

ORIGINAL ARTICLE

# *PDGFRB* mutants found in patients with familial infantile myofibromatosis or overgrowth syndrome are oncogenic and sensitive to imatinib

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Recently, germline and somatic heterozygous mutations in the platelet-derived growth factor receptor  $\beta$  (*PDGFRB*) have been associated with familial infantile myofibromatosis (IM), which is characterized by soft tissue tumors, and overgrowth syndrome, a disease that predisposes to cancer. These mutations have not been functionally characterized. In the present study, the activity of three *PDGFRB* mutants associated with familial IM (R561C, P660T and N666K) and one *PDGFRB* mutant found in patients with overgrowth syndrome (P584R) was tested in various models. The P660T mutant showed no difference with the wild-type receptor, suggesting that it might represent a polymorphic variant unrelated to the disease. By contrast, the three other mutants were constitutively active and able to transform NIH3T3 and Ba/F3 cells to different extents. In particular, the germline mutant identified in overgrowth syndrome, P584R, was a stronger oncogene than the germline R561C mutant associated with myofibromatosis. The distinct phenotypes associated with these two mutations could be related to this difference of potency. Importantly, all activated mutants were sensitive to tyrosine kinase inhibitors such as imatinib, nilotinib and ponatinib. In conclusion, the *PDGFRB* mutations previously identified in familial IM and overgrowth syndrome activate the receptor in the absence of ligand, supporting the hypothesis that these mutations cause the diseases. Moreover, imatinib seems to be a promising treatment for patients carrying these mutations. To our knowledge, these are the first confirmed gain-of-function point mutations of *PDGFRB* in human cancer.

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## INTRODUCTION

Infantile myofibromatosis (IM), although rare, is one of the most prevalent tumors of soft tissue in childhood.<sup>1,2</sup> It is characterized by firm nodules located in the skin, subcutaneous soft tissues, bones, muscles and viscera,<sup>3,4</sup> which generally develop before the age of 2 years.<sup>5,6</sup> The tumor cell morphology is intermediate between smooth muscle cells and fibroblasts.<sup>1,3,6</sup> There are two main forms of IM: the most common is the solitary form with a single nodule whereas a second form with multiple lesions in the bones and soft tissues also exists.<sup>2,3,5</sup> IM has a good prognosis and regression often occurs, except in the rare cases of visceral involvement, which is lethal in up to 70% of the cases.<sup>3,6</sup> Familial cases of IM have been reported with both recessive and dominant modes of inheritance.<sup>1,7,8</sup> Currently, definitive guidelines for treatment of IM with visceral involvement do not exist.<sup>2</sup>

Recently, point mutations in platelet-derived growth factor (PDGF) receptor  $\beta$  (*PDGFR $\beta$* ) were associated with familial IM.<sup>8,9</sup> PDGF receptors, *PDGFR $\alpha$*  and *PDGFR $\beta$* , are receptor tyrosine kinases that stimulate cell survival, proliferation and motility.<sup>10</sup> They are encoded by two highly related genes, *PDGFRA* and *PDGFRB*, and are expressed in various cell types, including fibroblasts.<sup>11</sup> Both genes are essential for proper embryo development in mice.<sup>12,13</sup> *PDGFR* proteins are divided into three different parts: the extracellular ligand binding domain, the transmembrane  $\alpha$ -helix and the intracellular kinase domain. The activity of the kinase domain is controlled by the juxtamembrane domain, the activation loop and the C-terminal tail, which

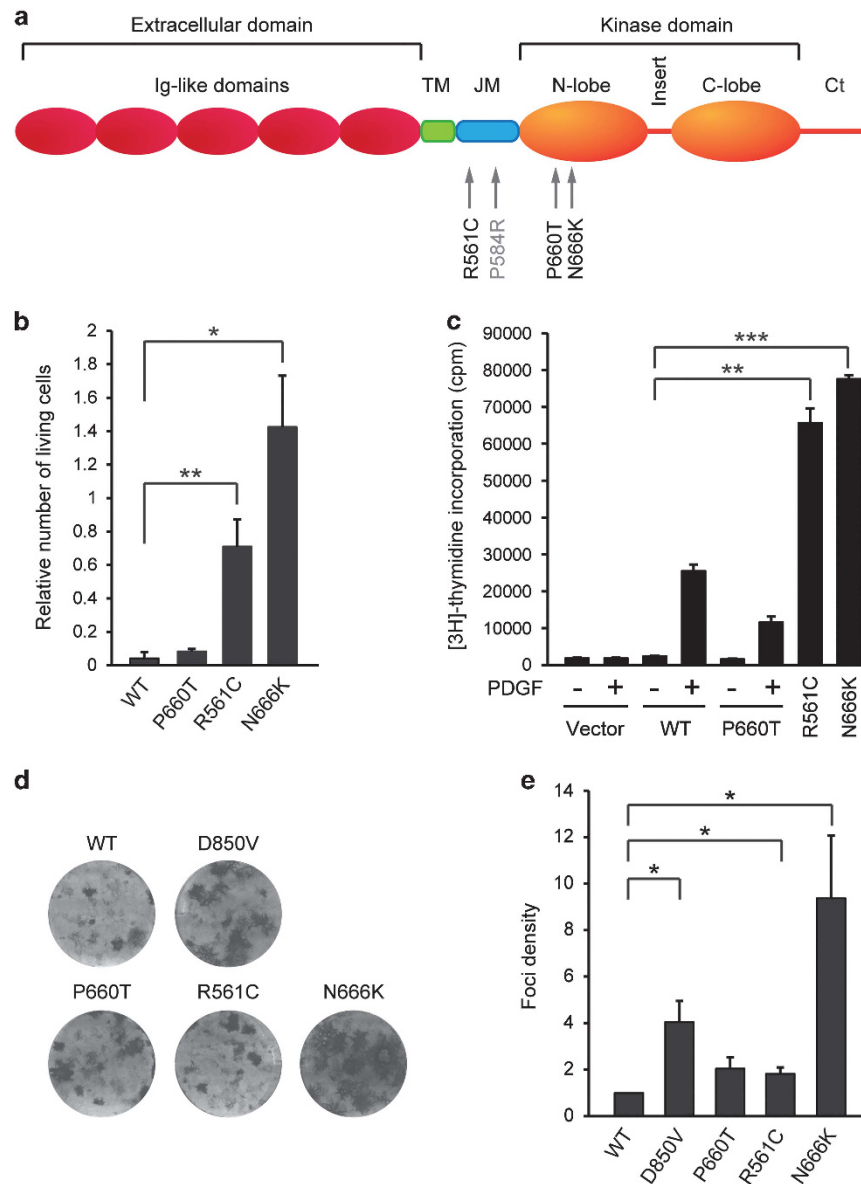
keep the receptor silent in the absence of ligand.<sup>14–16</sup> Upon PDGF binding, the receptor dimerizes and undergoes a conformational change, which activates the kinase domain. *PDGFRs* *trans*-autophosphorylate on tyrosine residues, leading to the binding of Src homology 2-domain-containing proteins, and subsequently, to the activation of signaling pathways, such as mitogen-activated protein (MAP) kinases, PLC $\gamma$  (phospholipase C $\gamma$ ), signal transducers and activators of transcription (STAT) and PI3K (phosphatidylinositol-3 kinase).<sup>10,14,17</sup>

Martignetti *et al.*<sup>8</sup> identified two germline missense mutations in *PDGFR $\beta$*  in eight families with members diagnosed with familial IM: the R561C substitution was present in seven families whereas the P660T was present in only one family.<sup>8</sup> The R561 residue is located in the juxtamembrane domain of the receptor while the P660 residue is found in the kinase domain (Figure 1a). The R561C mutation was found in four additional families with IM in a second study.<sup>9</sup> Interestingly, in one of the affected individuals, a somatic mutation located in the kinase domain, N666K, was found in addition to the germline one.<sup>9</sup> All these mutations associated with familial IM are heterozygous.<sup>8,9</sup> Their impact on the receptor function has not been demonstrated experimentally.

Germline mutations in *PDGFR $\beta$*  have also been associated with overgrowth syndromes, which are characterized by accelerated linear growth with increased risks of cancer and cognitive disorders.<sup>18</sup> In a recent study, a germline mutation in the juxtamembrane domain of *PDGFR $\beta$*  (Figure 1a), P584R, was identified in two girls presenting an overgrowth phenotype with

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**Figure 1.** The R561C and N666K mutants, but not P660T, transform cells. **(a)** Schematic representation of PDGFR $\beta$  and localization of the mutations identified in familial IM (black) and overgrowth syndrome (gray). Ig-like, immunoglobulin-like; TM, transmembrane domain; JM, juxtamembrane domain; N-lobe, N-terminal lobe; C-lobe, C-terminal lobe; Ct, C-terminal tail. **(b)** Ba/F3 cells stably expressing wild-type (WT) PDGFR $\beta$ , P660T, R561C or N666K were washed twice and cultured without IL-3 for 2 days. Living cells were counted on day 2 compared with day 0. As a control, cells were also seeded in the presence of IL-3 and the number of living cells on day 2 was similar in each condition (data not shown). **(c)** Ba/F3 cells stably expressing WT PDGFR $\beta$  or P660T and selected cells expressing R561C or N666K were washed twice and treated with control medium or PDGF-BB. Cell proliferation was assessed after 24 h by measuring the incorporation of [ $^3$ H]-thymidine. As a control, [ $^3$ H]-thymidine incorporation was similar in every cell lines seeded with IL-3 (data not shown). **(b, c)** One representative experiment out of four is shown with s.d. **(d)** NIH3T3 cells were transfected with WT PDGFR $\beta$ , D850V, P660T, R561C or N666K in triplicate and processed as described.<sup>27</sup> Three weeks after transfection, cells were fixed in ice-cold methanol for 20 min and foci were stained with 0.2% crystal violet (Sigma-Aldrich, Saint-Louis, MO, USA) in 20% ethanol for 20 min. **(e)** Foci density was quantified by using the Quantity One software (Bio-Rad) and normalized to 1 for the wild-type receptor. The average of three independent experiments is represented with s.e.m. (one-sided Student's *t*-test). \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

severe neurological problems.<sup>19</sup> Interestingly, one of these two patients presented a myofibroma on her mandibula.<sup>19</sup>

Although PDGFRA mutations have been associated with various types of cancers such as gastrointestinal stromal tumors (GIST), glioblastoma and inflammatory fibroid polyps,<sup>10,16,20</sup> the occurrence of PDGFRB activating point mutations have not been confirmed yet. Furthermore, mice carrying a weak activating point mutation in PDGFR $\beta$ , D849N, are viable and show no sign of tumor

development.<sup>21</sup> Only rare PDGFRB fusions with different partner genes were reported in myeloid neoplasms associated with hypereosinophilia.<sup>16,20,22,23</sup> These patients can be efficiently treated with imatinib, a tyrosine kinase inhibitor, which was initially developed for BCR-ABL-positive chronic myeloid leukemia.<sup>20</sup> By contrast, loss-of-function mutations in PDGFR $\beta$  have a role in idiopathic basal ganglia calcification (or Fahr disease).<sup>24–26</sup>

The aim of the present work was to characterize the PDGFR $\beta$  mutations associated with familial IM and overgrowth syndrome. We demonstrate that the somatic N666K mutation and, to a lesser extent, the germline R561C substitution activate PDGFR $\beta$  and allow cell transformation. In contrast, PDGFR $\beta$  P660T behaves like the wild-type receptor. The germline mutation P584R associated with overgrowth elicits a stronger response than the R561C substitution. Finally, activated mutants are sensitive to tyrosine kinase inhibitors such as imatinib.

## RESULTS

The N666K mutant and, to a lesser extent, the R561C mutant transform cells

We first assessed the transforming potential of three familial IM-associated PDGFR $\beta$  mutations, namely P660T, R561C and N666K (Figure 1a), in two different cell lines.

First, we tested their ability to transform Ba/F3 cells, which need interleukin (IL)-3 in the culture medium to survive and proliferate, except if they express an oncogene. This is a widely used and very sensitive model of cell transformation.<sup>27,28</sup> We stably expressed the empty vector, wild-type PDGFR $\beta$  or its mutants (P660T, R561C or N666K). The expression of the different receptors at the cell surface was verified by flow cytometry (Supplementary Figure 1). Then, cells were washed and cultured in a medium without IL-3. We determined the number of cells that were still alive 2 days after IL-3 removal (Figure 1b). We observed that the relative number of living Ba/F3 cells expressing the R561C and N666K mutants was higher than that of cells expressing the wild-type receptor and the P660T mutant. About 2 weeks after IL-3 removal, we obtained selected cell lines steadily growing without IL-3 for the N666K mutant but not for the wild-type receptor or the P660T mutant. Regarding R561C, we also obtained IL-3-independent selected cell lines, but the process was slower: it took more than 4 weeks. The proliferation of these cells was assessed by quantifying the incorporation of [<sup>3</sup>H]-thymidine (Figure 1c). Selected cells expressing the R561C and N666K mutants proliferated in the absence of exogenous stimulation, whereas cells expressing the wild-type receptor and the P660T mutant only proliferated when they were stimulated with PDGF. These results were confirmed by a colony formation assay in the absence of cytokines (Supplementary Figure 2). Selected cells expressing the N666K mutant gave rise to more colonies than those expressing the R561C mutant, while the wild-type receptor and the P660T mutant did not allow colony formation.

To further establish the transforming potential of the mutants, we performed a foci formation assay in NIH3T3 fibroblasts that were transfected with the wild-type receptor or one of the mutants. PDGFR $\beta$  D850V, which corresponds to the gastrointestinal stromal tumor-associated D842V mutation in PDGFR $\alpha$ <sup>29</sup> and to the activating D849V substitution in murine PDGFR $\beta$ ,<sup>30</sup> was used as a positive control. As expected, the wild-type receptor did not give rise to many foci whereas cells transfected with the D850V mutant formed robust foci (Figure 1d). Interestingly, the N666K mutant gave rise to a large number of foci, even more than the D850V mutant. Regarding the two other substitutions, R561C and P660T, it was difficult to assess whether they were different from the wild-type receptor by naked eye (Figure 1d). Nevertheless, the quantification of three independent experiments showed that the increase in foci density was significant for the R561C mutant, as well as D850V and N666K, compared with the wild-type receptor (Figure 1e).

Altogether, these results show that the N666K mutant transforms Ba/F3 and NIH3T3 cells while R561C is a weaker oncogene. On the contrary, the P660T mutant does not induce cell transformation in our assays.

The R561C and N666K mutants are constitutively activated

We next tested whether the mutations constitutively activated the receptor kinase activity. Receptors from stably transfected Ba/F3 cells were immunoprecipitated and their expression and phosphorylation levels were assessed by western blot (Figure 2a).

Wild-type PDGFR $\beta$  and the mutants were expressed at a similar level, except the N666K mutant whose expression was higher. As expected, two major bands were present for the different receptors: the upper one represents the mature glycosylated form whereas the lower one is the immature form of the receptor located in the endoplasmic reticulum.<sup>31</sup> The wild-type and the P660T receptors were phosphorylated only in response to PDGF. In contrast, the R561C and N666K mutants were constitutively phosphorylated (Figure 2a). Noticeably, the phosphorylation of R561C was not detectable in some of the selected cell lines used in replicate experiments.

We next analyzed the activation of different signaling pathways by western blot (Figure 2b). In agreement with their ability to transform cells and their constitutive activation, we observed that the R561C and N666K mutants induced the phosphorylation of ERK1/2, PLC $\gamma$ , STAT3, STAT5 and Akt in the absence of PDGF stimulation. STAT phosphorylation by the R561C mutant was weaker than that induced by the N666K. The P660T mutant behaved as the wild-type receptor. Interestingly, phosphorylated STAT3 and STAT5 were barely detectable in stimulated cells expressing the wild-type receptor or the P660T mutant (Figure 2b). This was consistent in all independent selected cell lines.

We confirmed these results in luciferase reporter assays. MCF7 and HT-1080 cells were transfected with wild-type or mutant PDGFR $\beta$  and with a reporter construct sensitive to MAP kinases (Figure 2c) or to STAT transcription factors (Figure 2d). In line with western blot experiments, the R561C and N666K mutants constitutively activated MAP kinases and STAT, whereas the P660T mutant showed no significant difference with the wild-type receptor (Figures 2c and d).

Taken together, our results demonstrate that the R561C and N666K mutants, in the absence of PDGF, activate signaling pathways that are normally activated by the stimulated wild-type receptor. Furthermore, these two mutants activate STATs to a greater extent than the stimulated wild-type and P660T receptors.

The overgrowth-associated P584R mutant is more active than R561C

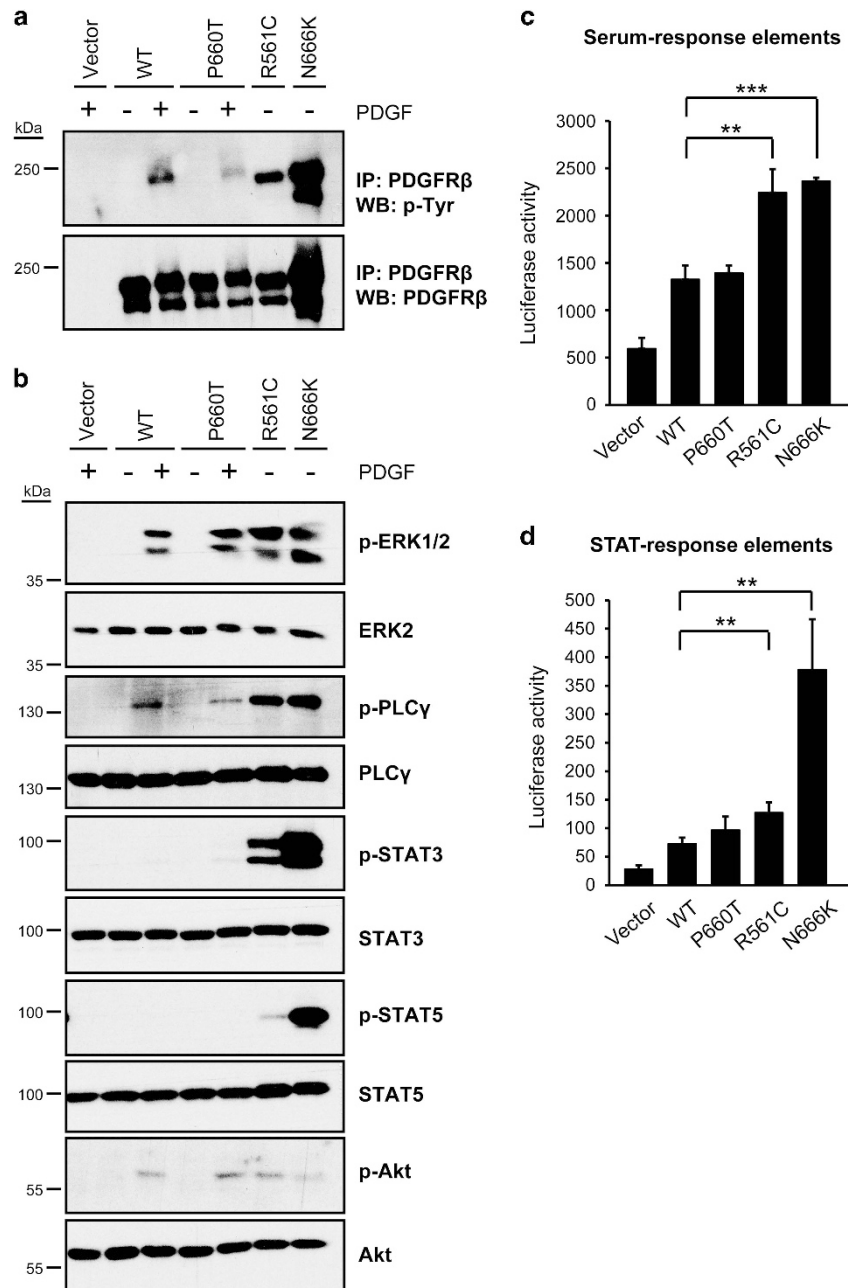
In the course of the present project, the overgrowth-associated P584R substitution was described. It is a germline mutation, like the familial IM-associated R561C mutation. We compared the R561C and P584R substitutions to understand how two mutations located in the same part of PDGFR $\beta$ , the juxtamembrane domain, could give rise to different phenotypes.

We first verified whether the P584R substitution had an impact on PDGFR $\beta$  activity by performing luciferase reporter assays. We observed that the P584R mutant was able to activate MAP kinases and STAT reporters in the absence of PDGF to a greater extent than R561C (Figures 3a and b). To confirm this difference, we performed a foci formation assay in NIH3T3 cells that showed that the P584R mutant gave rise to more foci than the R561C mutant (Figure 3c). We also expressed P584R in Ba/F3 cells. After verifying the cell surface expression of the mutant by flow cytometry (Supplementary Figure 1), we counted the number of living cells after IL-3 removal (Figure 3d). Surprisingly, 3 days after IL-3 removal, the P584R mutation conferred a modest survival advantage compared with the wild-type receptor. In contrast, the relative number of cells expressing the R561C mutant was much higher, in agreement with Figure 1b. However, about 3 weeks after IL-3 removal, we obtained selected cells for the

P584R mutant, while it took more than 4 weeks to obtain IL-3-independent selected cell lines for the R561C mutant. These cells were able to proliferate in the absence of PDGF stimulation (data not shown). Thus, both mutations stimulated Ba/F3 cells proliferation with different kinetics. Furthermore, in a colony formation assay, selected cells expressing the P584R mutant gave

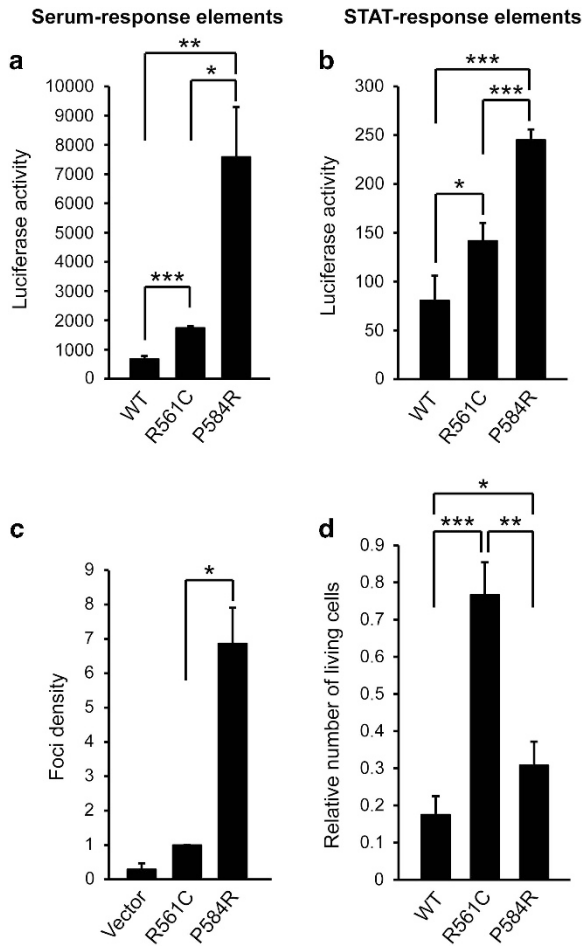
rise to a higher number of colonies, which were also more spread, compared with those expressing the R561C mutant (Supplementary Figure 2).

Taken together, our results show that the overgrowth-associated P584R mutation activates PDGFR $\beta$  to a greater extent than the R561C substitution.



**Figure 2.** PDGFR $\beta$  R561C and N666K are constitutively phosphorylated and constitutively activate signaling pathways. **(a)** Analysis of the phosphorylation and expression levels of the wild-type and mutated receptors by western blot. Ba/F3 cells stably expressing WT PDGFR $\beta$  or P660T and selected cells expressing R561C or N666K were starved for 4 h and stimulated with PDGF-BB for 15 min or left untreated. PDGFR $\beta$  was immunoprecipitated and analyzed by western blot experiments using an anti-phosphotyrosine antibody. Membranes were re-probed with an anti-PDGFR $\beta$  antibody. As a negative control, cells expressing the empty vector were used. Representative blots are shown ( $n=4$ ). **(b)** Analysis of the phosphorylation of ERK1/2, PLC $\gamma$ , STAT3, STAT5 and Akt by western blot. Ba/F3 cells stably expressing WT PDGFR $\beta$  or P660T and selected cells expressing R561C or N666K were starved for 4 h and stimulated with PDGF-BB for 15 min or left untreated. The phosphorylated protein was first analyzed and the membrane was re-probed with an antibody targeting the corresponding protein. Similar results were obtained in four independent experiments. **(c)** MCF7 cells were co-transfected with WT or mutated PDGFR $\beta$ ,  $\beta$ -galactosidase and an SRE promoter controlling luciferase expression. Cells were washed 4 h after transfection and left in the incubator for 24 h. The results are expressed as a ratio between luciferase and  $\beta$ -galactosidase activity. One representative experiment out of four is shown with s.d. **(d)** HT-1080 cells were processed as in **(c)**, except that the luciferase expression was controlled by a STAT-driven promoter (pGRR5). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .





**Figure 3.** The P584R mutant found in overgrowth syndrome is more active than the R561C. (a) MCF7 cells were co-transfected with WT PDGFR $\beta$ , R561C or P584R,  $\beta$ -galactosidase and an SRE promoter controlling luciferase expression and processed as described in (b). One representative experiment out of three is shown with s.d. (b) HT-1080 cells were processed as in (a), except that the luciferase expression was controlled by a STAT-driven promoter (pGRR5). (c) NIH3T3 cells were transfected with the empty vector, R561C or P584R in triplicate and processed as described.<sup>27</sup> Foci were stained as described in Figure 1e and normalized to 1 for the R561C mutant. The average of three independent experiments is represented. (d) Ba/F3 cells stably expressing WT PDGFR $\beta$ , R561C or P584R were processed as in Figure 1b except that they were cultured for 3 days. The histogram represents the relative number of living cells on day 3 compared with day 0. One representative experiment out of two is shown with s.d. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

The R561C, N666K and P584R mutants can induce cancer development *in vivo*

Our data indicate that two familial IM-associated mutations, R561C and N666K, and the overgrowth-associated mutation P584R constitutively activate PDGFR $\beta$ . To further assess the oncogenic properties of these mutations, we used a previously described *in vivo* model of cancer development.<sup>32</sup> Selected Ba/F3 cells expressing the R561C, N666K or P584R mutant were tumorigenic *in vivo* when intravenously injected into immunodeficient BALB/c *rag2*<sup>-/-</sup> mice, as shown by monitoring survival (Figure 4a). The mice that received cells expressing the N666K and P584R mutants developed the disease faster than those injected with cells expressing the R561C mutant, whereas the wild-type receptor and the P660T mutant had no effect. As expected, increased spleen

weight correlated with poor survival and constitutive activity of PDGFR $\beta$  mutants (Figure 4b). Splenomegaly was not observed in mice injected with cells expressing the wild-type receptor and the P660T mutant.

In conclusion, our *in vivo* data support the *in vitro* results demonstrating that the R561C, N666K and P584R mutants are active and could cause the disease. Furthermore, this assay confirms that the R561C mutant is less active than N666K and P584R.

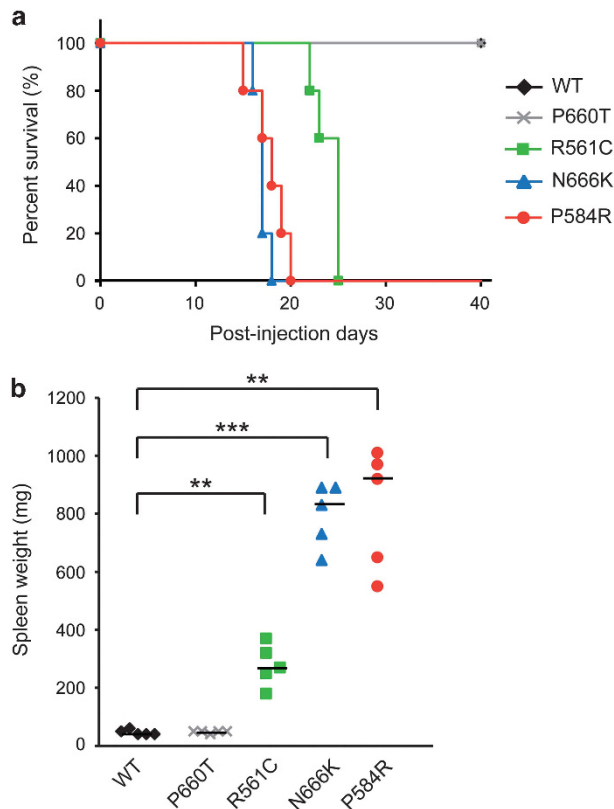
R561C, N666K and P584R are sensitive to tyrosine kinase inhibitors. Our results suggest that blocking the PDGF receptor activity might offer a therapeutic option for myofibromatosis and overgrowth. Therefore, we tested whether activated mutants were sensitive to imatinib, a first-generation tyrosine kinase inhibitor.

Firstly, we tested the effect of imatinib in a luciferase reporter assay responsive to MAP kinases (Figure 5). The luciferase activity induced by the activated mutants R561C, N666K and P584R was much reduced upon imatinib treatment. As expected, the D850V mutation was resistant to this tyrosine kinase inhibitor, in agreement with published reports regarding the corresponding mutation in *PDGFRA*.<sup>33,34</sup>

To better characterize the sensitivity of the three activated mutants to imatinib, we performed a [<sup>3</sup>H]-thymidine incorporation assay with increasing doses of imatinib. At a concentration of 1  $\mu$ M, a concentration corresponding to the plasma level of imatinib in patients,<sup>35,36</sup> cell proliferation induced by the stimulated wild-type receptor or by the activated mutants R561C, N666K and P584R was abolished (Figure 6a), in line with the decrease in signaling observed in luciferase experiments (Figure 5). Interestingly, the R561C and P584R mutants were consistently more sensitive to imatinib than the stimulated wild-type receptor, whereas the N666K mutant was slightly less sensitive (Figure 6a). This was confirmed by the calculation of the half maximal inhibitory concentration (IC<sub>50</sub>) (Figure 6d). Importantly, IL-3-dependent cell proliferation was not affected by imatinib, which selectively blocks PDGFRs and a few other kinases such as ABL (Figure 6a, gray line).

Since N666K was less sensitive to imatinib, we tested two other inhibitors approved for chronic myeloid leukemia: nilotinib, a second-generation inhibitor, and ponatinib, a third-generation inhibitor. In both cases, the R561C and P584R mutants were more sensitive than the stimulated wild-type receptor (Figures 6b and c). This was confirmed by a lower IC<sub>50</sub> (Figure 6d). According to the dose-response curve and the calculation of the average IC<sub>50</sub> (Figures 6b and d), the sensitivity of the N666K mutant to nilotinib did not differ significantly from that of the wild-type receptor. However, N666K was less sensitive to ponatinib than the wild-type receptor (Figure 6c). Nevertheless, ponatinib inhibited N666K at lower concentrations compared with the two other compounds (Figure 6d).

These results were confirmed by the analysis of the phosphorylation level of receptors and downstream signaling mediators by western blot (Figure 7). As expected, we observed that phosphorylation of the wild-type receptor in response to PDGF and phosphorylation of ERK1/2 were progressively decreased with increasing doses of imatinib (Figure 7a), nilotinib (Figure 7b) and ponatinib (Figure 7c). In agreement with the results obtained in proliferation assays, the constitutive phosphorylation of R561C and P584R was abolished with lower doses of inhibitors, in comparison with the wild-type receptor. In contrast, the constitutive phosphorylation of N666K was less sensitive to all inhibitors than the PDGF-induced phosphorylation of the wild-type receptor. For the three mutants, the decrease in constitutive signaling following the treatment with inhibitors was in line with the decrease in receptor phosphorylation. None of the inhibitors affected phosphorylation of ERK1/2 and STAT5 induced by IL-3, demonstrating the specificity of the treatment (Figure 7d).



**Figure 4.** The R561C, N666K and P584R mutants induce cancer development *in vivo*. Two million Ba/F3 cells stably expressing the wild-type receptor or the P660T mutant or selected Ba/F3 cells expressing the R561C, N666K or P584R mutant were intravenously injected into BALB/c *rag2*<sup>-/-</sup> mice (5 per group). **(a)** Mice survival was followed for 50 days. **(b)** Spleens were isolated at the time of killing and weighed. The black line represents the median. \*\**P* < 0.01; \*\*\**P* < 0.001.

Taken together, these results indicated that R561C, N666K and P584R are sensitive to specific tyrosine kinase inhibitors.

## DISCUSSION

In this study, we show for the first time that *PDGFRB* mutations found in patients with familial IM or overgrowth syndrome are of an activating character and sensitive to appropriate tyrosine kinase inhibitors.

Our results demonstrate that both the R561C and N666K mutations promote the PDGFR active conformation in the absence of ligand. Indeed, they allowed the transformation of two different cell lines (Ba/F3 and NIH3T3 cells), constitutively activated signaling pathways that are normally induced by the wild-type receptor in response to PDGF and promoted tumorigenesis *in vivo*. Furthermore, N666K seemed to be a stronger activating mutation compared with R561C. In agreement with the observation that the N666K mutant generated selected Ba/F3 cell lines faster than the R561C mutant, N666K induced a stronger activation of STAT and had a greater ability to transform NIH3T3 cells. This may be related to the fact that N666K is a somatic mutation while R561C is a germline one, which must be compatible with the embryo development. In this respect, Linhares *et al.*<sup>37</sup> described an asymptomatic R561C carrier and suggested that an additional mutation was required for a full phenotypic penetrance.

In contrast, we were not able to show any impact of the P660T substitution on PDGFR $\beta$  activity, even if it was predicted to be damaging by different prediction algorithms.<sup>8</sup> However, we

cannot exclude the possibility that the techniques that we used were not sensitive enough to detect a difference between the wild-type receptor and P660T. Our results correlate with the fact that P660T has been described as a polymorphism in one individual. The residue P660, located in the kinase domain, is not well conserved among PDGFR family members, by contrast to N666. Altogether, these observations do not support the involvement of the P660T mutation in the disease.

Regarding the germline *PDGFRB* mutation associated with overgrowth, our results show that the P584R mutation also confers a gain of function. P584R presented a higher activity than the germline familial IM-associated R561C mutation in luciferase, cell transformation and *in vivo* assays. This difference in the level of receptor activation could explain how two activating germline mutations located in the same domain of the receptor (juxtamembrane domain) could lead to different phenotypes. In this respect, P584R was associated with one case of myofibroma in one of the reported overgrowth patients. Future studies will have to decipher whether the P584R mutation could also specifically impact a particular signaling pathway downstream of PDGFR $\beta$ , compared with R561C. Indeed, different studies demonstrated involvement of the PI3K in overgrowth syndromes.<sup>38,39</sup> The discovery of a link between an activating PDGFR $\beta$  mutation and overgrowth is also reminiscent of the growth retardation effect of imatinib in children with chronic myeloid leukemia.<sup>40</sup>

Finally, we demonstrated that *PDGFRB* activating mutations associated with familial IM and severe overgrowth syndrome, which is associated with various neurological symptoms, are sensitive to tyrosine kinase inhibitors such as imatinib, nilotinib and ponatinib. Noticeably, their sensitivity differs slightly from the wild-type receptor. It could be due to the different localization of the mutations in the receptor. In particular, the R561C and P584R mutations may confer a higher sensitivity to imatinib, which could be given at a lower dose, similar to that given to patients with hypereosinophilia associated with *PDGFRA* fusion, with fewer side effects. Nevertheless, potential benefits must be carefully balanced with the side effects of a long-term imatinib treatment in children.<sup>40</sup>

To conclude, we show that *PDGFRB* mutations associated with familial IM and overgrowth syndromes are activating and sensitive to tyrosine kinase inhibitors. Our data support the hypothesis that these mutations cause these two diseases and suggest a potential treatment for severe cases. *PDGFRB* should thus be systematically sequenced in suspected cases of familial IM and overgrowth. The difference between the two phenotypes could be related to the mutation potency. To our knowledge, these are the first confirmed gain-of-function point mutations of *PDGFRB* in a human cancer.

## MATERIALS AND METHODS

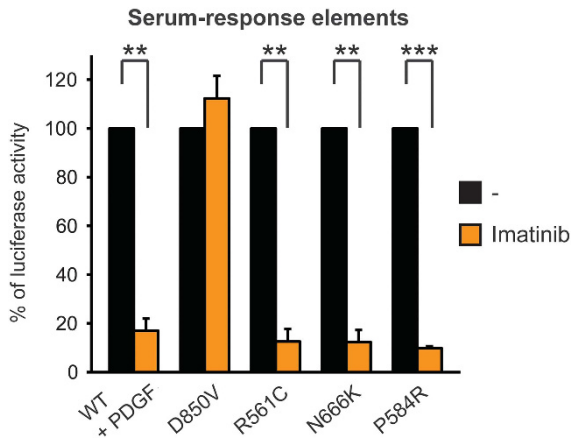
### Reagents and antibodies

PDGF-BB was purchased from PeproTech (Rocky Hill, NJ, USA). Imatinib and nilotinib were purchased from LC Laboratories (Woburn, MA, USA). Ponatinib was purchased from Selleck Chemicals (Munich, Germany). IL-3 was produced as described.<sup>32,41</sup> See Supplementary Materials and Methods for detailed descriptions of antibodies.

### Cell culture

The NIH3T3 (All) cell line was purchased from ATCC (Manassas, VA, USA) and cultured on collagen-coated plates (30  $\mu$ g/ml, Purecol, Advanced Biomatrix, San Diego, CA, USA) in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% decomplexed fetal calf serum (FCS), 1 mM sodium pyruvate and antibiotics (50 U/ml penicillin and 50  $\mu$ g/ml streptomycin). HT-1080 cells, derived from a human fibrosarcoma, were purchased from ATCC and were grown in Iscove's Modified Dulbecco's Medium (Gibco, Life Technologies) supplemented with 10% FCS.<sup>26</sup> MCF7 cells<sup>33</sup> derived from a human breast adenocarcinoma were cultured in DMEM supplemented

with 10% FCS. IL-3-dependent Ba/F3 cells,<sup>33</sup> a murine bone marrow-derived pro-B cell line, were grown in DMEM supplemented with 10% FCS and IL-3 (500 U/ml).



**Figure 5.** The R561C and N666K mutants are sensitive to imatinib. MCF7 cells were co-transfected with different vectors containing WT PDGFR $\beta$  or PDGFR $\beta$  mutants,  $\beta$ -galactosidase and an SRE promoter controlling luciferase expression. Cells were washed 4 h after transfection and treated or not with 500 nM imatinib for 24 h. In addition, cells expressing the wild-type receptor were stimulated with PDGF-BB. The histogram represents the mean of the ratio between luciferase and  $\beta$ -galactosidase activity of three independent experiments with s.e.m. The condition without imatinib was used as a reference. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

### Site-directed mutagenesis

After cloning of wild-type PDGFR $\beta$  in pcDNA3.1 (Invitrogen, Life Technologies) and pEF/myc/cyto (Invitrogen, Life Technologies), the point mutations D850V, P660T, R561C, N666K and P584R were introduced in each vector by site-directed mutagenesis according to the QuickChange XL-II kit protocol (Stratagene, La Jolla, CA, USA). In all cases, the whole insert was sequenced.

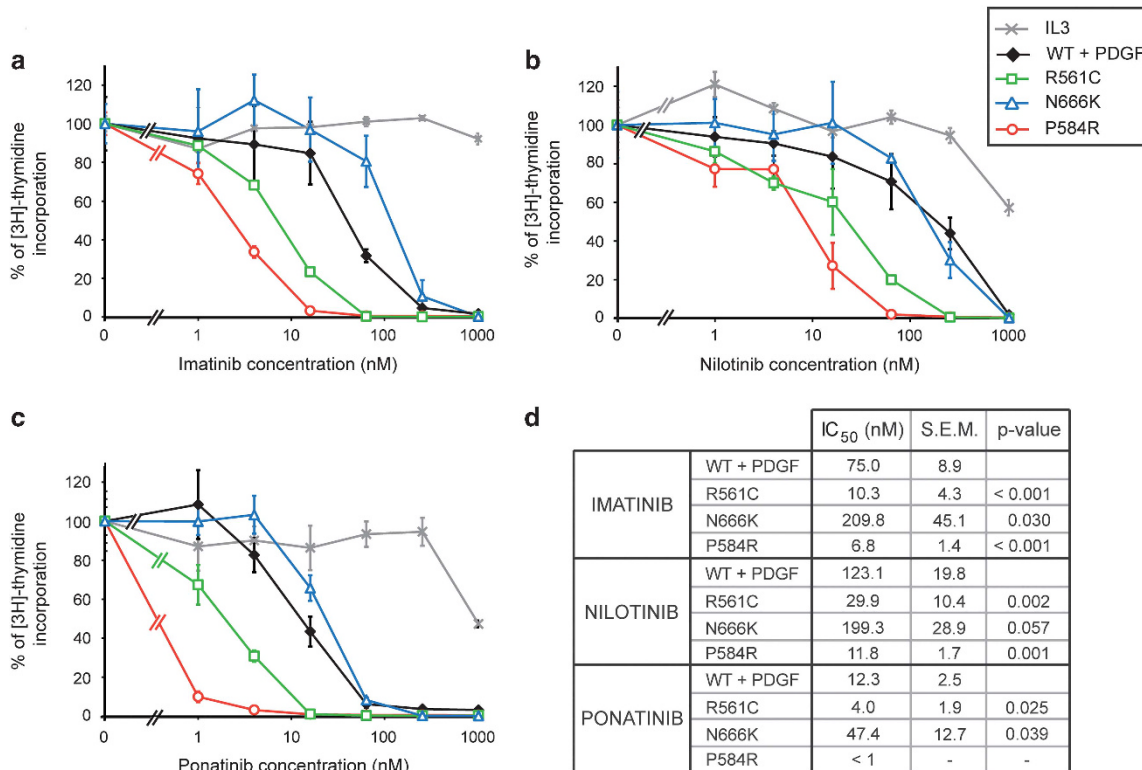
### Production of Ba/F3 stable cell lines expressing PDGFR $\beta$

Ten million Ba/F3 cells were electroporated (200 V, 75 Ohm, 1300  $\mu$ F) with 50  $\mu$ g pEF/myc/cyto-PDGFR $\beta$  wild-type, R561C, P660T, N666K or P584R and seeded in 15-cm dishes. Twenty-four hours after transfection, 500 000 electroporated cells were transferred to 10-cm dishes and selected with 3 mg/ml G418. After approximately 2 weeks of selection, Ba/F3 cells stably expressing the receptors were sorted by flow cytometry. Briefly, cells were stained with a primary anti-PDGFR $\beta$  antibody (AH 17.2, 5  $\mu$ g/ml) for 40 min at 4  $^{\circ}$ C, washed and incubated with a secondary antibody conjugated to phycoerythrin for 40 min at 4  $^{\circ}$ C in the dark. Finally, stained cells were sorted by FACSaria III (BD Biosciences, Erembodegem, Belgium).

Sorted Ba/F3 cells stably expressing the R561C, N666K and P584R mutants were then selected in DMEM supplemented with 10% FCS without IL-3.

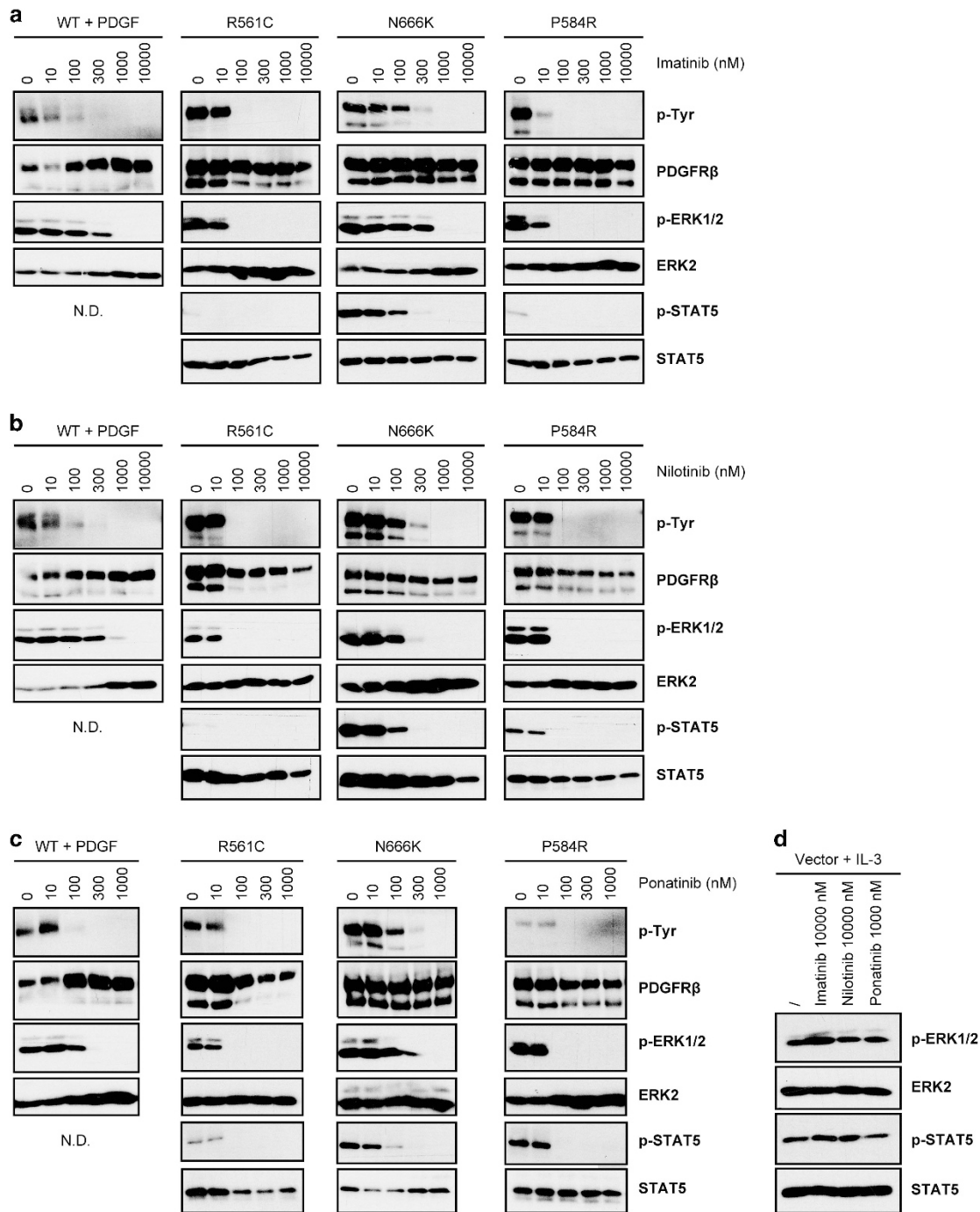
### Living cell count

Ba/F3 cells stably expressing wild-type PDGFR $\beta$  or one of its mutants were washed twice and seeded at 400 000 cells/well in 6-well plates in a medium without IL-3 in triplicate. On days 2 and 3, the number of living cells was assessed in a B $\ddot{u}$ rker chamber. As a control, cells were seeded in a medium with IL-3.



**Figure 6.** PDGFR $\beta$  mutations affect sensitivity to tyrosine kinase inhibitors. (a–c) Ba/F3 cells stably expressing WT PDGFR $\beta$  and selected cells expressing R561C, N666K or P584R were washed twice and treated with increasing concentrations of imatinib (a), nilotinib (b) or ponatinib (c). In addition, cells expressing the wild-type receptor were stimulated with PDGF-BB. As a negative control, cells expressing the wild-type receptor were also seeded in the presence of IL-3 (gray line). Cell proliferation was assessed after 24 h by measuring the incorporation of [<sup>3</sup>H]-thymidine. One representative experiment is shown with s.d. ( $n \geq 3$ ). Treatment with corresponding concentrations of vehicles had no effect on cell proliferation (data not shown). (d) IC<sub>50</sub> (nM) was calculated for each inhibitor and each mutant. The table represents the mean of at least three independent experiments.





**Figure 7.** Tyrosine kinase inhibitors decrease receptor phosphorylation and signaling downstream of PDGFR $\beta$  mutants. (**a–c**) Analysis of the phosphorylation of the activated PDGFR $\beta$  mutants, as well as the activation of ERK1/2 and STAT5, in response to imatinib (**a**), nilotinib (**b**) or ponatinib (**c**) treatment. Western blot experiments on Ba/F3 cells expressing WT PDGFR $\beta$  and selected cells expressing R561C, N666K or P584R were performed as described in Figure 2, except that cells were treated with increasing doses of imatinib, nilotinib or ponatinib during starvation. We were not able to observe any phosphorylation of STAT5 downstream of the PDGF-stimulated wild-type receptor (ND, not detectable). The maximal concentration used for ponatinib was 1  $\mu$ M because higher doses were toxic (data not shown). (**d**) As a control, the IL-3-induced phosphorylation of ERK1/2 and STAT5 in Ba/F3 cells transfected with the empty vector and incubated with the three inhibitors was also analyzed by western blot. (**a–d**) Two experiments were performed with similar results.

#### [<sup>3</sup>H]-thymidine incorporation assay

Ba/F3 cells stably expressing wild-type PDGFR $\beta$  or PDGFR $\beta$  P660T and selected Ba/F3 cells expressing PDGFR $\beta$  R561C, N666K or P584R were washed twice with DMEM supplemented with 10% FCS to remove IL-3 and were seeded at 10 000 cells/well in 96-well plates. Then, 20 ng/ml PDGF-BB,

imatinib, nilotinib, ponatinib, IL-3 or vehicle was added in the appropriate wells. Twenty hours later, 0.5  $\mu$ Ci [<sup>3</sup>H]-thymidine (GE Healthcare, Diegem, Belgium) was added in each well for 4 h and [<sup>3</sup>H]-thymidine incorporation was measured using a TopCount instrument (Perkin Elmer, Waltham, MA, USA) as described.<sup>33</sup>



### Foci formation assay

NIH3T3 cells were transfected with wild-type PDGFR $\beta$  or one of its mutants (0.55  $\mu$ g) with Lipofectamine 2000 as recommended by the manufacturer (Invitrogen, Life Technologies) and processed as described.<sup>27</sup>

### Western blot

Transfected Ba/F3 cells were washed twice with DMEM supplemented with 10% FCS to remove IL-3 and were seeded in 6-well plates (2.10<sup>6</sup>–4.10<sup>6</sup> cells/well), with or without increasing doses of imatinib, nilotinib or ponatinib. Four hours after IL-3 withdrawal, cells were stimulated with IL-3 or 25 ng/ml PDGF-BB or left untreated. After 15 min of stimulation, cells were washed with PBS. Cell lysates and western blots were performed as previously described.<sup>26</sup>

For immunoprecipitation experiments, cell lysates were produced as described above. Then, they were first incubated with an anti-PDGFR $\beta$  antibody (Santa Cruz Biotechnology) overnight on wheel at 4 °C, and Protein A/G UltraLink Resin (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h 30 min on wheel at 4 °C. Beads were washed twice with lysis buffer and once with PBS, and were resuspended in 2 × Laemmli buffer for western blot analysis.

### Luciferase assays

MCF7 cells were seeded at 150 000 cells/well in 12-well plates. The day after, cells were co-transfected by using TurboFect Transfection Reagent (Thermo Scientific, Thermo Fisher Scientific) or FuGENE HD Transfection Reagent (Promega, Leiden, The Netherlands), as recommended by the manufacturers, with different vectors: 0.5  $\mu$ g pcDNA3.1-PDGFR $\beta$  (wild-type or mutant), 0.5  $\mu$ g of a plasmid in which the luciferase expression is controlled by serum-response elements (pSRE)<sup>42</sup> and 0.5  $\mu$ g pEF1- $\beta$ -galactosidase (Invitrogen, Life Technologies). Four hours after transfection, cells were washed with PBS, fresh medium was added and 20 ng/ml PDGF-BB and/or 500 nM imatinib were added in the appropriated wells or cells were left untreated.

HT-1080 cells were seeded at 175 000 cells/well in 12-well plates. The day after, cells were co-transfected with different vectors: 0.5  $\mu$ g pcDNA3.1-PDGFR $\beta$  (wild-type or mutant), 0.5  $\mu$ g of a plasmid containing a luciferase reporter controlled by a STAT-sensitive promoter (pGRR5)<sup>43</sup> and 0.5  $\mu$ g pEF1- $\beta$ -galactosidase (Invitrogen, Life Technologies), as described above. Four hours after transfection, cells were washed with PBS and starved in serum-free medium.

Cells were lysed 24 h after transfection and the luciferase activity was assessed as described<sup>33</sup> by using GloMax (Promega). The  $\beta$ -galactosidase activity was assessed as described.<sup>26</sup>

### Tumorigenicity assay in BALB/c *rag2*<sup>-/-</sup> mice

Two million Ba/F3 cells stably expressing the wild-type receptor or the P660T mutant or selected Ba/F3 cells expressing the R561C, N666K or P584R mutant were intravenously injected in 9-week-old female BALB/c *rag2*<sup>-/-</sup> mice (Taconic Biosciences Inc., Hudson, NY, USA). Five mice per condition were tested. Survival was followed and isolated spleens were weighed after death. Mice that received cells expressing the wild-type receptor or the P660T mutant were still alive 50 days after injection and were killed on that day. Animal procedures were performed in accordance with the guidelines of the Ethics Committee on Animal Experimentation of the UCL Faculty of Medicine.

### Statistics

Experiments were performed at least three times (unless otherwise stated) and produced similar results. When the average of the different experiments is represented, standard error of the mean (s.e.m) is indicated while if one representative experiment is shown, s.d. is specified. Normal distribution was tested and statistical analyses were performed using Student's *t*-test (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001).

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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