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Audrey Sirvent, A. Boureux, V. Simon, C. Leroy, Serge Roche. The tyrosine kinase Abl is required for Src-transforming activity in mouse fibroblasts and human breast cancer cells.. *Oncogene*, 2007, 26 (52), pp.7313-7323. 10.1038/sj.onc.1210543 . hal-00189107

HAL Id: hal-00189107

<https://hal.science/hal-00189107>

Submitted on 20 May 2008

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The tyrosine kinase Abl is required for Src transforming activity in mouse fibroblasts and human breast cancer cells

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Running head: Abl in Src oncogenic activity

Key words: Src, Abl, ERK5, Rac, cell transformation, breast cancer

ABSTRACT

The cytoplasmic tyrosine kinase Src has been implicated in signal transduction induced by growth factors and integrins. Src also shows oncogenic activity when is deregulated. Accumulating evidence indicates that the tyrosine kinase Abl is an important substrate for Src signalling in normal cells. Here we show that Abl is also required for Src-induced transformation of mouse fibroblasts. Abl does not mediate tyrosine phosphorylation of Stat3 and Shc, two important regulators of Src oncogenic activity. In contrast, Abl controls the activation of the small GTPase Rac for oncogenic signalling and active Rac partly rescued Src transformation in cells with inactive Abl. Moreover, Abl mediates Src-induced ERK5 activation to drive cell transformation. Finally, we find that Abl/Rac and Abl/ERK5 pathways also operate in human MCF7 and BT549 breast cancer cells, where neoplastic transformation depends on Src-like activities. Therefore, Abl is an important regulator of Src oncogenic activity both in mouse fibroblasts and human cancer cells. Targeting these Abl-dependent signalling cascades may be of therapeutic value in breast cancers where Src-like function is important.

INTRODUCTION

The cytoplasmic tyrosine kinase Src plays important roles in signal transduction induced by a number of extracellular stimuli, including growth factors and integrins (Bromann et al., 2004). Src regulates activated cell growth, survival and migration, and shows oncogenic activity when is deregulated. This was first shown with vSrc, an active and viral form of the cellular counterpart responsible for the oncogenic activity of the Rous sarcoma virus in chicken. Later, the middle T antigen of polyoma virus (mT) was shown to induce Src oncogenic activity in rodents. By associating with Src, the mT viral protein prevents phosphorylation of the negative regulatory tyrosine at the C-terminus (Y527 in the chicken sequence) leading to constitutive kinase activity. Indeed, a kinase that harbours mutation of Tyr527 to Phe exhibits similar enzymatic and biological activities (Martin, 2001). Elevated Src activity is found in human colon and breast cancers. How Src is activated during tumourigenesis is still an open question, since very few activating mutations have been reported. Instead, overexpression and/or protein stabilization may contribute to its deregulation, although alternative mechanisms can be expected (Yeatman, 2004). A number of reports show a role of Src in tumour progression and metastasis (Ishizawar & Parsons, 2004). Accordingly, Src has become a potential therapeutic target in these cancers with several pharmacological inhibitors in clinical trials (Chen et al., 2006).

The molecular mechanism by which Src mediates cell transformation has been extensively studied in rodents, although not completely defined (Dilworth, 2002; Frame, 2004). Src is thought to phosphorylate specific substrates that activate constitutive signalling required for cell transformation. For example, Src phosphorylates the adaptor Shc, leading to Grb2/SOS complex recruitment to the membrane and activation of the Ras pathway. However, active Src induces only a modest activation of the Ras effectors Extracellular

Regulated Kinases (ERK) 1 and 2 implicating additional pathways. Indeed, Src also phosphorylates Stat3 on Tyr705 and the regulatory p85 subunit of phosphoinositide 3'kinase (PI3K). Both Stat3 and PI3K activities are required for cell transformation. The small GTPase Rac of the Rho family has been also implicated in Src oncogenic signalling (Minden et al., 1995; Servitja et al., 2003). It may function by activation of Jun N-terminal Kinase (JNK) and p38 ERK, which phosphorylate the transcription factors Jun and Stat3 (Turkson et al., 1999). Finally, ERK5 has been recently shown to participate in cell transformation via Src by an undefined mechanism (Barros & Marshall, 2005).

Compelling evidence shows that the tyrosine kinase Abl is an important substrate for Src signalling in normal cells (Pendergast, 2002). Abl resembles Src at its N-terminus while its large C-terminus has nuclear localisation sequences and protein and DNA interaction domains. Interestingly, Abl function depends on its sub-cellular localisation. Nuclear Abl regulates the cell response to DNA damage while cytoplasmic Abl participates in intracellular signalling induced by growth factors, cytokines and integrins (Daniels et al., 2004; Furstoss et al., 2002; Lewis et al., 1996; Plattner et al., 1999; Ushio-Fukai et al., 2005; Yang et al., 2006; Zipfel et al., 2004). For example, the Platelet Derived Growth Factor (PDGF)-induced Abl activation is required for DNA synthesis and F-actin assembly (Furstoss et al., 2002). Catalytic activity requires phosphorylation of Tyr245 and Tyr412 by Src (Hantschel & Superti-Furga, 2004) and this is necessary for morphological changes and mitogenesis (Furstoss et al., 2002). Abl activity required for cytoskeleton rearrangement also implicates the phospholipase C (PLC) γ , albeit by an unknown mechanism (Plattner et al., 2003). We and others have identified Rac as an important downstream component of Abl signalling, we have further shown that Abl mediates Src-induced c-myc expression and cell-cycle progression via Rac/JNK and Rac/NADPH oxidase signalling modules (Boureux et al., 2005).

Like Src, Abl exhibits some oncogenic activity when is deregulated. Active and cytoplasmic alleles induce rodent cell transformation, although not as efficiently as oncogenic Src (Zou & Calame, 1999). Bcr-Abl oncoprotein is responsible for 95% of human chronic myeloid leukaemia. Indeed, the Abl inhibitor Imatinib is now largely used to treat this type of leukemia (Krause & Van Etten, 2005). Moreover, Abl might be involved in solid tumours, since Srinivasan and Plattner have reported elevated Abl and Arg activities in human breast cancer cells (Srinivasan & Plattner, 2006). This deregulation was dependent upon tyrosine kinases of the Src and the EGF receptor families that are implicated in mammary carcinoma. Abl deregulation may participate in cell invasion, as illustrated with the highly aggressive breast cell-line MDAMB 435S. Abl was reported to regulate the epithelial to a mesenchyme transition of colon cancer cells induced by PDGF, which may involve Src (Yang et al., 2006). Based on these data, we surmised that Abl may be an important Src substrate for oncogenic activity. Here we show that Abl is required for Src-induced transformation of mouse fibroblasts, implicating both Rac and ERK5 for oncogenic signalling. Moreover, our data also implicate this signalling network in growth control of human breast cancer cells.

RESULTS SECTION

Abl is required for Src-induced transformation of mouse fibroblasts.

The contribution of Abl on the ability of Src to transform NIH 3T3 cells was first explored. To this end, we used the mT antigen for Src activation since the Src-mT complex has been found very efficient in foci induction (Dilworth, 2002). SrcY527F exhibited lower biological activity in this assay, probably due to active protein degradation (not shown). Wild-type Src alone is regulated in fibroblasts and does not induce foci while mT co-expression strongly induced cell transformation (Figure 1a). As expected, this response was dependent on Src activity as transformation was strongly reduced by the Src-like inhibitor SU6656 or by expressing the SrcYF-K⁻ inactive kinase (Figure 1b). mT alone also induced foci in a Src-like dependent manner while not as efficiently as Src-mT co-expression (unpublished data). Interestingly, the Abl inhibitor Imatinib strongly affected foci formation. This effect was observed with an IC₅₀ of about 2.5 μ M and a maximum of 10 μ M. This data was consistent with the concentration range observed for inhibition of phosphorylated active Abl (Tanis et al., 2003). Imatinib has been described to target c-Kit and PDGF receptors (Krause & Van Etten, 2005) suggesting a role for these kinases during cell transformation. However, this was unlikely as AG-1296, a specific inhibitor of these receptors (Kovalenko et al., 1997) had no effect on Src transformation. Similarly, Imatinib had no effect on Src-induced phosphorylation in transformed cells and kinase activity of the Src-mT complex (Figure 1e and f). A function of Abl on Src transformation was next confirmed with dominant negative approaches (Figure 1b). Kinase dead Abl-K⁻ strongly inhibited cell transformation unlike Abl. While not as active as Abl-K⁻, kinase inactive form of Arg, the other member of the Abl family, also reduced foci formation suggesting that it might be also implicated in Src transforming activity. Similar results were obtained with Abl-PP mutants in which Pro242

and Pro249 were replaced to Glu. These mutations stabilize the kinase in an open conformation and target the protein in cell cytoplasm (Furstoss et al., 2002). These inhibitory effects suggested that the cytoplasmic pool of Abl was required for Src oncogenic activity. Indeed, a similar inhibition was obtained with Abl2YF-NLS⁻, in which nuclear localisation signals and Src phosphorylation sites have been inactivated. Therefore, Tyr412 and Tyr245 are required for cytoplasmic signalling during Src transformation. The function of Abl was further confirmed with a siRNA approach. Down-regulation of Abl also reduced Src-induced cell transformation (Figure 1c). Finally, the observed Abl function was not specific to the transformation assay or the cell type used as Imatinib also reduced anchorage-independent growth of vSrc transformed Balbc 3T3 (Figure 1d).

Previously, we reported that SrcY527F phosphorylates Abl required for kinase activation (Furstoss et al., 2002). Whether the Src-mT complex activates Abl by a similar mechanism was then addressed (Figure 1e). Expression of mT in HEK 293 cells induced a large increase in cytoplasmic Abl activity concomitant with protein tyrosine phosphorylation. Both events were inhibited by the Src-like inhibitor SU6656 and mutation of Tyr245 and Tyr412 to Phe. This indicates that Src-mT-induced Abl activation requires phosphorylation on Tyr245 and Tyr412. Similar data were obtained in mT- and SrcY527-transformed fibroblasts (Figure 1f).

Abl does not mediate Shc and Stat3 tyrosine phosphorylation.

Tyrosine phosphorylation of Stat3 and Shc regulate Src-induced cell transformation (Bromberg et al., 1998; Nicholson et al., 2001). Whether these phosphorylations require Abl in Src-transformed cells was next addressed. As shown in Figure 2a, Imatinib had no effect on Shc phosphorylation on Tyr239 and Tyr240 and Stat3 on Tyr705. Nevertheless, these phosphorylations were required for Src-mT-induced foci as shown by the inhibitory effects of

Shc and Stat3 mutants in which these residues have been replaced by Phe (Shc2Y/2F and Stat3Y705F) (Figure 2b). Tyr315 is another reported phosphorylation site of Shc required for Src-mT-induced foci (Nicholson et al., 2001). However, Imatinib did not affect Shc tyrosine phosphorylation at all (Figure 2a), suggesting that Abl does not phosphorylate this residue. We concluded that Src is the major kinase responsible for Stat3 and Shc tyrosine phosphorylation and that Abl regulates alternative signalling during cell transformation.

Abl regulates Rac activation for Src-induced transformation.

Whether Abl regulates Src-induced Rac activation was next addressed. SrcY527F increased Rac activity when expressed in HEK 293 cells and this was inhibited by Imatinib treatment or expression of Abl-K⁻ (Figure 3a). These results were confirmed on the capacity of Rac to induce JNK activity. Indeed, Src also activated JNK in an Abl-dependent manner. Rac and JNK activities were also strongly increased in cells stably transformed by SrcY527F and inhibited by Imatinib treatment (Figure 3b). The Abl inhibitor also reduced phosphorylation of JNK substrates in vivo as shown on Stat3-Ser727 and Jun-Ser63 phosphorylation. Therefore, Abl mediates Src-induced Rac signalling.

The biological impact of Rac on Abl signalling was next addressed. Several lines of evidence suggest that small GTPases of the Rho family were required for Src-mT to induce cell transformation (Urich et al., 1997; Servitja et al., 2003; Urich et al., 1997; and data not shown). The contribution of Rac was shown by reduction of cell transformation obtained with the dominant negative RacN17 or Rac1 specific siRNA (Figure 3c). It should be noticed that the siRNA strategy was less efficient than the dominant-negative approach while highly effective in Rac1 down-regulation. This effect may be attributed to the temporary inhibition of the protein level during cell transformation assays (ie 12 days) and/or the contribution of Rac3 that was not targeted by our siRNA. We next explored the role of Rac in Abl

transforming signalling. As shown in Figure 3d, constitutive active RacV12 partially overcame the inhibitory effect of the kinase dead Abl-K⁻. A partial rescue was also obtained with a constitutive active form of the Rac GEF Vav2Δ, that maintains a high level of active Rac in vivo. Therefore, Rac is an important component of Abl oncogenic signalling but additional effectors are expected for full biological activity. Finally, the contribution of the Rac effectors JNK was also analysed on Src transformation (Figure 3e). Inactivation of the JNK pathway by expression of kinase dead forms of upstream kinases (MKK4 and MKK7) or by treatment of cells with the JNK inhibitor SP603580 strongly reduced foci formation. Finally, the incidence of an Abl-JNK pathway was also evaluated on Src oncogenic activity (Figure 3f). Surprisingly, none of JNK or p38 ERK activators gave a significant rescuing effect in cells with inactive Abl. We concluded that, while JNK activities are required for Src transformation, they are not sufficient for Abl signalling.

Abl regulates ERK5 activation for Src-induced transformation.

We then sought additional pathways that contribute to Abl function. Expression of Abl-PP and a constitutive active form of the upstream kinase MKK5 in HEK 293 cells induced a large increase in ERK5 activity (Figure 4a). SrcY527F also induced ERK5 activation (Barros & Marshall, 2005), although not as strongly as Abl-PP. Interestingly, this response was inhibited by Imatinib treatment or expression of the kinase dead Abl-K⁻ indicating that Abl mediates Src-induced ERK5 activity. Similarly, Imatinib treatment affected ERK5 activity that was largely increased in Src 527 fibroblasts (Figure 4b). The role of Abl/ERK5 signalling was next addressed on Src-induced transformation (Figure 4c). The dominant negative ERK5AEF in which the activation phosphorylation sites have been replaced with non-phosphorylatable residues reduced Src-mT-induced foci. Conversely, forced expression of constitutive active ERK5 activator MKK5-DA fully restored Src-mT

transforming activity both in cells over-expressing inactive Abl and in cells treated with a sub-maximal dose of Imatinib (Figure 4d). Therefore, ERK5 mediates Abl signalling required for Src transformation.

Abl/Rac and Abl/ERK5 pathways also operate in human breast cancer cells.

Finally, we wished to evaluate the importance of Abl/Rac and Abl/ERK5 pathways in human cancer. Our unpublished data indicate that Src kinases control growth of breast cancer cell lines, where neoplastic transformation depends on the oestrogen receptor (ER⁺) or the absence of ErbB2 (ErbB2⁻). The role of Abl on cancer cell growth was then addressed in MCF7 (ER⁺) and BT549 (ER⁻, ErbB2⁻) cell-lines. As illustrated in Figure 5a, the specific Src-like inhibitor SU6656 reduced standard cell growth. This was observed in conditions where the inhibitor reduced Src activities by more than 50% (Figure 5b). Whether Abl is a downstream element of Src function in these cells was next investigated. Src pharmacological inhibition induced a reduction of Abl activity by 50%. Imatinib induced a similar reduction on Abl activity and affected standard cell growth. This effect was linked to inhibition of Abl kinases as (i) related targets PDGF receptors or c-Kit were not detected in these cells (Srinivasan & Plattner, 2006) and (ii) AG-1296 had no effect on cell growth (Figure 6a). Similarly, SU6656 and Imatinib strongly affected anchorage-independent cell growth, unlike AG-1296 (Figure 5c). The specificity of this inhibition was also illustrated by the inability of these inhibitors to affect growth of the SKBR3 cell-line that overexpresses ErbB2 (not shown). We concluded that a Src/Abl pathway may regulate transforming activities of these breast cancer cells in vitro.

To confirm oncogenic activities of these kinases, the role of Rac and ERK5 was analysed on neoplastic cell growth. First, we observed that Src and Abl regulate Rac1 activity as shown by the reduction of the GTPase active level in cells incubated with respective

inhibitors (Figure 6a). Abl inhibition had a lower impact suggesting that Src may activate additional pathways for Rac1 activation. Interestingly, pharmacological inhibition of Rac strongly reduced standard cell growth (Figure 6b) in concordance with a role of Rac1 and Rac3 in BT549 cell growth (Chan et al., 2005). Moreover, Rac inhibition also reduced anchorage-independent cell growth (Figure 6e) implicating Rac in their transforming activity. Similarly, we observed that Src and Abl kinases regulate ERK5 activity (Figure 6c). Since no specific inhibitor is currently available, the role for ERK5 was evaluated by down-regulating the kinase with a RNA interference approach (Figure 6d) (Garaude et al., 2006). Cells were infected with retroviruses expressing shRNA specific to ERK5 that allows more than 50% reduction in both kinase activity and protein level. In these conditions, a partial, but significant reduction of standard cell growth was observed and anchorage-independent cell growth was strongly reduced (Figure 6e). We concluded that Rac and ERK5 are required for growth of MCF7 and BT549 cell-lines. Additionally, these data reinforce the notion of a Src/Abl signalling pathway involved in neoplastic transformation of breast cancer cells.

DISCUSSION

Abl plays an important role for Src signalling in normal cells and here we show that it is also required for Src oncogenic activity: Abl inhibition by pharmacological, dominant-negative or siRNA approaches, all reduced Src-induced foci. While Abl has a partial effect on cell-cycle progression (not shown), its role in foci induction may primarily involve modulation of cell contact-induced growth inhibition. Additionally, Abl may also impact on anchorage-independent growth as illustrated in transformed mouse fibroblasts and human breast cancer cells. It should be mentioned that this tyrosine kinase has been reported as a negative regulator towards cell transformation (Suzuki et al., 2004). This raises the idea that it may have positive or negative function depending on genetic background or the cell-type

used. For example, while the absence of Abl promoted SV40-induced cell growth, reintroduction of the corresponding gene accelerated cell growth after few passages.

Our report also shed new light on the Abl signalling pathways involved in Src transforming activity. First, we show that Src directly phosphorylates cytoplasmic Abl on Tyr245 and Tyr412 that will stabilize the kinase in open and active conformation necessary for cell transformation. Interestingly, residual activity was observed in the absence of phosphorylation surmising alternative regulatory mechanisms. Accordingly, PLC and PI3K inhibitors also affected mT-induced Abl activation (not shown) suggesting that phosphoinositides may also participate in Abl regulation (Plattner et al., 2003). How these lipids impact on Abl activity is still an open question that needs further clarification. Second, we show that Rac1 and ERK5 are important downstream components of Abl signalling. These observations are in close agreement with the reported role of Rac in Src transforming activity (Servitja et al., 2003; Urich et al., 1997). The molecular mechanism by which Abl regulates Rac is still unclear, but the likely hypothesis implicates phosphorylation of a specific GEF. Potential candidates may include Vav2, SOS1 and Tiam2 [(Servitja et al., 2003; Sini et al., 2004) and Fig 3d]. The existence of an Abl/Rac pathway is ascertained by the requirement of JNK in Src transformation. JNK signalling may implicate phosphorylation of Stat3 for maximal transcriptional activity (Turkson et al., 1999). This means an Abl/Rac/JNK/Stat3 signalling cascade important for cell transformation. Nevertheless, the importance of this pathway has not been established since the role of Stat3-Ser727 has not been validated on Src transformation. Clearly additional Abl pathways are expected in this response. Accordingly, we have identified ERK5 as a novel Abl downstream component required for Src transformation. The pivotal role of ERK5 has been illustrated by the capacity of MKK5-DA to rescue transformation of cells with inactive Abl. How Abl impacts on ERK5 activation is not known but our data favour a mechanism implicating catalytic activation rather than

protein stabilization as previously reported (Buschbeck et al., 2005). Abl may probably interact with upstream kinases, alternatively Abl-induced ERK5 may implicate Rac. Recent reports suggest that ERK5 participates on Src-induced cytoskeleton rearrangement (Barros & Marshall, 2005), Jun and cyclin D induction required for cell-cycle progression (Kato et al., 1997; Mulloy et al., 2003) and NF κ B activation leading to cell survival (Garaude et al., 2006). Probably all these effects may contribute to Src transforming activity.

Our study also suggests that Abl plays a functional role in breast cancer progression. This notion is in agreement with inhibitory effects observed with Imatinib in some breast cancer cells (Roussidis et al., 2004; Srinivasan & Plattner, 2006). Pharmacological inhibition did not correlate with expression of related targets implicating an Abl-like activity in neoplastic cell transformation. This has been further confirmed in MDAMB 435S cells by down-regulation of Abl level with a siRNA approach (Srinivasan & Plattner, 2006). Therefore, Abl inhibition may be of therapeutic value in mammary carcinoma. In this regard, a preclinical study reported preferential sensitivity to the dual Src/Abl inhibitor dasatinib for breast cancer cells of basal-type scored negative for ERbB2 and that have undergone the epithelial to a mesenchymal transition (Finn et al., 2007). We also noticed that while a high dose of Imatinib gave significant inhibitory effect on cell growth, it only gave a partial reduction on Abl activity in vitro. This may be explained by the low affinity of the inhibitor for phosphorylated and active form of the kinase (Tanis et al., 2003). Inhibitors that efficiently inhibit activated Abl may be of better therapeutic value. This notion has been recently validated with inhibitors of the EGF receptor family and suggests that a lack of efficient catalytic inhibition is sufficient to induce therapy failure (Sergina et al., 2007). Finally, our study also shows that a Src/Abl/Rac/ERK5 operates in breast cancer cells. This notion is illustrated with the inhibitory effect observed with Rac and ERK5 inactivation. Therefore, targeting this signalling pathway may be a therapeutic benefit to treat this cancer.

MATERIALS AND METHODS

Reagents, antibodies and DNA constructs. SU6656, AG-1296, NSC23766 and SP600125 were from Calbiochem (La Jolla, CA). Imatinib was a gift from B. Willi (Novartis Pharma AG, Basel, Switzerland). [γ - 32 P]ATP was purchased from Amersham. Src family kinases (SFK; cst1) and mT (762) antibodies have been described in (Franco et al., 2006). Antibodies specific to Stat3 and Ras were from Upstate Biotechnology, pJNK, JNK1, ERK5, pSer₆₃-Jun, pY₇₂₇Stat3, pY₂₃₉/Y₂₄₀Shc and Jun from Cell Signaling Technology, Abl (Ab3) from Calbiochem (La Jolla, CA), Rac1 from United Biomedical, FLAG from Sigma Aldrich (St Quentin, France). 4G10 and anti- α tubuline were from P. Mangeat and N. Morin respectively (CRBM, Montpellier, Fr). Constructs expressing Src, Abl, Ras and Stat3 were described in (Furstoss et al. 2002, Veracini 2006). Shc2YF (Y239F, Y240F) was generated by mutagenesis as in (Veracini et al., 2006). Constructs encoding Gst-Jun, FLAG-JNK1, FLAG-ERK5 and ERK5AEF (ERK5T218A/Y220F) were from R. Hipkind (IGMM, Montpellier, FR), RacV12 and RacN17 from P. Fort (CRBM, Montpellier, FR), Vav2 Δ (184-868) from X.R. Bustelo (CSIC-University of Salamanca, Spain), MKK4-DN (MKK4S220A/T224A), MKK4-DA (MKK4 S220E/T224E), MKK6-DA (MKK6S207E/T211E), MKK7-DA (MKK7 β 1T275A/S277A) were described in (Boureux et al., 2005), MKK6-DN and MKK7-DN were from J. Raingeaud (Inserm U641, Chatenay-Malabry, FR). MKK5-DA (MEK5S313D/T317D), pSuper-neo coding for shRNA ERK5 and shRNA control were from R. Hipkind (IGMM, Montpellier, FR). Gst-MEF2C was a gift of C. Tournier (University of Manchester, UK) and Gst-Jun of R. Hipkind (IGGM, Montpellier, FR). Gst-Crk has been described in (Furstoss et al., 2002).

Cell culture, transfections and infections. Balbc 3T3, Balbc 3T3 transformed by vSrc (vSrc-3T3) (Franco et al., 2006), NIH 3T3 transformed by SrcY527F-3T3 (Src527)(gift of S.A. Courtneidge, The Burnham Institute for Medical Research, La Jolla, CA), NIH 3T3

transformed by mT (mT-3T3) (gift of Kurt Ballmer-Hofer, Paul Scherrer Institute, Switzerland), NIH 3T3 and HEK 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS). BT549 and MCF7 were maintained respectively in DMEM F12 and RPMI supplemented with 10% FCS. Cell culture, transfections and retroviral infections were described previously (Franco et al., 2006). For drug treatment, cells were treated with the indicated inhibitor (or DMSO as a vehicle) for 5 hours in growth medium supplemented with 1% FCS. siRNA duplexes (25nM) targeting mouse Abl (Dharmacon), Rac1 (Deroanne et al., 2003) and controls (Scramble) were transfected using calcium phosphate as described in (Boureaux et al., 2005). Knockdown of Rac1 expression was done using specific siRNA as described in (Deroanne et al., 2003). EKR5 down-regulation was obtained by infection (Franco et al., 2006) of wild-type (mock) or shRNA ERK5 expressing retroviruses (Garaude et al., 2006)

Cell growth and transforming assays. Standard cell growth was performed in 2% FCS medium using 24 well plates (50 000 cells/well) and measured by Sulforhodamide B staining according to manufacturer's instructions (Sigma Aldrich, St Quentin, FR). Focus formation was performed using NIH 3T3 cells transfected with indicated construct using Lipofect-AMINE reagent (Invitrogen) and maintained in 10% FCS for 12-14 days. After staining with crystal violet (1%), foci larger than 0.5 mm in diameter were visually scored. For anchorage-independent cell growth, 0.67% agar in medium was layered on the bottom of a 12 wells plate and 1 000 (vSrc-3T3), 2 000 (MCF7) or 4 000 (BT549) cells/well were seeded on the top of this layer in 0.33% agar-medium. After 18-21 days colonies having >50 cells were scored positive. Inhibitors or vehicle (DMSO) were added every 3 days.

Biochemistry. Cell lysate, immunoprecipitation, Rac1 pull-down and western-blotting were performed as previously described (Boureaux et al., 2005; Veracini et al., 2006). In vitro

kinase assays for SFK, Abl, ERK5 and JNK were described in (Boureux et al., 2005; Kayahara et al., 2005; Veracini et al., 2006).

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Acknowledgments: We thank K. Ballmer-Hofer (Paul Scherrer Institute), X.B. Bustelo (University of Salamanca), S.A. Courtneidge (the Burnham Institute for Medical research), A. Pandiella (University of Salamanca), J. Raingeaud (INSERM U641) and B. Willi (Novartis Pharma) for various reagents. We thank C. Tournier (University of Manchester) and R. Hipkind (CNRS UMR 5535) for constructs and helpful discussion, and our colleagues for critical reading the manuscript. This work was supported by the CNRS, ARC, AICR and INCa. AB was supported by AICR, CL by INCa, AS by Laboratoires Servier and SR by INSERM.

TITLES AND LEGENDS TO FIGURES

Figure 1: Abl is a Src substrate required for its transforming activity. (a-c) Src-mT-induced foci require Abl kinase activity. **(a)** An example of inhibition of Src-mT-induced foci by Imatinib. **(b)** Statistical analysis of Src-mT-induced foci formation in cells treated with Imatinib or expressing dominant-negative mutants of Abl. **(c)** Down-regulation of Abl expression reduced Src-mT-induced foci. The levels of immunoprecipitated Abl after transfection of cells with indicated siRNA are shown. Src tyrosine kinases (SFK) levels from whole cell-lysates (WCL) are also shown. NIH 3T3 cells were transfected with indicated constructs, siRNA or empty control vectors (mock), and treated with inhibitors as shown. After 14 days of growth, foci were stained and scored as in “Materials and methods”. Foci formation (% of foci obtained relative to foci induced by Src-mT) is represented as the mean \pm SD of ≥ 3 independent experiments **(d)** Imatinib inhibits anchorage-independent cell growth of vSrc-transformed cells (vSrc-3T3). Indicated cells were seeded in soft-agar with 5 μ M SU6656, 10 μ M Imatinib, or vehicle (DMSO). An example (left panel) and quantification (right panel) of growth in soft-agar (means of colonies scored positive \pm SD, n=3) are shown **(e)** mT-induced Abl activation implicates phosphorylation on Tyr245 and Tyr412 by Src. Left panels: activity and tyrosine phosphorylation of immunoprecipitated Abl from HEK 293 cells transfected with indicated constructs and treated with SU6656 as shown. Right panels: tyrosine phosphorylation and catalytic activity of immunoprecipitated Abl from NIH 3T3 control cells (3T3) or cells stably transformed by mT (mT-3T3) and treated with inhibitors as indicated. In vitro kinase activity of the immunoprecipitated Abl was assessed using Gst-Crk as a substrate. Phosphorylation of Gst-Crk (32 P-GSt-Crk), levels of Abl, mT and tyrosine phosphorylated Abl (pY-Abl) are shown. Immunoprecipitated mT (ip: 762) was subjected to in vitro kinase assay. Phosphorylated mT (32 P-mT), Src (32 P-Src) and PI3K (32 P-PI3K) are shown. **(f)** Abl is activated and phosphorylated in SrcY527F transformed cells. Abl was

immunoprecipitated from NIH 3T3 cells stably transformed (Src527) or not (3T3) by SrcY527F and treated with inhibitors as indicated. Left panel: levels of Abl, tyrosine phosphorylated Abl and in vitro catalytic activity are shown. Right panel: tyrosine phosphorylation content of indicated cell-lysates (WCL) is shown.

Figure 2: Abl does not phosphorylate Shc and Stat3, two important effectors of Src transforming activity. (a) Abl inhibition does not affect Src-induced Stat3 and Shc tyrosine phosphorylation. NIH 3T3 control cells (3T3) and cells transformed by SrcY527F (Src527) were treated with inhibitors as indicated. The levels of Shc, pY-Shc, pY₂₃₉/pY₂₄₀Shc, Stat3 and pY₇₀₅Stat3 are shown. **(b)** Tyrosine phosphorylation of Shc and Stat3 are required for Src-mT-induced foci. NIH 3T3 cells were transfected with indicated constructs or empty control vectors (mock) and assayed for foci induction as in Figure 1. Foci formation (% of foci obtained relative to foci induced by Src-mT) is shown as means \pm SD of ≥ 3 independent experiments.

Figure 3: The small GTPase Rac is an important Abl downstream effector for Src transforming activity. (a, b) Abl regulates Src-induced Rac1 and JNK activation. Transfected HEK 293 **(a)** and NIH 3T3 cells stably transformed or not with SrcY527F **(b)** were treated with kinase inhibitors as shown. JNK1 and Rac1 activities were measured as in “Materials and methods”. Levels of active Rac1 (Rac1-GTP), Rac1, SrcY527F and Abl mutants are shown. In vitro JNK1 activity is shown and was assessed from the immunoprecipitated protein using Gst-Jun as a substrate. Phosphorylated Gst-Jun (³²P-Gst-Jun), levels of JNK1, Abl and Src are also shown. JNK activity in vivo is also revealed by phosphorylation of Jun at Ser63 and Stat3 at Ser727 in transformed cells. **(c-f)** Rac is an element of Abl signalling for Src-mT-induced foci. **(c)** Rac1 is required for Src-mT-induced

foci. The level of Rac1 is shown in NIH 3T3 cells transfected for 72 hours with indicated siRNA. **(d)** Rac activation partly rescues foci induction in cells expressing Abl-K⁻. **(e)** JNK is required for Src-mT-induced foci. **(f)** Constitutive activation of JNK and p38 ERK are not sufficient to rescue transformation in cells treated with Imatinib. NIH 3T3 cells were transfected with indicated constructs, siRNA or empty control vectors (mock), and treated or not with indicated drugs. After 14 days of growth, foci formation was scored as described in “Materials and methods”. Results (means \pm SD of ≥ 3 independent experiments) are expressed as described in Figure 1. Drug concentrations were 0.5 μ M (SP603580) and 2.5 μ M (Imatinib).

Figure 4: ERK5 is another important element of Abl signalling for Src transforming activity. **(a)** Abl regulates Src-induced ERK5 activity. HEK 293 cells were co-transfected with indicated constructs or empty vectors (mock) and treated with 10 μ M Imatinib or vehicle (DMSO). In vitro kinase assay of the immunoprecipitated FLAG-ERK5 was done as described in “Materials and methods”. Autophosphorylated ERK5 (³²P-ERK5) and phosphorylation of the exogenous substrate MEF2C (³²P-MEF2C) is shown. Levels of ERK5, Abl, Src and MEK5 are shown from whole cell-lysates (WCL). **(b)** Imatinib inhibits ERK5 activity in SrcY527F-transformed cells. Indicated cells were treated with pharmacological inhibitors as shown and in vitro kinase assay of the immunoprecipitated ERK5 was assessed. Autophosphorylated ERK5 (³²P-ERK5), phosphorylation of its substrate MEF2C (³²P-MEF2C) and ERK5 levels are shown. **(c)** ERK5 is required for Src-mT-induced foci and **(d)** MEK5-DA rescues foci induction in cells with inactive Abl. NIH 3T3 cells were transfected with indicated constructs or empty vectors (mock), and treated or not with indicated drugs. After 14 days of growth, foci formation was scored as described in “Materials and methods”. Results (means \pm SD of ≥ 3 independent experiments) are expressed as described in Figure 1.

Figure 5: Src and Abl activities control MCF7 and BT549 breast cancer cell growth. (a)

Imatinib and SU6656 reduce breast cancer cell growth. Indicated cells were treated with 5 μ M SU6656, (\blacktriangledown), 10 μ M Imatinib (\blacktriangle) or vehicle (\blacksquare , DMSO) in growth medium supplemented with 2% FCS. Time-course of cell growth (arbitrary units) represented as the mean \pm SD (n=3) is shown. **(b)** SU6656 inhibits Abl activity in breast cancer cells. Indicated cells were treated with vehicle (DMSO), 5 μ M SU6656 or 10 μ M Imatinib as shown. In vitro kinase activity of the immunoprecipitated Abl was assessed using Gst-Crk as a substrate. Phosphorylation of Gst-Crk (32 P-GSt-Crk), levels of Abl and quantification of relative kinase activity are shown. In vitro kinase assay of immunoprecipitated SFK was assessed using denatured enolase as a substrate. Phosphorylation of enolase (32 P-Enolase), autophosphorylation of SFK (32 P-SFK), kinase levels and quantification of relative kinase activities are shown. **(c)** Imatinib and SU6656 reduce anchorage-independent cell growth. Indicated cells were seeded in soft-agar with 5 μ M SU6656, 10 μ M Imatinib, or vehicle (DMSO) as described in “Materials and methods”. After 21 days, the number of colonies was scored and the mean \pm SD (n=3) is shown. * $P < 0.05$ and ** $P < 0.01$ using a student’s *t*-test.

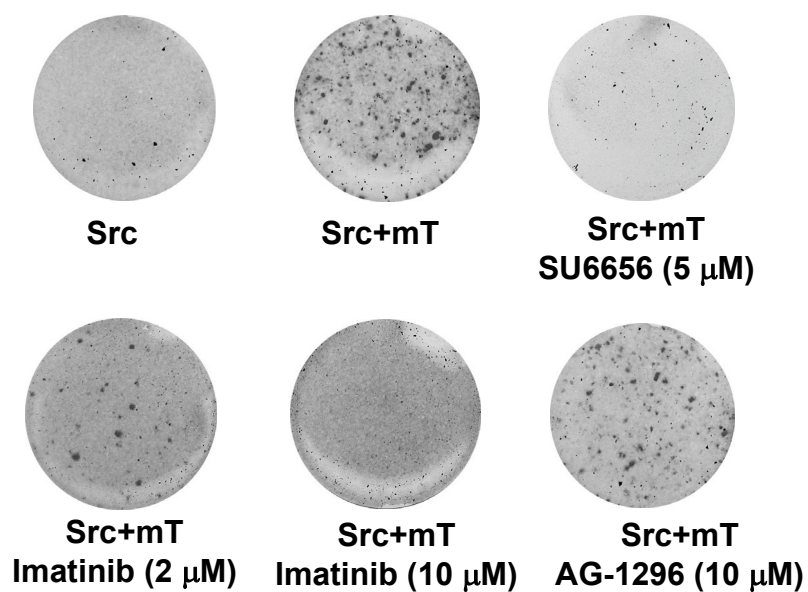
Figure 6: Src and Abl kinases regulate Rac and ERK5 activities, also required for breast cancer cells growth. (a)

Imatinib and SU6656 reduce Rac1 activity. Cells were treated with 100 μ M NSC23766, 5 μ M SU6656, 10 μ M Imatinib or vehicle (DMSO). Active Rac1 (Rac1-GTP) was purified and analysed as in “Materials and methods”. The levels of Rac1 and active Rac1 are shown. **(b)** The Rac1 inhibitor NSC23766 regulates cell growth. Indicated cells were treated with 100 μ M NSC23766 (\blacktriangle), 10 μ M AG-1296 (\blacktriangledown) or vehicle (\blacksquare , DMSO) in growth medium supplemented with 2% FCS. Time-course of cell growth (arbitrary units) represented as the mean \pm SD (n=3) is shown. **(c)** SU6656 and Imatinib inhibit ERK5 activity. In vitro

ERK5 activity of cells were treated with 5 μ M SU6656, 10 μ M Imatinib or vehicle (DMSO) Autophosphorylated ERK5 (32 P-ERK5), phosphorylation of MEF2C (32 P-MEF2C), levels of α -tubulin, immunoprecipitated ERK5 and quantification of relative kinase activities are shown. **(d)** ERK5 down-regulation reduces cell growth. Standard cell growth (means \pm SD, n=3) of cells infected with retroviruses encoding shRNA ERK5 (\blacktriangle) or shRNA control (\blacksquare) was analysed. Levels and in vitro ERK5 activity of cells infected with indicated retroviruses (top panel). Phosphorylated MEF2C (32 P-MEF2C), relative kinase activity and level of α -tubulin are shown. **(e)** ERK5 down-regulation reduces anchorage-independent cell growth. Growth in soft-agar (mean of colonies scored \pm SD, n=3) of cells infected with indicated retroviruses or treated with 100 μ M NSC23766, 10 μ M AG-1296 or vehicle (DMSO) as shown. * P < 0.05 and ** P < 0.01 using a student's t -test

Figure 1a, b

a



b

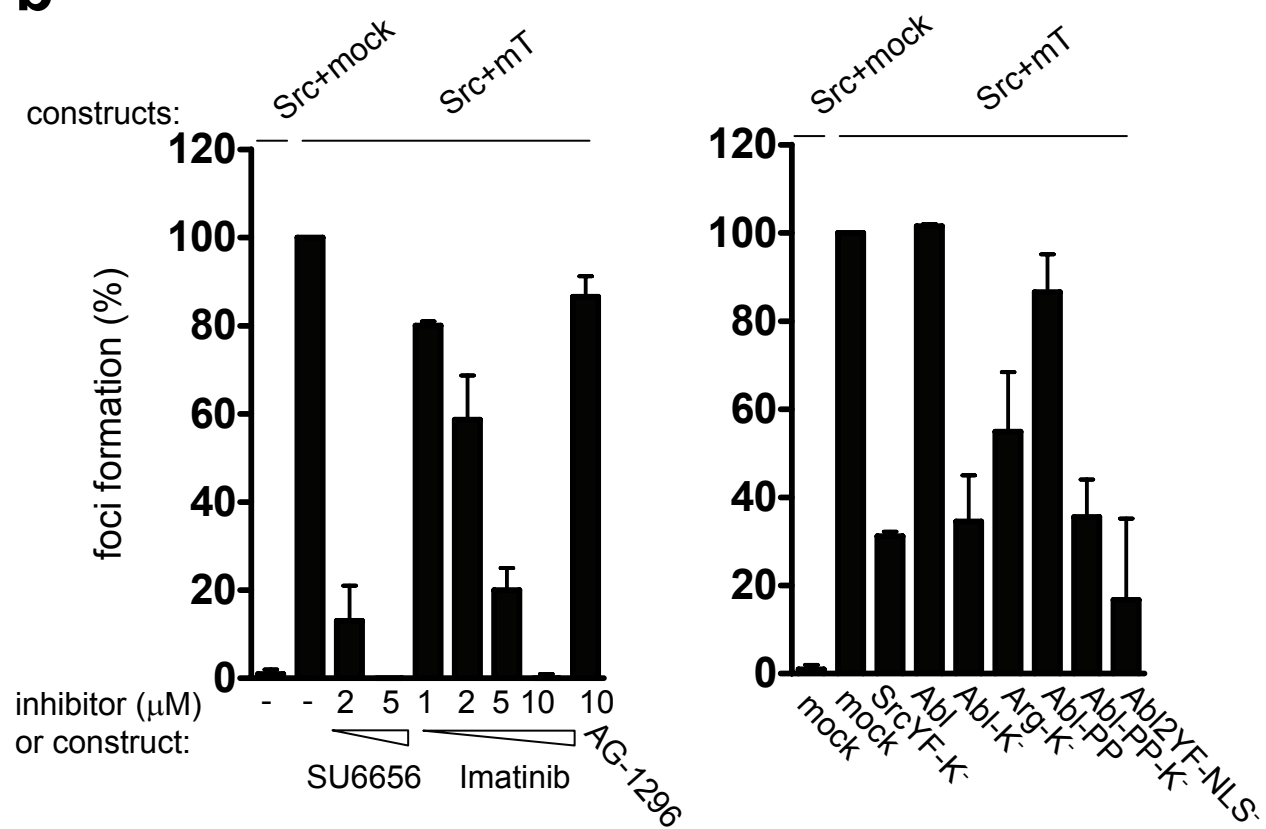


Figure 1c, d

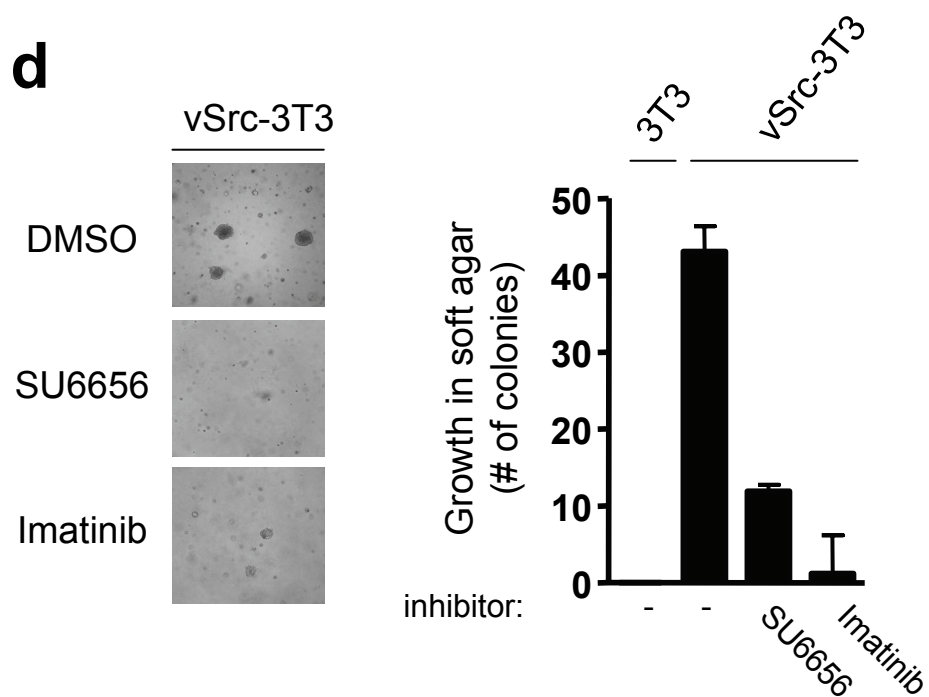
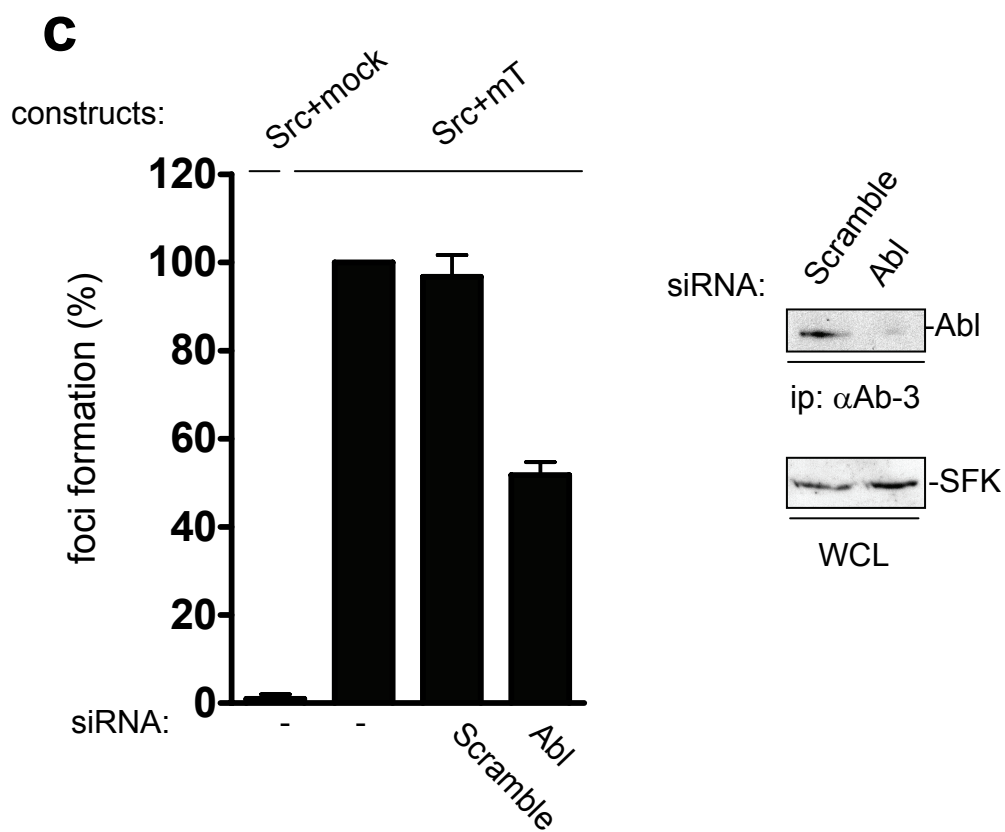
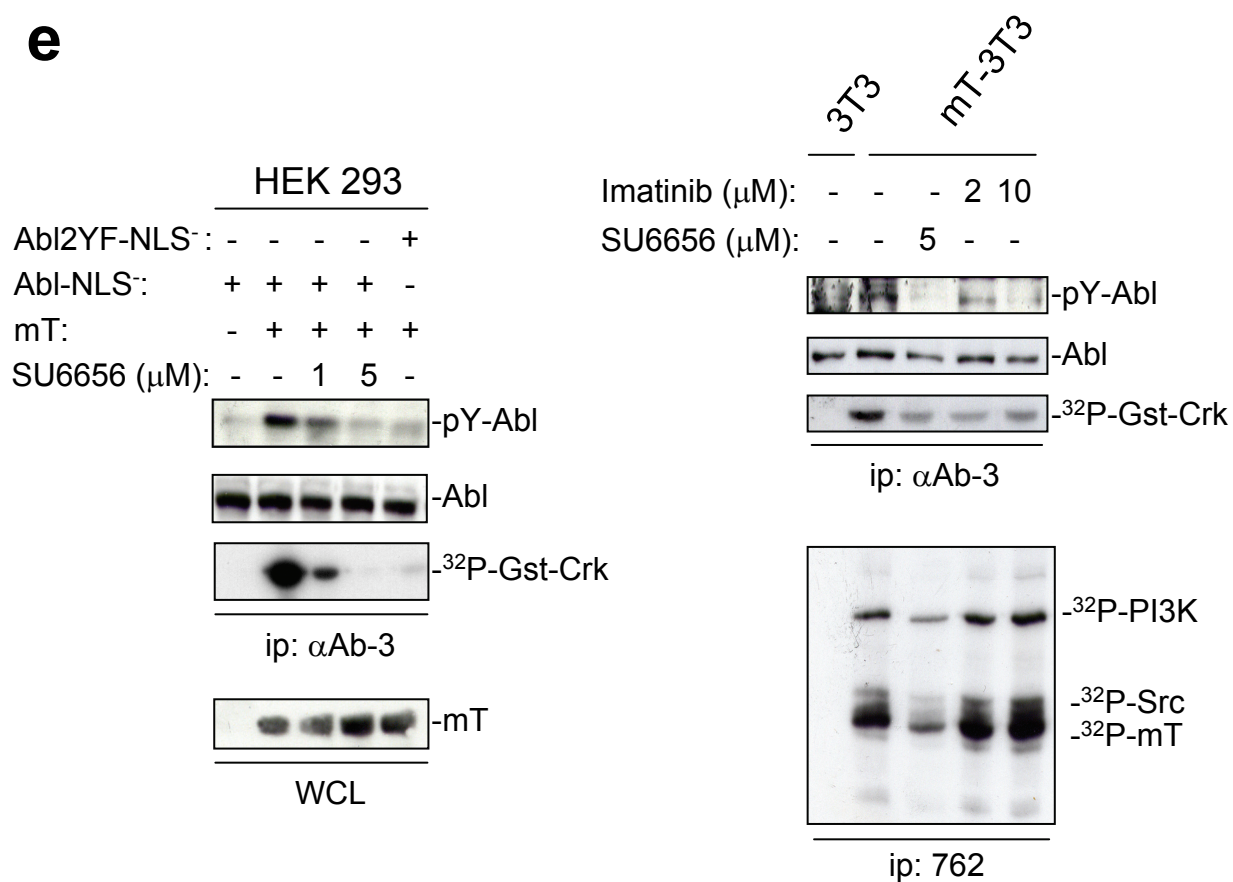


Figure 1e, f

e



f

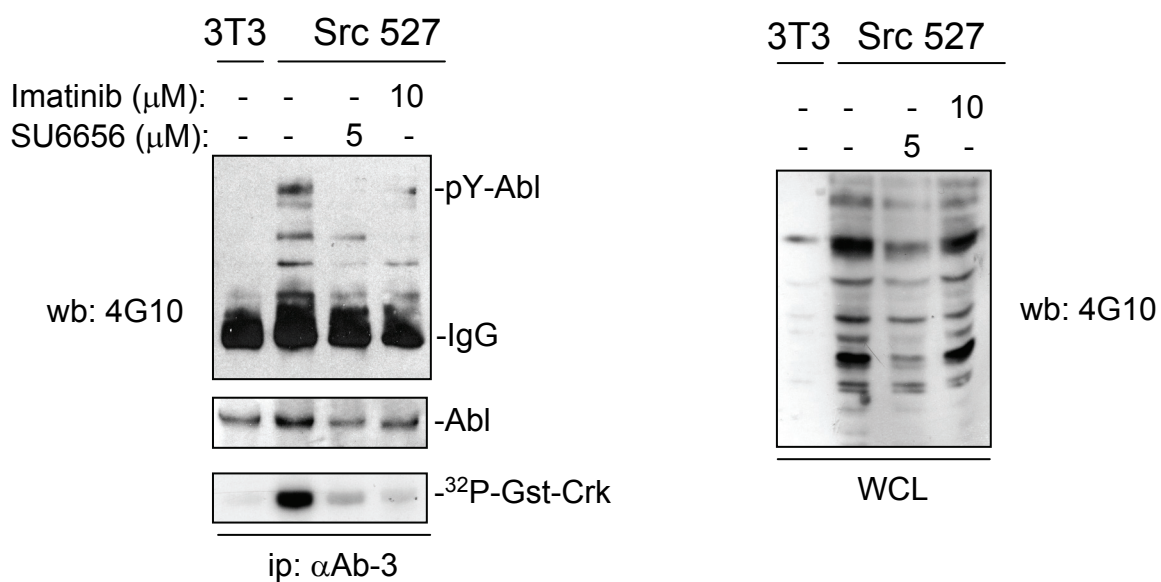
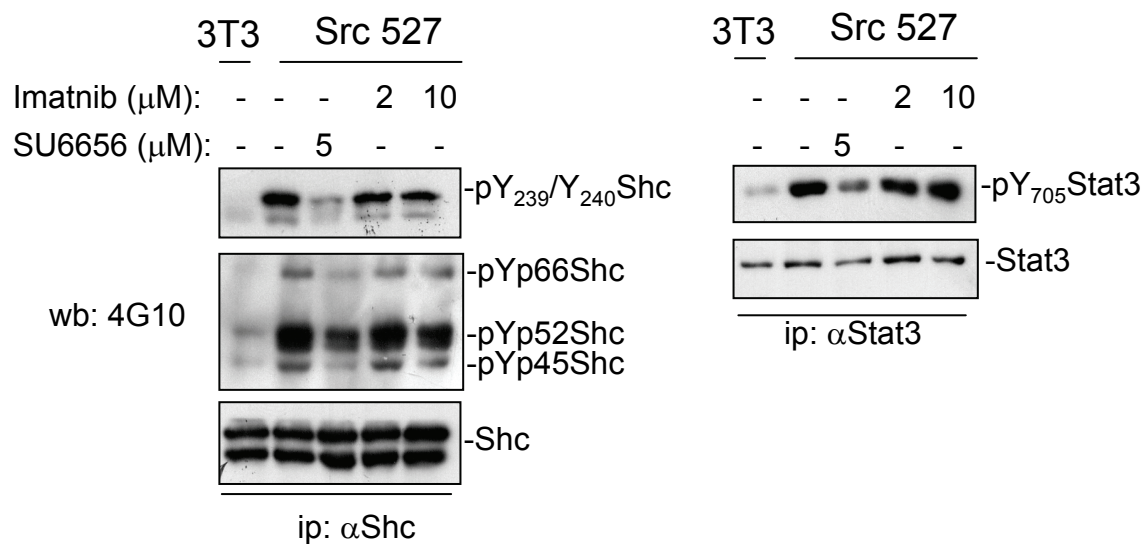


Figure 2a, b

a



b

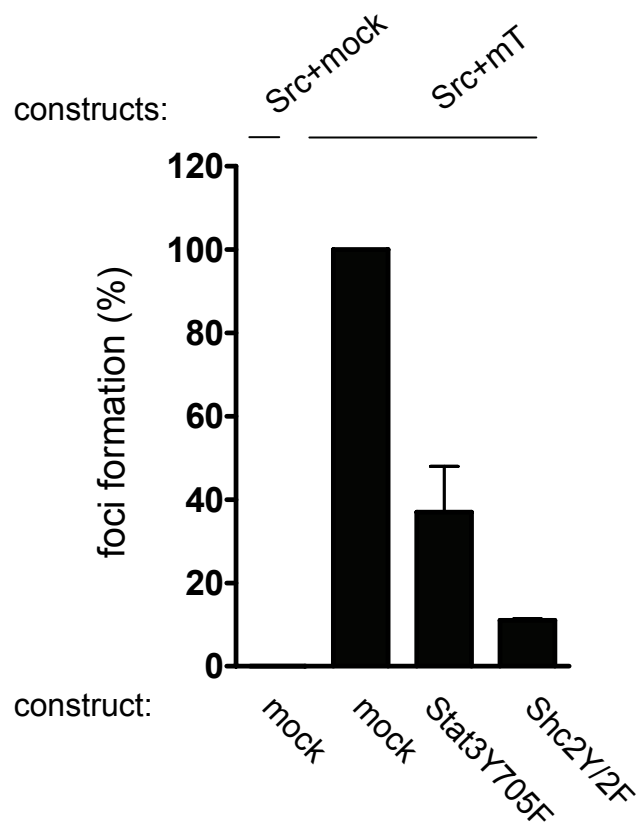


Figure 3a, b

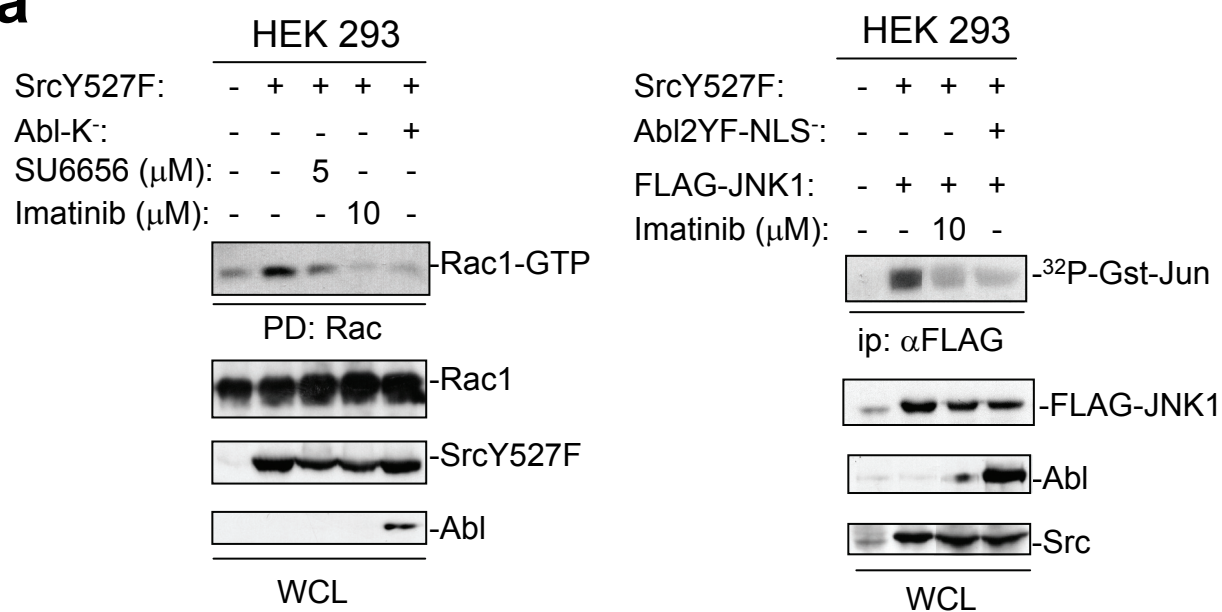
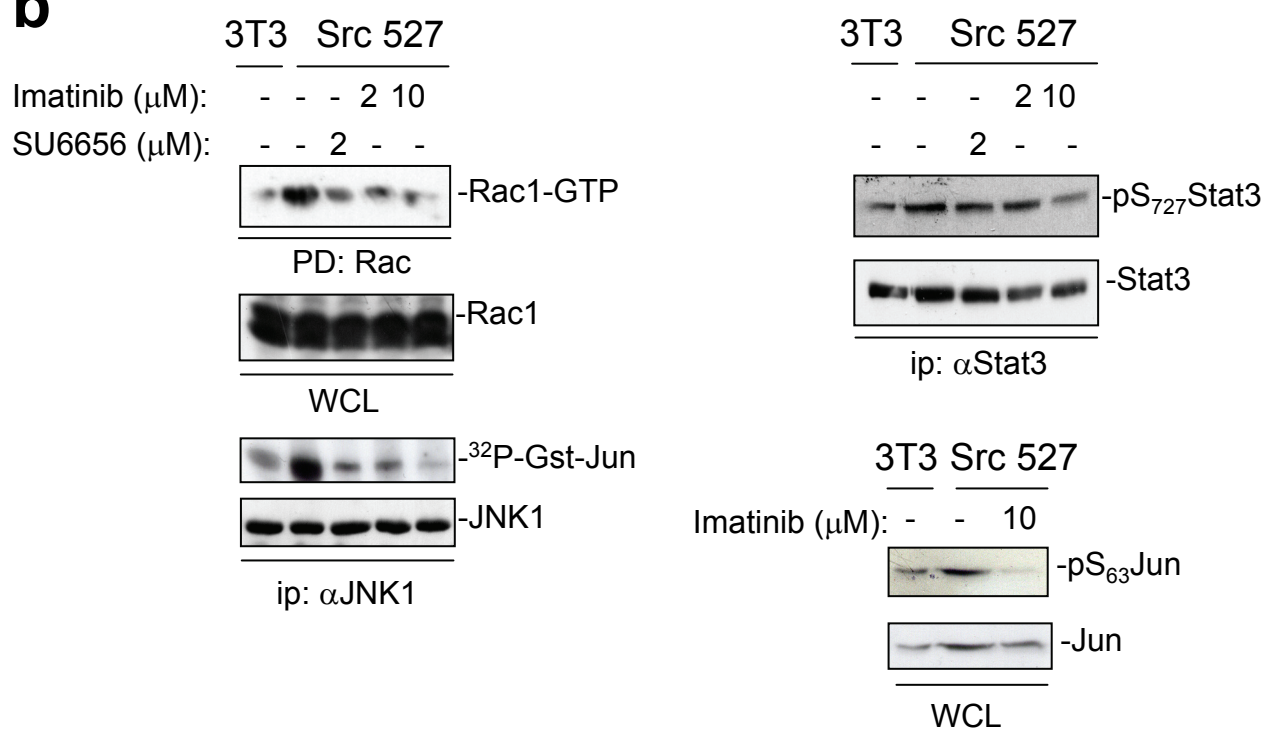
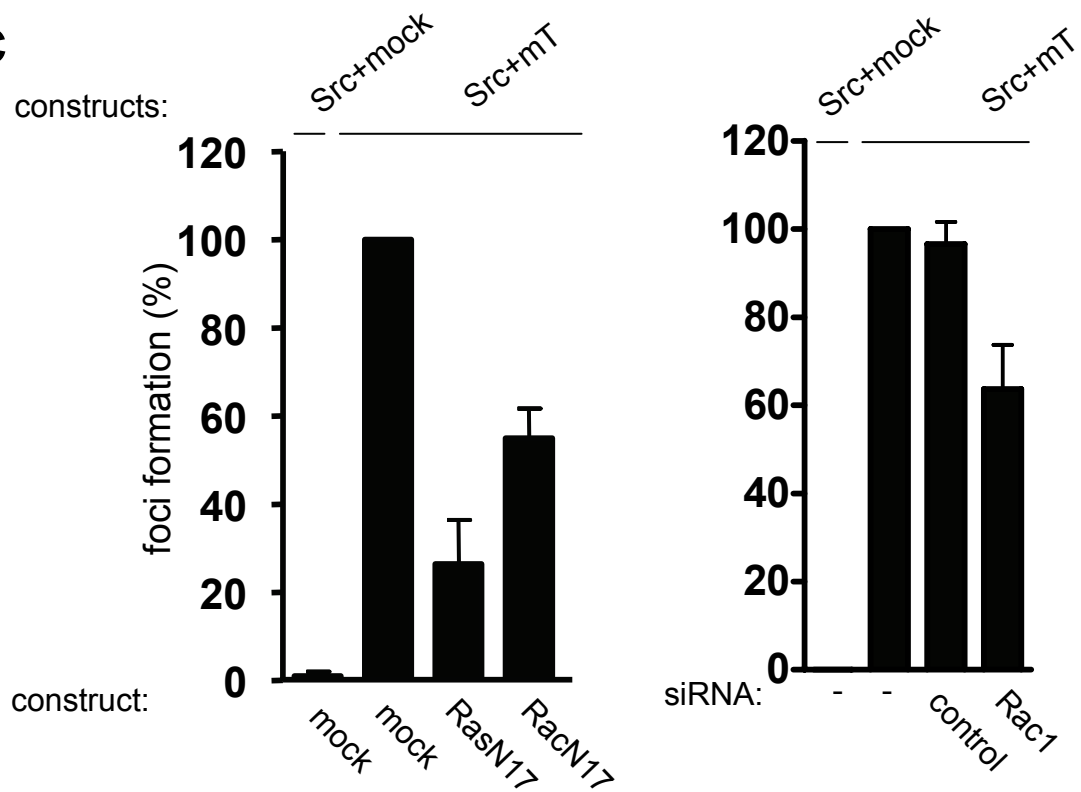
a**b**

Figure 3c, d

c



d

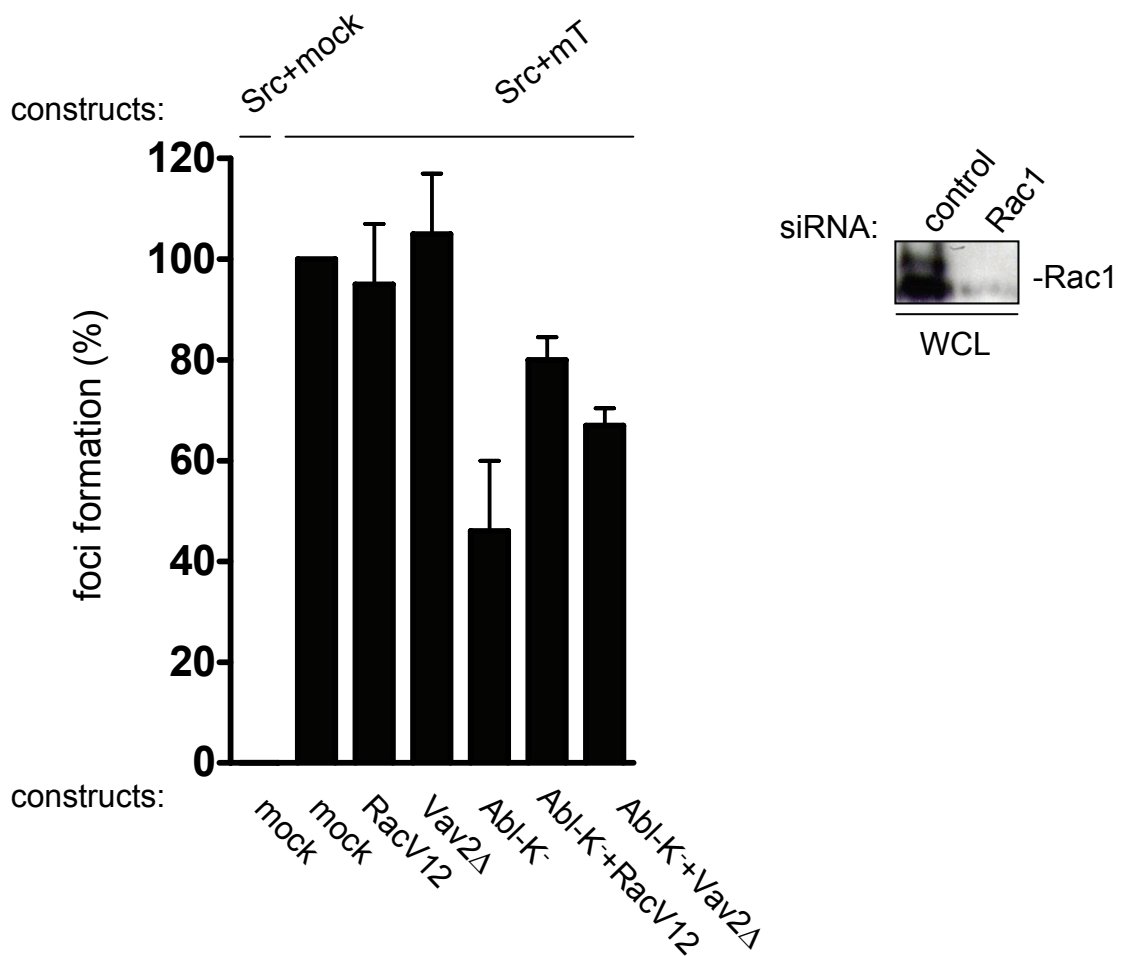
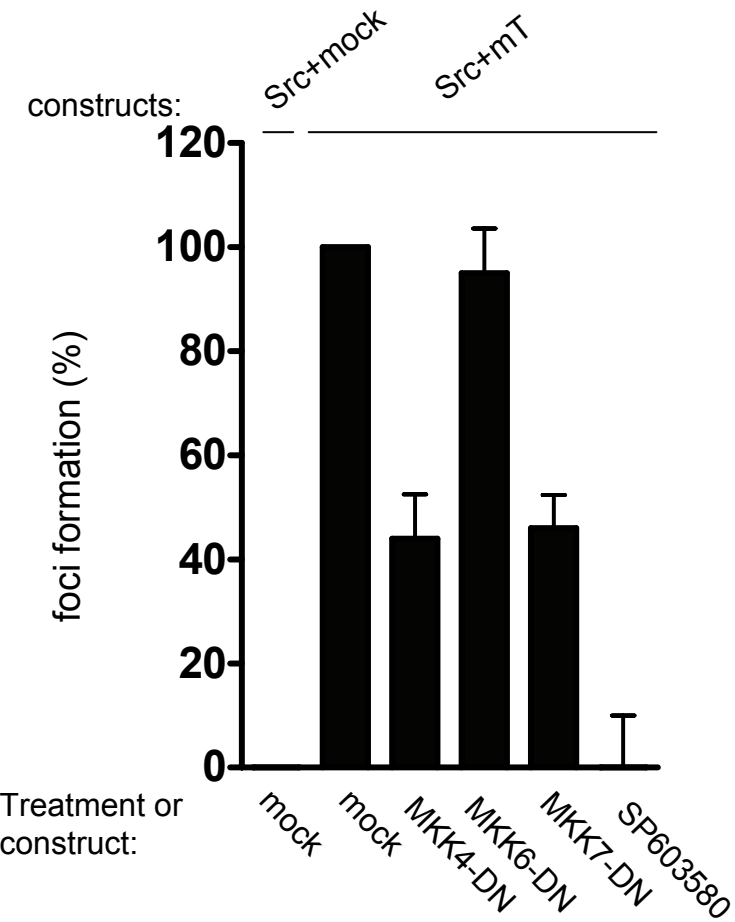


Figure 3e, f

e



f

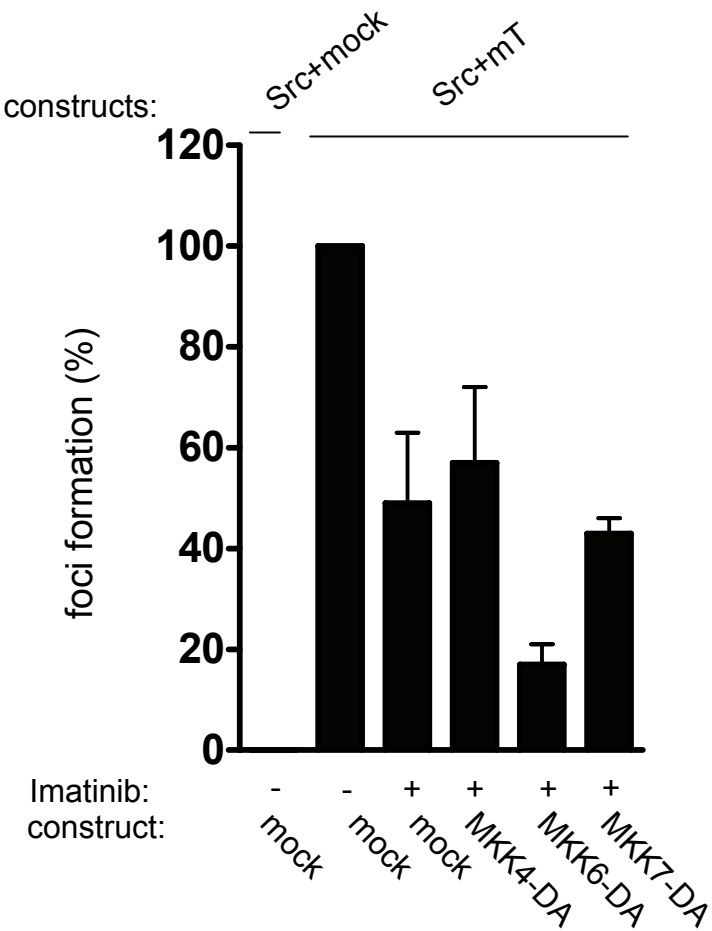


Figure 4a, b

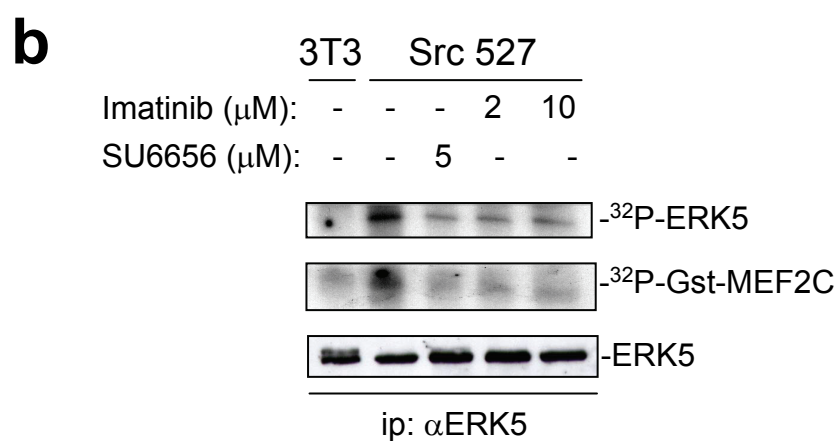
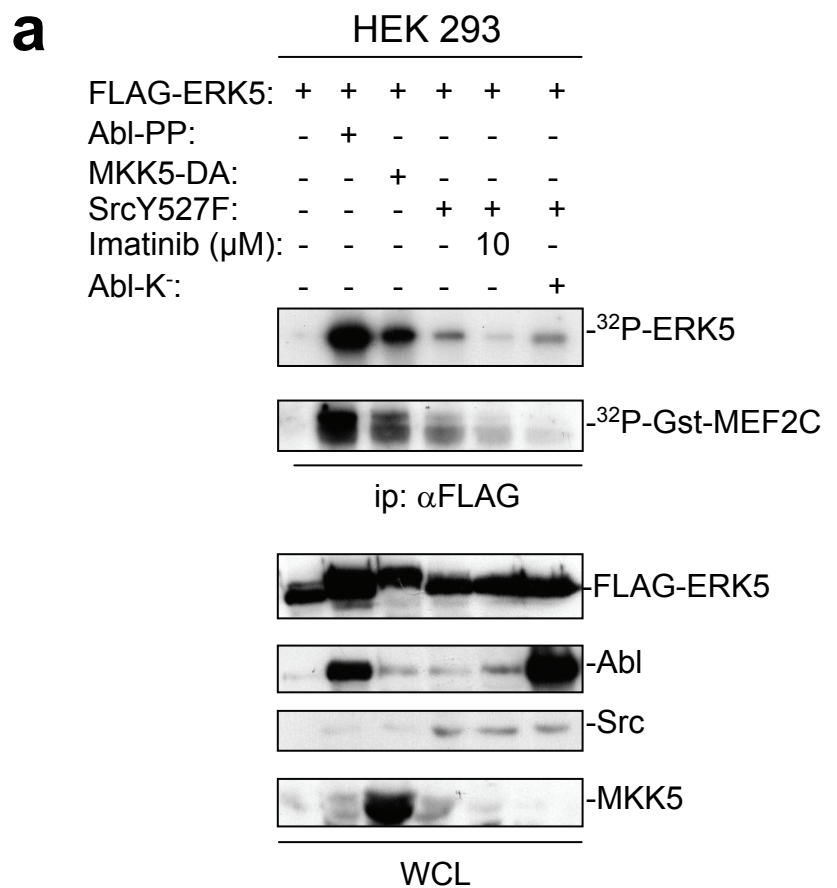
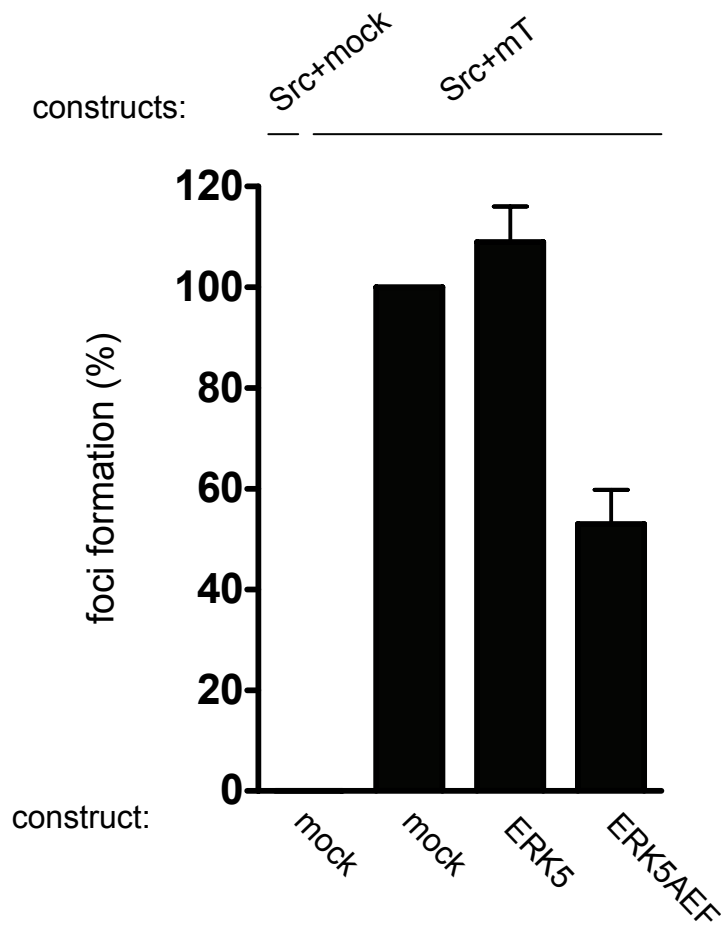


Figure 4c, d

c



d

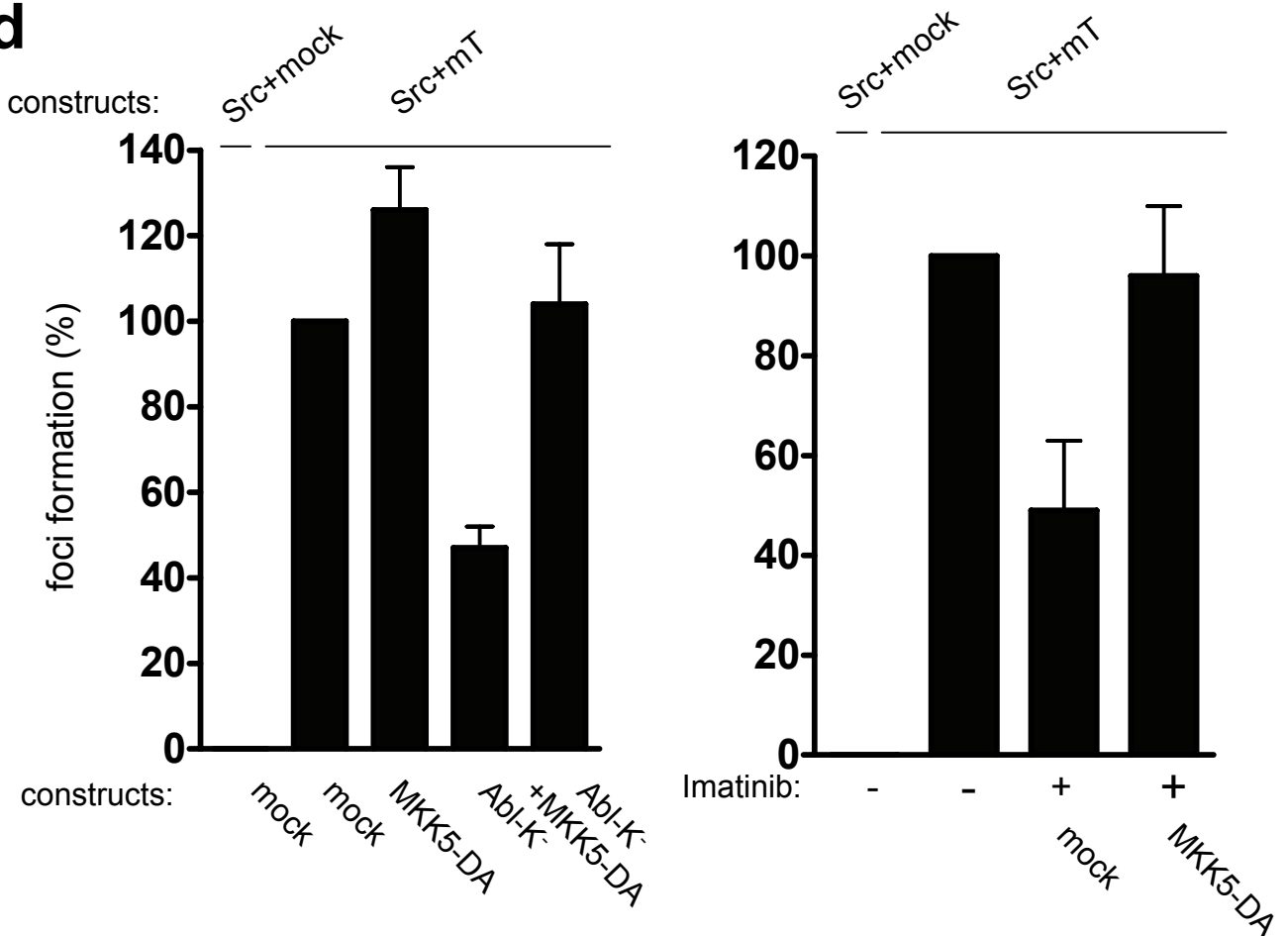
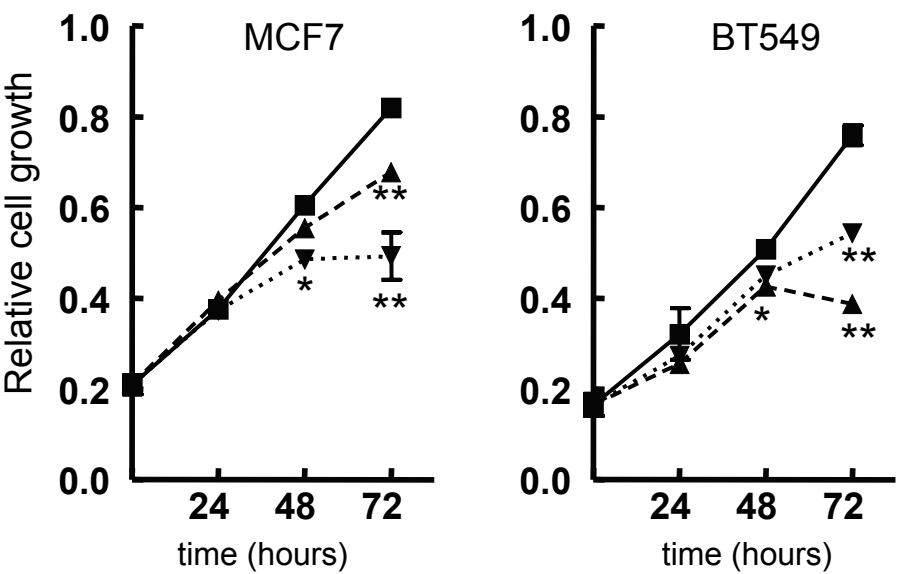
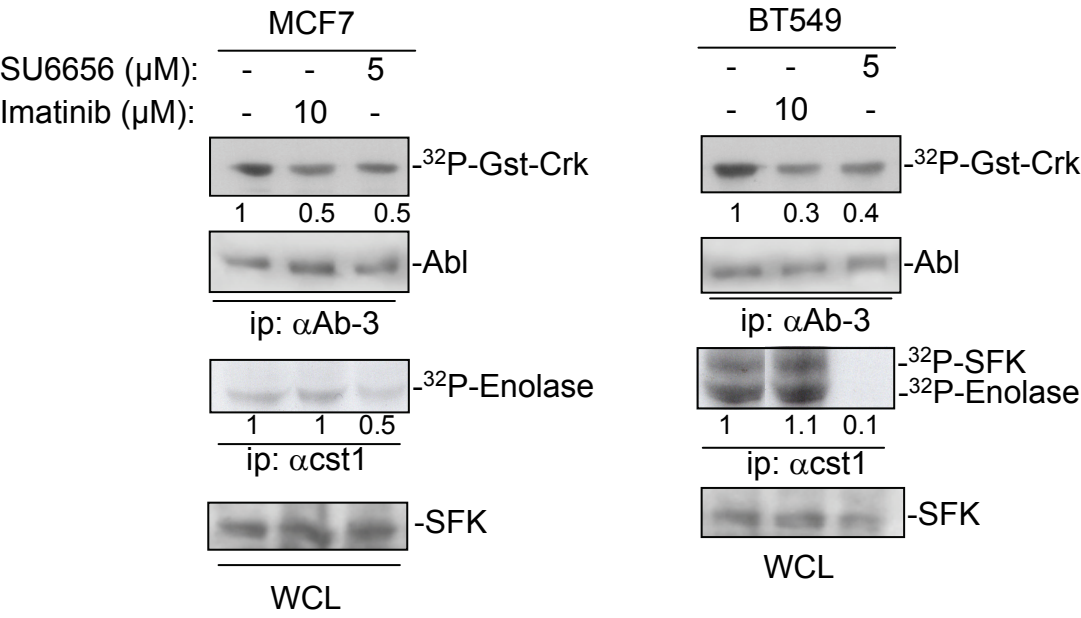


Figure 5

a



b



c

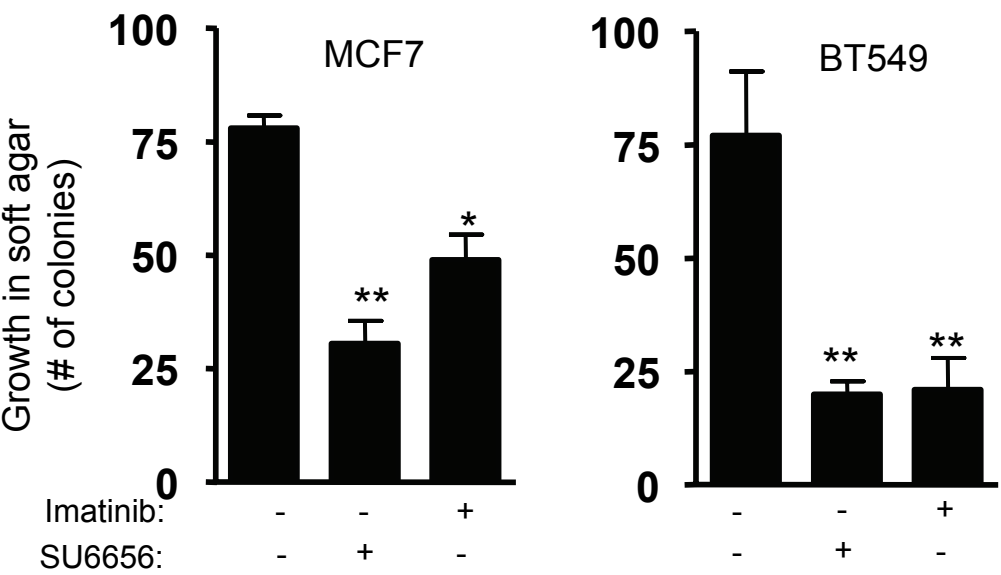
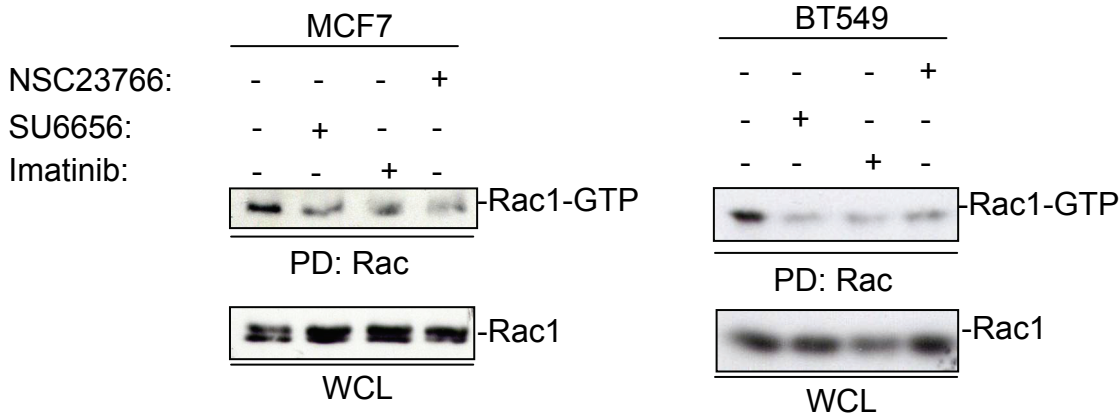
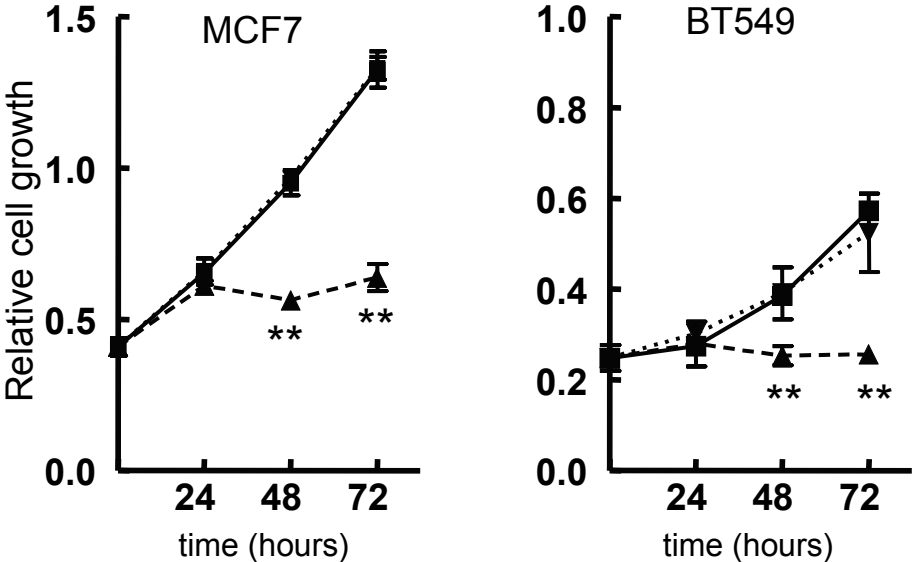


Figure 6a-c

a



b



c

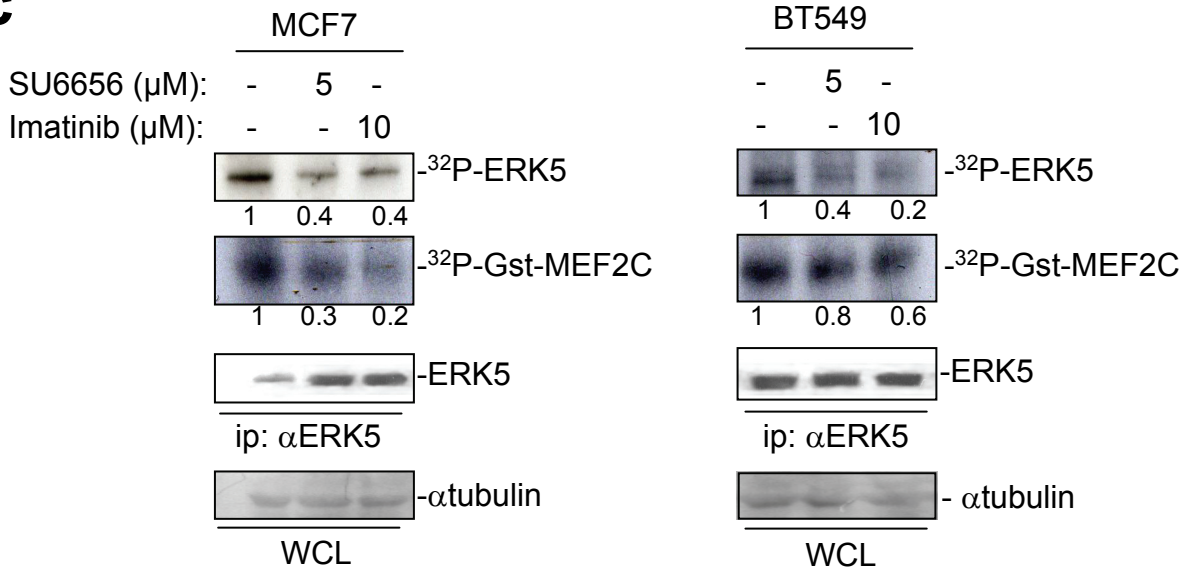
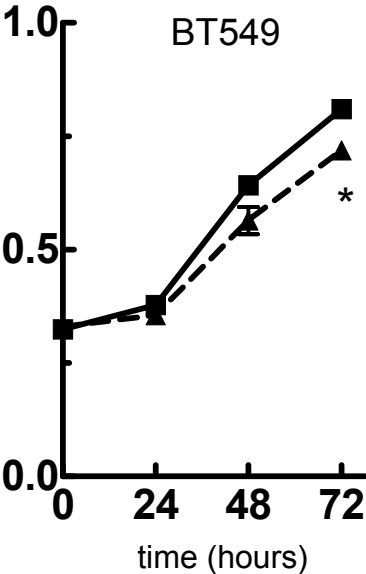
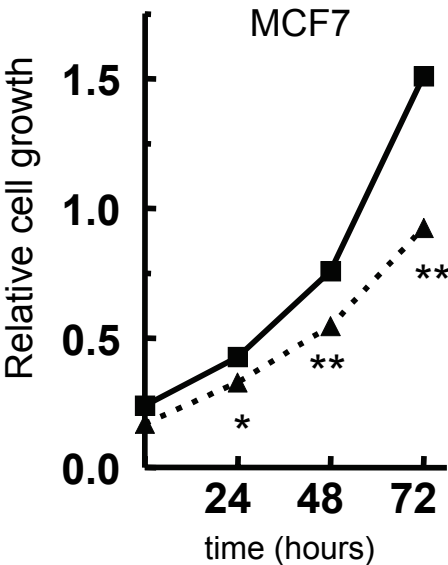
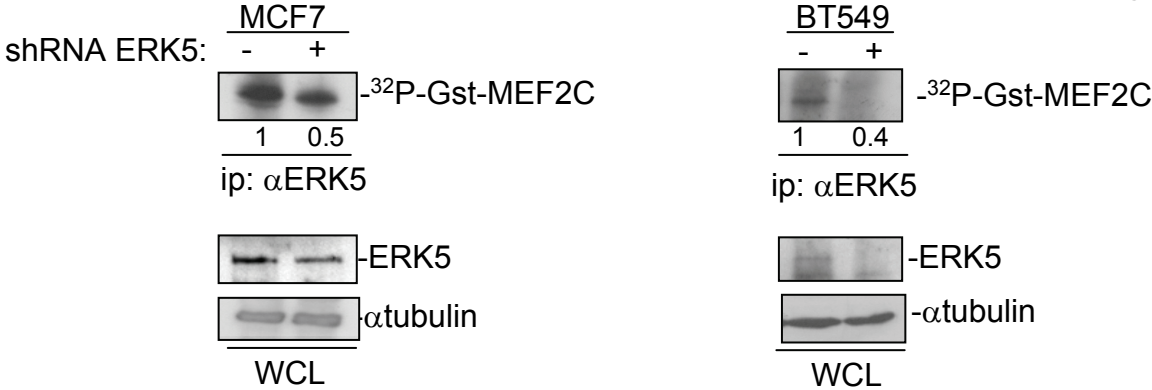


Figure 6d, e

d



e

