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A PINOID-Dependent Binary Switch in Apical-Basal PIN Polar Targeting Directs Auxin Efflux

Jiří Friml,¹ Xiong Yang,^{2,3} Marta Michniewicz,¹ Dolf Weijers,^{1,2} Ab Quint,² Olaf Tietz,⁴ René Benjamins,^{2,6} Pieter B. F. Ouwkerk,² Karin Ljung,⁵ Göran Sandberg,⁵ Paul J. J. Hooykaas,² Klaus Palme,⁴ Remko Offringa^{2*}

Polar transport-dependent local accumulation of auxin provides positional cues for multiple plant patterning processes. This directional auxin flow depends on the polar subcellular localization of the PIN auxin efflux regulators. Overexpression of the PINOID protein kinase induces a basal-to-apical shift in PIN localization, resulting in the loss of auxin gradients and strong defects in embryo and seedling roots. Conversely, *pid* loss of function induces an apical-to-basal shift in PIN1 polar targeting at the inflorescence apex, accompanied by defective organogenesis. Our results show that a PINOID-dependent binary switch controls PIN polarity and mediates changes in auxin flow to create local gradients for patterning processes.

The plant signaling molecule auxin plays a central role in a wide variety of development processes. A major determinant in auxin-mediated plant growth is the directed transport of auxin from foci of biosynthesis to sites of action. This polar auxin transport mediates vectorial gradients that underlie tropic growth responses and provide positional cues for apical-basal patterning, organogenesis, and vascular differentiation (1–4). The molecular characterization of the *Arabidopsis thaliana pin-formed* (*pin1*) mutant, which is defective in auxin transport and develops pin-like inflorescences, led to the identification of the PIN family of transporter-like membrane proteins. A substantial amount of data demonstrates that PIN proteins are important regulators of polar auxin transport that possibly function

as auxin efflux carriers (4). PIN proteins display asymmetric subcellular localization at the plasma membrane, which determines the direction of polar auxin transport and thus establishes the local auxin gradients that influence different developmental processes. The polarity of PIN proteins can be rapidly modulated in response to external or developmental cues (1, 3, 5), a process that is enabled by continuous GNOM ARF GEF-dependent cycling of PINs between endosomes and the plasma membrane (6) (GNOM, *Arabidopsis* GNOM protein; ARF, ADP ribosylation factor; GEF, guanine nucleotide exchange factor).

Loss-of-function mutants of the protein serine-threonine kinase PINOID (PID) display apical organogenesis defects similar to those of the *pin1* mutant (7). Constitutive overexpression of PID (*35S::PID*), but not of the kinase-negative MPID (*35S::MPID*), leads to hypocotyl and root agravitropy and to loss of the primary root meristem function (8, 9). The collapse of the root meristem in *35S::PID* seedlings, which is characterized by the loss of meristem initials followed by terminal differentiation, is restricted to the primary root and is preceded by a reduction in auxin-responsive *DR5::GUS* expression (Fig. 1, A and B) (9). Measurements of indole-3-acetic acid (IAA) in intact root tips showed that IAA levels are significantly reduced in *35S::PID* primary root tips as compared to wild-type root tips (Fig. 1E). In

contrast, free IAA concentrations in lateral root tips of 8- to 11-day-old *35S::PID* and wild-type seedlings do not differ significantly (Fig. 1E), and accordingly the *DR5::GUS* expression peak is unchanged (Fig. 1, C and D). These data confirmed that *PID* overexpression results in reduced auxin accumulation in the primary root tip, thereby causing a reduction in the *DR5* expression peak in the root meristem and eventually the collapse of this structure. Treatment with the auxin efflux inhibitor naphthylphthalamic acid (NPA) restores the *DR5* expression peak and prevents root meristem collapse (9), whereas treatment with auxin itself has no effect. These data suggest that PID is a regulator of NPA-sensitive polar auxin transport.

We used the timing of root collapse as an assay to address whether PID action on auxin transport occurs through PIN efflux regulators. *35S::PID* plants were crossed with loss-of-function mutants of *PIN* genes known to mediate root development, these being *PIN2*, *PIN3*, and *PIN4* (5, 10, 11). In the *pin2/eir1-1* and *pin4* mutant backgrounds, the *35S::PID*-mediated root collapse was significantly delayed, whereas the *pin3* mutation resulted in a mild delay in root collapse around 4 to 5 days after germination (Fig. 1F). Both *pin2* and *pin4* mutations result in increased auxin concentrations in the root: *pin2* elevates auxin concentrations because of the lack of redistribution of auxin via basipetal transport from the root tip to the elongation zone (12), and *pin4*-elevated auxin levels result from the absence of a focused PIN4-driven auxin sink in the first columella tier (11).

These results imply that PID gain of function changes auxin concentrations in the root tip through the PIN proteins. Conceivably, PID could regulate either the expression of PIN proteins, the polarity of their subcellular localization, or their activity. Because an activity assay for PIN proteins is so far not available, we focused on testing the expression and subcellular localization of PIN proteins, by immunolocalizing various PIN proteins in primary and lateral root tips of wild-type and *35S::PID* lines. This showed that the tissue-specific expression domains of the PIN proteins are unchanged in *35S::PID* root tips. Although quantitative changes cannot be excluded, this suggests that regulation of *PIN* gene expression or protein stability is not a primary target of PID action. *35S::PID* expression did, however, lead to a basal-to-apical shift in the subcellular polarity of PIN proteins. This apical shift was most ap-

¹Developmental Genetics, Center for Molecular Biology of Plants, University Tübingen, Auf der Morgenstelle 3, D-72076 Tübingen, Germany. ²Developmental Genetics, Institute of Biology, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, Netherlands. ³College of Life Sciences, Peking University, Beijing 100871, China. ⁴Albert-Ludwigs-Universität, Biologie II, Schaenzlestrasse 1, D-79104 Freiburg, Germany. ⁵Umeå Plant Science Center, Department of Forest and Plant Physiology, Swedish University of Agricultural Sciences, S 901 83 Umeå, Sweden. ⁶Institute of Applied Genetics and Cell Biology, BOKU—University of Natural Resources and Applied Life Sciences, Muthgasse 8, A-1190 Vienna, Austria.

*To whom correspondence should be addressed. E-mail: offringa@rubim.leidenuniv.nl

parent in cells where PINs normally show basal subcellular polarity, such as PIN2 in the cortex (Fig. 2, A and B versus C); PIN4 in the epidermis, cortex, endodermis, and vascular cells (Fig. 2, E and F versus G and H); and PIN1 in the endodermis and stele (fig. S1, A versus B). PIN localization in *35S::PID* lines remains unchanged in cells, where PIN proteins are localized apically in the wild type; for example, PIN2 in epidermal cells (Fig. 2, A and B versus C).

The *35S::PID*-induced basal-to-apical shift requires the active PID protein kinase, because it is not observed in the *35S::MPID* line (fig. S1C), and the shift is dose-dependent, because it is most pronounced in root tips of the strong overexpression line *35S::PID-21* (Fig. 2H) as compared to the intermediate line *35S::PID-10* (Fig. 2G). To

exclude the possibility that the basal-to-apical PIN polarity shift is a secondary consequence of changes in patterning before meristem collapse, we examined PIN localization in NPA-rescued root tips of 3-day-old *35S::PID-21* seedlings. NPA treatment itself did not have any pronounced effect on the subcellular polarity of PIN localization (fig. S1D), and the basal-apical shift was still observed in the rescued *35S::PID* root meristems (fig. S1E). Also, a clear *35S::PID*-induced basal-to-apical shift of PIN2 could be observed in the cortex of lateral root meristems that, in contrast to the primary root meristem, remain functional and do not collapse (Fig. 2D). To provide additional evidence for the immediate causality between ectopic PID expression and the shift in subcellular PIN polarity, we used an estrogen(4-hydroxytamoxifen)-inducible two-component expression system to drive ectopic *PID* expression. Tamoxifen-dependent expression of a coregulated *GUS* reporter gene was detectable in roots after 12 hours of induction (13), followed by strong constitutive expression 24 hours after induction (fig. S1G). The basal-to-apical shift of PIN

polarity was clearly visible in seedlings 12 hours after treatment with tamoxifen (Fig. 2J) and became more pronounced after 24 hours (Fig. 2, K and L). Neither incubation of *pINTAM* \gg *PID* seedlings in medium alone (fig. S1H) nor treatment of seedlings of the *pINTAM-GUS* control line with tamoxifen (Fig. 2I) induced changes in PIN polarity.

These data demonstrate that *PID* overexpression leads to apical retargeting of basally localized PIN proteins, and they explain the effect of *PID* overexpression on root meristem activity. In the wild-type root meristem, basal localization of PIN1, PIN2, and PIN4 in subepidermal cell types directs the auxin supply to the root tip and creates the auxin maximum needed for maintenance of root meristem activity. The *35S::PID*-induced basal-to-apical polarity shift of both PIN2 and PIN4 results in a unidirectional auxin flux away from the root tip. This depletes the primary root meristem of auxin (Fig. 1E) resulting in loss of the auxin maximum and root meristem collapse. The reason for the insensitivity of lateral roots to the *PID*-mediated shift in PIN polarity is unclear, but it could be due to increased

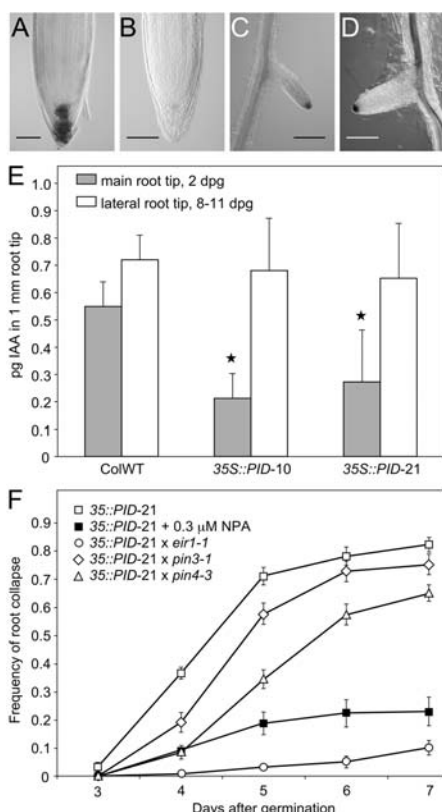


Fig. 1. Ectopic *PID* expression results in PIN-mediated changes in auxin homeostasis in the primary root meristem. (A to D) *DR5::GUS* expression in wild-type [(A) and (C)] and *35S::PID* [(B) and (D)] primary [(A) and (B)] and lateral [(C) and (D)] roots. *GUS* stainings were performed for 1 hour in (A) and (B) and for 4 hours in (C) and (D) (21). (E) Auxin measurements in 1-mm tips of the primary and lateral roots of wild-type and *35S::PID* seedlings (21). Dpg, days post germination. The asterisk marks values that are significantly different from the wild type (t test, $P < 0.05$). (F) Collapse of *35S::PID-21* primary root meristems is significantly delayed by NPA and in *pin2/eir1-1* and *pin4* mutant backgrounds (21). Scale bar, 60 μM in (A) and (B), 0.3 mm in (C), and 0.2 mm in (D).

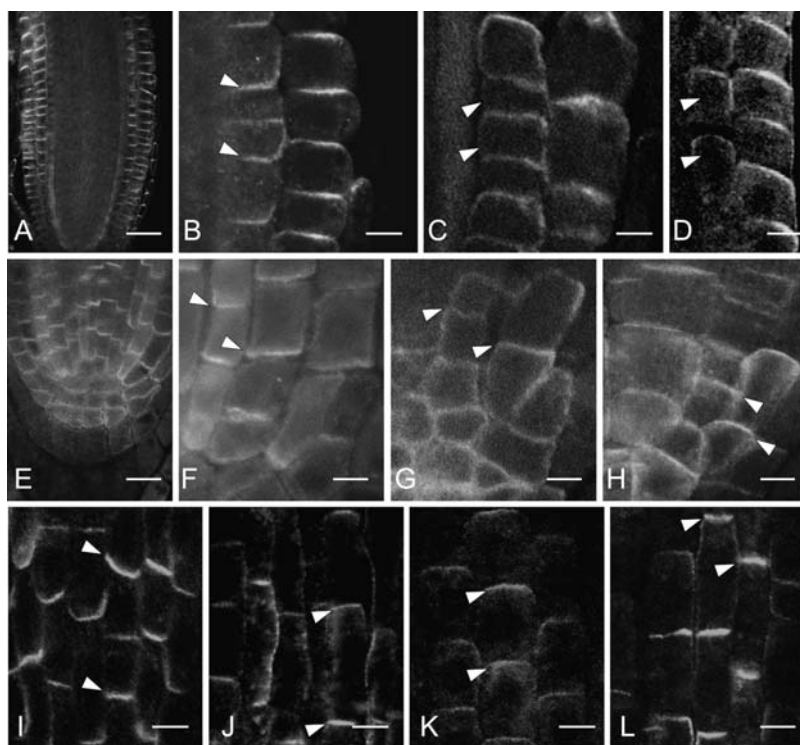


Fig. 2. *PID* overexpression induces a basal-to-apical shift in PIN polarity. (A to H) Immunolocalization of PIN2 [(A) to (D)] and PIN4 [(E) to (H)] in the primary root [(A) to (C) and (E) to (H)] and the lateral root (D) of wild-type control seedlings [(A), (B), (E), (F)] and of seedlings of the intermediate and strong *PID* overexpression lines *35S::PID-10* (G) and *35S::PID-21* [(C), (D), and (H)], respectively (21). (I to L) The effect of inducible overexpression of *PID* on the subcellular localization of PIN1 in roots of 3-day-old seedlings. (I) *pINTAM* \gg *GUS* control seedlings after 48 hours of incubation in tamoxifen-containing medium. *pINTAM* \gg *PID* seedlings after 12 (J), 24 (K), and 36 (L) hours of tamoxifen treatment are shown (21). Arrowheads indicate apical or basal subcellular PIN polarity [(B) to (D) and (F) to (L)]. Images are representative of at least 10 observed samples. Scale bar, 40 μM in (A), 3 μM in (B) to (D) and (F) to (L), and 15 μM in (E).

auxin biosynthesis in lateral root meristems (14). Alternatively, the exclusive collapse of *35S::PID* primary root meristems could relate to their embryonic origin.

PIN-dependent auxin gradients are responsible for both the establishment and the maintenance of the primary root meristem during embryogenesis (1). Root pole initiation depends on an apical-to-basal auxin flow in young globular embryos, which requires basally localized PIN proteins and GNOM activity (15). As a consequence, auxin accumulates in the hypophysis, which in response is specified as the root meristem precursor (1). As in postembryonic *35S::PID* roots, a basal-to-apical switch in PIN1 localization was also observed in *35S::PID* embryos; however, this switch occurred only in post-globular stages (Fig. 3B) after hypophysis specification. In line with this, *35S::PID* embryos showed mild defects in root meristem patterning only at maturity (fig. S2, A to C). Because these mild embryonic phenotypes may be due to low *35S* promoter activity during early embryo development, we expressed *PID* under the control of the *RPS5A* promoter, which is highly active in young embryos (16, 17). Accordingly, young globular *RPS5A* \gg *PID* embryos showed severe defects in PIN polar localization (Fig. 3C). The complete basal-to-apical shift of PIN1 polarity in *RPS5A* \gg *PID* embryos is clearly different from the random PIN1 polar localization observed in *gnom* mutants (15). The auxin-responsive *DR5rev::GFP* reporter (1) showed that *RPS5A* \gg *PID* globular- and heart-stage embryos are incapable of establishing an auxin maximum in the hypophysis. In fact, green

fluorescent protein (GFP) expression is excluded from the hypophysis and instead accumulates in the embryo proper (Fig. 3, D and E, and fig. S2, D and E). Altered PIN1 polarity and *DR5rev::GFP* misexpression in *RPS5A* \gg *PID* embryos is accompanied by misspecification of the hypophysis, as demonstrated by defective cell divisions (Fig. 3G). After germination, *RPS5A* \gg *PID* seedlings showed strong patterning defects, including the absence of a root and defective cotyledons (Fig. 3H), which are strikingly similar to *gnom* and quadruple *pin1*, *pin3*, *pin4*, *pin7* loss-of-function phenotypes (1). These data imply that the *PID*-mediated basal-to-apical shift in PIN polarity in the embryo reverses the apical-basal auxin flow, leading to accumulation of auxin in the apical regions instead of the hypophysis. As a consequence, the apical-basal axis (root pole) and bilateral symmetry (cotyledons) of the embryo are not properly specified.

The data described above show that *PID* gain of function induces a basal-to-apical shift in PIN polarity, resulting in alterations in auxin distribution that lead to corresponding developmental defects. Next we examined the effect of *PID* loss of function on PIN polarity, using transgenic lines carrying a functional *PIN1::GFP* fusion construct (3) (fig. S3, A to E). In both *pid* and *pin1* mutants, inflorescence development, including organ initiation at the apex, is severely disrupted. PIN1 is localized apically in epidermal cells below the very apex in these regions in the wild type (3, 18) (fig. S3, D and E). Strikingly, PIN1:GFP is localized to the basal side of epidermal cells in the same regions in different *pid* mutant allele backgrounds

(Fig. 4, A to C). This apical-to-basal reversal of PIN1 polarity is unlikely to be an indirect consequence of the altered inflorescence structure, because PIN1:GFP does not localize basically in morphologically comparable, NPA-induced, pin-shaped inflorescences of wild-type or *35S::PID* plants (Fig. 4, D to G). These data indicate that *pid* loss of function results in basal targeting of PIN proteins, leading to failure in the establishment of local auxin accumulation required for proper apical organ formation (3). The identification of *PID* as a regulator of polar PIN1 localization at the shoot apex explains the similarity in development of the *pid* and *pin1* mutants and places *PID* at a central position in polar auxin transport regulation.

The phenotypes of *PID* gain- and loss-of-function plants initially suggested a role for this protein kinase in the regulation of auxin signaling (8) or auxin transport (9); however, the mechanism and target(s) of *PID* action remained elusive. Data presented here now indicate that *PID* is a regulator of polar auxin transport at the level of PIN protein local-

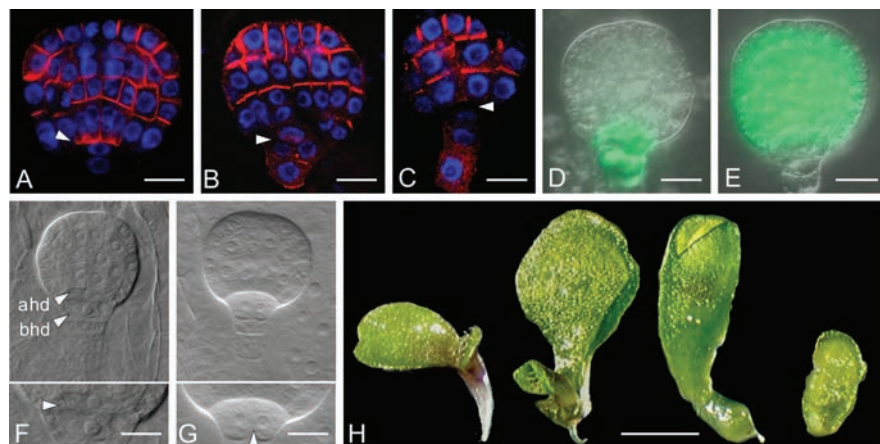


Fig. 3. Ectopic *PID* expression in embryos. (A to C) PIN1 immunolocalization in triangular-stage wild-type (A) and *35S::PID* (B) embryos and in a globular-stage *RPS5A* \gg *PID* (C) embryo. The absence of basally located PIN1 in cells above the hypophyseal cell group (arrowhead) is indicative of the basal-to-apical shift in PIN1 polarity. (D and E) *DR5rev::GFP* expression in globular wild-type (D) and *RPS5A* \gg *PID* (E) embryos (21). (F and G) Differential interference contrast images of wild-type (F) and *RPS5A* \gg *PID* (G) globular embryos (21). The plane of cell division in the hypophyseal region is indicated by an arrowhead. (H) One-week-old *RPS5A* \gg *PID* F1 seedlings showing defects in basal patterning and bilateral symmetry formation. Images are representative of at least five observed samples. Scale bar, 10 μ M in (A) to (E), 5 μ M in (F) and (G), and 3 mm in (H).

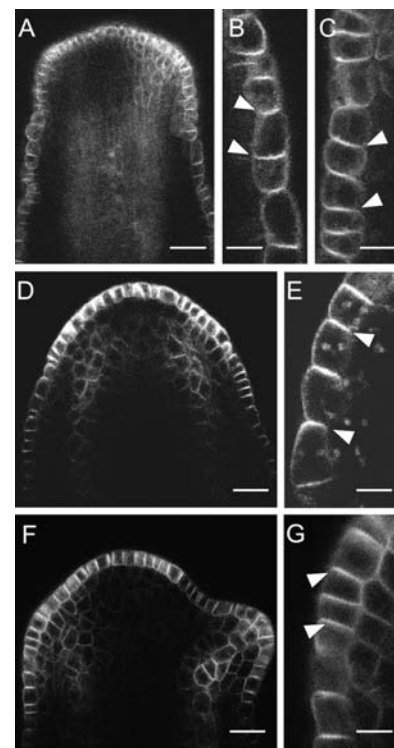


Fig. 4. Localization of the PIN1:GFP fusion protein in inflorescence apices in wild-type and mutant *Arabidopsis*. (A to C) Confocal sections of pin-formed inflorescences of the *pid::En310* [(A) and (B)] and *pid::En197* (C) loss-of-function alleles and of NPA-grown wild-type [(D) and (E)] and *35S::PID-21* [(F) and (G)] plants (21). Arrowheads indicate apical or basal subcellular PIN1:GFP polarity [(B), (C), (E), and (G)]. Images are representative of at least 10 observed samples. Scale bar, 20 μ M in (A), (D), and (F), and 5 μ M in (B), (C), (E), and (G).

ization. Rapid changes in the polarity of PIN proteins in response to developmental or environmental cues have been shown to redirect auxin flow and mediate multiple developmental processes (1, 3, 5), but the mechanism underlying polarity control is largely unresolved. These rapid relocations are enabled by constitutive cycling of PIN proteins. GNOM, an endosomal regulator of vesicle budding that mediates this process, is required for the recycling but does not seem to directly determine the polarity of PIN targeting, because *gnom* mutants show randomized changes in PIN1 polar localization (15). In line with this more general function of GNOM in polar auxin transport, most, if not all polar auxin transport-dependent developmental processes are disturbed in *gnom* mutants (19). In contrast, the role of PID in controlling PIN polarity is more specific. It appears that PID acts as a binary switch, with subthreshold PID levels leading to basal PIN localization and above-threshold PID levels leading to apical PIN localization. Consistently, the defects observed in both loss- and gain-of-function of *PID* lines can each be ascribed to a reversion in auxin flow. The observation that auxin controls cellular PID levels (9) raises the intriguing possibility that PID is involved in a feedback mechanism by which auxin influences the polarity of its own flow. Such a regulatory network has been proposed in the classical

“canalization” model to explain the self-organizing properties of vascular tissue differentiation (20) and would also account for the dynamic auxin-dependent changes in auxin redistribution underlying phyllotaxis (3, 18). Even though endogenous PID acts in a limited subset of PIN-dependent development processes, many more cells are competent to respond to PID overexpression. In *Arabidopsis*, PID belongs to a family of 23 protein kinases, the members of which are differentially expressed. It is therefore likely that ectopic PID expression reveals functions that are normally represented by these different PID family members. Although further experimentation is needed to determine whether PIN proteins are direct targets of PID, it is clear that PID-dependent phosphorylation is an essential intermediate step in the control of polar PIN protein targeting, and thus of directional auxin flow regulating patterning processes.

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Materials and Methods
Figs. S1 to S3
References

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Harnessing Chaperones to Generate Small-Molecule Inhibitors of Amyloid β Aggregation

Jason E. Gestwicki, Gerald R. Crabtree, Isabella A. Graef*

Protein aggregation is involved in the pathogenesis of neurodegenerative diseases and hence is considered an attractive target for therapeutic intervention. However, protein-protein interactions are exceedingly difficult to inhibit. Small molecules lack sufficient steric bulk to prevent interactions between large peptide surfaces. To yield potent inhibitors of β -amyloid ($A\beta$) aggregation, we synthesized small molecules that increase their steric bulk by binding to chaperones but also have a moiety available for interaction with $A\beta$. This strategy yields potent inhibitors of $A\beta$ aggregation and could lead to therapeutics for Alzheimer's disease and other forms of neurodegeneration.

Aggregation of $A\beta$ peptide, generated by proteolytic cleavage of amyloid precursor protein (APP), is a key event in the pathogenesis of Alzheimer's disease (1–3). Genetic studies on Alzheimer's disease implicate mutations

in APP and in genes involved in APP processing (4) in disease development. Because genetic factors that contribute to Alzheimer's disease also promote $A\beta$ aggregation, inhibition of polymerization may have therapeutic benefit. However, this process has proven extremely difficult to prevent (5, 6). One hurdle to overcome is the poor capacity of low molecular weight drugs to interfere with the protein-protein interactions that generate toxic $A\beta$ aggregates. Blocking protein-

protein interactions is a general problem and, despite the importance of these interactions in biologic processes, few inhibitors have been identified (7, 8). The binding energy that drives protein-protein contacts is typically distributed over a large area and, unlike enzymes, these surfaces lack a defined “hotspot” for pharmacological intervention. Additionally, protein surfaces possess a high level of plasticity that can serve to accommodate small molecules, thereby avoiding inhibition.

To approach this problem, we envisioned a Trojan Horse strategy in which a small bifunctional molecule would gain access to the relevant biologic compartment, bind tightly to a chaperone, and thereby gain the steric bulk needed to disrupt a protein-protein interaction (Fig. 1A). Chaperones are ideally suited for this approach, as they bind folding or misfolded proteins via exposed hydrophobic surfaces.

In this strategy, one end of the bifunctional molecule binds the FK506 binding protein (FKBP) chaperone family of peptidyl prolyl cis-trans isomerases. FKBP are encoded by 23 different genes, are highly expressed in all mammalian cells (9, 10), and are good candidates for recruitment by bifunctional molecules (11–15). The other end

Department of Pathology, Howard Hughes Medical Institute, Stanford University Medical School, Stanford, CA 94305, USA.

*To whom correspondence should be addressed.
E-mail: graef@cmgm.stanford.edu