

Structural insight into guanylyl cyclase receptor hijacking of the kinase–Hsp90 regulatory mechanism

Reviewed Preprint

Revised by authors after peer review.

[About eLife's process](#)

Reviewed preprint version 2

July 12, 2023 (this version)

Reviewed preprint version 1

May 2, 2023

Posted to bioRxiv

March 16, 2023

Sent for peer review

February 25, 2023

Nathanael A Caveney , Naotaka Tsutsumi, K Christopher Garcia 

Departments of Molecular and Cellular Physiology and Structural Biology, Stanford University School of Medicine, Stanford, CA 94305, USA • Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305, USA • Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan

 https://en.wikipedia.org/wiki/Open_access

 <https://creativecommons.org/licenses/by/4.0/>

Abstract

Membrane receptor guanylyl cyclases play a role in many important facets of human physiology, from regulating blood pressure to intestinal fluid secretion. The structural mechanisms which influence these important physiological processes have yet to be explored. We present the 3.9 Å resolution cryo-EM structure of the human membrane receptor guanylyl cyclase GC-C in complex with Hsp90 and its co-chaperone Cdc37, providing insight into the mechanism of Cdc37 mediated binding of GC-C to the Hsp90 regulatory complex. As a membrane protein and non-kinase client of Hsp90–Cdc37, this work shows the remarkable plasticity of Cdc37 to interact with a broad array of clients with significant sequence variation. Further, this work shows how membrane receptor guanylyl cyclases hijack the regulatory mechanisms used for active kinases to facilitate their regulation. Given the known druggability of Hsp90, these insights can guide the further development of membrane receptor guanylyl cyclase-targeted therapeutics and lead to new avenues to treat hypertension, inflammatory bowel disease, and other membrane receptor guanylyl cyclase-related conditions.

eLife assessment

In this **important** study, the human membrane receptor guanyl cyclase GC-C was expressed in hamster cells, co-purified in complex with endogenous HSP90 and CDC37 proteins, and the structure of the complex was determined by cryo-EM. The study shows that the pseudo-kinase domain of GC-C associates with CDC37 and HSP90, similarly to how the bona fide protein kinases CDK4, CRAF and BRAF have been shown to interact. The methodology used is state of the art and the evidence presented is **compelling**.

Cyclic GMP (cGMP) is an important second messenger for signaling in mammalian physiology, with roles in platelet aggregation, neurotransmission, sexual arousal, gut peristalsis, bone growth, intestinal fluid secretion, lipolysis, phototransduction, cardiac hypertrophy, oocyte maturation, and blood pressure regulation (Potter, 2011 [↗](#)). Largely, cGMP is produced in response to the activation of guanylyl cyclases (GC), a class of receptors that contains both heteromeric soluble receptors (α_1 , α_2 , β_1 and β_2 in humans) and five homomeric membrane receptors (GC-A, GC-B, GC-C, GC-E, GC-F in humans). Of note are the membrane receptor guanylyl cyclases (mGC) GC-A and GC-B, also known as natriuretic peptide receptor A and B (NPR-A and NPR-B), respectively, and GC-C, all of which have been a focus of therapeutic development. In the case of NPR-A and B, their role in regulating blood pressure in response to natriuretic peptide hormones (ANP, BNP, CNP) has led to the exploration of agonists for use in the treatment of cardiac failure (Kobayashi et al., 2012 [↗](#)). Meanwhile, GC-C is the target of clinically approved laxative agonists, linacotide and plecanatide (Miner, 2020 [↗](#); Yu & Rao, 2014 [↗](#)), which increase intestinal fluid secretion.

These membrane receptor GCs consist of an extracellular ligand binding domain (ECD), which acts as a conformational switch to drive intracellular rearrangements to activate the receptor (X. L. He et al., 2001a [↗](#)); a transmembrane region (TM); a kinase homology domain or pseudokinase domain (PK); a dimerization domain; and a guanylyl cyclase (GC) domain, which acts to produce cGMP. The PK domain is largely thought to be involved in scaffolding and physical transduction of the extracellular rearrangements to the GC domain, in some respects similar to the role of the PK domain in the Janus kinases of the cytokine signaling system (Glassman et al., 2022 [↗](#)). In addition, the PK domains of mGCs are regulated through phosphorylation (Potter & Garbers, 1992 [↗](#); Potter & Hunter, 1998 [↗](#); Vaandrager et al., 1993 [↗](#)) and via association with heat shock proteins (Hsp) (Kumar et al., 2001 [↗](#)).

While the role of the phosphorylation state on mGC activity has been explored in relative detail, how the heat shock protein 90 (Hsp90) is able to regulate mGC activity is largely unknown. It has been shown that GC-A activity can be regulated through the association of Hsp90 and the co-chaperone Cdc37 (Kumar et al., 2001 [↗](#)). The chaperone Cdc37 is known to assist in the Hsp90 regulation of around 60% of active kinases, both in soluble and membrane receptor form (Taipale et al., 2012 [↗](#)). Given the sequence and structural similarities between the PK domains of mGCs and the active kinase domains which Hsp90–Cdc37 regulates, it is possible that mGCs have evolved to hijack the regulatory mechanisms that are more broadly deployed for active kinases.

Here we report the 3.9 Å resolution structure of the GC-C–Hsp90–Cdc37 regulatory complex. In this structure, the core dimer of Hsp90 forms its canonical closed conformation, while Cdc37 and the C-lobe of the GC-C PK domain asymmetrically decorate the complex. The client (GC-C) is unfolded into the channel formed at the interface between the Hsp90 dimers. To our knowledge, this is the first structure of a membrane protein client of Hsp90 and the first structure of a non-kinase client of the Hsp90–Cdc37 regulatory system. This work provides pivotal understanding of the mechanism and structural basis of kinase fold recruitment to the Hsp90–Cdc37 regulatory complex. This increased understanding can guide the further development of mGC-targeted therapeutics and lead to new avenues to treat hypertension, inflammatory bowel disease (IBD), and other mGC-related conditions. In addition, the general insights into recruitment of Hsp90–Cdc37 clients can guide the further development of Hsp90 targeting therapeutics in cancer treatment.

Structure of the GC-C-Hsp90-Cdc37 regulatory complex

Membrane receptor guanylyl cyclases have been largely recalcitrant to structural analysis by x-ray crystallography and electron microscopy, apart from various crystal structures of both liganded and unliganded ECDs (X. L. He et al., 2001b [↗](#); X. lin He et al., 2006 [↗](#); Ogawa et al., 2004 [↗](#), 2010 [↗](#); van den Akker et al., 2000 [↗](#)). Given the relative disparity of our structural understanding, we sought to develop a stable construct to image and gain crucial understanding of the regulatory and functional aspects of mGCs which occur intracellularly. By replacing the ligand-responsive ECD with a homodimeric leucine zipper, we mimic the ligand activated geometry of the ECD (X. L. He et al., 2001b [↗](#)), while reducing complexity of the imaged complex and increasing stability (**Figure 1A** [↗](#)). This complex was recombinantly expressed in mammalian cells, purified with anti-FLAG affinity chromatography, and vitrified on grids for cryoEM analysis.

The purified sample had a substantial portion of imaged particles for which the native regulatory heat shock protein, Hsp90, and its co-chaperone, Cdc37, are bound. The *Cricetulus griseus* HSP90β and Cdc37 show remarkable sequence conservation in comparison to the human equivalents, at 99.7 and 94.2% identity, respectively. This native pulldown strategy contrasts with the structures of Hsp90-Cdc37 in complex with soluble kinases (García-Alonso et al., 2022 [↗](#); Oberoi et al., 2022 [↗](#); Verba et al., 2016 [↗](#)), for which Hsp90 and Cdc37 had to be overexpressed to obtain complex suitable for imaging. Three-dimensional reconstruction of our GC-C-Hsp90-Cdc37 particles generated a 3.9 Å resolution map of the regulatory complex (**Figure 1** [↗](#), **Figure 1—figure supplements 1** [↗](#) and **2** [↗](#)). A second, unsharpened map from subsequent heterogenous refinement resolves additional density for the dimerization domain, extending outward from the PK domain (**Figure 1B** [↗](#), **Figure 1—figure supplement 1** [↗](#)).

The resultant GC-C-Hsp90-Cdc37 complex is a hetero-tetramer formed by one resolved monomer of the GC-C receptor bound to a dimer of Hsp90 and one Cdc37 co-chaperone (**Figure 1D** [↗](#)). As observed with most Hsp90-client structures, the bulk of the complex is composed of the C2 pseudosymmetric, ATP bound, closed state Hsp90 dimer. Building on this dimeric core, the Cdc37 protrudes outward from one side with its characteristic long, coiled-coil, α-hairpin. On one face of the Hsp90 dimer core, Cdc37 interacts with the PK domain of GC-C, while an extended β-sheet wraps around to the other face, lying across and extending a β-sheet in the middle domain (MD^{Hsp90}) of one Hsp90 monomer. At the opposite face, the globular and α-helical Cdc37 middle domain (MD^{Cdc37}) is formed. The C-lobe of the GC-C PK domain packs against the N-terminal region of Cdc37 on one face of the dimeric Hsp90 core, with the N-lobe unfolding through the dimer core to interface with the MD^{Cdc37} on the opposite face. N-terminal to the PK N-lobe is the TM region, density for which was unobserved in our reconstructions. C-terminal to the PK C-lobe, we observe some poorly resolved density for the likely mobile dimerization domain in our unsharpened map. This would precede the GC domain, which is not observed in the density of our reconstructions (**Figure 1B** [↗](#)). Together, we can use our understanding of mGC topology and our reconstruction to orient the complex as it would sit on a membrane (**Figure 1B** [↗](#)), providing insight into how Hsp90 is able to access and regulate membrane protein clients. No density is observed for the second GC-C of the dimer, though it is sterically unlikely that an additional regulatory complex is forming on the second GC-C in a concurrent fashion, given the large size of the first Hsp90-Cdc37 and the requisite proximity of the second GC-C. In addition, this disruption of the native state of GC-C, as observed in our structure, would likely leave GC domains out of each other's proximity, precluding their catalytic activity while Hsp90 is bound.

Cdc37 mediated GC-C recruitment and Hsp90 loading

Despite the recognized plasticity of Cdc37 co-chaperone binding to approximately 60% of kinases (Taipale et al., 2012 [↗](#)), the importance of the Hsp90-Cdc37 complex for pseudokinase domain-containing proteins in the human proteome is not well studied. Thus, the structural basis for how

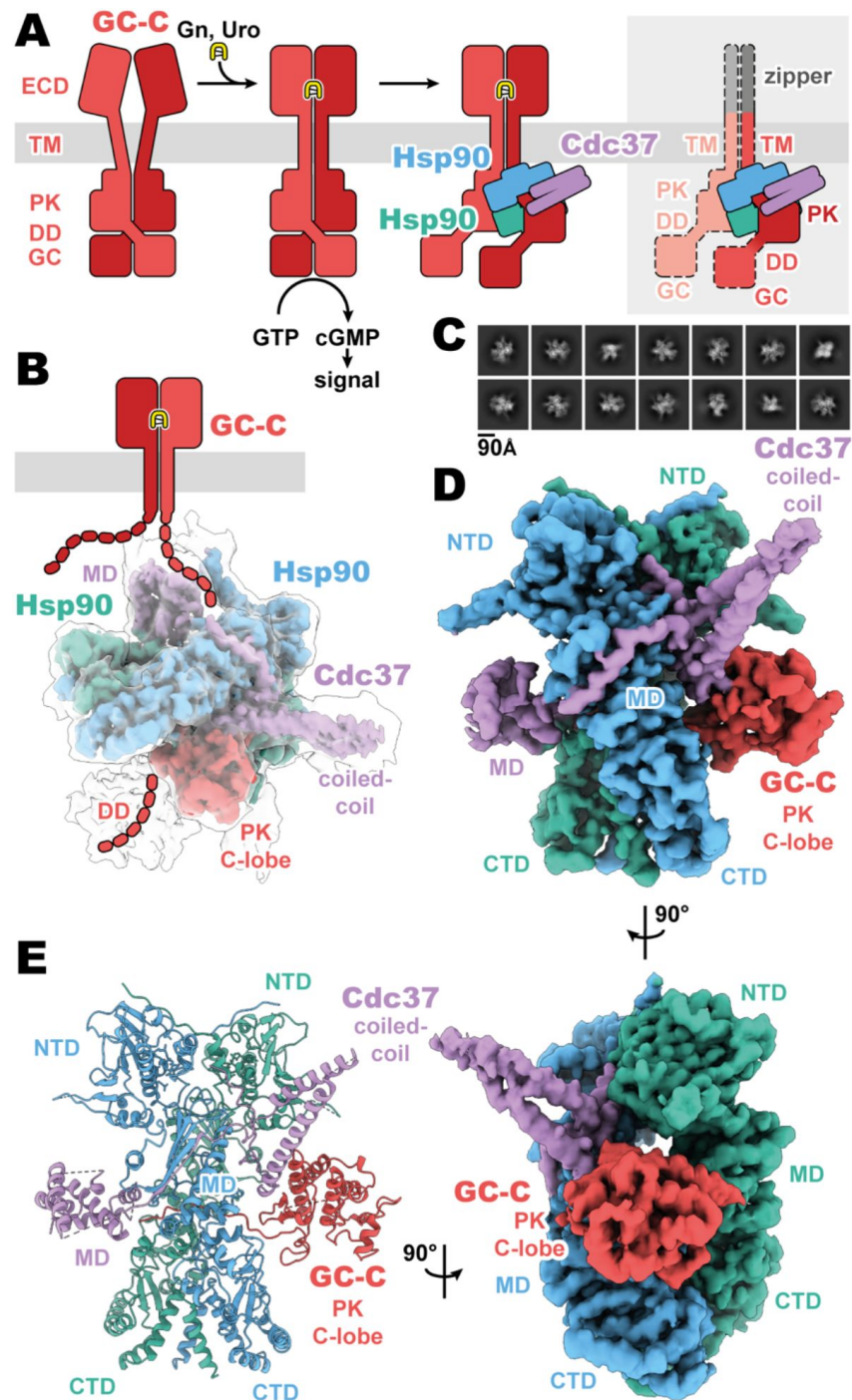


Figure 1.

Composition and cryoEM structure of the GC-C-Hsp90-Cdc37 regulatory complex.

(A) Cartoon representation of the components of GC-C signaling and Hsp90-Cdc37 regulation and the zippered and activated GC-C. GC-C is colored in red, guanylin/uroguanylin (Gn/Uro) in yellow, Hsp90 in blue and teal, and Cdc37 in purple. Extracellular domains (ECD), transmembrane domain (TM), pseudokinase domain (PK), dimerization domain (DD), and guanylyl cyclase domain (GC) are labelled. In the rightmost cartoon, the regions unobserved in the cryoEM density are in a lighter shade with a dashed outline. (B) The refined and sharpened cryoEM density map of GC-C-Hsp90-Cdc37, colored as in A, with a transparent overlay of an unsharpened map with additional DD density resolved. Cdc37 coil-coiled and middle domain (MD) are labelled. (C) Reference free 2D averages for the GC-C-Hsp90-Cdc37 complex. (D) The refined and sharpened cryoEM density map of GC-C-Hsp90-Cdc37, colored as in A and B, labelled with all domains as in A and B, with the addition of Hsp90 N-terminal domain (NTD), middle domain (MD), and C-terminal domain (CTD). (E) Ribbon representation of a model of GC-C-Hsp90-Cdc37 complex, colored and labelled as in A, B, and C.

Cdc37 can recruit GC-C to the Hsp90 regulatory complex is of particular interest. In our structures, we see that Cdc37 is displacing the N-lobe of the pseudokinase domain of GC-C, binding to the C-lobe at the N-C interface, and guiding the unfolded N-lobe into the Hsp90 dimer (**Figure 2**). The Cdc37–GC-C interface is relatively modest in size, with a calculated mean surface area of 689 Å² (as calculated by PISA (Krissinel & Henrick, 2007)). This interface is partly driven to form via charge complementarity, with positive contributions from a cluster of arginine residues on Cdc37 (R30, 32, 39) at the periphery of the interaction interface interacting with D609 and the polar residues Y580 and T586 (**Figure 2B**). Beyond this, the interface is likely largely driven via shape-complementarity, due to a minimal contribution from hydrogen bonding, salt-bridge formation, and aromatic packing contributions – in line with the ability of Cdc37 to chaperone such a diverse array of clients and client sequences.

As the unfolded PK N-lobe extends away from Cdc37, it enters the channel formed at the interface between the dimer of Hsp90 (**Figure 2C**). Here, GC-C residues 528 to 544 (VKLDTMIFGVIEYCERG) lie across the upper region of the Hsp90 CTDs, which form the floor of the channel. These CTDs form the bulk of the interaction interface as the unfolded N-lobe passes through this channel, yet there are minor contributions from the loop regions of the β -sheet from the MD^{Hsp90} which extend downward into this channel region. The unfolded region is relatively poorly resolved in the density, with some reconstructions from earlier refinement having no resolvable density in this channel region – indicative of the low stability and high mobility for the unfolded N-lobe as it passes through this region.

Conservation of Cdc37 mediated Hsp90 regulation

The core structural principles of Cdc37 mediated client recruitment to Hsp90 appear to remain constant across its large range of client diversity. Across other client–Hsp90–Cdc37 complexes with canonical soluble kinase clients (Cdk4, RAF1, B-raf) (García-Alonso et al., 2022; Oberoi et al., 2022; Verba et al., 2016), we see a conserved role for Cdc37 in client recruitment by associating with the C-lobe at the N-, C-lobe interface (**Figure 2—figure supplement 1A, B**). In these complexes, we see high levels of structural conservation for the Hsp90–Cdc37 (C α RMSDs of 1.4–3.3 Å for Hsp90 and 1.5–2.5 Å for Cdc37), while the client is structurally most homogenous at the interface with Cdc37, though less structurally conserved overall (C α RMSDs of 3.5–11.6 Å). Perhaps unsurprisingly, GC-C is one of the most divergent of these clients from a sequence perspective (**Figure 2—figure supplement 1C**), with sequence homology between the GC-C PK domain and the other client kinase domains ranging from 19 to 25% identity and 31 to 41% homology. This highlights the plasticity required of this system which can service such a vast array of clients across a broad range of sequence variation, yet more restricted fold architecture.

Discussion

The present cryoEM structure of GC-C–Hsp90–Cdc37 resolves the loading of GC-C, via its PK domain and interaction with Cdc37, to the Hsp90 core dimer (**Figure 1, 2**). This complex shows significant structural similarity to the mechanism that regulates soluble active kinases (García-Alonso et al., 2022; Oberoi et al., 2022; Verba et al., 2016) and presumably membrane receptor kinases in the human proteome. This structural and mechanistic conservation is largely driven by the co-chaperone Cdc37, which serves as the central binding platform for these clients by associating to the fold of the kinase (or pseudokinase in the case of mGC) domain, relatively independent of sequence identity. A model whereby recruitment is largely driven by both the fold complementarity and the specific stability properties of the kinase fold has been proposed previously (Taipale et al., 2012). In this model, instability of a fully folded kinase domain results in partial unfolding of the C-lobe, leading Cdc37 to bind the partially unfolded state. Given the lack of functional and sequence conservation for GC-C as a client of Cdc37, our data largely fits with this model for client recruitment. It is likely that the pseudokinase domains

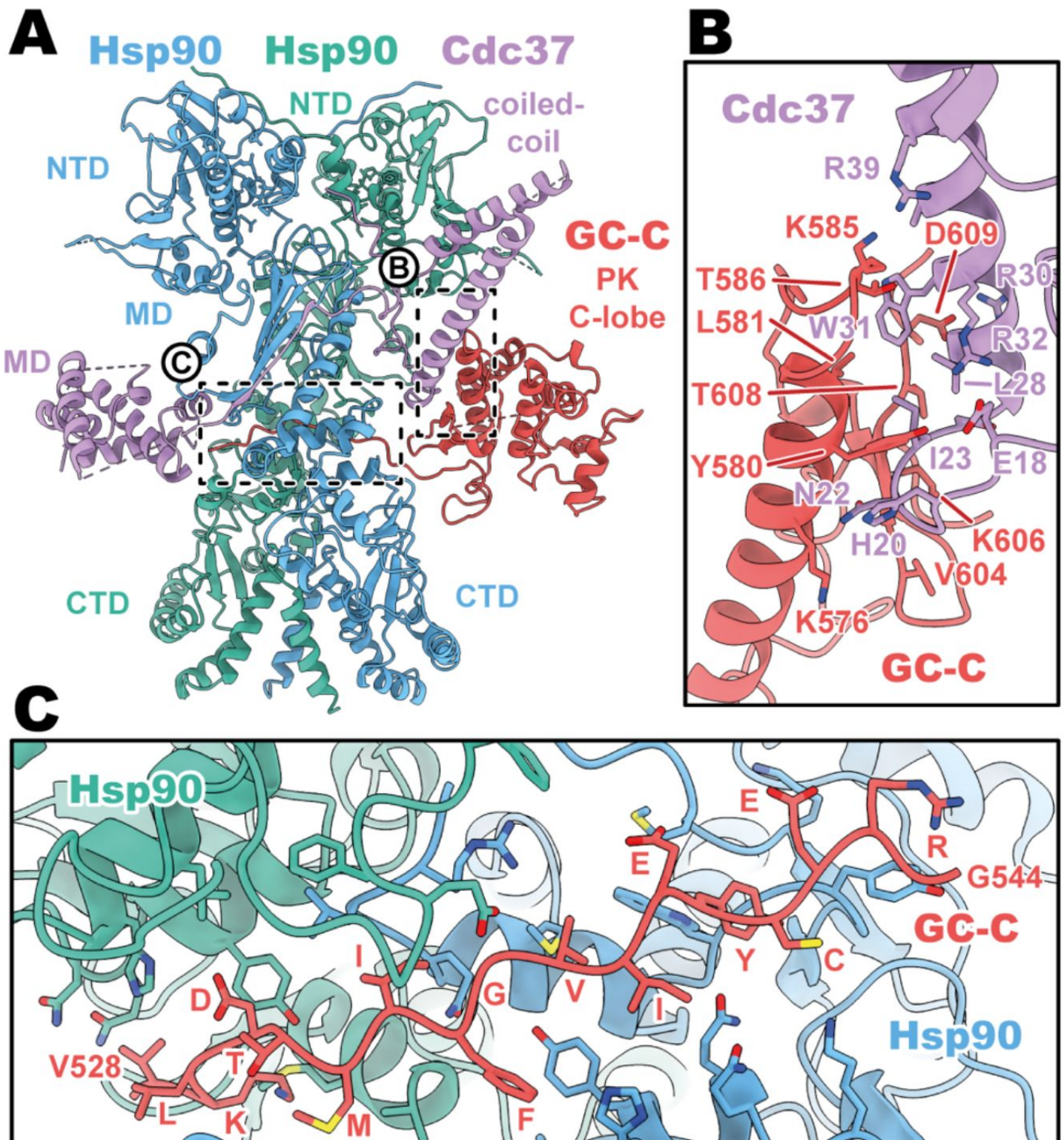


Figure 2.

Cdc37 mediated GC-C recruitment and Hsp90 loading interfaces.

(A) Ribbon representation of a model of GC-C-Hsp90-Cdc37 complex. GC-C is colored in red, Hsp90 in blue and teal, and Cdc37 in purple. Pseudokinase (PK), coil-coiled, middle (MD), C-terminal (CTD), and N-terminal (NTD) domains are labelled. (B) The Cdc37-GC-C interface in ribbon representation, with interacting residues drawn in sticks, colored as in A. (C) The unfolded N-lobe of GC-C PK domain as it passes between the Hsp90 dimer, in ribbon representation, with interacting residues drawn in sticks, colored as in A and B. This region's sequence is: VKLDTMIFGVIEYCERG.

of mGC have largely evolved to facilitate regulatory mechanisms for these receptors, both via their phosphorylation and by hijacking the regulatory mechanisms used by active soluble and membrane receptor kinases.

In the case of GC-A, previous work has shown that it associates with the Hsp90–Cdc37 complex to regulate GC activity (Kumar et al., 2001 [↗](#)). The authors showed that adding geldanamycin, an Hsp90 inhibitor, reduces the overall cGMP output of cells in response to ANP stimulation while also reducing the association of the Hsp90 to GC-A. While this initially may seem counterintuitive, this data fits with a model of ligand-induced activity potentiating the instability of the PK domain, which then facilitates binding of the regulatory complex to “re-fold” GC-A for further catalysis and cGMP production – in a core regulatory complex structurally similar to that which we observe for GC-C in this work (**Figure 2—figure supplement 2** [↗](#)). In the case of the Hsp90 inhibitor, this would release the Hsp90 and only allow full catalytic activity for the receptor until the receptor falls into the partially unfolded state, as the Hsp90 would no longer be able to re-engage at the C-lobe when inhibited (**Figure 2—figure supplement 2** [↗](#)).

Interestingly there may be an additional layer of regulation involved, with crosstalk between the phosphorylation and Hsp90 regulatory mechanisms of mGC. The phosphatase PP5 is known to interact with the Hsp90–Cdc37 system and dephosphorylate Hsp90, Cdc37, and the system’s kinase clients (Oberoi et al., 2022 [↗](#)). PP5 has been implicated in this role for mGC (Chinkers, 1994 [↗](#)), though this interaction was unable to be detected by a pull-down in a second study (Kumar et al., 2001 [↗](#)). In this way, mGC association with the Hsp90–Cdc37 complex could result in multiple fates and resultant activity profiles for the receptor. When the PK of an activated mGC falls into a destabilized state, this would result in the recruitment of the Hsp90–Cdc37. First, the regulatory complex could refold the receptor to maintain the activity of the receptor (**Figure 2—figure supplement 2 i** [↗](#)). In another scenario, the Hsp90–Cdc37 complex could additionally recruit PP5 to dephosphorylate the mGC (**Figure 2—figure supplement 2 ii** [↗](#)). Particularly in the case of GC-A and GC-B, and to some extent GC-C (Potter & Garbers, 1992 [↗](#); Potter & Hunter, 1998 [↗](#); Vaandrager et al., 1993 [↗](#)), this would impair the signaling activity of the mGC, though this could be rescued through the kinase re-association and phosphorylation. In a final scenario, the binding of the Hsp90–Cdc37 complex could result in the association of ubiquitin E3 ligases (Schopf et al., 2017 [↗](#)) (**Figure 2—figure supplement 2 iii** [↗](#)), which would ubiquitinate the mGC client, leading to the removal of the receptor.

The regulation of mGC is influenced by a network of factors working in harmony to ensure proper signaling and physiological response for these important receptors. The structure of the core regulatory complex shown in this work is key to many facets of mGC regulation. We hope that the structural basis for the Hsp90 regulatory platform for mGC will drive renewed investigation into these diverse mechanisms and lead to the therapeutic manipulation of these mechanisms to improve mGC targeting therapies.

Methods

Cloning and protein expression

For cryo-EM studies, a construct containing an HA secretion signal (MKTIIALSYIFCLVFA), a FLAG peptide (DYKDDDD), linker and 3C cleavage site (KGSLEVLFGPG), GCN4 homodimeric zipper (RMKQLEDKVEELLSKNYHLENEVARLKKLVGER), human GC-C regions corresponding to the small extracellular linker region, TM, and GC domain (residues 399-1,053), a second linker and 3C cleavage site (AAALEVLFGPGAA), a Protein C epitope tag (EDQVDPRLIDGK), and an 8x His tag were cloned into a pD649 mammalian expression vector. This construct contains all domains of the native GC-C, with the exception of the ECD. Protein was expressed using ExpiCHO Expression

System Kit (Thermo Fisher). Briefly, ExpiCHO cells were maintained in ExpiCHO Expression Media at 37°C with 5% CO₂ and gentle agitation, and transiently transfected by the expression construct and cultured according to the manufacturer's protocol. Cells were pelleted and stored at -80°C.

Protein purification

Cells were resuspended in 20 mM HEPES-Na pH 8.0, 300 mM NaCl, 1 mM TCEP, protease inhibitor cocktail (Sigma), and benzonase (Sigma). Cells were lysed by Dounce homogenizer and cellular debris were pelleted by low-speed centrifugation at 500 x g. Membranes were collected by centrifugation at 46,000 x g and stored at -80°C until use. Membranes were thawed and solubilized with the addition of 1% n-dodecyl β -D-maltoside (DDM) and 0.1% cholesteryl hemisuccinate (CHS) (10:1) (Anatrace). Debris and unsolubilized membranes were pelleted by centrifugation at 46,000 x g. The supernatant was subsequently used in FLAG affinity chromatography. The supernatant was applied to M1 anti-FLAG resin. The resin was washed with 20 bed volumes of 20 mM HEPES-Na pH 8.0, 300 mM NaCl, 1 mM TCEP, 0.005% lauryl maltose neopentyl glycol (LMNG), 0.0005% CHS (10:1) (Anatrace), and 5 mM ATP. The protein complex was eluted with the addition of 200 μ g/mL of FLAG peptide (DYKDDDD) (GenScript). Protein was subsequently concentrated to >2 mg/mL and used for cryo-EM imaging.

Cryo-electron microscopy

Aliquots of 3 μ L of complex were applied to glow-discharged 300 mesh UltrAuFoil® (1.2/1.3) grids. The grids were blotted for 3 seconds at 100% humidity with an offset of 3 and plunge frozen into liquid ethane using a Vitrobot Mark IV (Thermo Fisher). Grid screening and dataset collection occurred at Stanford cEMc on a 200 kV Glacios microscope (Thermo Fisher) equipped with a K3 camera (Gatan). Movies were collected at a magnification corresponding to a 0.9273 Å per physical pixel. The dose was set to a total of 58.8 electrons per Å². Automated data collection was carried out using SerialEM with a nominal defocus range set from -0.8 to -2.0 μ M.

Image processing

All processing was performed in cryoSPARC (Punjani et al., 2017 [\[link\]](#)) unless otherwise noted (**Figure 1—figure supplement 1** [\[link\]](#)). 8,788 movies were motion corrected using patch motion correction. The contrast transfer functions (CTFs) of the flattened micrographs were determined using patch CTF and an initial stack of particles was picked using Topaz picker (Bepler et al., 2019 [\[link\]](#)). Successive rounds of reference-free 2D classification were performed to generate a particle stack of 165,635 particles. These particles were then used in ab-initio reconstruction, followed by non-uniform refinement (Punjani et al., 2020 [\[link\]](#)) and finally local refinement with a loose mask around the entire complex. This resulted in a 3.9 Å reconstruction of the GC-C-Hsp90-Cdc37 complex. These particles were also used in a 4 class heterogeneous refinement to pull out a volume containing some resolved density for the dimerization domain of GC-C.

Model building and refinement

The Cdk4-Hsp90 β -Cdc37 (PDB 5FWK), PP5-B-Raf-Hsp90 β -Cdc37 (PDB 7ZR5), and AlphaFold models for GC-C (Jumper et al., 2021 [\[link\]](#); Mirdita et al., 2022 [\[link\]](#)) were docked into the map using UCSF Chimera X (Pettersen et al., 2021 [\[link\]](#)). A resultant hybrid model was then manually curated to contain the correct *Cricetulus griseus* sequences for Hsp90 β -Cdc37 and run through Namdinator (Kidmose et al., 2019 [\[link\]](#)). This was followed by automated refinement using Phenix real space refine (Adams et al., 2010 [\[link\]](#)) and manual building in Coot (Emsley & Cowtan, 2004 [\[link\]](#)). The final model produced a favorable MolProbity score of 2.14 (Chen et al., 2010 [\[link\]](#)) with 0.4% Ramachandran outliers (**Table 1** [\[link\]](#)). Model building and refinement software was installed and configured by SBGrid (Morin et al., 2013 [\[link\]](#)).

	GC-C–Hsp90–Cdc37 complex		GC-C–Hsp90–Cdc37 complex
	PDB XXXX		with DD density
	EMD-XXXXX		
Data collection and processing			
Nominal magnification		45,000	
Acceleration voltage (kV)		200	
Electron exposure (e ⁻ /Å ²)		58.8	
Defocus range (μm)		0.8 to 2.0	
Pixel size (Å)		0.9273	
Symmetry imposed		C1	
Final particle images	165,635		48,283
Map resolution FSC threshold		0.143	
Map resolution (Å)	3.9		6.3
Refinement			
Initial model used (PDB)	5FWK, 7ZR5, AlphaFold		
Model resolution FSC threshold (Å)	0.5		
Model resolution (Å)	4.2		
Model Composition			
Non-hydrogen atoms	13,478		
Protein residues	1,654		
Ligands	2		
<i>B</i> -factors (Å ²)			
Protein	119.49		
Ligand	102.85		
R.m.s. deviations			
Bond lengths (Å)	0.004		
Bond angles (°)	0.914		
Validation			
MolProbity score	2.14		
Clashscore	13.88		
Rotamer outliers (%)	0.67		
Ramachandran plot			
Favoured (%)	92.0		
Allowed (%)	7.6		
Outliers (%)	0.4		

Table 1.

CryoEM data collection, refinement, and validation statistics.

Data Availability

Cryo-EM maps and atomic coordinates for the GC-C-Hsp90-Cdc37 complex have been deposited in the EMDB (EMD-XXXXX) and PDB (XXXX). Material availability: The plasmids used in this study are available from KCG (kcgarcia@stanford.edu) by request.

Acknowledgements

We thank Liz Montabana and Stanford cEMc for microscope access for data collection. We thank Paul LaPointe and Kevin Jude for insightful discussion of Hsp90 structure and regulatory mechanisms. NAC is a CIHR postdoctoral fellow. KCG is an investigator with the Howard Hughes Medical Institute. KCG is supported by National Institutes of Health grant R01-AI51321, the Mathers Foundation, and the Ludwig Foundation.

Author Contributions

NAC contributed to conceptualization, methodology, investigation, analysis, writing – original draft, review, and editing. NT contributed to conceptualization, methodology, investigation, writing – review, and editing. KGC contributed to conceptualization, supervision, writing – review and editing, and funding acquisition.

Competing Interests

The authors declare no competing interests.

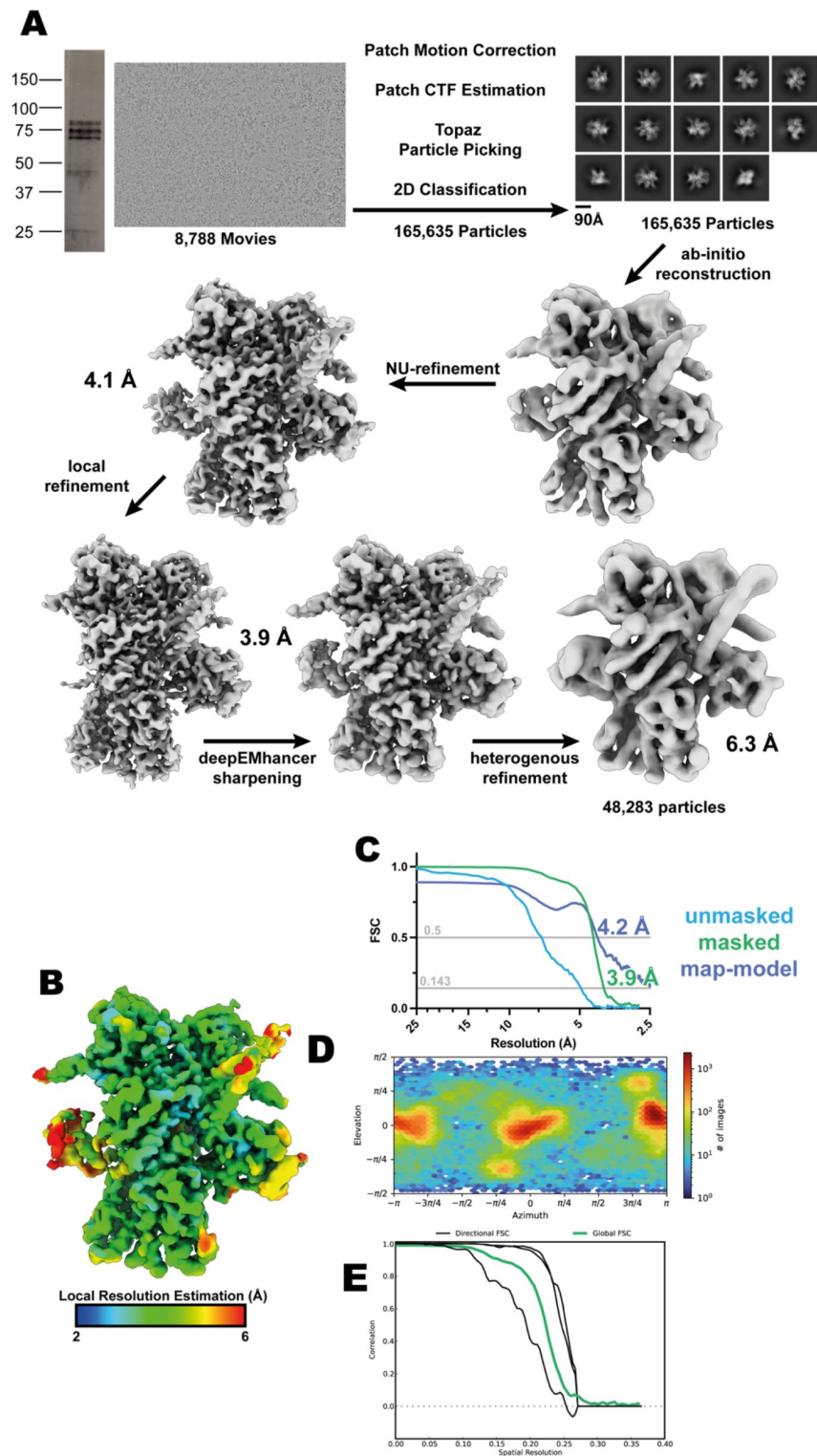


Figure 1—figure supplement 1.

GC-C-Hsp90-Cdc37 complex cryoEM data processing.

(A) Workflow for cryoEM data processing. SDS-PAGE gel, representative micrograph, reference free 2D averages, and cryoEM maps at the various stages of processing. (B) Local resolution estimation of the finalised cryoEM map. (C) FSC curve of the reconstruction using gold-standard refinement calculated from unmasked and masked half maps. Map-model FSC curve. (D) Orientational distribution of the reconstruction. (E) Directional FSC curves from 3DFSC (Aiyer et al., 2021 [DOI](#)).

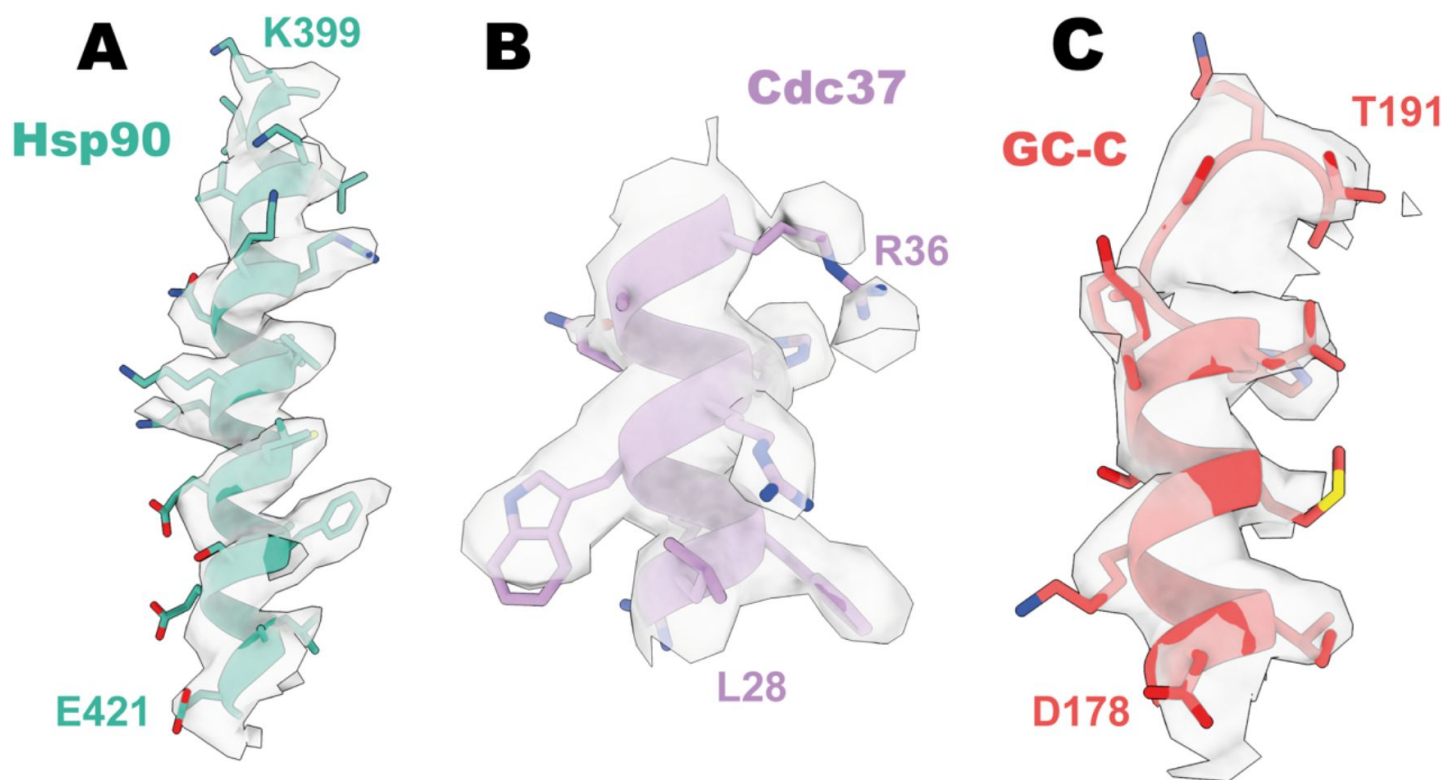


Figure 1—figure supplement 2.

Representative density of GC-C-Hsp90-Cdc37.

(A) Representative density of Hsp90. (B) Representative density of Cdc37. (C) Representative density of GC-C.

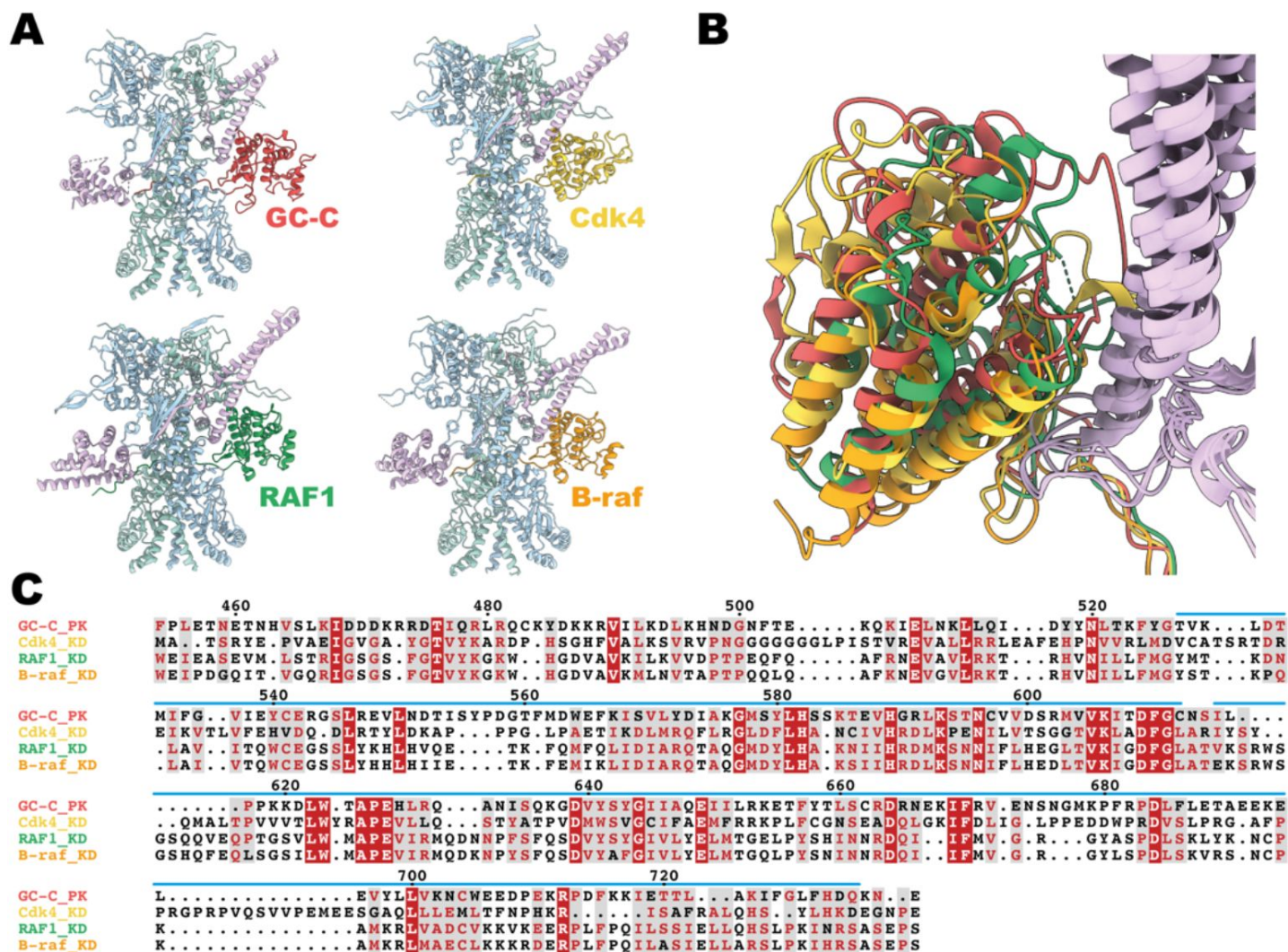


Figure 2—figure supplement 1.

Conservation of Cdc37 mediated Hsp90 regulation.

(A) Ribbon representation of a models of client-Hsp90β-Cdc37 complexes. GC-C is colored in red, Cdk4 in yellow (5FWK), RAF1 in green (7Z37), B-raf in orange (7ZR0), Hsp90β in light blue and teal, and Cdc37 in light purple. (B) A structural overlay of the structures in A. (C) A sequence alignment of the pseudokinase domain of GC-C and the kinase domains of Cdk4, RAF1, and B-raf. Sequence numbering per GC-C, with a blue line depicting regions resolved in the cryoEM density.

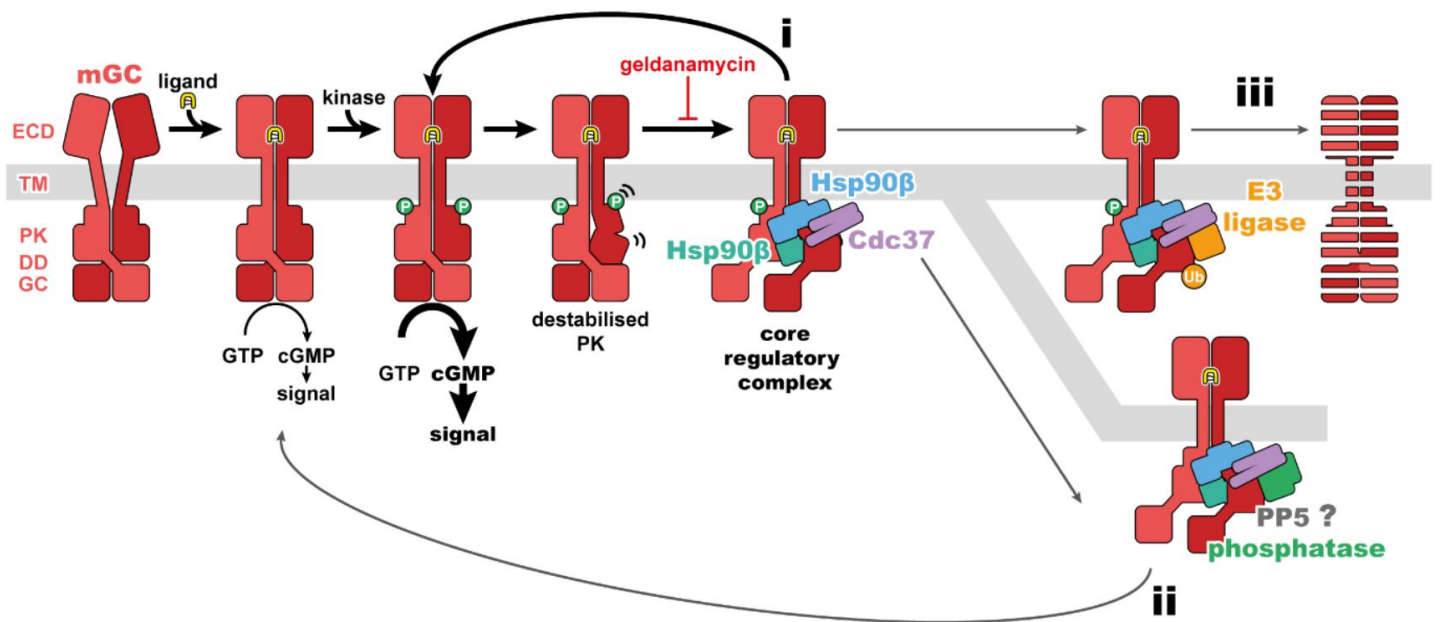


Figure 2—figure supplement 2.

Regulatory mechanisms for mGC activity.

A schematic of mGC ligand induced activity, phosphorylation, and destabilization, leading to formation of the mGC–Hsp90–Cdc37 complex structurally characterized in this work. This core regulatory complex would then lead to refolding of the PK and reactivation of the receptor (i), recruitment of PP5 and dephosphorylation of the receptor (ii), or recruitment of E3 ligases and removal of the receptor (iii). An mGC is depicted in red, ligand in yellow, Hsp90 in blue and teal, Cdc37 in purple, a phosphatase in green, and an E3 ligase in orange.

References

- Adams P. D., Afonine P. V., Bunkóczi G., Chen V. B., Davis I. W., Echols N., Headd J. J., Hung L. W., Kapral G. J., Grosse-Kunstleve R. W., McCoy A. J., Moriarty N. W., Oeffner R., Read R. J., Richardson D. C., Richardson J. S., Terwilliger T. C., Zwart P. H. (2010) **PHENIX: A comprehensive Python-based system for macromolecular structure solution** *Acta Crystallographica Section D: Biological Crystallography* **66**:213–221 <https://doi.org/10.1107/S0907444909052925>
- Aiyer S., Zhang C., Baldwin P. R., Lyumkis D. (2021) **Evaluating Local and Directional Resolution of Cryo-EM Density Maps** *Methods in Molecular Biology* **2215**:161–187 https://doi.org/10.1007/978-1-0716-0966-8_8/COVER
- Bepler T., Morin A., Rapp M., Brasch J., Shapiro L., Noble A. J., Berger B. (2019) **Positive-unlabeled convolutional neural networks for particle picking in cryo-electron micrographs** *Nature Methods* **16**:1153–1160 <https://doi.org/10.1038/s41592-019-0575-8>
- Chen V. B., Arendall W. B., Headd J. J., Keedy D. A., Immormino R. M., Kapral G. J., Murray L. W., Richardson J. S., Richardson D. C. (2010) **MolProbity: All-atom structure validation for macromolecular crystallography** *Acta Crystallographica Section D: Biological Crystallography* **66**:12–21 <https://doi.org/10.1107/S0907444909042073>
- Chinkers M. (1994) **Targeting of a distinctive protein-serine phosphatase to the protein kinase-like domain of the atrial natriuretic peptide receptor** *Proceedings of the National Academy of Sciences* **91**:11075–11079 <https://doi.org/10.1073/PNAS.91.23.11075>
- Emsley P., Cowtan K. (2004) **Coot: Model-building tools for molecular graphics** *Acta Crystallographica Section D: Biological Crystallography* **60**:2126–2132 <https://doi.org/10.1107/S0907444904019158>
- García-Alonso S., Mesa P., Ovejero L., de la P., Aizpurua G., Lechuga C. G., Zarzuela E., Santiveri C. M., Sanclemente M., Muñoz J., Musteanu M., Campos-Olivas R., Martínez-Torrecuadrada J., Barbacid M., Montoya G. (2022) **Structure of the RAF1-HSP90-CDC37 complex reveals the basis of RAF1 regulation** *Molecular Cell* **82**:3438–3452 <https://doi.org/10.1016/J.MOLCEL.2022.08.012>
- Glassman C. R., Tsutsumi N., Saxton R. A., Lupardus P. J., Jude K. M., Christopher Garcia K. (2022) **Structure of a Janus kinase cytokine receptor complex reveals the basis for dimeric activation** *Science* **376**:163–169 <https://doi.org/10.1126/science.abn8933>
- He X. L., Chow D. C., Martick M. M., Garcia K. C. (2001) **Allosteric activation of a spring-loaded natriuretic peptide receptor dimer by hormone** *Science* **293**:1657–1662 https://doi.org/10.1126/SCIENCE.1062246/SUPPL_FILE/1062246S2_THUMB.GIF
- He X. L., Chow D. C., Martick M. M., Garcia K. C. (2001) **Allosteric activation of a spring-loaded natriuretic peptide receptor dimer by hormone** *Science* **293**:1657–1662 https://doi.org/10.1126/SCIENCE.1062246/SUPPL_FILE/1062246S2_THUMB.GIF
- He X. Lin, Dukkupati A., Garcia K. C. (2006) **Structural Determinants of Natriuretic Peptide Receptor Specificity and Degeneracy** *Journal of Molecular Biology* **361**:698–714 <https://doi.org/10.1016/J.JMB.2006.06.060>

- Jumper J., Evans R., Pritzel A., Green T., Figurnov M., Ronneberger O., Tunyasuvunakool K., Bates R., Žídek A., Potapenko A., Bridgland A., Meyer C., Kohl S. A. A., Ballard A. J., Cowie A., Romera-Paredes B., Nikolov S., Jain R., Adler J., Hassabis D. (2021) **Highly accurate protein structure prediction with AlphaFold** *Nature* **596**:583–589 <https://doi.org/10.1038/s41586-021-03819-2>
- Kidmose R. T., Juhl J., Nissen P., Boesen T., Karlsen J. L., Pedersen B. P. (2019) **Namdirector - Automatic molecular dynamics flexible fitting of structural models into cryo-EM and crystallography experimental maps** *IUCr* **6**:526–531 <https://doi.org/10.1107/S2052252519007619/EH5002SUP3.PDF>
- Kobayashi D., Yamaguchi N., Takahashi O., Deshpande G. A., Fukui T. (2012) **Human Atrial Natriuretic Peptide Treatment for Acute Heart Failure: A Systematic Review of Efficacy and Mortality** *Canadian Journal of Cardiology* **28**:102–109 <https://doi.org/10.1016/j.cjca.2011.04.011>
- Krissinel E., Henrick K. (2007) **Inference of Macromolecular Assemblies from Crystalline State** *Journal of Molecular Biology* **372**:774–797 <https://doi.org/10.1016/j.jmb.2007.05.022>
- Kumar R., Grammatikakis N., Chinkers M. (2001) **Regulation of the Atrial Natriuretic Peptide Receptor by Heat Shock Protein 90 Complexes** *Journal of Biological Chemistry* **276**:11371–11375 <https://doi.org/10.1074/jbc.M010480200>
- Miner P. B. (2020) **Miner, P. B. (2020). Plecanatide for the treatment of constipation-predominant irritable bowel syndrome. 10.1080/17474124.2020.1722101, 14(), 71–84. 10.1080/17474124.2020.1722101 Plecanatide for the treatment of constipation-predominant irritable bowel syndrome 14:71–84** <https://doi.org/10.1080/17474124.2020.1722101>
- Mirdita M., Schütze K., Moriwaki Y., Heo L., Ovchinnikov S., Steinegger M. (2022) **ColabFold: making protein folding accessible to all** *Nature Methods* **19**:679–682 <https://doi.org/10.1038/s41592-022-01488-1>
- Morin A., Eisenbraun B., Key J., Sanschagrin P. C., Timony M. A., Ottaviano M., Sliz P. (2013) **Collaboration gets the most out of software** <https://doi.org/10.7554/ELIFE.01456>
- Oberoi J., Guiu X. A., Outwin E. A., Schellenberger P., Roumeliotis T. I., Choudhary J. S., Pearl L. H. (2022) **HSP90-CDC37-PP5 forms a structural platform for kinase dephosphorylation** *Nature Communications* **2022 13:1** **13**:1–13 <https://doi.org/10.1038/s41467-022-35143-2>
- Ogawa H., Qiu Y., Ogata C. M., Misono K. S. (2004) **Crystal structure of hormone-bound atrial natriuretic peptide receptor extracellular domain. Rotation mechanism for transmembrane signal transduction** *Journal of Biological Chemistry* **279**:28625–28631 <https://doi.org/10.1074/JBC.M31322200/ATTACHMENT/31436558-57B4-4F07-AC3C-698401425198/MMC1.ZIP>
- Ogawa H., Qiu Y., Philo J. S., Arakawa T., Ogata C. M., Misono K. S. (2010) **Reversibly bound chloride in the atrial natriuretic peptide receptor hormone-binding domain: Possible allosteric regulation and a conserved structural motif for the chloride-binding site** *Protein Science* **19**:544–557 <https://doi.org/10.1002/PRO.332>
- Pettersen E. F., Goddard T. D., Huang C. C., Meng E. C., Couch G. S., Croll T. I., Morris J. H., Ferrin T. E. (2021) **UCSF ChimeraX: Structure visualization for researchers, educators, and developers** *Protein Science* **30**:70–82 <https://doi.org/10.1002/pro.3943>

- Potter L. R. (2011) **Guanylyl cyclase structure, function and regulation** *Cellular Signalling* **23**:1921–1926 <https://doi.org/10.1016/J.CELLSIG.2011.09.001>
- Potter L. R., Garbers D. L. (1992) **Dephosphorylation of the guanylyl cyclase-A receptor causes desensitization** *Journal of Biological Chemistry* **267**:14531–14534 [https://doi.org/10.1016/S0021-9258\(18\)42069-8](https://doi.org/10.1016/S0021-9258(18)42069-8)
- Potter L. R., Hunter T. (1998) **Identification and Characterization of the Major Phosphorylation Sites of the B-type Natriuretic Peptide Receptor** *Journal of Biological Chemistry* **273**:15533–15539 <https://doi.org/10.1074/JBC.273.25.15533>
- Punjani A., Rubinstein J. L., Fleet D. J., Brubaker M. A. (2017) **CryoSPARC: Algorithms for rapid unsupervised cryo-EM structure determination** *Nature Methods* **14**:290–296 <https://doi.org/10.1038/nmeth.4169>
- Punjani A., Zhang H., Fleet D. J. (2020) **Non-uniform refinement: adaptive regularization improves single-particle cryo-EM reconstruction** *Nature Methods* **17**:1214–1221 <https://doi.org/10.1038/s41592-020-00990-8>
- Schopf F. H., Biebl M. M., Buchner J. (2017) **The HSP90 chaperone machinery** *Nature Reviews Molecular Cell Biology* **18**:345–360 <https://doi.org/10.1038/nrm.2017.20>
- Taipale M., Krykbaeva I., Koeva M., Kayatekin C., Westover K. D., Karras G. I., Lindquist S. (2012) **Quantitative Analysis of Hsp90-Client Interactions Reveals Principles of Substrate Recognition** *Cell* **150**:987–1001 <https://doi.org/10.1016/J.CELL.2012.06.047>
- Vaandrager A. B., van der Wiel E., de Jonge H. R. (1993) **Heat-stable enterotoxin activation of immunopurified guanylyl cyclase C. Modulation by adenine nucleotides** *Journal of Biological Chemistry* **268**:19598–19603 [https://doi.org/10.1016/S0021-9258\(19\)36558-5](https://doi.org/10.1016/S0021-9258(19)36558-5)
- van den Akker F., Zhang X., Miyagi M., Huo X., Misono K. S., Yee V. C. (2000) **Structure of the dimerized hormone-binding domain of a guanylyl-cyclase-coupled receptor** *Nature* **406**:6791 **406**:101–104 <https://doi.org/10.1038/35017602>
- Verba K. A., Wang R. Y. R., Arakawa A., Liu Y., Shirouzu M., Yokoyama S., Agard D. A. (2016) **Atomic structure of Hsp90-Cdc37-Cdk4 reveals that Hsp90 traps and stabilizes an unfolded kinase** *Science* **352**:1542–1547 https://doi.org/10.1126/SCIENCE.AAF5023/SUPPL_FILE/AAF5023S4.MOV
- Yu S. W. B., Rao S. S. C. (2014) **Advances in the management of constipation-predominant irritable bowel syndrome: The role of linaclotide** *Therapeutic Advances in Gastroenterology* **7**:193–205 https://doi.org/10.1177/1756283X14537882/ASSET/IMAGES/LARGE/10.1177_1756283X14537882-FIG2.JPEG

Author information

Nathanael A Caveney

Departments of Molecular and Cellular Physiology and Structural Biology, Stanford University School of Medicine, Stanford, CA 94305, USA

For correspondence: ncaveney@stanford.edu

ORCID iD: [0000-0003-4828-3479](https://orcid.org/0000-0003-4828-3479)

Naotaka Tsutsumi

Departments of Molecular and Cellular Physiology and Structural Biology, Stanford University School of Medicine, Stanford, CA 94305, USA, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305, USA, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan
ORCID iD: [0000-0002-3617-7145](https://orcid.org/0000-0002-3617-7145)

K Christopher Garcia

Departments of Molecular and Cellular Physiology and Structural Biology, Stanford University School of Medicine, Stanford, CA 94305, USA, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305, USA

For correspondence: kcgarcia@stanford.edu

ORCID iD: [0000-0001-9273-0278](https://orcid.org/0000-0001-9273-0278)

Editors

Reviewing Editor

Mohamed Trebak

University of Pittsburgh, United States of America

Senior Editor

Volker Dötsch

Goethe University, Germany

Reviewer #1 (Public Review):

Membrane receptor guanylyl cyclases are important for many physiological processes but their structures in full-length and their mechanism are poorly understood. Caveney et al. determined the cryo-EM structure of a highly engineered GC-C in a complex with endogenous HSP90 and CDC37. The structural work is solid and the structural information will be useful for the membrane receptor guanylyl cyclases field and the HSP90 field.

Reviewer #2 (Public Review):

Caveney et al have overexpressed an engineered construct of the human membrane receptor guanyl cyclase GC-C in hamster cells and co-purified it with the endogenous HSP90 and CDC37. They have then determined the structure of the resultant complex by single particle cryoEM reconstruction at sufficient resolution to dock existing structures of HSP90 and CDC37, plus an AlphaFold model of the pseudo-kinase domain of the guanylyl cyclase. The novelty of the work stems from the observation that the pseudo-kinase domain of GC-C associates with CDC37 and HSP90 similarly to how the bona fide protein kinases CDK4, CRAF and BRAF have been previously shown to interact.

Reviewer #3 (Public Review):

A detailed understanding of how membrane receptor guanylyl cyclases (mGC) are regulated has been hampered by the absence of structural information on the cytoplasmic regions of these signaling proteins. The study by Caveney et al. reports the 3.9Å cryo-EM structure of the human mGC cyclase, GC-C, bound to the Hsp90-Cdc37 chaperone complex. This structure represents a first view of the intracellular functional domains of any mGC and answers

without doubt that Hsp90-Cdc37 recognizes mGCs via their pseudokinase (PK) domain. This is the primary breakthrough of this study. Additionally, the new structural data reveals that the manner in which Hsp90-Cdc37 recognizes the GC-C PK domain C-lobe is akin to how kinase domains of soluble kinases docks to the chaperone complex. This is the second major finding of this study, which provides a concrete framework to understand, more broadly, how Hsp90-Cdc37 recruits a large number of other diverse client proteins containing kinase or pseudokinase domains. Finally, the Hsp90-Cdc37-GC-C structure offer clues as to how GC-C may be regulated by phosphorylation and/or ubiquitinylation by serving as a platform for recruitment of PP5 and/or E3 ligases.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Membrane receptor guanylyl cyclases are important for many physiological processes but their structures in full-length and their mechanism are poorly understood. Caveney et al. determined the cryo-EM structure of a highly engineered GC-C in a complex with endogenous HSP90 and CDC37. The structural work is solid and the structural information will be useful for the membrane receptor guanylyl cyclases field and the HSP90 field. However, a detailed characterization of the protein sample is lacking. Moreover, the physiological significance of this structure is not fully exploited by supporting experiments and the mechanistic insight is currently limited.

We thank Reviewer #1 for constructive reviews and agree that this work forms the basis for future exploration by the guanylyl cyclase and HSP90 fields.

1. The characterization of the protein sample is lacking. SDS-PAGE would be useful to identify potential proteolysis, leading to the dissociation of GC dimer. Further size-exclusion chromatography would be helpful to estimate the molecular weight of the complex and to determine if only GC-C monomer is purified.

We have included a representative SDS-PAGE gel in our revised version of the manuscript (Figure 1—figure supplement 1). While we agree that SEC could be beneficial to further explore the stoichiometry of the imaged sample, we see no significant degradation of the guanylyl cyclase via SDS-PAGE, and therefore believe that the zippered construct would remain dimeric. Relatively poor yields of these samples precluded further exploration in this regard.

1. The orientation distribution of the particles is not homogenous in Fig. S1D. It would be helpful to present the 3DFSC curve to evaluate the effect of preferred orientation on the reconstruction.

While the orientational distribution is not perfectly uniform, the provided angles allowed for sufficient reconstruction of maps with no notable anisotropy. We have included 3DFSC curves in our revised version of Figure 1—figure supplement 1.

1. Description of protein expression details is lacking. Did the author use transient transfection, stable cell line or virus-mediated transduction?

We have clarified that these cells were expressed using transiently transfected ExpiCHO cells.

1. HSP90 binds ATP and is often co-purified with endogenous ATP/ADP. Is there ATP or ADP present in the sample/cryo-EM maps? Is the conformation of NBD similar to ATP-bound HSP90? The author needs to include the description/figures about the nucleotide state of HSP90.

There is clear density for present nucleotide in our reconstruction. Given the mechanistic role for ATP turnover in the release of HSP90 client (Young, Hartl, 2000 – PMID 11060043) and the resolved density, we believe the identity for this nucleotide is ATP. We have added comment to this regard in the revised manuscript: “...the C2 pseudosymmetric, ATP bound, closed state Hsp90 dimer.”

1. The catalytic domains of GC have to be dimerized to perform cyclase function. The presence of only one GC-PK monomer in the cryo-EM structure indicates the structure does not represent an active state of GC. These results suggest the GC expressed in this way is not functional. The authors need to explain why most of the GC protein is trapped in this inactive form.

Indeed, we do believe that this regulatory state is non-functional, as observed for active kinases. We have clarified this in the revised manuscript: “In addition, this disruption of the native state of GC-C, as observed in our structure, would likely leave GC domains out of each other’s proximity, precluding their catalytic activity while Hsp90 is bound.”

1. The GC-C construct used here is a highly engineered “artificial” construct, which has not been fully characterized in this work. Does this construct have similar activity as the activated wt GC-C? Does the protein (this engineered construct) expressed in CHO cells show activity?

While our original goal in developing this construct was to create an imageable construct that was locked in the active state, our current interpretation of the data is that the leucine-zipper induced, putative active geometry leads to the majority of this construct falling into the regulatory state with HSP90 binding. We make no claim to have resolved an active conformation in this work, yet believe that this state is of note due to the previously unresolved nature of these regulatory complexes for guanylyl cyclase receptors.

1. Are the residues on the interface between GC and HSP conserved in other members of membrane receptor guanylyl cyclases? Would mutations on this interface affect the activity of GC?

Given the role this structure plays in our understanding that HSP90 client recruitment is largely not driven by specific residue interactions and the ~30% identity of GC-C to NPR-A and NPR-B, we do not believe that mutations that do not significantly change the stability or fold of the PK domain would significantly modify recruitment to HSP.

1. The authors propose that targeting HSP90 would tune the activity of GC. Is there any experimental data supporting this idea?

Based on the work of Kumar et al., 2001 (PMID 11152473), we do believe that there is a functional link between HSP90 recruitment and GC activity. We hope that this work will spark further exploration of these concepts.

1. The model in Fig. S3 is largely speculative due to the lack of supporting functional data. In addition, it would be better to change the title to “structure of the protein kinase

domain of guanylyl cyclase receptor in complex with HSP90 and cdc37" because the mechanistic insight is limited.

We agree that our supplemental figure is more speculative. We have referenced this in the discussion section of the manuscript and put this figure in the supplement to ensure that this is understood to be more speculative in nature.

Reviewer #2 (Public Review):

Caveney et al have overexpressed an engineered construct of the human membrane receptor guanyl cyclase GC-C in hamster cells and co-purified it with the endogenous HSP90 and CDC37. They have then determined the structure of the resultant complex by single particle cryoEM reconstruction at sufficient resolution to dock existing structures of HSP90 and CDC37, plus an AlphaFold model of the pseudo-kinase domain of the guanylyl cyclase. The novelty of the work stems from the observation that the pseudo-kinase domain of GC-C associates with CDC37 and HSP90 similarly to how the bona fide protein kinases CDK4, CRAF and BRAF have been previously shown to interact.

The experimentation is limited to the cryoEM analysis, and is lacking additional studies that would give deeper insight into the oligomeric nature - if any - of the GC-C when bound to HSP90-CDC37 as compared to the free protein. This is relevant, as the dimerization domain downstream of the pseudokinase, is evident in the maps - albeit not well resolved - and it is not clear whether it is still able to mediate dimerization with a second free or HSP90-CDC37bound GC-C. It would also be good to see some experimentation that asks whether association with HSP90-CDC37 inhibits the guanyl cyclase activity. It is clear from previous work that HSP90-CDC37 silence the kinase activity of their bound client kinases, but in this case the catalytic guanyl cyclase is not directly associated with the chaperone complex and may still be able to function.

Given the geometry of the interaction, the dimerization domain of the GC would likely be monomerized, albeit with global dimerization remaining – contributed by the ECD, or in our case the liganded-ECD mimicking leucine zipper. Experimentally, it has been shown in live cells (Kumar et al., 2001, PMID 11152473) that the HSP90 association is required for maximal GC-A function. This is likely due to some sort of resetting nature to the associating to allow further activity, as opposed to activity during the association – given the latter is unlikely based on our structure, where the two GC domains would not be able to form the active dimerized state. Further dissection of this, while outside the scope of the current work, is of great interest.

Although the sequence alignment presented in SuppFig 2 shows that GC-C conserves the classic DFG motif that plays a critical role in the regulation of most kinases, the numbering of the sequence is absent, making it very difficult to relate this to the structural detail shown in Fig 2B. This needs to be clarified, as the interaction of CDC37-Trp31 with the DFG motifs and downstream activation loops in CRAF and BRAF have been proposed as important features of the selectivity of these kinases for the HSP90-CDC37 system, and it would be good to be able to see clearly how much of this is also conserved in the GC-C pseudokinase domain interaction. For example, is the much shorter activation segment (DFG -> APE) ordered in the complex or disordered?

We have clarified Figure 2—figure supplement 1 with additional numbering. While we agree that the DFG motif may play a role in recognition, only the first residue of this motif is interacting with CDC37 in our structure, so it may be likely that the role of this motif is more structural in maintaining a CDC37 complementary fold, as opposed to direct residue interactions. Additionally, many kinases which are not regulated by CDC37/HSP90 contain this

motif. The shorter DFG-APE of GC-C is traceable with the exception of N613, S614, I615, though the density in this region reflects this loop not being well stabilized.

It was not easy to follow what was in the sample used for cryoEM. The cloning of the guanylyl cyclase (GC) component is described in the methods and they have shown some illustrations in fig 1 but a proper numbered figure of the domain organisation clearly showing domain boundaries and linker segments is really needed for a reader not familiar with the structure of GCs, especially since they have replaced the ECD with a leucine zipper in their construct. It is important to show a domain figure of what this construct looks like as well, as from the illustrations in fig 1 for examples its hard to see what's PK, DD, GC domains. It would also be helpful to see in the supplementary a gel of complex they put on the grids, to make it clearer what exactly the sample is and to reassure that the GC-C domains that are not resolved in the cryoEM are nonetheless present in the sample.

We have added in a gel figure to the supplement and clarified the content of the imaged construct in the methods section: “This construct contains all domains of the native GC-C, with the exception of the ECD.”

Overall there is only minimal proposal of mechanism or biological function based on the structure. The speculation in the Discussion of two fates - PP5 dephosphorylation or E3 ligase recruitment, is not supported by any experimentation, which is reasonable for speculation, but is also not underpinned by reference to any previously published work suggesting that these additional processes may be important. In the absence of any work by the authors can they put these speculations more in context with previously published work that supports the importance of these processes specifically for GC regulation?

We have ensured that these potential pathways only appear in the discussion section. It has been observed, for instance by Oberoi et al., 2022 that phosphatases can act on all components of a HSP90–CDC37–client system. Given there are well characterized phosphorylation sites for membrane GC receptors, we believe this is worth discussing in this manuscript, to stimulate further exploration of these mechanisms in the field. In addition, it has been reported that many E3 ligases are recruited to HSP90 complexes and can degrade rather non-specifically. It has been shown that one can generate PROTAC-like molecules to target non-specific clients to HSP90–E3 ligase machinery for degradation (Li et al., 2023). Given this proximity induced nature to E3 degradation of HSP90 clients, it would be highly likely that, at least in some cases, mGCs would be degraded by this mechanism as well.

Reviewer #3 (Public Review):

A detailed understanding of how membrane receptor guanylyl cyclases (mGC) are regulated has been hampered by the absence of structural information on the cytoplasmic regions of these signaling proteins. The study by Caveney et al. reports the 3.9Å cryo-EM structure of the human mGC cyclase, GC-C, bound to the Hsp90-Cdc37 chaperone complex. This structure represents a first view of the intracellular functional domains of any mGC and answers without doubt that Hsp90-Cdc37 recognizes mGCs via their pseudokinase (PK) domain. This is the primary breakthrough of this study. Additionally, the new structural data reveals that the manner in which Hsp90-Cdc37 recognizes the GC-C PK domain C-lobe is akin to how kinase domains of soluble kinases docks to the chaperone complex. This is the second major finding of this study, which provides a concrete framework to understand, more broadly, how Hsp90-Cdc37 recruits a large number of other diverse client proteins containing kinase or pseudokinase domains. Finally, the Hsp90-Cdc37-GC-C structure offer clues as to how GC-C may be

regulated by phosphorylation and/or ubiquitinylation by serving as a platform for recruitment of PP5 and/or E3 ligases.

Comments:

1. The authors used an interesting approach to obtain the GC-C-Hsp90-Cdc37 complex. Flagtagged human GC-C was overexpressed in CHO cells with the expectation of co-purifying endogenous hamster homologs of Hsp90 and Cdc37. There are several points worth noting:

a) It is not clear from the data presented (Figure 1C, Suppl Fig 1A) or the Methods the percentage of particles in the cryo-EM specimen that represent the GC-C-Hsp90-Cdc37 complex. Presumably, some fraction of GC-C isolated will not be associated with Hsp90Cdc37. If a very large portion of GC-C is associated with Hsp90-Cdc37, it would be good to explain why this is to be expected. Are 2D/3D classes corresponding to the activated GC-C dimer found? If not, why?

While we see some traces of GC-C not bound by Hsp90, there is, in the least, a significant alignment bias for the Hsp90 bound complex. We believe that the engineered construct, which we designed to be locked in a putative active conformation, is going through catalytic cycles to some point where the regulatory mechanism is kicking in. It may be that for proper resetting of the receptor, the receptor needs to cycle back through an unliganded, inactive conformation, which our leucine zipper construct is unable to allow, thus locking our GC in the regulatory complex, though this is speculation.

b) Figure 1A suggests that GC-C is phosphorylated before recruitment of Hsp90-Cdc37. What is the phosphorylation status of the GC-C specimen that was imaged by cryo-EM?

We had placed the P in grey in this figure to represent the potential for the active state to be phosphorylated. For GC-C in particular, the phosphorylation state does not affect activity as much as GC-A and GC-B for example. We have removed this P from the figure for clarity.

c) The resolution of the cryo-EM map (3.9 Å) is too low for unambiguous identification of proteins. Please provide more precise justification for the claim that the densities observed do in fact correspond to hamster Hsp90 and Cdc37.

While we agree that the resolution is limiting for protein identification, the fact that we are using a very stringent FLAG purification allows confidence in the ID for our target, GC-C. For Hsp90 and Cdc37, we are confident that they are endogenous hamster Hsp90 and Cdc37, given the large structural similarity observed in comparison to prior Hsp90/Cdc37/client complex structures, and the ID/register well confirmed by the placement of bulky residues.

d) The authors state that human GC-C pulls down hamster Hsp90-cdc37 but soluble kinases cannot, despite the high sequence identity between human and hamster Hsp90-cdc37. Is this because GC-C recognition is more promiscuous? Can this difference be understood in light of the new structural information presented?

“This native pulldown strategy contrasts with the structures of Hsp90–Cdc37 in complex with soluble kinases (García-Alonso et al., 2022; Oberoi et al., 2022; Verba et al., 2016), for which Hsp90 and Cdc37 had to be overexpressed to obtain complex suitable for imaging.”

It is our understanding, from reading the papers cited above, that Hsp90/Cdc37 needed to be overexpressed to obtain these samples for imaging. We use a different strategy because our sample does not require overexpression of Hsp90 and Cdc37. This may be because of

something specific to hamster cells, which were (presumably) not tested in the above studies, or it could be something specific to do with GC-C.

1. A large portion of the enforced GC-C dimer was not visible in the cryo-EM maps. It is not easy to learn from Figure 1 exactly which parts of the GC-C construct was sufficiently ordered and observed structurally. Please improve Figure 1.

We have adjusted Figure 1 to better depict what is observed in the cryoEM density.

1. On page 4, the authors claim that they are able to orient the GC-C-Hsp90-Cdc37 complex "as it would sit on a membrane" and referred to Figure 1B. It is not clear what is implied here. Does Hsp90-Cdc37 binding constrain the complex to face the inner leaflet of the membrane in a specific orientation as shown in Figure 1B? If true, this could potentially have important functional implications. Please illustrate how this was deduced based on the information available.

Given the observed density for the PK domain, which is membrane proximal, we can safely assume that the TM would be located immediately above this region. Given the size of Hsp90 and assuming the soluble Hsp90 must sit below the membrane, we can determine, with some accuracy the relative orientation of this complex next to the membrane. This orientation is depicted in Figure 1B.

1. Also on page 4, it is stated that it is sterically unlikely an additional Hsp90-Cdc37 complex is associated with the other copy of GC-C in the leucine zippered dimer. It is not obvious to the reader how this may be the case. An additional figure could help make this more clear. Additional biochemical evidence will also help. The absence of GC-C-Hsp90-Cdc37 dimers in cryo-EM micrographs can also support the argument.

We have clarified this: "is sterically unlikely that an additional regulatory complex is forming on the second GC-C in a concurrent fashion, given the large size of the first Hsp90-Cdc37 and the requisite proximity of the second GC-C."

1. Some comments on Figure 2:

a) NTD and CTD are mislabeled in Figure 2A.

Thank you for catching this, we have fixed this.

b) The authors should show cryo-EM density to support their modeling of GC-C in Figures 2B and C.

We have provided maps and models to the reviewer and will release these maps and models upon publication so that all relevant densities can be interpreted to their fullest extent by readers. In addition, we have added representative density panels to Figure 1-figure supplement 2.

1. The authors claim that Hsp90-Cdc37 clients are more similar structurally near the cdc37 interface. Please illustrate this with additional figures. Suppl. Figure 2 is inadequate for this purpose.

We have added a structural overlay to Figure 2—figure supplement 1A to illustrate this.

The authors can also consider adding a more detailed discussion comparing the interactions between the pseudokinase/kinase C-lobe and Cdc37 in known structures. Is

shape/charge complementarity a universal feature of cdc37-dependent kinase/pseudokinase recruitment? It would be interesting to also consider if it would be possible to predict which of the ~60 human pseudokinases are possible Hsp90-Cdc37 clients. New structural findings from this study and publicly available AI-predicted protein structures could help.

While the use of AI to predict pseudokinase interactions would indeed be interesting, we believe this is outside the scope of this work. Given methodology is in place for determination of kinase clients for Hsp90 (Taipale et al., 2012), this could be an additional route to obtain this information in future work.

Reviewer #2 (Recommendations For The Authors):

In Figure 1B the authors show a large unaccounted-for region of density which they speculate may be due to the dimerization domain. That this is lost in the sharpened maps suggests that it is more mobile than the core which probably dominates the automatic mask generation used by cryoSPARC. It would be very interesting to try and resolve this region further by using focussed classification and refinement - probably in RELION. This would add further novelty, as so far in the three HSP90-CDC37 kinase complexes previously described, little is seen outside the C-terminal lobe of the kinase (or in this case pseudokinase) lobe.

Given the structurally uncharacterized nature of the DD and GC domains for mGCs, using computational means to further our understanding of these regions was attempted. Across several software packages, these attempts were unsuccessful. We will be uploading these micrographs to EMPIAR shortly after publication, which will allow for other groups to re-process this data as they see fit and as new software techniques emerge in this rapidly developing field. We believe that the partially unfolded nature of the PK domain is providing too much of a hinge point prior to the DD for the software to be able to resolve this currently.