Mechanism of GTPase activation of a prokaryotic small Ras-like

2 **GTPase MgIA by an asymmetrically interacting MgIB dimer**

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- 10 Running title: GTPase activation by an asymmetrically interacting dimer
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- 12 Abbreviations: GAP: GTPase Activating Protein; *mant*: 2'/3'-O-(N-methyl
- 13 anthraniloyl; GDP: guanosine di-nucleotide; GMPPNP: guanosine-5'-[(β, γ) -
- 14 imido]triphosphate; GTP: guanosine 5'-tri-phosphate; GTPγS: guanosine:5'-O-
- 15 [gamma-thio]triphosphate; LDH: lactate dehydrogenase; PCR: Polymerase Chain
- 16 Reaction; PDB: Protein Data Bank; P_i: inorganic phosphate; PK: pyruvate kinase;
- 17 MgIA: Mutual gliding protein A; MgIB:Mutual gliding protein B, NADH: nicotinamide
- 18 adenine dinucleotide.

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21 Abstract

22 Cell polarity oscillations in *Myxococcus xanthus* motility are driven by a prokaryotic 23 small Ras-like GTPase, MgIA, which switches from one cell pole to the other in 24 response to extracellular signals. MgIA dynamics is regulated by MgIB, which 25 functions both as a GAP (GTPase activating protein) and a GEF (guanine nucleotide exchange factor) for MgIA. With an aim to dissect the role of asymmetry in the dual 26 GAP and GEF activities of MgIB, we generated a functional MgIAB complex by co-27 28 expressing MgIB with a linked construct of MgIA and MgIB. This strategy enabled us to generate mutations of individual MgIB protomers (MgIB₁ linked to MgIA or MgIB₂) 29 30 and delineate their role in GEF and GAP activities. We establish that the C-terminal 31 helix of MglB₁, but not MglB₂, stimulates nucleotide exchange through a site away 32 from the nucleotide-binding pocket, confirming an allosteric mechanism. Interaction 33 between the N-terminal β -strand of MgIB₁ and β_0 of MgIA is essential for the GEF 34 activity of MgIB. Specific residues of MgIB₂ which interact with Switch-I of MgIA partially contribute to its GAP activity. Thus, the role of the MglB₂ protomer in the 35 36 GAP activity of MgIB is limited to restricting the conformation of MgIA active site 37 loops by steric hindrance. The direct demonstration of the allosteric mechanism of GEF action provides us new insights into the regulation of small Ras-like GTPases, a 38 39 feature potentially present in many uncharacterized GEFs.

40 Keywords: Prokaryotic small Ras-like GTPase MgIA, MgIB, asymmetry, GAP,
41 Roadblock domain, GEF, allostery.

Introduction 42

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Polarity determination and its regulation are critical to several vital cellular processes 44 like signal transduction, cell growth and motility¹. Myxococcus xanthus is a Gram-45 46 negative soil bacterium which has been used as a model organism to study polarity reversals that characterize its gliding motility pattern². Polarity reversals are 47 48 associated with spatial oscillations of the intracellular proteins as the bacterium switches its direction of movement. Frz chemosensory system and the small Ras-like 49 GTPase MgIA are two main players in the regulation of these oscillations $^{3-5}$. 50

Small Ras-like GTPases play a key role in determining cell polarity⁶. These GTPases 51 usually act as molecular switches alternating between its GTP-bound active 52 conformation ('ON' state) and the GDP-bound inactive conformation ('OFF' state). 53 54 This conformational transition reorients residues of the GTPase catalytic pocket, which constitutes the conserved Switch loops (Switch-I and Switch-II) which further 55 56 signal downstream effectors. Most of these GTPases are associated with their 57 respective GTPase Activating Proteins (GAPs), which assist GTP hydrolysis, and 58 Guanine nucleotide Exchange Factors (GEFs), which stimulate nucleotide exchange⁷. MgIA, a prototypic member of the prokaryotic small Ras-like GTPase 59 60 family, localizes to the leading pole of the cell in its GTP-bound state, and the cell reverses polarity when it relocates^{8,9}. MgIB, which consists of a Roadblock/LC7 (RbI) 61 domain, acts as a GAP for MgIA¹⁰, and predominantly localizes to the opposite pole. 62 We recently demonstrated that MgIB also exerts a nucleotide exchange effect on 63 MgIA, thus performing the dual role of a GAP and a GEF¹¹. Such a dual mechanism 64 65 requires to be tightly regulated in the cell to prevent a futile cycle of MgIA GTP hydrolysis. But this mechanism of regulation of MgIB activity in light of cell polarity 66

67 reversal is not clearly understood. Interestingly, this was in contrast to another report 68 of MgIB acting only as a GAP and not as a GEF for MgIA¹². A complex of proteins 69 comprising the response regulator, RomR along with its partner RomX was 70 established as the GEF for MgIA ¹⁴. Recently an activator of MgIB, namely RomY 71 was identified which further stimulates the GAP activity of MgIB ¹³. MgIB also acts as 72 GAP for SofG, another small Ras-like GTPase involved in Myxococcus xanthus 73 motility¹⁵.

74 The structure of the MgIA-MgIB complex revealed that a homodimer of MgIB interacts with one molecule of MgIA (MgIA: MgIB = 1:2) resulting in an asymmetric 75 interaction between the two molecules^{10–12}. In the asymmetric complex, the two 76 77 protomers of MalB (hereafter referred to as MalB₁ and MalB₂) interact with different 78 residues of the MgIA monomer (Fig. 1A). Binding of MgIB reorients the Switch-I and 79 Switch-II loops of MgIA. As a result of this conformational change, Thr-54, which completes the Mg²⁺ coordination in MgIA, and Arg-53, which is the catalytic residue 80 81 near the nucleotide-binding pocket, are optimally positioned for hydrolysis. This is a mechanism of indirect GAP activity^{10,11,16}, where the GAP does not provide any of 82 the active site residues, but functions by orienting the active site residues of the 83 GTPase. 84

Another feature of the asymmetric MgIA-MgIB complex (PDB ID: 6IZW) is that the Cterminal α -helix (Ct-helix) of MgIB₁ binds in a pocket formed by the α_5 helix and the extended β_2 - β_3 loop of MgIA (Fig. 1B). According to our earlier observations, the Cthelix of MgIB is the GEF-active region¹¹. A C-terminal truncated construct of MgIB (last 20 amino acids of MgIB truncated, hereafter termed as MgIB^{Δ Ct})¹¹ interacted only with MgIA-GTP state, compared to the full-length MgIB which has an affinity for

MgIA also in the GDP bound state. It was also observed that the Ct-helix of MgIB is responsible for the nucleotide exchange activity on MgIA¹¹, by stimulating the release of GDP. In the crystal structure, the Ct-helix is ordered in the MgIB₁ monomer only. The corresponding residues in MgIB₂ are not ordered in the crystal structure, indicating that they are flexible.

96 The N-terminal residues of the two protomers contribute to an additional asymmetric feature. In MglB₁, the first helix of the Rbl/LC7 fold extends from Glu-11 whereas, in 97 98 MglB₂, the helix initiates from Tyr-8. The preceding residues 1–7 are disordered in 99 MglB₂ (Fig. 1A). However, the residues 2-7 of MglB₁ constitute a β -strand that forms 100 hydrogen bonds with the β_0 strand of MgIA which extends the central β -sheet in the 101 MgIA fold (Fig. 1C). These residues of MgIB thus form part of its binding interface 102 with MgIA. Hence, it is interesting to find whether this strand plays a role in orienting 103 MgIB optimally with respect to MgIA.

The existence of dual GAP and GEF activities by MgIB necessitates that the activities need to be tightly regulated to control the ON and OFF states of MgIA in the cell. Comparison of the crystal structures of MgIA-GTP equivalent states with and without MgIB (PDB ID: 6IZW, 6H17) shows that Switch-I takes a different conformation in the absence of MgIB ^{11,12}. Asn-37 and Arg-98 of MgIB₂ interact with Switch-I residues of MgIA in the MgIB-bound conformation and it is plausible that the interactions might specifically regulate MgIB GAP activity (Fig. 1D).

111 The most common mechanism of GEF activity for small Ras-like GTPases involves 112 direct binding of GEF residues at the vicinity of the nucleotide-binding pocket, such 113 that nucleotide and/or Mg²⁺ are evicted from the pocket¹⁷. The disordered C-terminal

114 end of the MglB₂ protomer lies close to the nucleotide-binding pocket of MglA and could drive nucleotide exchange by directly interacting with the switch regions of 115 MgIA. Interestingly, the C-terminal end (last 20 residues) of MgIB contains a stretch 116 117 of conserved negatively charged residues (Fig. 1A), which might compete with the 118 binding of phosphate moieties of the nucleotide to MgIA. This plausible mechanism of direct GEF activity is very similar to the TRAPPI complex, where a C-terminal 119 120 extension of Bet3p, which contains conserved glutamate, stretches to the nucleotidebinding pocket of the Rab GTPase Ypt1p¹⁸. 121

Alternatively, the ordered Ct-helix from the MglB₁ protomer in the crystal structure of 122 123 the MgIAB complex (6IZW), which lies opposite to the MgIA nucleotide-binding 124 pocket, suggests a unique allostery-based mechanism that can indirectly regulate 125 nucleotide exchange by MgIA. A comparison of MgIA-GDP (PDB ID: 5YMX) bound 126 and MgIAB-GMPPNP (PDB ID: 6IZW) bound structures reveals a registry shift of two amino acids and a unique flipping of the β_2 strand in MgIA (β -screw movement), 127 which exposes the hydrophobic side chains towards the MgIB binding interface^{10–12}. 128 129 If the interaction of the Ct-helix of the MgIB₁ protomer stabilizes the conformation of MgIA with the flipped β_2 -strand, this might favour MgIA to bind GTP over GDP, 130 thereby resulting in nucleotide exchange ¹². The flipped conformation also orients 131 Asp-58, the Walker B residue that assists Mg²⁺ coordination in the GTP-bound 132 state¹⁹. 133

For a mechanistic understanding of GEF action, it is critical to decipher whether the C-terminal region of $MglB_1$ or $MglB_2$ drives nucleotide exchange by MglA. With this goal, we successfully designed and purified, and biochemically characterized complexes of MglAB with C-terminal residues deleted from either MglB₁ or MglB₂.

138 Consequently, we establish that the Ct-helix from MgIB₁ allosterically drives 139 nucleotide exchange by MgIA, while the C-terminal residues from MgIB₂ are not 140 essential for GEF activity. The observation highlights a novel strategy of GEF 141 function based on allosteric control of a conformation favourable for GTP binding. 142 Such a mechanism of regulation by targeting the β_2 - β_3 loop of small Ras-like GTPases is potentially found in the GEF action of many prominent small Ras-like 143 144 GTPase families such as Rag and Arf GTPases, which share many common features with the MgIA family of Ras-like GTPases¹⁹. Furthermore, truncating the N-145 146 terminal β-strand of MgIB significantly reduces the GEF activity of MgIB, keeping the 147 GAP activity intact. This shows that the asymmetric interaction of the N-terminal β -148 strand of MglB₁ with the MglA central β-sheet is important for orienting the MglB Ct helix. On the other hand, mutations of Asn-37 and Arg-98 to alanines (MgIB^{N37R98A}) 149 150 keep the GEF activity intact. Instead, it reduces the GAP activity of MgIB as these 151 residues are involved in sterically orienting the switch loops of MgIA to drive GTP 152 hydrolysis. Hence, we attribute the GAP activity of MgIB to the MgIB dimeric interface 153 consisting predominantly of the MglB₂ protomer. Therefore, our study functionally 154 dissects the contributing factors to the contrasting GAP and GEF activities performed 155 by MgIB.

156 **Results**

157 **Design of the linked constructs for a functional asymmetric MgIAB complex**

158 To delineate the functional asymmetry between the two MgIB protomers, it was 159 essential to generate an asymmetric dimer of MgIB, in which only one of the MgIB 160 protomers (either MglB₁ or MglB₂) possessed the C-terminal residues. Because the 161 asymmetry in MgIB dimer arises only upon interaction with MgIA, we designed a 162 construct linking the C-terminus of MgIA with the N-terminus of MgIB (Fig. 2A). The 163 C-terminus of MgIA was proximal to the N-terminus of MgIB₁ protomer. Hence, we predicted that a short linker between MgIA and MgIB will suffice to form a functional 164 165 MgIA-MgIB₁ linked construct (Fig. 2A). The amino acid linker between MgIA and 166 MgIB was chosen such that it is flexible enough to allow the functional complex 167 formation but sufficiently rigid to disallow excessive free rotation between MgIA and 168 MglB. Hence, a Gly-Ser linker (GS) between MglA and MglB was designed based on 169 the positions of the C-terminal and N-terminal ends of MgIA and MgIB₁ respectively in the crystal structure of the MgIAB complex (PDB ID: 6IZW; Fig. 2A) and the crystal 170 171 packing in MgIA-GDP structure, where the C-terminal hexahistidine tag formed a β -172 strand which continued the central β -sheet of MgIA (PDB ID: 5YMX; Fig. 2A).

Purification from expression trials of the MgIA-link-MgIB₁ construct, without coexpression with MgIB, was unsuccessful because we could obtain only a negligible amount in the soluble fraction. This is possible because a dimeric MgIA-link-MgIB₁ might be in negligible amounts, resulting in an exposed MgIB dimer interface for the majority of the molecules. Hence, we adopted a co-expression strategy of MgIA-link-MgIB₁ and MgIB. Co-expression from a single plasmid was carried out where MgIB₂

and MgIA-link-MgIB₁ respectively were expressed as an operon, designed based on the *mgIBA* operon in *Myxococcus xanthus* genome (Fig. 2B; See Methods). In a coexpression system, an MgIB monomer could interact with the exposed MgIB dimer interface of the MgIA-link-MgIB₁ construct. Henceforth, a complex containing a linked MgIA-link-MgIB₁ construct is represented by an enclosure within '{' & '}' (for example, {AB^{WT}}; Fig 2B), while the absence of '{' and '}' represents a complex formed by mixing purified batches of the different proteins (eg. AB^{WT}).

MgIA-link-MgIB₁ and MgIB₂ were designed with a C-terminal hexahistidine tag 186 (GSHHHHHH) and a Strep-tag (WSHPQFEK), respectively, which could enable the 187 purification of the linked complexes by sequential affinity chromatography (Ni-NTA 188 and StrepTrap respectively) (Fig. 2C)²⁰. This purification strategy was further applied 189 for the C-terminal truncated constructs of MglB₁, and MglB₂, respectively, to 190 191 generate the asymmetric complexes comprising MgIB with and without the Cterminal residues ($\{AB_1^{\Delta Ct}B_2\}$, $\{AB_1B_2^{\Delta Ct}\}$; Fig. 2C). $\{AB^{WT}\}$, a linked construct of wild 192 type MgIB with MgIA, and $\{AB^{\Delta Ct}\}$, a linked construct of C-terminal truncated MgIB 193 194 with MgIA, functioned as controls. Any dissociation of the complexes would lead to the elution of MgIB dimer as a separate fraction from the Strep-Trap column due to 195 the higher affinity (MgIB dimer contains two Strep-tags, compared to the linked 196 197 complex with one Strep-tag).

198 Sequential affinity chromatography yielded asymmetric linked complexes

 $\{AB^{WT}\}\$ was purified by sequential affinity-based chromatography (Ni-NTA followed by StrepTrap) which ensured successful isolation of the complex (Fig. 3A). For $\{AB^{WT}\}\$, the majority of the linked complex eluted from the StrepTrap column in the 202 initial injection volumes of D-desthiobiotin (fraction I in Fig. 3A), with a negligible 203 amount of MgIB dimers (Fig. 3A) at later fractions (fraction II; MgIB dimers will have higher affinity to the column due to the presence of two Strep-tags per dimer). 204 205 Consequently, we proceeded with purifying all the other linked constructs with the respective C-terminal deletions (Fig. 3B-E). $\{AB_1B_2^{\Delta Ct}\}\$ behaved similarly to $\{AB^{WT}\}\$ 206 through Ni-NTA and StrepTrap columns (Fig. 3B). Interestingly, fraction II of elution 207 for $\{AB_1^{\Delta Ct}B_2\}$ (Fig. 3C) and $\{AB^{\Delta Ct}\}$ purifications (Fig. 3D) contained more prominent 208 bands corresponding to MglB₂ and bands of MglA-link-MglB₁, suggesting a 209 210 possibility of shuffling of protomers following Ni-NTA elution.

211 The presence of C-terminal residues of MgIB contributes to an aberrant movement in SEC¹¹, and hence the molecular weights of the elution peaks in SEC were further 212 confirmed using size exclusion chromatography coupled with multi-angle light 213 scattering studies (SEC-MALS; Fig. 4A, 4B). The single peak of {AB^{WT}} (peak 1 214 215 eluted at 14.2 ml; Fig. 4A) corresponded to a molar mass of around 53 kDa, close to the expected mass of the complex (tabulated in Fig. 4C). The homogenous single 216 peak of $\{AB_1B_2^{\Delta Ct}\}$ (peak 1 eluted at 14.9 ml) corresponded to an average molar 217 mass of 56 kDa (Fig 4A, 4C). The difference in elution volume with respect to {AB^{WT}} 218 219 despite the small difference in molecular weight could be attributed to the absence of aberrant movement without the MglB₂ Ct-helix in {AB₁B₂ $^{\Delta Ct}$ }. 220

However, the purified $\{AB_1^{\Delta Ct}B_2\}$ complex separated into two peaks in SEC (Fig. 4B). For $\{AB_1^{\Delta Ct}B_2\}$, the first peak (peak 1; eluted at 12.2 ml; Fig. 4B) corresponded to a molar mass of 112 kDa which interestingly corresponded to the mass of a dimeric $\{AB_1^{\Delta Ct}B_2\}$ complex. However, the peak was not symmetric and showed a long tail till the second peak (peak 2; eluted at 14.2 ml; Fig. 4 B) correlating with a full-length 226 MglB dimer (36 kDa) (Fig. 4C). This tailing nature of the peak corresponded to a 227 decreasing trend in the molar mass distribution, which could not be reliably estimated beyond 12.6 ml. This probably indicated the presence of a heterogeneous 228 mix of monomeric and dimeric $\{AB_1^{\Delta Ct}B_2\}$ complexes. The dissociation of protomers 229 was higher in the case of $\{AB^{\Delta Ct}\}$ which had a higher intensity of the MglB^{ΔCt} dimer 230 231 peak (labelled as peak 2; eluted at 15.6 ml) and a smaller peak corresponding to $AB^{\Delta Ct}$ (peak 1; eluted at 15.1 ml with a molar mass of approximately 55 kDa) (Fig. 232 233 4B, 4C). Thus, the sequential affinity purification and SEC results of the linked 234 complexes showed that the presence of the MglB₁ helix is critical for a stable 235 complex consisting of MgIA-link-MgIB₁ and MgIB₂. This is consistent with our 236 previous observation of interaction between GDP-bound MgIA and MgIB only in the presence of the MgIB C-terminal residues¹¹. From the observation of a stable 237 complex only when MglB₁ C-terminal helix is present, this stabilization effect is 238 239 probably a contribution from the MglB₁ C-terminal helix while the absence of it 240 destabilises the complex.

241 The presence of elution peaks corresponding to the molecular weight of MglB₂ dimers (either MgIB or MgIB^{ΔCt} in {AB₁^{$\Delta Ct}B₂} and {AB^{<math>\Delta Ct$}} purification, respectively)</sup> 242 proved that in the absence of the MglB₁ helix, dissociation of the linked MglAB 243 complex resulted in free MgIB₂ protomers from the complex. The incompatibility and 244 steric hindrance of MgIB dimer, especially MgIB₂ protomer, with the MgIA-GDP 245 conformation, might result in destabilization of the dimer interface and its 246 247 dissociation, which consequently might form MgIB dimers or trans oligomers (as observed in $\{AB_1^{\Delta Ct}B_2\}$ complex) with MgIA-link-MgIB₁ in solution. Hence, we 248

conclude that the MglB₁ Ct-helix interaction with MglA is essential for the formation
 of the stable and functionally relevant linked MglAB complexes.

251 The absence of MgIB₂ Ct-helix does not affect the GTPase activity of MgIA.

252 Following the purification of the linked MgIAB complexes, the GTPase activity of the 253 linked complexes was quantified using an NADH-coupled enzymatic assay to monitor GDP release. It was observed that $\{AB^{WT}\}$ showed comparable k_{obs} values to 254 the unlinked AB^{WT} complex which indicated the formation of a functional linked 255 complex (Fig. 5A, Table 1). Interestingly, $\{AB_1B_2^{\Delta Ct}\}$ exhibited GTP hydrolysis rates 256 257 comparable to that of the full-length complexes (linked and unlinked). This shows the 258 C-terminal residues of the MglB₂ protomer did not play a major role in increasing the net GTPase activity of MgIA (Fig. 5A, Table 1). The least activity was observed for 259 $\{AB_1^{\Delta Ct}B_2\}$ and $\{AB^{\Delta Ct}\}$ complexes both of which had the MglB₁ Ct-helix truncated. 260 261 Incidentally, the activity was equivalent to that displayed by the complex formed by MgIA and MgIB^{ΔCt} (AB^{ΔCt}) where the Ct-helix was absent in the MgIB dimer. 262 Comparable activities of unlinked MgIA and MgIB^{ΔCt} complex and linked {AB₁^{$\Delta Ct}B₂}</sup>$ 263 264 and $\{AB^{\Delta Ct}\}$ suggest that the concentrations of the MgIA domain is similar in all these 265 cases and the reduced activity is probably due to the absence of the MgIB₁ Ct-helix. Hence the reduced activity for $\{AB_1^{\Delta Ct}B_2\}$ indicates that the MglB₂ C-terminal 266 residues did not contribute to an increase in GTPase activity of MgIA. Thus, the 267 deletion of MgIB₂ C-terminal residues did not significantly affect MgIA GTP 268 269 hydrolysis, while the presence of the MglB₁ helix was preferentially critical for accelerating the rate. 270

271 MglB₁ Ct-helix is sufficient for nucleotide exchange in MglA.

272 Next, we performed nucleotide exchange assays with fluorescently labelled mant-GDP/GMPPNP. We first investigated *mant*-GDP binding to the linked complexes 273 (labelled as Stage I) followed by its dissociation (labelled as Stage II) with excess 274 unlabelled GDP (Fig. 5B). $\{AB^{WT}\}\$ and $\{AB_1B_2^{\Delta Ct}\}\$ showed very fast association with 275 *mant*-GDP ($k_{on} = 2.7 \ \mu M^{-1} min^{-1}$ and 3.2 $\mu M^{-1} min^{-1}$, respectively). The association 276 rates of {AB₁^{Δ Ct}B₂} were slower, similar to the unlinked AB^{WT} complex ($k_{on} = 0.9 \mu$ M⁻ 277 ¹min⁻¹). {AB^{WT}} and {AB₁B₂^{Δ Ct}} showed higher rates of exchange than unlinked AB^{WT} 278 (Table 2A), probably owing to the tethering of MgIB to MgIA which increases the 279 probability of the MgIB Ct-helix association with MgIA. $\{AB_1^{\Delta Ct}B_2\}$ shows exchange 280 with a slower k_{on} (0.92 μ M⁻¹min⁻¹) than AB^{WT}. This exchange activity is probably due 281 282 to the combined effect of the presence of a linked MglB₁ and from the *trans* activity 283 by the full-length MglB₂ dimer, formed post-dissociation of the complex as indicated by the SEC-MALS study. The slowest k_{op} of 0.45 μ M⁻¹min⁻¹ was exhibited by {AB^{Δ Ct}} 284 because both the linked MgIB₁ protomer and the MgIB₂ dimer, formed from the 285 286 dissociated protomers, lacked the C-terminal residues. This was similar to what was observed in unlinked $AB^{\Delta Ct}$ (0.6 $\mu M^{-1}min^{-1}$). The rates of exchange for dissociation of 287 *mant*-GDP by adding unlabelled GDP (k_{off}) were again highest for {AB^{WT}} and 288 $\{AB_1B_2^{\Delta Ct}\}\$ (0.26 min⁻¹ and 0.33 min⁻¹, respectively) followed by $\{AB_1^{\Delta Ct}B_2\}\$ (0.18 min⁻¹ 289 ¹). {AB^{Δ Ct}} (k_{off} = 0.09 min⁻¹) exhibited slower exchange as the exchange of GDP was 290 not facilitated in the absence of the MgIB Ct-helix, similar to unlinked $AB^{\Delta Ct}$. 291

Similar nucleotide exchange experiments were performed with *mant*-GMPPNP followed by dissociation with excess unlabelled GTP (Fig. 5C) to further resolve the effect of the MglB Ct-helix deletions. { AB^{WT} } and { $AB_1B_2^{\Delta Ct}$ } showed very fast association and dissociation rates of *mant*-GMPPNP and hence values of k_{on} and k_{off} 296 could not be estimated. This could be because the linked MgIB₁ Ct-helix in both these complexes drove nucleotide exchange efficiently. $\{AB_1^{\Delta Ct}B_2\}$, without a Ct-helix 297 in the MglB₁ protomer, exhibited slower exchange kinetics as unlinked AB^{WT} with 298 measurable k_{on} and k_{off} values of 2.2 μ M⁻¹min⁻¹ and 0.18 min⁻¹, respectively. {AB^{Δ Ct}} 299 exhibited exchange rates similar to unlinked AB^{Δ Ct}, however, due to the lack of 300 stability of the complex we observed inconsistent signals for mant-GMPPNP binding 301 302 across replicates. Unlike in GTPase assays (where a GTP concentration of 1 mM 303 was used), a lower concentration of mant-GMPPNP (800 nM) failed to stimulate GTP binding and stabilization for this complex. Hence, we failed to obtain reliable 304 estimates of k_{on} . k_{off} was negligibly low (0.07 min⁻¹). All these results indicate that in 305 $\{AB^{WT}\}\$ and $\{AB_1B_2^{\Delta Ct}\}\$, the exchange of GDP with GTP is driven by the MglB₁ Ct-306 307 helix, which interacts away from the nucleotide-binding pocket of MgIA.

308 N-terminal β-strand of MglB₁ helps orient the Ct helix driving nucleotide

309 exchange

310 Next, we attempted to dissect the role of the asymmetric interaction of the N-terminal 311 region of MgIB₁ with MgIA. Residues 2-7 were deleted in MgIB, which constituted the β -strand in MglB₁ that interacts with β_0 -strand of MglA. This strand might play a role 312 313 in orienting MgIB and facilitating optimal interaction with MgIA. This construct was named MglB^{ΔN}. Size exclusion chromatography confirmed that MglB^{ΔN} was well 314 315 folded and formed a dimer like MgIB (Fig. 6A). Next, we compared the GTPase activity of MgIA in presence of MgIB^{ΔN}. MgIA GTP hydrolysis rate in the presence of 316 the mutant MgIB was reduced as compared to that with MgIB^{WT} (Fig. 6B, Table 1). 317 k_{obs} was similar to that with MglB^{ΔCt} (Table 1) which had only GAP and no GEF 318

319 activity due to the absence of the Ct helix. We further checked the GDP exchange rates of MgIA in presence of MgIB^{ΔN} using fluorescently labelled *mant* GDP (Fig. 6C). 320 We observed that the k_{on} and k_{off} rates (0.8 μ M⁻¹min⁻¹ and 0.09 min⁻¹, respectively) 321 322 are similar to MgIA alone which proved that the GEF activity of MgIB^{ΔN} was abolished (Table 2B). We performed the same assay with excess concentrations of 323 MglB mutants (MglA:MglB of 1:4) and observed similar results (Supplementary Fig 324 325 2). This validates that the reduction of the GEF activity of mutant MgIB is not due to 326 reduced affinity to MgIA. We also observed GDP exchange rates using fluorescently labelled mant GMPPNP. MglB^{ΔN} showed a slower k_{on} rate similar to MglB^{ΔCt} (0.8 μ M⁻ 327 ¹min⁻¹) (Table 2C). All these results showed that the deletion of the N-terminal β -328 329 strand in MgIB hindered the optimal interaction of the MgIB₁ Ct helix with MgIA 330 thereby abrogating the GEF activity.

331 Asn-37 and Arg-98 of MglB are essential for MglB GAP activity.

332 It is evident from the structure of the MgIA/MgIB complex (GTP bound; PDB: 6IZW; 333 Fig. 1A) that the interaction of MgIB reorients Switch-I region of MgIA for active hydrolysis thereby functioning as a GAP. Since the residues Asn-37 and Arg-98 of 334 MglB₂ interact with the Switch-I residues of MglA (Fig. 1D), these residues might be 335 336 critical for Switch-I reorganization and holding it in its active conformation thereby 337 contributing to the MgIB GAP function. A double mutant was, hence, designed where we mutated Asn-37 and Arg-98 to alanines (MglB^{N37R98A}), to investigate the role of 338 339 these residues in activating MgIA GTP hydrolysis. The mutation did not affect the 340 folding or oligometric status of the purified protein as inferred from size exclusion chromatography (Fig. 6A). However, the GTPase stimulation rate of MgIA by 341 MalB^{N37R98A} was slightly reduced as compared to wild-type MglB as evident from the 342

343 observed GTP hydrolysis rates (Fig. 6B, Table 1). To further dissect the role of the 344 mutations, we performed a nucleotide exchange assay with mant GDP and competed it out with unlabelled GDP (Fig. 6C). We observed that the k_{on} rate of MgIA 345 with MglB^{N37R98A} (2.7 μ M⁻¹min⁻¹) was slightly higher than with MglB (1.3 μ M⁻¹min⁻¹) 346 347 (Table 2B). However, the k_{off} rate was similar to MgIA+MgIB (0.2 min⁻¹). However, the k_{on} and k_{off} rates were comparable to MgIA+MgIB when we performed the same 348 349 assay with an excess of MgIB mutants (MgIA: MgIB 1:4) (Supplementary Fig. 2). This showed that the GEF activity of MgIB^{N37R98A} was intact. In similar assays done with 350 351 mant GMPPNP and competition with unlabelled GTP, we observed a similar trend where the k_{on} rate of MgIA with MgIB^{N37R98A} (1.5 μ M⁻¹min⁻¹) was again similar to that 352 353 observed for MgIA+MgIB (1.8 μ M⁻¹min⁻¹) (Table 2C). Hence it was concluded that the 354 interactions of Asn-37 and Arg-98 of the MglB₂ protomer with the MglA Switch-I 355 region were important for accelerating the MgIB GAP activity. All these results 356 highlight that the MgIB dimeric interface predominantly consisting of the MgIB₂ 357 protomer is the GAP active region of MgIB whereas the Ct-helix of the MgIB₁ 358 protomer is the GEF active region.

359 **Discussion**

We successfully designed linked constructs of the MgIAB complex with MgIB Ct-helix truncations to dissect the functional asymmetry of the interaction between MgIA and MgIB. Our study employs a unique approach to dissect the functional basis of an asymmetric interaction of a multimeric regulator of a GTPase. We have succeeded in resolving the respective GAP and GEF active regions of the MgIB, *in vitro*, which can eventually throw light into how this dual function of MgIB is spatially resolved in a cell exhibiting polarity reversals.

Earlier studies showed that the Ct-helix truncated construct of MgIB acts as a GAP ¹⁰ 367 and the C-terminal region contributes to the GEF activity¹¹. $\{AB_1^{\Delta Ct}B_2\}$, which lacked 368 the MgIB₁ Ct-helix, possessed hydrolysis rates similar to $\{AB^{\Delta Ct}\}$ where the helix was 369 truncated in both the MgIB protomers. In nucleotide exchange assays, both {AB^{WT}} 370 and $\{AB_1B_2^{\Delta Ct}\}$ exhibited efficient GDP and GTP exchange as both the complexes 371 had MglB₁ helix interacting with MglA. The accelerated rates of exchange in {AB^{WT}} 372 373 and $\{AB_1B_2^{\Delta Ct}\}$ could be attributed to the linked complex which increases the 374 probability of the interaction of the MgIB₁ Ct-helix with the helix binding pocket of MgIA. In {AB₁^{Δ Ct}B₂}, the dissociated MgIB₂ could dimerise and the Ct-helix was 375 probably capable of interacting with MgIA in trans. This resulted in GDP and GTP 376 exchange rates of $\{AB_1^{\Delta Ct}B_2\}$ similar to unlinked MgIA/MgIB complex where the 377 378 interaction of MgIA with MgIB depended on the diffusion of the two components. 379 Hence, the comparison of $\{AB^{WT}\}\$ and $\{AB_1B_2^{\Delta Ct}\}\$ shows that the GEF-active component is the MglB₁ helix that acts via the helix binding pocket on MglA which 380 381 lies opposite to the nucleotide-binding pocket.

382 Another component of the asymmetric interaction is the β -strand of the MglB₁, an 383 extension of the Rbl/LC7 fold of the MgB₁ protomer interacting with the β_0 strand of 384 the central β -sheet of MgIA. This interaction could potentially help in maintaining a 385 correct orientation of MgIB with respect to MgIA. The β_0 strand is unique for the MgIA family. Deletion of interacting β -strand in MgIB does not affect the stability of MgIB. 386 However, it does reduce the GTPase rate of MgIA as compared to that of MgIB^{WT}. 387 The GAP activity was intact but the GEF activity of MqIB^{ΔN} was affected. We 388 389 compared the conformations of residues 1 to 7 in the structure of MgIB only (PDB ID: 390 6HJM Chains A, B) and MgIAB complex (PDB ID: 6IZW). MgIB₁ forms the N-terminal 391 β -strand only in the presence of MgIA. In MgIB₂, the first α -helix of Rb/LC7 is longer 392 compared to MgIB₁. If MgIB₁ also had a continuous longer α -helix, it might hinder the 393 interaction of the Ct-helix of MgIB₁ onto MgIA (Fig. 1B). We speculate that the 394 formation of the N-terminal β - strand of MglB₁ and further its interaction with MglA 395 helps to avoid clashing of N-terminal region of MgIB and Ct-helix of MgIB. Also, 396 deletion of this strand probably hinders the optimal interaction of MgIB with MgIA 397 thereby affecting the GEF activity of MgIB.

Asn-37 and Arg-98 residue mutants of MgIB were designed to dissect the role of these residues in orienting the Switch region of MgIA. The double mutation reduced the GTPase stimulation of MgIB. However, the nucleotide exchange activity was intact despite the mutation. Hence, these residues from MgIB₂ protomer interacts and orients the Switch-I residues of MgIA promoting MgIB GAP activity. MgIB contains a roadblock domain (RD), which is the ancestor of the Longin domain, a characteristic domain present in GEFs of eukaryotic small Ras-like GTPases. MgIB

405 RD forms a dimer symmetrically to form an extended β-meander and forms a 406 platform, which interacts asymmetrically with one MgIA molecule²¹.

407 The mutation of Asn-37 and Arg-98 residues of MgIB was designed to dissect the 408 role of these residues in orienting the Switch region of MgIA. The double mutation 409 reduced the GTPase stimulation of MgIB. However, the nucleotide exchange activity 410 was intact despite the mutation. Hence, these residues from MglB₂ protomer 411 interacts and orients the Switch-I residues of MgIA promoting MgIB GAP activity. 412 MglB contains a roadblock domain (RD), which is the ancestor of the Longin domain, a characteristic domain present in GEFs of eukaryotic small Ras-like GTPases. MglB 413 414 RD forms a dimer symmetrically to form an extended β-meander and forms a platform, which interacts asymmetrically with one MgIA molecule²¹. It is interesting to 415 compare such an asymmetric interaction in the MgIAB complex to that of a 416 417 symmetric interaction in Rag GTPase dimer. Uniquely, Rag GTPases have an RD in cis towards its C-terminus, analogous to MgIA-link-MgIB₁ in our study. This RD 418 mediates the dimerization of two subunits²². However, the interface analogous to the 419 420 interface between MgIA and MgIB dimer does not exist in Rag GTPases. The RD dimer forms a platform to support the respective GTPase domains such that each of 421 them can be independently activated to a conformation facilitating GTP hydrolysis 422 (GAP activity)^{23,24}. Extensions to the RD domain functions as a GEF in eukaryotes 423 through direct interaction at the nucleotide-binding pocket¹⁸, however, we show that 424 the extension to the MgIB RbI domain functions through an independent allosteric 425 426 mechanism involving the C-terminal helix (Fig. 7).

427 The recently identified module comprising RomR-RomX has been shown to act as a 428 GEF for MgIA¹⁴. It recruits MgIA-GTP to the leading pole of the cell thereby

429 establishing the front-rear polarity. Interestingly, both MgIB and RomR-RomX 430 localise in a bipolar asymmetric fashion with the bigger cluster at the lagging pole. 431 However, the GAP activity of MgIB is speculated to be dominating at the lagging 432 pole. This is further stimulated by the presence of an MglB activator, RomY at the lagging pole¹³. It will be interesting to decipher the role of the MglB-Ct helix in MglA 433 434 activation in presence of this co-GAP, RomY. Further, there is a possibility that the 435 GAP and GEF domains of MgIB are spatially regulated by interaction with other interactors such as MgIC^{25,26}. Recent reports show conformational changes in MgIB 436 dimers when complexed with MgIA/RomY and MgIC²⁷. These could be critical in 437 438 regulating the GAP activity of MgIB at the lagging pole. Further, there could be yet 439 unknown factors sequestering the GEF active MglB₁ Ct-helix, spatially resolving its GAP and GEF functions. Disengaging the Ct-helix of MgIB is essential to separate 440 441 the GAP and GEF activities of MgIB, thereby retaining MgIB as a GAP only. In such 442 a disengaged state, which could be driven by post-translational modifications or 443 other interaction partners within the cell, MgIB loses its GEF function and also 444 detaches from MgIA once GTP hydrolysis takes place. Together, this can facilitate 445 the exclusion of MgIA from the lagging pole and promote its recruitment to the leading pole and the Agl-Glt complexes by the RomR-RomX, MglB and other 446 447 effectors.

Allostery is the mechanism of activation of an enzyme by interacting at a region other than its active site. In this case, through our design of linked complexes of MgIAB, we conclusively prove that MgIB is an allosteric activator of nucleotide exchange from MgIA. Our study validates that the Ct-helix of MgIB₁ interacts with the helix binding pocket consisting of the β_2 - β_3 loop of MgIA which is the allosteric regulator

453 site, thereby stabilizing the flipped conformation of MgIA following the β -screw 454 movement. Such a conformation of MgIA possesses a nucleotide-binding pocket in an 'open' conformation ready to accommodate GTP, and the GDP dissociates easily. 455 456 Consequently, when GTP binds, it associates tightly with the catalytic pocket which 457 is further hydrolysed by the GAP activity of MgIB. This is a unique mechanism - most 458 of the GEFs of eukaryotic GTPases interact directly with the residues of the GTPase active site ¹⁶ to drive nucleotide exchange. It is possible that many mechanistically 459 460 uncharacterized GEFs of eukaryotic small Ras-like GTPases could also act by an 461 allosteric mechanism involving the inter-switch region. We predict that the families of 462 small Ras-like GTPases which exhibit an inter-strand movement such as Arf, 463 septins, and Rag GTPases are likely to exhibit these allosteric mechanisms of GEF activity^{19,28,29}. 464

465 Materials and Methods

466 **Cloning**

Restriction-free cloning ³⁰ was used to generate the constructs of MgIA-link-MgIB 467 with C-terminal hexahistidine tag in *pHis17-Kan^R* (*mgIA-link-mgIB-H*₆, refer Addgene 468 plasmid #78201 for vector backbone, *amp^R* cassette replaced with kanamycin 469 470 resistance using specific primers: pHis17 Kan^r). Further MgIA-link-MgIB^{Δ Ct} with Cterminal hexahistidine tag in *pHis17-Kan^R* was cloned (*mgIA-link-mgIB*^{$\Delta Ct}-H_6$). The</sup> 471 472 primers used are enlisted in Table 3. Similarly, MgIB and MgIB^{ACt} were cloned with a C-terminal Strep-tag in pHis17-Amp^R. MgIA and MgIB constructs used were as 473 reported in ¹¹. The clones were subjected to *DpnI* (New England Biolabs Inc.) 474 475 digestion followed by transformation. Positive clones were selected on a suitable 476 antibiotic (kanamycin and ampicillin respectively) containing plates and checked by 477 the consequent release of correct-sized fragments following double digestion with Ndel and BamHI (New England Biolabs Inc.). All the clones were confirmed by 478 479 sequencing.

480

Firstly, MglB-Strep and MglA with the intervening operon sequence was cloned 481 482 (mglB-Strep-Operon-mglA). The operon sequence was synthesized using forward 483 and reverse primers (Table 3) using overlap extension (Refer to Fig. S1 for details). This product was used as a megaprimer to be inserted in *pHis17 mglA-link-mglB-H*₆ 484 485 construct as the template at the 5' end of the *mglA* sequence. MglA has an internal Xhol restriction site. We performed double digestion using the internal Xhol and 486 HindIII (on the vector backbone at the 3' end of the gene) restriction sites on mglB-487 Strep-Operon-mgIA and mgIA-link-mgIB-H₆ constructs to generate the vector and 488

insert, respectively, for the ligation reaction (enzymes from New England Biolabs Inc.). *mglB-Strep-Operon-mglA* vector treated with TSAP (Promega) and the digested *mglA-link-mglB-H*⁶ insert was ligated at the MglA *Xhol* restriction site using T4 DNA Ligase, (New England Biolabs Inc). The clones were transformed and screened similarly using double digestion with *Ndel* and *HindIII*. This generated the $\{AB^{WT}\}$ construct.

495

For $\{AB_1^{\Delta Ct}B_2\}$, $mglB^{\Delta Ct}$ -Strep-Operon-mglA was PCR amplified using the same overlap extension primers as before, using $mglB^{\Delta Ct}$ -Strep construct. This product was digested using N-terminal *Ndel* and internal *Xhol* and inserted into a similarly digested mglA-link-mglB-H₆ vector. $\{AB_1B_2^{\Delta Ct}\}$, and $\{AB^{\Delta Ct}\}$ were generated using combinations of *Ndel* and *Xhol* digested inserts of mglB-Strep-Operon-mglA and $mglB^{\Delta Ct}$ -Strep-Operon-mglA, respectively, and ligated to a digested *Ndel* and *Xhol* mglA-link-mglB^{\Delta Ct}-H₆ template.

503 MglB Δ N, and MglB^{N37,R98A} mutations were incorporated in MglB using primers listed in 504 Table 3. The clones were subjected to *DpnI* (New England Biolabs Inc.) digestion 505 followed by transformation. Positive clones were selected on ampicillin-containing 506 plates and checked by the consequent release of correct-sized fragments following 507 double digestion with *NdeI* and *BamHI* (New England Biolabs Inc.). Both clones were 508 confirmed by sequencing.

509

510 **Protein Expression**

511 The plasmids were transformed in BL21DE3 (for $\{AB^{WT}\}, \{AB_1^{\Delta Ct}B_2\}, \{AB_1B_2^{\Delta Ct}\}$) and 512 BL21AI (for $\{AB^{\Delta Ct}\}, Mg|B^{\Delta N}, Mg|B^{N37R98A}$) strains of *E. coli* respectively. The cultures 513 were grown in respective antibiotic-containing media (1X LB with 0.1 mg/ml ampicillin or 0.05 mg/ml kanamycin) and were kept in shaking conditions at 37°C.
The cultures were induced with 0.5 mM IPTG (for BL21DE3) and 0.2% L-Arabinose
(for BL21AI) once it reached the exponential phase of growth (between 0.6-0.8 OD).
The cultures were incubated overnight at 18°C. The samples were subjected to 15%
SDS-PAGE to observe the overexpressed band of protein of interest.

519 **Protein purification**

520 Purifications of MgIA and MgIB were performed as described in reference 11¹¹

<u>His-tagged purification:</u> For the MgIAB linked constructs, harvested cells were resuspended in the lysis buffer L (50 mM Tris pH 8.0, 200 mM NaCl and 10% glycerol) at 4 °C. Consequently, the samples were centrifuged at 39,000 g for 45 minutes. The lysate was loaded on a 5 ml HisTrap HP (GE Healthcare) column. The loading buffer used was 200 mM NaCl, 50 mM Tris pH 8.0 and the elution buffer used was 200 mM NaCl, 50 mM Tris pH 8.0, 500 mM Imidazole. Protein was eluted using a stepwise gradient of 2%, 5%, 10%, 20%, 50%, and 100% of elution buffer.

528 StrepTrap: Elute from HisTrap consisting of the respective protein fractions were centrifuged at 39,000 g for 15 minutes and the supernatant was loaded on a 5 ml 529 530 StrepTrap HP column (GE Healthcare). The binding buffer used was 150 mM NaCl, 50 mM Tris pH 8.0 and the elution buffer used was 150 mM NaCl, 50 mM Tris pH 531 532 8.0, 2.5 mM desthiobiotin. Elution buffer was injected into the column in 2 rounds of 533 5 ml each (Fraction I and II, respectively). The fractions with the protein were pooled, 534 concentrated and injected into Superdex-75 column (GE Healthcare) to elute with a 535 final buffer containing 50 mM NaCl, 50 mM Tris pH 8.0 (A50).

In the case of $\{AB^{\Delta Ct}\}$, only HisTrap and StrepTrap were performed followed by washing off desthiobiotin using 50mM NaCl, 50mM Tris pH 8.0 (A50) buffer during protein concentration and consequently stored.

Size Exclusion chromatography: The column was equilibrated with 50mM NaCl, 540 50mM Tris pH 8.0 (A50) buffer. Concentrated protein after StrepTrap step 541 (approximately 2-3 mg/ml, 200 μ L of protein for analytical runs) was injected into the 542 Superdex 75 size exclusion column (GE Healthcare) (volume less than 900 μ L). UV 543 absorbance at 280 nm was observed to monitor the elution of the protein. The 544 respective fractions were pooled, concentrated and stored.

Anion Exchange (MonoQ): For MgIB mutants, the protocol was followed the same as MgIB purification. However, following Superdex 75, the protein was impure. Hence it was injected in MonoQ 4.6/100 PE (GE Healthcare). Buffers used for binding and elution were Buffer A (50 mM Tris [pH 8.0], 50 mM NaCl) and Buffer B (50 mM Tris [pH 8.0], 1 M NaCl), respectively. A linear gradient of Buffer A ranging from 0% to 50% Buffer B over 20 column volumes was injected and the fractions containing the protein were pooled and concentrated.

552 SEC-MALS

553 Size exclusion chromatography coupled with Multi-angle light scattering (SEC-554 MALS) experiments enabled the accurate mass estimation of the linked complexes. 555 Superdex 200 Increase 10/300 GL column was used for SEC which was connected to an Agilent HPLC unit having an 18-angle light scattering detector (Wyatt Dawn 556 557 HELIOS II) and a refractive index detector (Wyatt Optilab T-rEX). The experiments 558 were performed at room temperature. The column was equilibrated with A50 (50 mM 559 NaCl, 50 mM Tris pH 8.0) buffer at 0.4 ml/min. BSA at 2 mg/ml was used for the 560 calibration of the system. The purified linked complexes (approximately 5 mg/ml, 110 561 μ L) were consequently loaded to estimate the molecular weight of the eluted peaks. 562 The Zimm model implemented in ASTRA software was used for the curve fitting and sestimation of molecular weights. GraphPad Prism was used to average molar massfrom fitted plots.

565 NADH-coupled GTP hydrolysis assay

NADH coupled enzymatic assay ³¹, similar to the protocol used in Baranwal, et al, 566 2019¹¹, was used to measure GTP hydrolysis activity. A master mix was prepared in 567 568 A50 buffer (50 mM NaCl, Tris pH 8.0) containing 600 µM NADH, 1 mM phosphoenol 569 pyruvate, 5 mM MgCl₂, 1 mM GTP and pyruvate kinase and lactate dehydrogenase 570 enzyme mix (~25 U/ml). All components were mixed to a 200 µl reaction volume and 571 added to Corning 96 well flat bottom plate. The reactions were initiated by adding 572 purified complexes or a mix of MgIA/MgIB (mutants) in 1:2 ratios to a final concentration of 10 µM MgIA. A decrease in NADH absorbance was measured at 573 574 340 nm using a Varioskan Flash (4.00.53) multimode plate reader. The absorbance 575 was measured every 20 s for 7200 s. The initial time point and absorbance of buffer components were subtracted from all readings. NADH absorbance was converted to 576 577 GDP produced using a slope obtained from a standard curve containing known concentrations of NADH. GraphPad Prism was used for data analysis and plotting 578 579 the k_{obs} values.

580 Nucleotide exchange assay

581 The kinetic measurements for the linked complexes were performed on Fluoromax-4 582 (Horiba) where the intensity of fluorescence emission by *mant*-labelled GDP/GMPPNP (Jena Bioscience, Germany) at 440 nm was monitored after the 583 excitation at 360 nm (protocol similar to the one used in Baranwal, et al, 2019¹¹). The 584 585 sample volume was 200 µl in a guartz cuvette (10 × 2 mm path length) and 586 excitation and emission slit widths of 2 nm. *m*-GDP/GMPPNP (final concentration 800 nM) was present in buffer A50 (50 mM Tris pH 8.0, 50 mM NaCl, 5 mM MqCl₂). 587

588 The protein mix, i.e., a final concentration of 3 μ M of the respective linked complexes 589 was added in the cuvette at 400 seconds after stabilization of the signal from only mant-nucleotide. Consequently, the fluorescence was recorded for 1,400 590 591 seconds where the increase in fluorescence intensity reflected the nucleotide-binding 592 kinetics. At 1,800 seconds, the mant-labelled nucleotide was competed out with an 593 excess of unlabelled GDP/GTP (final concentration 500 µM), resulting in the release 594 of mant-labelled nucleotide from the protein which manifested as a decrease of 595 fluorescence intensity. For plotting the relative intensities from the measurements, 596 each value was divided by the average of the first 200 readings (400 seconds). 597 These accumulations and decay reactions were fitted to exponential binding 598 equations as given below in GraphPad Prism to estimate the k_{on} and k_{off} values.

599 $P + N_1 \rightarrow PN_1 + N_2 \rightarrow PN_2 + N_1$, where P represents protein, N₁ is the labelled 600 nucleotide, N₂ is the unlabelled nucleotide, and PN denotes the protein-nucleotide 601 complex.

602 For estimation of
$$k_{on}$$
, $PN_t = PN_{max} (1 - e^{-kt})$.

For estimation of
$$k_{off}$$
, $PN_t = PN_{max} - PN_{min} (e^{-kt}) + PN_{min}$

Here, PN_t represents the amount of the complex at time t, PN_{max} is the maximum amount of the complex upon association, and PN_{min} is the minimum amount of the complex after dissociation.

607 High-Pressure Liquid Chromatography

The protein samples (100 μ M each) were diluted with 50mM NaCl, 50mM Tris pH 8.0 buffer and then heat denatured at 75 °C. The heated protein sample was spun at 21,000g for 10 min. Then the supernatant was filtered with a 0.22- μ m cellulose 611 acetate filter (Corning), and the sample was loaded on the DNAPac PA200 ion exchange column (Thermo Fisher). Buffer A (2 mM Tris (pH 8.0)) was used as a 612 613 binding buffer. The runs were performed at a flow rate of 0.4 ml/min, with linear 614 gradients of 0% to 20% Buffer B (2 mM Tris (pH 8.0), 1 M NaCl) for 3 CV (column volumes), 20%–50% Buffer B for 3 CV and 100% B for 2 CV. The sample injection 615 616 volume was 100 µl. The absorbance at 260 nm, indicative of the presence of GDP, 617 was plotted against conductivity. The GDP standard was run with 100 µl of a 500 µM 618 filtered GDP solution.

619

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629 Author Contributions

- 630 SC: Designed and performed all the experiments and wrote the manuscript
- 631 MK: Performed cloning and initial purification of MgIB mutants MgIB^{N37R98A} and

 $MglB^{\Delta N}$ and reviewed the manuscript.

633 PG: Conceptualized the study, supervised the experiments and analyses, and re-

634 viewed and edited the manuscript

- 635 CrediT:
- 636 SC: Methodology; Investigation; writing original draft;
- 637 MK: Investigation; Writing review and editing
- 638 PG: Conceptualization; Methodology; Funding acquisition; Project administration;
- 639 Supervision; Writing review and editing

640 **Conflict of Interest**

641 The authors declare no conflict of interest.

643 Figures



644

Figure 1: Asymmetric features of MgIAB complex

A. Structure of the *M. xanthus* MgIA-MgIB complex (PDB ID: 6IZW) (MgIA in green, MgIB₁ in magenta and MgIB₂ in light blue) highlighting the MgIB₁ Ct-helix interaction with MgIA (blue dotted box) labelled as allosteric interaction and MgIB₂ Ct-helix interaction with MgIA nucleotide-binding pocket (black dotted box) labelled as direct interaction. The residues at the C and N-termini ('C' and 'N' respectively) and the breaks of protein chains (residue numbers) are labelled for all the chains. The amino

652	acid sequence of the Ct-region of the two MgIB protomers is shown below with the
653	disordered residues in the crystal structure and the Ct-helix sequence labelled. In the
654	inset, a schematic representation, as depicted in later figures, of the MgIAB complex
655	is shown.

- 656 **B.** Interaction of MgIB₁ Ct- helix (magenta) (start and end residue numbers labelled)
- 657 with MgIA (green) helix binding pocket. MgIA α_5 helix and the β_2 - β_3 loop have been 658 labelled.
- 659 **C.** Interaction between MglB₁ N-terminal β -strand and β_0 -strand of MglA which 660 extends the MglA β -sheet constituting the central Ras fold.
- 661 D. Interaction between Asn-37 and Arg-98 residues of the MglB₂ protomer with
- 662 Switch-I region (dark purple) of MgIA. Switch-I and Switch-II residues are labelled in
- 663 dark purple and peach respectively.



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665

666 Figure 2: Design of the linked constructs.

A. Orientation of N- and C-termini of MgIA and MgIB led to the design of a linked construct. (Insets) Structure of the *M. xanthus* MgIA MgIB complex (PDB ID: 6IZW; top; box in dashed outline) rotated sideways and zoomed in (bottom left; box in solid outline) to highlight the respective termini positions. A short Gly-Ser linker (light orange) between the C-terminus of MgIA and the N-terminus of MgIB₁ will serve to connect MgIA and MgIB. The position of a similar Gly-Ser linker from the C-terminal end of MgIA to its C-terminal hexahistidine tag (purple; as observed in the crystal packing of MgIA-GDP structure PDB ID: 5YMX) superposed with the N-terminal βstrand of MgIB₁ is shown to demonstrate the feasibility of interaction.

676 **B.** Strategy for co-expressing constructs of MgIB₂ (grey) and MgIA-link-MgIB₁ (green-677 pink) (MgIA: green; Linker: yellow; MgIB₁: pink) with an intervening DNA sequence 678 from the operon (dark blue). The possible combinations of complexes thus formed are shown. The linked constructs include {AB^{WT}} and those with respective 679 truncations of MgIB₁ and MgIB₂ C-terminal residues ($\{AB_1^{\Delta Ct}B_2\}, \{AB_1B_2^{\Delta Ct}\}$ and 680 $AB^{\Delta Ct}$ respectively). MglB₂ (outlined in blue) and MglA-link-MglB₁ (outlined in dark 681 682 red) are tagged with Strep (S: WSHPQFEK) and hexahistidine tag (H) respectively. 683 **C.** Strategy of purification of the asymmetric complexes through sequential affinity

684 chromatography steps (Ni-NTA and StrepTrap).



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686

687 Figure 3: Purification of linked complexes

A, **B**, **C**, **D**. SDS-PAGE showing the StrepTrap elution profiles of the linked complexes of $\{AB^{WT}\}$, $\{AB_1^{\Delta Ct}B_2\}$, $\{AB_1B_2^{\Delta Ct}\}$ and $\{AB^{\Delta Ct}\}$ respectively. The first lane labelled as Inp is the load (obtained after Ni-NTA purification), and the lane labelled FT represents flow through (unbound protein). M is the protein ladder labelled with the molecular weights (in kDa). Fractions I and II represent the 5 eluted fractions of 1 ml each from each injection of 5 ml of 2.5 mM desthiobiotin containing elution buffer. The major constructs eluted as 'fractions I' are represented via the schematic.

E. SDS PAGE showing the purified complexes from the linked constructs (cropped images of single lanes from figure panels A – D compiled for ease of comparison).
The upper band corresponds to MgIA-link-MgIB₁ and the lower band is of MgIB₂.
Corresponding schematics of each construct are shown on the left and top of the lanes, and the sizes are labelled on the right.



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-		-		
		-		

	Pea	k 1 (AB co	omplex)	P	eak 2 (B ₂ di	imer)
Construct	Eluti on Volu me (ml)	Expected molar mass (kDa)	Observed molar mass (kDa)	Elution Volum e (ml)	Expected molar mass (kDa)	Observed molar mass (kDa)
{AB ^{wt} }	14.2	58.5	53	NA	-	-
$\{AB_1B_2^{\Delta Ct}\}$	14.9	56.1	56	NA	-	-
{AB1 ^{ACt} B2}	12.2	56.1	112.9	14.2	36.7	36
{AB ^{∆Ct} }	15.1	53.7	55.6	15.6	31.9	31

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Figure 4: The C-terminal residues of MgIB contribute to the differential stability of the linked complexes.

A, B SEC-MALS (Superdex-200 profiles for SEC carried out at room temperature ~ 25 °C) for the linked constructs: $\{AB^{WT}\}\$ blue and $\{AB_1B_2^{\Delta Ct}\}\$ magenta in panel A and $\{AB_1^{\Delta Ct}B_2\}\$ green and $\{AB^{\Delta Ct}\}\$ brown in panel B. The left and the right y axes represent the refractive index and molar mass (kDa) respectively for both plots. Molar masses are represented by thicker lines as compared to refractive indices.

709 **C.** Table enlisting the elution volumes and corresponding average molar masses 710 (expected and observed; Peaks 1 and 2 correspond to AB₁B₂ complex and B₂ dimer,

711 respectively as labelled in panels A and B) for the linked complexes as concluded

712 from SEC-MALS.



- 713
- 714

Figure 5: Constructs with MglB₁ helix stimulate higher GTP hydrolysis rates of
MglA and nucleotide exchange,

717 **A.** Comparison of k_{obs} values of {AB^{WT}} (blue), {AB₁^{$\Delta Ct}B_2$ </sup>} (green), {AB₁B₂^{ΔCt}} 718 (magenta), {AB^{ΔCt}} (brown), unlinked AB^{WT} (purple), AB^{ΔCt} (coral). The mean and 719 95% confidence intervals are shown by long and short horizontal lines, respectively 720 for each sample.

B. Kinetics of increase in *mant*-GDP fluorescence (region labelled as *m*-GDP) upon adding {AB^{WT}} (blue), {AB₁^{Δ Ct}B₂} (green), {AB₁B₂^{Δ Ct}} (magenta), {AB^{Δ Ct}} (brown), AB^{WT} (purple), AB^{Δ Ct} (coral), at 400 seconds (marked by the dashed line labelled

'+P'; Stage I), followed by a competition of *mant*-GDP by adding excess unlabelled
GDP at 1800 seconds (marked by the dashed line labelled '+GDP'; Stage II).

726 **C.** Kinetics of increase in *m*-GMPPNP fluorescence (region labelled as *m*-GNP) 727 upon adding $\{AB^{WT}\}$ (blue), $\{AB_1^{\Delta Ct}B_2\}$ (green), $\{AB_1B_2^{\Delta Ct}\}$ (magenta), $\{AB^{\Delta Ct}\}$ 728 (brown), AB^{WT} (purple), $AB^{\Delta Ct}$ (coral), at 400 seconds (marked by the dashed line 729 labelled '+P'; Stage I), followed by a competition of *mant*-GMPPNP by adding excess 730 unlabelled GTP at 1800 seconds (marked by the dashed line labelled '+GTP'; Stage 731 II).

- 732 The phases I and II represent association and dissociation for kon and koff
- radia estimation respectively (tabulated in Table 2A), and are demarcated by solid lines.







A. Size exclusion chromatography (Superdex-75) profiles of mutants of MglB 738 (purple), MglB^{ΔN} (cyan) and MglB^{N37A,R98A} (ochre). 739

B. Comparison of k_{obs} values of MgIB control (square) with MgIB^{WT} (purple) with 740 MglB asymmetry mutants MglB^{∆N} (cyan) and MglB^{N37R98A} (ochre) and respective 741 742 constructs with MgIA (circles).

743 The mean and 95% confidence intervals are shown by long and short horizontal 744 lines, respectively for each sample.

C. Kinetics of increase in *mant*-GDP fluorescence (region labelled as *m*-GDP) upon 745 adding MgIA (dark green), unlinked AB^{WT} (purple), MgIA with MgIB mutants, MgIB^{ΔN} 746 (cvan) and MgIB^{N37R98A} (ochre) at 400 seconds (marked by the dashed line labelled 747

'+P'; Stage I), followed by a competition of *mant*-GDP by adding excess unlabelled
GDP at 1800 seconds (marked by the dashed line labelled '+GDP'; Stage II).

D. Kinetics of increase in *mant*-GMPPNP fluorescence (region labelled as *m*-GMPPNP) upon adding MgIA (dark green), unlinked AB^{WT} (purple), MgIA with MgIB mutants, MgIB^{ΔN} (cyan) and MgIB^{N37R98A} (ochre) at 400 seconds (marked by the dashed line labelled '+P'; Stage I), followed by a competition of *mant*-GMPPNP by adding excess unlabelled GTP at 1800 seconds (marked by the dashed line labelled '+GTP'; Stage II).

The phases I and II represent association and dissociation for kon and koff estimation respectively (tabulated in Table 2B and 2C), and are demarcated by solid lines.



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761 Figure 7: Mechanism of dual GAP and GEF activity of MgIB

Asymmetric complex of MgIA (green) with MgIB₁ (magenta) and MgIB₂ (light blue) with interfaces annotated with the GAP and GEF functions of MgIB. C-terminal helix of MgIB₁ interacts with the MgIA helix binding pocket (blue) imparting the GEF activity. The absence of the N-terminal region of MgIB₁ (yellow) precludes MgIB₁ Ct helix interaction. The dimeric interface of MgIB (orange) stabilizes MgIA and promotes the GAP function.

Tables

- **Table 1:** List of observed k_{cat} (k_{obs} in min⁻¹, k_{obs} here is the amount of GDP released
- per minute per µM enzyme, for details, see Materials & Methods)

Construct	k _{obs} (min ⁻¹)
MgIA	0.009 ± 0.003 (n=3)
MgIB	0.02 ± 0.002 (n=3)
{AB ^{W1} }	0.19 ± 0.008 (n=13)
$\{AB_1^{\Delta Ct}B_2\}$	0.07 ± 0.008 (n=9)
$\{AB_1B_2^{\Delta Ct}\}$	0.16 ± 0.016 (n=10)
{AB ^{∆Ct} }	0.10 ± 0.014 (n=3)
AB ^{W1}	0.19 ± 0.02 (n=7)
AB ^{ΔCt}	0.08 ± 0.005 (n=3)
MgIAB ^{ΔN}	0.08 ± 0.006 (n=4)
MgIAB ^{N37R98A}	0.11 ± 0.004 (n=4)

Table 2: List of rates of association and dissociation (k_{on} (µM⁻¹min⁻¹) and k_{off} (min⁻¹) 778 respectively).

A. Linked Constructs

Construct	mant GDP-GDP exchange		mant GMPPNP-GTP exchange	
	k _{on} (μM ⁻¹ min ⁻¹)	k _{off} (min⁻¹)	k _{on} (μM ⁻¹ min ⁻¹)	k _{off} (min⁻¹)
{AB ^{w1} }	2.7 ± 0.1 (n=3)	0.26 ± 0.003 (n=3)	ND (n=3)	ND (n=3)
$\{AB_1^{\Delta Ct}B_2\}$	0.92 ± 0.01 (n=3)	0.18 ± 0.004 (n=3)	2.2 ± 0.5 (n=3)	0.18 ± 0.1 (n=3)
$\{AB_1B_2^{\Delta Ct}\}$	3.2 ± 0.11 (n=3)	0.33 ± 0.004	ND (n=3)	ND (n=3)

		(n=3)		
{AB ^{∆Ct} }	0.48 ± 0.02 (n=3)	0.09 ± 0.002 (n=3)	ND (n=3)	0.07 ± 0.03 (n=3)
AB ^{WI}	1.08 (n=1)	0.18 (n=1)	2.3 (n=1)	0.28 (n=1)
AB ^{ΔCt}	0.70 (n=1)	0.04 (n=1)	1.5 (n=1)	ND (n=1)

B. MgIB mutants (mant GDP-GDP exchange)

Construct	<i>k_{on}</i> (μΜ⁻¹min⁻¹)	k _{off} (min⁻¹)	<i>k_{on}</i> (μM⁻¹min⁻¹)	k _{off} (min⁻¹)
	1:2	1:2	1:4	1:4
А	0.7 (n=1)	0.1 (n=1)		
			0.8 (n=1)	0.06 (n=1)
AB ^{WI}	1.3 (n=1)	0.2 (n=1)		
			2.2 ± 0.2 (n=3)	0.1 ± 0.03 (n=3)
AB ^{ΔN}	0.8 ± 0.1 (n=3)	0.09 ± 0.01		
		(n=3)	1.0 ± 0.1 (n=3)	0.08 ± 0.01 (n=3)
AB ^{N37R98A}	2.7 ± 0.4 (n=3)	0.2 ± 0.03 (n=3)		
			2.7 ± 0.5 (n=3)	0.2 ± 0.04 (n=3)

C. **MgIB mutants** (*mant* GMPPNP-GTP exchange)

MgIA ND MgIA+MgIB $1.8 \pm 0.5 \text{ (n=3)}$ MgIA+MgIB ^{ΔN} $0.8 \pm 0.08 \text{ (n=3)}$ MgIA+MgIB ^{N37R98A} $1.5 \pm 0.08 \text{ (n=3)}$	Construct	<i>k_{on}</i> (μM ⁻¹ min ⁻¹)
MgIA+MgIB $1.8 \pm 0.5 \text{ (n=3)}$ MgIA+MgIB ^{ΔN} $0.8 \pm 0.08 \text{ (n=3)}$ MgIA+MgIB ^{N37R98A} $1.5 \pm 0.08 \text{ (n=3)}$	MgIA	ND
MgIA+MgIB ^{ΔN} 0.8 ± 0.08 (n=3) MgIA+MgIB ^{N37R98A} 1.5 ± 0.08 (n=3)	MgIA+MgIB	1.8 ± 0.5 (n=3)
MgIA+MgIB ^{N37R98A} 1.5 ± 0.08 (n=3)	MglA+MglB ^{∆N}	0.8 ± 0.08 (n=3)
	MgIA+MgIB ^{N37R98A}	1.5 ± 0.08 (n=3)

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801 Table 3: List of primers used

Plasmid	Primer Sequence
Constructs	
pHis17 <i>mglA-</i> <i>link-mglB H6</i>	MgIA-link-B-F 5'CCTCACCGAGCTCAAGAAGGGTGGTGGTTCCGGCACGCAA CTGGTGATGTACG3' MgIB H6-R 5'GCTTTTAATGATGATGATGATGATGGG3'
pHis17 <i>mglA-</i> <i>link-mglB^{∆Ct} H6</i>	MgIA-link-B-F 5'CCTCACCGAGCTCAAGAAGGGTGGTGGTTCCGGCACGCAA CTGGTGATGTACG3' MgIBΔCt H6-R 5'GCTTTTAATGATGATGATGATGATGGGATCCAGGACTGTCA GTCTTCT3'
pHis17 <i>mglB</i> <i>Strep</i>	MgIB-F 5'GTTTAACTTTAAGAAGGAGATATACAT3' MgIB-Strep-R 5'TTATTTTCGAACTGAGGATGAGACCAGGATCCCTCGCTGA AGAGGTTGTCG3'
pHis17 <i>mglB^{∆Ct} Strep</i>	MgIB-F 5'GTTTAACTTTAAGAAGGAGATATACAT3' MgIB-Strep-R 5'TTATTTTCGAACTGAGGATGAGACCAGGATCCAGGACTGT CAGTCTTCTTC3'
pHis17 mglB-Strep- Operon-mglA	T7-F 5'TAATACGACTCACTATAGGG3' Strep-Operon-MgIA-R 5'GGATGAGTAATTGATGAAGGACATTGGCTTCCCGGGTTATT TTTCGAACTGAGGATGAG3' Strep Operon MgIA-F 5'FCTCATCCTCAGTTCGAAAAATAACCCGGGAAGCCAATGTC CTTCATCAATTACTCATCC3' T7-R 5'GCTAGTTATTGCTCAGCGG3'
pHis17 <i>mglB</i> ₄ ^N	MgIB ∆N-F 5'GTTTAACTTTAAGAAGGAGATATACATATGTACGAAGAGGA GTTCACC3' MgIB H6-R 5'GCTTTTAATGATGATGATGATGATGGG3'
pHis17 <i>mglB</i> _{N37A R98A}	MgIB N37A-F 5'CCTCGTCGACAAGGCCGGGCAGCTCATCTCC3' MgIB R98A-R 5'CGACCAGCACGACCGCGCTGCCGACGATGG3'

pHis17 <i>mglA</i> . <i>mglB, mglB</i> ⊿ ^{Ct}	Refer Baranwal et al., 2019 ¹¹
pHis17 Kan ^r	pHisK-F 5'GCTAAAGTTGTTAAAGACTTTAAATTGCCGCGCGGCAGCCA CATG3'
	pHisK-R 5'GTATATATGAGTAAACTTGGTCTGACAGTTAGAAAAACTCAT CGAGCATC3'

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