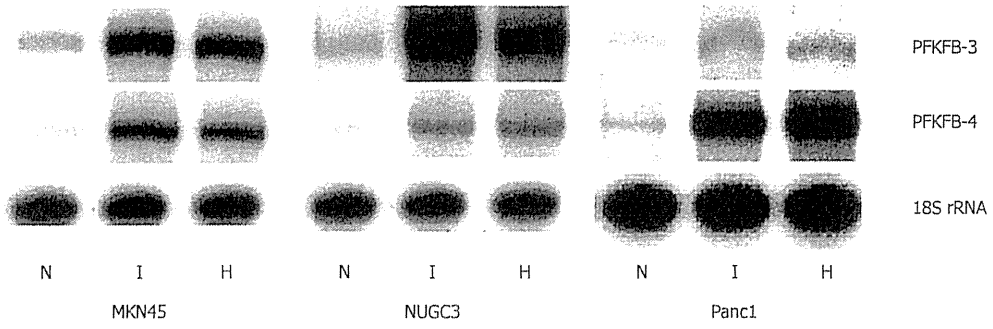

Figure 1 Effect of hypoxia (H) and hypoxia mimic dimethyloxalylglycine (I) on the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 mRNA in human gastric cancer cell lines MKN45 and NUGC3 and pancreatic cancer cell line Panc1. Measured by ribonuclease protection assay, N: Control (normoxic) cells[32]. PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

tive splice variants were identified in mouse brain and other tissues[60]. One of them has two ORFs (for 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase) as a result of insert after the 7th exon. Other alternative splice variants have inserts in kinase domain or a deletion in bisphosphatase domain[60]. Its functional significance is not elucidated as of yet.

At the same time, the analysis of PFKFB-3 and PFKFB-4 expression as well as its regulation by hypoxia in pancreatic and gastric cancer cells, which significantly differ from many other malignant cells, is needed for further advance our knowledge on the mechanisms of different tumors progression. Recently, it was shown that excess glucose induces hypoxia-inducible factor-1α in pancreatic cancer cells and stimulates glucose metabolism possibly through PFKFB as well as the migration of these cancer cells and that hypoxia strongly up-regulates the expression of PFKFB-4 and PFKFB-3[32,61].

PFKFB-4 AND PFKFB-3 GENE EXPRESSIONS IN PANCREATIC AND GASTRIC CANCER CELL LINES AND MOLECULAR MECHANISMS OF ITS REGULATION

It was shown that different *PFKFB* genes are expressed in human pancreatic and gastric cancer cells and are up-regulated in hypoxic condition[32]. Hypoxia induces the expression of these genes through transcription factor HIF binding sites to hypoxia responsible element (HRE) of *PFKFB-4* and *PFKFB-3* genes, because deletion or point mutation in these HRE eliminates the hypoxic regulation of *PFKFB-4* and *PFKFB-3* genes[25,31,36,37]. As shown in Figure 1, the expression of PFKFB-4 mRNA is detectable at very low level in both MKN45 and NUGC3 gastric cancer cell lines growing under normal condition. The expression of PFKFB-3 mRNA in these cell lines was significantly higher as compared to the PFKFB-4 mRNA[32].

Exposure of MKN45 and NUGC3 gastric adenocarcinoma cells to hypoxia or dimethyloxalylglycine, which suppress prolyl hydroxylase enzymes, significantly enhanced the expression of *PFKFB4* as well as *PFKFB3* genes ($P < 0.001$; Figures 1 and 2). At the same time, the expression of PFKFB-3 mRNA in Panc1 pancreatic cancer cell line ($P < 0.01$) growing under normal condition was lower as compared to gastric cancer cell lines, but for PFKFB-4-slightly higher[32]. Moreover, the basal level of PFKFB-4 mRNA expression as well as its hypoxia responsiveness was more robust as compared to PFKFB-3 mRNA[32]. Thus, there is a difference in the basal level of these two variants of PFKFB mRNA between gastric and pancreatic cancer cells as well as in its sensitivity to hypoxia and dimethyloxalylglycine.

At the same time, the protein level of PFKFB-4 isoenzyme in non-treated gastric adenocarcinoma cells was much higher as compared to pancreatic cancer cells (Figure 3). Moreover, the protein level of PFKFB-4 in NUGC3 gastric cancer cells is significantly higher than in MKN45 cells. Hypoxia and dimethyloxalylglycine strongly enhances the expression of PFKFB-4 at protein level in pancreatic and gastric cancer cell lines. This increase of PFKFB-4 protein expression varies in different cancer cell lines possibly because these gastric and pancreatic cells have significantly different constitutive level of PFKFB-4 protein. Moreover, no strong correlation is present between mRNA and protein of PFKFB-4 in the pancreatic and gastric malignant cells in normoxic as well as in hypoxic condition[32]. This data agrees with results of previous investigations[31].

It is interesting to note that the level of PFKFB-4 mRNA and protein differs in different mammary gland adenocarcinoma cell lines both in normal condition and after hypoxic exposure[31]. Thus, the level of PFKFB-4 mRNA is more pronounced in the T47D malignant cell line as compared to MCF7 cells both in normal condition and after hypoxia. At the same time, the protein level of PFKFB-4 is much higher in the MCF7 cells *vs* T47D cell line in normal condition as well as after hypoxic exposure. Other cell lines (SKBR3 and MDA-MB-468) have similar level of PFKFB-4 mRNA expression both in normal condition and upon hypoxia. Unexpectedly, in MDA-MB-468 mammary gland adenocarcinoma cells PFKFB4 protein was detected at negligible level at the same ex-

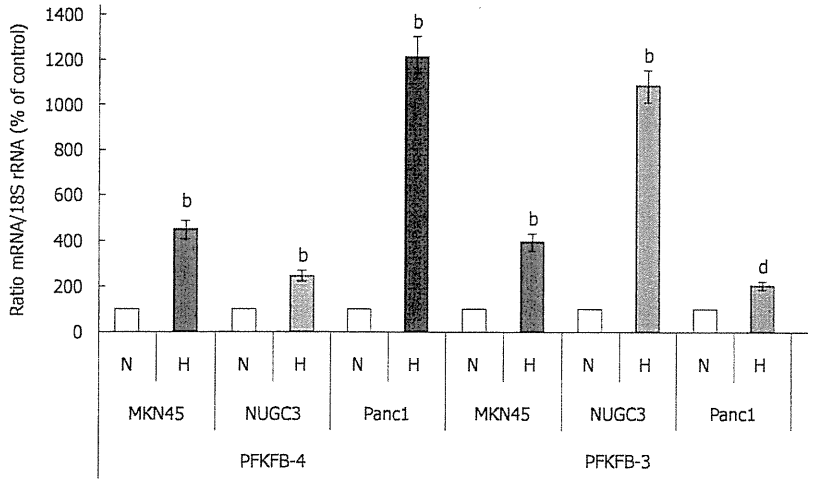

Figure 2 Quantification of ribonuclease protection assay of the effect of hypoxia (H) on the expression level of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 and -3 mRNAs in human gastric (MKN45 and NUGC3) and pancreatic (Panc1) cancer cell lines. $^{b}P < 0.01$ *vs* control cells; $^{d}P < 0.01$ *vs* control cells[32]. N: Normoxic (control) cells. PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

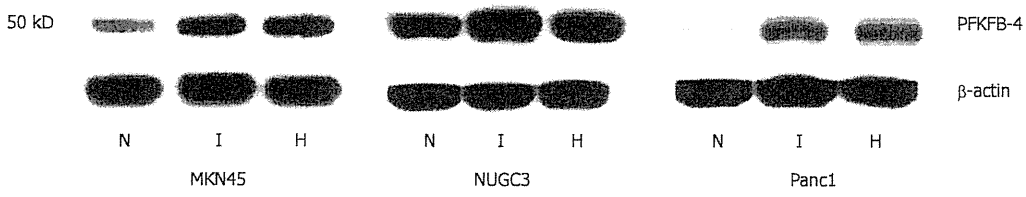

Figure 3 Western blot analysis of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 protein in human gastric (MKN45 and NUGC3) and pancreatic (Panc1) cancer cell lines: Effect of hypoxia (H) and dimethyloxalylglycine (I)[32]. PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

perimental conditions, which were used for SKBR3 and other mammary gland adenocarcinoma cell lines[31]. In contrast, the level of PFKFB-4 mRNA is correlated with corresponding protein level both in SKBR3 and BT549 cell lines.

Really, the protein level of PFKFB-4, which in Panc1 pancreatic cancer cells is much lower as compared to gastric cancer cells and correlates with stronger induction of both PFKFB-4 mRNA and protein expressions upon hypoxia. At the same time, the hypoxia-induced PFKFB-4 protein level in Panc1 cells is in fact lower when compared to the level of this protein in NUGC3 cells. Moreover, the high PFKFB-4 protein level in NUGC3 gastric cancer cells is correlated with the lower induction of PFKFB-4 mRNA and protein expressions upon hypoxia; on the other hand, the level of PFKFB-4 protein in hypoxia-treated cells is very high as compared to the levels of PFKFB-4 protein in both Panc1 and MKN45 control or hypoxia-treated cells[32]. This difference between mRNA and protein levels of PFKFB-4 which in the different pancreatic and gastric cancer cells is possibly related to the mechanisms controlling PFKFB4 protein stability. However, the precise molecular mechanism for these discrepancies is complex and possibly includes PFKFB-4 enzyme posttranslational modification or its stability in a cell-specific manner and warranties further detailed investigation.

The induction of PFKFB-3 mRNA expression in the NUGC3 gastric cancer cell line by hypoxia and dimethyloxalylglycine is more pronounced as compared to PFKFB-4 mRNA expression. It is important to note that the expression PFKFB-3 mRNA in the Panc1 pancreatic cancer cells has the lowest hypoxia responsiveness as compared to both gastric cancer cell lines. We have previously shown that the hypoxic induction of PFKFB-3 mRNA expression in mammary gland cancer cells is more robust in MCF7 and T47D breast cancer cells (estrogen receptor-positive cell lines) as compared to SKBR-3 and MDA-MB-468 cells (estrogen receptor-negative cell lines)[31]. The dissimilar sensitivity of the *PFKFB-3* gene expression to induction by hypoxia was also shown for other cell lines, like HeLa, Hep3B, RPE, and fibroblasts, while induction of Glut1 mRNA by hypoxia was similar in all these cell lines[25].

It is interesting to note that the induction of PFKFB-4 mRNA expression by hypoxia was simulated by dimethyloxalylglycine in different pancreatic and gastric as well as in many other cancer cell lines[25,27,31,32]. Dimethyloxalylglycine, a specific inhibitor of prolyl hydroxylases, is an oxoglutarate analog, which protects the HIF-1α protein from proteasomal degradation and significantly increases its level[59]. Suppression of prolyl hydroxylase enzymes can induce the level and functional activity of HIF-1α under normoxia and mimics hypoxic condition[62]. Induction of PFKFB-4 mRNA synthesis by hypoxia is mediated by the hypoxia responsive element located in the promoter region of this gene which is similar to the same elements, described in different hypoxia responsive genes, including *PFKFB-3* gene[25,36,63,64].

As shown in Figure 4A, hypoxia increases the expression level of PFKFB-3 and PFKFB-4 as well as VEGF and Glut1 genes in both gastric and pancreatic cancer

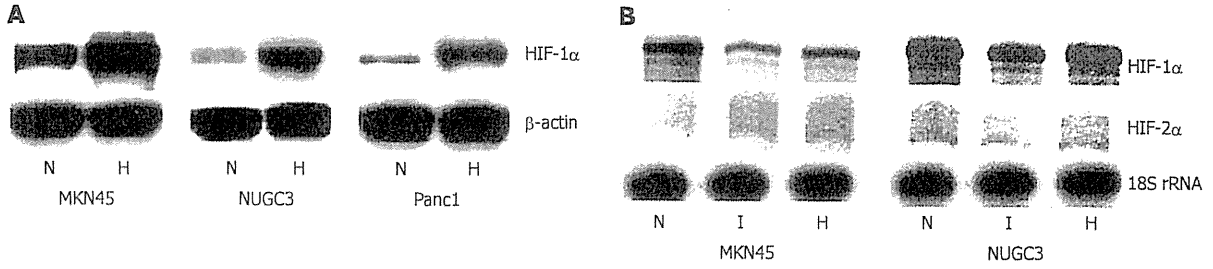

Figure 4 Expression of hypoxia inducible factor-1α protein (Western blotting; A) and hypoxia inducible factor-1α and hypoxia inducible factor-2α mRNA (ribonuclease protection assay; B) in human gastric (MKN45 and NUGC3) and pancreatic (Panc1) cancer cell lines: effect of hypoxia (H) and dimethyloxalylglycine (I)[32]. HIF: Hypoxia inducible factor.

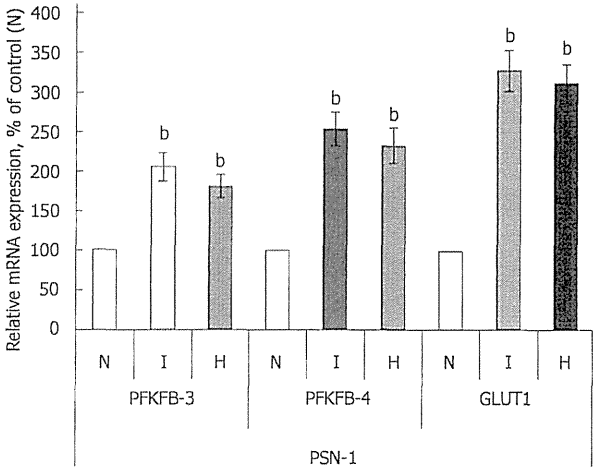

Figure 5 Effect of hypoxia (H) and hypoxia mimic dimethyloxalylglycine (I) on the expression level of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, -4, and GLUT1 mRNAs (measured by qPCR) in human pancreatic (PSN-1) cancer cells. $n = 4$; $^bP < 0.01$ *vs* control cells. N: Normoxic (control) cells; PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

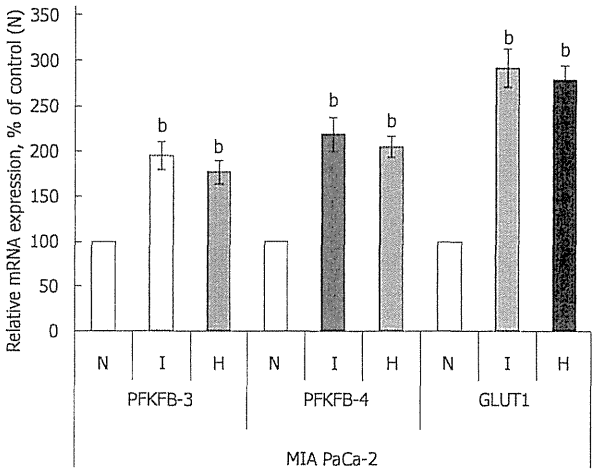

Figure 6 Effect of hypoxia (H) and hypoxia mimic dimethyloxalylglycine (I) on the expression level of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, -4, and GLUT1 mRNAs (measured by qPCR) in human pancreatic (MIA PaCa-2) cancer cells. $n = 4$, $^bP < 0.01$ *vs* control cells. N: Normoxic (control) cells; PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

cell lines and these changes are correlated with enhanced level of HIF-1α protein[32]. This data argues with HIF-1α-dependent mechanism of the induction of these genes under hypoxia. At the same time, the constitutive level of HIF-1α as well as HIF-2α mRNA expression and their induction by hypoxia (Figure 4B) is dissimilar in the MKN45 and NUGC3 gastric adenocarcinoma cells[32]. Thus, the level of HIF-1α mRNA expression is decreased in both gastric cancer cell lines treated by hypoxia or dimethyloxalylglycine, but no significant changes of HIF-2α mRNA expression were found in the NUGC3 gastric cancer cell line under hypoxia. However, the expression of HIF-2α mRNA in the MKN45 gastric cancer cells was slightly induced by dimethyloxalylglycine as well as hypoxia. A similar pattern of the expression of HIF-1α and HIF-2α mRNAs in the A_{549} lung adenocarcinoma cell line and many other cancer cell lines treated by hypoxia was shown[25,64-66].

It is important to note that there is an inverse correlation between induction of HIF-1α mRNA and protein expressions upon hypoxic exposure. These observations suggest that the increase in HIF-1α protein expression was not reflected at the mRNA level. Moreover, the expression of HIF-1α mRNA is significantly decreased both under hypoxia and dimethyloxalylglycine action. It is possible that this discrepancy between HIF-1α mRNA and protein levels, which was found in different gastric, pancreatic and many other cancer cell lines, is related to the divergence in mechanisms which control the stability of HIF-1α mRNA and protein. Thus, the hypoxic induction of HIF-1α protein expression is a result of its stabilization, possibly mediated by specific prolyl hydroxylase enzymes, oxygen- and iron-dependent, which utilize oxoglutarate as a co-substrate[62,67,68]. At the same time, the decreased expression of HIF-1α mRNA both under hypoxia and dimethyloxalylglycine action is possibly mediated by suppression of transcription or by its increased degradation[65].

The expression mRNA level of PFKFB variant 3 and 4 was also investigated in two other pancreatic cancer cell lines: PSN-1 and MIA PaCa-2 (Figures 5 and 6). Hypoxia strongly induces ($P < 0.001$) the expression of both *PFKFB4* and *PFKFB3* genes in PSN-1 and MIA PaCa-2 cancer cells being more robust for *PFKFB4* gene. Moreover, hypoxic induction of *PFKFB4* and *PFKFB3* gene expressions in both these pancreatic cancer cell lines cor-

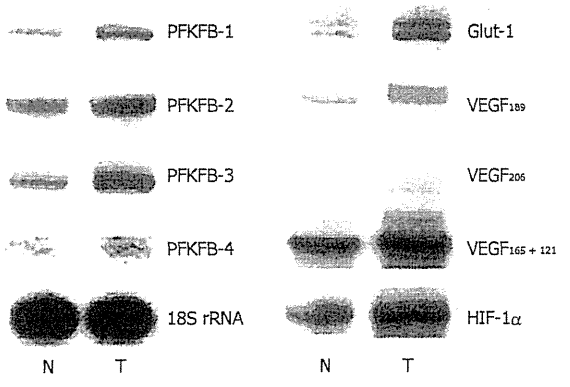

Figure 7 Representative polyacrylamide gel autoradiograph employed in a typical ribonuclease protection assay of different 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase genes (*PFKFB-1*, *PFKFB-2*, *PFKFB-3*, and *PFKFB-4*), GLUT1, hypoxia inducible factor-1α, and different alternative splice variants of VEGF-A in gastric malignant tumors (T) and non-malignant tissue counterparts (N) from same patients. The 18S rRNA expressions were used as control of RNA quantity used for analysis[32]. HIF: Hypoxia inducible factor; PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

relates with a strong increase of *GLUT1* gene expression ($P < 0.001$).

In conclusion, this chapter provides evidence that PFKFB-4 and PFKFB-3 mRNA are expressed in different cancer cell lines, including pancreatic and gastric adenocarcinoma cells, and strongly respond to hypoxia possibly through a HIF- dependent mechanism using active HIF-binding sites in *PFKFB4* and *PFKFB3* genes. At the same time, no clear correlation is existent between different variants of PFKFB mRNA expressions and its protein levels in different cancer cell lines both in normoxic and hypoxic conditions. Moreover, hypoxic induction of HIF-1α protein level correlates with a reduction of HIF-1α mRNA expression in these cell lines. Thus, there is an opposite correlation between hypoxic regulation of PFKFB-4 mRNA and protein levels in different hypoxia-treated adenocarcinoma cells *in vitro*[31,32,65]. It is possible that permanent degradation of HIF-1α protein in normoxic condition support high level expression of corresponding mRNA needed for synthesis of this protein. At the same time, stabilization of HIF-1α protein under hypoxia suppresses transcription of this gene or/and initiates the degradation of HIF-1α mRNA.

EXPRESSION OF DIFFERENT PFKFB IN HUMAN GASTRIC, COLON, LUNG, AND BREAST MALIGNANT TUMORS

There is data that PFKFB-4 and PFKFB-3 mRNA and protein expressions are significantly increased ($P < 0.001$) in gastric cancers as compared to corresponding non-malignant tissue counterparts from the same patients (Figures 7 and 8)[32]. This increase in the expression of these PFKFB genes in gastric malignant tumors correlates with the up-regulation of HIF-1α and known HIF-dependent genes GLUT1 and VEGF ($P < 0.001$). Moreover, the expression of PFKFB-1 and PFKFB-2 mRNA is also increased in gastric cancer tissue. It is interesting to note that the expression level of different PFKFB mRNAs in non-malignant stomach tissue was highest for PFKFB-2, much less for PFKFB-3 and slight for PFKFB-4 and PFKFB-1[32].

As shown in Figure 9, PFKFB-4 and PFKFB-3 mRNA are also overexpressed ($P < 0.001$) in lung, colon, and breast cancers as compared to corresponding non-malignant tissue counterparts from the same patients being more pronounced for PFKFB-4 in lung and breast tumors[30,33,69]. Up-regulation of PFKFB-2 mRNA is also shown for lung cancers[30]. Moreover, the level of GLUT1 and VEGF mRNA expressions is also significantly increased ($P < 0.001$) in all these cancer tissues, especially GLUT1 in lung and colon tumors (Figure 10)[30,33]. Western analysis of PFKFB-4 and PFKFB-3 proteins as well as HIF-1α protein clearly demonstrated its up-regulation in all analysed tumors with more pronounced changes in PFKFB-4 protein in lung and breast cancers (Figure 11)[30,32,33]. Thus, overexpression of PFKFB-3, PFKFB-4 and PFKFB-2 is observed in various human cancers through various mechanisms: by a combination of hypoxia inducible transcription factors (for PFKFB-4 and PFKFB-3), activation of oncogenic proteins and the loss of tumor suppressor function[29-33,37].

Recently, it was shown that amino acid activates AKT-dependent PFKFB2 phosphorylation at Ser-483 and that this activation was mediated by the PI3K and p38 signaling pathways[70]. Furthermore, AKT inactivation blocked PFKFB2 phosphorylation and fructose-2,6-bisphosphate production, thereby suggesting that the above signaling pathways converge at AKT kinase. Moreover, MACC1 (MUC1, mucin 1, cell surface associated) may affect tumor metabolism partly through expression and phosphorylation of PFKFB2[71].

At the same time, the protein level of both PFKFB-4 and PFKFB-3 is significantly different in non-malignant lung, breast, colon, and gastric tissues being more pronounced for colon and gastric tissues. It is interesting to note that the level of PFKFB-4 protein in all studied cancers (lung, breast, colon, and gastric) was also higher as compared to the PFKFB-3 isozyme (Figure 11). Thus, the main protein isoform of PFKFB enzyme family expressed in lung, breast, colon, and gastric malignant tumors is PFKFB-4.

Moreover, there is data that hypoxia is needed for tumor progression and initiates the endoplasmic reticulum stress for induction of neovascularization and apoptosis inhibition[72-76]. It is known that some PFKFB enzymes are components of the endoplasmic reticulum stress and participate in proliferation processes[77]. Recently, it was shown that PFKFB-3-driven glycolysis participates in vessel sprouting process which strongly depends upon endoplasmic reticulum stress[73,78,79]. Thus, endothelial cells relied on glycolysis rather than on oxidative phosphorylation for ATP production and loss of the glycolytic activator PFKFB3 in endothelial cells impaired

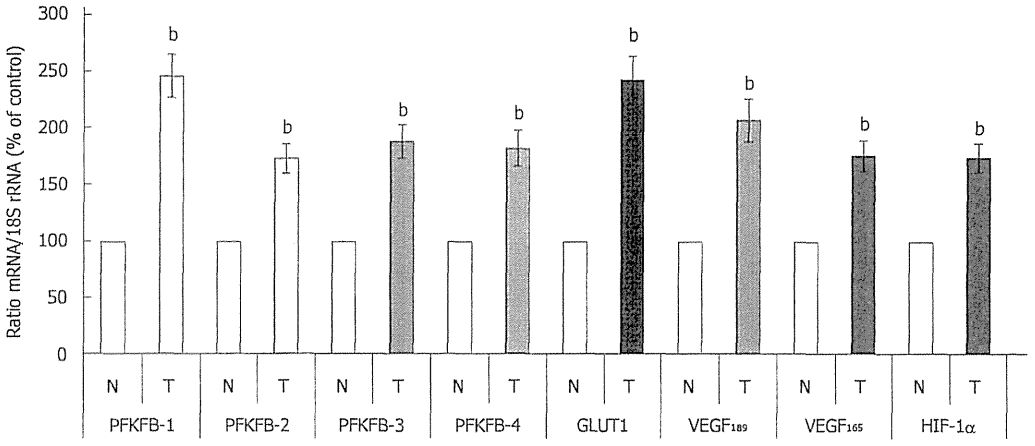

Figure 8 Quantification of ribonuclease protection assay of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-1, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4, GLUT1, hypoxia inducible factor-1α, and splice variants of VEGF-A mRNA in human gastric malignant tumors (T) and corresponding non-malignant tissue (N) from the same patients. $^bP < 0.01$ *vs* control cells[32]. HIF: Hypoxia inducible factor; PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; VEGF: Vascular endothelial growth factor.

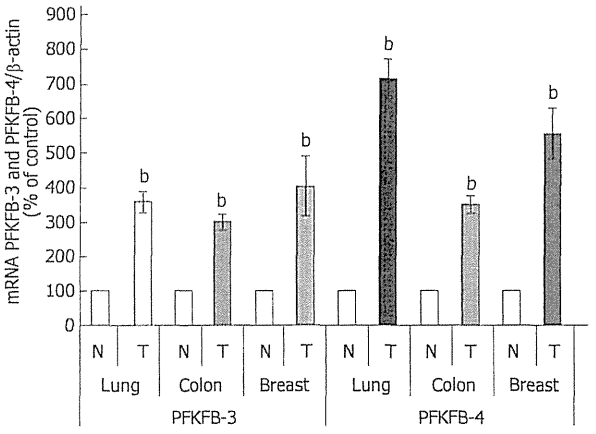

Figure 9 Quantification of ribonuclease protection assay of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 mRNA expressions in lung, colon, and breast malignant tumors (T) and corresponding non-malignant tissue counterparts (C). Values of PFKFB-3 and PFKFB-4 mRNA expressions were normalized to 18S rRNA; n = 15-20, $^bP < 0.01$ *vs* non-malignant tissues[30,33]. PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

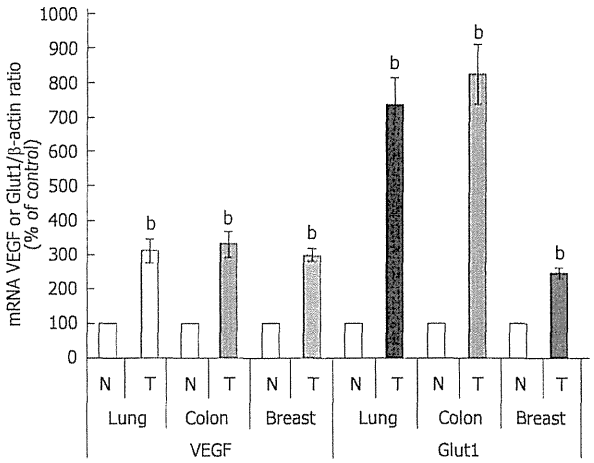

Figure 10 Quantification of ribonuclease protection assay of VEGF and Glut1 mRNA expressions in lung, colon, and breast malignant tumors (T) and corresponding non-malignant tissue counterparts (C). Values of VEGF and Glut1 mRNA expressions were normalized to 18S rRNA; n = 15-20; $^bP < 0.01$ *vs* non-malignant tissues[30,33]. PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; VEGF: Vascular endothelial growth factor.

vessel formation[78,80]. Moreover, the glycolytic activator PFKFB3 regulates stalk cell proliferation and renders endothelial cells more competitive to reach the tip[81].

The induction of different PFKFB as well as tumor angiogenesis and growth is realized not only through activation of transcription factor HIF[7,14,34,73,74,82-86]. For PFKFB3 it was shown that its transcription as well as allosteric activation is promoted by MAPK pathway[87]. Many growth factors may contribute to cancer progression, including pancreatic cancer, through induction of the expression of genes without hypoxia responsive elements[88]. Moreover, hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation and growth[89]. Recently, it was shown that clathrin heavy chain promotes growth and angiogenesis of pancreatic adenocarcinoma, which is an aggressive disease with a high mortality rate, through the regulation of HIF-1α and VEGF signaling and that hypoxia-induced pancreatic cancer cells invasion is also mediated by transcription factor HIF[85,90]. One of the key functions of clathrin heavy chain protein is to bind with the HIF-1α protein, increasing the stability of this protein and facilitating its nuclear translocation, thereby regulating the expression of VEGF. Thus, suppression of clathrin heavy chain either by shRNA or by specific antibody inhibited pancreatic adenocarcinoma growth and angiogenesis[85].

A better understanding of the impact of *PFKFB* gene networks regulation on glycolysis as well as cell cycle control, apoptosis and cell survival promises to shed light on the emerging association between PFKFB-3, PFKFB-4,

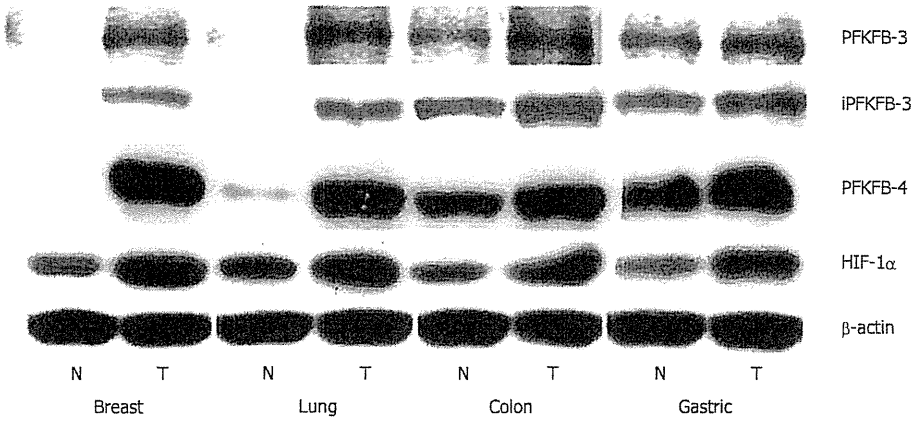

Figure 11 Representative Western blot analysis of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4, and hypoxia inducible factor-1α protein levels in breast, lung, colon, and stomach malignant tumors (T) and non-malignant (control) tissues counterparts (N) from same patients. The actin was used to ensure equal loading of the sample[30,32,33]. HIF: Hypoxia inducible factor; PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

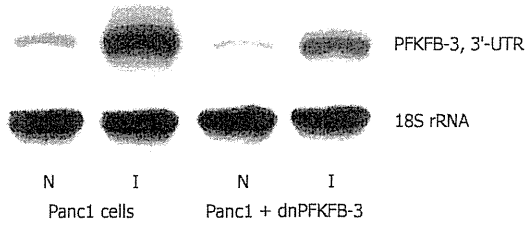

Figure 12 Representative polyacrylamide gel autoradiograph employed in a typical ribonuclease protection assay of endogenous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 mRNA in pancreatic carcinoma cell line Panc1, stable transfected by pcDNA3.1(+) vector (Panc1 cells) or by dominant/negative 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (Panc1 + dnPFKFB-3) in normoxic (N) condition and after treatment of Panc1 cells with dimethyloxalylglycine, inhibitor of prolyl hydroxylase (I; 1 mmol/L for 6 h). The 18S rRNA antisense probe was used as control of analyzed RNA quantity[89]. PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

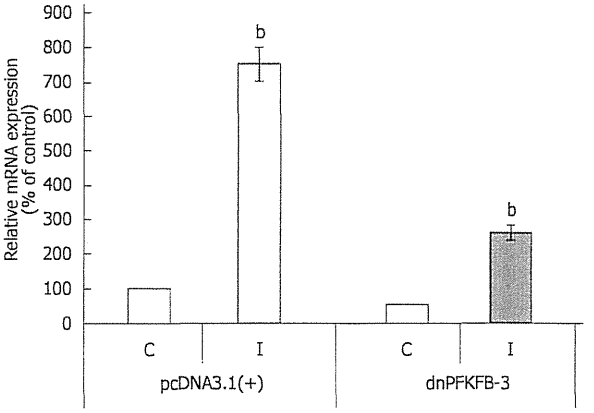

Figure 13 Quantification of ribonuclease protection assay of endogenous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 mRNA expression in pancreatic carcinoma cell line Panc1, stable transfected by pcDNA3.1(+) vector or dominant/negative 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 in normoxic (control) condition (C) and after treatment of Panc1 cells with dimethyloxalylglycine, inhibitor of prolyl hydroxylase (I). $n = 5$; $^{b}P < 0.01$ *vs* control[89]. dnPFKFB-3: Dominant/negative 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3.

cell proliferation and cancer. These provide rationale for the development of agents that selectively inhibit the PFKFB3 and PFKFB-4 enzymes as antineoplastic agents. Recently was shown that inhibition of PFKFB-3 activity suppresses glycolytic flux and tumor growth by rapid induction of apoptosis in transformed cells[91,92]. It is possible, that the stimulation of glycolysis in cancer cells results by multimodal mechanism of stress stimuli affecting PFKFB3 transcriptional regulation and kinase activation by protein phosphorylation[41]. Moreover, the glycolytic enzyme PFKFB3 regulates autophagy and inhibition of PFKFB3 in tumor cells would induce autophagy as a pro-survival mechanism and inhibitors of autophagy could increase the anti-tumor effects of PFKFB3 inhibitors[93,94].

Previously, we have shown that suppression of PFKFB-3 and PFKFB-4 expression ($P < 0.05$-0.01) in pancreatic Panc1 and PSN-1 cancer cells by dominant/negative technology also decreases VEGF expression ($P < 0.05$) and proliferation rate ($P < 0.05$) of these cells (Figures 12-14)[95]. For this aim we introduced point mutation in ATP-binding domain of 6-phosphofructo-2-kinase part of PFKFB-3 as well as PFKFB-4 cDNA and cloned in pcDNA3.1 vector. Pancreatic Panc1 and PSN-1 cancer cells were stable transfected with dnPFKFB-3 and dnPFKFB-4 constructs and studied the expression of endogenous PFKFB-3, PFKFB-4, and VEGF-A mRNAs in these cells as well as its proliferation rate. It was shown that both dnPFKFB-3 and dnPFKFB-4 suppress the expression of endogenous PFKFB-3, PFKFB-4, and VEGF-A mRNAs as well as cell proliferation in pancreatic cancer cells[95]. Results of this investigation agree with the role of PFKFB3-driven glycolysis in vessel sprouting[78,80,81] and demonstrate possibility to apply the dominant-negative strategy for suppression of tumor cells glycolysis and proliferation through reduction of the expression of PFKFB-3 and PFKFB-4 enzymes.

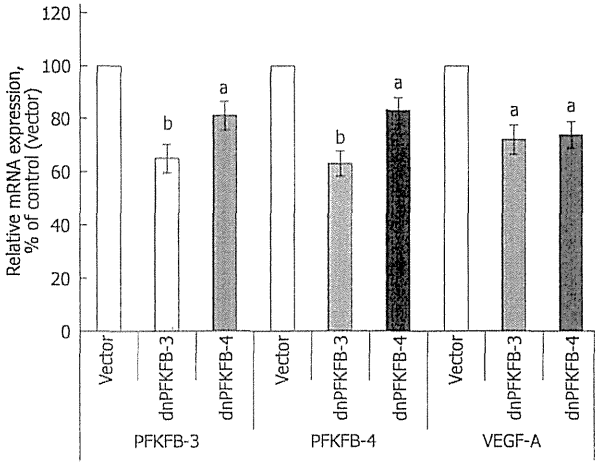

Figure 14 Endogenous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4, and vascular endothelial growth factor mRNA expressions in pancreatic carcinoma cell line PSN-1, stable transfected with pcDNA3.1(+) vector (Vector), dominant/negative 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, and dominant/negative 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4, measured by ribonuclease protection assay. $n = 5$; $^aP < 0.05$ *vs* control; $^bP < 0.01$ *vs* control[89]. PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; VEGF: Vascular endothelial growth factor.

Thus, PFKFB-4 as well as PFKFB-3 participates in the regulation of glycolysis and promotes tumor growth and survival of cancer cells[34,52-54,91]. Moreover, targeting PFKFB3 by specific inhibitors is a perspective therapeutic strategy against cancer[91,92]. It was also shown that blocking of PFKFB4 induces reactive oxygen species and cancer cell death and that targeting PFKFB4 may also, therefore, present new therapeutic opportunities[52,53].

CONCLUSION

PFKFB-3 and PFKFB-4 play a significant role in the regulation of glycolysis and cancer growth by inducing cell proliferation and survival. The *PFKFB-4* and *PFKFB-3* genes have active HIF-responsible elements and its expression is increased in different malignant tumors and strongly induced in various cancer cell lines under hypoxia. The expression of *PFKFB-4* and *PFKFB-3* genes as well as its hypoxia responsibility was also shown for pancreatic (Panc1, PSN-1 and MIA Paca-2) and gastric (MKN45 and NUGC3) cancer cell lines. The highest constitutive expression level of PFKFB-4 protein was found in the NUGC3 adenocarcinoma cells and lowest in the Panc1 cells, with the maximum response to hypoxia in the pancreatic adenocarcinoma cells. Moreover, the hypoxia responsiveness of PFKFB-3 and PFKFB-4 mRNA expressions in pancreatic and gastric cancer cell lines is correlated with the increased level of HIF-1α protein and enhanced expression of *VEGF* and *GLUT1* genes. At the same time, the basal expression level of HIF-1α as well as HIF-2α mRNA and their hypoxia responsiveness are variable in these cancer cells as well as in many other cancer cell lines. The overexpression of different PFKFB was also shown in gastric, colon, lung, and breast cancer tissues. It is interesting to note that the protein level of PFKFB-4 in colon and gastric malignant tumors as well as non-malignant tissue counterparts was greater as compared to the variant 3 of PFKFB. Both PFKFB-4 and PFKFB-3 isoenzymes are overexpressed in different malignant tumors and undergo changes in their metabolism that contribute to the proliferation and survival of cancer cells. A better understanding of the impact of *PFKFB* gene networks regulation on cell cycle control and glycolysis as well as nutrient balance at the molecular, cellular and system levels promises to shed light on the emerging association between PFKFB-3, PFKFB-4, cell proliferation and cancer. These provide rationale for the development of agents that selectively inhibit the PFKFB3 and PFKFB-4 enzymes as antineoplastic agents.

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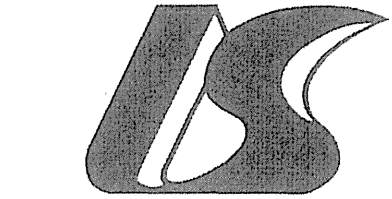
P- Reviewer: Ma DX, Paydas S **S- Editor:** Qi Y **L- Editor:** A
E- Editor: Ma S



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ISSN 1007-9327

nature COMMUNICATIONS

ARTICLE

Received 20 Aug 2014 | Accepted 30 Oct 2014 | Published 10 Dec 2014 DOI: 10.1038/ncomms6715 OPEN

Oncogenic Kit signals on endolysosomes and endoplasmic reticulum are essential for neoplastic mast cell proliferation

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Kit is a receptor-type tyrosine kinase found on the plasma membrane. It can transform mast cells through activating mutations. Here, we show that a mutant Kit from neoplastic mast cells from mice, Kit(D814Y), is permanently active and allows cells to proliferate autonomously. It does so by activating two signalling pathways from different intracellular compartments. Mutant Kit from the cell surface accumulates on endolysosomes through clathrin-mediated endocytosis, which requires Kit's kinase activity. Kit(D814Y) is constitutively associated with phosphatidylinositol 3-kinase, but the complex activates Akt only on the cytoplasmic surface of endolysosomes. It resists destruction because it is under-ubiquitinated. Kit(D814Y) also appears in the endoplasmic reticulum soon after biosynthesis, and there, can activate STAT5 aberrantly. These mechanisms of oncogenic signalling are also seen in rat and human mast cell leukemia cells. Thus, oncogenic Kit signalling occurs from different intracellular compartments, and the mutation acts by altering Kit trafficking as well as activation.

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The *Kit* proto-oncogene encodes a type III receptor tyrosine kinase (RTK), a class of proteins that includes platelet-derived growth factor receptors (PDGFR), Fms, and Fms-like tyrosine kinase 3 (Flt3)[1–3]. Kit is expressed on mast cells, interstitial cells of Cajal, haematopoietic cells, germ cells and melanocytes[4]. On stimulation with stem cell factor (SCF), Kit triggers many signalling events at the plasma membrane (PM), resulting in cell proliferation, survival and differentiation[5].

Kit is composed of five *N*-glycosylated immunoglobulin domains in the N-terminal extracellular portion that bind SCF, as well as a transmembrane domain, and an intracellular C-terminal tyrosine kinase domain[6]. The binding of SCF autophosphorylates Kit on specific tyrosine residues. Kit then binds to other cytoplasmic enzymes containing Src hohomogy-2 (SH2) domain, and this complex phosphorylates other proteins[3–6]. This process activates the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) pathway, the Ras-Raf-Erk cascade and Src kinases, which regulate cell growth, gene expression and cytoskeletal structures[7–10].

In many mast cell neoplasms and gastrointestinal stromal tumours, Kit has gain-of-function mutations, causing permanent, ligand-independent activation of the receptor[11–14]. In human neoplastic mast cell disorders such as mastocytosis and mast cell leukemia, Kit often has an Asp816Val substitution (D816V) in the kinase domain[12,13]. Similar mutations are also found in rat mast cell leukemia (RBL-2H3; D817Y) and a mouse mastocytoma (P815; D814Y)[13]. Their permanent activation of the PI3K-Akt pathway causes neoplastic transformation. When mutant Kit activates the PI3K-Akt pathway, this suppresses apoptotic proteins such as Bim, resulting in cell survival[15–18]. When mutant Kit phosphorylates signal transduction and activator of transcription (STAT) proteins, they move from the cytoplasm to the nucleus and cause transcription of proto-oncogenes such as *c-myc*[19–21].

Cancer-causing mutants of RTKs, such as Met(D1246N) and Flt3-internal tandem duplication (Flt3-ITD), can cause incorrect signalling not just from the PM, but also from intracellular compartments, because their activated kinase domain is always exposed[22–26]. These mutations also change the trafficking and degradation of the receptors, because they change glycosylation and ubiquitination, and receptors accumulate in the wrong compartments[22–27]. Signalling by oncogenic RTKs on intracellular compartments has been implicated in their transforming ability, but the mechanism of signalling by mutant Kit is not fully understood.

We recently established two mast cell lines, RCM and R, from mouse splenocytes. RCM cells proliferate without cytokines, generate tumours *in vivo* and express a mutant Kit, Kit(D814Y). R cells require cytokines to proliferate and express wild-type Kit (Kit(wt)). This scenario allows us to compare Kit(wt) with Kit(D814Y) in an identical cellular background.

To explore how Kit(D814Y) transduces oncogenic signals, we studied what pathways it activates, from various subcellular compartments, using immunofluorescence confocal microscopy, vesicle immunoprecipitation and chemical inhibition of intracellular trafficking.

In mice cells, Kit(D814Y) from the PM accumulates on endolysosomes through clathrin-mediated endocytosis (CME); this occurs in a kinase activity-dependent manner. It then forms a complex with PI3K, and activates Akt, leading to cell proliferation. Also, soon after Kit(D814Y) is synthesized, it appears in the endoplasmic reticulum (ER), where it causes oncogenic activation of STAT5. Two other mast cell lines, HMC-1 and RBL-2H3, from humans and rats, gave similar results. Our findings demonstrate that Kit signalling from subcellular compartments is necessary for the neoplastic proliferation of mast cells.

Results

Kit^{D814Y} causes autonomous proliferation of mouse RCM cells. We recently established two mast cell lines from mouse splenocytes, RCM cells and R cells, bearing c-Kit and FcεRI. RCM cells grow without cytokines and develop tumours *in vivo* (Fig. 1a). These cells show constitutively tyrosine-phosphorylated 145- and 160-kDa proteins, identified as the Kit tyrosine kinase (Fig. 1b,c; see also Fig. 4b). Furthermore, Kit's kinase domain has an Asp814Tyr (D814Y) mutation (Fig. 1d), which keeps the kinase permanently active[12,13,21].

Immunoprecipitation assays confirmed that Kit(wt) in R cells and pt18 cells[28] was activated in a ligand-dependent manner, whereas Kit(D814Y) was phosphorylated and associated with the PI3K p85 subunit without SCF (Fig. 1e; see also Fig. 3i), and thus was permanently active. Glutathione *S*-transferase-pulldown (GST-pulldown) assays showed that the C-terminal or N-terminal SH2 domains of p85 (SH2c or SH2n) were associated with SCF-stimulated Kit(wt) and Kit(D814Y) (Fig. 1f). Next, we treated RCM cells with PKC412, an inhibitor of Kit tyrosine kinase[29]. Similar to previous reports[16,29], PKC412 inhibited the phosphorylation of Kit(D814Y) and p85's association with Kit(D814Y), and also inhibited cell proliferation in a dose-dependent manner (Fig. 1f,g and Supplementary Fig. 1; see also Fig. 4c). We next performed RNA interference experiments to suppress Kit(D814Y). Western blotting showed 80–85% less Kit(D814Y) protein after transfection with Kit1 and Kit2 small interfering RNAs (siRNAs) for 20 h (Fig. 1h, left). Knockdown of Kit(D814Y) also suppressed cell proliferation (Fig. 1h, right). Thus, the kinase activity is required for autonomous proliferation.

Kit^{D814Y} localizes to endolysosomes. Kit mutants exogenously expressed in CHO, NIH3T3 or HEK293 cells are found mainly in the Golgi apparatus or ER in an immaturely glycosylated form[30–32]. To investigate Kit's glycosylation state, we treated Kit(D814Y) from RCM cells with endoglycosidase H, which digests immature high-mannose forms, but not mature complex-glycosylated forms. Figure 2a shows that most Kit(D814Y), like Kit(wt), was present in a complex-glycosylated form. Kit(wt) and Kit(D814Y) both shifted to a non-glycosylated form following the complete digestion of N-linked glycans by peptide-N-glycosidase F.

Next, we investigated the subcellular localization of Kit in paraformaldehyde-fixed (PFA-fixed) cells by immunofluorescence confocal microscopy. Consistent with previous reports[9,33,34], in pt18 and R cells, most Kit(wt) was at the PM, whereas in RCM cells Kit(D814Y) was mainly on vesicular structures (Fig. 2b). We investigated these structures and calculated Pearson's correlation coefficients (Pearson's R) between Kit and various markers: calnexin (ER), TGN46 (Golgi), EEA1, CD63 (endosome), LAMP1 and cathepsin D (endolysosome). As cathepsin D and CD63 could not be detected by immunofluorescence in PFA-fixed cells, we fixed RCM cells with methanol to visualize those proteins. Although Kit's distributions were largely similar between the different methods (Supplementary Fig. 2a,b), methanol caused fluorescence loss in the cytosol and nucleus (compare Fig. 2c with 2d). Kit(D814Y) co-localized with LAMP1 significantly, and with cathepsin D-positive vesicles somewhat, suggesting it is mainly present on endolysosomes (Fig. 2c,d,g). Moreover, green fluorescent protein-tagged (GFP-tagged) Kit(D814Y) was co-localized with LAMP1, and with fluorescent dextran, which is incorporated into endocytic compartments[35] (Fig. 2e). Kit(D814Y) surrounded cathepsin D as well as dextran (Fig. 2d,e, magnified images), indicating that the kinase domain is exposed on the cytoplasmic surface of endolysosomes.

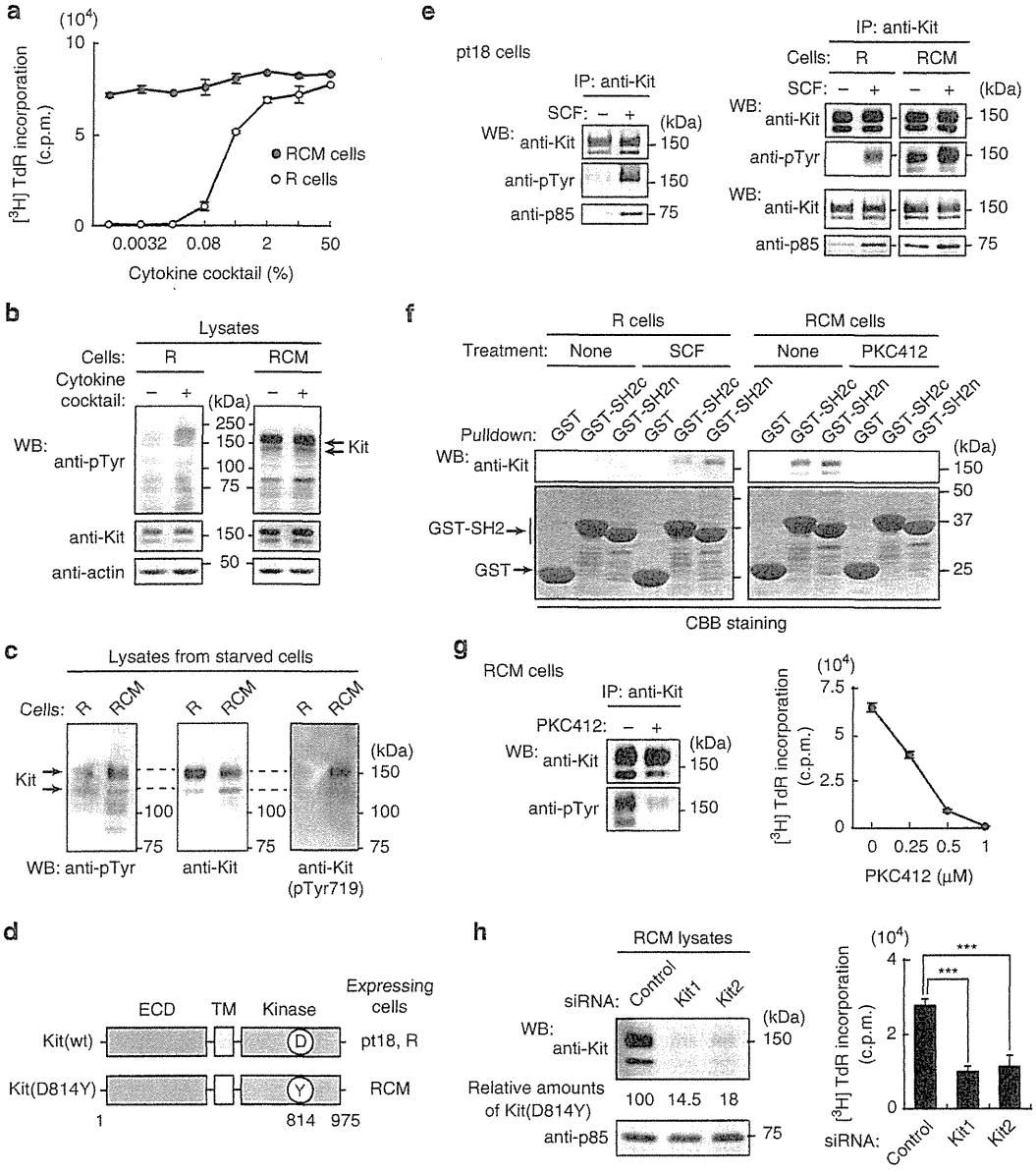


Figure 1 | Kit(D814Y) is essential for autonomous proliferation of mouse RCM cells. (**a**) RCM and R cells were cultured in the indicated cytokine cocktail for 48 h. Proliferation was assessed by [^{3}H]-thymidine incorporation into R (open circles) and RCM cells (filled circles). Results (c.p.m.) are means ± s.d. ($n=3$). NB: RCM cells proliferated without the cytokine cocktail. (**b**,**c**) Expression of constitutively phosphorylated Kit in RCM cells. Starved R and RCM cells were treated with the cytokine cocktail for 5 min. Lysates were immunoblotted with anti-phosphotyrosine (anti-pTyr), anti-Kit, anti-Kit(pTyr719) and anti-actin. Arrows indicate constitutively tyrosine-phosphorylated protein corresponding to Kit in RCM cells. (**d**) Schematic representations of normal Kit and Kit(D814Y) showing the extracellular domain (ECD), the transmembrane domain (TM), the kinase domain, Asp814 in the kinase domain (D in black) and the tyrosine mutation at 814 (Y in red). (**e**) Constitutive activation of Kit(D814Y). Starved pt18, R, and RCM cells were treated with 50 ng ml^{-1} SCF for 5 min. Kit was immunoprecipitated then immunoblotted. (**f**) GST-pulldown. RCM or R cells were treated with 1 μM PKC412 (Kit kinase inhibitor) for 24 h or 50 ng ml^{-1} SCF for 5 min, respectively. Kit was pulled down with GST-SH2c or GST-SH2n, and amounts of Kit pulled down was assayed by immunoblotting. GST proteins were visualized by Coomassie staining. (**g**) Effect of PKC412 on the kinase activity of Kit(D814Y). RCM cells treated with 1 μM PKC412 were cultured for 24 h. Anti-Kit immunoprecipitates were immunoblotted. The graph shows the levels of [^{3}H]-thymidine incorporation into RCM cells at the indicated PKC412 concentrations. Results (c.p.m.) are means ± s.d. ($n=3$). (**h**) RCM cells were transfected with control siRNA or Kit siRNAs (Kit1 or Kit2) and cultured for 20 h. Lysates were immunoblotted with anti-Kit and anti-p85. Amounts of Kit(D814Y) are expressed relative to control cell lysate, after normalization with p85. The graph shows the levels of [^{3}H]-thymidine incorporation into RCM cells. Results (c.p.m.) are means ± s.d. ($n=3$). Data were subjected to one-way ANOVA with Dunnett's multiple comparison *post-hoc* test. ***$P<0.001$.

Next, we compared the distribution of Kit(D814Y) and recycling endosomes using cholera toxin subunit B (CTXB; a recycling endosome marker)[36]. Kit's localization to recycling endosomes was significantly less than that to endolysosomes (Fig. 2f,g), and a second marker of recycling endosomes, Rab11, confirmed this (Supplementary Fig. 2c). These results indicate that Kit(D814Y) traffics to endolysosomes rather than recycling to the PM.

Kit^{D814Y} moves to endolysosomes by kinase-mediated endocytosis. In RCM cells treated for 24 h with the kinase inhibitor PKC412, there was more Kit(D814Y) at the PM and less in endolysosomes (Fig. 3a). Treatment did not cause accumulation of Kit(D814Y) on either ER or Golgi. Following removal of inhibitor, Kit(D814Y) moved within 60 min from the PM, via endosomes, to endolysosomes (Supplementary Fig. 3a). These results suggest that Kit's kinase activity is needed for endocytosis, but not for biosynthetic traffic to the PM. Figure 3b shows that 24-h treatment with an inhibitor of lysosomal proteases (NH_4Cl) resulted in retention of Kit(D814Y) inside RCM cells, indicating that endocytosed Kit(D814Y) is targeted to lysosomes, and subsequently degraded. In the presence of cycloheximide, an inhibitor of protein synthesis, Kit(D814Y) was degraded within 5 h, whereas transferrin receptor (TfR), which undergoes recycling, was unchanged (Fig. 3c), suggesting that Kit traffics constitutively to lysosomes.

In endocytosis of growth factor receptors, for example, of epidermal growth factor (EGF) and transforming growth factor β (TGFβ), clathrin plays a critical role in their stability[37,38]. Accordingly, receptor-mediated endocytosis is divided into two major categories: non-clathrin endocytosis (NCE) and CME[37–39]. To study the role of clathrin in Kit(D814Y) endocytosis, we treated RCM cells with hypertonic sucrose, thereby blocking the formation of clathrin-coated pits[40]. Within 3 h, this reduced the protein levels of mature Kit(D814Y) and TfR without affecting the cytosolic protein p85 (Fig. 3d,e). Similar results were obtained from RCM cells treated with pitstop2, a selective inhibitor of CME[41] (Fig. 3f). To confirm that, with CME blocked, Kit and TfR were being processed by NCE, we treated cells for 3 h with pitstop2 plus filipin, or with sucrose plus filipin, which blocks NCE by disrupting lipid rafts[37,42]. Kit(D814Y) and TfR were then found at the PM, presumably protected from rapid degradation (Fig. 3g,h and Supplementary Fig. 3b). In cells treated with filipin alone, the protein levels and localization of Kit(D814Y) were unaffected (Fig. 3g,h and Supplementary Fig. 3b,c). Similar to sucrose and pitstop2, knockdown of the assembly polypeptide-2 α subunit (AP2α), which is required for CME[39], decreased the protein levels of Kit(D814Y) and TfR (Supplementary Fig. 3d). Thus, the major pathway for Kit(D814Y) endocytosis is CME.

In NCE, ubiquitination of receptors and other endocytic cargo is important for association with the ESCRT (endosomal sorting complexes required for transport) machinery, for rapid incorporation into lysosomes and for degradation[39,43–45]. As Kit(D814Y) mainly undergoes CME not NCE, we hypothesized that it may not be fully ubiquitinated. When Kit(wt) was stimulated with SCF, in pt18 and R cells it became fully ubiquitinated, and was degraded within 30 min (Figs 3i and 6g; see also Fig. 3l), consistent with previous reports[31–34,45]. In sharp contrast, in RCM cells ubiquitination of Kit(D814Y) was much lower, regardless of the presence or absence of SCF. When CME was blocked, Kit(D814Y) became substantially ubiquitinated (Fig. 3j), presumably as it then trafficked via NCE. These results suggest that, in RCM cells, most Kit(D814Y) undergoes CME depending on its kinase activity, and accumulates on the cytoplasmic surface of endolysosomes, but not in a fully ubiquitinated state. In support of this, degradation of Kit(D814Y) was significantly slower than that of activated Kit(wt) in the presence of cycloheximide (Fig. 3k).

Next, to investigate the mechanism of endosome-to-lysosome trafficking of Kit, we knocked down tumour-susceptibility gene 101 (Tsg101), a component of ESCRT[43,44,46]. Figure 3l shows that in R cells treated with SCF for 30 min, Tsg101 knockdown inhibited the degradation of Kit(wt) but not of TfR (see also Supplementary Fig. 5e). These results suggest that Kit(wt) is incorporated into lysosomes in a manner that is dependent on ESCRT, similar to EGFR[39,43,46]. By contrast, knockdown decreased Kit(D814Y) in RCM cells (Fig. 3m). As ESCRT depletion enhances ESCRT-independent transport into lysosomes[46], our results indicate that Kit(D814Y) is incorporated into lysosomes in a manner that is independent of ESCRT.

Activation of Akt and STAT5 is necessary for proliferation of RCM cells. Neoplastic transformation of mast cells involves phosphorylation and activation of Akt, STAT proteins, Erk1/2 and JAKs[4,13,15–21]. In RCM cells, Akt and STAT5, but not Erk1/2, STAT3 and JAK2, were constitutively phosphorylated (Supplementary Fig. 4a,b).

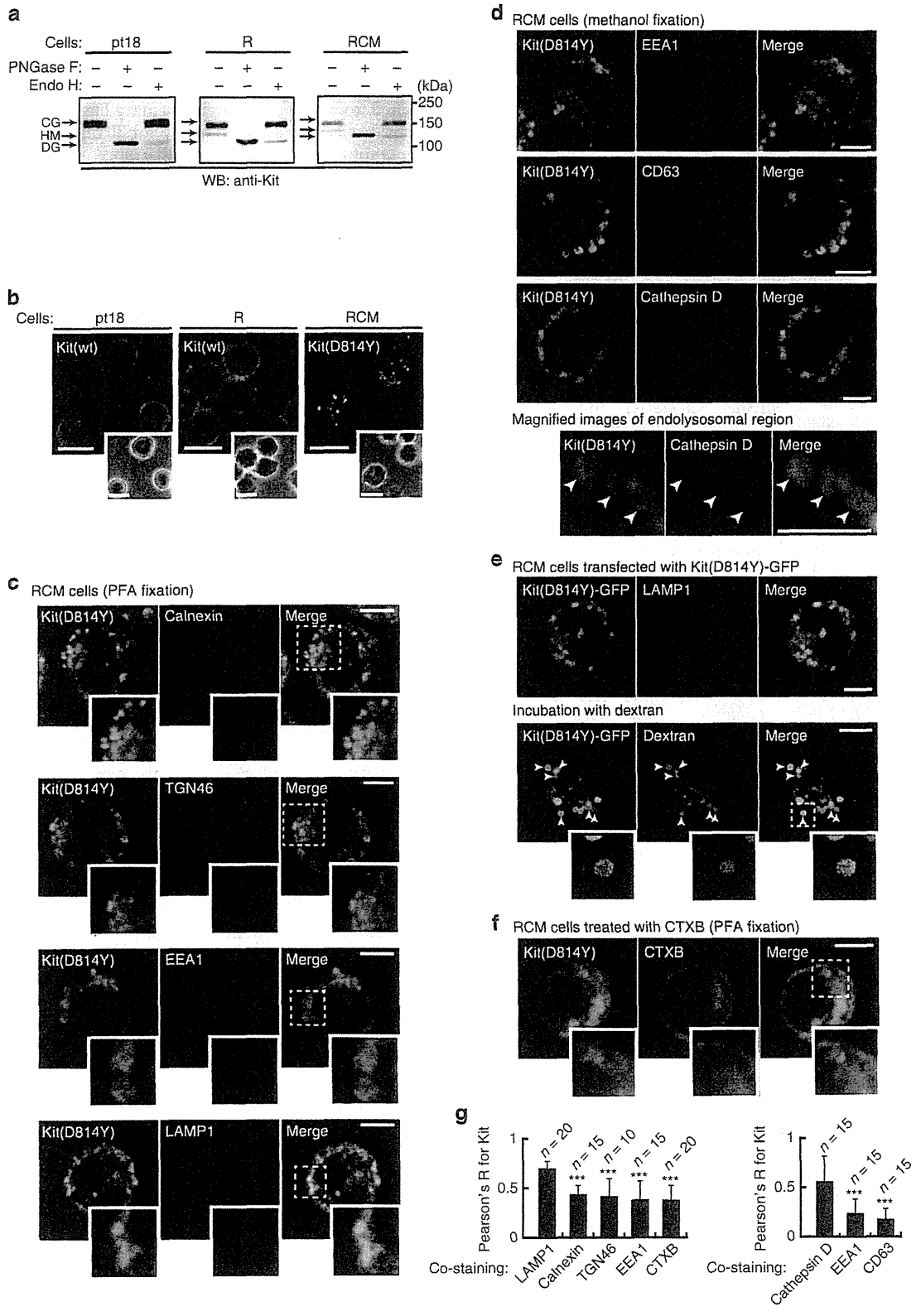
To investigate whether it was Kit(D814Y) that had activated Akt and STAT5, we inhibited Kit(D814Y) by PKC412 or siRNA-mediated knockdown. Both treatments inhibited the activation of Akt and STAT5 (Fig. 4a,b). We further found that the PI3K p85 subunit was co-immunoprecipitated with Kit(D814Y), and that PKC412 blocked this association (Fig. 4c; see also Fig. 1f, right). In this cell line, a PI3K inhibitor LY294002 suppressed Akt activation in a dose-dependent manner (Fig. 4d), as reported previously[7,19,47]. Thus, Kit(D814Y) activates Akt through association with PI3K.

Next, we tested whether Akt activation was required for autonomous proliferation. In RCM cells, the inhibitor Akti repressed the activation of Akt without affecting STAT5. It also caused a dose-dependent suppression of cell proliferation (Fig. 4e). Although the PI3K-Akt pathway has a role in RTK trafficking[35,48,49], no redistribution was seen here on treatment with either LY294002 or Akti (Fig. 4f). This shows that the PI3K-

Figure 2 | Kit(D814Y) localizes to endolysosomes in mouse RCM cells. (**a**) Glycosylation of Kit(D814Y). Lysates from pt18, R and RCM cells were treated with peptide N-glycosidase F (PNGase F) or endoglycosidase H (endo H), then immunoblotted with anti-Kit. CG, complex-glycosylated form; DG, deglycosylated form; HM, high mannose form. NB: most Kit(D814Y) was a mature complex-glycosylated form. (**b**) Localization of normal Kit and Kit(D814Y). RCM, R or pt18 cells were fixed with PFA and stained with anti-Kit. Phase contrast images are shown. Bars, 10 μm. NB: normal Kit accumulated at the PM; Kit(D814Y) at vesicular structures. (**c–g**) Localization of Kit(D814Y) to endolysosomes. (**c**) PFA-fixed or (**d**) methanol-fixed RCM cells were double-stained with anti-Kit (green) in conjunction with the indicated antibody (red). Insets show the magnified images of the boxed area. Representative images of Kit-positive endolysosomes containing cathepsin D are shown. Bars, 5 μm. CD63, cluster of differentiation 63; EEA1, early endosome antigen-1; LAMP1, lysosome-associated membrane protein-1; TGN46, *trans*-Golgi network 46. (**e**) Endolysosomal localization of Kit(D814Y)-GFP. RCM cells transfected with Kit(D814Y)-GFP. (*Upper panels*) Cells were stained with anti-LAMP1 (*endolysosome marker; red*). (*Lower panels*) For visualizing endocytic compartments, cells were incubated with 1 mg ml^{-1} AF647-dextran for 1 h. Expressed protein and dextran were visualized by GFP (green) and AF647 fluorescence (blue). Magnified images of the boxed area are shown. Arrowheads indicate Kit-positive endolysosomes that contain dextran. Bars, 5 μm. NB: Kit(D814Y) surrounded cathepsin D and dextran. (**f**) For visualizing recycling endosomes, RCM cells were incubated for 1 h with 5 μg ml^{-1} AF647-cholera toxin subunit B (AF647-CTXB) and stained with anti-Kit (green). The toxin was visualized with AF647 fluorescence (blue). Magnified images of the boxed area are shown. Bar, 5 μm. (**g**) Pearson's coefficients (Pearson's R) were calculated by intensity correlation analysis of Kit(D814Y) versus organelle markers. Results are means ± s.d. ($n = 10 \sim 20$). Data were subjected to one-way ANOVA with Dunnett's multiple comparison *post-hoc* test. ***$P < 0.001$.

Akt pathway has an essential role in cell proliferation but does not influence Kit(D814Y) trafficking.

We next suppressed STAT5 function by utilizing STAT5 inhibitor (STAT5i) or knockdown. Both treatments inhibited the proliferation of RCM cells (Fig. 4g,h). Knockdown had no effect on the activation of Akt. On the contrary, proliferation was unaffected by Erk1/2 inhibition with U0126 (Supplementary Fig. 4c). Taken together, these results suggest that Kit(D814Y) constitutively activates the PI3K-Akt pathway and STAT5, and that these activations are essential for autonomous proliferation.



Kit^{D814Y} activates Akt through PI3K only on endolysosomes. Activated RTKs, such as EGFR, Met, and PDGFR can transduce signals not only on the cell's outer membrane, but also on endocytic compartments[23,24,49–52]. To examine whether endolysosomes serve as a platform for oncogenic Kit signalling, we purified endolysosomes from RCM cells by immunoprecipitation, using an anti-LAMP1 antibody. Figure 5a shows that Kit(D814Y), Akt and p85, but not STAT5, were found in the endolysosomal fraction. As active Akt is bound to phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$), which is generated by PI3K, through its pleckstrin homology (PH) domain[3,53], it is likely that Akt and Kit(D814Y)-PI3K become associated in endolysosomes. To test this hypothesis, we investigated whether $PI(3,4,5)P_3$ was generated on endolysosomes, by expression of a GFP-tagged Akt PH domain (PH-GFP)[54]. In RCM cells most PH-GFP was on vesicles but not at the PM, whereas PH(R25C)-GFP, a non-$PI(3,4,5)P_3$-binding mutant, was in the cytoplasm (Fig. 5b). As PH-GFP could not be detected after immunofluorescence, we directly visualized endocytic compartments by fluorescent dextran. Figure 5c shows that they were significantly co-localized with PH-GFP. Furthermore, phosphorylated Akt was found on vesicles where Kit localized (Fig. 5d). These results suggest that Akt is activated by Kit(D814Y) predominantly on endolysosomes.

Next, we investigated whether Kit(D814Y) must localize to endolysosomes to activate Akt. We used bafilomycin A1 (BafA1), which blocks endosomal trafficking without affecting internalization and recycling[44,55]. After 24 h, Kit(D814Y) was rare in endolysosomes (LAMP1-positive), but common in endosomes (EEA1-positive) (Fig. 5e and Supplementary Fig. 5a), indicating that trafficking from endosomes to endolysosomes is blocked. Figure 5f shows that BafA1 treatment also prevented Kit(D814Y) degradation. BafA1 did not affect Kit(D814Y)'s kinase activity (Fig. 5f, left). It reduced activation of Akt but not of STAT5 (Fig. 5f). BafA1 did not suppress the association of Kit(D814Y) with p85 and did not affect Kit phosphorylation at Tyr719 (Fig. 5f, left and Supplementary Fig. 5b), a binding site for p85 (refs 7,8,53). Therefore, Kit(D814Y) presumably associates with PI3K before reaching endolysosomes. Treatment with NH_4Cl, (inhibits lysosomal proteases) for 24 h preserved Kit(D814Y) from degradation, but did not affect its localization to endolysosomes (Fig. 5e, bottom). In contrast to BafA1, NH_4Cl had no effect on the activation of Akt (Fig. 5f, right), suggesting that Kit must localize to endolysosomes to activate Akt. In support of this conclusion, when Kit(D814Y) trafficking to endolysosomes was blocked by BafA1, p85 and Akt did not co-fractionate with the endolysosomal membrane (Supplementary Fig. 5c).

To examine whether the inhibition of Akt by BafA1 resulted from apoptosis, we performed immunoblotting for cleaved caspase-3, an apoptosis marker[20]. Figure 5g shows that BafA1 treatment for 24 h induced cleavage of caspase-3, but treatment for 3 h did not. The treatment for 3 h also inhibited Akt (Fig. 5g), indicating that the effect of BafA1 on Akt activity resulted from inhibition of Kit trafficking, but not from apoptosis.

Kit(wt) is also transported through the endocytic pathway after SCF binding[33,34]. To examine whether Kit(wt) also activated a PI3K-Akt pathway on endolysosomes, we treated R cells with BafA1 for 3 h and then stimulated them with SCF. Akt remained active up to 60 min after SCF stimulation, regardless of the presence or absence of BafA1 (Fig. 5h). Similar results were obtained from pt18 cells (Supplementary Fig. 5d). Figure 5i shows that in R cells stimulated with SCF for 30 min, p85 and Akt were absent from endolysosomes. Furthermore, in SCF-stimulated R cells, phosphorylated Akt and PH-GFP were not detected in endolysosomal vesicles (Fig. 5j,k). Also, accumulation of Kit(wt) on endosomes following Tsg101 knockdown did not activate Akt (Supplementary Fig. 5e), suggesting that Kit(wt) activates the PI3K-Akt pathway transiently, presumably when bound to the PM. However, we could not exclude the possibility that Kit(wt) activates Akt soon after endocytosis.

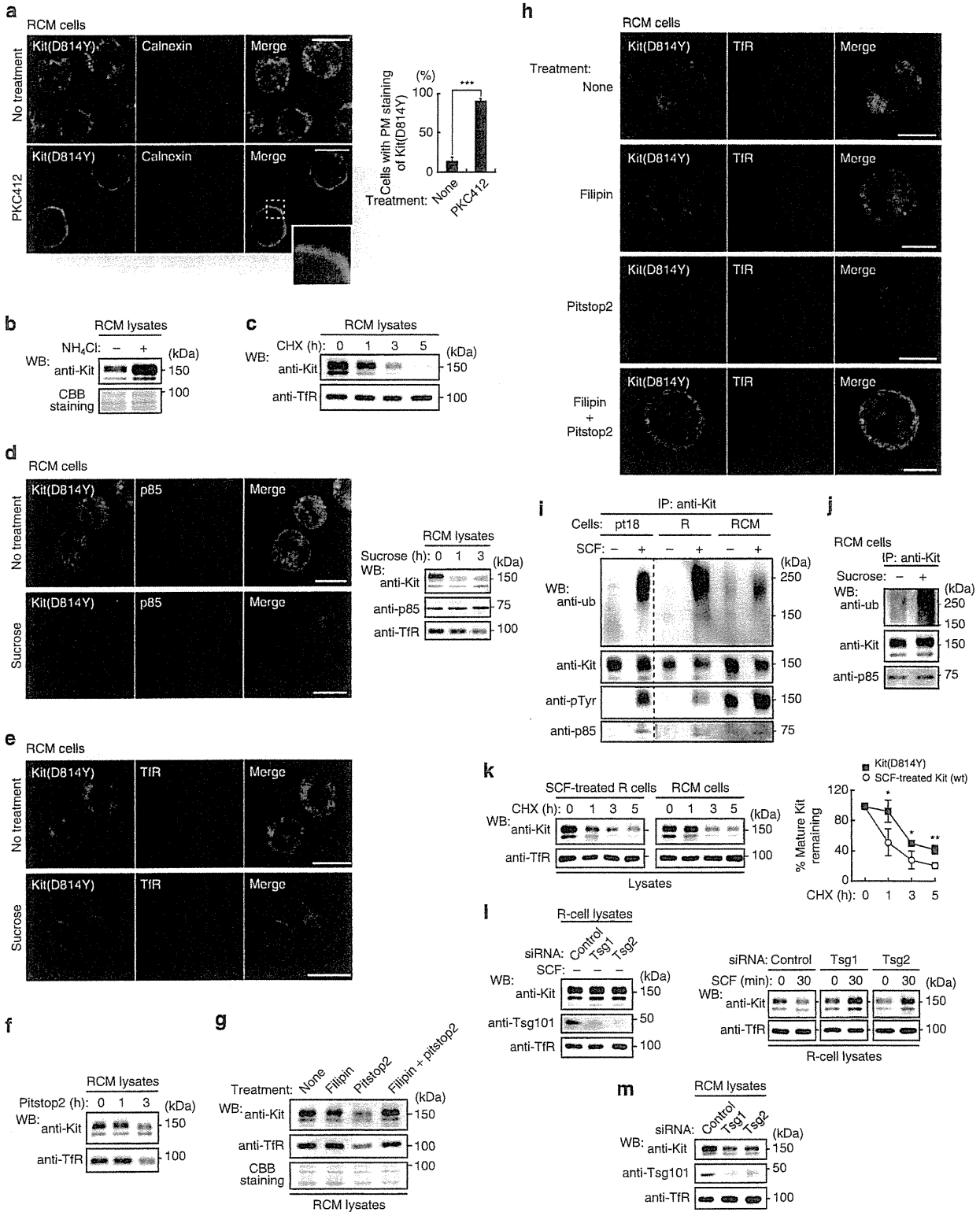
Partially glycosylated Kit^{D814Y} on the ER activates STAT5. Cytochalasin D, an inhibitor of endocytosis through actin depolymerization[35,36], prevented the activation of Akt without influencing Kit(D814Y)'s kinase activity and p85's association with Kit(D814Y), but the treatment did not affect STAT5 activity (Supplementary Fig. 5b,f). These results suggest that Kit(D814Y) activates STAT5 from a different subcellular compartment. Thus, we determined where in the cell Kit(D814Y) activates STAT5. First, we examined whether exocytic transport of Kit(D814Y) from the Golgi apparatus towards the PM was required. We treated RCM cells with monensin, which inhibits export from the Golgi by blocking intra-Golgi transport[56]. After 24-h treatment, Kit(D814Y) co-localized with the Golgi marker GM130 (Fig. 6a). Figure 6b shows that monensin treatment resulted in expression of partially glycosylated Kit(D814Y). Monensin had no effect on the autophosphorylation of Kit(D814Y), or on p85's association with Kit(D814Y) (Fig. 6b, left and Supplementary Fig. 5b). Moreover, monensin abolished Akt activation, presumably because Kit could no longer locate to endolysosomes. STAT5 activation was not affected (Fig. 6b, right), suggesting that Kit activates STAT5 on the Golgi and/or ER, not at the PM.

Next, we used tunicamycin to inhibit ER export of Kit(D814Y) by blocking protein glycosylation[26,57]. Tunicamycin suppressed trafficking from ER to Golgi, and Kit(D814Y) accumulated on the ER (Fig. 6c, middle panels). Tunicamycin-treated cells also expressed non-glycosylated Kit(D814Y) (Fig. 6d, top). Like monensin, tunicamycin did not stop the autophosphorylation of Kit(D814Y) or p85's association with Kit(D814Y), but did

Figure 3 | Kit(D814Y) traffics from the plasma membrane to endolysosomes through its kinase activity and clathrin-mediated endocytosis. (**a**) RCM cells cultured in the presence of 1 μM PKC412 (Kit inhibitor) for 24 h and stained with anti-Kit (green) and anti-calnexin (ER marker; red). Bars, 10 μm. The graph shows the percentage of cells with predominant PM localization of Kit(D814Y). Results (%) represent means ± s.d. from three independent experiments ($n>200$ cells). ***$P<0.001$, Student's *t*-test. (**b**,**c**) RCM cells were cultured in the presence of (**b**) 20 mM NH_4Cl (inhibits lysosomal proteases) for 24 h or (**c**) 200 μg ml^{-1} cycloheximide (inhibits protein synthesis) for the indicated periods, then immunoblotted. Total protein levels were confirmed by Coomassie staining. (**d**–**f**) Inhibition of clathrin-mediated endocytosis by sucrose or pitstop2. RCM cells were treated with (**d**,**e**) 0.45 M sucrose or (**f**) 50 μM pitstop2. After 3 h, cells were stained with antibody. Bars, 10 μm. (**f**) Immunoblots. (**g**,**h**) Inhibition of non-clathrin endocytosis by filipin. RCM cells treated with 1 μg ml^{-1} filipin and/or 50 μM pitstop2 were cultured for 3 h. (**g**) Lysates were immunoblotted. Total protein levels were confirmed by Coomassie staining. (**h**) Cells were stained with anti-Kit (cyan) and anti-TfR (red). Bars, 5 μm. (**i**,**j**) Ubiquitination of Kit(D814Y). Starved pt18, R and RCM cells were treated with 50 ng ml^{-1} SCF (**i**) or 0.45 M sucrose (**j**) for 5 min. Anti-Kit immunoprecipitates were immunoblotted. Ub: ubiquitin. (**k**) RCM or SCF-treated R cells were cultured in 200 μg ml^{-1} cycloheximide (CHX) for the indicated periods, then immunoblotted. The graph shows the percentage of mature Kit remaining after CHX treatment. Results (%) represent means ± s.d. from three independent experiments. *$P<0.05$; **$P<0.01$, Student's *t*-test. (**l**,**m**) R cells or RCM cells were transfected with Tsg101 siRNAs (Tsg1 or Tsg2) and cultured for 24 h. Lystes from R cells treated with 50 ng ml^{-1} SCF for 30 min (**l**) or RCM cells (**m**) were immunoblotted.

prevent Akt activation (Fig. 6d, top and Supplementary Fig. 5b). STAT5 activation was again enhanced (Fig. 6d, right), indicating that Kit(D814Y) activates STAT5 on the ER.

Brefeldin A (BFA) also inhibits protein export from the ER[26,30,58]. On 16-h BFA treatment, as with tunicamycin, partially glycosylated Kit(D814Y) accumulated on the ER and STAT5 became active (Fig. 6c,d). STAT5 was seen in the nucleus and on cytosolic reticular structures, but not at the PM (Fig. 6e, top). BFA treatment significantly enhanced the co-localization of Kit(D814Y) with STAT5 on the reticular structures (Fig. 6e, bottom), further suggesting that Kit(D814Y) activates STAT5 on the ER. Figure 6f shows that the effects of BFA and tunicamycin



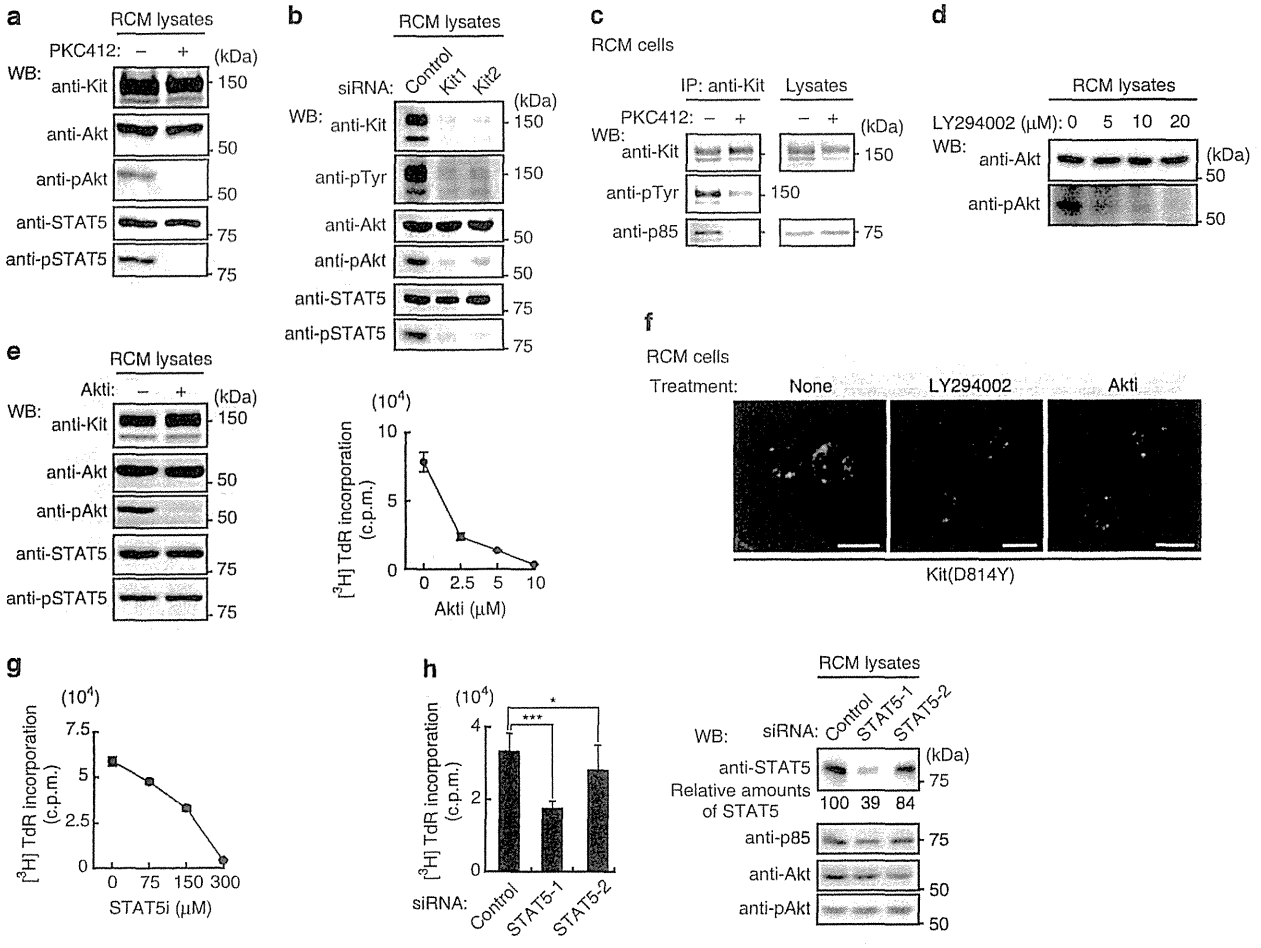


Figure 4 | In mouse RCM cells, Akt and STAT5 must be permanently active for autonomous proliferation. (**a**,**b**) Constitutive activation of Akt and STAT5 by Kit(D814Y). (**a**) RCM cells were treated with 1 μM PKC412 (Kit kinase inhibitor) for 24 h, lysed, then immunoblotted. (**b**) RCM cells transfected with control siRNA or Kit siRNAs were cultured for 20 h, then immunoblotted. (**c**) Association of Kit(D814Y) with PI3K. RCM cells were treated with 1 μM PKC412 for 24 h. Anti-Kit immunoprecipitates (left) and lysates (right) were then immunoblotted. NB: Kit(D814Y) was dissociated from p85 by PKC412 treatment. (**d**) Activation of Akt by PI3K. RCM cells were treated with the PI3K inhibitor LY294002. Lysates were then immunoblotted. (**e**) Role of Akt in proliferation of RCM cells. RCM cells treated with 10 μM Akti (Akt inhibitor) were cultured for 24 h. (Left) Lysates were then immunoblotted. (Right) [^{3}H]-thymidine incorporation. Results (c.p.m.) represent means ± s.d. ($n=3$). (**f**) Effect of LY294002 and Akti on Kit(D814Y) localization. RCM cells were treated with 20 μM LY294002 or 10 μM Akti for 24 h and stained with anti-Kit. Bars, 10 μm. (**g**,**h**) Role of STAT5 in proliferation of RCM cells. (**g**) [^{3}H]-thymidine incorporation into RCM cells treated with the STAT5 inhibitor STAT5i for 24 h. Results (c.p.m.) are means ± s.d. ($n=3$). (**h**) RCM cells transfected with control siRNA or STAT5 siRNAs (STAT5-1 and STAT5-2) were cultured for 24 h. (Left) Bar graph shows the levels of [^{3}H]-thymidine incorporation into RCM cells. Results (c.p.m.) are means ± s.d. ($n=11$). Data were subjected to one-way ANOVA with Dunnett's multiple comparison *post-hoc* test. $^*P<0.05$; $^{***}P<0.001$. (Right) Lysates were immunoblotted. Amounts of STAT5 are expressed relative to control cell lysate after normalization with p85.

Figure 5 | Kit(D814Y) traffics to endolysosomes to activate Akt in mouse cells. (**a**) Endolysosomes from RCM cells were immunoprecipitated with control or anti-LAMP1, then immunoblotted. (**b**,**c**) RCM cells were transfected with PH-GFP or PH(R25C)-GFP. (**b**) Expressed proteins were visualized by GFP fluorescence. Phase contrast images are shown. Bars, 10 μm. (**c**) Cells were cultured for 1 h with 1 mg ml^{-1} AF647-dextran (a marker for endocytic compartments). Bars, 10 μm. The graph shows Pearson's R correlation coefficient between dextran and expressed proteins. Results are means ± s.d. from 19 or 21 cells. $^{**}P<0.01$, Student's *t*-test. (**d**) RCM cells were co-stained for Kit(D814Y) (green) and phosphorylated Akt (pAkt; red). Bar, 5 μm. (**e**–**g**) RCM cells were cultured with 100 nM BafA1 (blocks endosomal trafficking) or 20 mM NH_4Cl (blocks proteases) for 24 h. (**e**) Cells stained with anti-Kit (green) and anti-LAMP1 (endolysosome marker; red). Bars, 10 μm. The graph shows Pearson's R correlation coefficient between Kit(D814Y) and LAMP1. Results are means ± s.d. ($n=10\sim22$). Data were subjected to one-way ANOVA with Dunnett's multiple comparison *post-hoc* test. $^{***}P<0.001$; NS, not significant. (**f**) Immunoblots of anti-Kit immunoprecipitates and cell lysates. (**g**) Immunoblots, RCM cells treated with 100 nM BafA1 for the indicated periods. (**h**) After 3-h treatment with 100 nM BafA1, R cells were stimulated with 50 ng ml^{-1} SCF for the indicated periods in the presence of BafA1, then immunoblotted. The graph shows the percentage of pAkt after nomalization with control at 5-min stimulation. Results (%) represent means ± s.d. from three independent experiments. BafA1 did not affect pAkt significantly (Student's *t*-test). (**i**) Immunoblots, endolysosomes from R cells treated with 50 ng ml^{-1} SCF as indicated. (**j**,**k**) R cells were stimulated with 50 ng ml^{-1} SCF for 5 or 60 min. Expressed proteins, Kit(wt) or pAkt are shown. Bars, 10 μm. The graph shows the number of Kit(+) vesicles with pAkt. Results (%) represent means ± s.d ($n=8\sim14$). NS, not significant; $^{***}P<0.001$, Student's *t*-test. ND = not detected.