Activation of tyrosine kinases by mutation of the gatekeeper threonine

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Protein kinases targeted by small-molecule inhibitors develop resistance through mutation of the 'gatekeeper' threonine residue of the active site. Here we show that the gatekeeper mutation in the cellular forms of c-ABL, c-SRC, platelet-derived growth factor receptor-α and -β, and epidermal growth factor receptor activates the kinase and promotes malignant transformation of BaF3 cells. Structural analysis reveals that a network of hydrophobic interactions—the hydrophobic spine—characteristic of the active kinase conformation is stabilized by the gatekeeper substitution. Substitution of glycine for the residues constituting the spine disrupts the hydrophobic connectivity and inactivates the kinase. Furthermore, a small-molecule inhibitor that maximizes complementarity with the dismantled spine (compound 14) inhibits the gatekeeper mutation of BCR-ABL-T315I. These results demonstrate that mutation of the gatekeeper threonine is a common mechanism of activation for tyrosine kinases and provide structural insights to guide the development of next-generation inhibitors.

Deregulated protein kinases have been linked to numerous diseases including cancer and diabetes as well as inflammation. The targeted inhibition of protein tyrosine kinases is now well established as an effective therapeutic regimen for chronic myeloid leukemia (CML) and several solid tumors[1–4]. Many small-molecule kinase inhibitors have exploited a conserved threonine residue within the ATP binding site for binding specificity[5]. This threonine controls access of the inhibitors to a hydrophobic pocket deep in the active site that is not contacted by ATP, hence leading to its designation as a 'gatekeeper' residue[6]. Substitution of the gatekeeper threonine residue with bulky side chains is a common mechanism of resistance to pharmacological ATP-competitive kinase inhibitors[7,8].

Imatinib has been used successfully to inhibit BCR-ABL in CML[9], c-KIT in gastrointestinal stromal tumor (GIST)[10] and platelet-derived growth factor receptor-α (PDGFRA) in hypereosinophilic syndrome (HES)[11,12]. The first imatinib-resistant mutation described in CML patients was an isoleucine substitution at the gatekeeper residue Thr315 (numbered according to the sequence for the type Ia isoform of c-ABL)[13]. The T315I mutation has been detected in imatinib-naïve CML patients and accounts for ∼20% of the total burden of clinical resistance[14]. Mutation at the analogous position to Thr315 in other imatinib targets such as c-KIT (Thr670) and PDGFRA (Thr674) have been linked to imatinib resistance in patients with GIST and HES, respectively[15,16]. Similarly, the gatekeeper mutation T790M in EGFR causes resistance to gefitinib and erlotinib, and has been detected in lung cancer patients before drug treatment and in the germ line of a family pedigree with several cases of lung cancer[17,18]. The association of gatekeeper-residue mutations with malignancy even in the absence of treatment with kinase inhibitors implies a role in activation of the transforming function of several protein tyrosine kinases. However, the mechanism for this oncogenic activation has not been defined.

Recent inferences drawn from crystallographic and chemical genetic studies suggest that ABL and SRC are regulated in a similar fashion and have highly conserved tertiary structures[19–26]. Interestingly, mutation of the gatekeeper residue has been noted in the sequence of v-SRC from several independent strains of avian Rous sarcoma virus (RSV), but the mechanism responsible for cellular transformation was not clearly defined[27–29]. On the basis of the strong correlation between substitution of the gatekeeper threonine and oncogenic activation of v-SRC, and on structural considerations gleaned from our previous studies with dual SRC-ABL kinase inhibitors[30], we reasoned that substitution of the gatekeeper threonine residue with bulkier residues would be a common mechanism of activation of tyrosine kinases.

In this study, we show that the substitution of a bulkier hydrophobic residue for the gatekeeper threonine in the human tyrosine kinases activates tyrosine phosphorylation. *In silico* modeling of the native and gatekeeper variants of different kinases and crystallographic analysis of SRC-T341I suggest that an isoleucine substitution for the gatekeeper threonine stabilizes a 'hydrophobic spine'[31] that is a characteristic feature of the active state of several kinases. In support

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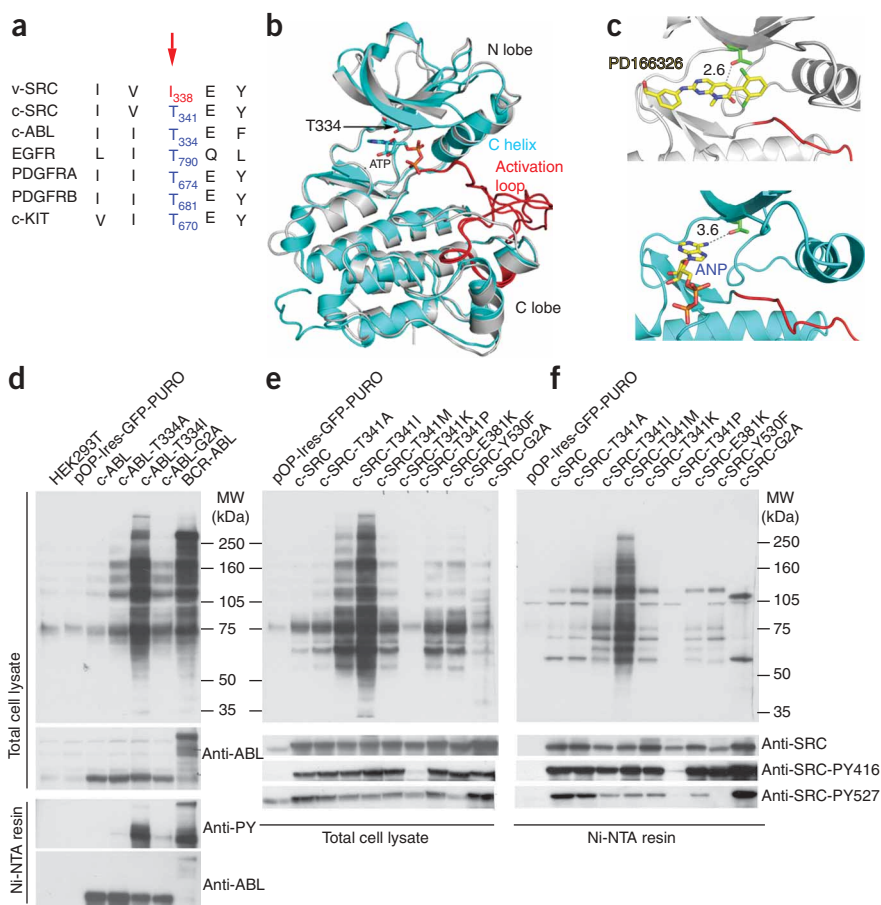
Figure 1 Sequence conservation and structural features of the gatekeeper residue threonine in tyrosine kinases and activation of kinase activity by gatekeeper residue mutation. (**a**) Sequence alignment of the kinase domain hinge region and conservation of the gatekeeper residue in v-SRC, c-SRC, c-ABL and several receptor tyrosine kinases. (**b**) Structural alignment of inactive c-SRC (cyan; PDB 1Y57) and c-ABL (gray; PDB 2G2I) showing the conformational similarities; the kinase hinge region is marked by the arrow. (**c**) An enlarged view of the ABL active site bound to PD166326 (above; PDB 1OPK) and an ATP analog (below; PDB 2G2F), showing the specific interaction of PD166326 but not ATP with the gatekeeper threonine. (**d**) Immunoblot analysis of HEK293T cells expressing BCR-ABL and different variants of c-ABL. Above, total cell lysates probed with anti-phosphotyrosine antibody (anti-PY). The blot was stripped and reprobed with anti-ABL. Below, immunoblots of Ni-NTA–purified histidine-tagged c-ABL proteins probed with anti-PY, followed by stripping and reprobing with anti-ABL antibody. (**e**) Immunoblot analysis of HEK293T cells expressing different c-SRC kinase variants. Above, total cell lysates probed with anti-PY. The blots were stripped and reprobed with anti-SRC, anti-phosphotyrosine-416 (anti-SRC-PY416) and anti-phosphotyrosine-527 (anti-SRC-PY527) antibodies. (**f**) Immunoblot of Ni-NTA–purified c-SRC kinase variants probed with anti-PY followed by stripping and reprobing as described in **e**. MW, molecular weight.

of this hypothesis, we show that disruption of hydrophobic connectivity either pharmacologically or by mutagenesis of residues constituting the spine can effectively inhibit the kinase activity of the gatekeeper variants of c-ABL and BCR-ABL.

RESULTS

Gatekeeper mutations in c-SRC and c-ABL activate the kinase

The gatekeeper residue threonine is found in many tyrosine kinases (**Fig. 1a**). It lies in the hinge region between the N and C lobes of the kinase (**Fig. 1b**), where it controls access to a hydrophobic pocket that helps anchor kinase inhibitors to the active site (**Fig. 1c**). Because v-SRC and BCR-ABL are already deregulated, enzymatically activated protein kinases, we examined the effect of mutation of the gatekeeper residue on the kinase function of c-SRC and c-ABL isoform1b (the gatekeeper threonine is residue 334).
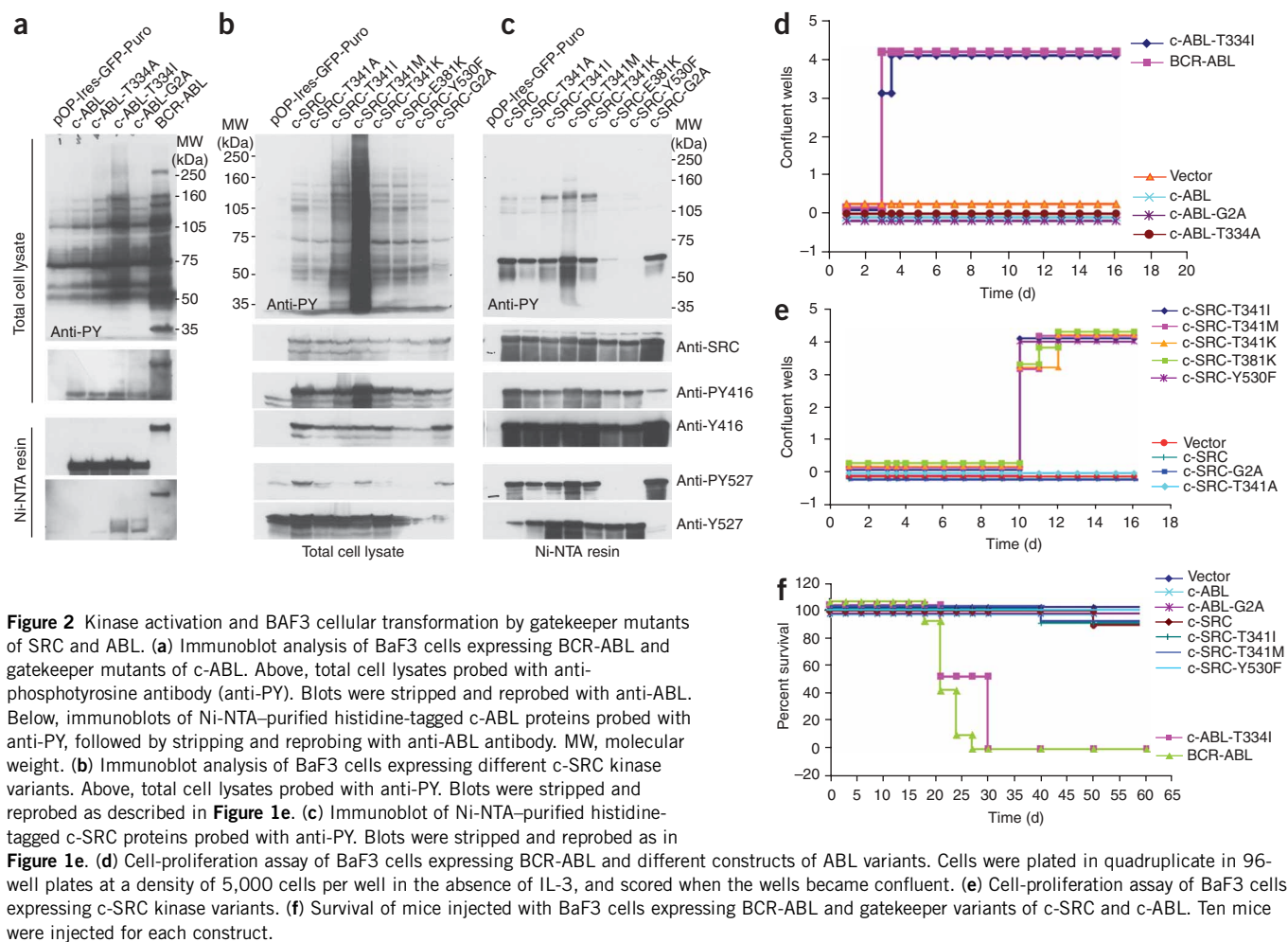
Transient expression in human embryonic kidney cells (HEK293T) of C-terminally His-tagged T334I, T334A and G2A c-ABL mutants revealed elevated kinase activity as compared to native c-ABL (**Fig. 1d**). The myristoylation-defective G2A mutant of c-ABL, which activates the c-ABL kinase[23], was included as a positive control for kinase activation. The T334I variant induced equivalent levels of total cellular phosphotyrosine in cell lysates as BCR-ABL, whereas T334A and G2A substitutions had weaker effects (**Fig. 1d**). After purification of c-ABL and its variants by nickel-resin, we likewise observed increased autophosphorylation of ABL variants, with T334I showing the greatest degree of phosphotyrosine (**Fig. 1d**, below).

Similarly, we found that substitution of isoleucine and methionine at the homologous gatekeeper threonine residue in c-SRC (T341) induced high levels of total cellular phosphotyrosine and increased

tyrosine autophosphorylation; methionine substitution, which likewise introduces hydophobicity, has a more dramatic effect than isoleucine substitution (**Fig. 1e,f**). To further explore the structural consequences of amino acid substitution at the gatekeeper threonine, we created the T341K and T341P mutations. Lysine, which introduces a strong positive charge into the enzymatic active site, caused reduced kinase activation relative to isoleucine and methionine. Proline, which is expected to kink the polypeptide backbone at hinge region, inactivated the enzyme (**Fig. 1e,f**). These observations demonstrate the sensitivity of the enzyme to amino acid substitutions at the threonine residue of the hinge region. Immunoblotting with phosphospecific antibodies against Tyr416 of c-SRC, which is phosphorylated in the enzymatically active state, and Tyr527, which is phosphorylated in the inactive state, revealed that several gatekeeper variants of SRC are expressed as catalytically active enzymes.

Gatekeeper variants of c-ABL and c-SRC transform BaF3 cells

Next, we sought to determine whether kinase-activated variants of c-ABL and c-SRC would show similar patterns of autophosphorylation and induce leukemic transformation in BaF3 cells, a cytokine-dependent hematopoietic progenitor line that has been widely used in assays of oncogenicity by activated tyrosine kinases[32,33]. We expressed gatekeeper mutants and other variants of c-ABL and c-SRC in BaF3 cells by retroviral transduction and puromycin selection. Immunoblotting of total cell protein lysates and purified c-ABL and c-SRC showed a comparable pattern of kinase activation as HEK293T cells for all variants except c-SRC-G2A, which showed partial kinase activation in HEK293T cells but not in BaF3 cells (**Fig. 2a–c**).

Figure 2 Kinase activation and BAF3 cellular transformation by gatekeeper mutants of SRC and ABL. (**a**) Immunoblot analysis of BaF3 cells expressing BCR-ABL and gatekeeper mutants of c-ABL. Above, total cell lysates probed with anti-phosphotyrosine antibody (anti-PY). Blots were stripped and reprobed with anti-ABL. Below, immunoblots of Ni-NTA–purified histidine-tagged c-ABL proteins probed with anti-PY, followed by stripping and reprobing with anti-ABL antibody. MW, molecular weight. (**b**) Immunoblot analysis of BaF3 cells expressing different c-SRC kinase variants. Above, total cell lysates probed with anti-PY. Blots were stripped and reprobed as described in **Figure 1e**. (**c**) Immunoblot of Ni-NTA–purified histidine-tagged c-SRC proteins probed with anti-PY. Blots were stripped and reprobed as in **Figure 1e**. (**d**) Cell-proliferation assay of BaF3 cells expressing BCR-ABL and different constructs of ABL variants. Cells were plated in quadruplicate in 96-well plates at a density of 5,000 cells per well in the absence of IL-3, and scored when the wells became confluent. (**e**) Cell-proliferation assay of BaF3 cells expressing c-SRC kinase variants. (**f**) Survival of mice injected with BaF3 cells expressing BCR-ABL and gatekeeper variants of c-SRC and c-ABL. Ten mice were injected for each construct.

Expression of BCR-ABL and activated PDGFRB, PDGFRA, FMS-like tyrosine kinase-3 (FLT3), EGFR and Janus kinase-2 (JAK2) induces interleukin-3 (IL-3)-independent proliferation of BaF3 cells, which correlates tightly but not absolutely with malignant transformation[32,34–37]. We therefore tested BaF3 cells expressing c-ABL and c-SRC variants for their capacity to survive and proliferate when cultured in the absence of IL-3. Like BCR-ABL, c-ABL-T334I supported robust survival in short-term assays (**Supplementary Fig. 1** online) and rapid IL-3–independent proliferation of BaF3 cells in prolonged cell culture (**Fig. 2d**). In contrast, cells expressing the weakly activated c-ABL variants T334A and G2A or overexpressing native c-ABL showed no detectable enhanced survival or proliferation (**Fig. 2** and **Supplementary Fig. 1**). Cells expressing the activated c-SRC variants T341I, T341M, T341K, T381K and Y530F demonstrated only modest degrees of survival (**Supplementary Fig. 1**) and delayed IL-3–independent cell growth after 10 d of culture (**Fig. 2e**). The c-SRC-G2A and c-SRC-T341A cells showed neither survival nor proliferation (**Fig. 2e** and **Supplementary Fig. 1**). Taken together with the autophosphorylation data, these results demonstrate a correlation between the level of kinase activation and cellular transformation for c-ABL and further suggest that c-ABL is more effective at transforming this hematopoietic cell line than activated variants of SRC. The longer latency of transformation by SRC kinase variants may be due to the fact that activated SRC kinase alone is not sufficient for

transformation and may require additional epigenetic or genetic alterations to transform the BaF3 cells.

To determine whether the *in vitro* assays of IL-3–independent cell survival and proliferation correlated with the development of leukemia *in vivo*, we injected BaF3 cells expressing the c-ABL and c-SRC variants into syngenic Balb/c mice and observed for leukemia induction, with BCR-ABL–expressing BaF3 cells serving as a control. Mice injected with c-ABL-T334I or BCR-ABL–transformed BaF3 cells died within 4 weeks with peripheral blood leukocytosis and massive splenomegaly. In contrast, none of the other variants of c-ABL or c-SRC developed leukemia (**Fig. 2f**). These data demonstrate that mutation at the gatekeeper threonine can activate the intrinsic tyrosine kinase activity of both c-ABL and c-SRC, but only activated variants of c-ABL induce leukemia in BaF3 cells, consistent with a long-standing observation that activated SRC is ineffective at transforming BaF3 cells[38].

Gatekeeper mutation in receptor tyrosine kinases is activating
Transient expression of the receptor tyrosine kinase PDGFRB and its gatekeeper mutation variants (T681A, T681I and T681M) in HEK293T cells showed enhanced kinase autophosphorylation and increased tyrosine phosphorylation of total cellular protein. Like the c-SRC kinase, introduction of the methionine residue (PDGFRB-T681M) generated the most active kinase (**Supplementary Fig. 2a**

online). For PDGFRA, only the T674M variant was associated with kinase activation, as demonstrated by autophosphorylation of purified protein (**Supplementary Fig. 2b**). For unknown reasons, we were unable to achieve adequate expression of EGFR in HEK293T cells.

We next expressed these receptor tyrosine kinase mutants in BaF3 cells. Immunoblotting of total cell lysates and partially purified proteins showed kinase activation for each of the gatekeeper variants of PDGFRB (T681A, T681I and T681M; **Fig. 3a**), PDGFRA (T674I and T674M; **Fig. 3b**) and EGFR (T790I, T790M and L858R; **Fig. 3c**). For these experiments, EGFR-L858R served as a control, because EGFR activation and BaF3 cell transformation has been described previously for this mutation[36]. The results of these gatekeeper mutagenesis studies revealed that, like c-ABL and c-SRC, PDGFRB is maximally activated by the substitution with bulkier residues (isoleucine and methionine) and more modestly activated by alanine substitution (**Fig. 3a**), whereas PDGFRA and EGFR can be activated only by the substitution with isoleucine or methionine but are tolerant of substitution with smaller side chain substitutions such as alanine (**Fig. 3b,c**). Unlike BCR-ABL or c-ABL-T334I, none of the receptors

bearing gatekeeper mutations supported short term IL-3–free survival in BaF3 cells (**Supplementary Fig. 3** online). Like c-SRC-T341M/I, BaF3 cells carrying receptors bearing the gatekeeper variants PDGFRB-T681I, PDGFRB-T681M; PDGFRA-T674I, PDGFRA-T674M; EGFR-T790I, EGFR-T790M and EGFR-L858R showed delayed but robust growth of BaF3 cells after 9–10 d (**Fig. 3d–f**). The longer latency of transformation by these receptor kinase variants may be due to the fact that the activated kinase alone is not sufficient for transformation and may require additional epigenetic or genetic alterations to transform the BaF3 cells. These data demonstrate that the gatekeeper mutations activate the kinase activity in receptor tyrosine kinases and can transform the cytokine-dependent cell line BaF3, although with longer latency than for activated variants of c-ABL.

A hydrophobic spine is assembled during kinase activation

Crystal structures of protein kinases in their active and inactive conformations have provided a general understanding of their regulation via intramolecular autoregulatory interactions[39]. Typically,
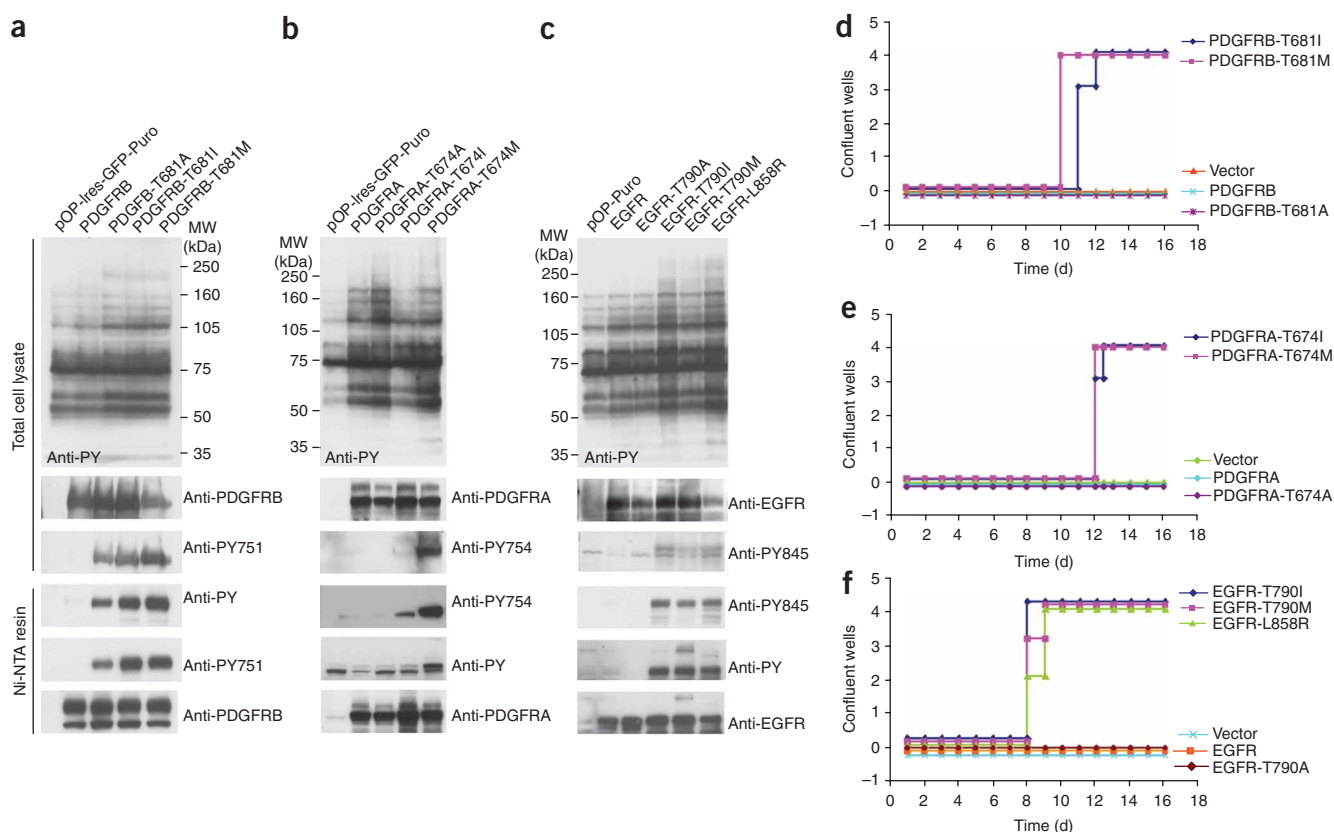

Figure 3 Kinase activation and BAF3 cellular transformation by gatekeeper residue mutation of receptor tyrosine kinases. (**a**) Immunoblot analysis of BaF3 cells expressing gatekeeper mutants of PDGFRB. Above, total cell lysates probed with anti-phosphotyrosine antibody (anti-PY). Blots were stripped and reprobed with anti-PDGFRB antibody and anti–PDGFRB-phosphotyrosine-751 (anti-PY751). Below, immunoblots of Ni-NTA–purified histidine-tagged PDGFRB proteins probed with anti-PY, followed by stripping and reprobing with anti-PDGFRB antibody and anti-PY751. (**b**) Immunoblot analysis of BaF3 cells expressing gatekeeper mutants of PDGFRA. Above, total cell lysates probed with anti-PY. Blots were stripped and reprobed with anti-PDGFRA antibody and anti–PDGFRA-phosphotyrosine-754 (anti-PY754). Below, immunoblots of Ni-NTA–purified histidine-tagged PDGFRA proteins probed with anti-PY, followed by stripping and reprobing with anti-PDGFRA antibody and anti-PY754. (**c**) Immunoblot analysis of BaF3 cells expressing gatekeeper mutants of EGFR. Above, total cell lysates probed with anti-PY. Blots were stripped and reprobed with anti-EGFR antibody and anti–EGFR-phosphotyrosine-845 (anti-PY845). Below, immunoblots of Ni-NTA–purified histidine-tagged EGFR proteins probed with anti-PY, followed by stripping and reprobing with anti-EGFR antibody and anti-PY845. (**d–f**) Cell-proliferation assays of BaF3 cells expressing wild-type and gatekeeper mutants of PDGFRB (**d**), PDGFRA (**e**) and EGFR (**f**). Cells were plated in quadruplicate in 96-well plates at a density of 5,000 cells per well in the absence of IL-3 and scored when the wells became confluent.
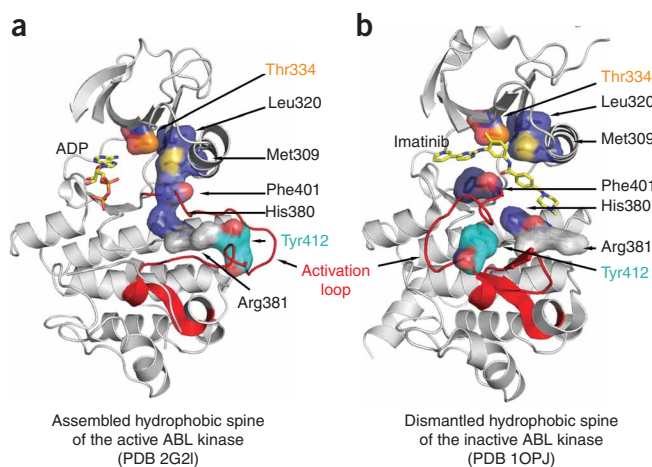
a

b

Assembled hydrophobic spine
of the active ABL kinase
(PDB 2G2I)

Dismantled hydrophobic spine
of the inactive ABL kinase
(PDB 1OPJ)

Figure 4 The active conformation of ABL is stabilized by a hydrophobic spine linking the gatekeeper threonine to the activation loop. (**a**) The surface projections of the hydrophobic spine that assembled during ABL kinase activation in ABL-ADP conformation (shown in blue; PDB 2G2I). The gatekeeper residue, Thr334, is shown as an orange surface. (**b**) The surface projections of the dismantled hydrophobic spine in the inactive kinase conformation of ABL–imatinib (PDB 1OPJ). Imatinib binds to the inactive kinase which is stabilized by the DFG-out conformation caused by dismantling of the hydrophobic spine.

activation involves changes in the position and orientation of the activation loop and the catalytic c-helix. A surface comparison of active kinase structures has revealed a network of highly conserved hydrophobic or amino-aromatic interactions called the hydrophobic spine, which stabilizes the active kinase conformation[31].

In ABL kinase, the spine comprises four residues, Leu320, Met309, Phe401 and His380, which form a chain of hydrophobic interactions from the N lobe via the active site to the Tyr412 of the activation loop (**Fig. 4a**). These interactions are disrupted in the inactive state (**Fig. 4b**). The gatekeeper residue Thr334 is situated near the tip of the hydrophobic spine. Our modeling analysis suggests that substitution of a bulkier hydrophobic residue at this position would stabilize

the active state by strengthening the spine. In our model, Leu403 serves as a bipositional switch that positions Asp400 of the DFG motif in the correct orientation to enable catalysis (**Supplementary Fig. 4a,b** online). In the active state the backbone of Leu403 coordinates Arg381 of the catalytic HRD motif, whereas in the inactive state the sidechain of Leu403 stabilizes Phe401 in the catalytically quiescent 'DFG-out' conformation. Furthermore, computational modeling of the published structures of SRC and EGFR revealed similar hydrophobic interactions in SRC (**Supplementary Fig. 5** online) and EGFR (**Supplementary Fig. 6** online), where the gatekeeper residue sits at the top of the spine, stabilizing the active state. The active SRC and EGFR kinases maintain a hydrophobic spine from the gatekeeper threonine to Tyr419 and Tyr869 of the activation loop, respectively (**Supplementary Figs. 5b** and **6b**). These interactions are disrupted in the inactive state (**Supplementary Figs. 5d** and **6d**). As in ABL, Leu410 of SRC and Leu858 of EGFR serve as a bipositional switch for kinase regulation[40,41]. Altogether, these *in silico* analyses suggest that gatekeeper mutants stabilize the active state of the kinase by contributing to the hydrophobic spine.

Figure 5 The hydrophobic spine in active and inactive SRC kinases. (**a**) Crystal structure of chicken c-SRC-T338I bound to ATPγS. SRC-T338I is homologous to T334I in human c-SRC. Chicken SRC residues are numbered according to the human c-SRC kinase numbering. The residues Leu328, Met317, Phe408 and His387, which constitute the hydrophobic spine, are shown in blue. The gatekeeper isoleucine residue is shown in orange. The activation loop is shown in red. (**b**) The inactive conformation of chicken c-SRC (PDB 2SRC), colored as in **a**. (**c**) Active site of Lck kinase (PDB 1QPC) in the active state bound with AMP-PNP shown in yellow. Gatekeeper residue Thr316 and the catalytic Lys273 are shown as green surfaces. The water molecules sandwiched between the residues Thr316 and Lys273 are shown as red circles. The interactions of lysine with ANP and the catalytic Glu288 are shown; bond distances are presented in angstroms. (**d**) Active site of SRC-T341I kinase domain (PDB 3DQW) bound with ATPγS shown in yellow. The surfaces of the side chains for residues Ile341 and Lys298 are shown in green. Interactions of Lys298 with Glu310 and ATPγS are mapped and the bond distances are indicated in angstroms. (**e**) Active site of insulin receptor kinase (IRK; PDB entry 1GAG) bound with ANP shown in yellow. The surfaces of the side chains for residues Met1076 and Lys1030 are shown in green. Interactions of catalytic Lys1030 with Glu1047 and ANP are mapped, and the bond distances are indicated in angstroms.

a

b

c-SRC-T341I
active kinase

Wild type c-SRC
inactive kinase

c

d

e

Active site of native Lck
(active state)
PDB 1QPC

Active site of SRC-T341I
(active state)
PDB 3DQW

Active site of native IRK
(active state)
PDB 1GAG