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Autoinhibition and Structure of P-Rex2

By

LAUREN ANDERSON
DISSERTATION

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in the

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DAVIS

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2026

*For M.M.M. for always making space for me to follow my aspirations
and W.A. & M.M. for your unconditional love*

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I don't know how, but we did it! Long nights, failed experiments, equipment failures, THREE lab floods, and countless other trials by fire and we finally did it. Through all of this I have become a scientist, historian, author, leader and now “doctor.” Furthermore, I have accumulated a long list of folks who have made completing my PhD possible to whom I am so wholeheartedly grateful.

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Abstract

Dbl Rho guanine-nucleotide exchange factors (RhoGEFs) promote normal cell function by regulating small GTPases, yet their misregulation can contribute to cancer progression and metastasis. Among these, the phosphoinositol-3,4,5-trisphosphate (PIP₃)-dependent Rac exchanger (P-Rex) subfamily, which includes P-Rex1 and P-Rex2, plays a central role connecting GTPase response to signaling through Gβγ and PIP₃. Although P-Rex1 has been extensively characterized, the molecular mechanisms governing P-Rex2 regulation and the broader determinants of differences within the P-Rex family remain poorly understood. Taking an integrative structural biology approach, these gaps have begun to be investigated. Cryo-EM helped resolve the first structure of full-length P-Rex2 to moderate resolution, revealing P-Rex structure is well-conserved between the two proteins, but the position of the N-terminal module relative to the C-terminal core is considerably different between the two, indicating potential differences in regulatory preference. Furthermore, HDX-MS and SEC-SAXS data support structural and biochemical differences between P-Rex proteins, uncovering that not all regulatory mechanisms observed in P-Rex1 are maintained in P-Rex2. Throughout this research, exclusively freshly purified protein was used in all experiments as attempts to freeze P-Rex proteins were unsuccessful. Freezing RhoGEFs for biochemical studies is commonplace in the field but is supported by little experimental validation on the effects of freezing which may bias activity measurements. In response to these observations, isolated DH/PH tandems of P-Rex1, P-Rex2, and PRG were tested under different cryoprotectant conditions and activity and thermostability were monitored over time after flash freezing. These studies revealed that freezing and cryoprotectant addition individually can alter enzymatic activity and increase experimental variability within short timeframes, even when overall protein conformation appears unchanged. No single cryoprotectant was able to maintain both activity and thermostability across all proteins tested, highlighting the unpredictable and protein-specific effects of freezing RhoGEFs and the need for careful validation of studies using frozen RhoGEF proteins. Altogether, this work advances our understanding of

P-Rex2 regulation, reveals mechanistic diversity within P-Rex RhoGEFs, and identifies critical variables which influence RhoGEF characterization. Moreover, these insights have important implications for the development of therapeutic strategies targeting these enzymes.

Table of Contents

Acknowledgement	iii
Abstract.....	v
Chapter 1. Introduction	1
Figure	8
Chapter 2. GEF me a break: the consequences of freezing Rho guanine-nucleotide exchange factor catalytic domains	9
Abstract.....	9
Introduction	10
Results.....	12
Insights into Dbl RhoGEF DH/PH biochemical differences that could potentially confer differences in stability.....	12
Glycerol and sucrose increase variation in activity measurements of freshly purified RhoGEF DH/PH proteins	13
Isolated DH/PH protein activity is widely affected by freezing	14
Thermostability of DH/PH proteins generally does not change at early time points post-freezing...	16
Long-term freezing does not affect the global structure of P-Rex2 DH/PH	17
Discussion.....	18
Materials and Methods.....	20
Cloning and Plasmids	20
Protein production, purification, and handling	20
GTPase Exchange Activity Assay	22
Differential Scanning Fluorimetry	23
Size exclusion chromatography coupled to small angle x-ray scattering (SEC-SAXS).....	23
Tables	24
Figures.....	25
Chapter 3. P-Rex2 exhibits unique structural features and regulatory mechanisms distinct from the closely related RhoGEF P-Rex1	37
Abstract.....	37
Introduction	38
Results.....	41
The overall structure of P-Rex2 resembles that of P-Rex1 with differences in the positioning of the N-terminal module.....	41
Structural differences in the proposed phosphatase active site and G β γ -binding elements.....	43

IP ₄ binding does not enable resolution of the P-Rex2 IP4P subdomain	44
3.3.4. Binding of IP ₄ has no effect on the tertiary structure of P-Rex2.....	45
P-Rex1 is more active than P-Rex2 against Rac1	46
The P-Rex2 DH/PH takes on a single, compact conformational state independent of DEP1	47
The P-Rex2 DEP1 domain plays a role in maintaining autoinhibition.....	48
Discussion.....	49
Experimental procedures.....	54
Sequence Identity Comparison to P-Rex1	54
Cloning and plasmids	54
Protein Production and Purification	54
Cryo-EM Grid Preparation and Data Acquisition	56
Cryo-EM Data Processing.....	57
Model Building, Refinement, and Validation.....	59
Hydrogen-Deuterium Exchange Mass Spectrometry	60
GTPase Exchange Activation Assay	61
Statistical Analysis.....	62
Size-Exclusion Chromatography Small-Angle X-ray Scattering.....	62
Tables	64
Figures.....	66
Chapter 4. Conclusions and Future Directions	93
References	98

Chapter 1. Introduction

Cell morphology and migration are fundamental processes underlying development, immune response, and wound healing. Dysregulation of these processes contributes to numerous diseases, most notably cancer metastasis, in which cells acquire the ability to invade surrounding tissues and migrate to distant sites¹. At the cellular level these behaviors are driven by dynamic remodeling of the actin cytoskeleton, which enables cells to generate force, change shape, and respond to extracellular cues. A central class of regulators of actin dynamics is the Rho family of small GTPases, which function as molecular switches cycling between an inactive GDP-bound state and an active GTP-bound state. When activated, Rho GTPases such as Rac1, Cdc42, and RhoA coordinate actin polymerization, branching, and contractility through downstream effectors^{2,3}. Although these proteins are essential for cytoskeletal regulation, their intrinsic rates of nucleotide exchange are low, necessitating upstream regulatory proteins to control their activation. Rho guanine-nucleotide exchange factors (RhoGEFs) catalyze the exchange of GDP for GTP on GTPases, thus promoting their activation. In humans, approximately 80 RhoGEFs have been identified with the majority of them belonging to the diffuse B-cell lymphoma (Dbl) family⁴. In addition to their catalytic activity, RhoGEFs act as signaling hubs, interacting with membrane receptors, lipid second messengers, and scaffolding proteins to bridge extracellular signals and localized GTPase activation. Through these roles, RhoGEFs serve as key intermediates linking upstream signaling pathways to cytoskeletal remodeling and cell behavior.

Given their central role in regulating cell migration and morphology, dysregulation of Rho GTPase signaling pathways is frequently associated with disease. In cancer, aberrant activation of these pathways contributes to uncontrolled proliferation, altered cell morphology, and enhanced migratory and invasive capacity⁵. Direct pharmacological targeting of actin or Rho GTPases has proven challenging. For example, actin is essential for normal cellular function and its inhibition is often toxic. Actin inhibitors like latrunculin prevent actin repolymerization, resulting in rapid loss of actin structures in the

cell which is dangerous to cell health⁶. On the other hand, GTPases have had few successful inhibitors, many with weak binding affinity in the μM range⁷, largely due to a lack of pharmacologically druggable pockets. Apart from Sotorasib, an allele-specific KRAS^{G12C} inhibitor for non-small cell lung cancer, the development of selective inhibitors of small GTPases is lagging⁸. As a result, attention has shifted toward upstream regulators like RhoGEFs, which may provide more selective opportunities for therapeutic intervention. Notably, genetic and functional studies have shown that loss of specific RhoGEFs can be tolerated due to functional redundancy, suggesting that these proteins may represent more tractable drug targets⁹.

Most Dbl RhoGEFs share a conserved catalytic core consisting of a tandem Dbl homology (DH) domain and pleckstrin homology (PH) domain. The DH domain directly engages the GTPase and facilitates nucleotide exchange, while the adjacent PH domain can contribute to membrane localization and regulatory interactions. Structural studies have extensively characterized isolated DH/PH tandems, revealing a conserved architecture in which the DH domain forms a six helix bundle that interfaces with the GTPase¹⁰. Despite this progress, the DH domain alone presents a challenging target for small molecule inhibition due to its relatively flat and conserved interaction interface, which lacks well-defined binding pockets and is similar across Dbl RhoGEF family members.

Beyond the DH/PH catalytic core, Dbl RhoGEFs contain a diverse array of additional regulatory domains, often referred to as accessory domains. These domains mediate interactions with proteins, lipids, and signaling molecules, and play critical roles in controlling the activity, localization, and specificity of RhoGEFs¹⁰. Importantly, the assortment of accessory domains are comparatively far more variable than the conserved DH/PH tandem, contributing to the functional diversity of Dbl RhoGEFs and offering opportunities for selective targeting. However, in contrast to the well-studied catalytic core, the structural and mechanistic roles of accessory domains remain poorly understood.

A major limitation in the field is that most structural and biochemical studies have focused on isolated domains or truncated constructs rather than full-length proteins. While these approaches have provided important insights into catalytic mechanism, they often fail to capture the regulatory interactions between domains that govern RhoGEF activity *in vivo*. Emerging evidence indicates that accessory domains can exert both positive and negative regulatory effects, sometimes through long-range intramolecular interactions that are not apparent in isolated constructs. A key example highlighting the limitations of studying truncated constructs is the Dbl RhoGEF Ect2 by its BRCT domains. Early biochemical studies demonstrated that BRCT domains play a dual role both negatively regulating and promoting Ect2 activity depending on the stage of the cell cycle¹¹. These conclusions were largely based on experiments using isolated BRCT domains and the DH/PH catalytic tandem, which suggested that BRCT-mediated interactions could modulate access to the catalytic core. Subsequent structural work resolved the BRCT domains and revealed that a Rac GTPase-activating protein can disrupt Ect2 autoinhibition¹², providing further mechanistic insight but still in a partially reconstituted system. However, it was not until structures incorporating both the BRCT domains and the DH/PH tandem were determined that the physical basis of this regulation became clear, demonstrating that the BRCT domains, in conjunction with the PH domain, occlude the GTPase binding interface¹³. Additional studies have further shown that individual BRCT domains play a unique functional role to coordinate inhibition and activation in Ect2¹⁴. While we still lack a full-length structure of Ect2, our understanding of its regulation increases the closer we get to this goal. Thus, it is likely much of our understanding of RhoGEF regulation will require structural characterization of full-length RhoGEFs and their multidomain architectures.

While using full-length RhoGEFs is the next step to understanding their complex structures and dynamics, full-length RhoGEFs are experimentally challenging to study. These proteins are typically large, multidomain, and conformationally dynamic, which complicate their expression, purification, and

structural characterization. In many cases, successful expression requires eukaryotic systems such as insect or mammalian cells, and their inherent flexibility can limit the applicability of high-resolution structural methods such as X-ray crystallography¹⁵. In addition to these challenges, simple changes in handling RhoGEF constructs in biochemical studies present a significant barrier to reproducing some studies. In part, this may stem from differences in buffer composition and cryopreservation strategies, but these differences and their potential effects on the protein's biochemical properties are underappreciated, but critically important, for proper characterization of these enzymes. Developing robust methods detailing the handling and storage of these proteins is an essential step toward making full-length RhoGEFs more experimentally tractable.

The phosphatidylinositol-3,4,5-trisphosphate (PIP₃)-dependent Rac exchanger (P-Rex) subfamily of Dbp RhoGEFs provides a valuable model for understanding the complexities of multidomain RhoGEF regulation. This subfamily consists of two members, P-Rex1 and P-Rex2, which share a similar domain organization comprising an N-terminal DH/PH tandem followed by two DEP, two PDZ, and inositol polyphosphate 4-phosphatase (IP4P) domains. Each of these proteins respond to signals from G protein coupled receptor and receptor tyrosine kinase signaling pathways through activation via the Gβγ subunits of heterotrimeric G proteins and the lipid PIP₃. P-Rex1 is among the best-characterized RhoGEFs and represents one of the first examples for which full-length structural information has been obtained^{15–20}.

Structural studies of P-Rex1 progressed in a stepwise manner with early work focusing on isolated domains and later moving toward full-length architectures. Early crystal structures of the P-Rex1 DH/PH tandem in complex with Rac1 and Cdc42 revealed how the DH domain engages different GTPases and suggested the presence of recognition elements that contribute to substrate specificity^{16,17}. Notably, these structures also exhibited differences in the relative orientation of the DH and PH domains as a pronounced bend between the two domains (Fig. 1.1A-B), indicating conformational flexibility at

this site. Additionally, these studies suggested that surfaces on the PH domain could participate in protein-protein interactions beyond lipid binding based on the observed crystal contacts¹⁶. Structural characterization of the isolated PH domain (Fig. 1.1C) identified the binding site for PIP₃ by way of the soluble headgroup, IP₄¹⁶. This structure also revealed a nonspecific membrane interaction element within the β3/β4 loop, adjacent to the IP₄ binding site. All together, these findings provided important insight into how P-Rex1 may associate with membranes, achieve GTPase specificity, and binds PIP₃. However, these studies were limited to truncated constructs and therefore could not capture the contributions of the C-terminal accessory domains or provide insight into Gβγ-mediated activation.

Advances in cryo-electron microscopy (cryo-EM) from the “resolution revolution” enabled the study of larger, more flexible multidomain proteins such as P-Rex1, which had proven challenging targets for X-ray crystallography¹⁵. Using this approach, the structure of P-Rex1 in complex with Gβγ (Fig. 1.1D) revealed that Gβγ-mediated activation involves extensive interactions beyond the catalytic core. In particular, the second DEP domain, both PDZ domains and IP4P domain contribute to the Gβγ binding interface¹⁸. These studies also refined our understanding of P-Rex1 domain architecture. PDZ domains are common protein interaction modules and adopt a canonical fold with 6 β-strands interlaced with a short α-helix and a long α-helix where the N- and C-termini are in close proximity²¹. In P-Rex1, the first PDZ domain closely resembles the canonical PDZ fold, yet the second PDZ domain displays a non-canonical structure where an additional long helix is inserted between the βE and βF strands. Further insights were also gained into domains uncommon in Dbl RhoGEFs. DEP domains, which are relatively rare among Dbl RhoGEFs, adopt a fold with a structurally conserved helical core with less conserved β-hairpin arm²² and historically have been associated with membrane interactions. In the P-Rex1–Gβγ structure, only the second of the two DEP domains was resolved, leaving the structure and position of the first DEP domain unknown. However, the extensive binding interface of the DEP2 domain with Gβγ still provided clues to the orientation of P-Rex1 to the membrane. Given this position DEP2 could

reasonably engage the lipid bilayer, implicating DEP2 as a membrane binding component. The IP4P domain is unique to the P-Rex subfamily. Although initially predicted to be of low complexity with weak sequence homology to inositol polyphosphate 4-phosphatase— for which it was named— the IP4P domain was found to adopt a well-defined and complex fold resembling that of *Legionella* phosphoinositide phosphatases¹⁸. While P-Rex1 IP4P lacks phosphatase activity, this domain is a critical scaffolding domain in P-Rex1, participating in protein-protein interactions with other accessory domains to form the C-terminal core. Despite the use of full-length constructs in these studies, significant portions of P-Rex1 remained unresolved due to conformational flexibility, highlighting challenges inherent in studying these proteins.

More recently, two independent full-length structures of P-Rex1 successfully resolved all major domains by stabilizing flexible domains of the proteins through distinct strategies that anchored the PH domain to a four-helix bundle (4HB) extending from the IP4P domain^{15,20} (Fig. 1.1E). These structures confirmed earlier hypotheses that the PH domain participates in protein-protein interactions; however, rather than interacting with external partners, the PH domain forms intramolecular contacts that contribute to the overall architecture and regulation of the protein. Furthermore, these structures along with biochemical characterization enabled the identification of two negative regulatory mechanisms that maintain autoinhibition. These findings underscore the importance of studying multidomain architectures to understand RhoGEF regulation. Moreover, we expect that the process of gaining these kinds of full-length structural insights on other full-length RhoGEFs will be expedited due to advances in structure determination methods, namely cryo-EM, which enabled the rapid advances in P-Rex1. Throughout the evolution of understanding P-Rex1 regulation, much of it has been assumed to be the same in P-Rex2; however, these proteins exhibit distinct functional properties which have not yet been investigated. For example, P-Rex1 can activate multiple Rho GTPases, whereas P-Rex2 displays more restricted specificity toward Rac1²³. Additionally, P-Rex2 also uniquely interacts with the tumor

suppressor PTEN, yet P-Rex1 does not^{24,25}. The basis for these differences remains incompletely understood. Given our deep understanding of P-Rex1 structure and autoinhibitory mechanisms, P-Rex2 represents a logical next target for advancing our understanding of RhoGEF structure and function.

In this dissertation, I address these gaps through careful characterization of handling RhoGEF catalytic domains, determining the structure of full-length P-Rex2, and characterizing P-Rex2 autoinhibitory mechanisms. Specifically, I aim to identify structural and regulatory differences between P-Rex1 and P-Rex2 to contextualize why these proteins seem to have different biochemical properties. Additionally, I aim to identify common trends in the freezing and handling of Dbp RhoGEFs using activity and thermostability to measure changes over time. These studies will provide foundational and robust ways for groups studying RhoGEFs to properly characterize these proteins prior to *in vitro* biochemical studies and promote reproducibility in the field. Additionally, these studies provide new insight into how accessory domains regulate RhoGEF activity, reveal mechanistic differences between the closely related P-Rex proteins, and establish a framework for investigating other multidomain RhoGEFs. More broadly, this work advances our understanding of how signaling inputs are integrated to control cytoskeletal dynamics and cell behavior.

Figure

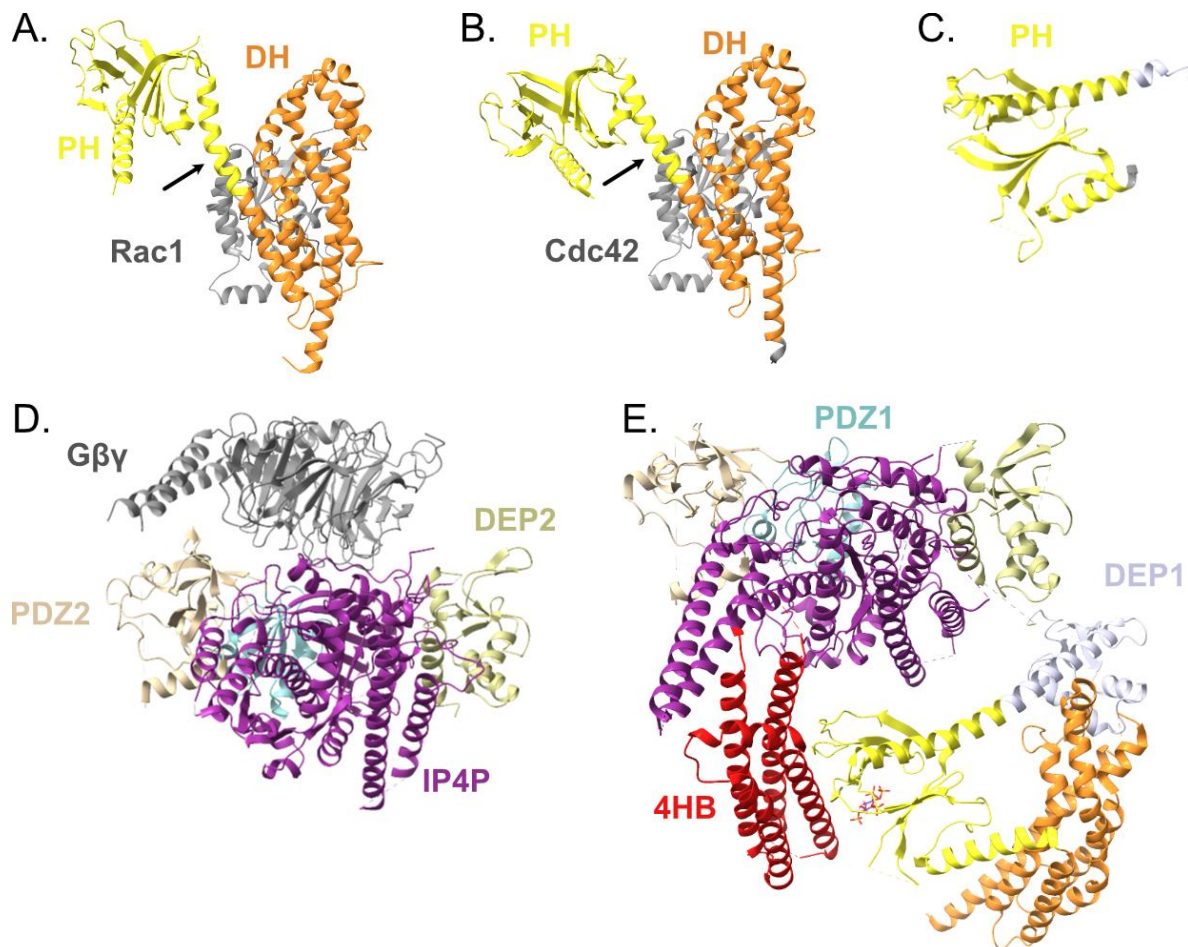


Figure 1.1. Evolution of P-Rex1 Structures. A) P-Rex1 DH/PH complexed with Rac1 (PDB: 4YON) and B) P-Rex1 DH/PH complexed with Cdc42 (PDB: 5F11) highlighting the flexibility at junction between the DH and PH domains (black arrow). C) Isolated, ligand-free P-Rex1 PH domain (PDB: 5D27). D) P-Rex1 complexed with soluble Gβγ (PDB: 6PCV). While a full-length P-Rex1 construct was used, only the C-terminal core (DEP2-IP4P) was able to be resolved due to flexibility of the unresolved domains. E) Autoinhibited P-Rex1 (PDB: 8TUA) stabilized by the addition of IP₄.

Chapter 2. GEF me a break: the consequences of freezing Rho guanine-nucleotide exchange factor catalytic domains

This chapter contains text which has also been published on BioRxiv DOI: 10.64898/2026.04.08.717323.

This work contains contributions from Emma Barpal, Herra Mendoza, and Jennifer Cash.

Abstract

Purified proteins are routinely flash frozen for use in functional and structural studies, providing a convenient way to reproduce results across complex experiments. Rho guanine-nucleotide exchange factors (RhoGEFs) are no exception to this practice, yet the effects of freezing on their activity and stability remain largely uncharacterized. This gap potentially affects the characterization of these important enzymes and how results are interpreted with respect to their prospective use as therapeutic targets. Here, we tested the isolated DH/PH tandems of P-Rex1, P-Rex2, and PRG under different cryoprotectant conditions and monitored activity and thermostability over time after flash freezing. Our results show a clear divergence between the activity of fresh and frozen purified RhoGEF protein samples in as little as one week for some conditions. Specifically, the variability in data collected on frozen samples was greatly increased. Despite these differences, thermostability seems to be preserved for much longer timepoints across RhoGEFs. Moreover, despite eventual changes in both activity and thermostability with respect to freezing, there are no obvious changes in global conformation between fresh and frozen samples of the isolated P-Rex2 DH/PH tandem. From our data, there are few generalizable trends between the different RhoGEFs and no single cryoprotective agent tested was a silver bullet to preserve both activity and thermostability across RhoGEFs. Overall, our findings emphasize the unpredictable effects of freezing RhoGEFs. As such, RhoGEF freezing should be carefully characterized for each protein and critically viewed when comparing analyses between different studies.

Introduction

Dbl Rho guanine-nucleotide exchange factors (RhoGEFs) are important regulators of small Rho GTPases which control cell movement and proliferation^{2,3}. Common across most Dbl RhoGEFs is a tandem catalytic Dbl homology (DH) domain and pleckstrin homology (PH) domain which are structurally well-characterized (Fig. 2.1). The DH domain fold is composed of six α -helices forming an oblong helical bundle¹⁰ with the C-terminal helix extending into that of the PH domain. The PH domain is a beta sandwich flanked by an amphipathic helix. Well-conserved helices in the DH domain bind directly to the switch regions of the GTPase to allow for catalytic activity (Fig. 2.2). Currently, there are at least 78 structures in the Protein Data Bank from X-ray crystallography, cryo-electron microscopy, and nuclear magnetic resonance that contain a Dbl RhoGEF DH domain²⁶. Of the publications associated with these structures, only about half explicitly include freezing as part of their standard purified protein storage protocol, and the rest are ambiguous on storage conditions. These proteins are routinely frozen with 5-10% glycerol or without cryoprotectant, yet there have not been systematic reports on either the consequences of freezing these proteins or the possible effects of cryoprotective agents (CPAs) on their activities, potentially impacting rigor and reproducibility in the field.

In addition to their normal biological function, RhoGEFs are implicated in driving a variety of diseases, including cancer, diabetes, hypertension, and neurodegenerative diseases^{24,27}. Guanine nucleotide exchange activity assays^{20,27,28} and thermostability measurements²⁹ employing purified proteins are commonly used to characterize disease-relevant RhoGEF variants and evaluate potential chemical inhibitors, informing our understanding of how RhoGEFs can propel disease. Before deriving conclusions from these types of *in vitro* experiments, it is essential to ensure that the involved protein samples are well-behaved and functionally intact. Unless these samples are exclusively freshly prepared, understanding how freezing and storage conditions affect the activity and stability of a specific RhoGEF

protein is critical for interpreting experimental results and ensuring reliable biochemical characterization of that RhoGEF.

Cryopreservation of proteins through flash freezing in liquid nitrogen (LN₂) and subsequent storage at ultralow temperatures is common practice to preserve biological materials. However, physical consequences of ice formation, like cold-induced unfolding and cryoconcentration, have been shown to disrupt protein function³⁰. While rapid freezing of enzymes with LN₂ has been widely recommended for preservation of activity^{31,32} some proteins are damaged in this process^{33,34}. CPAs like glycerol³⁵ and sucrose are broadly effective at mitigating these effects, but still some proteins remain sensitive to damage despite the addition of CPA.

Based on anecdotal evidence, our lab noticed that purified RhoGEF proteins may be sensitive to freezing and wanted to further probe this observation. We selected three fairly well-studied RhoGEFs, P-Rex1, P-Rex2, and PDZ-RhoGEF (PRG), and purified their tandem DH/PH domains. We then examined how their activities and thermostabilities change without and with different CPAs, both before and after freezing. These proteins differ in GTPase specificity and biochemical characteristics, allowing us to explore whether CPA and freezing effects may be generalizable across the Dbp RhoGEF family. Our results reveal that freezing alters activity measurements unpredictably within each GEF protein sample and, furthermore, even the addition of CPA to samples, without freezing, increases the spread of activity measurements collected and can artificially increase activity. Furthermore, we demonstrate that, at least with P-Rex2 DH/PH, changes in activity and thermostability from freezing or addition of CPA are not reflected as conformational changes that can be detected with size-exclusion chromatography coupled with small-angle X-ray scattering (SEC-SAXS). The underlying mechanisms responsible for freezing- and/or cryoprotectant-induced changes to GEF function remain unknown, but these findings underscore that these factors can unpredictably influence RhoGEF function. Thus, assays using frozen proteins without

accompanying characterization of the effects of freezing and CPA on those proteins should be interpreted with caution.

Results

Insights into Dbl RhoGEF DH/PH biochemical differences that could potentially confer differences in stability

The Dbl RhoGEF family of proteins are characterized by a DH catalytic domain that binds GTPases and is almost always followed by a PH domain¹⁰. Despite this conserved architecture, the biochemical characteristics of this conserved domain tandem can be divergent, as RhoGEFs exhibit varying specificities for different GTPases. To investigate how biochemically disparate RhoGEF DH/PH domains may be, we chose four commonly studied RhoGEF proteins, P-Rex1, P-Rex2, PDZ-RhoGEF (PRG), and p63RhoGEF and one RhoGEF for which there is very little biochemical information available, ARHGEF39. P-Rex1 and P-Rex2 are two closely related family members, each sharing the same overall domain architecture and specificity for Rac GTPases³⁶. PRG and p63RhoGEF are G protein-regulated family members specific for RhoA³⁷. ARHGEF39 is unique within the family, as the expressed protein consists of only the DH/PH domain tandem without any additional accessory domains, suggesting that it could represent a relatively stable isolated DH/PH. It is reportedly specific for RhoA³⁸, but this protein is vastly understudied. For our comparison of these five proteins, we analyzed their DH/PH structures and corresponding electrostatic surface potentials, calculated their isoelectric points (pI), and constructed a structure-based sequence alignment (Fig. 2.1 & 2.2). AlphaFold3³⁹ was used to predict the structure of the DH/PH tandem for P-Rex2 and ARHGEF39, as there are currently no published structures of the DH/PH tandems of these proteins.

Although the overall structures of these DH/PH domains are very similar, they exhibit some notable differences (Fig. 2.1 & 2.2). In comparing sequence identity, the closely related P-Rex1 and P-Rex2 are 71% identical, but the identities of the other DH/PH domains to one another is only around 20%, even

for RhoGEFs that share RhoA specificity (Fig. 2.2). In comparing surface electrostatics, most of the five DH domains are very similar in the character of their GTPase-binding sites apart from ARHGEF39, which is less electropositive in nature (Fig. 2.1; left column of right panel). In contrast, on the surface of the DH/PH domains opposite the GTPase-binding site, there is much more variability in electrostatic surface potential (Fig. 2.1; right column of right panel). Additionally, while the isoelectric points of P-Rex1, P-Rex2, and PRG are very similar at a calculated 8.5-8.7, the pI of p63RhoGEF and ARHGEF39 are much different at 6.7 and 9.8, respectively. This has important implications for working with the isolated DH/PH domains, as a purified protein is less stable in a buffer with a pH very close to the pI of the protein, suggesting that a purification buffer that works well for one of these proteins may destabilize another one. Collectively, this suggests that even though isolated RhoGEF DH/PH tandem domains are structurally similar overall, they can exhibit biochemical differences that might impact their behavior and stability in purified form.

Glycerol and sucrose increase variation in activity measurements of freshly purified RhoGEF DH/PH proteins

Due to the biochemical similarity of P-Rex1 and P-Rex2 DH/PH domains and the divergence of PRG, we selected these three for analysis. We also chose to use isolated DH/PH tandems rather than full-length RhoGEFs to examine effects on only the catalytic core of the GEF and to limit confounding variables like intramolecular interactions from additional regulatory domains. Working with the isolated DH/PH also offers the technical advantage of utilizing bacterial expression, enabling production of large amounts of protein which allows for longitudinal analyses of biological replicates. In our hands, we observed that the activities of these proteins change after freeze-thaw.

First, we wanted to establish any broad effects on these proteins from the addition of cryoprotectant agents (CPAs) and determine if CPAs affect baseline activity measurements of freshly purified P-Rex1, P-Rex2, and PRG DH/PH tandems. Glycerol and sucrose are commonly used, powerful

CPAs which promote folding and protein stability through preferential exclusion of the CPA from the protein hydration-shell, improving preservation of protein function^{40–43}. Thus, we analyzed protein activity of samples prepared without cryoprotectant, with 5-20% glycerol, or with 5-10% sucrose immediately after purification.

To test the activity of each RhoGEF DH/PH tandem in each CPA condition, we used a GEF activity assay which reports the increase in association between a soluble GTPase and a fluorescent, non-hydrolyzable GTP analogue (mant-GTP) (Fig. 2.3). For each biological replicate, activity was normalized to the cryoprotectant-free condition. P-Rex1 shows no significant changes in activity with respect to CPA condition, but the 95% confidence interval (CI) strongly increases across all conditions (Fig. 2.3A), suggesting the presence of CPA increases variability between replicates and makes data interpretation more difficult. P-Rex2 exhibits increased activity in 20% glycerol and 5% sucrose conditions as well as a strong increase in CI across all CPA conditions (Fig. 2.3B). PRG shows increased activity with 10% glycerol and both sucrose conditions (Fig. 2.3C). PRG presents an increased CI in most CPA conditions except 5% sucrose, although it is unclear why the data are much tighter in this condition as compared to the other conditions or samples. Together, these data indicate that the addition of glycerol or sucrose broadly increases variation in the measured activities of the proteins tested. However, these conditions do not uniformly affect activity across different proteins with any distinguishable patterns, even among closely related RhoGEFs.

Isolated DH/PH protein activity is widely affected by freezing

It is common practice to freeze purified proteins and store them for later use, providing convenience over purifying them fresh for each experiment. However, some purified enzymes have been reported to lose some or all of their activity during the freeze-thaw process, including α -amylase⁴⁴ catalase³², phosphofructokinase⁴⁵, and lactate dehydrogenase⁴⁶. To assess how RhoGEFs may be affected, we

measured the activities of the isolated RhoGEF DH/PH tandems in each CPA condition after flash freezing and storage at -80°C for varying periods of time. We also assessed these samples by SDS-PAGE. After up to six months, we found no significant changes to the proteins as visualized by SDS-PAGE, indicating that there is no detectable degradation or irreversible aggregation of these samples upon freezing and storage (Fig. 2.4).

After one month, the activity of frozen P-Rex1 DH/PH without CPA and low levels of glycerol or sucrose decreases (Fig. 2.5). However, higher concentrations of glycerol (10-20%) and 10% sucrose seem to mitigate this effect. By the six-month timepoint, only the 10% glycerol sample consistently maintained comparable levels of P-Rex1 DH/PH activity to the fresh sample. We also observed an unexpected phenomenon where, in conditions with low levels of CPA, after an initial drop, P-Rex1 activity rises back to levels indistinguishable from the fresh sample at the six-month timepoint.

Compared to P-Rex1, P-Rex2 DH/PH is much less resilient to freezing in most of the tested conditions. After one month, P-Rex2 DH/PH activity decreased in all storage conditions except 10% sucrose (Fig 2.5). After six months, the 10% sucrose sample maintained activity levels comparable to the fresh protein sample. Interestingly, we observed that the activity of P-Rex2 DH/PH stored in low CPA concentration conditions returned to near-fresh activity levels at six months, similar to P-Rex1. While the differences in the mean activities of these samples are statistically insignificant, the spread of these data are wider than that of the fresh sample as shown in the 95% confidence intervals (CI) of the data (Fig. 2.6).

PRG DH/PH activity was tested over a shorter period than the P-Rex proteins. Despite this, PRG revealed a wide spread between data (Fig. 2.6). While PRG DH/PH activity is preserved in most conditions within the 4 week time frame, there are strange trends within these data, making it unlike either P-Rex protein and difficult to interpret. For example, PRG DH/PH stored in 5% glycerol shows opposite trends

between biological replicates where one shows a dramatic increase in activity after freezing while others decreases in activity. The 20% glycerol condition shows a similar occurrence.

Altogether, these data show that freezing affects each GEF in different ways with no clear or generalizable trends. Both P-Rex DH/PH samples are affected by freezing, generally manifested as decreases in activity, but surprisingly do not have the same responses across the different CPA sample conditions. PRG DH/PH, on the other hand, does not seem to be as greatly affected by freezing within a one month timeframe. However, PRG shows even greater changes in variability of activity measurements than either P-Rex sample, and perturbations from freezing in certain glycerol conditions lead to increases in activity, unlike P-Rex samples. Overall, RhoGEF DH/PH activity measurements seem to be widely impacted by freezing.

Thermostability of DH/PH proteins generally does not change at early time points post-freezing

Next, we wanted to assess the protein quality and stability at each of these time points to help explain the changes and variation in activity measurements. We characterized how each storage condition affects RhoGEF thermostability over the time course tested using differential scanning fluorimetry (DSF). To our surprise, protein thermostability did not correspond to observed changes in activity. Prior to freezing, the melting temperature of freshly purified P-Rex1 DH/PH across conditions with and without CPA remained consistent at ~38°C and was preserved after one month of storage (Fig. 2.7). At the six month time point, there is a significant decrease in thermostability across all conditions along with an increase in variability between measurements. With unfrozen P-Rex2 DH/PH, melting temperature increases with 20% glycerol from 41°C to 43°C but remains consistent within sucrose-containing conditions at ~41°C (Fig. 2.7). Around the six month time point post-freezing, P-Rex2 DH/PH thermostability in the no CPA condition decreases. Higher concentrations of glycerol seem to exacerbate the loss of thermostability, decreasing melting temperature by 2-3°C by six months. Unfrozen PRG DH/PH

exhibits a melting temperature of $\sim 35^{\circ}\text{C}$, and, similar to P-Rex2, this increases in the presence of 20% glycerol to $\sim 36.5^{\circ}\text{C}$. The thermostability of PRG DH/PH is relatively unaffected by freezing in most conditions over the 4 week time frame with the exceptions of the 20% glycerol condition where melting temperature dropped within one week and both sucrose conditions where melting temperatures dropped after four weeks of storage.

Long-term freezing does not affect the global structure of P-Rex2 DH/PH

Changes to thermostability and activity after storage at -80°C led us to investigate whether or not the global structure of the DH/PH tandem itself was changing, leading to these differences. To do this, we utilized size exclusion chromatography coupled with small-angle X-ray scattering (SEC-SAXS). Because P-Rex2 DH/PH activity and thermostability were affected upon freezing without CPA but preserved in 5% sucrose, we chose these two conditions and let the samples mature at -80°C for 6 months before assessing them using SEC-SAXS. We compared these samples to data previously collected by our lab on a freshly purified P-Rex2 DH/PH sample⁴⁷ (SASDZV5). Both the CPA-free and the 5% sucrose samples eluted in a single symmetric peak (Fig. 2.8A). There is a slight delay in the elution of the frozen CPA-free sample and furthermore in the 5% sucrose sample, but this could be due to differences in column interaction. In both samples, there are no signs of aggregation or repulsion at low q in either the scattering intensity plots (Fig. 4A-B) or Guinier analyses (Fig. 2.8B-C). Dimensionless Kratky analysis also confirmed that, after freezing and storage, both samples maintain a compact and folded structure with well-defined peaks corresponding to globular proteins and near-identical profiles to the fresh P-Rex2 DH/PH (Fig. 2.9C). The molecular weight calculated for each sample corresponds to that of a P-Rex2 DH/PH monomer expected to be ~ 44 kD (Table 2.1). Interestingly, the correlation volume of fresh P-Rex2 DH/PH was reported at 41.6 kD, but the frozen CPA-free and 5% sucrose samples were 42.5 and 39.3 kD, respectively. Given the comparable radius of gyration (R_g) between these samples (Table 2.1), it is unlikely that the difference in molecular weight is significant. Finally, the pair distance distribution function, $P(r)$, indicated D_{max} values

of 81.6 Å (CPA-free) and 80.1 Å (5% sucrose) (Fig. 2.9D, Table 2.1), again demonstrating that there are not any significant global differences in conformation, shape, or oligomeric state. Therefore, the changes that lead to decreases in thermostability and activity are not necessarily related to large scale conformational changes and may be due to other sources of damage.

Discussion

In this study, we examined how flash freezing and storage at ultralow temperatures affect the RhoGEF activity and thermostability of isolated DH/PH tandems from P-Rex1, P-Rex2, and PRG. We also evaluated whether or not glycerol or sucrose could preserve these properties during storage. The answers to these questions are complicated: each isolated DH/PH tandem responded to different freezing and storage conditions in unique ways. Even closely related RhoGEFs, P-Rex1 and P-Rex2, did not produce the same response to the same CPA conditions. One of the few widely generalizable trends we observed is that both the addition of cryoprotectants and prolonged storage of these purified RhoGEFs increases variability and reduces reproducibility of activity measurements. Compounding this phenomenon, the mant-GTP exchange assay itself presents technical challenges that can further complicate data interpretation. Perturbations in how the assay is performed, including but not limited to the order in which reagents are mixed, how quickly the initiated reaction is measured, and selection of RhoGEF concentration in the assay, can lead to variable results. Because of this, we spent substantial time optimizing how we perform the assay to make it as consistent as possible, as evidenced by low variability within unfrozen samples, especially those without CPA. Even after optimization of this workflow and attentiveness to consistent handling techniques, adding CPA to or freezing RhoGEF samples make the resulting data more difficult to interpret. Moreover, the amount of cryoprotectant carried into the activity assay is minimal. Even at the highest glycerol concentration tested, the final concentration added to the reaction is less than 0.5%. Thus, it is unlikely that cryoprotectants directly affect the assay itself but rather influence the apparent biochemical qualities of the proteins.

We also found that a loss of activity and/or increase in variability does not necessarily correspond to a decrease in thermostability. Moreover, despite clear changes in activity and thermostability in P-Rex2 DH/PH, SEC-SAXS analysis revealed no detectable changes in overall conformation or flexibility compared to fresh protein. Given the limitations of SEC-SAXS for resolving structural changes as a low-resolution method, this suggests that more subtle shifts in regulatory or dynamic states may underlie the observed effects, requiring more high-resolution solution-based methods like hydrogen-deuterium exchange mass spectrometry to determine what causes these changes. While the results were not definitive, the absence of clear trends among GEFs and cryoprotectants in preserving activity and thermostability serves as an important reminder that protein handling conditions must be carefully characterized to ensure reproducibility.

While there are few generalizable trends between GEFs, these data indicate potential appropriate frozen storage conditions for individual RhoGEFs. For example, P-Rex1 DH/PH stored in 10% glycerol appears to retain catalytic activity for up to six months despite a noticeable reduction in thermostability over the same period. This suggests that glycerol may help preserve function even with subtle structural destabilization. For P-Rex2 DH/PH, 10% sucrose may serve as a suitable cryoprotectant under the conditions tested. Furthermore, these CPA concentrations could represent suitable storage conditions for full-length P-Rex proteins as well. However, these practices need to be validated with equally rigorous work with the full-length proteins before adopting them into protocols. In contrast, PRG maintained both activity and thermostability for at least one month while frozen under CPA-free conditions.

Together, these findings caution against assuming that frozen RhoGEFs retain their functional properties and emphasize that biochemical assays using frozen protein should be interpreted carefully. Moreover, these findings complicate direct comparisons between studies using frozen proteins, especially those from different labs where published information on protein handling and storage is not clear or provided. Our study calls attention to potential alarming issues around reproducibility in the field of

biochemically characterizing purified RhoGEF proteins. More broadly, this work serves as a cautionary reminder that not all proteins— regardless of how well-conserved or similar in function— are affected the same by freezing, even under the same conditions.

Materials and Methods

Cloning and Plasmids

P-Rex1 DH/PH, P-Rex2 DH/PH, and soluble Rac1 expression constructs were described previously^{16,47,48}. pDONR221 PRG was obtained courtesy of Center for Personalized Diagnostics through DNASU (HsCD00860223)⁴⁹. PRG DH/PH (residues 729-1081) were cloned into a pMALc2H₁₀T vector with a Tobacco Etch Virus (TEV)-cleavable N-terminal maltose binding protein (MBP)-10xHis tag. pLV_DORA_RhoA_IRES was generated by the Hayer lab and gifted to us through Dr. Sean Collins⁵⁰. Soluble RhoA (residues 1-189, excluding the CAAX site) was cloned into the same pMALc2H₁₀T vector with a TEV-cleavable N-terminal MBP-10xHis tag.

Protein production, purification, and handling

Soluble Rac1 and RhoA were expressed in Rosetta (DE3) pLysS *E. coli* cells and purified as previously described¹⁶.

P-Rex and PRG DH/PH constructs also were expressed in Rosetta (DE3) pLysS *E. coli* cells as N-terminal His-tagged MBP fusion proteins. Cells were grown in Terrific Broth to an OD₆₀₀ of 0.8, induced with 0.1 mM IPTG at 16-20 °C, harvested after 16-20 hours, and then flash frozen and stored at -80 °C. Cell pellets were thawed and resuspended in 9 ml 20 mM HEPES (pH 8), 300 mM NaCl, 0.1 mM EDTA, 2 mM DTT and protease inhibitor cocktail (125 nM aprotinin, 6.8 μM leupeptin, 0.15 nM soybean trypsin inhibitor, 1 μM E-64, and 1 μM bestatin) per 1 g cell pellet. Cells were then homogenized with a dounce and lysed with an Avestin EmulsiFlex-C5 High Pressure Homogenizer. Lysate was clarified by

ultracentrifugation at 40,000 rpm for 45 min in a Type 45 Ti rotor. The supernatants were then filtered through a glass fiber filter.

P-Rex DH/PH clarified supernatant was incubated with Ni-NTA resin for 30-60 min. Resin was washed with buffer A [20 mM HEPES (pH 8), 300 mM NaCl, 2 mM DTT] followed by buffer A containing 10 mM imidazole, and proteins were eluted with buffer A containing 250 mM imidazole. Elutions were then simultaneously dialyzed into buffer containing 20 mM HEPES (pH 7), 200 mM NaCl, 2 mM DTT, and 10% glycerol and treated with TEV protease overnight to remove the N-terminal His-tagged MBP at a 1:2 molar ratio of TEV:MBP-P-Rex DH/PH. The cleaved MBP-His was then captured by an additional pass over Ni-NTA resin. The flow through was then processed over a HiTrap SP Sepharose Fast Flow column in 20 mM HEPES (pH 7) and 2 mM DTT and eluted over an NaCl gradient from 0-0.5 M over 4 CV. Fractions containing P-Rex DH/PH were pooled and concentrated to ~2 mg/ml, diluted to 1 mg/ml with buffer [20 mM HEPES (pH 7), 100 mM NaCl, and 2 mM DTT] containing 0-20% glycerol or 0-10% sucrose, and thoroughly and gently pipet mixed. Fresh protein was promptly used for assays where DSF was conducted within 15 minutes and the GEF activity assay was performed within 30-90 minutes of sample assembly. The protein was then immediately flash frozen in IN_2 and stored at -80 °C in 60 μL aliquots. Frozen P-Rex1 DH/PH samples were thawed on ice and then thoroughly pipet mixed. Frozen P-Rex2 DH/PH samples were thawed under cold running water, thoroughly pipet mixed, and then placed on ice.

PRG clarified supernatant was incubated with amylose resin for 1 hour. Resin was washed with buffer A and eluted with buffer A containing 10 mM maltose. Elutions were then simultaneously dialyzed into buffer containing 20 mM HEPES (pH 8), 100 mM NaCl, and 2 mM DTT and treated with TEV protease overnight to remove the N-terminal His-tagged MBP at a 1:6 molar ratio of TEV:MBP-PRG DH/PH. The dialyzed sample was then incubated with Ni-NTA resin for 1 hour to capture the cleaved MBP-His. The flow through was then processed over ENrich SEC 70 in 20 mM HEPES (pH 8), 200 mM NaCl, 2 mM DTT. Fractions containing PRG DH/PH were pooled and concentrated to ~2-5 mg/ml and diluted to 1 mg/ml

with buffer [20 mM HEPES (pH 8), 200 mM NaCl, 2 mM DTT] containing 0-20% glycerol or 0-10% sucrose. Fresh protein was promptly used for assays where DSF was conducted within 15 minutes and the GEF activity assay was performed within 30-90 minutes of sample assembly. The protein was then immediately flash frozen in LN_2 and stored at -80°C in 82 μL aliquots. Frozen PRG DH/PH samples were thawed at 4°C , thoroughly pipet mixed, and then placed on ice.

GTPase Exchange Activity Assay

A FRET-based guanine nucleotide exchange assay was used to assess the activity of the fresh and frozen isolated DH/PH protein samples. P-Rex DH/PH samples were diluted from 1 mg/ml ($\sim 22.7\ \mu\text{M}$) to 0.1 mg/ml with assay buffer [20 mM HEPES (pH 8), 100 mM NaCl, 5 mM MgCl_2 , 1 mM DTT, and 5% glycerol]. PRG DH/PH samples were diluted from 1 mg/ml ($24.15\ \mu\text{M}$) to 0.06 mg/ml ($1.625\ \mu\text{M}$) with assay buffer. A final concentration of 100 nM P-Rex1 or 200 nM P-Rex2 DH/PH was added to 2 μM Rac1 or 100 nM PRG DH/PH was added to 2 μM RhoA in assay buffer. The reaction was then initiated by adding 0.8 μM 2'/3'-O-(N-methyl-anthraniloyl)-guanosine-5'-diphosphate (mant-GTP), a non-hydrolyzable form of GTP. The reactions were excited at 280 nm and the fluorescence intensity at 450 nm was measured in 30s intervals on a SpectraMax M5 (Molecular Devices; San Jose, CA) plate reader for a total of 15 min. Fluorescence curves were fit to a one-phase association model using GraphPad Prism (San Diego, CA). The k value obtained from the one-phase association model was used to represent GEF activity. A range of DH/PH protein concentrations were empirically tested to determine the most appropriate one to use. Because of instrumentation limitations which restricted obtaining initial rates in this assay, lower DH/PH concentrations that strongly fit to a one-phase association equation were favored. In all RhoGEF activity assays, each biological replicate was normalized within itself. For the analysis of CPAs on freshly purified RhoGEF activity (Fig. 1), the freshly purified CPA-free sample was used for normalization within each biological replicate. For the analysis of freezing in each CPA condition (Fig. 2), the unfrozen purified protein in each CPA condition was used for normalization within each biological replicate. We selected this

strategy to analyze the trends in RhoGEF activity from CPA-addition or freezing and to minimize the effect of slight variation between biological replicates in data interpretation.

Differential Scanning Fluorimetry

Experiments were performed on a CFX96 Real-Time PCR System. Purified P-Rex DH/PH was diluted to a final concentration of 0.5 mg/ml in a buffer containing 20 mM HEPES (pH 8), 100 mM NaCl, and 2 mM DTT. Purified PRG DH/PH was diluted to a final concentration of 0.6 mg/ml in a buffer containing 20 mM HEPES (pH 8), 200 mM NaCl, and 2 mM DTT. 10X Sypro or Prolite Orange dye was added to each condition, and fluorescence was monitored across temperatures from 22 °C to 95 °C, incrementing by 1 °C per minute. Fluorescence signal was measured in the dedicated FRET channel as per manufacturer specifications. The protein melting temperature (T_m) was determined by fitting the fluorescence data to a Boltzmann sigmoidal curve and calculating the inflection point in GraphPad Prism.

Size exclusion chromatography coupled to small angle x-ray scattering (SEC-SAXS)

Purified P-Rex2 DH/PH was frozen without cryoprotectant or with 5% sucrose and stored at -80 °C for six months. Samples were thawed under cold water, gently pipet mixed, and then thoroughly buffer exchanged into 20 mM HEPES (pH 7), 200 mM NaCl, 2 mM DTT, and 1% glycerol in a 10 kD Amicon concentrator using 2 minute 8,000 x g spins. During buffer exchange, both samples showed signs of sample precipitation. To remove aggregated protein, samples were spun for 10 minutes at 14,000 x g and decanted to separate soluble from pelleted protein. Soluble samples were shipped to the Structurally Integrated Biology for Life Sciences (SIBYLS) beamline for data collection and analyzed as previously described⁴⁷.

Tables

Table 2.1. SEC-SAXS data analyses

	P-Rex2 DH/PH – no CPA SASDB: SASDZT5	P-Rex2 DH/PH – 5% sucrose SASDB: SASDZS5
Guinier analysis		
R _g (Å)	25.83 ± 0.25	25.58 ± 0.27
I(0)	11.14 ± 0.07	10.74 ± 0.08
Q _{min} * R _g	0.346	0.497
Q _{max} * R _g	1.311	1.337
Molecular weight analysis		
Porod Volume (Å ³)	55000	48200
MW (Vc) (kDa)	42.5	39.3
Shape (S&S)	Compact	Compact
D _{max} (Å)	81.6	80.1
P(r) analysis		
D _{max} (Å)	85.0	84.0
R _g (Å)	26.1 ± 0.18	26.18 ± 0.17

Figures

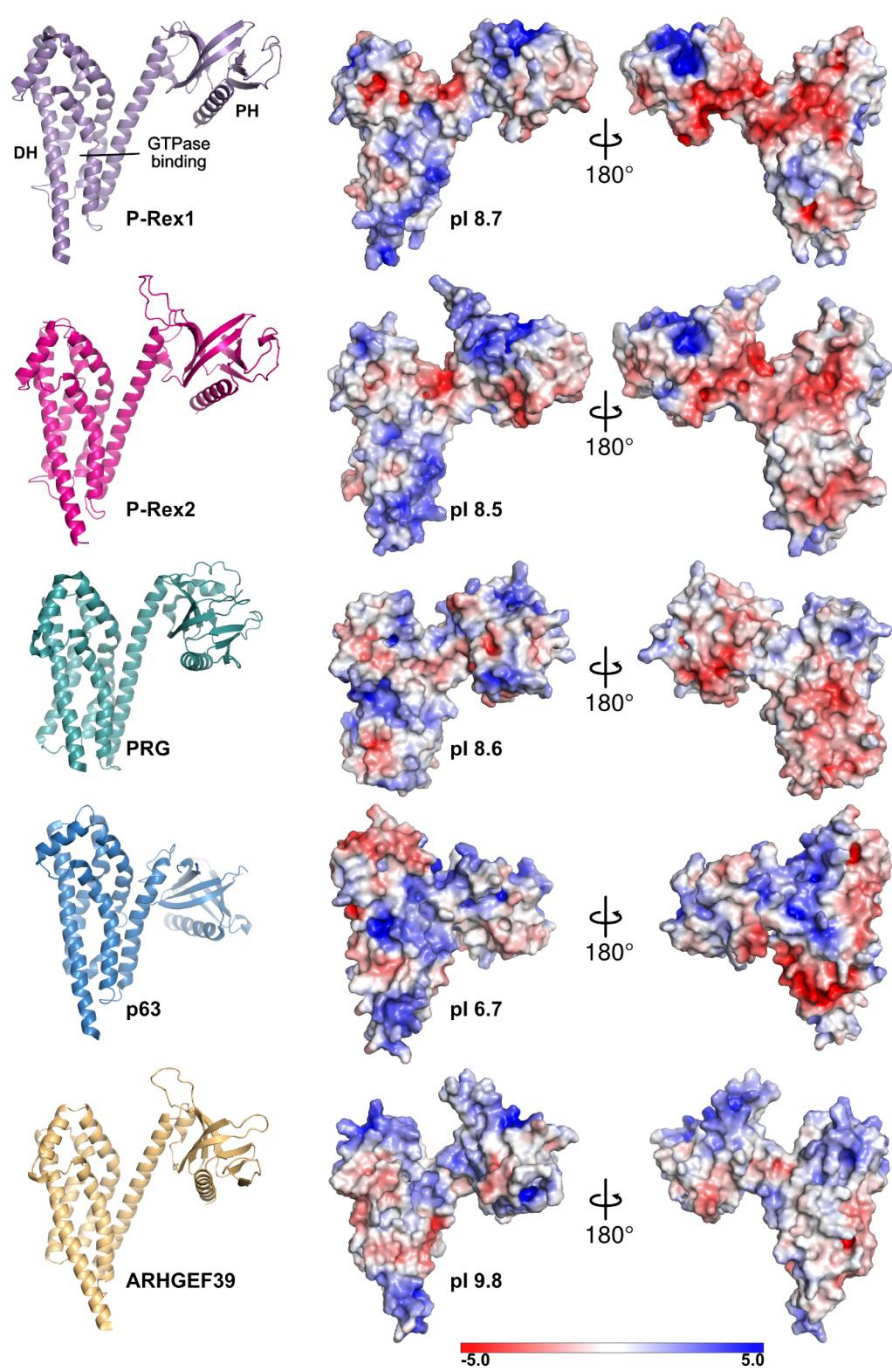


Figure 2.1. Comparison of structures and characteristics of DH/PH tandem domains from select Dbl RhoGEFs. The isolated DH/PH tandem domains of P-Rex1 (PDB: 5FI1), P-Rex2 (AlphaFold3 prediction), PRG (PDB: IXCG), p63RhoGEF (PDB: 2RGN), and ARHGEF39 (AlphaFold3 prediction) are shown with structural features labeled. For those from experimental structures, only the DH/PH tandem is shown; the other components of each structure were removed. The right panel shows the electrostatic surface potential of

each DH/PH calculated using Adaptive Poisson-Boltzmann Solver (APBS)⁵¹ electrostatics within PyMOL (The PyMOL Molecular Graphics System, Version 3.1.8)⁵² on a range of +/- 5.0, with blue indicating electropositive and red electronegative. In this panel, the view of the structure on the left in surface is the same view of the structure as is shown in the left panel in cartoon. Also shown for each is the isoelectric point (pI) calculated using ProtParam. The sequence used for each RhoGEF is shown in Figure 2.2.

<u>α1 helix</u>					Percent identity matrix					
p63	150	EEEQKKKALERSMYVLSELVET	171		p63	100	17	20	21	23
PRG	731	-----REIDRQEVINELFVT	745		PRG	19	100	17	20	19
ARHGEF39	12	VQEQRARWERKRACTARELLET	33		ARHGEF39	20	19	100	21	19
PRex1	39	AARESERQLRLRLCVLNEILGT	60		PRex1	21	20	21	100	71
PRex2	13	SAKDLEKQLRLRVCVLSELQKT	34		PRex2	23	19	19	71	100

<u>α1 helix</u>		<u>α1' helix</u>		<u>α2 helix</u>			
p63	EKMYVDDLQIVEGYMATMAAQGVPE---	S-LRGRDRIVFGNIQQIYEWHRDYFLQELQR	227				
PRG	EASHLRTLRLVLDLIFYQRMKKEN-----	IMPREELARLFPNLPPELIEIHNSWC-EAMKK	798				
ARHGEF39	ERRYQEQLGLVATYFLGILKAKG-----	TLRPPERQALFGSWELIYGASQELL-PYLEG	86				
PRex1	ERDYVGTTLRFLQSAFLHRIRQNVADSVEKGLTEENVKVLFSNIEDILEVHKDFL-AALEY	119					
PRex2	ERDYVGTLEFLVSAFLHRMNQCAASKVDKNVTEETVKMLFSNIEDILAVHKEFL-KVVEE	93					

<u>α3 helix</u>		<u>α4 helix</u>			
p63	CLKDPD---WLAQLFIKHE-----	RRLHMYVVYCQNKPKSEHVVSFEGDS--Y---F--	271		
PRG	LREEGPPIIKEISDLMLARFDGPAREELQQVAAQFCSYQSIALELIKTKQRKESRFQLFMQ	858			
ARHGEF39	G-----CWGQGLEGFC-----	RHLELYNQFAANSERSQTTLQEQLKKNKGFRRFVR	132		
PRex1	CLHPEPQSQHELGNVFLKFK-----	DKFCVYEEYCSNHEKALRLLEVEL-NKIPTVRAFL	173		
PRex2	CLHPEPNAQQEVGTCFLHFK-----	DKFRIYDEYCSNHEKAQKLLLEL-NKIRTIRTFL	147		

<u>α4 helix</u>		<u>α5 helix</u>		<u>α6 helix</u>			
p63	--EELRQQLGHRLLQNDLLIKPVQIRIMKYQLLLKDFLKYYNRAGMDTADLEQAVEVMCFV	329					
PRG	--EAESHQPCCRLLQRLDLIISEMQRITKYPLLESIIKHTEGGTSEHEKLCRARDQCREI	916					
ARHGEF39	--LQEGRPFGGLQDQLPLPLQRLQYENLVVALAENTGPNSPDHQQLTRAARLISSET	190					
PRex1	SCMLLGGRKTTDIPLEGYLLSPIQRICKYPLLLKELAKRTPGKHPDHPAVQSALQAMKTV	233					
PRex2	NCMLLGGRKNTDVPLEGYLVTPIQIRICKYPLILKELLKRTPRKHSDYAAVMEALQAMKAV	207					

<u>α6 helix</u>		<u>αN helix</u>		<u>β1 strand</u>			
p63	PKRCN---DMMTLGRLRGFEGKLTAAQK-----	LLGQDTFWVTE--PEAGGL	371				
PRG	LKYVNEAVKQ TENRHRLEGYQKRLDATALEASNPAAEFKSLDLTTRKMIHEGPLTWRI	976					
ARHGEF39	AQRVHTIGQKQKNDQHLRRVQALLSGRQA-----	KGLTSGR-WFLRQGWLLVVP	238				
PRex1	CSNINETKRQMEKLEALEQLQSHIEGWEG-----	SNLTDICTQLLLQGTLL-LKI	281				
PRex2	CSNINEAKRQMEKLEVLEEWQSHIEGWEG-----	SNITDTCTEMLMCGVL-LKI	255				

<u>β2 strand</u>		<u>β3 strand</u>		<u>β4 strand</u>		<u>β5 strand</u>	
p63	LSSRGRERRVFLFEQIIIFSEALGGG-----	VRGGTQPGYVYKNSIKVSCLGLEGN	422				
PRG	SKDKTLDLHVLLLEDLLVLLQKQDEKLLKCHSKTAVGSSDSKQTFSPVLKLNALVIRSV	1036					
ARHGEF39	PHGEPRPRMFFLFTDVLMAKPRPPLHLLRSGTFAC----	KALYPMAQCHLSRV-----	288				
PRex1	SAGNIQERAFFLFDNLLVYCKRKSRTVTSKSKSTKRTKSINGSLYIFRGRINTEVMEVENV	341					
PRex2	SSGNIQERVFFLFDNLLVYCKRKHRLKNS-----	KASTDGHRYLFRGRINTEVMEVENV	310				

<u>β6 strand</u>		<u>β7 strand</u>		<u>αC helix</u>			
p63	LQGDPCRFAITSR-----	GPEGGIQRYVLQAADPAISQAWIKHVAQILESQRD	470				
PRG	ATD-KRAFF---IICT-----	SKLGPPQIYELVALTSSDKNTWMELLEAVRNATR	1083				
ARHGEF39	-----FGHSGGPC-GG-LLSLSPHEKLLLMSTDQEELSRWYHSLTWAISSQKN	335					
PRex1	EDG-TADYHSNGYTVTNGWKIHNTAKNKFVCMAKTAEKQKWLDAIREREQRES	396					
PRex2	DDG-TADFHSSGHIVVNGWKIHNTAKNKFVCMAKTPEEKHEWFEAILKERERRKG	365					

Figure 2.2. Sequence alignment of the DH/PH structures shown in Figure 2.1. Alignments were performed using Clustal Omega and then manually adjusted based on inspection of the alignment of each structure to that of P-Rex1. Percent identities are of just the sequence regions shown and calculated by Clustal Omega. Associated secondary structure is annotated based on that of P-Rex1. Loops are left unlabeled.

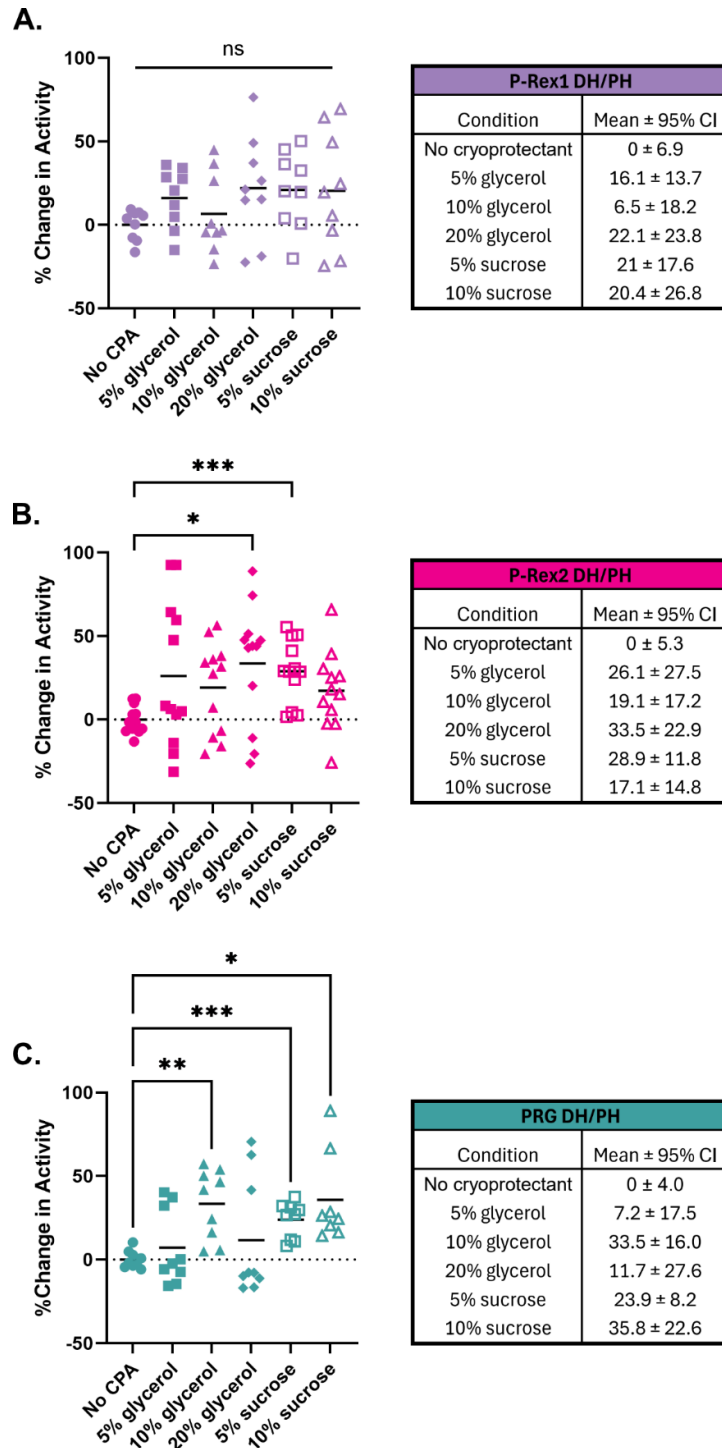


Figure 2.3. In unfrozen samples, glycerol and sucrose generally increase the variability of activity measurements of isolated RhoGEF DH/PH domain tandems. Following purification, all isolated DH/PH tandems were prepared in different cryoprotectant conditions. One hundred nM P-Rex1 DH/PH (A) and 200 nM P-Rex2 DH/PH (B) were assessed against Rac1, while 100 nM PRG DH/PH (C) was assessed against

RhoA. For each biological replicate, activity was normalized to the activity of the no cryoprotectant sample to evaluate the changes in activity solely from the addition of cryoprotectant. Statistical significance was determined using Brown-Foresythe and Welch ANOVA test with a Dunnett's T3 multiple comparisons test with individual variances computed for each comparison. ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

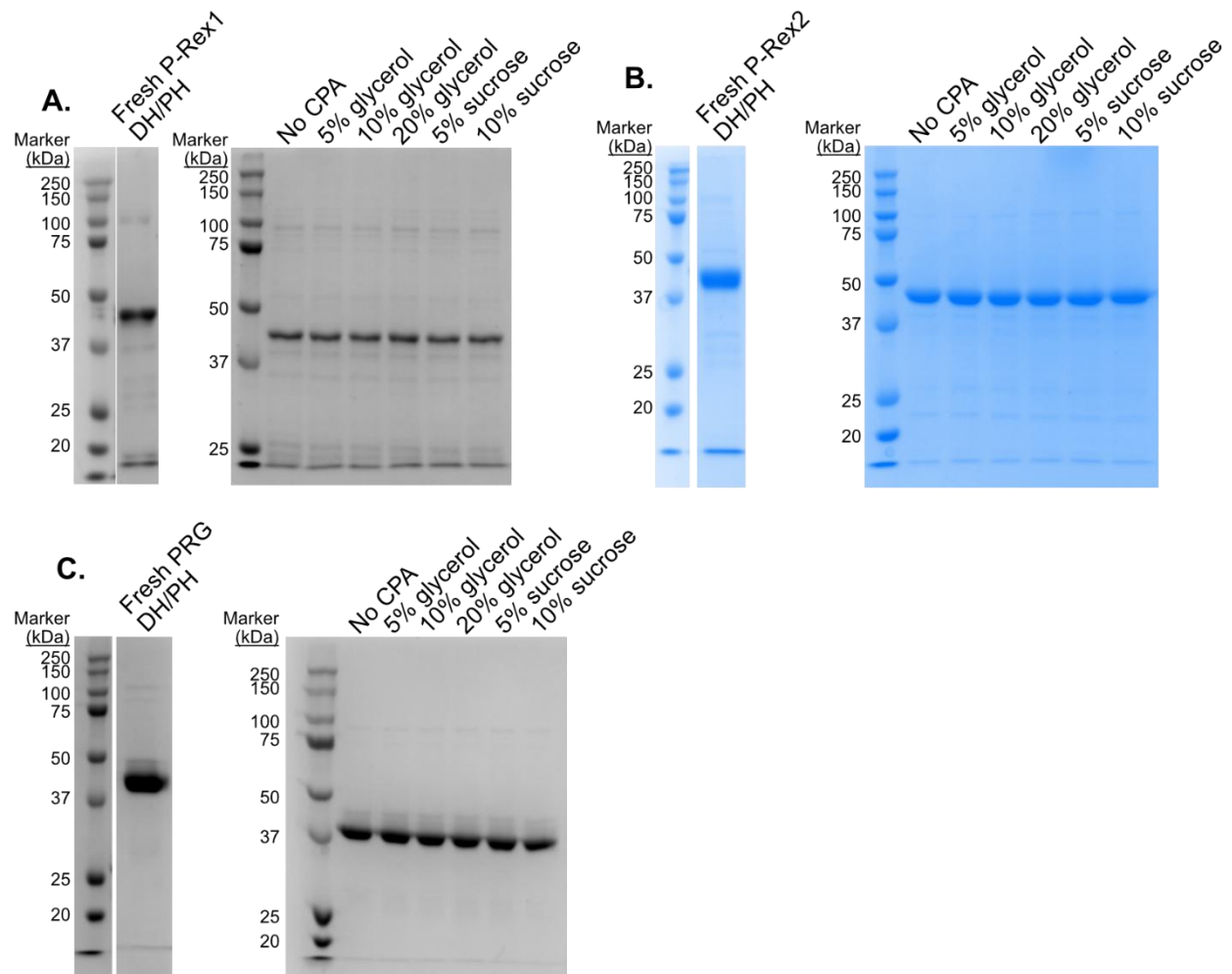


Figure 2.4. RhoGEF DH/PH protein samples run on SDS-PAGE after freezing compared to fresh. A. P-Rex1 fresh and frozen for 6 months samples. B. P-Rex2 fresh and frozen for 6 months samples. C. PRG fresh and frozen for 2 weeks samples.

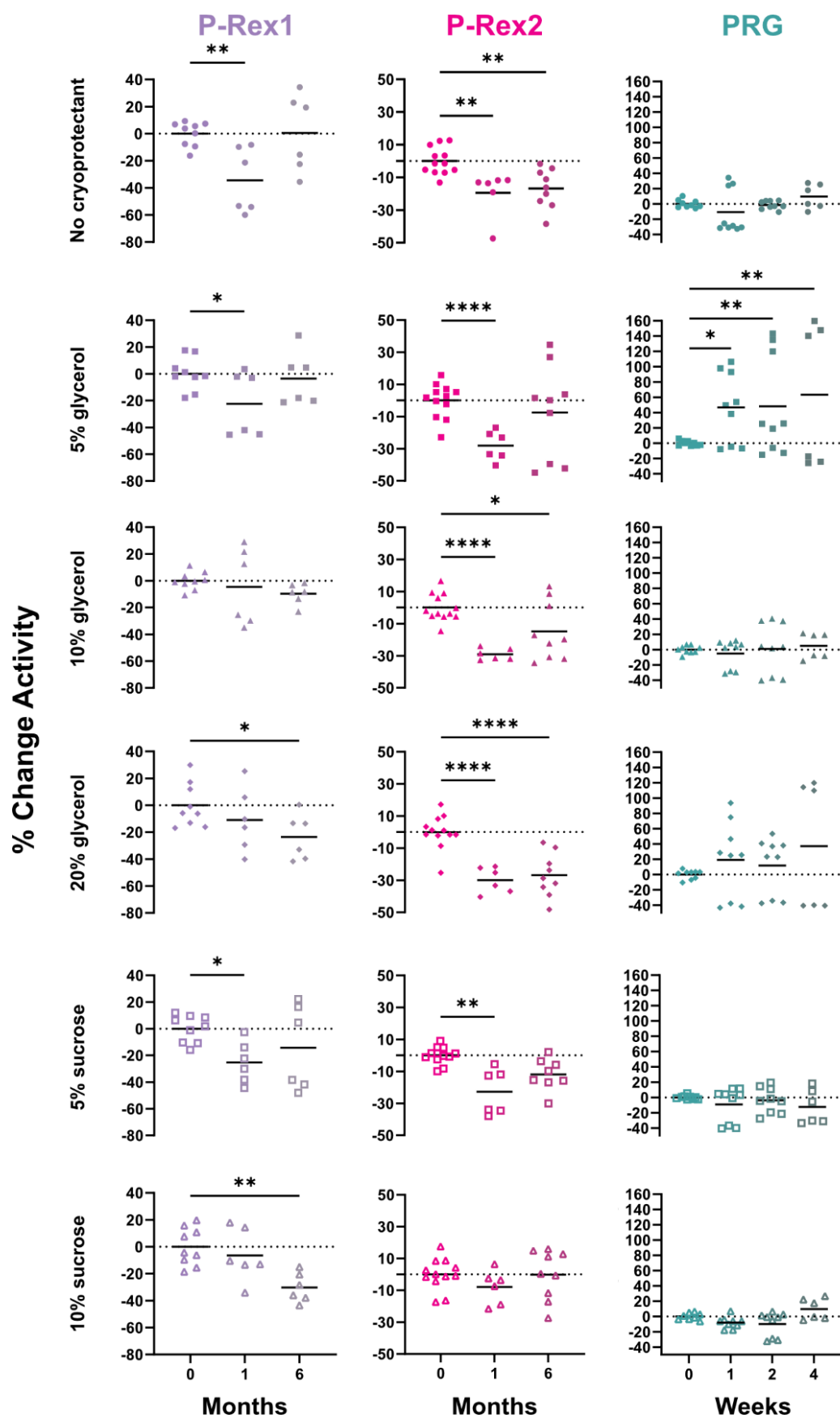


Figure 2.5. Isolated RhoGEF DH/PH activity changes over time and/or becomes less reproducible after

freezing. One hundred nM P-Rex1 and 200 nM P-Rex2 DH/PH GEF activity was assessed against Rac1 while 100 nM PRG DH/PH GEF activity was assessed against RhoA. Each protein under each CPA condition was tested over various time points after freezing. Each biological replicate was normalized to the activity of the freshly purified protein. Ninety-five percent confidence intervals (CI) of these data are shown in Fig. S4. Statistical significance was determined using a two-way ANOVA test with a post hoc Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$

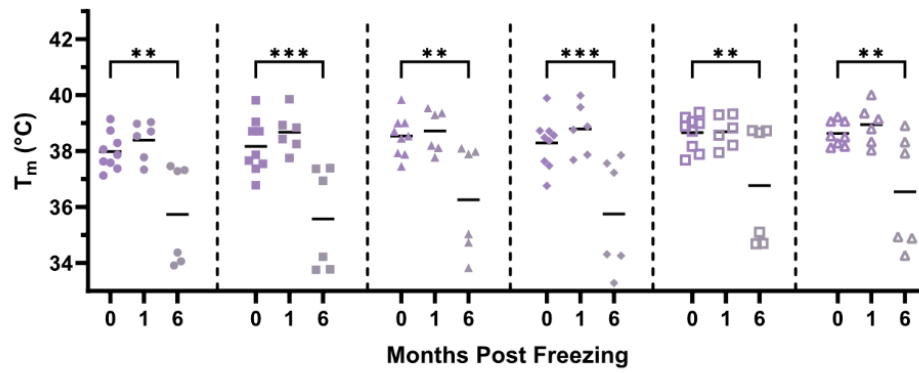
P-Rex1 DH/PH		
Condition	Time Post Freezing (Months)	Mean $\pm$ 95% CI
No cryoprotectant	0	0 $\pm$ 6.9
	1	-34.4 $\pm$ 25.1
	6	0.5 $\pm$ 30
5% glycerol	0	0 $\pm$ 9.3
	1	-22.3 $\pm$ 25.2
	6	-3.5 $\pm$ 20.8
10% glycerol	0	0 $\pm$ 5.1
	1	-4.5 $\pm$ 30
	6	-9.7 $\pm$ 8.1
20% glycerol	0	0 $\pm$ 12.5
	1	-10.8 $\pm$ 24.9
	6	-23.4 $\pm$ 17.8
5% sucrose	0	0 $\pm$ 7.7
	1	-25.2 $\pm$ 16.2
	6	-14.1 $\pm$ 33.5
10% sucrose	0	0 $\pm$ 10.6
	1	-6.4 $\pm$ 20.4
	6	-30.1 $\pm$ 11.4

P-Rex2 DH/PH		
Condition	Time Post Freezing (Months)	Mean $\pm$ 95% CI
No cryoprotectant	0	0 $\pm$ 5.3
	1	-19.4 $\pm$ 14.6
	6	-16.7 $\pm$ 9.2
5% glycerol	0	0 $\pm$ 6.7
	1	-28.1 $\pm$ 9.5
	6	-7.4 $\pm$ 22.4
10% glycerol	0	0 $\pm$ 5.4
	1	-29 $\pm$ 3.7
	6	-14.8 $\pm$ 13.8
20% glycerol	0	0 $\pm$ 6.6
	1	-29.9 $\pm$ 8.4
	6	-26.8 $\pm$ 10.4
5% sucrose	0	0 $\pm$ 3.3
	1	-22.7 $\pm$ 14.9
	6	-11.9 $\pm$ 8.2
10% sucrose	0	0 $\pm$ 6.2
	1	-7.9 $\pm$ 11.1
	6	-0.2 $\pm$ 11.9

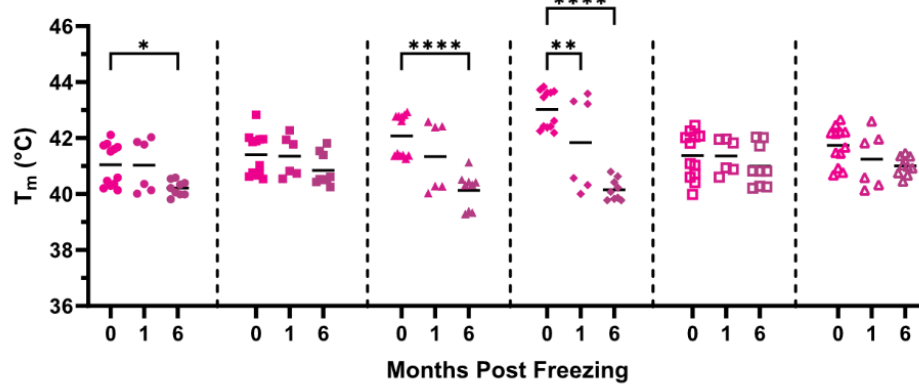
PRG DH/PH		
Condition	Time Post Freezing (Weeks)	Mean $\pm$ 95% CI
No cryoprotectant	0	0 $\pm$ 3.9
	1	-10.6 $\pm$ 22.5
	2	-1.5 $\pm$ 4.1
	4	9.5 $\pm$ 16.7
5% glycerol	0	0 $\pm$ 2.5
	1	46.6 $\pm$ 35.3
	2	48.2 $\pm$ 50.4
10% glycerol	0	0 $\pm$ 4
	1	-5.2 $\pm$ 14.2
	2	0.8 $\pm$ 25.8
20% glycerol	0	0 $\pm$ 4.5
	1	18.9 $\pm$ 38.8
	2	11.8 $\pm$ 28.5
5% sucrose	0	0 $\pm$ 1.9
	1	-9.2 $\pm$ 17.5
	2	-3.9 $\pm$ 12.7
10% sucrose	0	0 $\pm$ 3.7
	1	-8.1 $\pm$ 5.8
	2	-9.7 $\pm$ 12.2
	4	9.8 $\pm$ 14.5

Figure 2.6. Average and spread of activity data in CPA conditions over time from Figure 2.5.

P-Rex1 DH/PH



P-Rex2 DH/PH



PRG DH/PH

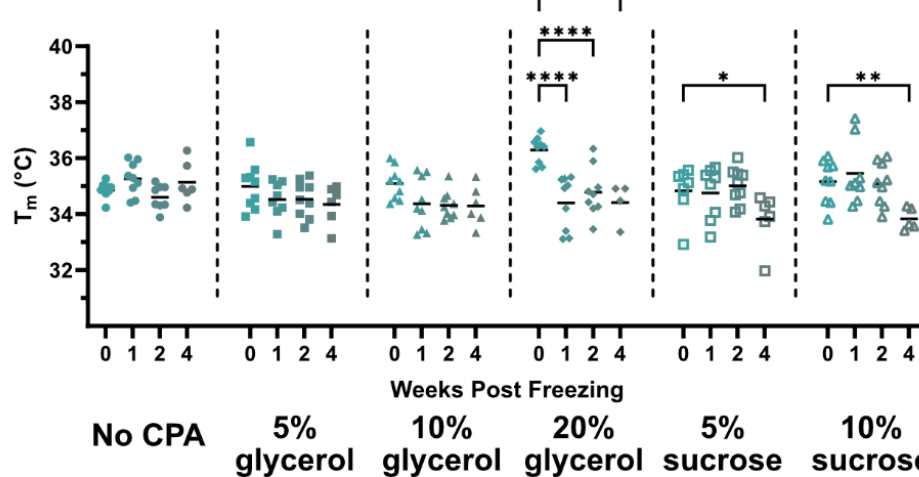


Figure 2.7. Thermostability of DH/PH tandems begins to drop at late time points. P-Rex1 DH/PH, P-Rex2 DH/PH, and PRG DH/PH thermostability was tested with differential scanning fluorimetry (DSF) across cryoprotectant conditions at various time points. Statistical significance was determined using a two-way ANOVA test with a post hoc Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$

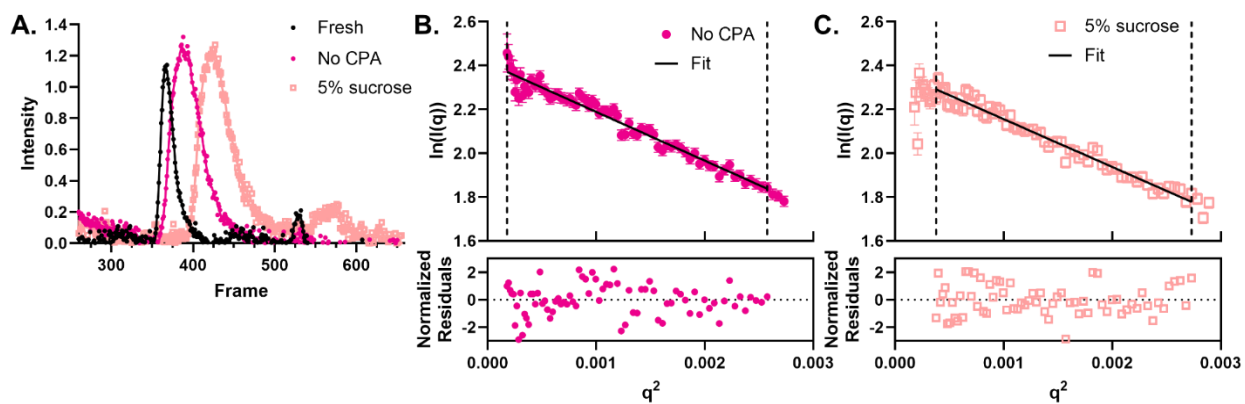


Figure 2.8. Frozen P-Rex2 DH/PH elution profiles and Guinier analyses. A. Elution profiles of frozen P-Rex2 DH/PH without CPA (magenta) and with 5% sucrose (peach) compared to a previously published freshly purified P-Rex2 DH/PH sample (SASDZV5)⁴⁷. B-C. Guinier analyses and normalized residuals of frozen P-Rex2 DH/PH samples.

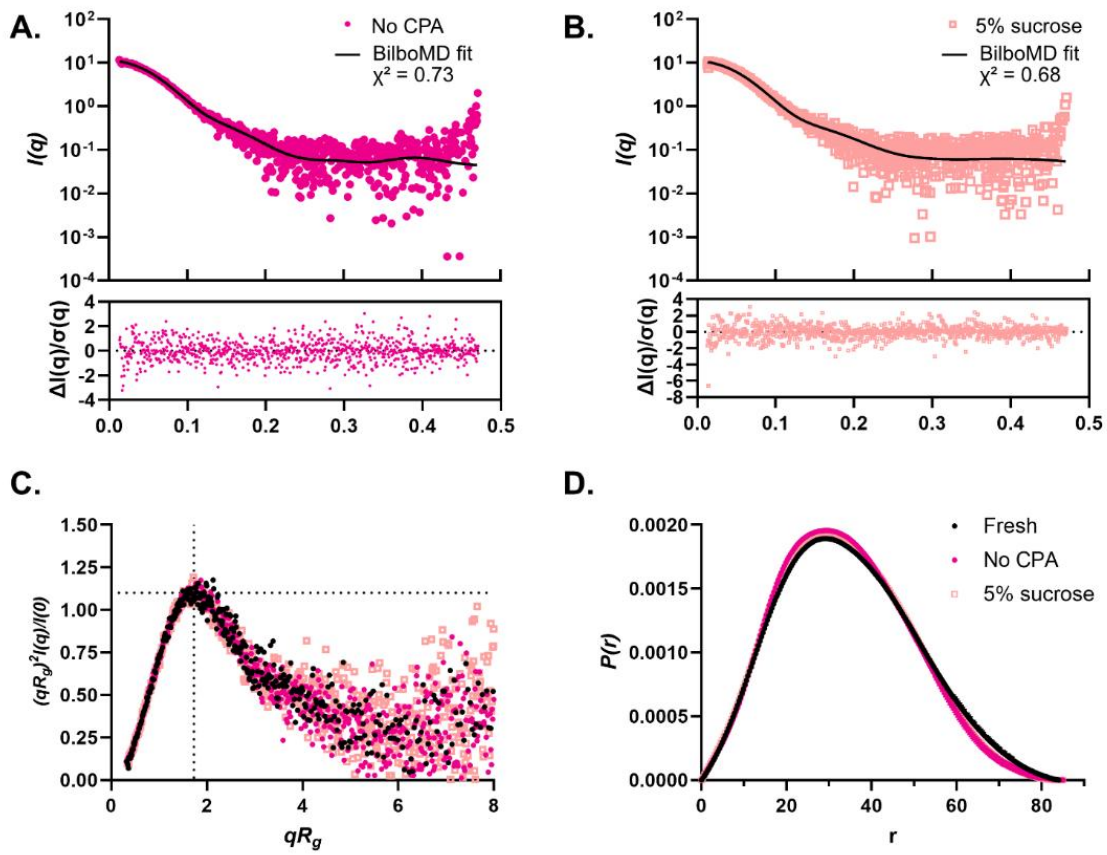


Figure 2.9. SEC-SAXS of frozen P-Rex2 DH/PH does not reveal large changes to protein conformation.

Scattering intensity plots of P-Rex2 DH/PH after freezing in (A) no CPA and (B) 5% sucrose after 6 months of storage. C. Dimensionless Kratky plot comparing freshly purified P-Rex2 DH/PH (black circles) and frozen P-Rex2 DH/PH samples with no CPA (magenta circles) or 5% sucrose (peach squares). There are no significant differences in shape between the fresh or frozen samples. D. Pair distance distribution function of freshly purified P-Rex2 DH/PH and samples frozen without CPA or 5% sucrose.

Chapter 3. The Rho guanine-nucleotide exchange factor P-Rex2 exhibits unique structural features and regulatory mechanisms distinct from the closely related RhoGEF P-Rex1

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This work contains contributions from Rohan Marde, Grace Muma, Veda Nayak, Chi Phan, Sheng Li, and Jennifer Cash.

Abstract

Rho guanine-nucleotide exchange factors (RhoGEFs) activate small GTPases to drive cytoskeletal rearrangement, cell motility, and proliferation. The phosphatidylinositol-3,4,5-trisphosphate (PIP₃)-dependent Rac exchanger (P-Rex) subfamily of RhoGEFs includes P-Rex1 and P-Rex2 which, when misregulated, contribute to cancer progression and metastasis. P-Rex activity is controlled by accessory domains that maintain the protein in a cytosolic, autoinhibited state until activated by the lipid PIP₃ and G protein $\beta\gamma$ subunits. While P-Rex1 autoinhibition has been structurally and biochemically characterized, P-Rex2 has remained largely unexplored. Furthermore, despite high sequence similarity and domain conservation, P-Rex homologs differ in substrate specificity and regulatory interactions, and the molecular basis for these divergences is unknown. Here, we have taken an integrative structural biology approach to investigate these gaps. Using cryo-EM, we determined the first structure of full-length P-Rex2 to moderate resolution, revealing that, while the overall structure closely resembles that of P-Rex1, there is a substantial repositioning of the N-terminal module relative to the C-terminal core. This may play a key role in precluding the intramolecular interactions between the N- and C-terminal domains that are observed in autoinhibited P-Rex1. Hydrogen-deuterium exchange mass spectrometry revealed that, unlike P-Rex1, P-Rex2 dynamics are unaffected by IP₄, the headgroup of PIP₃. SEC-SAXS data support that the N-terminal module itself is less dynamic, and biochemical assays show that P-Rex2 may be differently regulated by autoinhibition, likely through a mechanism divergent from P-Rex1. These findings uncover unique features in the molecular mechanisms of P-Rex2 regulation.

Introduction

Rho guanine-nucleotide exchange factors (RhoGEFs) accelerate the exchange of GDP for GTP on small Rho GTPases like Rac and Cdc42 to direct actin cytoskeleton rearrangement^{4,37}. This activity allows them to drive cell migration and invasion which, when misregulated, can contribute to various diseases including cancer, resulting in metastasis⁷. Members of the Dbl family of RhoGEFs, which contains over 70 members, share a conserved Dbl homology (DH) catalytic domain followed by a pleckstrin homology (PH) domain that together form the catalytic core¹⁰. Although the DH/PH tandem is well characterized structurally^{16,53,54}, the variety of accessory domains that accompany the catalytic core across and within different RhoGEFs introduces complicated, and comparatively vastly understudied, regulatory controls on GEF activity. These include regulation through autoinhibition, membrane localization, and protein-protein interactions^{23,37}. As a result, the complex molecular mechanisms governing the activity of each of these multidomain proteins are poorly understood.

The phosphatidylinositol (3,4,5)-trisphosphate (PIP₃)-dependent Rac exchanger (P-Rex) subfamily of Dbl RhoGEFs includes two members, P-Rex1 and P-Rex2. P-Rex1 is abundantly expressed in leukocytes and neurons whereas P-Rex2 is strongly expressed in brain, lung and liver cells^{55–58}. *PREX2* additionally gives rise to a splice variant, P-Rex2b, which is mainly expressed in heart tissue and retains all identifiable domains except the IP4P domain which is truncated and ends with a short alternative sequence⁵⁹. Misregulation of P-Rex family proteins is associated with varied pathologies. While P-Rex1-driven diseases, like prostate and breast cancers, are linked to overexpression^{60–62}, P-Rex2-driven diseases are propelled by mutation. For example, more than 23% of human hepatocellular carcinoma and 38% of squamous cell carcinoma samples harbor non-silent somatic mutations in P-Rex2^{63,64}. Additionally, mutations in P-Rex2 can lead to metastatic melanoma⁶⁵ and pancreatic cancers⁶⁶ or disrupt

PI3K signaling pathways to drive diabetes and insulin resistance⁶⁷. In contrast, single and double knock-out of *PREX1* and *PREX2* genes in mouse models produces healthy, viable mice with very mild neutrophilia and a characteristic pigmentation phenotype^{58,68}. Collectively, these data suggest that P-Rex is an excellent therapeutic target. Thus, characterizing the molecular mechanisms involved in P-Rex1 and P-Rex2 regulation and defining the differences between the two are important for guiding efforts to rationally design isoform-specific P-Rex modulators, both for use as tools in understanding RhoGEF regulation and for potential treatment of diseases.

P-Rex1 and P-Rex2 show 56% sequence identity and 68% sequence similarity overall, and both proteins share a conserved domain architecture consisting of an N-terminal DH/PH tandem followed by two Dishevelled, Egl-10 and Pleckstrin (DEP) domains, two Postsynaptic density protein 95, Discs large protein, and Zonula occludens-1 (PDZ) domains, and a C-terminal inositol polyphosphate-4-phosphatase-like (IP4P) domain (Fig. 3.1A). The P-Rex1 N-terminus forms a multi-domain module composed of the DH, PH, and DEP1 domains which is flexibly tethered to the rest of the protein (Fig. 1B). The DH domain, which engages GTPases directly, is highly conserved as are the other domains within the N-terminal module (Fig. 3.1A). P-Rex1 also contains a C-terminal “core” composed of the tightly associated DEP2, PDZ1, PDZ2, and IP4P domains which exhibit the highest sequence divergence between the two proteins (Fig. 3.1A&B). Furthermore, a flexibly tethered subdomain of ~250 residues extends out from the IP4P domain and forms a 4-helix bundle (4HB).

Both enzymes are maintained in a cytosolic, autoinhibited state with limited basal activity until they are recruited to the cell membrane and synergistically activated by the lipid PIP₃ and the Gβγ subunits of heterotrimeric G proteins^{55,59,69}. Structural studies of P-Rex1 have begun to clarify how accessory domains facilitate autoinhibition and how Gβγ and PIP₃ relieve this^{15,16,18–20,70,71}. A two-pronged autoinhibitory mechanism is mediated by interdomain contacts that restrict the DH domain from interacting with GTPases^{15,20}. One interaction is formed by the DEP1 domain docking against the

DH domain through a hydrophobic pocket of residues to stabilize a compact, low activity state¹⁹ (Fig. 3.1B). A second interaction is mediated through the PH domain engaging the 4HB of the IP4P^{15,20}. PIP₃ binding to the PH domain disrupts the PH–IP4P contact— an effect that the soluble headgroup of PIP₃, IP₄, does not reproduce²⁰. In fact, IP₄ actually stabilizes the PH–IP4P autoinhibitory conformation. Gβγ, which binds to the surface formed by the DEP2, PDZ1, PDZ2 and IP4P domains, likely contributes to activation by promoting membrane localization, but this has not been conclusively determined¹⁸. Together, PIP₃ and Gβγ bind to synergistically activate P-Rex1 and fully relieve autoinhibition. Despite the wealth of structural information available on P-Rex1^{15–20}, there is only a single reported structure of a piece of P-Rex2: the isolated PH domain⁷².

While the close sequence similarity and shared domain architecture suggest that P-Rex2 adopts a structure similar to P-Rex1, there are notable differences between the two in substrate specificity and regulation^{23,73}. For example, P-Rex1 can activate all Rac-like and some Cdc42-like GTPases *in vitro*, but only Rac-like GTPase activation has been observed *in vivo*^{53,55,73,74}. On the other hand, P-Rex2 specificity *in vitro* seems to be limited to Rac1^{69,75}. Additionally, the tumor suppressor protein phosphatase and tensin homolog (PTEN) has been shown to form a co-inhibitory interaction with P-Rex2, but not P-Rex1^{24,25,56}. Furthermore, P-Rex1 contains a membrane localization element in the β3/β4 loop of its PH domain that is missing in P-Rex2. When this element is swapped into the corresponding loop of P-Rex2, it enhances membrane localization of P-Rex2¹⁶. Collectively, this evidence suggests that there may be differences in the molecular features of P-Rex1 and P-Rex2 that lead to these unique properties. In support of this, cross-linking mass spectrometry data on P-Rex2²⁵ do not entirely correlate with the autoinhibited structure of P-Rex1, as P-Rex1 homologs of some cross-linked P-Rex2 residues are too far apart— much beyond the length of the crosslinker utilized (Fig. 3.2A). These data point to potential structural differences between the two proteins that have not yet been described.

Here, we investigated the structure and autoinhibitory mechanisms of P-Rex2 using an integrative structural biology approach. We determined a moderate-resolution cryo-electron microscopy (cryo-EM) structure of full-length P-Rex2 which showed that, although the N-terminal module and C-terminal core each individually exhibit a similar domain organization as compared to P-Rex1, the N-terminal module itself is positioned proximally to the core, with the DEP1 domain contacting the base of the IP4P domain. Furthermore, hydrogen-deuterium exchange mass spectrometry (HDX-MS) experiments show that, unlike P-Rex1, binding of IP₄ has no effect on the overall conformation of P-Rex2. Size exclusion chromatography coupled with small-angle X-ray scattering (SEC-SAXS) shows that although DEP1 promotes a compact overall architecture of the N-terminal module, it does not change the flexibility of the DH/PH tandem as previously described in P-Rex1. Despite these differences, mutagenesis and biochemical assays demonstrate that the DEP1 domain remains essential for autoinhibition by engaging the DH domain similarly to P-Rex1. This work reveals unique structural features that distinguish P-Rex2 from P-Rex1, providing insights into key molecular details that may confer regulatory differences between the two.

Results

The overall structure of P-Rex2 resembles that of P-Rex1 with differences in the positioning of the N-terminal module

To begin to understand the molecular differences in P-Rex2 as compared to P-Rex1, we determined the structure of full-length (FL) P-Rex2 using cryo-electron microscopy (cryo-EM) single particle analysis (Fig. 3.1C, 3.3-3.6, & Table 3.1). Similar to P-Rex1, P-Rex2 exhibits a strong preferred orientation on grids (Fig. 3.3C & 3.5), necessitating the collection of supplemental data at a 35° tilt to provide additional views of the particle. Still, this was not enough to completely overcome the preferred orientation problem, and this coupled with inherent flexibility within P-Rex2 led to anisotropy within our

maps. The overall domain organization of P-Rex2 resembles that of P-Rex1, composed of an N-terminal catalytic module (DH/PH-DEP1) and a C-terminal regulatory core (DEP2-IP4P) (Fig. 3.1C). Early-stage data processing led to a reconstruction of the whole particle with a global resolution of 3.4 Å according to cryoSPARC. This map was used to dock in starting models generated by AlphaFold (Fig. 3.4A & 3.5A). However, resolution of the N-terminal module was very low in comparison to the C-terminal core of the protein, in part due to flexibility of the N-terminus relative to the core. In subsequent data processing pipelines, we focused on generating maps of the C-terminal core and the N-terminal module independent of each other in attempts to achieve more interpretable maps. This led to two final maps: one in which the core was resolved to higher resolution overall, exhibiting a global resolution of 2.9 Å according to cryoSPARC (Fig. 3.4B & 3.5B), and one where the N-terminal module was more interpretable with a global resolution of 4.4 Å (Fig. 3.5C). However, these resolutions are overestimates, as the gold standard FSC curves have gradual slopes with rebounding peaks around 5 Å due to both the flexibility of P-Rex2 and its orientation bias (Fig. 3.5). Based on inspection of the maps, estimates from 3DFSC⁷⁶ are likely more accurate with the resolution of the core and N-terminal module at 3.6 Å and 6.8 Å, respectively (Fig. 3.5B&C).

Like in autoinhibited P-Rex1, the P-Rex2 DH domain is connected to the PH domain through a bent helix, while the PH domain is connected to the DEP1 domain through a more linear, continuous helix, and the DEP1 domains docks onto the end of the DH helical bundle (Fig. 3.1C, 3.6B-D, & 3.7). The C-terminal core of P-Rex2 is composed of the second DEP domain, both PDZ domains and the IP4P domain where the IP4P domain is the central component and is decorated with the other accessory domains at peripheral ends. A β -strand connects DEP2 to PDZ1 and is part of a large β -sheet that twists through the center of the IP4P domain from one side to the opposite. The domain organization within each the N-terminal module and C-terminal core is conserved between P-Rex1 and P-Rex2, exhibiting an

RMSD C α of 1.4 Å each (Fig. 3.7). However, the P-Rex2 IP4P subdomain, observed in the autoinhibited P-Rex1 structures, is not resolved in any of our 2D class averages or 3D reconstructions.

Despite the overall structural similarity in P-Rex1 and P-Rex2, there is a significant shift in the N-terminal module relative to the core such that the P-Rex2 DEP1 domain is positioned proximal to the α H helix at the base of the core rather than distally to the side (Fig. 3.7B & C). To quantify this relative shift, the α H helices were aligned (RMSD C α of 0.32 Å), and the centers of mass (COMs) of each N-terminal domain were used to measure the distances between homologous domain pairs in the N-terminus. Comparing P-Rex1 and P-Rex2, the DH, PH, and DEP1 domains are shifted by 19 Å, 30 Å, and 22 Å, respectively (Fig. 3.7B). The P-Rex2 DEP1 domain COM is ~16 Å closer to the base of the α H helix than the P-Rex1 DEP1 COM (Fig. 3.7C). Additionally, the angle of the DEP1 COM from the α H helix decreases from 115° in P-Rex1 to 93° in P-Rex2, resulting in an overall more compact structure of P-Rex2 (Fig. 3.7C). Given this substantial relative shift in the N-terminal module, we mapped the existing P-Rex2 cross-linking mass spectrometry data²⁵ onto the P-Rex2 structure to assess how well the data corroborated the N-terminal module position (Fig. 3.2). Distances between residues in the N-terminal module cross-linked to residues in the core are mostly within reason, based on the length of the cross-linker used, especially in comparison to the distances between homologous residues in P-Rex1 (Fig. 3.2). However, there are some exceptions. For example, K337 and K364 in the PH domain are too far away from K1442 in the IP4P domain. These cross-links also occur at low frequency in comparison to the others, which could suggest that these represent non-specific intermolecular interactions between different P-Rex2 molecules. Alternatively, given its flexible nature, these cross-links may represent transient or low-population conformations of P-Rex2 that are not represented in our final cryo-EM maps.

Structural differences in the proposed phosphatase active site and G β γ -binding elements

When the structure of the P-Rex1 C-terminal core was first determined in complex with Gβγ, it was discovered that the IP4P domain is structurally very similar to *Legionella* bacterial phosphoinositide 3-phosphatases¹⁸. Based on homology, the catalytic triad in P-Rex1 was proposed to be formed by Cys1583, Arg1589, and Asp1638 (Fig. 3.6F), although, to date, no substrates have been identified to confirm P-Rex1 phosphatase activity. P-Rex2 also shares this phosphatase fold; however, there are notable differences in the homologous residues that would form the catalytic triad. Although Cys1529 and Arg1535 are structurally conserved, Asp1584 is pointed away from the other triad residues, albeit map density in this area is weaker than in the rest of the “active site.” Intriguingly, Cys1529 seems to form a disulfide bond with Cys1536 even though the P-Rex2 cryo-EM sample was kept under reducing conditions at all times (Fig. 3.6E&G). The corresponding residue in P-Rex1 is Ser1590, which would not be able to form a disulfide bond (Fig. 3.6F). Although phosphatase activity has not yet been evaluated for P-Rex2, these differences would likely preclude any activity at this site.

On P-Rex1, Gbg binds the “top” of the C-terminal core, burying residues on the IP4P domain and both PDZ domains. Preceding the loop containing Asp1638 is a helix that forms part of this site and is important for engaging Gβγ²⁶ (Fig. 3.6F). In P-Rex2, the C-terminus of this helix and the beginning of the loop on which Asp1584 resides are disordered, and the remainder of the loop is structurally different from that of P-Rex1 (Fig. 3.6G). Additionally, parts of P-Rex1 PDZ1 and PDZ2 that are buried by Gbg are disordered in P-Rex2. Because these structural elements collectively are components of the Gβγ-binding site, these divergences could have implications in P-Rex2 interacting differently with Gβγ as compared to P-Rex1. However, Gbg binding to P-Rex2 has not yet been structurally characterized or quantitatively measured to determine if these structural differences lead to differences in binding affinity.

IP₄ binding does not enable resolution of the P-Rex2 IP4P subdomain

In P-Rex1, the IP4P 4HB subdomain extends from the C-terminal core and makes contacts with the N-terminal module, promoting autoinhibition^{15,20}. This closed, autoinhibited conformation is stabilized by binding of inositol-(1,3,4,5)-tetrakisphosphate (IP₄), the soluble head group of the lipid PIP₃, to the PH domain²⁰. Addition of IP₄ to the P-Rex1 cryo-EM sample facilitated resolution of both the 4HB and the N-terminal module by stabilizing the contact between the two, as these elements appear to be otherwise flexibly tethered to the core¹⁸. While the N-terminal module was clearly visualized in our P-Rex2 cryo-EM data, the IP4P subdomain was never resolved in any 2D class averages or 3D reconstructions despite extensive particle classification aimed at isolating subsets of particles where the subdomain might be observed (Fig. 3.3C & 3.4). The subdomain (~29 kD) was confirmed to be intact in the P-Rex2 protein sample via molecular weight estimation by SDS-PAGE (Fig. 3.3A). Seeking to resolve the P-Rex2 IP4P subdomain, we added 4-fold molecular excess of IP₄ to the P-Rex2 cryo-EM sample and collected a small dataset. However, this did not alter the resulting 2D class averages or 3D reconstructions, and we were still unable to resolve the subdomain (Fig. 3.8). This suggests that IP₄ binding does not affect the conformation of P-Rex2 as it does P-Rex1.

Binding of IP₄ has no effect on the tertiary structure of P-Rex2

To further examine the lack of effect of IP₄ on visualization of the IP4P subdomain, we conducted hydrogen-deuterium exchange mass spectrometry (HDX-MS) on P-Rex2 in the presence and absence of IP₄. HDX-MS measures deuterium uptake into a sample of protein on a per peptide basis, allowing assessment of the structure and dynamics of the protein. By comparing samples of P-Rex2 with and without IP₄, we can assess how its structure changes upon IP₄ binding. Like in our cryo-EM experiments, we used a 4-fold molar excess of IP₄ relative to P-Rex2. In P-Rex2, IP₄ binding protects the β 1/ β 2 and β 6/ β 7 loops as well as the β 6 strand from deuterium uptake through direct binding to the PIP₃-binding site (Fig. 3.9A & B). Similarly, IP₄ binding to P-Rex1 strongly decreases deuterium uptake in these structural elements (Fig. 3.9C; reanalyzed data from Ravala et al., 2024). However, it also

decreases exchange in the P-Rex1 PH $\beta 5/\beta 6$ loop and IP4P 4HB₁ and 4HB₂ helices by stabilizing the interaction between the PH domain and the 4HB²⁰ (Fig. 3.9C & E). Interestingly, the P-Rex2 PH $\beta 5/\beta 6$ loop also exhibits a strong decrease in deuterium uptake, yet P-Rex2 lacks any significant IP₄-dependent protection within the IP4P subdomain or any other regions of the protein (Fig. 3.9A-B & D). This suggests either that IP₄ has no effect on contacts between the N-terminal module and IP4P subdomain or that these interactions do not occur. In comparing the HDX-MS profiles of the IP4P subdomain of P-Rex2 and the P-Rex1 4HB in the absence of IP₄, the deuterium incorporation dynamics are very similar (Fig. 3.10)²⁰. This implies that these two regions may be structurally related to one another, although this has yet to be definitively determined.

P-Rex1 is more active than P-Rex2 against Rac1

The distinct structural organization of P-Rex2 prompted us to investigate how its basal GEF activity might be different from that of P-Rex1. Utilizing a guanine-nucleotide exchange assay that measures GEF activity through loading soluble Rac1 with a fluorescently-tagged, nonhydrolyzable mant-GTP, we compared the activities of P-Rex2 and P-Rex1 constructs. Both P-Rex1 and P-Rex2 exhibit concentration-dependent activity levels where, at higher concentrations, P-Rex1 activity on Rac1 exceeds that of P-Rex2. At 500 nM GEF, we observe a ~1.7-fold difference in activity between FL P-Rex1 and P-Rex2 (Fig. 3.11A). To tease out whether higher GEF activity arises from differences in specificity to Rac1 or autoinhibition from domains outside of the catalytic core, we purified the isolated P-Rex DH/PH tandems and analyzed the activity of each (Fig. 3.11B & 3.12A). Again, P-Rex2 DH/PH displayed notably lower activity than the P-Rex1 DH/PH fragment with the greatest difference in calculated k_{obs} at a single concentration being ~3-fold, showing that there is a difference in intrinsic activity of each for Rac1. Compared to the FL P-Rex constructs, the isolated DH/PH tandems of P-Rex1 and P-Rex2 showed ~10- and 6-fold increases in activity, respectively, highlighting that the domains outside of DH/PH do exert autoinhibition against the catalytic tandem. Together, these observations indicate that both specificity

for Rac1 and the autoinhibitory controls imposed by the accessory domains in the full-length protein likely play a role in measured differences in P-Rex1 and P-Rex2 activities. Because the fold differences in activities of the DH/PH and FL proteins are ~3 versus only ~1.7, respectively, it appears that P-Rex1 might be more tightly regulated by autoinhibition. To pick apart these two effects more thoroughly, we attempted to compare activities of these constructs against Rac2. However, although FL P-Rex1 showed about the same activity against Rac2 as Rac1, FL P-Rex2 showed almost no basal activity against Rac2 in our assay (data not shown). Therefore, although there are likely differences in basal activity and autoinhibition in P-Rex proteins with P-Rex1 possibly being more tightly regulated, divergences in GTPase specificity between the two make these differences difficult to interpret.

The P-Rex2 DH/PH takes on a single, compact conformational state independent of DEP1

Previous size exclusion chromatography coupled with small-angle X-ray scattering (SEC-SAXS) experiments showed that the P-Rex1 DH/PH exists in two conformational states— the extended and compact form— whereas the DH/PH-DEP1 construct favors a single, compact conformation¹⁹. To determine if the P-Rex2 DEP1 domain also induces compaction of the catalytic core, we performed SEC-SAXS analysis on purified P-Rex2 DH/PH and DH/PH-DEP1 constructs (Fig. 3.12A). The SAXS profiles of both P-Rex2 constructs were consistent with monodisperse, well-folded particles (Fig. 3.13A & B, Table 3.2). Dimensionless Kratky analysis confirmed that both P-Rex2 DH/PH and DH/PH-DEP1 are compact and folded, exhibiting nearly identical profiles with well-defined peaks characteristic of globular proteins (Fig. 3.13C). Guinier analysis yielded correlation volumes corresponding to molecular weights of 41.6 and 57.1 kDa, respectively, within reasonable agreement with the expected molecular weights (44 and 51 kDa) (Fig. 3.12B & C). The pair distance distribution functions, $P(r)$, indicated D_{max} values of 84 Å for P-Rex2 DH/PH and 105 Å for P-Rex2 DH/PH-DEP1 which suggests that the maximum particle dimension increases between the two constructs reasonably with the increase in mass (Fig. 3.13D). This is not the case in P-Rex1 where the maximum particle dimension of P-Rex1 DH/PH, ~105 Å, is roughly the same as

the DH/PH-DEP1, 104 Å¹⁹. Together, this suggests that the P-Rex2 DH/PH may not take on an extended state like the P-Rex1 DH/PH and that it favors compact states regardless of the presence of DEP1. To directly compare the data from P-Rex1 and P-Rex2, we analyzed the published P-Rex1 SAXS datasets (SASDHY9 and SASDHW9) and our P-Rex2 datasets using BilboMD⁷⁷ rather than the previously employed ensemble optimization method (EOM)⁷⁸. Using the AlphaFold server to generate an initial model of each construct and BilboMD to produce ensemble models which represent highly populated conformations in solution, we identified that both P-Rex2 constructs were best fit by a single-state model (Fig. 3.13A & B). However, when we performed the BilboMD analysis on the P-Rex1 data (Fig. 3.14), BilboMD did not reproduce clear indication of two discrete conformations of the P-Rex1 DH/PH, as previously shown with EOM analysis¹⁹, and only suggested a more elongated state corresponding to a larger apparent molecular weight than would be expected for DH/PH. In all, these data suggest that both P-Rex2 DH/PH and DH/PH-DEP1 exist predominantly in compact, folded conformations in solution, indicating potentially reduced conformational flexibility as compared to P-Rex1.

The P-Rex2 DEP1 domain plays a role in maintaining autoinhibition

Previously, the DEP1 domain was shown to play an important role in maintaining P-Rex1 autoinhibition through contacts with the DH domain^{15,20}. While P-Rex2 is structurally highly conserved within the N-terminal module, SEC-SAXS data alluded to potential differences in flexibility and conformation within the isolated DH/PH domain. This led us to investigate the role of DEP1 in P-Rex2 autoinhibition. We first compared the activities of P-Rex2 DH/PH, DH/PH-DEP1, and FL constructs using the guanine-nucleotide exchange assay with soluble Rac1 (Fig. 3.15A). As expected, P-Rex2 FL exhibited the lowest activity. The DH/PH-DEP1 construct exhibited intermediate activity between the full-length and DH/PH constructs, consistent with the DEP1 domain being involved in autoinhibition. Next, we introduced single point mutations in the DH/PH-DEP1 construct at the DH-DEP1 interface. Because the resolution of the N-terminal module in the EM structure was not high enough to resolve sidechains, we

focused on homologous residues that are important for maintaining P-Rex1 autoinhibition²⁰ and compared the activities of these variants to those of wild-type DH/PH-DEP1 and DH/PH (Fig. 3.15B-C & 3.16). Variants L151A, L152A, L434A, and L434F exhibited moderately increased activity, whereas I378A, I378F, and E424K showed the highest increases in activity. These data support that the hydrophobic pocket formed by Leu151, Leu152, Ile378, and Leu434 as well as a potential ionic contact at Glu424 collectively contribute to stabilization of the interface between the DH and DEP1 domains to promote autoinhibition (Fig. 3.15C). The K60A and K63A variants were generated due to the proximity of these residues to Glu424 in our predicted model before we obtained the cryo-EM structure of P-Rex2; however, we were unable to build these in our experimental model. The K60A variant did not express, and the L434 variants consistently exhibited low expression, which may reflect their importance in the stability of the N-terminal module (Fig. 3.16).

Discussion

Here, we presented the first structural characterization of autoinhibited P-Rex2 and revealed how its domain organization and regulatory features diverge from its close family member, P-Rex1 (Fig. 3.17). Our cryo-EM structure shows that while both the N- and C-termini are each individually structurally well-conserved between P-Rex1 and P-Rex2, the positions of these relative to one another are strikingly different between the two proteins. This major architectural dissimilarity may have significant consequences for how autoinhibition is achieved, pointing to potentially unique mechanisms being utilized in each isoform.

In P-Rex2, both the helix that spans the PH to DEP1 domains and the DEP1 domain itself are proximal to the base of the IP4P core. Docking of the N-terminal module to the core likely enables its resolution in our cryo-EM structure by tethering the otherwise likely flexible N-terminus. However, it is difficult to pinpoint the underlying cause of this structural difference between P-Rex proteins. The

surface of the P-Rex2 N-terminal module that interacts with the core is very similar to that of P-Rex1, suggesting that this is not the source of the divergence. One hypothesis is that the connection between the first and second DEP domains in P-Rex2 is more rigid and inflexible than that in P-Rex1, as this linker is composed of bulkier, less flexible residues in P-Rex2 (TFYPRNE) compared to P-Rex1 (TYKARSE). This is supported by clear map density representing the P-Rex2 DEP1-DEP2 connection, although it is not high enough resolution for us to be able to confidently model into it. Alternatively, the unique structural features at the base of the P-Rex2 core could promote the docking of the N-terminal module. In P-Rex1, the IP4P α H and α I helices are moderately well-defined and are connected by a disordered loop of ~14 residues (Fig. 3.18A). In P-Rex2, the homologous residues are much more well-resolved, forming a helical structure that packs very closely to the α H helix (Fig. 3.18B & C). Although the functional consequences of these structural features remain unclear, elucidating whether these elements contribute to autoinhibition could provide insight into regulatory surfaces that could potentially be exploited for P-Rex-specific inhibition.

Contrary to the P-Rex1 autoinhibitory mechanism, P-Rex2 does not exhibit a PH-IP4P subdomain interaction, with or without IP₄. IP₄ is an abundant negative regulator of neutrophil activation⁴⁰ as it can compete with PIP₃ to bind to PH domains, thereby blocking signaling downstream of PIP₃⁴¹. P-Rex1, which is strongly expressed in neutrophils⁵⁵, is likely inhibited in these cells through both this mechanism as well as IP₄ stabilization of the P-Rex1 autoinhibited conformation. P-Rex2, however, is absent from neutrophils and other immune cells. Instead, P-Rex2 is expressed in Purkinje neurons⁵⁸ and liver, lung, kidney and pancreas cells⁴² which utilize IP₄ in a slightly different manner. In rat hepatocytes, for example, as little as 0.5 μ M I(1,3,4,5)P₄ extends the duration of I(1,4,5)P₃-dependent Ca²⁺ signaling⁴³. While the concentration of IP₄ in these cells is unknown as it is quickly degraded in liver homogenate⁴⁴, given the sensitivity of the cells to small amounts of IP₄, the concentration of IP₄ in hepatocytes is likely much less than that in neutrophils (4 μ M)⁴⁵. This suggests

that the differential regulation of P-Rex1 and P-Rex2 by IP₄ may be an evolutionary biological adaptation to the specific cell types each protein is found in and the role IP₄ plays in that cell.

While our HDX-MS data suggest that the P-Rex2 IP4P subdomain may take on a similar tertiary structure as P-Rex1, the position of the N-terminal module makes it unlikely that the IP4P subdomain would be able to make the same contacts with the PH domain, as the P-Rex2 PH domain is positioned farther away from the C-terminal core. Although our present data do not support a clear role for the P-Rex2 IP4P domain in autoinhibition, it may help contextualize data pointing to a role in PTEN-induced inhibition. Cross-linking mass spectrometry of P-Rex2²⁵ shows limited intramolecular contacts between the IP4P subdomain and the N-terminal module. However, upon PTEN binding, there is a substantial increase in the number of cross links between the IP4P subdomain and the DH and PH domains. This raises the possibility that PTEN does not primarily inhibit P-Rex2 through direct engagement with the N-terminus as previously suggested⁵⁶, but instead binds to the PDZ2 and IP4P subdomain²⁵ to strengthen long-range interdomain interactions between the IP4P and DH/PH. In this context, it is notable that the pancreatic cancer-associated V432M mutation, which lies near the DH–DEP1 interface, escapes PTEN-driven inhibition in breast cancer cells despite retaining PTEN binding⁸⁵. Our biochemical assays using the isolated N-terminal module show only a minimal, non-significant increase in basal nucleotide exchange activity in the V432M variant, suggesting that this mutation does not drive disease through disruptions to autoinhibition from DEP1. However, these data imply that, in the full-length protein, the V432M mutation potentially changes how the N-terminal module interacts with the IP4P subdomain to disrupt PTEN-driven inhibition. While our understanding of the P-Rex2–PTEN coinhibitory complex is still limited, it would be valuable to investigate this mechanism further to identify how disruptions to this complex contribute to cancers and diabetes and, furthermore, to tease out why PTEN is a regulator of P-Rex2 and not P-Rex1.

We observed that the isolated P-Rex2 DH/PH exhibits slower nucleotide exchange on soluble Rac1 than that of P-Rex1. It is possible that this could be due to differences in specificity of the two GEFs for Rac1. Based on crystal structures of P-Rex1 DH/PH in complex with Rac1 or Cdc42, the $\alpha 4/\alpha 5$ loop and $\alpha 5$ helix of the DH domain may be involved in substrate specificity between different GTPases¹⁶. P-Rex2 maintains very high sequence identity in these regions with a few exceptions. At the GTPase-binding site, P-Rex2 Val160 within the $\alpha 4/\alpha 5$ loop and Val167, Thr168, and Ile179 of the $\alpha 5$ helix are different from the homologous residues found in P-Rex1 (Ile186, Leu193, Ser194, and Leu205, respectively) and potentially could account for the observed differences in nucleotide exchange on Rac1. We also showed that, of the two identified mechanisms that govern P-Rex1 autoinhibition, only the DH–DEP1 interface is conserved in P-Rex2. However, the DH–DEP1 interface alone does not fully account for autoinhibition in P-Rex2, as P-Rex2 FL exhibits significantly lower activity than DH/PH–DEP1. This suggests that other regions of the protein must also participate in achieving full autoinhibition. We also observed differences in the Gbg-binding sites between P-Rex1 and P-Rex2. However, because these regions are distal from both the P-Rex catalytic site and autoinhibitory regions, we do not believe these differences would influence P-Rex basal activity in the absence of Gbg. Further examination of the C-terminal accessory domains for their possible roles in autoinhibition is needed. Additionally, biochemical and biological exploration of the alternative spliceoform P-Rex2b, which largely lacks the IP4P domain, has been very limited but might provide insight into a P-Rex2 that more closely exhibits the higher activity of P-Rex1.

Because of the unexpected differences in structure and autoinhibitory regulatory mechanisms, it is possible that P-Rex1 and P-Rex2 respond differently to activation by PIP₃ and G $\beta\gamma$. For example, interactions between the P-Rex1 PH domain and IP4P subdomain contribute to autoinhibition and must be disrupted for activation²⁰, whereas the lack of such coupling in P-Rex2 implies that the PH domain may be more readily available to bind to PIP₃, allowing P-Rex2 to become activated at lower PIP₃

concentrations despite exhibiting lower basal activity than P-Rex1. There are also potential differences in Gβγ binding. In P-Rex1, the region around Gln1615-Leu1647 was identified to play a major role in binding Gβγ¹⁸. Within the homologous area in P-Rex2 (Lys1578-Pro1587), the structure diverges significantly. In another P-Rex1 element implicated in Gβγ binding (Met1582-Val1594), the homologous region in P-Rex2 appears to form an unusual disulfide bond that cannot form in P-Rex1 (Fig. 3.6F & G). These differences suggest that P-Rex2 may bind Gβγ with a different mechanism and/or affinity than P-Rex1, potentially eliciting a distinct activation response. However, our understanding of the activation mechanisms of P-Rex RhoGEFs is still largely unexplored. Future work investigating this is still needed to understand how P-Rex1 and P-Rex2 escape their individual autoinhibitory mechanisms.

In summary, this work has begun to uncover how P-Rex2 regulation is unique from P-Rex1 and how its structural elements could contribute to these differences. Although both proteins share high sequence similarity, P-Rex1 and P-Rex2 autoinhibition are achieved in part through distinct domain organization. This highlights opportunities to selectively target one isoform over the other through divergent regulatory surfaces outside of the DH domain via the rational design of modulators. Attempts at specifically targeting individual Dbl RhoGEFs have historically been unsuccessful due to the flat, broad DH domain that is highly conserved and binds structurally similar GTPases. More broadly, these findings are exciting because they are proof of principle that even closely related Dbl RhoGEFs within the same subfamily can exhibit clear differences in their structure and regulation, offering a framework for designing selective modulators that target Dbl RhoGEFs by utilizing their accessory domains. Future efforts integrating structural and cellular studies will be essential for translating these mechanistic insights into strategies for therapeutically targeting misregulated RhoGEF signaling and, furthermore, fine-tuning GTPase signaling to treat and prevent disease.

Experimental procedures

Sequence Identity Comparison to P-Rex1

Using our P-Rex2 model, we identified the residue boundaries of each of the seven domains: DH: 15-211, PH: 212-365, DEP1: 366-467, DEP2: 473-569, PDZ1: 590-672, PDZ2: 673-815, and IP4P: 816-1606.

The corresponding domain boundaries were identified in P-Rex1 based on structure comparison.

Sequence identity was calculated using The Sequence Manipulation Suite⁸⁶.

Cloning and plasmids

P-Rex1 full-length (FL), P-Rex1 DH/PH, and Rac1 DNA constructs were described previously^{16,18,19}. P-Rex2 pcDNA3.1/V5-His DNA was a gift from Ramon Parsons (Addgene plasmid #41555)²⁴. P-Rex2 FL was cloned into a pRK5 mammalian expression vector with a TEV-cleavable N-terminal GST tag and a C-terminal FLAG tag. P-Rex2 DH/PH (residues 1-377) and P-Rex2 DH/PH-DEP1 (residues 1-467) were each cloned into the pMALc2H₁₀T vector with a TEV-cleavable N-terminal MBP-10xHis tag. P-Rex2 DH/PH-DEP1 point mutations were generated using site-directed mutagenesis.

Protein Production and Purification

P-Rex1 and P-Rex2 FL were expressed in FreeStyle 293-F cells by transient transfection using polyethylenimine. Cells were harvested 48 hours post-transfection by centrifugation for 15 min at 1,000 x g, flash frozen in liquid nitrogen, and stored at -80 °C. Pellets were thawed and lysed in Cell Lytic M (Sigma) supplemented with 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), and a protease inhibitor cocktail (125 nM aprotinin, 6.8 μM leupeptin, 0.15 nM soybean trypsin inhibitor, 1 μM E-64, and 1 μM bestatin). After rocking at 4 °C for 15 min, lysates were clarified by ultracentrifugation at 40,000 rpm for 45 min at 4 °C in a Type 45 or 70 Ti rotor depending on the size of the sample. The supernatant was filtered through a glass fiber filter and incubated with 800 μL glutathione agarose resin per liter harvested cells for 1-2 hours at 4 °C with gentle rocking. The resin

was washed with 20 mM HEPES (pH 8), 200 mM NaCl, 1 mM EDTA, 2 mM DTT, and protease inhibitor cocktail. GST-tagged P-Rex proteins were eluted with 100 mM HEPES (pH 8), 300 mM NaCl, 1 mM EDTA, 1 mM DTT and 30 mM reduced glutathione. Overnight at 4°C, elution fractions were digested with TEV protease (1:1 molar ratio) and dialyzed against 20 mM HEPES (pH 8), 100 mM NaCl, 1 mM EDTA, and 2 mM DTT. Protein was then further purified over an ENrichQ (BioRad) anion exchange column in 20 mM HEPES (pH 8), 2 mM DTT and eluted with a NaCl gradient from 100 mM to 550 mM over 30 CV. Purified protein was pooled, concentrated, and immediately used for experiments.

Soluble Rac1 was expressed in Rosetta (DE3) pLysS *E. coli* cells and purified as previously described¹⁶. Truncated P-Rex constructs also were expressed in Rosetta (DE3) pLysS *E. coli* cells as N-terminal His-tagged MBP fusion proteins. Cells were grown in Terrific Broth to an OD₆₀₀ of 0.8, induced with 0.1 mM IPTG at 16 °C, harvested after 16-20 hours, and then flash frozen and stored at -80 °C. Cell pellets were thawed and resuspended in 9 ml 20 mM HEPES (pH 8), 300 mM NaCl, 0.1 mM EDTA, 2 mM DTT and protease inhibitor cocktail per 1 g cell pellet. Cells were then homogenized with a dounce and lysed with an Avestin EmulsiFlex-C5 High Pressure Homogenizer. Lysate was clarified by ultracentrifugation at 40,000 rpm for 45 min in a Type 45 Ti rotor. The supernatants were then filtered through a glass fiber filter and incubated with Ni-NTA resin for 30-60 min. Resin was washed with buffer (20 mM HEPES pH 8, 300 mM NaCl, 2 mM DTT) followed by buffer containing 10 mM imidazole, and proteins were eluted with buffer containing 250 mM imidazole. DH/PH and DH/PH-DEP1 elutions were then simultaneously dialyzed into buffer containing 20 mM HEPES (pH 7 or pH 8, respectively), 200 mM NaCl, 2 mM DTT, and 10% glycerol and digested with TEV protease overnight at a 1:2 molar ratio of TEV:MBP-fusion protein to remove the N-terminal His-tagged MBP. Cleaved MBP-His was then captured by an additional pass over Ni-NTA resin. The flow through was applied to a HiTrap SP Sepharose Fast Flow column (Cytiva) in 20 mM HEPES (pH 7) and 2 mM DTT and eluted over an NaCl gradient from 0-0.5 M over 4 CV.

P-Rex2 DH/PH-DEP1 mutants were expressed under the same conditions as the wild-type construct, but in small-scale 15 ml cultures. Cell pellets were resuspended in 1 ml 20 mM HEPES (pH 8), 300 mM NaCl, 0.1 mM EDTA, 2 mM DTT, and protease inhibitor cocktail. Resuspended cells were sonicated on ice (5 seconds on, 5 seconds off, for 60 seconds per cycle for 3 cycles) using a Q125 sonicator (Qsonica Sonicators), and lysates were clarified by centrifugation at 21,300 x g for 45 min at 4°C. The supernatants were incubated with Ni-NTA resin for 30-60 min at 4°C. The resin was washed and proteins eluted, cut with TEV, and dialyzed the same as the wild-type DH/PH-DEP1. Cleaved MBP-His was then captured by an additional pass over Ni-NTA resin. The resin was washed one additional time with a buffer containing 20 mM HEPES (pH 8), 300 mM NaCl, 2 mM DTT, and 10 mM imidazole. The combined flow through and wash were concentrated using a 10kD MWCO Amicon concentrator. Final protein concentrations were estimated by visualization on SDS-PAGE.

Cryo-EM Grid Preparation and Data Acquisition

P-Rex2 samples at 0.4 mg/ml were briefly incubated with 0.07 mM n-Dodecyl-Beta-Maltoside (DDM) before applying a 4 μ L sample to a glow discharged 300-mesh Quantifoil (1.2/1.3) grid, blotted for 4 or 6 seconds with a blot force of 20, and plunge-frozen into ethane cooled with liquid nitrogen using a Vitrobot (Thermo Fisher Scientific) set to 4 °C and 100% humidity. Data were collected at the Pacific Northwest Center for Cryo-EM (PNCC) using EPU (Thermo Fisher) on a Krios transmission electron microscope operating at 300 keV with a Falcon 4i direct electron detector (Thermo Fisher) and SelectrisX. Datasets were collected on an untilted grid (12,070 micrographs) with beam image shift (BIS) and on a grid tilted by 35° (7,722 micrographs) without BIS. Tilted data were collected to overcome a preferred orientation of the sample on grids.

For the IP₄ experiments, small datasets were collected on P-Rex2 samples at 0.7 mg/ml that were briefly incubated with 0.07 mM DDM in the presence or absence of 16.6 μ M IP₄ and frozen with

the same freezing conditions described above. Approximately 1300-1400 micrographs were collected for each dataset using a Glacios transmission electron microscope operating at 200 keV with a K3 direct electron detector (Gatan, Inc.) and then processed up through 2D classification. Both samples yielded classes with similar views without distinguishable differences in P-Rex2 conformation, mass present, or resolution (Fig. 3.3).

Cryo-EM Data Processing

Untilted and tilted datasets were preprocessed separately. Motion correction, contrast transfer function (CTF) estimation, and particle picking were performed in Warp⁸⁷. The default BoxNet2Mask model was re-trained on P-Rex2 particles for particle picking in these datasets. Particle stacks were then imported into cryoSPARC⁸⁸ for further processing.

In our first processing pipeline (Fig. 3.4A), the untilted and tilted datasets were each separately put through cursory 2D classification to sort out obvious junk in each particle stack. Using only the best particles with clear secondary structure features from the tilted dataset, ab-initio volumes were generated. The volume containing the whole particle (both N- and C-terminal components) was then used, in combination with decoy ab-initio volumes, in separate heterogeneous refinements for both the tilted and untilted particle stacks. Particles which sorted into the whole particle volume from both heterogeneous refinements were combined and underwent an additional round of heterogeneous refinement. A final round of 2D classification was used as a quality check where only the highest quality particles were used for non-uniform refinement (NU Refine). The resulting volume was used in a final heterogenous refinement, deriving the final particle stack. These the best volume and associated particles were used in non-uniform refinement and subsequent local CTF refinement in cryoSPARC; however, the maps generated from these jobs had odd features where recognizable helices looked step-like. In an attempt to resolve this, we used 3D refinement with Blush regularization in Relion 5.0⁸⁹ which

was fairly successful in improving the appearance of these helices. Finally, the refined map and particles underwent local refinement in cryoSPARC with a mask around the whole particle. The resulting map was put through density modification using EMReady⁹⁰ to improve interpretability of the map for use in model building (Fig. 3.6B&C).

In the final processing pipeline (Fig. 3.4B), each particle stack was separately cleaned through multiple rounds of 2D classification to sort out obvious junk, and then particle stacks from the untitled and tilted data were combined. Because of strong orientation bias despite the addition of tilted data, there were a few class averages that would dominate the views observed in 2D classification. Therefore, in an iterative 2D classification approach, these well resolved classes were separated for later use, obvious junk was discarded, and further 2D classification was done on the remaining particles, which helped to sort out more rare, good-quality particle views. After several rounds of this, we were left with a much larger cleaned particle stack than without using this approach. With these particles, ab-initio reconstruction yielded one volume where both the C-terminal core and the N-terminal module were resolved and 3 volumes where mainly only the core was resolved. Only the volume with clear density for the N-terminal module was used for downstream processing. This volume was processed through non-uniform refinement⁹¹. This reconstruction along with three decoy volumes created from “junk” particles were used in heterogeneous refinement against the cleaned particle stack, resulting in ~100k additional particles sorting into the “good” class. Additional non-uniform refinement of this stack and volume resulted in a 2.9 Å consensus map which featured primarily only the core (Fig. 3.5B). Given the apparent continuous flexibility of the N-terminal module, 3D Variability Analysis and 3DFlex were used to attempt to better resolve this region, but neither of these programs improved results. Instead, we used a binary approach where focused 3D classification was performed on the consensus map and its particles using a mask around the N-terminal module to sort out particles where the N-terminus is in a single position with respect to the C-terminus. The class containing a visible N-terminal module from this job was then

locally refined using the same mask around the N-terminal module which produced the N-term local refinement map (Fig. 3.5C). The consensus map and the map from local refinement of the N-terminal module were used to generate a composite map in Phenix, utilizing a 4.5 Å resolution cutoff (Fig. 3.6D).

Model Building, Refinement, and Validation

Primarily, two maps were used to build the P-Rex2 model, as each map was more interpretable in different areas. One of these was the composite map and the other was the density-modified map from EMReady, both of which are described above. Using the AlphaFold Server³⁹, P-Rex2 models of each the N-terminus (residues 1-472) and C-terminus (473-1606) were generated. Models were fit into the aligned maps using ChimeraX⁹², and the best fitting model for each the N- and C-terminus was chosen for further model building and modification. Parts of the model that were completely outside of map density were deleted in Coot 0.9.8.95⁹³, and then each model was run through rigid body real-space refinement in Phenix 1.21.2-5419⁹⁴ after adding hydrogens and with minimization_global enabled⁹⁵. Certain parts of the protein were rigid body fit into the map with little further modification, as resolution in these areas was low, including the DH, PH, and DEP1 domains. The density of the midpoint of the helix connecting the PH and DEP1 domains was sparse, and the EMReady map was particularly helpful in modeling this area. The connection between the DEP1 and DEP2 domains was clearly visible in the map but not at high enough resolution to build unambiguously since it was very different from this region in P-Rex1 and in the AlphaFold models. The part of the map representing the C-terminal core was the highest resolution and most interpretable part of the map. However, the PDZ2 domain was particularly disordered, and much of this domain was left unmodeled. The IP4P subdomain observed in the autoinhibited structure of P-Rex1, termed the 4HB, was completely missing from the P-Rex2 map, presumably because it is flexibly tethered to the core. There were also a number of loops in the IP4P domain that were left unmodeled due to sparse or missing density. Importantly, conclusions regarding the model were only drawn from regions of the structure that were well-supported by map density.

After this, the model was further modified and rebuilt in Coot using tight geometric restraints. Because map density was very sparse or low resolution in some regions, homology models P-Rex1–Gβγ (6PCV), P-Rex1•IP4 (8TUA), and P-Rex1 DH/PH–DEP1 (7RX9) were used to assist decision making during model building. In some areas, the AlphaFold P-Rex2 model was different from the homologous region in P-Rex1 but nicely fit the P-Rex2 map, while in others, the predicted and homology models clearly deviated from the path of the map. In regions where the map was ambiguous, the AlphaFold P-Rex2 model was left with limited modification when it fit the density reasonably well, corresponded to helical or β-strand regions, and was conserved in P-Rex1. In areas where the P-Rex2 map diverged from P-Rex1, such as in loops where the prediction was less reliable or the density path was not clear, the model was deleted. After building was complete, regions of the structure that formed helices or β-sheets were manually confirmed and annotated, and Phenix real-space refinement was run using secondary structure restraints. Model validation was performed in Phenix⁹⁵ (Table 1 & Fig. 3.6A). EMRinger was also run within Phenix⁹⁶. The associated web interface was used to run the 3D FSC program to calculate sphericity and generate a 3D FSC histogram and directional FSC plot^{76,97}. The Q score was calculated during the validation stage of data deposition⁹⁸. Structure figures were generated using ChimeraX⁹² and PyMOL⁵². Some software was accessed through membership in the SBCGrid Consortium⁹⁹.

Hydrogen-Deuterium Exchange Mass Spectrometry

Prior to conducting hydrogen/deuterium exchange experiments, optimal quench conditions were determined to generate the best enzymatic peptide coverage map for P-Rex2 FL, as previously described^{18,100}. Briefly, 3 μl purified P-Rex2 (2 mg/ml in 20 mM HEPES (pH 8), 150 mM NaCl, 2 mM DTT) was diluted with 9 μl of H₂O Buffer at 0 °C, and then mixed with 18 μl of ice cold quench buffers containing 0.1 M Glycine (pH 2.4), 16.6% glycerol and varying concentration of GuHCl (0.08, 0.8, 1.6 and 3.2 M). The quenched samples were subjected to on-line proteolysis and LC/MS analysis. The best sequence coverage of P-Rex2 was obtained using the 1.6 M GuHCl quench buffer.

P-Rex2 exchange stock solutions (10.8 μ M of P-Rex2 FL and 10.8 μ M of P-Rex2 FL with 45 μ M IP₄) were prepared in 8.3 mM Tris, 150 mM NaCl, pH 7.2 and kept on ice. HDX experiments were initiated by adding 50 μ l of exchange stock solutions (P-Rex2 FL or P-Rex2 FL-IP₄) to 150 μ l of D₂O buffer (8.3 mM Tris, 150 mM NaCl, pDread7.2) and incubating for various times intervals (10, 100, 1000, 10,000 and 100,000 sec) at 0 °C. At each time, 36 μ l of exchange mixture was withdrawn and quenched with 54 μ l of ice cold quench buffer (0.1 M Glycine (pH 2.4), 1.6 M GuHCl). Quenched samples were aliquoted and immediately frozen on dry ice. Non-deuterated and equilibrium deuterated control samples were also prepared for back exchange correction¹⁰¹.

All frozen samples were later loaded onto a cryogenic autosampler and automatically thawed at 4 °C before digestion on an immobilized pepsin column (16 μ l bed volume) at 25 μ l/min. Proteolytic fragments were collected on a trap column and separated on an Acclaim PepMap RSLC C18 reverse phase analytical column (ThermoSci, 0.3 x 50 mm, 2 μ m, 100 Å) using a linear acetonitrile gradient (5%-45% over 30 min). The effluent was directed into an Orbitrap Elite Mass Spectrometer (ThermoFisher Scientific, San Jose, CA). Instruments settings were optimized to minimize the back-exchange¹⁰². The data was acquired in either MS1 profile mode or data-dependent MS/MS mode. Peptide identification was done with the aid of Proteome Discoverer software (ThermoFisher). The centroids of the mass envelopes of deuterated peptides were calculated with HDExaminer (Sierra Analytics Inc, Modesto, CA) and then converted to corresponding deuterium incorporation with corrections for back-exchange¹⁰³.

GTPase Exchange Activation Assay

P-Rex GEF activity was analyzed through a FRET-based nucleotide exchange assay¹⁰⁴ measuring association of GTPase with fluorescently-labeled N-methyl-anthraniloyl-GTP (mant-GTP; Abcam), a nonhydrolyzable GTP analog, in a black 384-well plate (Greiner). P-Rex construct plus 2 μ M GTPase were added in 20 mM HEPES (pH 8), 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 5% glycerol at room

temperature. The reaction was initiated by adding 0.8 μ M mant-GTP and immediately measured for fluorescence at Ex = 280 nm, Em = 450 nm at room temperature in 30 second intervals for 15 min on a SpectraMax M5 (Molecular Devices) plate reader. Fluorescence curves were then fitted to a one-phase association model using GraphPad Prism.

Statistical Analysis

All statistical analyses were performed in GraphPad Prism software on data from at least three independent experiments. All GEF activity assay data were fit to a one-phase association model. The k value obtained was used to represent k_{obs} where applicable. All P-Rex2 DH/PH-DEP1 variants were normalized to P-Rex2 DH/PH-DEP1 WT and then evaluated for statistical significance. Statistical significance was determined using a one-way ANOVA test with a post hoc Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Size-Exclusion Chromatography Small-Angle X-ray Scattering

Small-angle X-ray scattering (SAXS) experiments were performed at the SIBYLS beamline 12.3.1 at the Advanced Light Source. This beamline has additional inline instrumentation and detectors coupled to a size exclusion column^{105,106}. Purified P-Rex2 DH/PH and DH/PH-DEP1 were concentrated to 2 mg/ml and then buffer exchanged into 20mM HEPES (pH 7), 200mM NaCl, 2mM DTT, and 1% glycerol SEC running buffer. The X-ray wavelength was set to 1.127 Å and the sample-to-detector distance to 2,100 mm, which gives scattering vectors (q) ranging from 0.01 Å⁻¹ to 0.4 Å⁻¹. The scattering vector is $q = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle. The SAXS flow cell was coupled to an Agilent 1260 Infinity HPLC system using a Shodex PROTEIN KW-802.5 SEC column equilibrated with the running buffer with a flow rate of 0.65 ml/min. For SAXS measurements, 2 sec X-ray exposures were collected continuously during a 25 min elution. All frames for analyses had one SAXS frame corresponding to the running buffer before the detection of a peak subtracted from each.

The radius of gyration (R_g) was calculated for each of the subtracted frames using the Guinier approximation: $I(q) = I(0) \exp(-q^2 R_g^2/3)$ with the limits $qR_g < 1.3$. The elution peak was compared to the integral of ratios to background and R_g relative to the recorded frame using the program RAW¹⁰⁷. Uniform R_g values across an elution peak represent a homogeneous sample. Final merged SAXS profiles, derived by integrating multiple frames at the elution peak, were used for further analyses. Calculated were the Guinier plot to provide information on the aggregation state, the volume of correlation (V_c) to estimate the molecular weight¹⁰⁸, and the pair distribution function $[P(r)]$ to calculate the maximal inter-particle dimension¹⁰⁹. AlphaFold Server³⁹ was used to generate models of P-Rex1 DH/PH (38-409), P-Rex2 DH/PH (1-377), P-Rex1 DH/PH-DEP1 (38-499) and P-Rex2 DH/PH-DEP1 (1-467). The resulting models were used with their corresponding intensity profiles in BilboMD⁷⁷ to determine the model fit to the intensity profile and potential conformational heterogeneity.

Data availability

The P-Rex2 EM data can be accessed as EMDB entries EMD-74547 (consensus map), EMD-74548 (local refinement of the N-terminal module), EMD-74549 (local refinement of the whole particle), and EMD-74550 (composite map). The P-Rex2 model has been deposited as PDB entry 9ZQ7. The SAXS data for P-Rex2 DH/PH and DH/PH-DEP1 can be accessed as SASDB entry SASDZV5 and SASDZU5, respectively.

Tables

Table 3.1. Cryo-EM Data Collection, Processing, and Modeling Statistics

Data Processing	Composite Map	Consensus Map	N-terminal Local Refinement	Whole Particle Local Refinement
EMDB accession code	EMD-74550	EMD-74547	EMD-74548	EMD-74549
Symmetry imposed	C1	C1	C1	C1
Particles in final map	-	215,042	101,178	128,298
Global Resolution (Å, at 0.143)	-	2.9	4.4	3.4
3DFSC sphericity	-	0.734	0.872	0.83
Map sharpening B factor (Å ²)	-	79.8	161.7	76.3
Data Collection & Pre-Processing	Untilted	Tilted	Atomic modeling	
EMPIAR accession code	XXXX		PDB accession code	9ZQ7
Micrographs (collected)	12,070	7,722	Model Composition	
Electron dose (e ⁻ /Å ²)	50	50	Chains	1
Frames per movie	1143	1206	Non-hydrogen atoms	8320
Voltage (kV)	300	300	Hydrogens	7704
Stage tilt (°)	0	35	Protein residues	1035
Pixel size (Å)	0.7296	0.7296	Water	0
Defocus range (µm)	0.7-2.2	0.7-2.2	Ligands	0
Micrographs (used)	8133	6959	B factors (Å ² , min/max/mean)	
Extracted particles	2,184,088	2,137,912	Protein	30/524/132
Final particles after 2D classification	1,390,781	1,585,976	Ligand	0
			Water	0
*Composite map			R.m.s. deviations	
			Bond lengths (Å)	0.003
			Bond angles (°)	0.477
			Validation	
			MolProbity score	1.63
			Clashscore	6.97
			Rotamer outliers (%)	1.53
			CaBLAM outliers (%)	0.86
			Ramachandran plot (%)	
			Favored	97.5
			Allowed	2.4
			Outliers	0.1
			Model vs. Data*	
			CC mask	0.58
			CC box	0.7
			CC peaks	0.44
			CC volume	0.6
			Map-to-model FSC (Å, at 0.5)*	3.4
			EMRinger score*	1.87
			Q-score*	0.37

Table 3.2. P-Rex2 SEC-SAXS data analyses

	P-Rex2 DH/PH (SASDZV5)	P-Rex2 DH/PH-DEP1 (SASDZU5)
Guinier analysis		
R _g (Å)	26.12 +/- 0.38	29.11 +/- 0.47
I(0)	9.68 +/- 0.11	13.27 +/- 0.17
Q _{min} * R _g	0.455	0.684
Q _{max} * R _g	1.312	1.301
Molecular weight analysis		
Porod Volume (Å ³)	52300	77200
MW (Vc) (kDa)	41.6	57.1
Shape (S&S)	Compact	Compact
D _{max} (Å)	81.1	96.0
P(r) analysis		
D _{max} (Å)	84	105.0
R _g (Å)	26.94 +/- 0.16	29.99 +/- 0.28

Figures

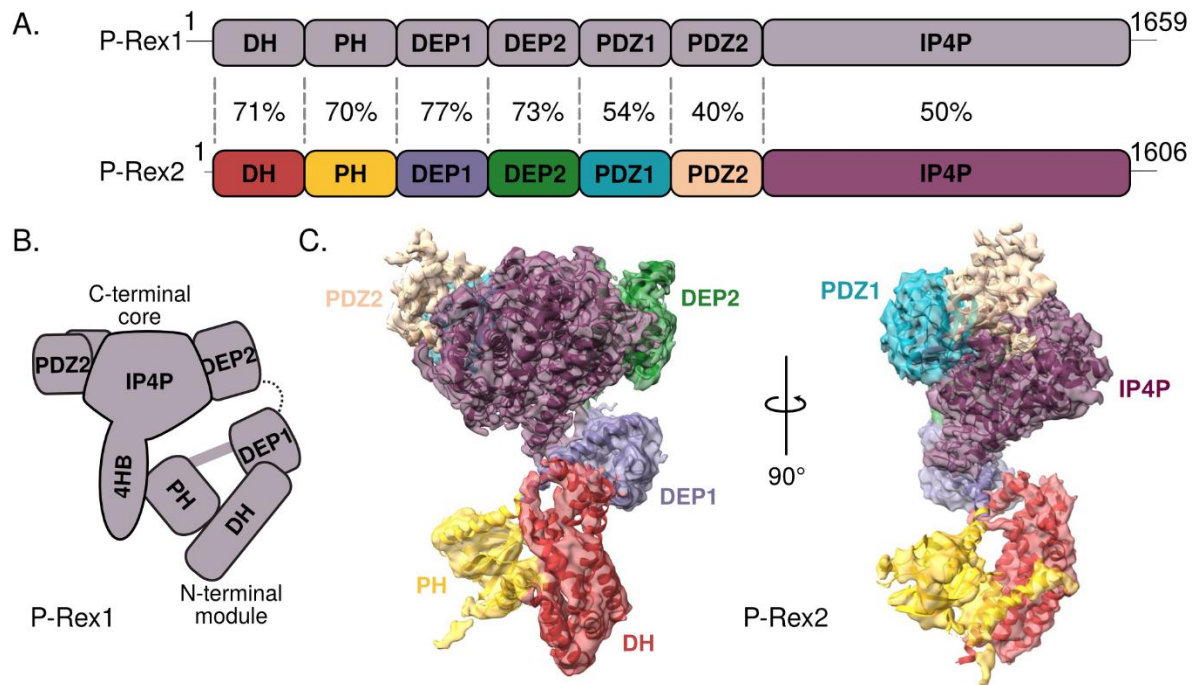


Figure 3.1. P-Rex2 exhibits a structure that is overall conserved with P-Rex1. A) Domain layout of P-Rex1 in gray and P-Rex2 colored by domain. The sequence identities between domains of the isoforms are labeled. B) Schematic of the domain organization of P-Rex1. C) Cryo-EM reconstruction of P-Rex2 (composite map) colored by domain with atomic model superimposed. The IP4P subdomain was not resolved in P-Rex2, unlike in P-Rex1.

A

P-Rex1 Homologous Residue 1	P-Rex1 Homologous Residue 2	Distance (Å)	P-Rex2 Residue 1	P-Rex2 Residue 2	Distance (Å)
141	938	97.7	115	904	Not modeled, unlikely to be within cross-linking distance
154	1272	61.9	128	1212	Unknown with current model
241	1272	48.3	215	1212	Unknown with current model
164	1502	32.6	138	1442	Not modeled, likely to be within cross-linking distance
395	1502	22.2	364	1442	35.8
368	1502	32.8	337	1442	56.5
418	513	33.7	387	481	20.8
415	1502	42.0	384	1442	15.9
418	1502	45.1	387	1442	17.0
429	1502	54.4	397	1442	29.9

B.

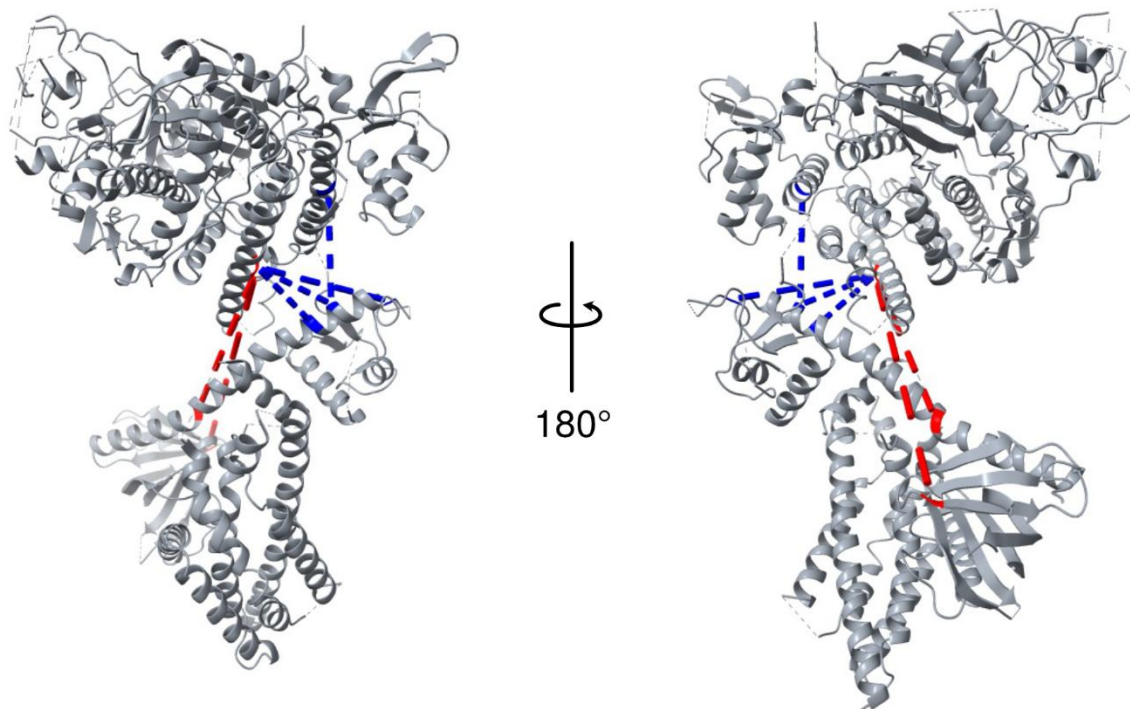


Figure 3.2. P-Rex2 cross-linking mass spectrometry (XL-MS) data agree with the cryo-EM structure of

P-Rex2. A) Using P-Rex2 cross-linking mass spectrometry data from D'Andrea et al. (2021), we analyzed cross-links between residues in the N-terminal module and the C-terminal core. Looking at homologous residues in P-Rex1, we calculated the distances between C α atoms of cross-linked residues using the structure of autoinhibited P-Rex1 (PDB: 8TUA). After obtaining the cryo-EM structure of P-Rex2, we performed the same calculations. B) P-Rex2 XL-MS data for cross-links between residues in the N-terminal module and the C-terminal core shown plotted onto the P-Rex2 structure. Blue dashes represent cross-linked residues that are <30 Å apart and red dashes represent cross-linked residues that are > 30 Å apart.

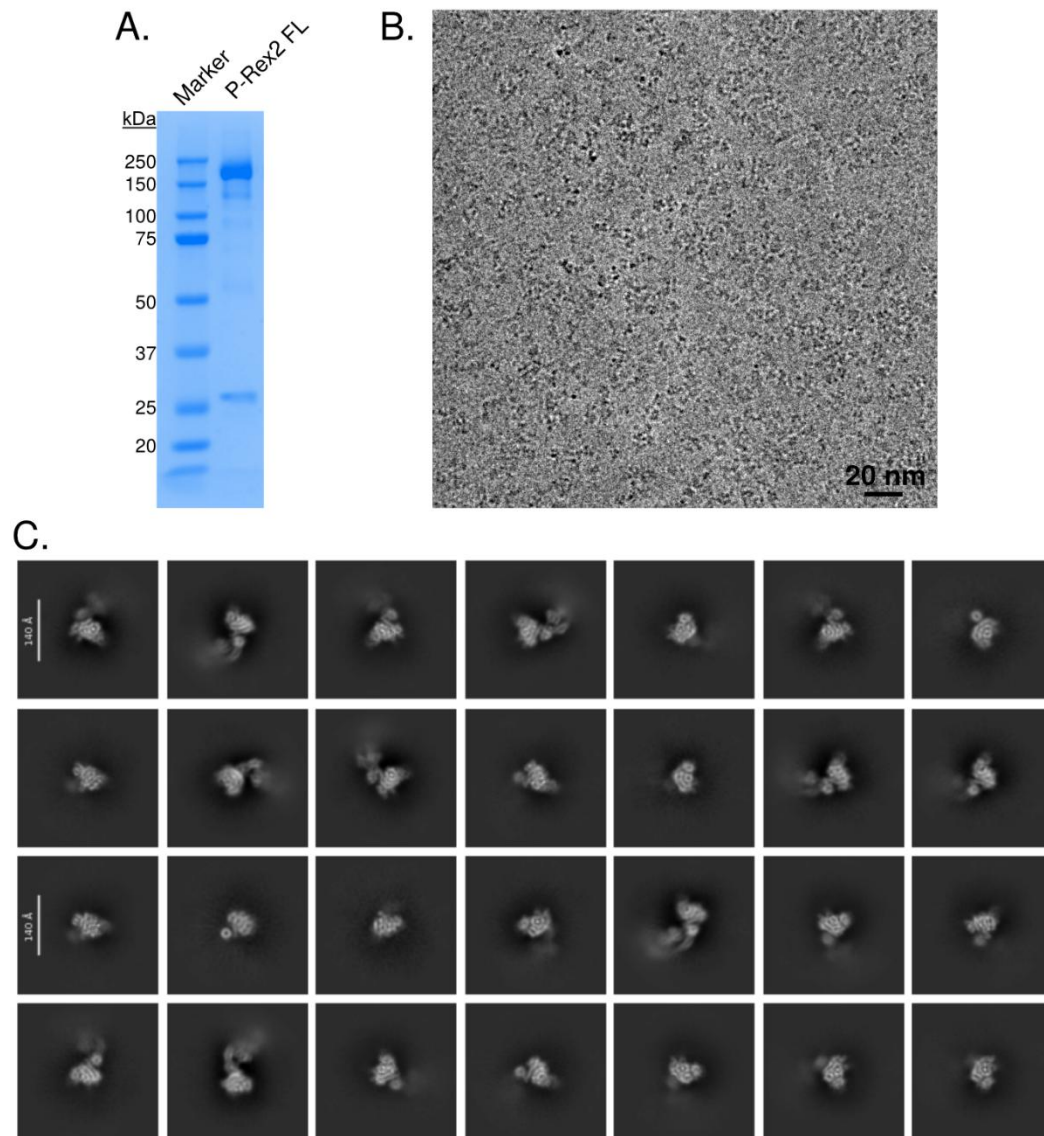
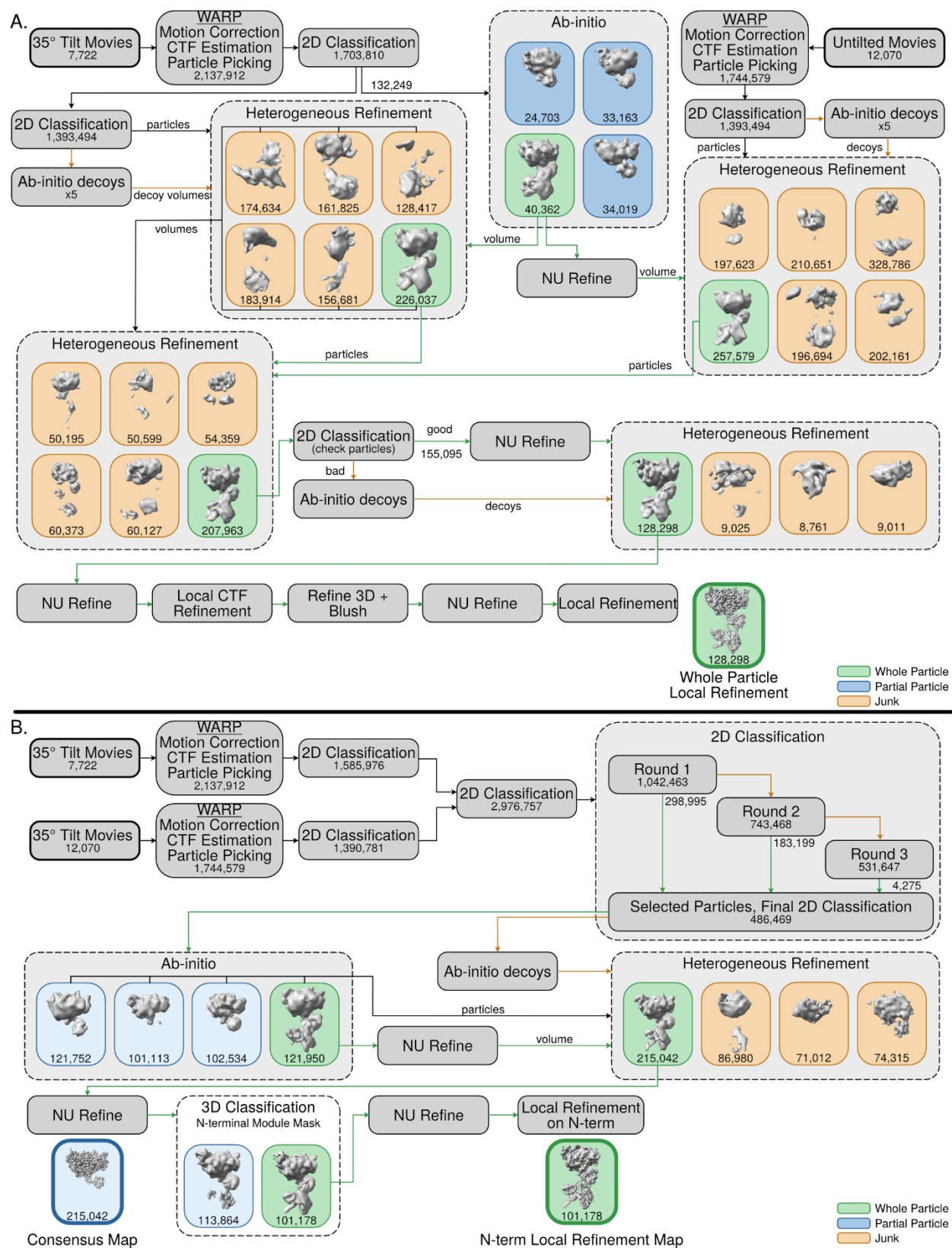


Figure 3.3. P-Rex2 cryo-EM sample quality. A) SDS-PAGE of purified P-Rex2 FL. B) Representative micrograph. C) Best 2D class averages from P-Rex2 data processing.



whole particle local refinement map (EMD-74549). B) Data processing pathway resulting in the consensus map (EMD-74547) and N-terminal local refinement map (EMD-74548). Final maps from both pipelines are outlined with a thick line.

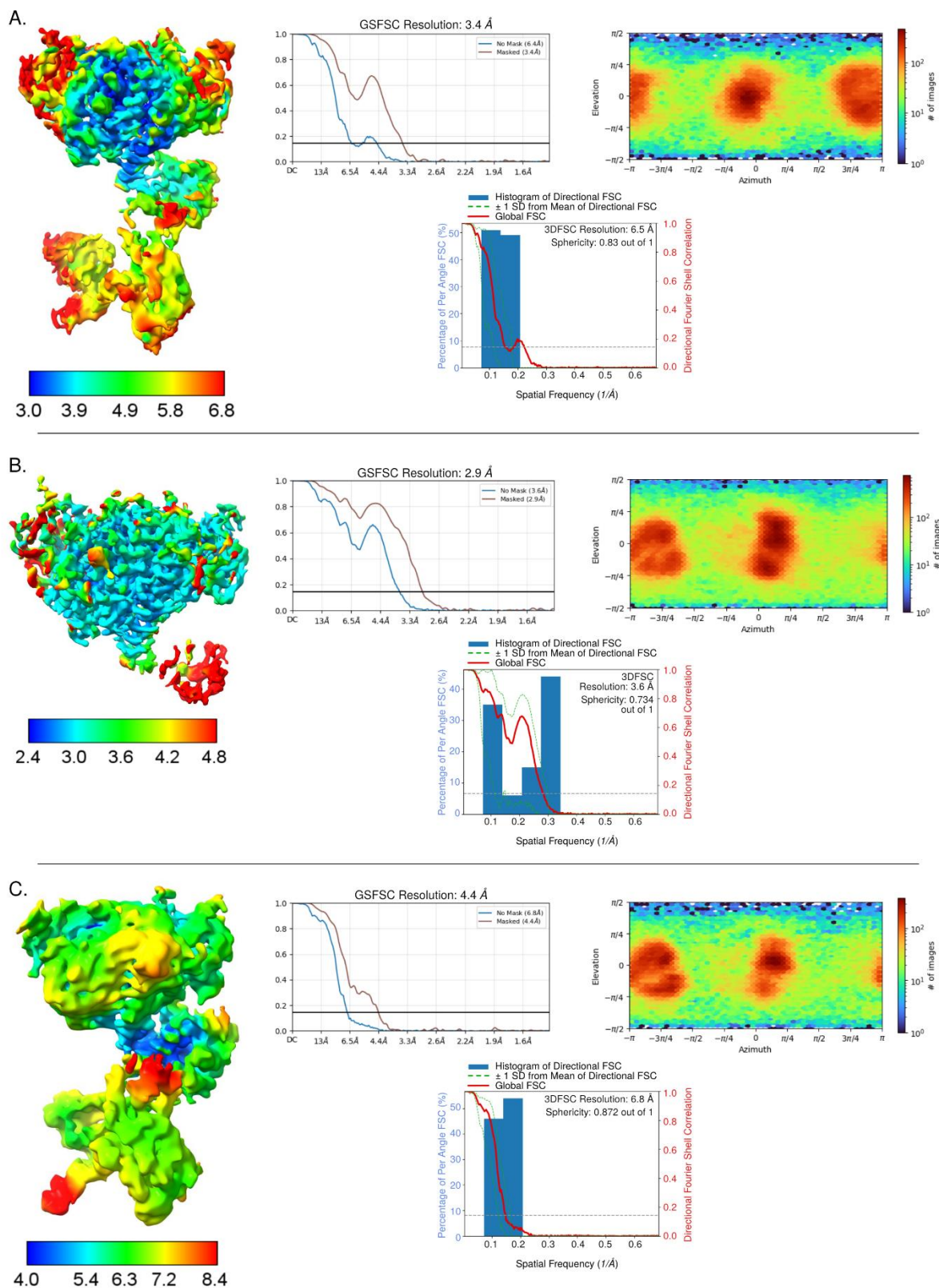


Figure 3.5. Resolution and quality of P-Rex2 cryo-EM maps used for model building. A) Map from local refinement of the whole particle, (B) consensus map, and (C) map from local refinement of the N-

terminal module colored by local resolution, the corresponding cryoSPARC gold standard FSC curve and viewing direction distribution plot, and a 3D FSC histogram and directional FSC plot calculated by the 3D FSC program (<https://3dfsc.salk.edu/>).

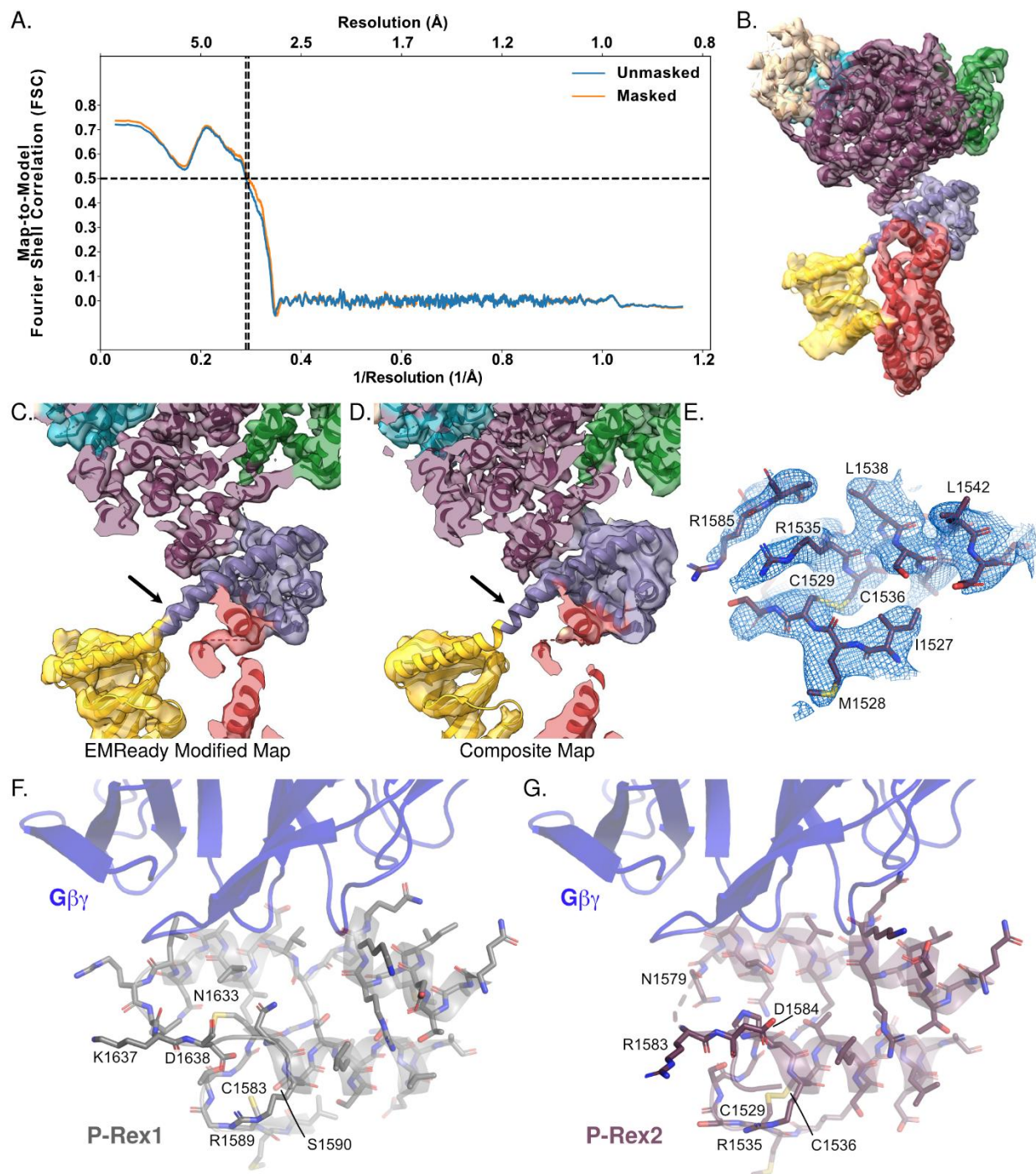


Figure 3.6. Interpretability of maps and fit of P-Rex2 model. A) Map-to-model FSC of the composite map. B) A density modified map generated with EMReady using the map from P-Rex2 whole particle local refinement. C-D) This map was especially useful in regions with poor resolution in the composite

map such as in the helix bridging the PH and DEP1 domains (black arrow). E) P-Rex2 residues representing part of the “catalytic triad” (C1529, R1535, and D1584) of the proposed pseudo-phosphatase site and composite map density representing them, showing that this area is well-resolved in the map. Also shown here is the disulfide bond formed between C1529 of the triad and C1536, which is homologous to S1590 in P-Rex1. F) Part of the P-Rex1 Gβγ-binding site, also showing the triad (C1583, R1589, and D1638), with Gβγ shown semi-transparently (PDB: 6PCV). G) The equivalent site in P-Rex2 with Gβγ superimposed, showing differences in the triad as well as disorder in the loop upstream of D1584.

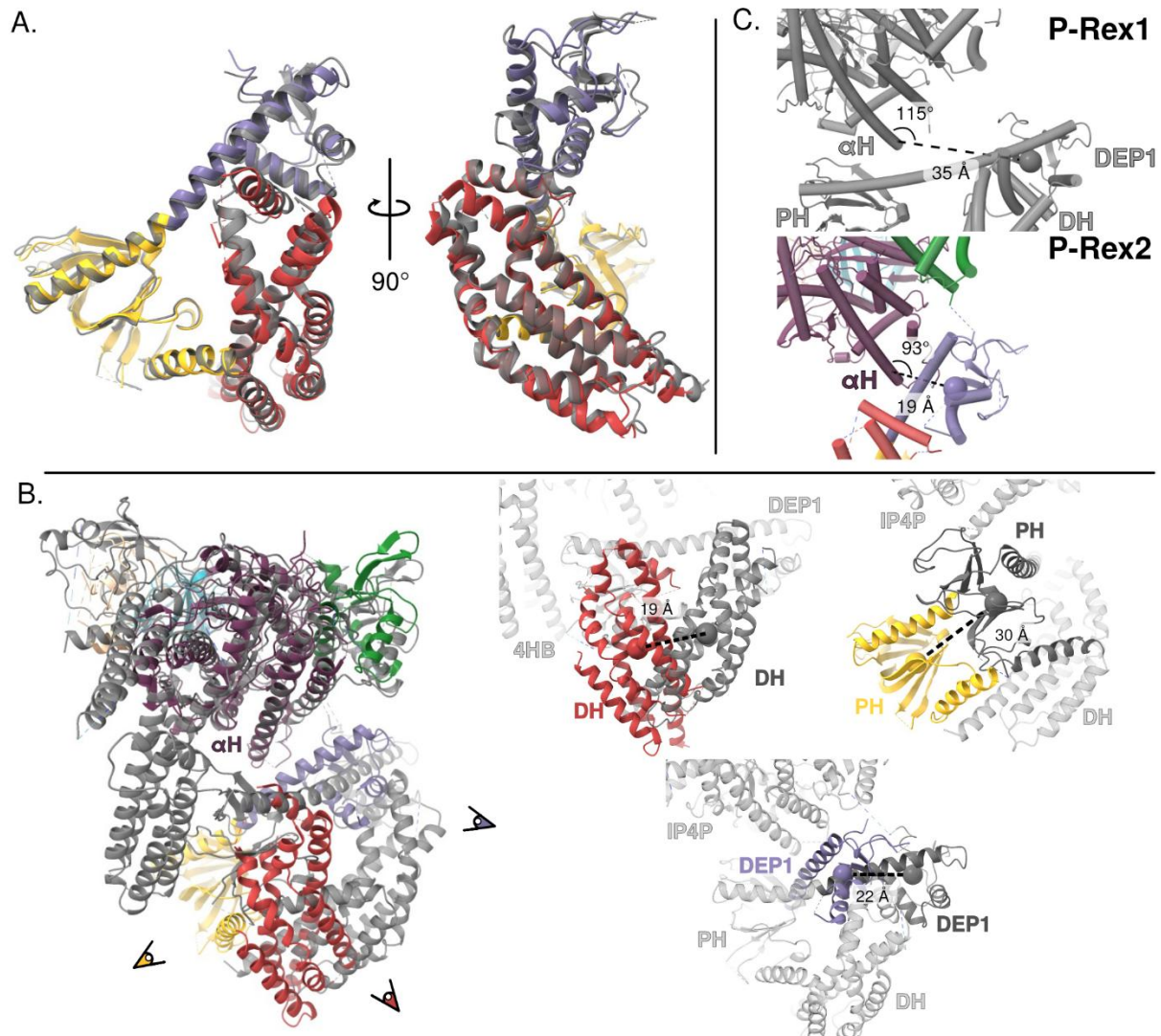


Figure 3.7. P-Rex2 shows a relative shift in the position of the N-terminal module as compared to P-Rex1. A) N-terminal modules of P-Rex1 (gray, PDB: 8TUA, residues 38-499) and P-Rex2 (colored according to domain, residues 15-467) aligned to one another. There are minimal differences in their overall architecture. B) P-Rex2 aligned to P-Rex1 through the α H helix in the IP4P domain (P-Rex1 1491-1513, P-Rex2: 1431-1453) using Coot least-squares (LSQ) superimposition. A close-up view of each N-terminal domain (noted by the eyeballs) is shown in the insets, with P-Rex1 shown in gray. Spherical markers denote the center of mass (COM) of each homologous domain, and the distance between these in the aligned structures is indicated (P-Rex1 DH 38-237, PH 238-396, DEP1 397-499; P-Rex2 DH 15-211, PH 212-365, DEP1 366-467). C) The distance between the COM of the DEP1 domain and L1513/L1453 of the α H

helix for P-Rex1 and P-Rex2, respectively, is shown. The measurement for an angle formed by a line connecting these two points and a line connecting L1513/L1453 to Q1491/A1431 of the α H helix for P-Rex1 and P-Rex2, respectively, is shown. Together, these describe the degree to which the N-terminal module is rotated and translated closer to the core in P-Rex2 as compared to P-Rex1.

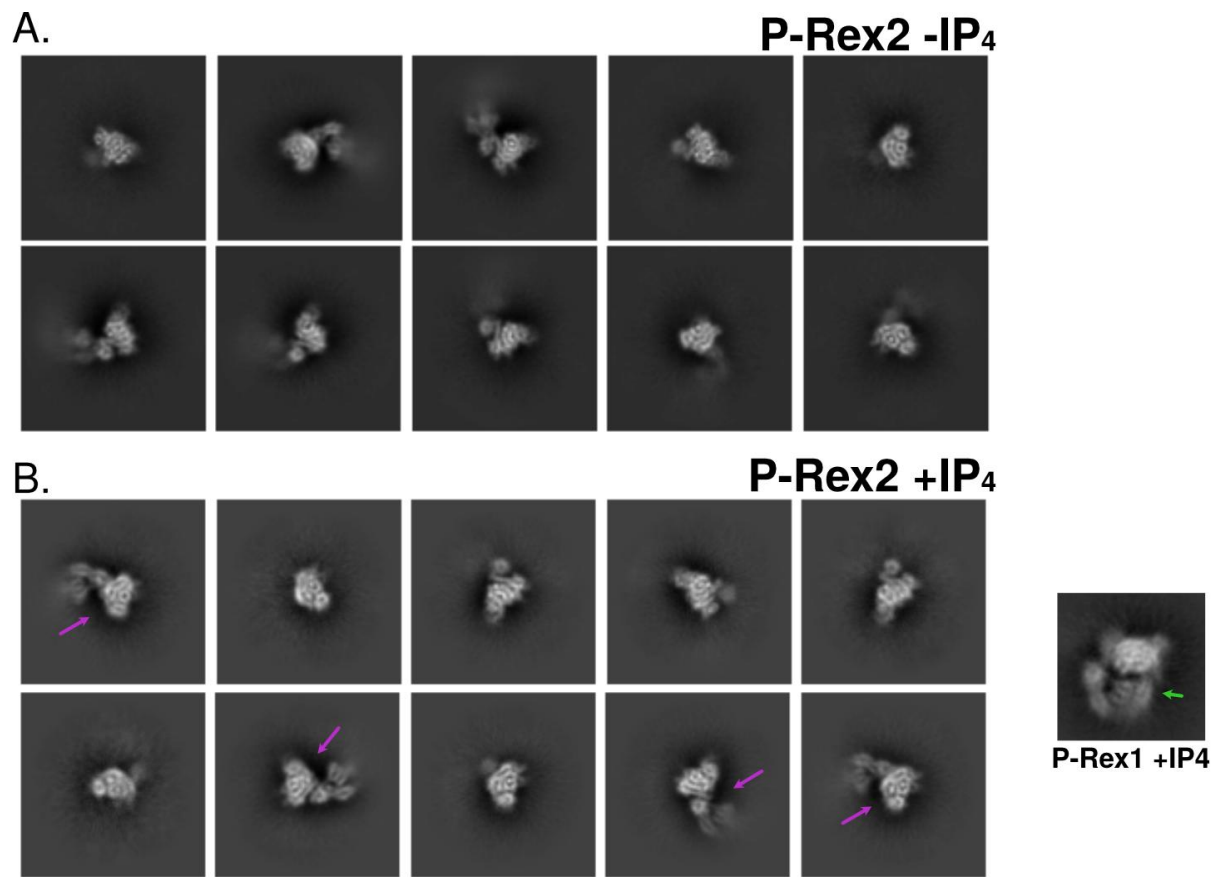


Figure 3.8. 2D class averages show that IP₄ binding does not stabilize a PH–IP₄P interaction to allow resolution of the P-Rex2 IP₄P subdomain. Two small datasets were collected on a Glacios on P-Rex2 samples in the A) absence and B) presence of IP₄. Datasets were processed in the same manner. A single 2D class average of P-Rex1 with IP₄ (Ravala et al., 2024) containing density for the IP₄P subdomain (green arrow) is included to highlight its absence in P-Rex2 with IP₄ class averages (magenta arrow).

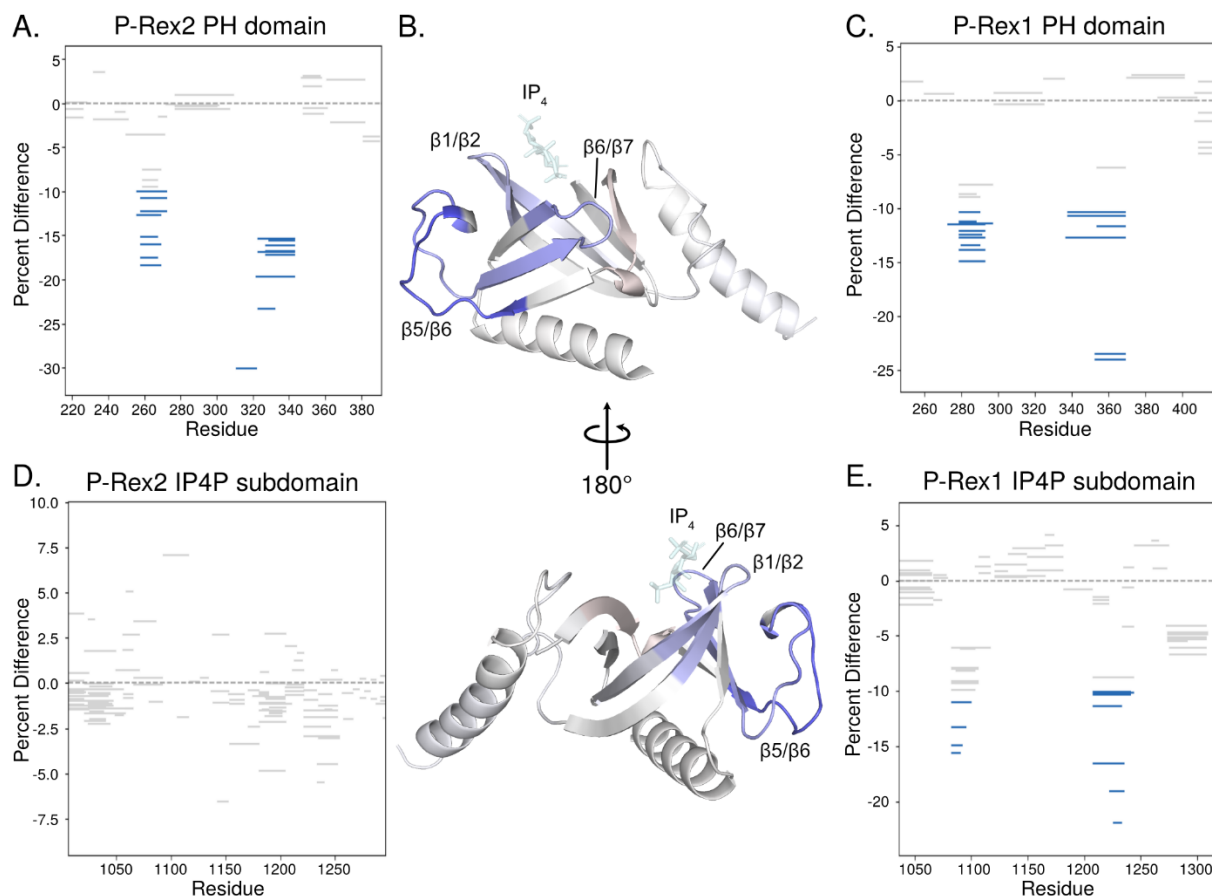


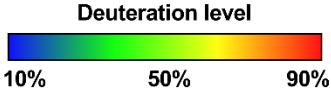
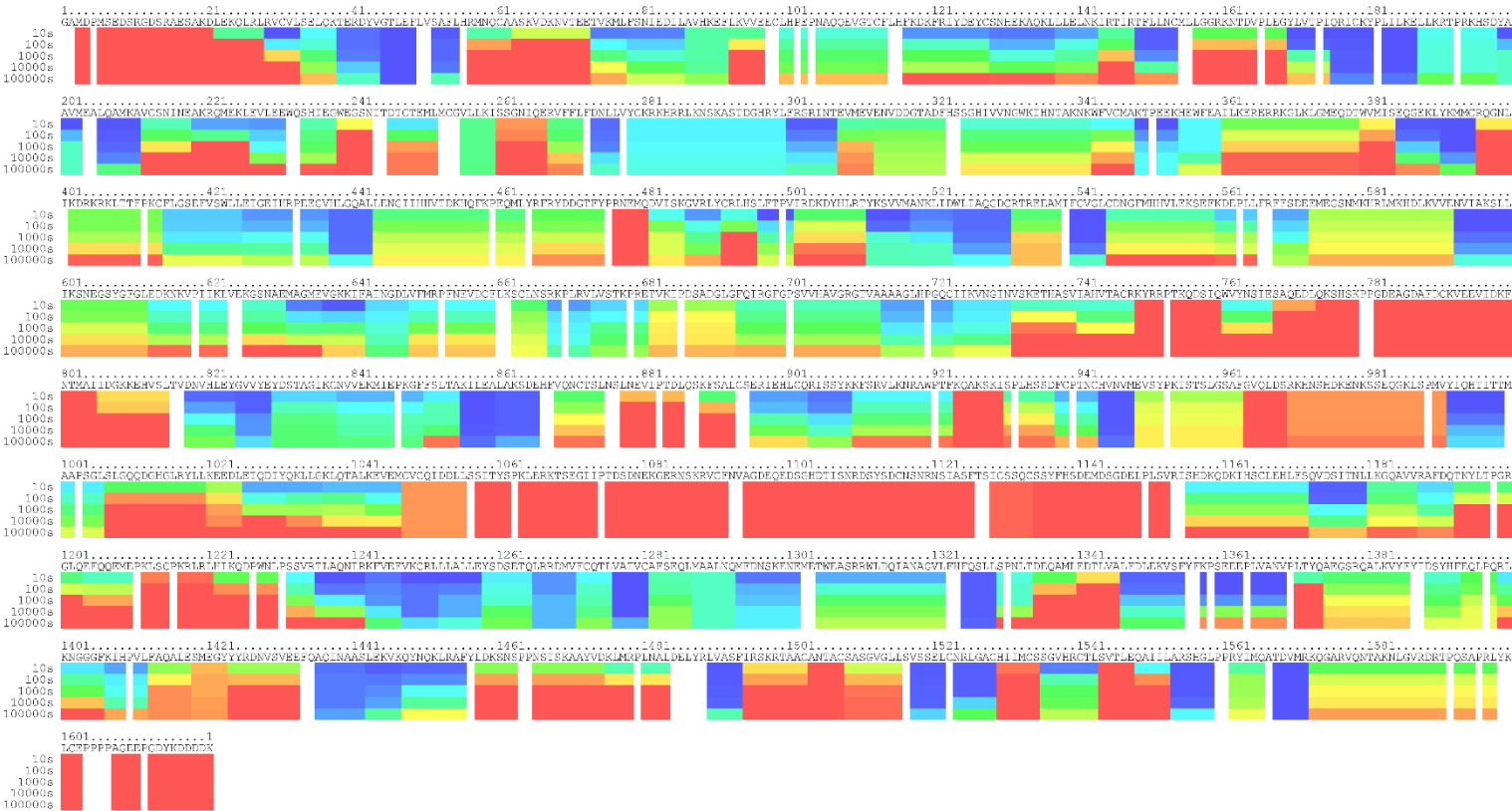
Figure 3.9. IP₄ does not alter the dynamics of the P-Rex2 IP4P subdomain, unlike P-Rex1. HDX-MS data

were collected on P-Rex2 in the presence and absence of IP₄. A) HDX-MS peptide data at the 10,000 s time point shown on a Wood's plot. Changes in protection greater than or equal to 10% are colored in blue. These changes indicate less deuterium uptake in the presence of IP₄. The PH domain of P-Rex2 (216-381) exhibits IP₄-dependent protection in the PIP₃-binding site. B) P-Rex2 PH model colored according to difference HDX-MS data at the 10,000 s time point. Blue and light red regions indicate less and more deuterium uptake, respectively, in the presence of IP₄. IP₄ is docked based on the P-Rex1 PH•IP₄ structure (PDB: 5D3X) and shown transparently in cyan. Loops in between β -strands are labeled. C) HDX-MS data from Ravala et al., 2024 shown on a Wood's plot for comparison. P-Rex1 PH (247-418) shows protection from deuterium uptake in the PIP₃-binding site in the presence of IP₄. D-E) The IP4P subdomain of P-Rex1 (1036-1316) shows increased protection from deuteration in the 4HB₁ and 4HB₂ helices. However, the P-

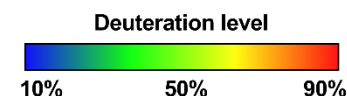
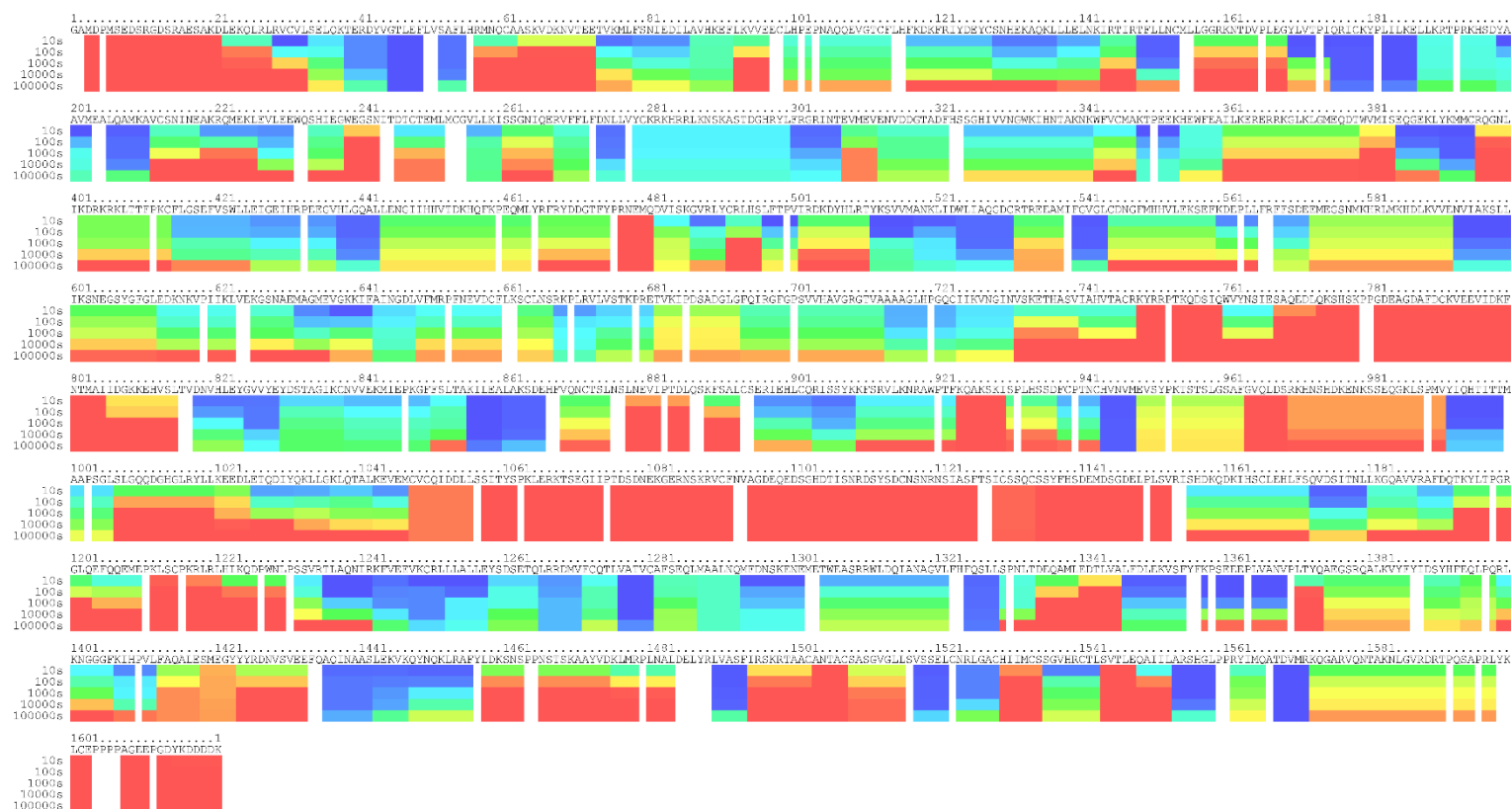
Rex2 IP4P subdomain (996-1296) does not show any significant change in deuteration uptake in the presence of IP₄.

Figure 3.10. HDX-MS data on P-Rex2 with and without IP₄. Ribbon maps representing HDX-MS experiments with P-Rex2 alone and bound to IP₄. Also shown are the changes in exchange rates that occur in P-Rex2 upon IP₄ binding.

Ribbon Map of P-Rex2 (% deuteration)



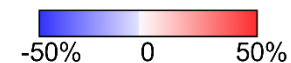
Ribbon Map of P-Rex2 in P-Rex2•IP₄ Complex (% deuteration)



Influence of IP₄ on Exchange in P-Rex2 (% deuteration)



Blue indicates regions that exchange slower in the presence of IP₄.
 Red indicates regions that exchange faster.



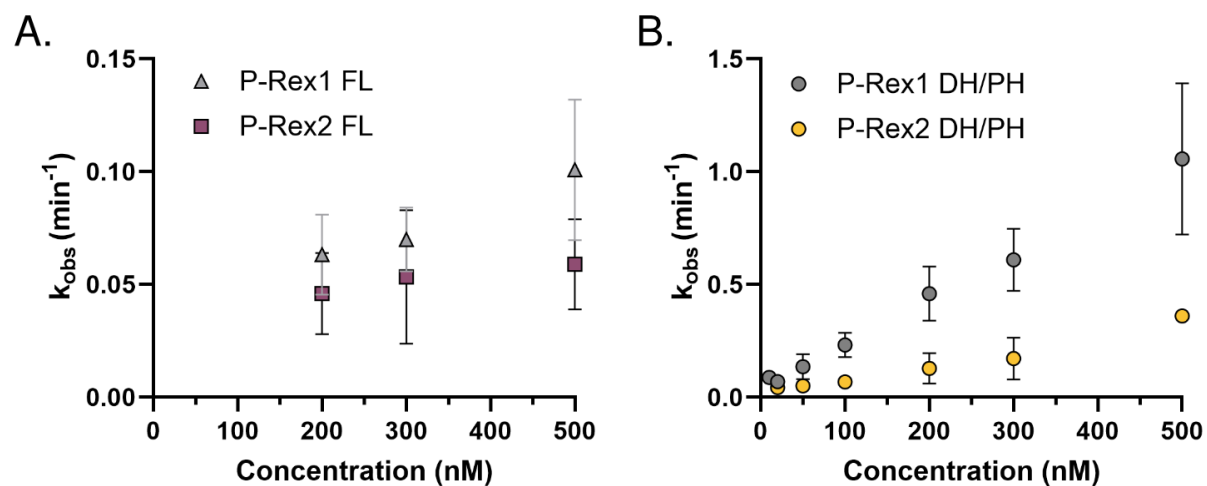


Figure 3.11. P-Rex2 exhibits lower basal activity against soluble Rac1 than P-Rex1. A) *In vitro* GEF activity assay with purified full-length P-Rex1 and P-Rex2. B) GEF activity of purified P-Rex1 and P-Rex2 DH/PH constructs assessed at different concentrations. Error bars indicate standard deviation.

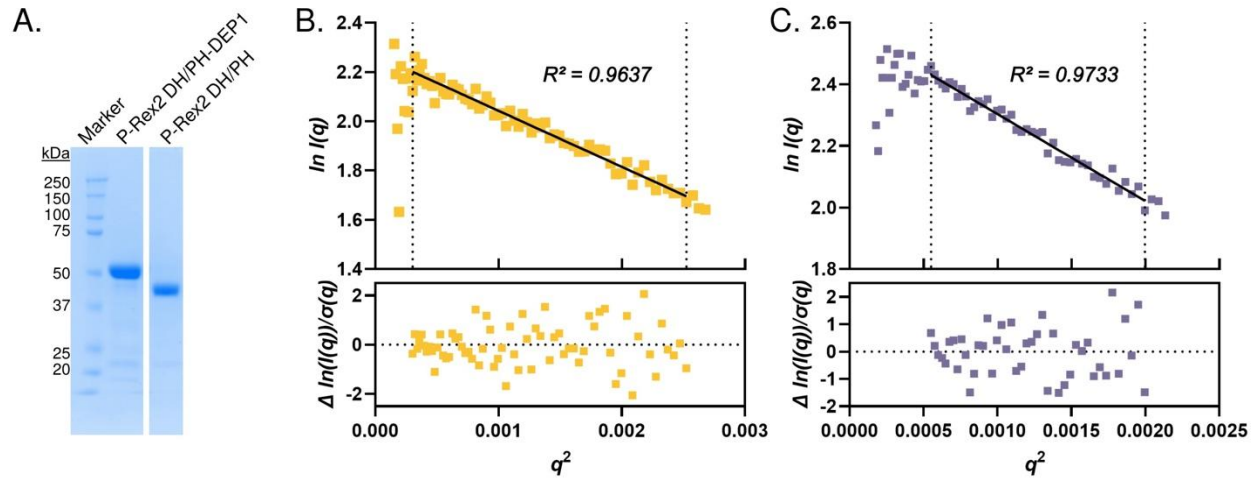


Figure 3.12. P-Rex2 SEC-SAXS data analysis. A) SDS-PAGE of purified P-Rex2 DH/PH and DH/PH-DEP1 constructs used in SEC-SAXS and GEF activity assay experiments. B) P-Rex2 DH/PH and C) P-Rex2 DH/PH-DEP1 Guinier plots.

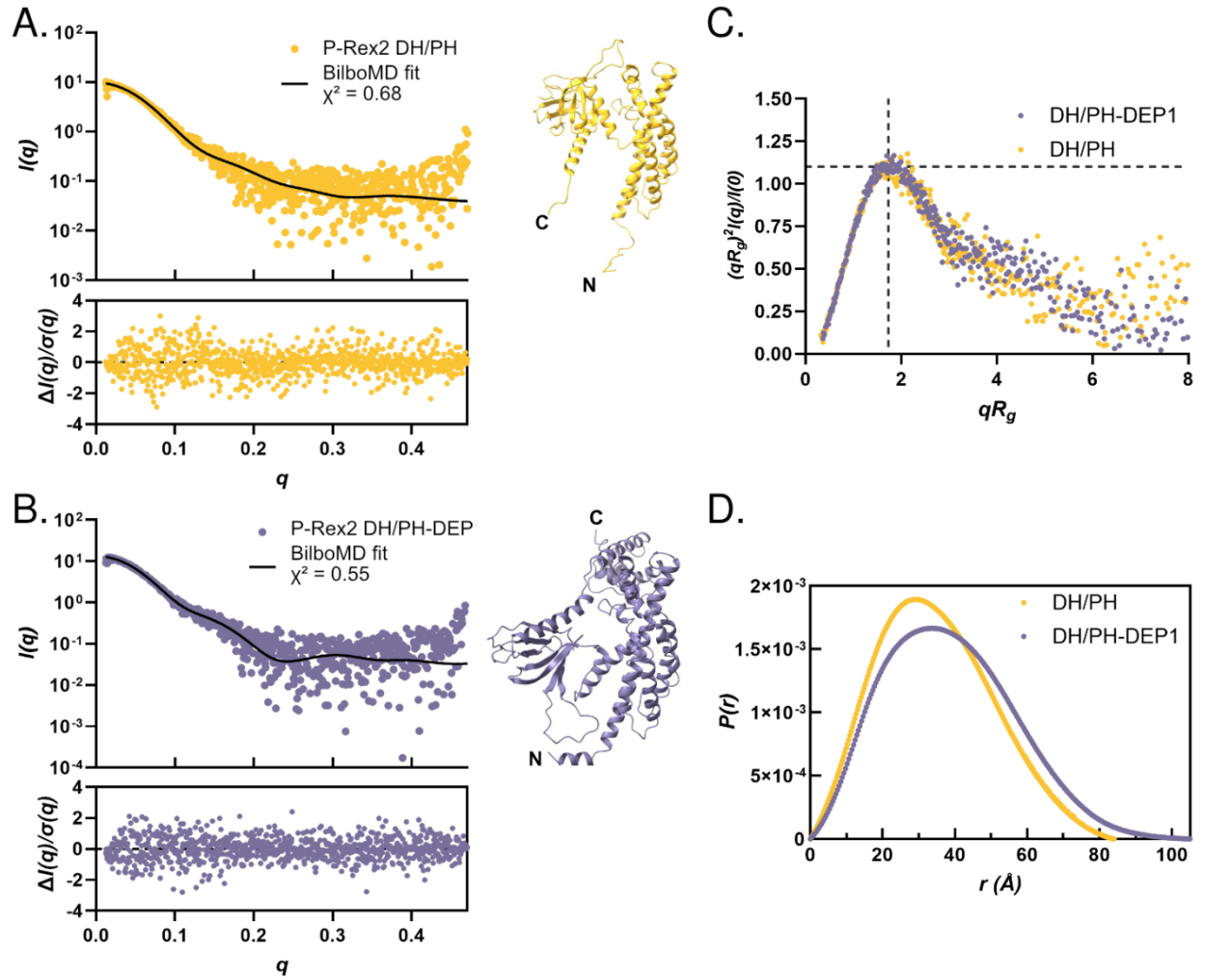


Figure 3.13. SEC-SAXS data show that P-Rex2 DH/PH takes on a single, compact state. A-B) Scattering intensity plots of P-Rex2 DH/PH (yellow) and DH/PH-DEP1 (purple) fit with the BilboMD model. The output BilboMD model is shown to the right of the plot. Normalized fit residuals are shown in the bottom panel. C) Dimensionless Kratky plot for P-Rex2 DH/PH and DH/PH-DEP1. There is no significant change in shape between the DH/PH and DH/PH-DEP1. D) Pair distance distribution function of P-Rex2 DH/PH and DH/PH-DEP1.

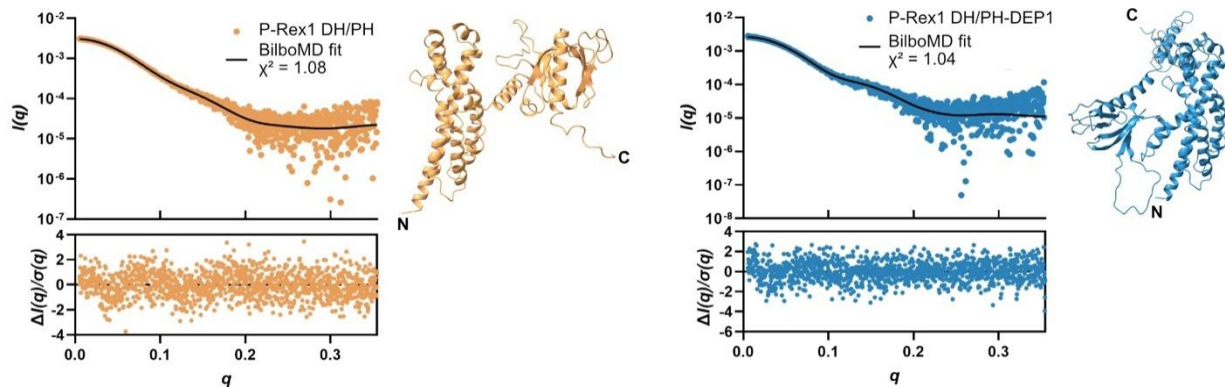


Figure 3.14. BilboMD re-analysis of P-Rex1 DH/PH and DH/PH-DEP1 SEC-SAXS data. Published SEC-SAXS datasets of P-Rex1 DH/PH and DH/PH-DEP1 (SASDHY9 and SASDHW9; Ravala et al., 2020) were analyzed using the BilboMD program. Scattering intensity plots of P-Rex1 DH/PH (orange) and DH/PH-DEP1 (blue) fit with the BilboMD model. The output BilboMD model is shown to the right of the plot. Normalized fit residuals are shown in the bottom panel.

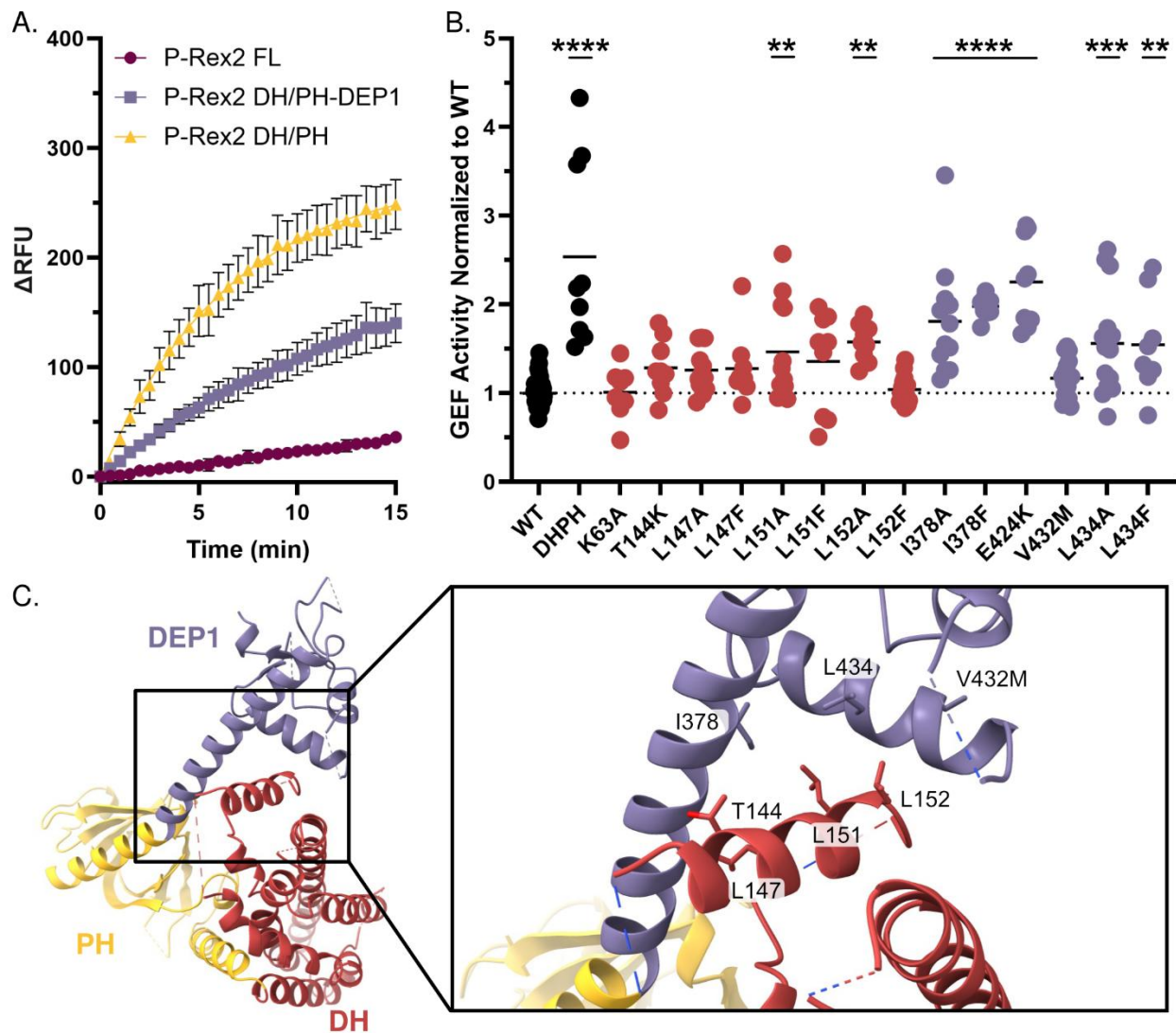


Figure 3.15. The P-Rex2 DEP1 domain exerts autoinhibition on the catalytic core via interactions with the DH domain. A) *In vitro* GEF activity assay with soluble Rac1 plus P-Rex2 FL (300 nM), DH/PH-DEP1 (300 nM), and DH/PH (200 nM). Error bars represent standard deviation. B) GEF activity assay with soluble Rac1 plus P-Rex2 DH/PH-DEP1 variants, colored according to the domain that the mutation lies in. C) Residues that were evaluated for their importance in maintaining autoinhibition through the DH-DEP1 interface.

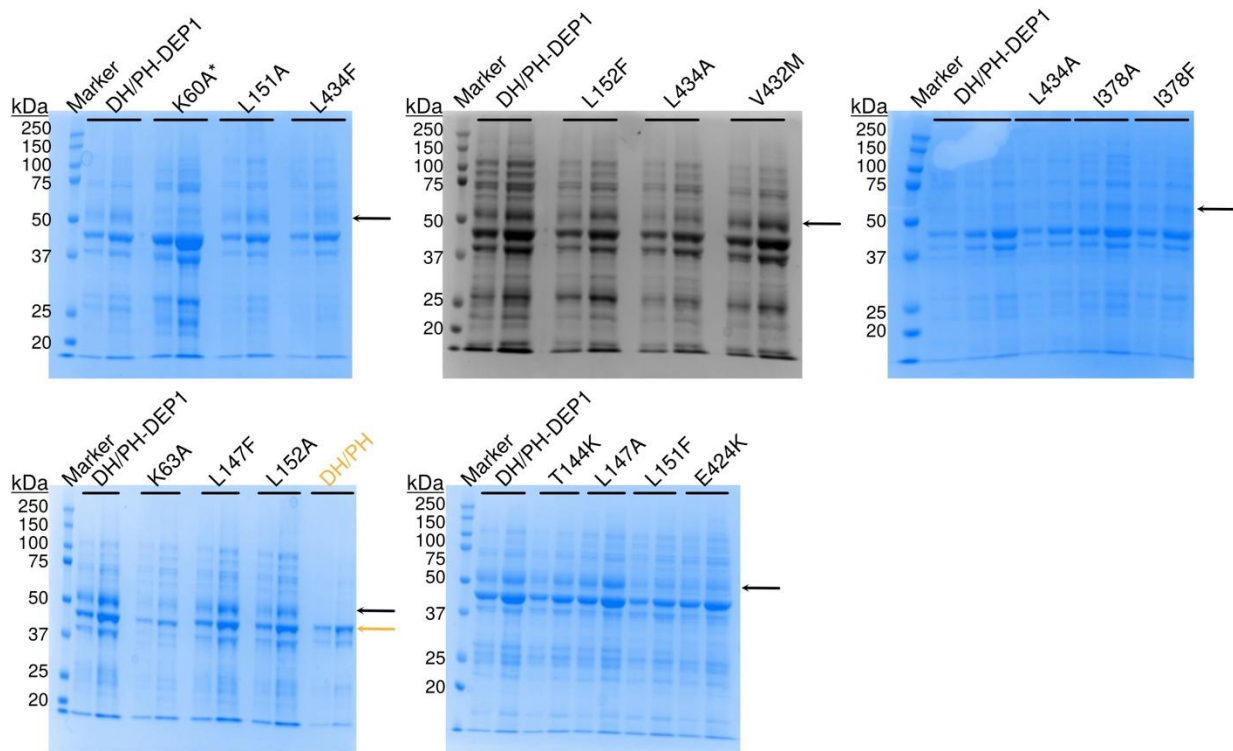


Figure 3.16. SDS-PAGE of P-Rex2 DH/PH-DEP1 and DH/PH proteins used in Figure 6 GEF activity assays.

The black arrow indicates the band that represents each P-Rex2 DH/PH-DEP1 construct (~51 kDa). The yellow arrow indicates the band that represents P-Rex2 DH/PH (~44 kDa). For each construct, two lanes were run with different sample volumes to aid in estimating sample concentration.

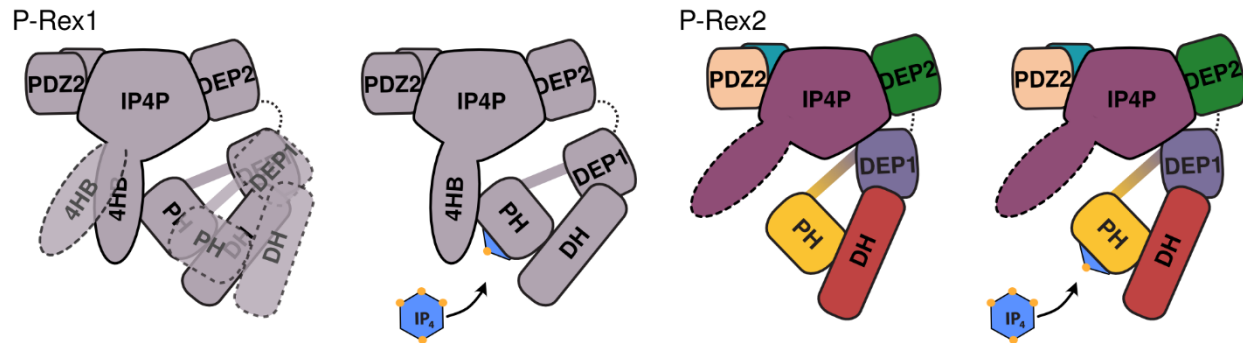


Figure 3.17. Overview of structural differences between P-Rex2 and P-Rex1. Although the structure of each the N-terminal module and C-terminal core are conserved between P-Rex2 and P-Rex1, the positioning of the two relative to one another is divergent. Furthermore, the autoinhibited form of P-Rex1 exhibits a conformation where the N-terminal module interacts with the 4HB subdomain of the IP4P domain, both of which appear to be otherwise flexibly tethered to the C-terminal core. This conformation is stabilized by binding IP₄. In contrast, the P-Rex2 N-terminal module docks to the bottom of the C-terminal core and lacks any interaction with the IP4P subdomain which was not resolved in our cryo-EM data, presumably because it is flexibly tethered to the core. IP₄ binding has no apparent effect on the structure or dynamics of P-Rex2 outside of the PIP₃-binding site.

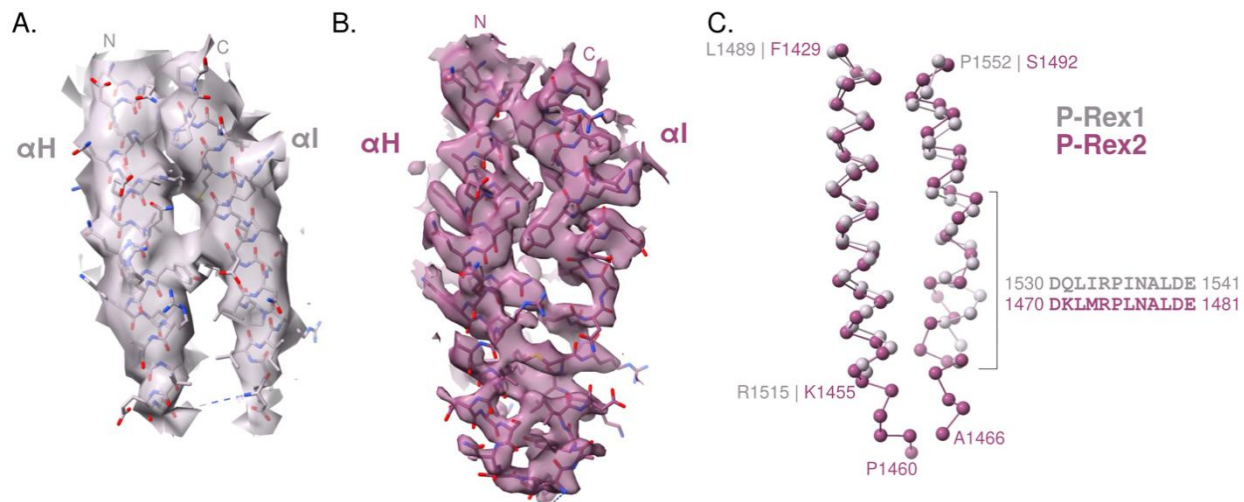


Figure 3.18. Comparison of the α H/ α I interface between P-Rex1 and P-Rex2. Cryo-EM maps with the models fit showing how the map interpretability in regions around the α H and α I helices in A) P-Rex1 (PDB: 8TUA, EMDB-41621) and B) P-Rex2 are significantly different. There is also greater resolvability in the loop connecting these features in P-Rex2. C) The isolated C α trace shows that this difference is most pronounced in the alternate structure from D1470 to E1481 in P-Rex2.

Chapter 4. Conclusions and Future Directions

This chapter will summarize the key research findings and discuss the value and contribution they add to the field. Additionally, this chapter will review the limitations of the research and describe the outstanding research questions in the field which have not yet been addressed. This study aimed to identify the structure and autoinhibitory mechanism of P-Rex2, compare these characteristics to the closely-related RhoGEF P-Rex1, and identify trends in RhoGEF storage and freezing techniques. Overall, this study found that P-Rex2 is structurally well-conserved with P-Rex1, mirroring the same domain arrangements within the N-terminal module and C-terminal core. However, it was surprising that the orientation of the N-terminal module with respect to the C-terminal core is different in P-Rex2 compared to P-Rex1, indicating a potential explanation for the observed functional and regulatory differences between the two proteins. After further exploration, we confirmed that while P-Rex2 shares an autoinhibitory mechanism within the N-terminal module, an IP_4 -dependent mechanism between the N- and C-terminal portions of the protein is not retained, reinforcing the idea that closely related RhoGEFs can diverge significantly in their regulatory strategies.

In parallel, this work highlights sample handling as an often-overlooked variable in the RhoGEF field. Analysis of flash freezing and cryoprotectant conditions demonstrates that RhoGEF activity can be significantly altered over relatively short periods of time and result in increased variability in these measurements. These findings importantly, yet unfortunately, revealed no universal storage condition between the RhoGEFs tested which emphasizes that even among closely related proteins, careful, protein-specific validation is required. Together, these results challenge a common assumption in the field that freezing does not alter the biochemical properties of RhoGEFs or that assumptions can be made about their functionality based on similarity alone. While actionable conclusions broadly applicable to the RhoGEF field are still unavailable at this time, this underscores the complexity of RhoGEF regulation and stability at both the molecular and methodological levels. These findings suggest

that RhoGEFs may broadly be sensitive to freezing and specific storage conditions. It is crucial going forward to thoroughly test the properties of frozen RhoGEF proteins and accurately describe and report any observed changes. By prioritizing characterization efforts, we will be able to improve rigor and reproducibly in the field.

These insights are particularly important given the growing interest in RhoGEFs as therapeutic targets. Rational inhibitor design has been limited by a lack of detailed structural and mechanistic understanding. The identification of distinct structural features and regulatory mechanisms in P-Rex2 provides a framework for selectively targeting individual P-Rex isoforms. In particular, regions that differ between P-Rex1 and P-Rex2, like the organization and dynamics of the N-terminal module, represent promising candidate sites for selective inhibition. Additionally, our results combined with work from the Ellisdon²⁵ and Parsons^{56,85} labs have also unlocked the potential to utilize our knowledge of P-Rex2–PTEN interactions to guide rational drug design. Emerging approaches, including generative protein design and AI-guided ligand discovery, may enable the development of molecules that exploit these newly identified structural features. Such tools could accelerate the creation of isoform-specific inhibitors with applications not only probing RhoGEF biology for understanding the *in vivo* roles of these proteins, but also in targeting diseases such as cancer and diabetes.

While this work establishes a structural and mechanistic framework for understanding P-Rex2 regulation and its divergence from P-Rex1, several limitations and unresolved questions remain. One limitation of this work is that we were unable to identify a universal cryoprotectant that improved storage and freeze-thaw resilience across all RhoGEFs tested. While this may indicate that a larger and more diverse sampling of RhoGEFs is needed to identify broader trends, additional cryoprotectants like ethylene glycol, inositol, sodium glutamate, glycine, proline, and sodium acetate⁴⁶ could also be evaluated in future studies or testing alternative storage approaches such as lyophilization. Furthermore, freezing and handling studies were conducted only on isolated DH/PH tandems rather

than full-length proteins. Preliminary success with P-Rex2 DH/PH stored in 10% sucrose suggests that similar approaches may stabilize the full-length protein for long-term storage, although this remains to be experimentally validated. If successful, these methods could reduce waste associated with proteins produced in expensive eukaryotic expression systems and improve experimental efficiency by enabling reliable use of freeze-thawed samples.

Another limitation of this study was the absence of density corresponding to the IP4P subdomain in the cryo-EM reconstruction. Although we currently hypothesize that this missing density reflects intrinsic flexibility within this region, our findings also suggest potential biological and structural explanations for why the subdomain remains dynamic and is not stabilized by IP₄ addition alone. In the future, formation of a P-Rex2–PTEN complex may provide a strategy for stabilizing this region and resolving its structure. Alternatively, approaches using crosslinked samples may be able to accomplish a similar goal.

This work also raises several fundamental questions regarding how P-Rex2 is regulated, integrated into signaling networks, and activated. One outstanding question centers on the contributions of the IP4P subdomain in P-Rex2 regulation which remains unclear. In P-Rex1, this subdomain clearly contributes to autoinhibition by promoting a closed conformation through interactions with the PH domain^{15,20}. In contrast, structural modeling and crosslinking data suggest that the P-Rex2 IP4P subdomain is unlikely to access the same surface of the PH domain as in P-Rex1²⁵. However, as discussed in Chapter 3, crosslinking between the IP4P subdomain and the N-terminal module increases upon PTEN addition, suggesting that PTEN binding may stabilize interactions that are otherwise transient or inaccessible. Rather than relying on an IP₄-dependent stabilization mechanism, P-Rex2 may require PTEN to stabilize interactions between the IP4P subdomain and the N-terminal module, leading to a closed, compact state mirroring that of P-Rex1. Furthermore, studies along these lines could contribute to a broader effort to understand why P-Rex2, but not P-Rex1, selectively

interacts with PTEN. The structural distinctions between P-Rex1 and P-Rex2 in combination with previous biochemistry on the P-Rex2-PTEN complex have begun to reveal potential mechanistic frameworks for this isoform-specific interaction, which has important implications for disease-relevant signaling pathways. The precise PTEN binding site on P-Rex2 still remains a contentious topic, but techniques such as HDX-MS and cryo-EM may be critical for resolving this long-standing question. In parallel, although substantial evidence suggests that P-Rex1 does not bind PTEN²⁵, identifying the molecular basis for this selectivity could reveal additional isoform-specific regulatory surfaces with therapeutic potential or even how some P-Rex2 mutations escape inhibition through PTEN.

The N-terminal module of P-Rex2 is more readily resolved by cryo-EM than the corresponding region of P-Rex1 in apo samples, suggesting that this module may be intrinsically less flexible. This difference may influence how this region regulates full-length basal activity and contributes to potentially distinct activation mechanisms. In P-Rex1, the N-terminal module has been proposed to extend along the plasma membrane surface during activation²⁰. In part this large conformational shift may be due to the flexible linker connecting the two DEP domains. However, the P-Rex2 linker connecting these domains is composed of bulkier, more rigid residues leading to much clearer density of this region compared to P-Rex1. We hypothesize that differences between these linkers could potentially alter membrane engagement or conformational rearrangements in P-Rex2. Future experiments involving sequence swaps between the corresponding linker regions in P-Rex1 and P-Rex2 and model membrane systems may help define the structural basis for these differences and determine whether the DEP1-DEP1 linker may also play a regulatory role with respect to activation and represent a potential therapeutic target.

The reduced activity observed between the isolated P-Rex2 N-terminal module and the full-length protein (Fig. 3.15A) suggest that the C-terminal accessory domains contribute to autoinhibition, although likely through mechanisms distinct from those described for P-Rex1. In P-Rex1, the IP4P

subdomain forms a secondary autoinhibitory interaction with the PH domain. However, the substantial decrease in activity observed upon inclusion of the P-Rex2 C-terminal domains suggests the presence of additional regulatory mechanisms that remain unresolved. Defining these mechanisms may reveal new regulatory interfaces and potential sites for therapeutic intervention. This question may also have biological relevance in the context of the P-Rex2 isoform P-Rex2b, which lacks most of the IP4P domain. Studying P-Rex2b or additional truncated constructs may help clarify how the C-terminal domains contribute to autoinhibition and activation, while also identifying regulatory elements unique to P-Rex2.

Although substantial effort has focused on defining the autoinhibitory mechanisms of P-Rex1 and P-Rex2, comparatively little is known about how these proteins transition into their active states. Understanding activation in the context of membrane association, PIP_3 signaling, and $\text{G}\beta\gamma$ binding will be essential for establishing how P-Rex proteins function under physiological conditions. Furthermore, defining these activation mechanisms will provide critical insight into how disease-associated mutations perturb normal regulation and contribution to pathological signaling.

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