

30. Beck, F. X., Schmolke, M., Guder, W. G., Dörge, A. & Thureau, K. Osmolytes in renal medulla during rapid changes in papillary tonicity. *Am. J. Physiol.* **262**, F849–F856 (1992).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

**Acknowledgements**

We thank J. Faulhaber and H. Ehmke for blood gas analysis; M. Bösl for blastocyst injection and implantation; M. Knipper for the prestin antiserum; M. Knepper for the aquaporin 2 antiserum; S. Gluck for the proton ATPase antiserum; M. Kolster, B. Dierkes and I. Öztürk for technical assistance; H. Voss for taking care of animals; and U. Koch for support. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Prix Louis-Jeanet de Médecine to T.J.J.

**Competing interests statement**

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to T.J.J. (e-mail: jentsch@zmnh.uni-hamburg.de).

**Structural determinants for GoLoco-induced inhibition of nucleotide release by G $\alpha$  subunits**

Randall J. Kimple\*, Michelle E. Kimple†, Laurie Betts\*†, John Sondek\*‡§ & David P. Siderovski\*‡§

\* Department of Pharmacology; † Department of Biochemistry and Biophysics; ‡ Lineberger Comprehensive Cancer Center; and § UNC Neuroscience Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA

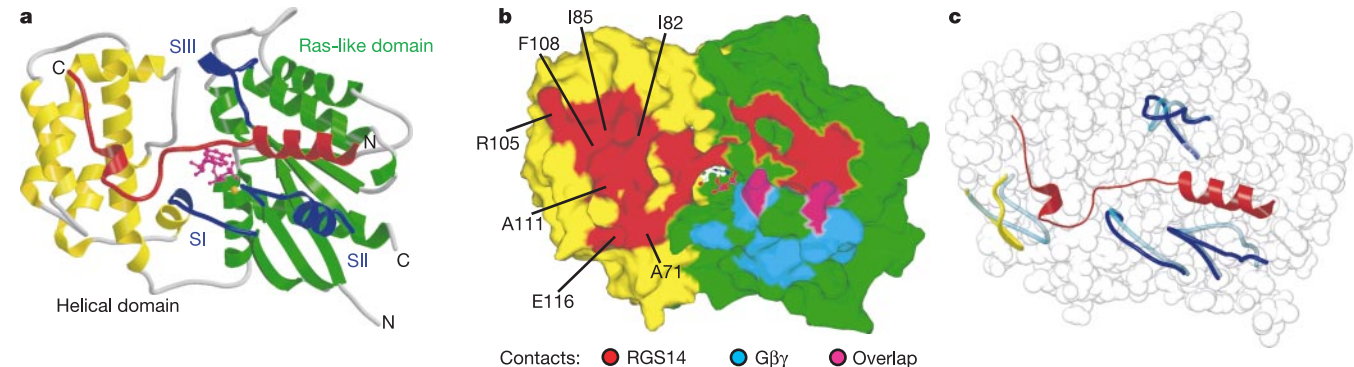
Heterotrimeric G-proteins bind to cell-surface receptors and are integral in transmission of signals from outside the cell. Upon activation of the G $\alpha$  subunit by binding of GTP, the G $\alpha$  and G $\beta\gamma$  subunits dissociate and interact with effector proteins for signal transduction. Regulatory proteins with the 19-amino-acid GoLoco motif<sup>1,2</sup> can bind to G $\alpha$  subunits and maintain G-protein subunit dissociation in the absence of G $\alpha$  activation<sup>3–7</sup>. Here we describe the structural determinants of GoLoco activity as revealed by the crystal structure of G $\alpha_{i1}$ -GDP bound to the GoLoco region of the ‘regulator of G-protein signalling’ protein

RGS14. Key contacts are described between the GoLoco motif and G $\alpha$  protein, including the extension of GoLoco’s highly conserved Asp/Glu-Gln-Arg triad into the nucleotide-binding pocket of G $\alpha$  to make direct contact with the GDP  $\alpha$ - and  $\beta$ -phosphates. The structural organization of the GoLoco-G $\alpha_{i1}$  complex, when combined with supporting data from domain-swapping experiments, suggests that the G $\alpha$  all-helical domain and GoLoco-region carboxy-terminal residues control the specificity of GoLoco-G $\alpha$  interactions.

In heterotrimeric G-protein signalling, cell surface receptors (GPCRs) are coupled to membrane-associated heterotrimers comprising a GTP-hydrolysing G $\alpha$  subunit and a G $\beta$ -G $\gamma$  dimer. G $\beta\gamma$  binds tightly to GDP-bound G $\alpha$ , enhancing the coupling of G $\alpha$  to the receptor and acting as a guanine nucleotide dissociation inhibitor (GDI) to inhibit spontaneous GDP release<sup>8,9</sup>. Agonist-promoted exchange of bound GDP for GTP alters the conformation of three G $\alpha$  ‘switch’ regions (I–III) and allows G $\beta\gamma$  dissociation and subsequent effector interactions by both  $\alpha$ -GTP and free G $\beta\gamma$ . Intrinsic, or RGS protein-accelerated<sup>10</sup>, GTP hydrolysis by G $\alpha$  returns the subunit to the GDP-bound state and allows G $\alpha\beta\gamma$  re-assembly and termination of effector interactions. GoLoco-motif proteins interact specifically with GDP-bound G $\alpha_{i/o}$ -class G $\alpha$  subunits, preventing both GDP release<sup>3–6</sup> and G $\beta\gamma$  re-assembly<sup>6,7</sup>, and thus permitting continued G $\beta\gamma$ -effector interactions in the absence of G $\alpha$  activation<sup>2</sup>. The GoLoco motif is present in RGS12, RGS14, LOCO, Purkinje-cell protein-2 (Pcp2) and Rap1GAP isoforms, and is repeated in tandem arrays within the *Drosophila* protein Rapsynoid (also known as Partner of Inscuteable, Pins) and its mammalian homologues AGS3 and LGN (ref. 1). During development of the *Drosophila* nervous system, the binding of Pins to G $\alpha_s$  and the resultant displacement of G $\beta\gamma$ , is thought to underlie mitotic spindle re-orientation, which is critical for the asymmetric cell divisions exhibited by embryonic neuroblasts and sensory organ precursor cells<sup>7</sup>. More recently, the Pins-related protein LGN has been shown to be essential for mitotic spindle assembly and organization in mammalian cells<sup>11</sup>.

To ascertain the structural determinants of selective  $\alpha$ -GDP binding and novel GDI activity exhibited by GoLoco proteins, we determined the crystal structure of the RGS14 GoLoco region bound to an adenylyl cyclase-inhibitory G $\alpha$  subunit ( $\alpha_{i1}$ -GDP). Diffraction data collected from a single crystal at 100 K were used to refine the structure to 2.7 Å resolution (Supplementary Information Table A) using the structure of  $\alpha_{i1}$ -GDP-Mg<sup>2+</sup> as a model for molecular replacement<sup>12</sup>.

Of the 36 amino acids of the R14GL peptide (residues r496–r530, numbered according to full-length rat RGS14), 35 are ordered



**Figure 1**  $\alpha_{i1}$ -GDP in complex with the RGS14 GoLoco region. **a**, Ribbon drawing of R14GL peptide (red) in contact with the Ras-like (green) and all-helical (yellow) domains of G $\alpha_{i1}$ . Also shown are the three switch regions of G $\alpha_{i1}$  (blue), GDP (magenta) and Mg<sup>2+</sup> (orange). **b**, Molecular surface of R14GL (red) and G $\beta\gamma$  (cyan) contacts on G $\alpha_{i1}$ -GDP, denoting

shared switch II residue contacts (magenta). Highlighted are G $\alpha_{i1}$  residues that contact the R14GL peptide and are different within G $\alpha_{i1}$ . **c**, Space fill model of  $\alpha_{i1}$ -GDP in its R14GL-bound conformation (switch regions in blue,  $\alpha$ B- $\alpha$ C loop in yellow) and G $\beta\gamma_{27}$ -bound conformation (in cyan).

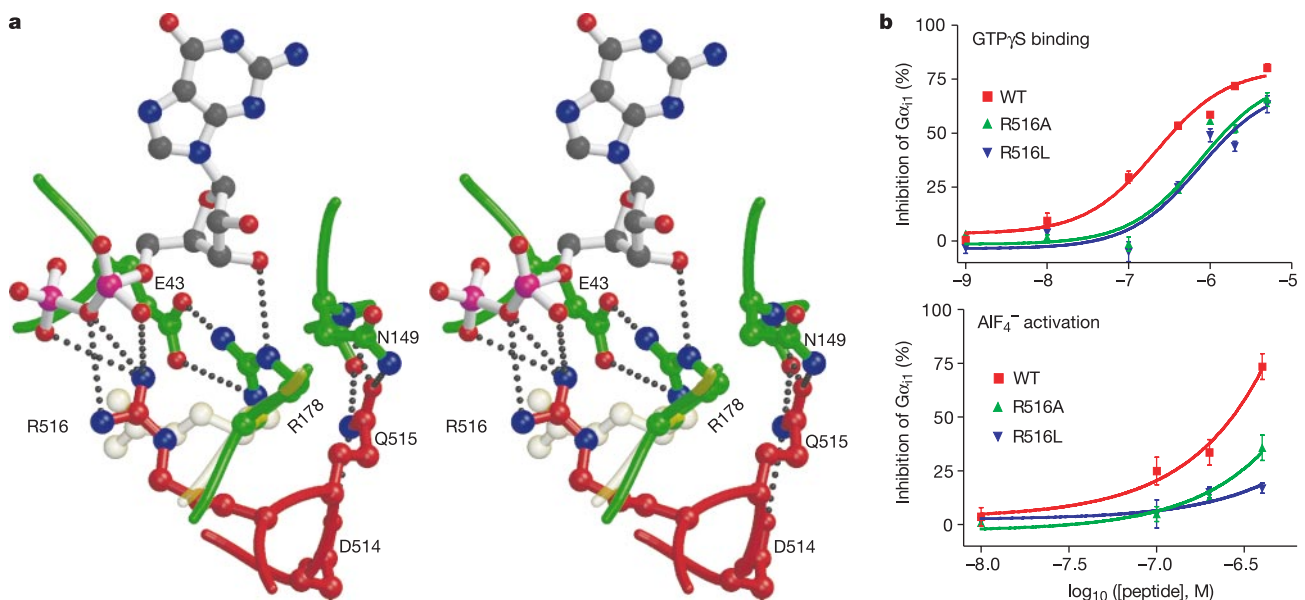
within the complex (Fig. 1a and Supplementary Information); more than half of the total surface area of the peptide is buried by the  $\text{G}\alpha_{i1}$  subunit (1,900 Å<sup>2</sup> buried out of 3,666 Å<sup>2</sup> total surface area; Supplementary Information Table B). The amino terminus of the R14GL peptide (r496–r508) forms an  $\alpha$ -helix that is sandwiched between switch II and the  $\alpha 3$  helix of the  $\text{G}\alpha_{i1}$  Ras-like domain (Fig. 1a); contacts between R14GL and switch II residues overlap with contacts made between  $\alpha_{i1}$ ·GDP and  $\text{G}\beta\gamma$  subunits (Fig. 1b). The side chain of Gln r508, which caps the  $\alpha$ -helix C terminus, is extensively buried within  $\text{G}\alpha_{i1}$ . Contacts made between  $\text{G}\alpha_{i1}$  and the N-terminal portion of the R14GL peptide (residues r496–r516) all involve residues that are identical between  $\text{G}\alpha_{i1}$  and  $\text{G}\alpha_o$ . The GoLoco peptide continues across the interdomain region between the Ras-like and all-helical domains in a more random conformation; in this region, conserved residues of the 19-amino-acid GoLoco motif contact both switch I and the bound guanine nucleotide. Surprisingly, the R14GL peptide C terminus (residues r517–r530), a region poorly conserved among GoLoco proteins, interacts extensively (660 Å<sup>2</sup> buried surface area) with the  $\alpha$ A and  $\alpha$ B helices of the  $\text{G}\alpha_{i1}$   $\alpha$ -helical domain (Fig. 1a). More surprisingly,  $\text{G}\alpha$  residues within this region that contact the GoLoco peptide (Ala 71, Ile 82, Ile 85, Arg 105, Phe 108, Ala 111 and Glu 116; Fig. 1b) are the only contact residues that differ between  $\text{G}\alpha_{i1}$  and  $\text{G}\alpha_o$  subunits.

Interactions with the R14GL peptide alter the conformationally flexible switches I and II relative to uncomplexed  $\alpha_{i1}$ ·GDP·Mg<sup>2+</sup> (not shown) and  $\text{G}\beta\gamma$ -bound  $\alpha_{i1}$ ·GDP·Mg<sup>2+</sup> (Fig. 1c). In particular, the significant deformation of switch II (for example, Arg 208 moves about 6 Å relative to heterotrimer configuration) would preclude coincident  $\text{G}\beta\gamma$  binding to GoLoco-complexed  $\alpha_{i1}$ ·GDP (Fig. 1c). This supports previous findings that  $\text{G}\beta\gamma$ - and GoLoco-binding are mutually exclusive<sup>2,6,7</sup>. The conformation of switch region III is also altered relative to the heterotrimer structure (Fig. 1c), and the  $\alpha$ B– $\alpha$ C loop of the  $\text{G}\alpha_{i1}$  helical domain, previously dubbed ‘switch IV’<sup>13</sup>, is displaced away from the Ras-like domain (for example, Ala 114 moves about 11 Å, and Glu 116 moves about 4 Å relative to the heterotrimer configuration) to accommodate

binding of the GoLoco C-terminal region.

The highly conserved Asp–Gln–Arg triad within the GoLoco motif participates directly in GDP binding by extending the arginine side chain into the nucleotide binding pocket (Fig. 2), highly reminiscent of the catalytic ‘arginine finger’ employed by GTPase-accelerating proteins (GAPs) for Ras-superfamily GTPases<sup>14–16</sup>. Gln r515 is positioned away from the GDP binding pocket and makes both side-chain and backbone interactions with Asp r514 of the peptide and Gln 147 and Asn 149 of the  $\text{G}\alpha$  subunit; these constraints placed on the Gln r515 conformation position the peptide to allow full insertion of the neighbouring Arg r516 residue into the GDP binding pocket. The guanidinium group of Arg r516 contacts the  $\alpha$ - and  $\beta$ -phosphates and the bridging oxygen between them (Fig. 2a). The role of Arg r516 in the GoLoco– $\text{G}\alpha$  interaction is underscored by the diminished GDI activity seen on mutation of Arg r516 to alanine (R516A) or leucine (R516L) (nearly a tenfold reduction in activity; Fig. 2b), or the complete loss of GDI activity when changed to phenylalanine<sup>17</sup>. Although the nonconservative replacement of arginine with the bulky, hydrophobic phenylalanine also results in greatly decreased binding affinity<sup>2,17</sup>, neither the R516L nor the R516A mutation decreased the affinity of the GoLoco– $\alpha_{i1}$ ·GDP interaction. Dissociation constants ( $K_D$ ) derived by a surface plasmon resonance biosensor for both mutants were comparable to that previously obtained for the wild-type RGS14 GoLoco region: for glutathione S-transferase (GST)–RGS14<sub>496–513</sub> [R516A],  $K_D = 52$  nM ( $\chi^2 = 5.2$ ); and for GST–RGS14<sub>496–513</sub> [R516L],  $K_D = 70$  nM ( $\chi^2 = 31.9$ ); compared with wild-type GST–RGS14<sub>496–513</sub> for which  $K_D = 65$  nM ( $\chi^2 = 3.7$ )<sup>5</sup>. Conservative mutation of a nucleotide-interacting arginine residue has been known to diminish biochemical activity without loss of binding affinity. Conservative mutations to the catalytic arginine finger of several GAPs, including p120-RasGAP<sup>14</sup>, NF1/neurofibromin<sup>15</sup>, RhoGAP<sup>16</sup>, and eIF5 (ref. 18), were previously reported to affect the ability to stimulate GTP hydrolysis without influencing complex formation with cognate GTPase partners.

Arg 178 within switch I of the  $\text{G}\alpha_{i1}$  subunit is critically involved in GTP hydrolysis by stabilizing the  $\gamma$ -phosphate leaving group<sup>19–21</sup>. In



**Figure 2** Role of the GoLoco motif Asp–Gln–Arg triad in GDI activity. **a**, Stereo view of the remodeled nucleotide binding pocket. The  $\text{G}\alpha_{i1}$  Arg 178 side chain (transparent yellow) is re-oriented (green) to form a salt bridge with Glu 43 of the  $\text{G}\alpha_{i1}$  subunit, thus allowing Arg r516 of the GoLoco motif (red) to approach and interact with the  $\alpha$ - and  $\beta$ -phosphate oxygens (and bridging oxygen) of GDP. **b**, GST–RGS14 GoLoco fusion proteins (GST–

RGS14<sub>496–531</sub>) bearing alanine (R516A) or leucine (R516L) substitutions show decreased GDI activity relative to the wild-type GST–RGS14<sub>496–531</sub> (WT), as measured both by BODIPY–GTP $\gamma$ S binding (left) and by AIF<sub>4</sub><sup>–</sup>-induced increase of intrinsic tryptophan fluorescence (right).



the crystals was adjusted to 20% glycerol by 2% (v/v) stepwise increases in glycerol concentration. A native data set was collected on a single crystal using an R-Axis IV++ detector with Rigaku RUH3R generator and Osmic Confocal Blue Optics at the UNC-CH X-Ray Facility. All diffraction data were processed using DENZO and SCALEPACK<sup>24</sup>. The structure of  $\alpha_{i1}$ -GDP·Mg<sup>2+</sup> (Protein Data Bank accession number 1BOF)<sup>12</sup>, excluding the first 30 amino acids, water molecules and sulphates, was used as a molecular replacement model for  $\alpha_{i1}$ -GDP-R14GL using the CCP4 program AMoRe<sup>25</sup>. The program O (ref. 26) was used for model building and the program CNS (with bulk-solvent correction)<sup>27</sup> was employed for simulated annealing and torsion angle refinement. Data collection and structure refinement statistics are shown in Supplementary Information Table A. Figures 1a, c, and 2a were created using MOLSCRIPT<sup>28</sup> and Raster3D<sup>29</sup>. Figure 1b was made using Spock<sup>30</sup> (<http://mackerel.tamu.edu/jon/spock>) and Raster3D.

### Biochemical assays

Assays of GDI activity and GoLoco-G $\alpha$  binding affinity were performed as previously described<sup>2</sup>. Briefly, to quantify GDI activity, initial rates of fluorescence increase on BODIPY-GTP $\gamma$ S binding by G $\alpha$ , or activation of intrinsic G $\alpha$  tryptophan fluorescence by AIF<sub>6</sub> was measured by real-time spectrofluorometry in the presence and absence of GoLoco-containing peptide or GST-fusion protein. Binding affinity of GST-GoLoco fusion protein for  $\alpha_{i1}$ -GDP was measured using an anti-GST surface plasmon resonance biosensor surface (BIACORE). Apparent dissociation constants were calculated using the 1:1 Langmuir (with mass transport) model as implemented by the BIAevaluation 3.0 program (BIACORE).

Received 29 October 2001; accepted 5 February 2002.

- Siderovski, D. P., Diversé-Pierluissi, M. A. & De Vries, L. The GoLoco motif: a G $\alpha$ -i/o binding motif and potential guanine-nucleotide exchange factor. *Trends Biochem. Sci.* **24**, 340–341 (1999).
- Takesono, A. *et al.* Receptor-independent activators of heterotrimeric G-protein signalling pathways. *J. Biol. Chem.* **274**, 33202–33205 (1999).
- De Vries, L. *et al.* Activator of G protein signalling 3 is a guanine dissociation inhibitor for G $\alpha$  i subunits. *Proc. Natl Acad. Sci. USA* **97**, 14364–14369 (2000).
- Natochin, M. *et al.* AGS3 inhibits GDP dissociation from G $\alpha$  subunits of the G<sub>i</sub> family and rhodopsin-dependent activation of transducin. *J. Biol. Chem.* **275**, 40981–40985 (2000).
- Kimple, R. J. *et al.* RGS12 and RGS14 GoLoco motifs are G $\alpha$  interaction sites with guanine nucleotide dissociation inhibitor activity. *J. Biol. Chem.* **276**, 29275–29281 (2001).
- Natochin, M., Gasimov, K. G. & Artemyev, N. O. Inhibition of GDP/GTP exchange on G $\alpha$  subunits by proteins containing G-protein regulatory motifs. *Biochemistry* **40**, 5322–5328 (2001).
- Schaefer, M., Petronoczi, M., Dorner, D., Forte, M. & Knoblich, J. A. Heterotrimeric G proteins direct two modes of asymmetric cell division in the *Drosophila* nervous system. *Cell* **107**, 183–194 (2001).
- Brandt, D. R. & Ross, E. M. GTPase activity of the stimulatory GTP-binding regulatory protein of adenylate cyclase, Gs. Accumulation and turnover of enzyme-nucleotide intermediates. *J. Biol. Chem.* **260**, 266–272 (1985).
- Higashijima, T., Ferguson, K. M., Sternweis, P. C., Smigel, M. D. & Gilman, A. G. Effects of Mg<sup>2+</sup> and the  $\beta\gamma$ -subunit complex on the interactions of guanine nucleotides with G proteins. *J. Biol. Chem.* **262**, 762–766 (1987).
- Neubig, R. R. & Siderovski, D. P. Regulators of G protein signalling as new central nervous system drug targets. *Nature Rev. Drug Discovery* **1**, 187–197 (2002).
- Du, Q., Stukenberg, P. T. & Macara, I. G. A mammalian Partner of inscuteable binds NuMA and regulates mitotic spindle orientation. *Nature Cell Biol.* **3**, 1069–1075 (2001).
- Coleman, D. E. & Sprang, S. R. Crystal structures of the G protein G<sub>i1</sub> complexed with GDP and Mg<sup>2+</sup>: a crystallographic titration experiment. *Biochemistry* **37**, 14376–14385 (1998).
- Mixon, M. B. *et al.* Tertiary and quaternary structural changes in G $\alpha$  induced by GTP hydrolysis. *Science* **270**, 954–960 (1995).
- Brownbridge, G. G., Lowe, P. N., Moore, K. J., Skinner, R. H. & Webb, M. R. Interaction of GTPase activating proteins (GAPs) with p21ras measured by a novel fluorescence anisotropy method. Essential role of Arg-903 of GAP in activation of GTP hydrolysis on p21ras. *J. Biol. Chem.* **268**, 10914–10919 (1993).
- Mittal, R., Ahmadian, M. R., Goody, R. S. & Wittinghofer, A. Formation of a transition-state analog of the Ras GTPase reaction by Ras-GDP, tetrafluoroaluminate, and GTPase-activating proteins. *Science* **273**, 115–117 (1996).
- Graham, D. L., Eccleston, J. F. & Lowe, P. N. The conserved arginine in rho-GTPase-activating protein is essential for efficient catalysis but not for complex formation with Rho.GDP and aluminum fluoride. *Biochemistry* **38**, 985–991 (1999).
- Peterson, Y. K. *et al.* Stabilization of the GDP-bound conformation of G $\alpha$  by a peptide derived from the G-protein regulatory motif of AGS3. *J. Biol. Chem.* **275**, 33193–33196 (2000).
- Paulin, F. E., Campbell, L. E., O'Brien, K., Loughlin, J. & Proud, C. G. Eukaryotic translation initiation factor 5 (eIF5) acts as a classical GTPase-activator protein. *Curr. Biol.* **11**, 55–59 (2001).
- Coleman, D. E. *et al.* Structures of active conformations of G $\alpha$ 1 and the mechanism of GTP hydrolysis. *Science* **265**, 1405–1412 (1994).
- Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E. & Sigler, P. B. GTPase mechanism of G proteins from the 1.7-Å crystal structure of transducin  $\alpha$ -GDP-AIF-4. *Nature* **372**, 276–279 (1994).
- Bourne, H. R. G proteins. The arginine finger strikes again. *Nature* **389**, 673–674 (1997).
- Wall, M. A., Posner, B. A. & Sprang, S. R. Structural basis of activity and subunit recognition in G protein heterotrimers. *Structure* **6**, 1169–1183 (1998).
- Remmers, A. E., Engel, C., Liu, M. & Neubig, R. R. Interdomain interactions regulate GDP release from heterotrimeric G proteins. *Biochemistry* **38**, 13795–13800 (1999).
- Otwiniowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–326 (1997).
- Navaza, J. AMoRe: an automated package for molecular replacement. *Acta Crystallogr. A* **50**, 157–163 (1994).
- Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. Improved methods for binding protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* **47**, 110–119 (1991).
- Brunger, A. T. *et al.* Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D* **54**, 905–921 (1998).
- Kraulis, P. J. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950 (1991).
- Merritt, E. A. & Bacon, D. J. Raster3D: Photorealistic molecular graphics. *Methods Enzymol.* **277**, 505–524 (1997).
- Christopher, J. A. SPOCK: *The Structural Properties Observation and Calculation Kit* (Program Manual) (The Center for Macromolecular Design, Texas A&M University, College Station, 1998).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

### Acknowledgements

We thank R. Neubig for supplying the G $\alpha$  chimaeras GoGiGo and GiGoGi, members of the Siderovski and Sondek laboratories for technical assistance and support, and T. K. Harden for comments and enthusiasm. R.J.K. is supported by a predoctoral fellowship from the National Institute of Mental Health. J.S. acknowledges support from the National Institutes of Health (NIH) and the Pew Charitable Trusts. D.P.S. is a Year 2000 Scholar of the EJLB Foundation, recipient of the Burroughs-Wellcome Fund New Investigator Award in the Pharmacological Sciences, and acknowledges additional grant support from the NIH.

### Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to D.P.S. (e-mail: dsiderov@med.unc.edu). The atomic coordinates have been deposited at the Research Collaboratory for Structural Bioinformatics under accession code 1KJY.