

Ligand recognition by SH3 and WW domains: the role of N-alkylation in PPII helices

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SH3 and WW domains are involved in a variety of intracellular signaling pathways. Recent work has shed light on the mechanism whereby these signaling modules recognize prolines in polyproline ligands, which has implications in the design of ligands selectively targeting these interactions.

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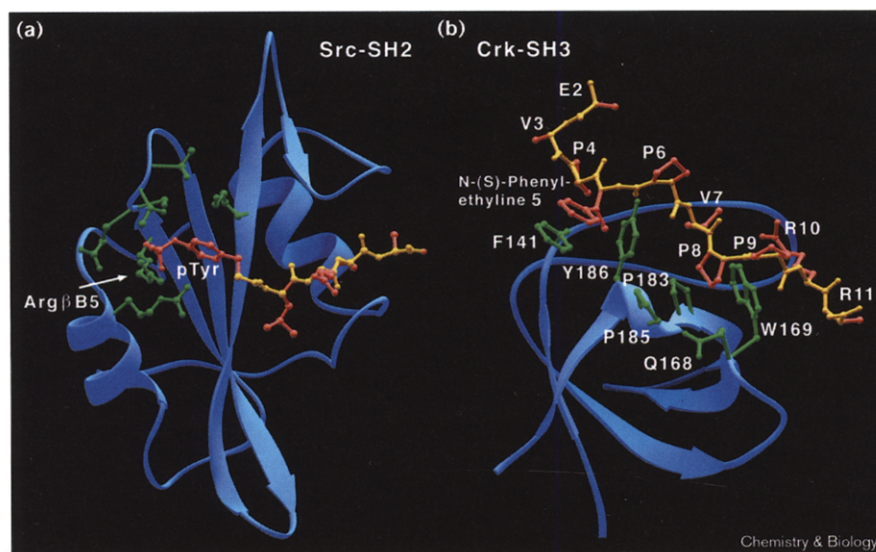
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Chemistry & Biology September 1999, 6:R241–R246
<http://biomednet.com/elecref/10745521006R0241>

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Signal transduction refers to the transmission of extracellular information, in a coordinated, spatially defined manner, to intracellular targets. Information transfer occurs primarily through changes in molecular interactions or covalent modification of proteins, peptides, lipids and other small molecules [1]. These lead to a variety of events including allosteric modulation of enzyme activity, formation of multimolecular assemblies or changes in intracellular localization. The result is often a net increase in the local concentration of primed molecules capable of further propagating the signal to additional downstream effectors. The majority of molecular interactions in signaling pathways are governed by a small set of conserved, noncatalytic protein domains. These modules, which include SH2, SH3, WW, PDZ, PTB and EH domains, each recognize a specific peptide motif in their targets (for recent reviews see [2,3]). The binding domains are arrayed in signaling proteins in a combinatorial fashion, along with various catalytic domains, enabling individual molecules to integrate information from multiple sources. Such integration gives rise to the highly interconnected nature of signaling networks in the cell. The molecular mechanisms of target recognition by the modular binding domains have been the focus of extensive studies and have been reviewed in detail elsewhere. Here we focus on the recent work by Nguyen *et al.* [4] on ligand recognition by SH3 and WW domains. The unexpected mode whereby these domains bind peptides has implications for the design of high-affinity and highly specific molecules targeting individual pathways. As a point of comparison, we begin by briefly reviewing ligand recognition by SH2 domains.

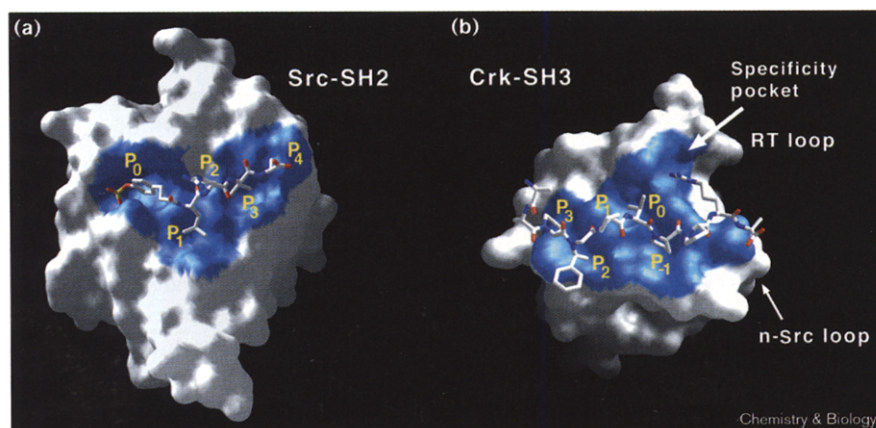
Among the most extensively studied signaling modules are the Src homology II (SH2) domains, commonly found in tyrosine kinase signaling pathways. These domains bind phosphotyrosine (pTyr or pY) in specific sequence contexts, thereby acting as sensors of tyrosine kinase activity. SH2 domains are compact structures of approximately 100 amino acids formed by a central β sheet inserted between two α helices (Figure 1a). Peptides are bound in an extended conformation perpendicular to the plane of the sheet at one edge, with the pTyr residue inserting into a cleft bordered by the sheet and the amino-terminal helix. Residues of the ligand carboxy-terminal to the pTyr are recognized by regions of the SH2 domain bordered by the β sheet and the carboxy-terminal helix. The affinity of physiological ligands is typically in the range 0.1–1 μ M [5]. Binding of a specific peptide involves two levels of recognition. First, phosphotyrosine must be recognized relative to tyrosine. This has been achieved through the construction of the pTyr binding pocket (Figure 1a). The most conserved feature of this cleft is a buried arginine sidechain (Arg β B5), whose guanidinium amino groups form bidentate hydrogen bonds to the phosphate moiety on pTyr [6]. Additional electrostatic and hydrophobic contacts with the pTyr sidechain vary among different SH2 domains. In all cases, however, the pTyr sidechain is nearly completely buried in the complex, virtually exhausting the binding potential of this residue. The complementary geometries and precise positioning of the SH2 β B5 arginine and ligand pTyr sidechains are key in the interaction (Figure 1a), rendering SH2 Arg(β B5)→Lys mutants or ligands containing nonaromatic phosphoserine and phosphothreonine residues ineffective binders to the wild-type partner [7]. Similarly, the burial of an unsatisfied charge on Arg(β B5) by nonphosphorylated ligands substantially decreases their affinities for the domain. The second level of specificity involves the ability of different SH2 domains to discriminate between different pTyr peptides. This specificity is conferred by three to five amino acids carboxy-terminal to the pTyr residue. A given SH2 domain will show preference for different amino acid types at the individual positions. These preferences are not strict, however, and are based on the character of the interaction (charged or hydrophobic) rather than the specific stereochemical properties of the sidechains. Many SH2 domains contain a shallow hydrophobic pocket that recognizes a hydrophobic sidechain of the ligand three residues carboxy-terminal to the pTyr (the +3 position, Figure 2a). Alternatively, a second smaller class of SH2 domains contain a more extended hydrophobic groove that recognizes aliphatic residues at the +1, +3 and +5 positions of the ligand (Figure 2a). As an example of the first

Figure 1

Ligand recognition by SH2 and SH3 domains. **(a)** Ribbon [33] diagram of Src SH2 (blue) complexed with a phosphotyrosine ligand, pYEEI (yellow backbone with red sidechains) [6]. Sidechains of residues in the phosphotyrosine binding pocket are shown in green. **(b)** Ribbon [33] diagram of Crk-SH3 (blue) in complex with a designed high-affinity ligand containing a N-(S)-phenylethyl group [4]. SH3 sidechains contacting the proline core of PPII ligands are shown in green, the ligand is shown in yellow with red sidechains. Residue numbering is according to [4], using single-letter amino acid code.

mode, the SH2 domains of Src and Lck bind peptides containing a pYEEI motif (where E is glutamate and I is isoleucine) [6,8]. The glutamic acid residues make loose contacts with positively charged solvent-exposed residues, whereas the isoleucine residue is bound in a well-defined cavity formed by two loops bordering the carboxy-terminal helix (Figure 1a). Variations in the amino acid sequence within these loops is thought to be at least in part responsible for determining specificity toward ligands. Evidence for this was provided by Marengere *et al.* [9] who show that a mutation of a threonine in Src-SH2 EF loop to a tryptophan residue (found at this position in Grb2 SH2) changed ligand specificity to resemble that of Grb2 SH2. Thus SH2 domains bind ligands through the conserved recognition of a pTyr followed by several carboxy-terminal residues, which impart specificity through less well-conserved interactions.

The structurally unrelated SH3 and WW domains bind polyproline motifs [1,10], which are functionally analogous to pTyr in SH2 recognition. Proline residues within these motifs make key contacts with conserved elements of the protein surfaces, while surrounding residues serve to confer specificity through interactions with more variable regions. WW domains are small (<40 residues) protein modules consisting of a slightly twisted three-stranded antiparallel β sheet [11]. A hydrophobic patch, created by aromatic and methyl-containing residues on one face of the sheet, forms the ligand binding surface [11]. SH3 domains are comprised of roughly 60 residues, forming two three-stranded antiparallel β sheets packed at approximately right angles with respect to one other (Figure 1b) [12]. A cluster of aromatic residues from three strands form a curved surface on one edge of the molecule, which, along with two adjacent loops, create the ligand-binding site

Figure 2

Grasp [34] surface representation of SH2 and SH3 domains complexed with ligands (stick models). **(a)** Src-SH2 domain bound to pTyr peptide [6]. **(b)** Crk-SH3 domain bound to a high-affinity ligand designed by Nguyen *et al.* [4]. The P₂ site contains an N-(S)-phenylethyl group instead of a proline sidechain in the wild-type peptide.

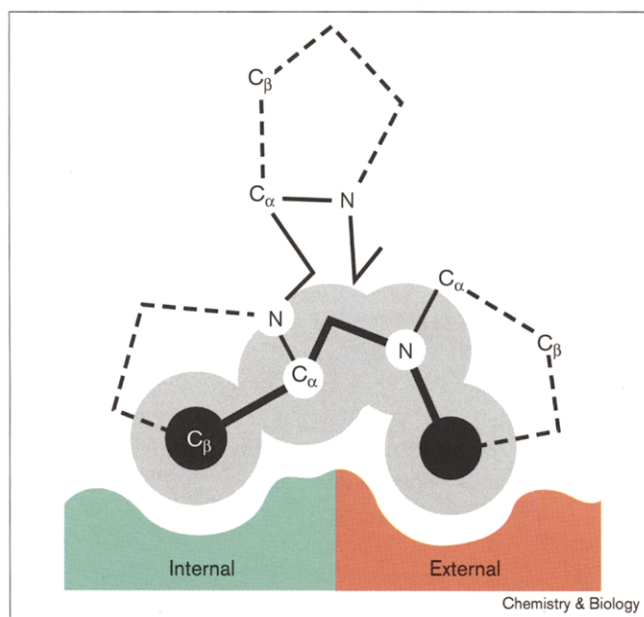
(Figures 1b,2b) [13,14]. X-ray and nuclear magnetic resonance (NMR) studies indicate that both SH3 and WW domains interact with proline-containing ligands in a left-handed type II helix (PPII helix) conformation [14,15]. The consensus binding sequences for these modules are xPxxP (SH3) and PPxY or PPLP (WW) (where P is proline, x any nonproline amino acid and L is leucine) [16,17], and binding is abolished in mutants where prolines are replaced by other amino acids [4,14,16]. The SH3 binding site is composed of three pockets, two of which bind xP elements in the recognition motif. Remarkably, ligands are capable of binding in either of two orientations having opposite directionality of the backbone [15,18]. Interactions of residues flanking the proline motif with the third pocket control orientation, and SH3 residues in the n-Src- and RT-loops ($\beta 3$ – $\beta 4$ and $\beta 1$ – $\beta 2$, respectively) lining this pocket are the primary determinants of ligand specificity (Figure 2b) [19,20]. Specificity of WW domains is conferred by contacts to the final residue in the consensus sequence of the ligands [11]. For both SH3 and WW domains, affinity for polyproline ligands is low, typically in the micromolar range [14,18]. Furthermore, despite the specificity constraints, individual SH3 domains are capable of binding, with varying degrees of promiscuity, a series of SH3 ligands [17,21], and the same ligand has been shown to bind both WW and SH3 domains *in vitro* [17].

To date, most studies of SH3-ligand specificity have focused on the nonproline residues in the PPII helices, as well as residues outside of these regions. Results from biased combinatorial peptide libraries of the form xxxPPx-Pxx as well as phage display libraries have been particularly informative in this regard [14,22,23]. Using the former, Chen *et al.* [22] first identified two classes of SH3 ligands, characterized by an arginine residue either three residues amino-terminal (class I) or two residues carboxy-terminal (class II) to the PxxP core. Subsequent structural and biochemical analyses established that this arginine residue forms energetically favorable contacts with an aspartic acid in the SH3 domain RT loop in both ligand classes, dictating opposite orientation of the backbone in the two cases [15,18]. Analogous data on the Abl SH3 domain have similarly established that residues amino-terminal to the PxxP core of a ligand derived from its partner, 3BP1, make energetically important contacts to the RT loop. Interactions with the specificity pocket has also been exploited in the design of nonpeptide libraries attached amino-terminally to a common LPPLP core [24,25]. These libraries led to identification of high-affinity small molecule-peptide hybrids that, based on structure determination of their complexes with an SH3 domain, recognize the specificity pocket in a manner distinct from natural ligands. Comparison of library-derived class I ligands for Src-, PI3K- and Abl-SH3 domains has also revealed different preferences for nonproline residues in individual xP elements in the core binding region.

Although Src and PI3K prefer LP in the amino-terminal element, Abl prefers PP; in the carboxy-terminal element, Src prefers LP whereas PI3K selects for RP [23]. In both cases, these differences have been attributed to variations in the n-Src loop of the SH3 domains, which contacts the first residue in xP elements of class I ligands. The culmination of these findings were reported by Pisabarro *et al.* [26] who have recently shown how an increase in affinity and specificity can be achieved by rationally optimizing the interactions of a ligand with both the specificity and proline binding pockets. On the basis of the crystal structure of the Abl-SH3 domain in complex with a native 3BP1 peptide, they designed a ligand that had a 30-fold higher affinity for Abl-SH3, and 150-fold greater selectivity toward the Fyn SH3 domain [26]. This was accomplished by replacing methionine and proline residues adjacent to the PPII helix by tyrosine and serine, respectively, as well as a leucine within the polyproline region by proline [26]. The crystal structure of the optimized ligand bound to the Abl-SH3 domain reveals that the hydroxyl group of the tyrosine aromatic ring forms favorable hydrogen bonds with serine and aspartate residues on the protein. In Fyn-SH3, the serine and aspartate residues correspond to an arginine and a glutamine residue, respectively. Together with replacement of a glycine (Abl-SH3) located in the RT loop by a threonine (Fyn-SH3), these larger amino acids would probably prohibit the binding of the tyrosine because of steric clashes, explaining the lower affinity observed for Fyn. The mutation to a serine enables the formation of an intraligand hydrogen bond between this residue and an adjacent proline. As a result, the two halves of the ligand are displaced with respect to each other, creating a more favorable positioning of the two regions for SH3 binding. Circular dichroism data indicate that the mutation of a solvent-exposed leucine residue to a proline at the p1 position (Figure 2b) stabilizes the PPII helix, resulting in entropic benefits for complex formation [26].

The importance of proline in SH3 and WW recognition has been attributed, in part, to the ability of this residue to induce PPII helices, providing optimal spacing of residues recognized by the binding surface of the domains. There is also evidence for a direct role in mediating unique contacts within the SH3 and WW domain binding sites. Lim *et al.* [15] have demonstrated by molecular modeling that, depending on the relative orientation of the ligand, residues in PPII helices pack with their C β atom directed either toward (internal packing) or away (external packing) from the protein surface (Figure 3). Correspondingly, the group bonded to the amide nitrogen, H or CH $_2$, is directed either away (internal packing) or toward (external packing) the interface. In the latter case, the hydrophobic pockets of the SH3 and WW domains would strongly disfavor interactions with nonproline residues, whose backbone amide protons would be targeted into the binding site. N-alkyl substitution of prolines, however, enables their

Figure 3



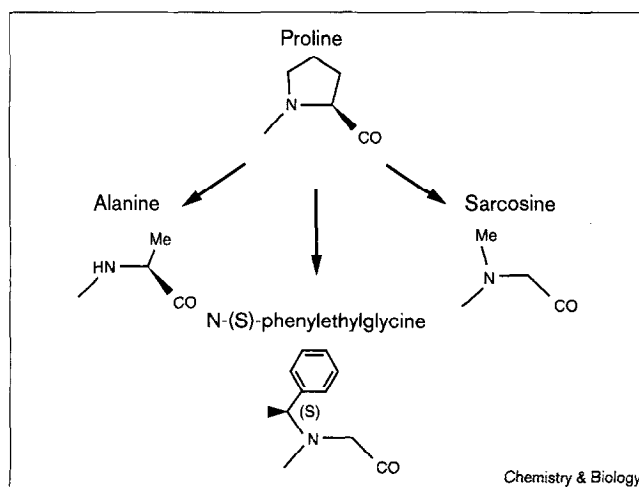
Schematic depiction of external versus internal binding modes of proline residues in PPII helices (see text). Adapted from [4].

sidechains to turn inward and make appropriate hydrophobic contacts with the domain surface (Figure 3).

In their recent publication, Nguyen *et al.* [4] further refined the role of proline in these interactions through elegant studies of synthetic SH3/WW ligands containing noncyclic N-alkylated amino acids. They designed a series of peptides in which residues in the proline-rich core of several natural SH3/WW ligands were individually replaced by either alanine or sarcosine (N-methyl glycine; Figure 4). Alanine mutants of conserved prolines show reduced affinity, whereas ligands containing sarcosine at these sites bind their target domains with affinities comparable to that of wild-type peptides, showing that the rigidity and shape of the proline ring are dispensable in the interaction [4]. They argued, therefore, that it is largely N-substitution at key positions in the ligand that is recognized by SH3 and WW domains rather than the particular geometric properties of the proline residue.

In contrast to earlier focus by others on the regions outside of polyproline motifs, Nguyen *et al.* [4] demonstrated how optimizing the contacts to the proline-binding regions of SH3 domains can be exploited as an alternative approach in the design of high affinity ligands. Having established the importance of N-substitution in PPII recognition, they synthesized a series of 12-mer SH3 ligand mutants in which either of the two conserved prolines in the xPxxP motif was replaced by a non-natural N-substituted amino acid. In fluorescence binding assays they found the affinity of most of these ligands for the carboxy-terminal SH3

Figure 4



Chemical structures of proline, alanine, sarcosine and N-(S)-phenylethylglycine.

domain of Sem5, the amino-terminal SH3 domains of Grb2 and Crk, and the SH3 domain of Src [4] to be comparable to wild type. Unfavorable binding was observed for N-alkyl residues with charged groups. This is consistent with the predicted binding mode in which externally packing residues orient their N-alkyl moieties toward the binding surface, which for SH3 and WW domains is hydrophobic. Conversely, several ligands bound the SH3 domains with higher affinities than the proline-containing analogs. The tightest binding ligand contained an N-(S)-phenylethyl group (Figure 4) at the p_{-1} position, and bound Grb2-SH3 with a K_D of 40 nM, two orders of magnitude higher than the affinity for the wild-type ligand. Moreover, this interaction is stereospecific, since the R-stereoisomer bound with a 10^3 -fold lower affinity. Only modest gains in affinity were observed for several other SH3 domains, indicating that use of N-alkyl amino acids can enhance specificity as well as binding affinity. Similar specificity differences were observed when the same functional group was placed at the p_2 position, with Crk-SH3 showing the highest affinity in this case. The X-ray structure of the Crk-SH3-peptide complex confirms that the ligand is bound in a PPII helix conformation (Figure 1b). The N-alkyl group of the mutant residue turns inward and contacts the SH3 domain surface, as expected for residues at external binding sites. The methyl group of the N-(S)-phenylethyl moiety inserts into the proline-binding site, occupying a position equivalent to the β -carbon of a proline residue in a natural ligand at the p_2 position [27]. The phenyl ring, however, is solvent exposed and no interactions with the SH3 domain are observed (Figure 2b). Interchanging the positions of the methyl and phenyl groups in the R-stereoisomer would lead to steric clashes because the size of the SH3-binding pocket cannot accommodate this ring system, explaining the low

affinity for the N-(R)-phenylethyl ligand. Interestingly, a similar ligand lacking the phenyl group showed only a modest gain in affinity for Crk-SH3, and no significant difference in affinity was measured for a ligand with a phenyl group but lacking the methyl moiety. These data imply that the phenyl ring serves primarily to restrict the N-alkyl group to a conformation favorable for binding probably as a result of minimizing pseudo allylic^{1,3} interactions with substituents at C α of the previous residue, and therefore has an entropic benefit for complex formation.

These findings encourage investigations to further enhance the binding affinity and selectivity of SH3 and WW domain ligands. The passive role of the aromatic ring in the N-(S)-phenylethyl group, for example, could be exploited by designing ligands with functional groups leading off the ring and making specific contacts with the surface of the SH3 domain. Nguyen *et al.* [4] have reported affinities for peptides containing a single N-alkyl residue. Further improvements in affinity and specificity could obviously be achieved by targeting both proline binding pockets simultaneously. Such ligands would have the two conserved prolines replaced with N-alkylated residues that individually show high affinity and selectivity for their respective binding sites. Improvement in specificity could also arise from the loss of orientational binding ambiguity observed for natural ligands. The near symmetrical sidechain geometry apparently enables proline residues to make energetically similar contacts with their targets, irrespective of binding orientation [15]. This will not be the case for N-substituted residues in general.

The question arises why SH3- and WW-domain-ligand interactions have evolved without higher specificity for their ligands, rendering signaling pathways utilizing these modules susceptible to cross-talk. One answer could be that high-affinity binding with slow off rates would make it difficult for other regulatory proteins to intervene, and signals would no longer be transient. High-affinity binding with high off rates has been observed in kinetics studies of several signaling systems, however, including SH2-pTyr and GTPase-effector binding [28,29]. Alternatively, because signaling modules can act as molecular adapters, bringing together multiple interacting partners, they should be considered within the framework of a coordinated cellular structure. Even though the individual domain-ligand interactions may be of low affinity and relatively promiscuous, cooperativity can greatly increase affinity and specificity in higher-order complex formation, potentially without sacrificing the rapid kinetic characteristic of the separate binding events [30]. Cowburn *et al.* [31] have demonstrated that linking low-affinity SH2 and SH3 domain binding peptides can yield high affinity ligands targeting tandem SH2-SH3 domains. In addition, the simultaneous accommodation of multiple binding units places more stringent requirements on target

ligands. Sites accessible to binding *in vitro*, might be occluded in such complexes. An interaction observed *in vitro* might never occur *in vivo* because of different subcellular localization of the interacting partners. Specificity is thus distributed over a number of determinants. Finally, ambiguity in ligand binding could in itself be a means by which signals are modulated. Depending on other queues, a stimulus may activate more than one signaling pathway, and this might be an efficient means of accomplishing this task. In such cases the dynamic properties of the signaling assemblies might be particularly important in determining the balance of outputs and the resulting state of the cell.

The work presented by Nguyen *et al.* [4] sheds further light into the mechanism whereby SH3 and WW domains recognize their ligands. Replacement of conserved proline residues by other N-substituted amino acids could yield ligands with improved selectivity and affinity toward individual SH3 and WW domains by making more extensive contacts with the surfaces of these domains. With the aid of such ligands, signaling through SH3 and WW domains could be selectively manipulated, elucidating the roles of the proteins involved, as well as the biological function of the individual pathways. From our increasing understanding of signal transduction pathways underlying diseases, targeting of specific protein-protein interactions has emerged as a means of therapy [32]. Drug delivery and specificity requirements necessitate therapeutic agents to be small molecules capable of traversing cell membranes in addition to being selectively bound by their targets. This, in turn, places stringent requirements on the binding sites of target proteins. The active sites of enzymes are designed to make extensive and highly specific contacts to small substrates, utilizing the resulting high binding energy for catalysis. These sites therefore provide suitable targets for inhibitory molecules. It has become evident, however, that SH3 and WW domains lack such sites, instead offering energetic binding potential spread across a relatively large surface area. Nguyen *et al.* [4] have detailed how this potential is distributed, giving further insight into the feasibility of developing drugs targeting signaling pathways utilizing SH3 and WW domains.

Acknowledgements

We thank Eduardo Torres for assistance in preparation of the figures.

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