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The Limb Bud Shh-Fgf Feedback Loop Is Terminated by Expansion of Former ZPA Cells

Paul J. Scherz,[1] Brian D. Harfe,[1,2] Andrew P. McMahon,[3] Clifford J. Tabin[1]*

Vertebrate limb outgrowth is driven by a positive feedback loop involving *Sonic Hedgehog* (*Shh*), *Gremlin*, and *Fgf4*. By overexpressing individual components of the loop at a time after these genes are normally down-regulated in chicken embryos, we found that *Shh* no longer maintains *Gremlin* in the posterior limb. *Shh*-expressing cells and their descendants cannot express *Gremlin*. The proliferation of these descendants forms a barrier separating the Shh signal from *Gremlin*-expressing cells, which breaks down the Shh-Fgf4 loop and thereby affects limb size and provides a mechanism explaining regulative properties of the limb bud.

Many key developmental decisions are made in response to secreted factors emanating from defined organizers or signaling centers. Whereas much has been learned about how signaling centers form, much less attention has been given to an equally important question: What is the mechanism that terminates their activity, assuring that the potent factors they produce do not interfere with subsequent development once they have fulfilled their function?

Two classic signaling centers are found in the limb bud. The distal outgrowth of the limb bud requires the activity of several members of the fibroblast growth factor (Fgf) family that are synthesized in a signaling center at the tip of the limb bud known as the apical ectodermal ridge (AER) (*1*, *2*). Fgf signaling also serves to maintain the expression of another critical secreted factor in the limb bud, *Sonic hedgehog* (*Shh*) which is produced by a second signaling center, the zone of polarizing activity (ZPA) in the posterior mesenchyme (*1*, *3*). *Shh* is responsible for patterning the anterior-posterior axis of the limb (*4*) and also acts to maintain the expression of several Fgf's in the overlying AER, including *Fgf4* (*1*, *3*), *Fgf9*, and *Fgf17* (*5*). *Shh* maintains *Fgf4* (and presumably *Fgf9* and *Fgf17*) by up-regulating *Gremlin* in the adjacent mesenchyme (*6*, *7*). *Gremlin* is a bone morphogenetic protein (Bmp) antagonist (*8*) that prevents Bmp's from down-regulating *Fgf4* (*9*, *10*). Importantly, the fourth member of the Fgf family expressed in the AER, *Fgf8*, is not directly dependent on *Shh* for its transcription (*11*). However, in the absence of *Gremlin*, the AER itself becomes disorganized and *Fgf8* is down-regulated (*12*). Thus, the signaling centers in the posterior and distal tip of the limb bud are interdependent for their activity, and this positive feedback loop is required for producing a normal limb structure.

The signaling loop between *Shh* and Fgf's operates throughout limb development until embryonic day 6 (E6) (stage 27) (*13*), when *Fgf4* and *Gremlin* cease to be expressed and *Shh* is down-regulated [*Shh* expression ceases at E7.5 (stage 29)] (*4*). Concomitantly, the rate of cell proliferation in the limb decreases (*14*). As critical as the feedback loop is to patterning, it is equally critical that it be terminated. This is highlighted by the effect of ectopically providing Shh late in limb development after the ZPA no longer exists. *Fgf8* expression is transiently maintained at the distal tips of each digit by factors secreted from the forming skeletal elements (*15*). If *Shh* is ectopically provided at this stage, *Fgf8* expression is prolonged (*15*), the digit rays continue to grow, additional phalanges are produced, and the limb becomes longer than normal (*15*, *16*). Thus, the breakdown of the Shh-Fgf feedback loop is critical to control the size of the limb. To try to understand how this breakdown occurs, we overexpressed *Shh*, *Gremlin*, and *Fgf4* before E6 (stage 27) and asked if any of them maintain the other genes in the loop after their normal down-regulation. A bead soaked in Fgf4 implanted in the posterior mesenchyme of E5 (stage 25) chick limb buds maintained *Shh* up-regulation for 48 hours until E7 (stage 28) (16 out of 27 limbs), when expression of *Shh* in contralateral controls was no longer detectable (Fig. 1, A and B), suggesting that a loss of responsiveness to Fgf4 is not the mechanism by which the feedback loop breaks down. Similarly, viral misexpression of *Gremlin* with an RCAS virus injected at E4 (stage 23) leads to a continued up-regulation of *Fgf4* (8 out of 26 limbs) and *Shh* (4 out of 10 limbs) 72 hours later at E7 (stage 28) (Fig. 1, C to F), indicating that Gremlin responsiveness is intact as the feedback loop degenerates. In contrast, a bead soaked in Shh and implanted in the posterior limb at E5 (stage 25) cannot maintain *Gremlin* (0 out of 12 limbs) or *Fgf4* (0 out of 10 limbs) expression 24 hours later (Fig. 1, G to J) This suggests that a loss in the ability of mesenchymal cells to respond to Shh by up-regulating *Gremlin* is the mechanism by which the Shh-Fgf feedback loop breaks down.

In control experiments, a Shh bead was implanted into the anterior limb at the same stage, and it ectopically maintained *Gremlin* expression (9 out of 16 limbs) (Fig. 1K). Thus, although Shh responsiveness is lost in the posterior limb, it is maintained in the anterior limb. This is less surprising in the context of previous studies showing that there is a zone in which *Gremlin* expression is excluded in the posterior limb (*7*, *17*). From E4 to E5.5 (stages 23 to 26), this zone of exclusion increasingly expands, such that a gap opens between the domains of *Shh* and *Gremlin* expression (Fig. 2, A and B). It is the cells within this domain that are unable to express *Gremlin* in response to ectopic Shh.

In a separate recombinase-based fate mapping study, we marked the descendants of cells that at one time expressed *Shh* in

[1]Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA. [2]Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, 1600 Southwest Archer Road, Gainesville, FL 32610–0266, USA. [3]Department of Molecular and Cellular Biology, The Biolabs, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed. E-mail: tabin@genetics.med.harvard.edu

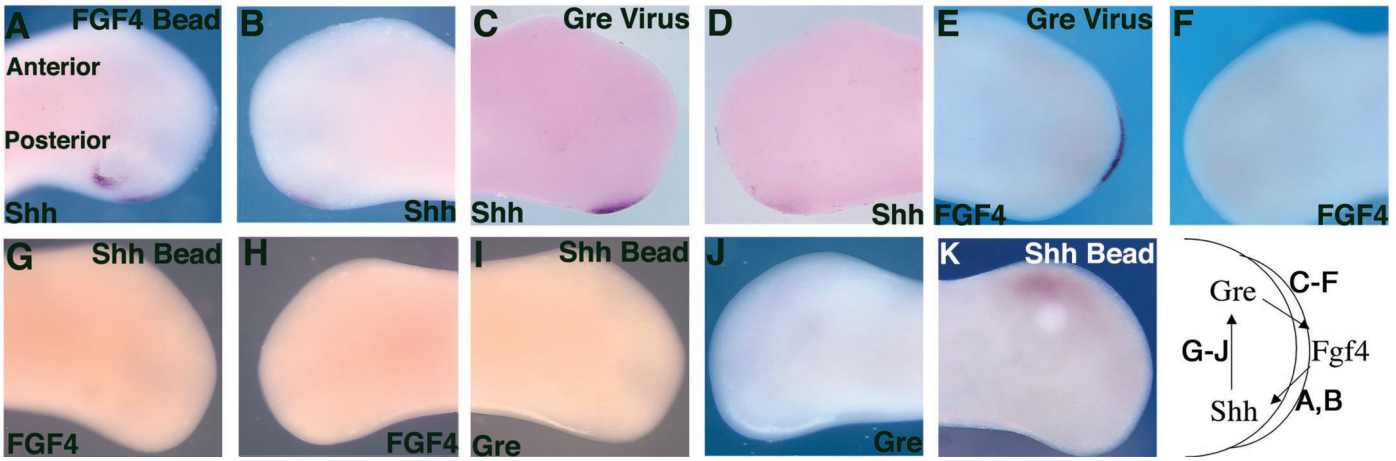

Fig. 1. The Shh-Fgf feedback loop is disrupted between *Shh* and *Gremlin*. (**A** and **B**) An Fgf4 bead (A) maintains *Shh* expression in chick limbs at E7 (stage 28), unlike controls (B). (**C** to **F**) Ectopic *Gremlin* viral expression maintains both *Fgf4* (C) and *Shh* (E) expression at E7 (stage 28) in chick limbs at higher levels than controls [(D) and (F)]. (**G** to **J**) A Shh bead implanted into the posterior chick limb is unable to maintain either *Gremlin* (G) or *Fgf4* (I) expression at E7 (stage 28) as in controls [(H) and (J)]. (**K**) A Shh bead maintains ectopic *Gremlin* expression in the anterior at E7 (stage 28). The diagram on the bottom right shows the step of the feedback loop being tested in each experiment.

the ZPA (*18*, *19*). Former *Shh*-expressing cells expand anteriorly to encompass a domain substantially larger than the ZPA, similar to the *Gremlin*-negative domain at a similar stage in the mouse (Fig. 2, C and D). To test whether the *Gremlin*-negative domain is indeed the population of cells that formerly expressed *Shh*, sections of such a mouse limb were stained both for β-galactosidase to visualize *Shh* descendants and by in situ hybridization to detect *Gremlin* expression at E11.5. As previously observed, the *Gremlin*-expressing cells were only found in superficial layers subjacent to the dorsal and ventral surface ectoderm (Fig. 2E) (*12*, *17*). *Gremlin*-expressing cells lie contiguous to *Shh* descendants but do not overlap with them (Fig. 2, E, G, and I). The conclusion that the former *Shh*-expressing cells cannot express *Gremlin* is particularly convincing because in some sections, groups of non-*Shh* descendants are mixed within the zone of *Shh* descendants (Fig. 2H). These cells express *Gremlin*, whereas neighboring *Shh* descendants do not (Fig. 2, F, H, and J), suggesting that the inability to express *Gremlin* is a cell-autonomous property of *Shh* descendants and not a general response to a posteriorly localized factor. This cell autonomy was further confirmed in transplantation experiments (fig. S1).

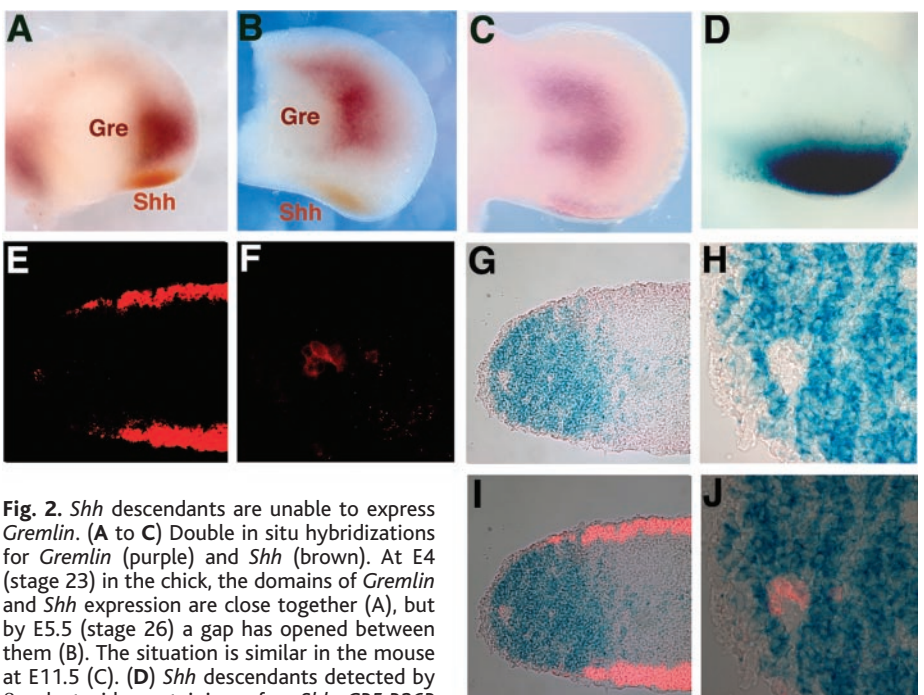

Fig. 2. *Shh* descendants are unable to express *Gremlin*. (**A** to **C**) Double in situ hybridizations for *Gremlin* (purple) and *Shh* (brown). At E4 (stage 23) in the chick, the domains of *Gremlin* and *Shh* expression are close together (A), but by E5.5 (stage 26) a gap has opened between them (B). The situation is similar in the mouse at E11.5 (C). (**D**) *Shh* descendants detected by β-galactosidase staining of a *Shh::CRE;R26R* mouse at E11.5 closely resemble the domain of posterior *Gremlin* exclusion. (**E** to **J**) An in situ hybridization in mouse limbs for *Gremlin* [(E) and (F)], β-galactosidase staining for *Shh* descendants [(G) and (H)] and the merged images [(I) and (J)]. At E11.5, *Gremlin* expression is contiguous but nonoverlapping with *Shh* descendants [(E), (G), and (I)]. Non-*Shh* descendants that are mixed into the *Shh* descendant domain express *Gremlin*, showing *Gremlin* repression is cell autonomous [(F), (H), and (J)].

These data suggest a model to explain the termination of the Shh-Fgf feedback loop during limb development. Cells that express *Shh* and their descendants are unable to express *Gremlin*. Initially, this is not a problem because Shh protein diffuses (*20*) and activates target genes such as *Ptc1* (*21*) at a substantial distance from the ZPA. However, as limb outgrowth proceeds, the proliferation of *Shh* descendants serves as a barrier between the source of Shh and cells that are able to produce *Gremlin* in response. By E6 (stage 27), the cells competent to express *Gremlin* become too distant from the source of Shh to receive an adequate dose of the protein to produce *Gremlin*, leading to a breakdown of the Shh-Fgf feedback loop. Consistent with this, *Fgf4* expression is first lost from the posterior end of the AER (fig. S2).

To obtain direct evidence that the downregulation of the loop is caused by the barrier of *Shh* descendants between *Gremlin* and *Shh* expressing cells, we removed a wedge of tissue containing *Shh* descendants from a chick limb bud and stapled the remaining posterior cells (including the cells actively producing Shh) to the remaining anterior limb at E5 (stage 25). We reasoned that removing these cells might

put the responsive tissue back within the range of Shh, thereby preserving the feedback loop. Indeed, by eliminating the tissue between *Shh*-expressing and *Gremlin*-competent cells, the feedback loop continues to function. *Shh* (three out of six limbs), *Gremlin* (seven out of nine limbs), and *Fgf4* (5 out of 15 limbs) are each up-regulated when compared with the unoperated contralateral limb for at least 24 hours after the endogenous Shh-Gremlin-Fgf feedback loop normally breaks down (Fig. 3, A to H). Thus, in the absence of this surgical manipulation, the barrier formed by the expansion of the former *Shh*-expressing cells provides an innate stopping mechanism for the Shh-Fgf4 feedback loop.

In these experiments, a wedge containing as much as a third of the distal limb mesenchyme is removed, and the resulting limbs are wild type both in structure and in size (Fig. 3, I and J). This is an example of regulative development, the general phenomenon that embryos are extremely resilient; in spite of considerable experimental manipulations, normal embryos can develop (*22*). Yet, how regulative growth is achieved has not received much attention. The model presented here provides a potential explanation: Once the ZPA is pinned to the remaining limb tissue, without a barrier to *Gremlin* induction, the feedback loop is restored and the posterior cells resume expansion. This continues until the barrier again reaches the critical width at which the loop breaks down. Because this width depends on the diffusion distance of Shh and not on the amount of tissue removed, the development is regulative, and hence the limbs grow to their normal extent. Consistent with this model, expansion of the posterior tissue is indeed restored following surgery, as can be seen in the gap which once again is established between the sites of *Shh* and *Gremlin* expression (compare Fig. 3, A and C). The renewed expansion of the posterior tissue was also directly verified with injections of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Fig. 3, K to M).

The mechanism by which the Shh-Fgf feedback loop is terminated in the limb also provides insight into size control in limb outgrowth. Controlling body size and establishing the proper relative sizes of the constituent body parts and organs are critical aspects of embryonic development. In spite of its importance [with a few exceptions, such as the control of muscle size by myostatin (*23*)], little is known about the control of differential size in vertebrates (*24*). As the limb bud grows out, the digit rays split into individual phalangeal elements in a proximal-to-distal sequence based on an intrinsic developmental program, such that new cartilaginous elements are formed distally, with a predefined spacing, as long as the digit ray keeps growing. The length of time that this process continues determines the length of the digits. Thus, dolphin digit rays grow for a longer period and form more phalanges than the limbs of land mammals (*25*).

As noted earlier, digits continue to grow and additional phalanges form when *Shh* and Fgf's are exogenously maintained independent of the normal requirement for positive feedback (*15*, *16*). Artificially maintaining *Gremlin* expression in the distal posterior limb also results in a continuation of *Shh* and *Fgf4* expression (Fig. 1, C to F). As a consequence, *Gremlin*-infected posterior digit primordia are 13.4% longer than the contralateral digit 4 (Fig. 3, N to P; five out of seven limbs). In these infected limbs, no chondrogenesis occurs in the extra outgrowth because of the Bmp-antagonistic effects of Gremlin. The expectation would be that if *Gremlin* expression were prolonged but did not proceed indefinitely (because of the viral promoter in this experimental setting), condensation would proceed normally, and longer digits would be formed.

Why are the former *Shh*-expressing cells refractory to *Gremlin* induction? It is possible that this nonresponsiveness is imposed by transcription factors expressed in the ZPA cells and their descendants. This is consistent with our finding that cells that never expressed *Shh* but are adjacent to or mixed with former *Shh*-expressing cells are capable of activating *Gremlin*. It is also possible that extremely high levels of Shh signaling, such as those normally attained only in an intracellular autocrine fashion by cells producing Shh, render cells unable to express *Gremlin*. This latter possibility may be supported by consideration of the situa-

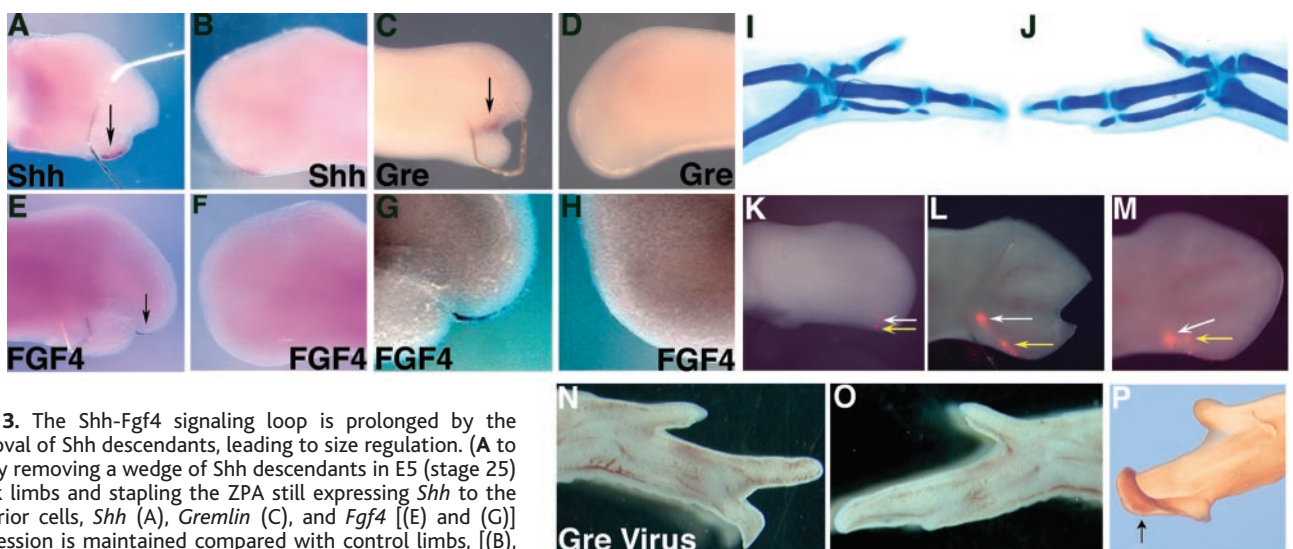

Fig. 3. The Shh-Fgf4 signaling loop is prolonged by the removal of Shh descendants, leading to size regulation. (**A** to **H**) By removing a wedge of Shh descendants in E5 (stage 25) chick limbs and stapling the ZPA still expressing *Shh* to the anterior cells, *Shh* (A), *Gremlin* (C), and *Fgf4* [(E) and (G)] expression is maintained compared with control limbs, [(B), (D), (F), and (H)] showing that the expansion of *Shh* descendants blocks the Shh-Fgf4 signaling loop. (G) and (H) are higher magnifications of (E) and (F), respectively. (**I** to **M**) Limbs in which Shh descendants are removed and the ZPA stapled to anterior cells show size regulation (I) to become comparable in anterior-posterior and proximal-distal patterning and size to contralateral control limbs (J). Limbs were injected with two adjacent dots of DiI (arrows) (K), and then either the *Shh* descendants were removed and the remaining tissue stapled (L) or the DiI-injected limbs were left unoperated (M). The cells in between the dots expand in the operated limbs, showing that *Shh*-expressing cells continue to proliferate until the distance between *Shh* and *Gremlin* expression lies outside the range of Shh diffusion again, thereby giving size regulation. (**N** to **P**) Ectopic expression of *Gremlin* in the posterior limb bud to E10 (stage 36) with the use of a virus, detected with an antibody, 3C2, against viral MA antigen (arrow) (P), gives rise to elongated growth of the posterior tissue (N) when compared with contralateral controls (O). Length of the ectopic growths were measured from the tip of the ectopic soft tissue to the proximal end of digit 4, which still forms cartilage.

tion where ectopic Shh protein is applied to the anterior limb bud (*26*). Recognizable posterior digits (albeit not as perfect as with a ZPA graft) are formed in response to Shh protein beads, which may imply that there is an induction of a *Gremlin*-refractory cell population by exceptionally high levels of Shh signaling adjacent to the beads. Alternatively, the termination of growth of these limbs could reflect the limited amount of Shh protein in the bead. Another series of experiments seemingly relevant to the current study involves grafts of irradiated ZPA cells. However, these experiments need to be reevaluated with modern tools (supporting online material text).

We focused on *Fgf4* as the AER component of the Shh-Gremlin-Fgf feedback loop. As noted above, *Fgf8*, *Fgf9*, and *Fgf17* are also expressed in the AER. *Fgf4*, *Fgf9*, and *Fgf17* have been shown to depend on Shh for their transcription (*5*), whereas Gremlin is indirectly responsible for maintaining *Fgf8* expression by affecting AER organization (*12*). Hence, the loss of expression and/or down-regulation of all four of these genes is likely to result when the expansion of the posterior limb bud exceeds the ability of Shh to maintain *Gremlin*. This provides a unique mechanism for terminating the activity of two key signaling centers in the limb, with important implications for the little-understood properties of regulative development and size control during embryogenesis.

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Overriding Imatinib Resistance with a Novel ABL Kinase Inhibitor

Neil P. Shah,[1] Chris Tran,[1,2] Francis Y. Lee,[3] Ping Chen,[3] Derek Norris,[3] Charles L. Sawyers[1,2]*

Resistance to the ABL kinase inhibitor imatinib (STI571 or Gleevec) in chronic myeloid leukemia (CML) occurs through selection for tumor cells harboring BCR-ABL kinase domain point mutations that interfere with drug binding. Crystallographic studies predict that most imatinib-resistant mutants should remain sensitive to inhibitors that bind ABL with less stringent conformational requirements. BMS-354825 is an orally bioavailable ABL kinase inhibitor with two-log increased potency relative to imatinib that retains activity against 14 of 15 imatinib-resistant BCR-ABL mutants. BMS-354825 prolongs survival of mice with BCR-ABL–driven disease and inhibits proliferation of BCR-ABL–positive bone marrow progenitor cells from patients with imatinib-sensitive and imatinib-resistant CML. These data illustrate how molecular insight into kinase inhibitor resistance can guide the design of second-generation targeted therapies.

Imatinib (STI571 or Gleevec) is a small-molecule inhibitor of the BCR-ABL tyrosine kinase that produces clinical remissions in chronic myeloid leukemia (CML) patients with minimal toxicity (*1*, *2*). Imatinib is now frontline therapy for CML, but resistance is increasingly encountered. Clinical resistance is primarily mediated by mutations within the kinase domain of BCR-ABL and, to a lesser extent, by amplification of the *BCR-ABL* genomic locus (*3*). Crystallographic studies revealed that imatinib binds to the adenosine triphosphate (ATP)–binding site of ABL only when the activation loop of the kinase is closed and thus stabilizes the protein in this inactive conformation (*4*). In addition, the normally smooth contour of the phosphate-binding loop of ABL is distorted by imatinib binding, adding further to the unique conformational requirements for optimal kinase inhibition. These conformation-specific binding requirements contribute to imatinib's selectivity, particularly with regard to the closely related kinase SRC, which imatinib does not inhibit. Structural studies of the pyrido[2,3-d]pyrimidine class of dual SRC-ABL inhibitors show that these compounds also bind to the ATP-binding site in ABL, but without regard for the position of the activation loop, which can be in the active or inactive conformation (*5*).

To date, mutations at 17 different amino acid positions within the BCR-ABL kinase domain have been associated with clinical resistance to imatinib in CML patients (*6*–*11*). The majority of amino acid substitutions are believed to cause resistance by impairing the ability of the kinase to adopt the specific closed conformation to which imatinib binds, although a small fraction directly interfere with drug binding (*9*). This insight raises the possibility that other small-molecule ABL kinase inhibitors, such as those that also inhibit SRC, might have activity against imatinib-resistant BCR-ABL mutants. Indeed, promising in vitro activity against a limited number of imatinib-resistant BCR-ABL isoforms has been seen for two compounds from the pyrido[2,3-d]pyrimidine class of dual SRC-ABL inhibitors (PD166326 and PD180970) (*12*, *13*).

BMS-354825 [N-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-

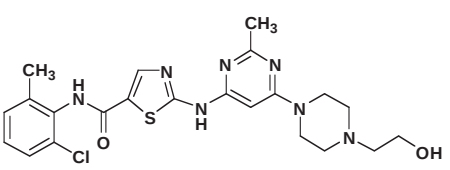

Fig. 1. Chemical structure of BMS-354825.

[1]Division of Hematology and Oncology, Department of Medicine, [2]Howard Hughes Medical Institute, The David Geffen School of Medicine, University of California, Los Angeles, CA, 90095, USA. [3]Bristol-Myers Squibb Oncology, Princeton, NJ, USA.

*To whom correspondence should be addressed. E-mail: csawyers@mednet.ucla.edu