these GO terms categories were A Kinase (PRKA) Anchor Protein 12 in PHM^{KIT-V559D} and P21 Protein-Activated Kinase 1 (PAK1) in PHM^{KIT-D816H}.

KIT activation through SCF is a well described process which consists of KIT dimerization and trans-phosphorylation of different residues [1]. Therefore, we manually checked if

SILAC captured differences in these phospho-sites. Two phospho-peptides mapped to KIT: pY-KIT⁷⁰³ and pY-KIT⁷⁴⁷. These residues are both known to be phosphorylated when SCF induces KIT activation [1]. Our results revealed that Y-KIT⁷⁰³ site was significantly more phosphorylated in PHM^{KIT-D816H} compared to PHM^{KIT-V559D} (Table 1).

Kinase prediction modeling revealed induced CK1 signaling in PHM^{KIT-V559D}, and induced PAK signaling in PHM^{KIT-D816H}

Phosphorylation is controlled by specific protein kinases and phosphatases. In this process, protein kinases modify proteins by adding phosphate groups to amino acid residues, while protein phosphatases remove these phosphate groups [14,15]. The importance of such kinases and phosphatases for cell physiology has been widely documented [16]. We used phospho-peptides identified with SILAC and the iGPS software to predict potential kinases active in each mutant protein species.

Non-SCF-stimulated cells—In non-SCF-stimulated cells, we found that 328 and 319 kinases were active in PHM^{KIT-V559D} and PHM^{KIT-D816H} respectively (Supplementary Table 3).

In both mutants, the top predicted kinases belonged to the group of the CMGC (cyclin-dependent kinase [CDK], mitogen-activated protein kinase [MAPK], glycogen synthase kinase [GSK3], CDC-like kinase [CLK])[17]. The most active kinase in both conditions was p38.

Next, we investigated variations in kinase activity in different KIT mutants. We first calculated the number of peptides that were phosphorylated by every kinase and compared these between different conditions using Yates' chi-squared test with the 2×2 contingency table [11]. None of the kinases were significantly different comparing non-SCF-stimulated PHM^{KIT-V559D} and PHM^{KIT-D816H} cells (Supplementary Table 4).

SCF-stimulated cells stimulated with SCF—The site-specific kinase-substrate prediction in cells stimulated with SCF found that 344 different kinases were predicted to be active in PHM^{KIT-V559D} and 343 in PHM^{KIT-D816H} mutant cells (Supplementary Table 5).

Similarly to non-SCF-stimulated cells, also in SCF-stimulated cells most active kinases in both conditions belonged to the CMGC family. Again, the most active kinase in both conditions was p38.

However, when testing for differences in kinase activation as described above, we found that 37 kinases had a different activity in PHM^{KIT-V559D} compared to PHM^{KIT-D816H} ($p < 0.05$). Table 2 lists kinases significantly more active in PHM^{KIT-V559D} and Table 3 those more active in PHM^{KIT-D816H}.

In PHM^{KIT-V559D}, the top hyperactive kinases were CK1 ϵ and δ , members of the casein kinase 1 (CK1) family; while among the top kinases overactive in PHM^{KIT-V559D} there were two members of the p21-activated kinases (PAKs) family: PAK4 and PAK2.

In order to validate our analyses, we selected substrates of kinases predicted to be over activated and checked their phosphorylation status. The top kinases in PHM^{KIT-V559D} were CK1 δ and ϵ which both phosphorylate β -catenin at serine 45 [18–20]. PAK4 is the top member of the PAKs family predicted to be overactive in PHM^{KIT-D816H} and it phosphorylates LIMK1 at threonine 508 [21]. Immunoblotting confirmed our prediction showing higher levels of phospho- β -catenin in PHM^{KIT-V559D}, and higher levels of phospho-LIMK1 in PHM^{KIT-D816H}. Interestingly, high levels of phospho- β -catenin were identified also in PHM^{KIT-K642E} when stimulated with SCF.

DISCUSSION

A percentage of melanomas has activating mutations in the KIT gene, which can occur in different domains: in the juxtamembrane domain, in the tyrosine Kinase I domain and also in the tyrosine kinase II domains [22]. The use of small molecules inhibitors directly targeting KIT, were ineffective in the treatment of patients with KIT mutant melanomas [23–26]. In order to identify targets for indirect blockade of KIT using small molecules inhibitors we sought to investigate the phospho-proteome of primary human melanocytes transduced with different KIT mutations. We investigated pathways known to be linked to KIT signaling and used SILAC to investigate global changes of the phosphoproteome.

Our results revealed that mutations in different KIT regions differentially affect KIT downstream signaling and thus may impact the biological characteristics of such protein species.

To test the hypothesis that mutations in different KIT regions lead to differences in downstream signaling we first used immunoblotting to investigate pathways which have previously been described to be modulated by KIT. Mutations in the KIT^{V559D} (juxtamembrane domain) and in KIT^{D816H} (tyrosine kinase II domain) led to a higher activation of PI3K/mTOR, MAPK and p-38 pathways compared to the mutation in KIT^{K642E} (kinase domain I) and to wild-type KIT (Figure 1C). This could be explained by structural changes in the KIT protein after mutagenesis. In fact, KIT is normally auto-inhibited by the juxtamembrane domain which folds and interacts with the kinase domains, prolonging the induction time to activation [27]. Mutations in the juxtamembrane domain (KIT^{V559D}) change the structure of KIT and decrease the inhibitory juxtamembrane domain binding affinity to the kinase domains [27]. Mutations in the tyrosine kinase II domain (KIT^{D816H}) cause a structural change in the activation loop, but also weaken the binding of the juxtamembrane domain to the kinase domain [28]. Thus, both KIT^{V559D} and KIT^{D816H} mutations have similar effects on KIT structure and functionality and disrupt auto-inhibitory mechanisms.

Previous studies showed that in neoplastic mast cells and in leukemia KIT^{D816H} mutations lead to STAT3 and STAT5 activation [29–31]. We found similar changes in STAT signaling using melanocytes strongly suggesting a distinct effect of the KIT mutation rather than a cell specific change. We found detectable levels of p-STAT3 in clones harboring the KIT^{D816H} mutation with and without SCF stimulation and in SCF-stimulated KIT^{V559D}. Therefore, the

JAK/STAT pathway could be a possible target for the treatment of melanomas harboring mutations in KIT^{V559} and KIT^{D816}.

Compared to immunoblotting, SILAC is a much more sensitive method for the analysis of global phospho-proteome changes in clones with different point mutations [32]. We used this technique to compare cells harboring mutations in the KIT^{V559D} and KIT^{D816H}.

Previous studies showed that KIT mutations affect the localization of the protein in the cell. Both, KIT protein bearing mutations in the juxtamembrane domain and in the tyrosine kinase II domain were described to be mainly localized in the cytoplasm of the cell and not on the cell surface [33,34]. However, we found that the stimulation with soluble SCF causes differences in clones with different mutations, suggesting the presence of mutant KIT proteins on the cell surface. Since SCF-dependent activation of KIT is the result of trans-phosphorylation of known KIT residues (Yuzava et al. Cell 2007), we investigated the phosphorylation status of different KIT mutants. We found two phosphorylation sites in KIT, both known to be phosphorylated when KIT is activated by SCF [1]: KIT⁷⁰³ and KIT⁷⁴⁷. The detected levels of p-KIT⁷⁰³ were significantly higher in KIT^{D816H} compared to KIT^{V559D} mutants. Our findings suggest, that SCF-dependent KIT activation is stronger in the presence of KIT^{D816H} mutation. This is in line with previous studies, which show that in cells expressing tyrosine kinase II domain mutations, SCF has an impact on cell signaling [35,36] and that surface-expressed KIT has an important biological role [37].

The analyses of kinase activity in different clones stimulated with SCF showed significant differences between the two mutations. In cells with KIT^{V559D} mutation, all overactive kinases belong to two families: CK1 and MAPK. Among them CK1 δ and ϵ were the two top overactive kinases. Their activity was confirmed by the high phosphorylation levels of their substrate β -catenin. Interestingly, we saw even higher levels of p- β -catenin in clones with the KIT^{K642E} mutation, which is usually found in KIT mutant melanoma. CK1 δ and ϵ are highly expressed in cancers and control tumor growth, apoptosis, metabolism and differentiation [38]. Selective inhibitors of CK1 δ/ϵ have been developed [39] and our results suggest that they could be used for the treatment of tumors harboring mutations in KIT^{V559} and KIT^{K642}.

In cells with a KIT^{D816H} mutation, the overactive kinases belong to STE and CAMK families, including several p21- activated kinases (PAKs). The activity of PAK4 was confirmed by high phosphorylation levels of its substrate LIMK1 in KIT^{D816H}. PAKs overexpression and activation is frequently seen in various human tumors driving cellular processes involved in cancer growth and progression [40]. Recently, specific inhibitors of these kinases were developed [40]. Our results suggest a possible application of these inhibitors for the treatment of tumors harboring KIT^{D816H} mutations.

Certainly, further studies of these compounds are needed to evaluate the (pre)-clinical effectiveness of these targeted approaches.

CONCLUSIONS

The heterogeneity of possible KIT alterations and the functional differences related to different mutations showed in our study, could explain the low clinical benefit of KIT inhibition in some cancers like melanoma. Our study shows how specific KIT mutations impact cell phosphor-proteome, and it suggests therapeutic options for tumors with specific KIT mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SIGNIFICANCE

Different type of cancers harbor mutations in the oncogene KIT. The use of small molecules inhibitors directly targeting KIT had a limited success in the treatment of patients with KIT mutant cancers. Our study describes specific phospho-proteome changes due to different KIT mutations, and provides targets of further therapeutic options.

HIGHLIGHTS

- Different KIT mutations lead to activation of different downstream pathways
- Single point mutations in KIT determine changes in the cell's phospho-proteome that are specific for each mutations site.
- Mutations in KIT⁵⁵⁹ lead to over activation of kinases members of the casein kinase 1 (CK1) family.
- Mutations in KIT⁸¹⁶ lead to over activation of kinases members of the p21- activated kinases (PAKs) family.

Figure 1. Different KIT mutations lead to activation of different downstream pathways
A) Schematic representation of KIT protein and its main oncogenic mutations. We induced point mutations in three different KIT domains using site directed mutagenesis and transfected primary human melanocytes (PHM) using a gateway/lenti virus transduction system. **B) Schematic representation of the KIT signaling cascade.** KIT signals through the MAPK pathway, the PI3K/mTOR pathway, the JAK/STAT pathway and the SRC proteins. Highlighted in yellow are the important members of each pathway whose activation (phosphorylation status) we explored in immunoblots. **C) Immunoblot analysis of important members of each signaling pathway.** PHM^{KIT-V559D} and PHM^{KIT-D816H} show higher levels of phospho-proteins in the PI3K/mTOR, MAPK and p38-MAPK pathways compared to PHM^{empty} and PHM^{KIT-K642E}. In PHM^{KIT-D816H} there is a higher phosphorylation level of STAT3. The SCF-stimulation differently affect the different clones.

Figure 2. Schematic representation of the SILAC workflow and numbers of phospho-peptides identified

PHM were transfected with empty vector, KIT^{V559D} and KIT^{D816H} and cultured with SILAC labeling aminoacids (light, middle and heavy). Cells lysates were performed from SCF stimulated and non-stimulated cells. Precipitation with pY antibodies was used to enrich pY sites. Mass spectrometry identified 3.655 p-peptides in the unstimulated cells and 3.774 in the cells stimulated with SCF.

Figure 3. Schematic representation of the SILAC bioinformatic analysis

We first calculated fold change differences in phospho-peptides abundance comparing two KIT mutated clones (PHM^{KIT-V559D}, PHM^{KIT-D816H}). For kinases prediction the ambiguous p-sites were excluded and analyses were run with iGPS kinases prediction model. For GO terms all p-peptides with a fold change lower than 2 were excluded and the analyses were run with DAVID.

A. *Unstimulated cells* **B. *Cells stimulated with SCF*** **Figure 4. Top ten GO terms enriched in each condition**

A) Non-stimulated cells. B) Stem-cell-factor (SCF) stimulated cells. Bars represent the number of proteins per GO term. GO terms ‘intracellular signaling cascade’, ‘protein transport’ and ‘protein localization’ were the three top terms in both clones (PHM^{KIT-V559D} and PHM^{KIT-D816H}) when non-stimulated with KITs` ligand SCF. Stimulation with SCF led to difference in most enriched GO terms when comparing PHM^{KIT-V559D} to PHM^{KIT-D816H}.

Figure 5. Validation of kinases prediction

β-catenin, substrate of CK1 δ/ε, is more phosphorylated in PHM^{KIT-V559D} and LIMK1, substrate of PAK4, is more phosphorylated in PHM^{KIT-D816H}.

Table 1
Phospho-peptides identified with SILAC that mapped to KIT in the set of cells stimulated with SCF

The phosphorylation of the tyrosine at the position KIT⁷⁰³ and KIT⁷⁴⁷ were more frequent in PHM^{KIT-D816H} compared to PHM^{KIT-V559D}

Peptide	Protein Name	p-position	$\log_2$ (PHM ^{KIT-V559} /PHM ^{empty})	$\log_2$ (PHM ^{KIT-D816H} /PHM ^{empty})	$\log_2$ (PHM ^{KIT-V559D} /PHM ^{KIT-D816H})
QEDHAEAAALY(Phospho)K	KIT	703	0,09617515	0,382111205	-0,285936054
IGSY(Phospho)IER	KIT	747	0,182567828	0,215969609	-0,033340178

Table 2

Kinases predicted to be significantly more active in PHM^{KIT-V559D}

Several members of the CK1 and MAPK families are more active in SCF-stimulated PHM^{KIT-V559D} compared to PHM^{KIT-D816H}.

Kinase	Predictor	PHM ^{KIT-V559D}		PHM ^{KIT-D816H}		chi-square	p-value
		#peptides phosphorylated by the kinase	#peptides phosphorylated by other kinases	#peptides phosphorylated by the kinase	#peptides phosphorylated by other kinases		
CK1e	CK1	24	967	11	1225	7,370248599	0,0066
CK1d	CK1	26	965	13	1223	6,999461306	0,0082
VRK2	CK1	26	965	13	1223	6,999461306	0,0082
VRK1	CK1	9	982	1	1235	6,660780936	0,0099
p38d	CMGC/MAPK	149	842	140	1096	6,363376264	0,0116
CK1a2	CK1	26	965	14	1222	6,10196088	0,0135
CK1g2	CK1	26	965	14	1222	6,10196088	0,0135
CK1g3	CK1	10	981	2	1234	5,861582913	0,0155
p38a	CMGC/MAPK	147	844	140	1096	5,706824311	0,0169
CK1g1	CK1	24	967	13	1223	5,49894639	0,0190
TTBK1	CK1	24	967	13	1223	5,49894639	0,0190
TTBK2	CK1	24	967	13	1223	5,49894639	0,0190
VRK3	CK1	24	967	13	1223	5,49894639	0,0190
Erk2	CMGC/MAPK	133	858	128	1108	4,693931917	0,0303
Erk3	CMGC/MAPK	114	877	107	1129	4,664878546	0,0308
JNK1	CMGC/MAPK	114	877	107	1129	4,664878546	0,0308
JNK3	CMGC/MAPK	114	877	107	1129	4,664878546	0,0308
Erk5	CMGC/MAPK	112	879	105	1131	4,604749554	0,0319
Erk1	CMGC/MAPK	111	880	104	1132	4,574821581	0,0324
CK2a1	Other/CK2	58	933	48	1188	4,273468354	0,0387
p38g	CMGC/MAPK	42	949	32	1204	4,150522396	0,0416
JNK2	CMGC/MAPK	115	876	110	1126	4,130732142	0,0421
p38b	CMGC/MAPK	115	876	110	1126	4,130732142	0,0421

Table 3
Kinases predicted to be significantly more active in PHM^{KIT-D816H}
Several members of the STE and CAMK families are more active in to PHM^{KIT-D816H} compared to PHM^{KIT-V559D}.

Kinase	Predictor	PHM ^{KIT-V559D}		PHM ^{KIT-D816H}		chi-square	p-value
		#peptides phosphorylated by the kinase	#peptides phosphorylated by other kinases	#peptides phosphorylated by the kinase	#peptides phosphorylated by other kinases		
NRK	STE/STE20	19	972	47	1189	6,148410177	0,0132
YSK1	STE/STE20	14	977	36	1200	4,967107717	0,0258
PAK4	STE/STE20	13	978	34	1202	4,830557183	0,0280
PAK2	STE/STE20	16	975	39	1197	4,792530401	0,0286
HPK1	STE/STE20	14	977	35	1201	4,50104747	0,0339
LOK	STE/STE20	18	973	41	1195	4,232277697	0,0397
STLK6	STE/STE20	12	979	31	1205	4,219774086	0,0400
KHS2	STE/STE20	19	972	42	1194	3,981863445	0,0460
MST4	STE/STE20	19	972	42	1194	3,981863445	0,0460
OSR1	STE/STE20	19	972	42	1194	3,981863445	0,0460
PAK5	STE/STE20	19	972	42	1194	3,981863445	0,0460
CASK	CAMK	0	991	7	1229	3,961348608	0,0466
SSTK	CAMK	0	991	7	1229	3,961348608	0,0466
TSSK1	CAMK	0	991	7	1229	3,961348608	0,0466