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# Specificity of Arl2/Arl3 signaling is mediated by a ternary Arl3-effector-GAP complex

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**Abstract Arl2 and Arl3, members of the Arf subfamily of small G proteins, are believed to be involved in ciliary and microtubule-dependent processes. Recently, we could identify RP2, responsible for a variant of X-linked retinitis pigmentosa, as the Arl3-specific GAP. Here, we have characterized Arl2/3 interactions. We show the formation of a ternary complex between Arl3, its cognate GAP RP2 and its retinal effector HRG4. This complex seems to be important for photoreceptor function.**

*Structured summary:*
MINT-6602303:
*ARL2* (uniprotkb:P36404) *binds* (MI:0407) to *HRG4* (uniprotkb:Q13432) by *pull down* (MI:0096)
MINT-6602333:
*ARL3* (uniprotkb:P36405) *binds* (MI:0407) to *CoD* (uniprotkb:Q9BTW9) by *pull down* (MI:0096)
MINT-6602347, MINT-6602369:
*RP2* (uniprotkb:O75695), *ARL3* (uniprotkb:P36405) and *HRG4* (uniprotkb:Q13432) *physically interact* (MI:0218) by *fluorescence polarization spectroscopy* (MI:0053)
MINT-6602195:
*PDE delta* (uniprotkb:O43924) and *ARL2* (uniprotkb:P36404) *bind* (MI:0407) by *fluorescence polarization spectroscopy* (MI:0053)
MINT-6602213:
*BART* (uniprotkb:Q8WZ55) and *ARL2* (uniprotkb:P36404) *bind* (MI:0407) by *fluorescence polarization spectroscopy* (MI:0053)
MINT-6602239:
*ARL3* (uniprotkb:P36405) and *BART* (uniprotkb:Q8WZ55) *bind* (MI:0407) by *fluorescence polarization spectroscopy* (MI:0053)
MINT-6602322:
*ARL2* (uniprotkb:P36404) *binds* (MI:0407) to *CoD* (uniprotkb:Q9BTW9) by *pull down* (MI:0096)
MINT-6602258:
*RP2* (uniprotkb:Q8IWN7) and *ARL3* (uniprotkb:P36405) *bind* (MI:0407) by *fluorescence polarization spectroscopy* (MI:0053)
MINT-6602233:
*ARL3* (uniprotkb:P36405) and *HRG4* (uniprotkb:Q13432) *bind* (MI:0407) by *fluorescence polarization spectroscopy* (MI:0053)
MINT-6602360:
*HRG4* (uniprotkb:Q13432), *ARL3* (uniprotkb:P36405) and *RP2* (uniprotkb:O75695) *physically interact* (MI:0218) by *molecular sieving* (MI:0071)
MINT-6602297:
*ARL2* (uniprotkb:P36404) *binds* (MI:0407) to *PDE delta* (uniprotkb:O43924) by *pull down* (MI:0096)
MINT-6602227:
*ARL3* (uniprotkb:P36405) and *PDE delta* (uniprotkb:O43924) *bind* (MI:0407) by *fluorescence polarization spectroscopy* (MI:0053)
MINT-6602204:
*HRG4* (uniprotkb:Q13432) and *ARL2* (uniprotkb:P36404) *bind* (MI:0407) by *fluorescence polarization spectroscopy* (MI:0053)



*Keywords:* Signal transduction; G proteins; Arf; GAP; Photoreceptor

## 1. Introduction

The ADP-ribosylation factor (Arf) subfamily belongs to the superfamily of Ras-related GTP-binding proteins (G proteins) and contains several members (Arf1-6, Sar1) which regulate membrane traffic [1]. The 19 currently known, less characterized Arf-like (Arl) proteins form a heterogeneous group of proteins with less sequence homology to each other and do not serve as cofactors in the ADP-ribosylation reaction of Gα proteins [2,3], but do share similar structural features with Arf proteins [4]. Not much is known about GEF and GAP regulators of Arls.

Arl2 and 3 are closely related and seem to play important roles in the regulation of microtubule-dependent processes, as indicated by mutations of Arl2/3 orthologues [5]. Additionally, it was shown that the GDP-form of Arl2 binds the tubulin-specific folding cofactor D (CoD) and that this interaction regulates tubulin folding, microtubule dynamics and the disassembly of the apical junctional complex [6–8]. Another tubulin-specific folding cofactor, CoC, is a GTPase-activating protein (GAP) for Arl3 [9]. *Arl3* was found in screens for ciliary genes [10,11] and localizes to the connecting cilium of human photoreceptor cells, in line with observations that ciliary proteins are responsible for a range of diseases like polycystic kidney disease or retinitis pigmentosa [12,13].

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In spite of apparently divergent functions, Arl2 and 3 share common interactors. BART (binder of Arl2) was the first interaction partner of Arl2/3 proteins identified [14]. The closely related PDEδ and HRG4 are involved in phototransduction processes and bind to Arl2 and 3 [15,16]. PDEδ is a prenyl-binding protein reported to transport prenylated proteins from the inner segment to the outer segment of rod photoreceptors [17]. In contrast to the ubiquitously expressed PDEδ, HRG4 is expressed to high levels specifically in photoreceptors and its deletion in mice leads to dysfunctions in the inner and outer segments of photoreceptor cells [18]. We have shown that the proposed Arl3 effector RP2 is actually a highly efficient and specific Arl3GAP with only limited activity on Arl2 [9]. RP2 is a protein mutated in patients with X-linked retinitis pigmentosa 2 [19] and displays sequence and functional homology to CoC.

Considering that apparently overlapping and specific functions, we investigated the interactions between Arl2/3 and BART, PDEδ, HRG4 and CoD to check whether they may have regulatory or effector-like functions and discovered the formation of a novel ternary complex formed by the G protein Arl3, RP2 and HRG4. Such a complex has previously been discovered only for Arf proteins. It suggests novel modes of regulation of the ciliary proteins Arl3 and RP2 with transport processes in the photoreceptor cells.

## 2. Materials and methods

### 2.1. Plasmids and proteins

Human PDEδ was cloned into BamHI/EcoRI of pGEX4T5-TEV, HRG4 into BamHI/EcoRI of pGEX4T3, BART into NcoI/BamHI of pGEX4T1-TEV vector and CoD into BamHI/NotI of pGEX4T5-TEV.

RP2 and His-tagged Arl2 and Arl3 were purified as described [20]. PDEδ, HRG4 and BART were similarly purified except that TEV protease was used instead of thrombin. CoD was expressed from pGEX4T5-TEV in Rosetta BL21 DE3. After overnight induction at 18 °C with 100 μM IPTG, cells were lysed using French press and sonification, after incubation with protease inhibitors and lysozyme (0.5 mg/ml suspension), in D-buffer (20 mM Tris–HCl [pH 7.5], 300 mM NaCl, 5% glycerol, 3 mM β-mercaptoethanol) + 0.25% Triton X-100, 0.25% NP-40. The lysate was applied to a GSH-column, washed, and the fusion protein cleaved over night with 600 μg TEV protease at 4 °C. CoD was further purified by gel permeation in D-buffer + 0.05% NP-40 on a Superdex S200 26/60 column.

### 2.2. Nucleotide exchange

After purification, both Arl2 and Arl3 are bound to GDP, as detected via HPLC. Exchange for the GTP analogs $GppCH_2p$/GppNHp and to mGppNHp (m, mant = *N*-methylanthraniloyl) was as described before [9]. Loading of Arl proteins with mGDP was achieved by incubating the low affinity complex Arl2/3-$GppCH_2p$ with a threefold excess of mGDP over night at 4 °C. His-tagged Arl2/3-mGDP was separated from $GppCH_2p$ and unbound mGDP by a Ni-NTA purification step. Nucleotide-free Arl2 protein was generated by incubating 1 mg Arl2-$GppCH_2p$ with 1 μl PDE (0.002 U) for 2 h at 8 °C.

### 2.3. Fluorescence measurements

Measurements were performed at 25 °C in M-buffer (20 mM Tris–HCl [pH 7.5], 50 mM NaCl, 5 mM $MgCl_2$, 3 mM β-mercaptoethanol). Fluorescence data were recorded with a Fluoromax-2 spectrophotometer (Jobin Yvon, Grasbrunn, Germany), with excitation and emission wavelengths of m-nucleotides at 366 nm and 450 nm, respectively.

Nucleotide dissociation was started by adding 100 μM of unlabeled nucleotide to 1 μM of mGppNHp- or mGDP-bound Arl2/3. Reactions were followed in a stopped-flow apparatus (Applied Photophysics, Leatherland, UK). To determine nucleotide association of Arl2, 200 nM of mGDP or mGppNHp were mixed with 0.5–4 μM of nucleotide-free Arl2 and fluorescence recorded. Arl2 concentrations were plotted against $k_{obs}$ and fitted to a linear function to yield rate constant. Curves were analyzed and fitted with the program GraFit 5.0 (Erithacus software).

Fluorescence polarization measurements were performed and analyzed as described [9]. For the polarization-based GTP-hydrolysis experiment of the triple complex, 1 μM Arl3$GppCH_2p$ was incubated with 1 μM mGTP or 1 μM mGppNHp for 1 h on ice.

### 2.4. Binding His-pull down assay

40 μg of GDP or GppNHp loaded His-tagged Arl2/3 in 150 μl S-buffer (20 mM Tris–HCl [pH 7.5], 300 mM NaCl, 5 mM $MgCl_2$, 3 mM β-mercaptoethanol)+100 μM GDP or GppNHp and 10 mM imidazol were applied onto Ni-NTA Spin Columns (QIAGEN, Germany). After washing, 40 μg of CoD, PDEδ or HRG4 in 150 μl S-buffer + 20 mM imidazole were applied. After washing, proteins were eluted and analyzed by gel electrophoresis.

### 2.5. Analytical gel filtration

A preformed complex of RP2 and Arl3 $Q71L_{17–177}$ in 1.5 molar excess was incubated with HRG4 for one hour at 4 °C in M-buffer with 5 mM GppNHp, and applied to a Superdex S75 10/30 column in M-buffer + 100 μM GppNHp. Fractions were analyzed by gel electrophoresis.

## 3. Results

### 3.1. Nucleotide affinity of Arl2 and Arl3

While studying Arl–partner interaction, inconsistencies about nucleotide affinities became apparent. It was reported previously that Arl2 has nucleotide affinities in the sub-micromolar range [21] while Arl3 has affinities for GDP and GTP of 0.24 and 48 μM, respectively [15,21]. At least the affinity for GTP is unusually weak for proteins of the Ras superfamily [22]. We thus re-determined nucleotide interactions of Arl2 and Arl3. Dissociations rate constants were obtained by standard protocol using fluorescent nucleotides (Table 1, see Section 2). Nucleotide dissociation rates of Arl2 are in the range of $10^{-2}$ $s^{-1}$, similar to earlier measurements [21], but are much slower for Arl3, indicative of much stronger binding affinity.

To determine the association rates for the GTP-analogue mGppNHp and mGDP, it is necessary to prepare nucleotide-free protein, to be independent of the dissociation of pre-bound GDP [23]. While Arl2 was stable in a nucleotide-free state, Arl3 precipitated during degradation of nucleotide. The association rates of Arl2 to both nucleotides are in the range of $10^5$ $M^{-1}$ $s^{-1}$ (Table 1). We assume similar values for Arl3 because they do normally not differ very much from $10^5$ to $10^6$ $M^{-1}$ $s^{-1}$ for different Ras-superfamily proteins. Even weakly binding analogues of GDP or weak binding mutants of Ras have similar association rate constants, and even the very weak binding ADP associates to Rab5 with a rate constant of $4 * 10^4$ $M^{-1}$ $s^{-1}$ [24,25]. The affinity of Arl3 for both

Table 1
Kinetic rate constants and affinities of Arl proteins to nucleotides

| | $k_{on}/M^{-1}\,s^{-1}$ | $k_{off}/s^{-1}$ | $K_D$/nM |
|---|---|---|---|
| Arl2*mGppNHp | $1.8 * 10^5$ | $1.7 * 10^{-2}$ | 94 |
| Arl2*mGDP | $2.7 * 10^5$ | $1.0 * 10^{-2}$ | 37 |
| Arl3*mGppNHp | nd | $5.9 * 10^{-4}$ | 3[a] |
| Arl3*mGDP | nd | $1.6 * 10^{-5}$ | 0.06[a] |

nd, not determined.
[a]Assuming the Arl2-$k_{on}$ values for Arl3.

GDP and GTP is thus estimated to be 0.06 nM and 3 nM, respectively instead of 48 μM for GTP determined previously [15], possibly due to refolding of unstable nucleotide-free protein or dissociation of prebound GDP.

### 3.2. Arl2/Arl3 and their interacting partner proteins

To analyse the specificity and nucleotide-dependency of Arl2/3 binding to interactors, we used fluorescence polarization with purified proteins (Fig. 1A and B). Results are summarized in Table 2. Except for the Arl3-specific GAP RP2, the interacting proteins bind to Arl2 and Arl3 with similar affinities, with a slight preference of PDEδ for Arl2. They behave like bona fide effector proteins, since they bind the GDP-form of the Arl proteins only with very low affinities ($K_d$ > 10 μM, data not shown).

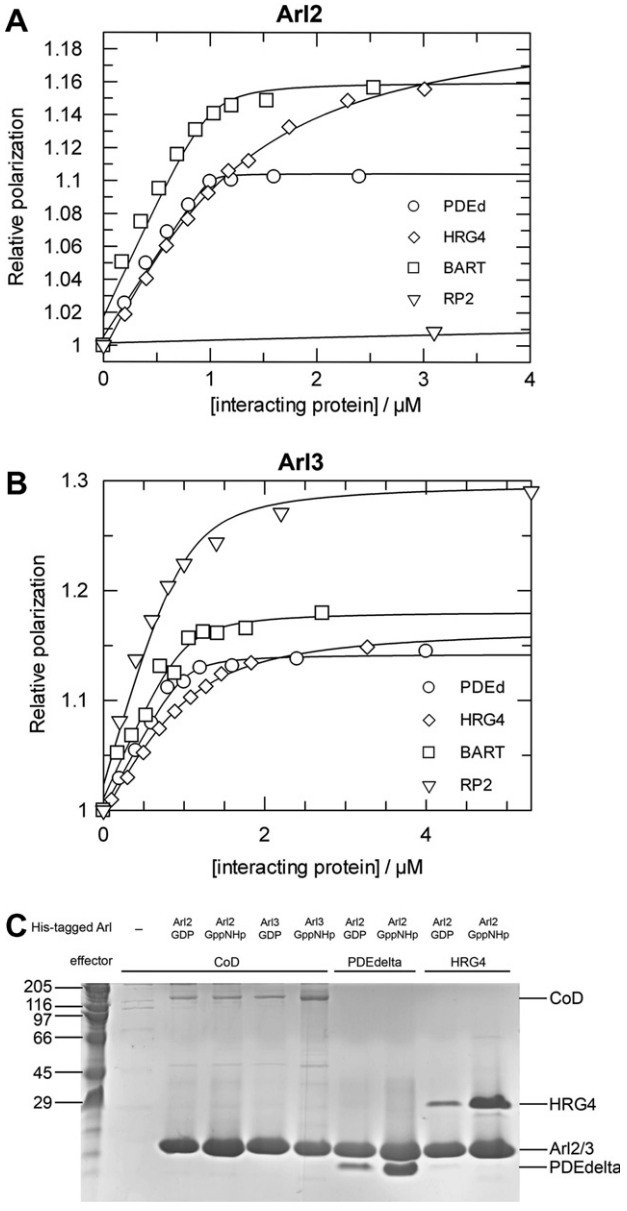


Fig. 1. Arl2/3-effector interactions. (A, B) mGppNHp-bound Arl2/Arl3 were titrated with increasing amounts of different interacting proteins as indicated and fluorescence polarization was measured. The data were fitted to a quadratic binding equation which is shown as sold line. (C) Electrophoresis showing the results of a pull down experiment of GDP- or GppNHp-bound histidine-tagged Arl2/3 with purified interacting proteins, as described in Section 2.

Table 2
Affinities of Arl2 and Arl3 to interacting proteins

| Kd/nM | RP2 | PDEδ | HRG4 | BART |
|---|---|---|---|---|
| Arl2*mGppNHp | >50000 | 2.7 | 580 | 24 |
| Arl3*mGppNHP | 95 | 29 | 270 | 52 |

The proposed binding between Arl2-GDP and Cofactor D could not be confirmed with fluorescence polarization due to unspecific, non-saturating increases in polarization (data not shown). In a qualitative pulldown-assay, CoD binding could be detected to both His-tagged Arl2/3 irrespective of bound nucleotide (Fig. 1C). The affinity is somewhat stronger for the interaction with Arl3-GppNHp. For an estimation of affinity, the amount of CoD precipitated with Arl correlates with the amount of HRG4 and PDEδ bound to the GDP-form of Arl2/3. This indicates an affinity of CoD to Arl proteins in the micromolar range.

Considering our previous experience with RP2 as being a GAP rather than an effector of Arl3 [9], we checked for a possible GAP- and GEF-activity. Except for RP2, no GAP or GEF activities were observed for the interaction partners, indicating that these proteins are true effectors (data not shown).

### 3.3. Formation of a ternary Arl3-RP2-HRG4 complex

In contrast to other Ras proteins, more components and features are required for efficient GTPase activity of Arfs. For example, Arf1 requires the presence of Coatomer in addition to the conventional ArfGAP for an efficient GTP-hydrolysis. We thus wondered whether this is also true for the Arl–ArlGAP interaction. When superimposing the structure of the Arl2-PDEδ effector [26] and the Arl3-RP2 GAP complexes, the resulting structural model shows that the interfaces of Arl3

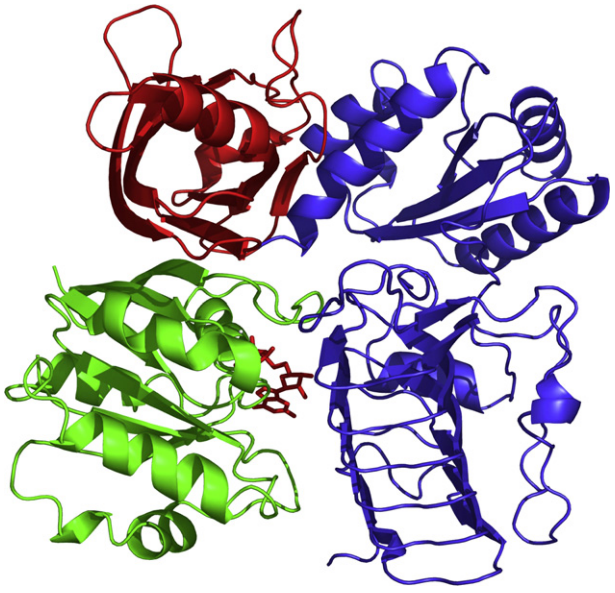

Fig. 2. Model of PDEδ fitting into a hypothetical ternary complex with Arl3-RP2. Structural overlay of the Arl3-RP2 complex with PDEδ from the Arl2-PDEδ complex, obtained by superimposition of Arl2 and Arl3.

with PDEδ and RP2 are separate from each other, with only minor clashes between them (Fig. 2). This indicates that Arl3 could in principle bind to RP2 and PDEδ simultaneously. We thus measured the RP2-stimulated GAP activity on Arl3GTP in the presence of PDEδ or its homologous protein HRG4 to see whether they are able to further stimulate GAP activity. However we could not detect any additional acceleration of GTP-hydrolysis (data not shown).

Guided by the structural observations, we wondered whether PDEδ and/or HRG4 are able to form ternary complexes with Arl3 and RP2. The interaction of RP2 to Arl3mGppNHp leads to a strong increase in fluorescence polarization signal of mGppNHp, as observed before [20]. Binding of another protein to the Arl3-RP2 complex should additionally increase the polarization signal, which is indeed observed after addition of HRG4, but not PDEδ (Fig. 3A). To confirm that HRG4, RP2 and Arl3 really form a ternary complex, we used size-exclusion chromatography. A ternary complex of RP2, $Arl3_{17–177}$ Q71L and HRG4 could be detected with an elution maximum at 8.8 ml (Fig. 3B), whereas excess Arl3-RP2 and non-bound HRG4 elute at 9.5 ml, as seen by electrophoresis of the eluted fractions (below). In contrast, PDEδ elutes as a monomer at 12.4 ml and does not bind to the Arl3-RP2 complex, which elutes at 9.7 ml (Fig. 3C).

These results show that the Arl3-RP2 complex is able to bind HRG4 and to distinguish HRG4 from its homolog PDEδ. To explain this specificity, we identified potential clashes between PDEδ and RP2 from the structural overlay (Fig. 4A, yellow). Regions in PDEδ potentially clashing with RP2 are absent from or not conserved in HRG4 (Fig. 4B), thus rationalizing the specificity of HRG4 binding.

Fluorescence polarization showed an increase in binding affinity of HRG4 to the Arl3-RP2 complex compared to Arl3 alone by almost 10-fold (Fig. 5A), possibly due to additional interactions between HRG4 and RP2 in the ternary complex. To test the possible biological significance of the ternary complex, catalytic amounts of RP2 were added to a 1:1 complex of Arl3-mGTP-HRG4. We detected a decrease of polarization signal down to the level of Arl3-mGTP, with a half-life time of less than 10 min, which is not observed when using the non-hydrolysable mGppNHp. RP2 thus induced hydrolysis of Arl3-GTP in the Arl3-HRG4 complex, leading

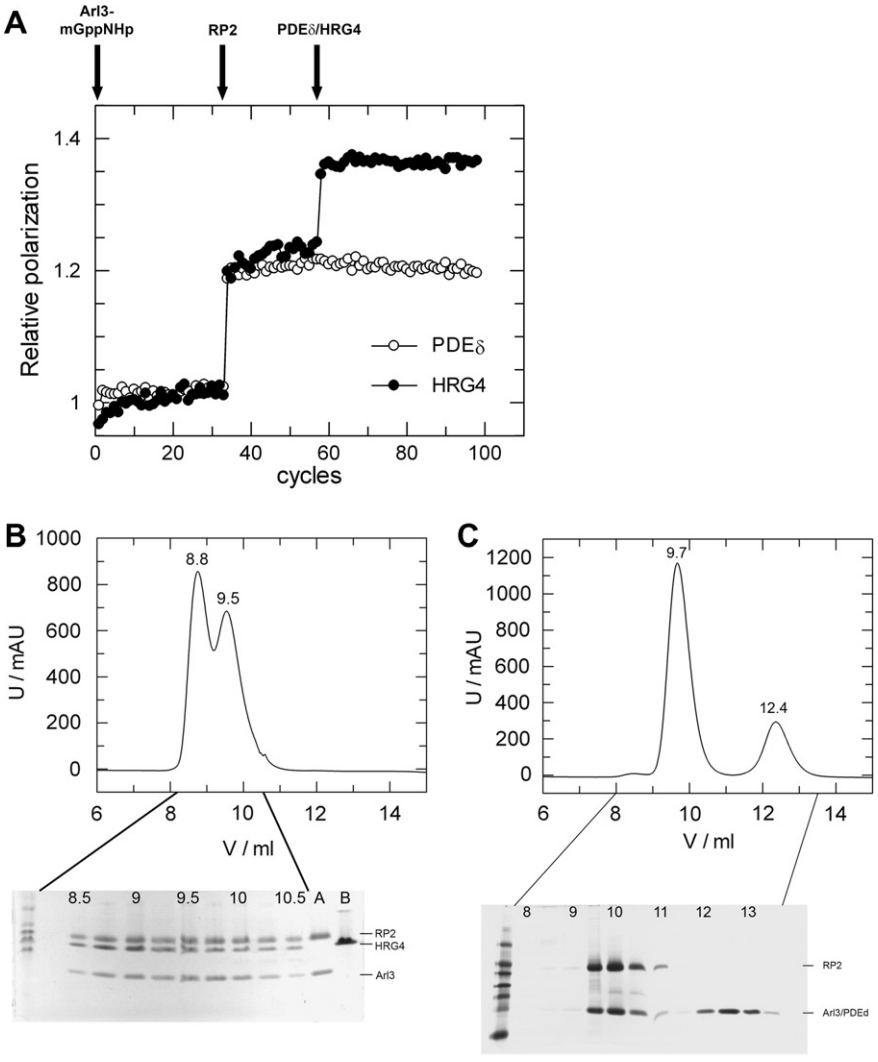


Fig. 3. Formation of a ternary complex between Arl3, RP2 and HRG4. (A) Fluorescence polarization of 1 μM Arl3-mGppNHp. RP2 (5 μM) and PDEδ or HRG4 (10 μM) were added at the indicated time points. (B,C) Interaction of the $Arl3_{17–177}$(Q71L)-GppNHp-RP2 complex with HRG4 (B) and with PDEδ (C): upper panel, gel permeation chromatography profile, lower panel, electrophoresis of chromatographic fractions.

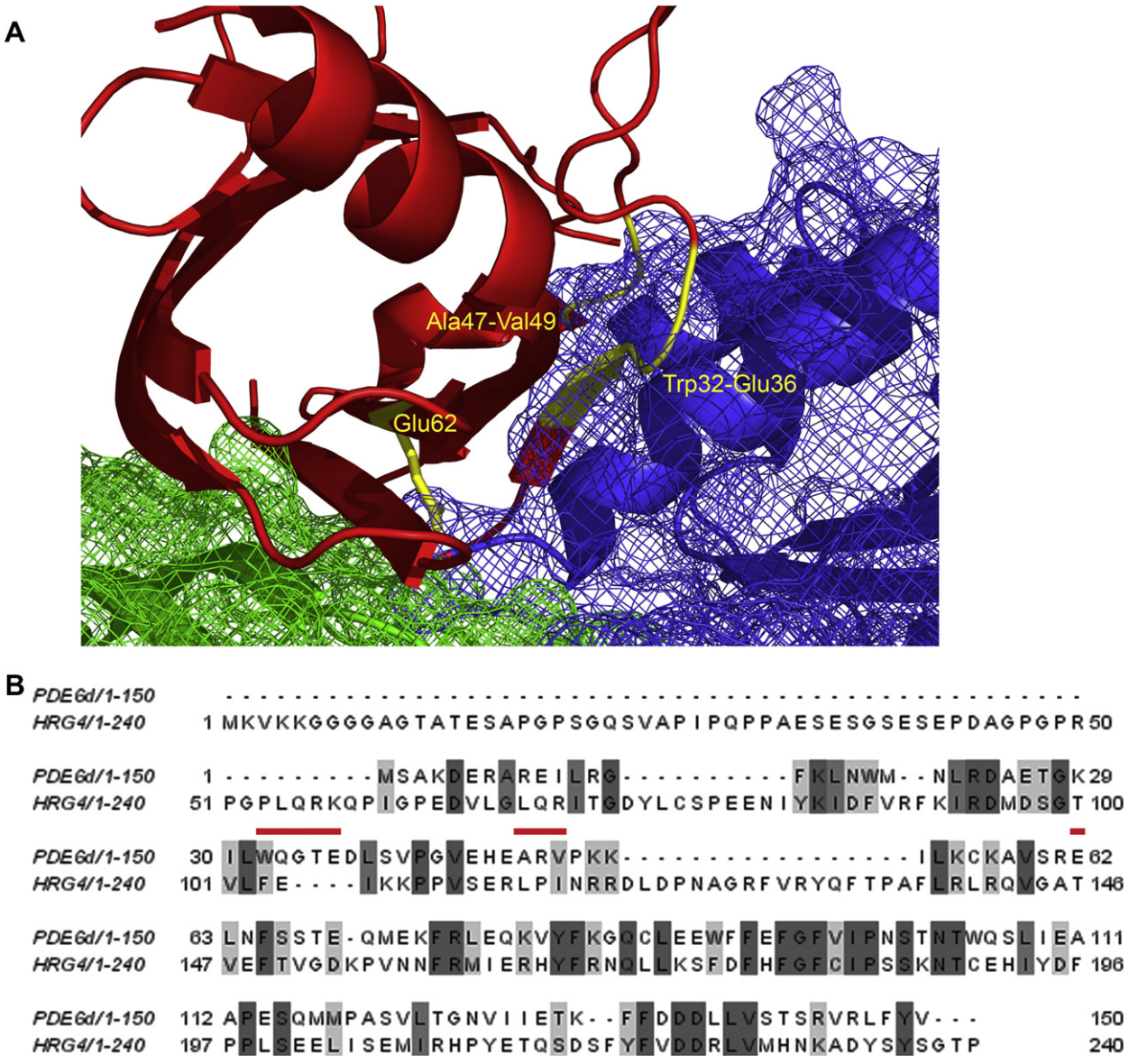


Fig. 4. Specificity of HRG4 vs. PDEδ in the triple complex. (A) Detailed view into the hypothetical PDEδ-RP2 interface using the structural superimposition of Fig. 2. Ribbon representation and the surface mesh of Arl3 and RP2 are colored green and blue, respectively. PDEδ is represented as a red ribbon, with residues of PDEδ potentially clashing with RP2 colored yellow. (B) Sequence alignment of human PDEδ and HRG4. PDEδ residues clashing with RP2 in the structural alignment are marked with red lines.

to the release of HRG4, which binds only weakly to Arl3-GDP.

## 4. Discussion

Previously, it was shown that only the Arl2 mutant T30N, but not the mutant Q70L binds to CoD [6]. It was concluded that Arl2-GDP and but not Arl2-GTP bind CoD. Using proteins saturated with the corresponding nucleotide, we show that there is no difference in CoD binding between Arl2-GDP and Arl2-GppNHp, while Arl3 shows some GTP-dependency of binding. This supports observations by us and others that the mutation of the P-loop Ser/Thr in the GxxxxGKS/T motif does not necessarily, as generally assumed, reflect an exclusive binding of GDP [24,27]. In general it should be kept in mind that the corresponding mutation, T30N in Arl2, destabilizes G proteins and also strongly affects the binding to effectors, as shown for Ras, where the S17N mutation no longer bind effector molecules [28,29].

*HRG4*, *Arl3* and *RP2* were all found in screens for ciliary genes [11]. They are important for the maintenance of photoreceptor cells, since knockouts of Arl3 or HRG4 cause strong retinal defects in mice and mutations of RP2 are causing retinitis pigmentosa [18,19,30]. The Arl3$^{-/-}$ deletion mice exhibit a mislocalization of rhodopsin and probably also other proteins that are important for phototransduction. Here, we have found that HRG4, Arl3 and RP2 form a specific ternary complex suggesting that they could be involved in a common pathway, such as the movement of components within the cilia. In contrast to other small G proteins like Ras, Rho or Rab, where down-regulation by GAP requires the prior dissociation of the

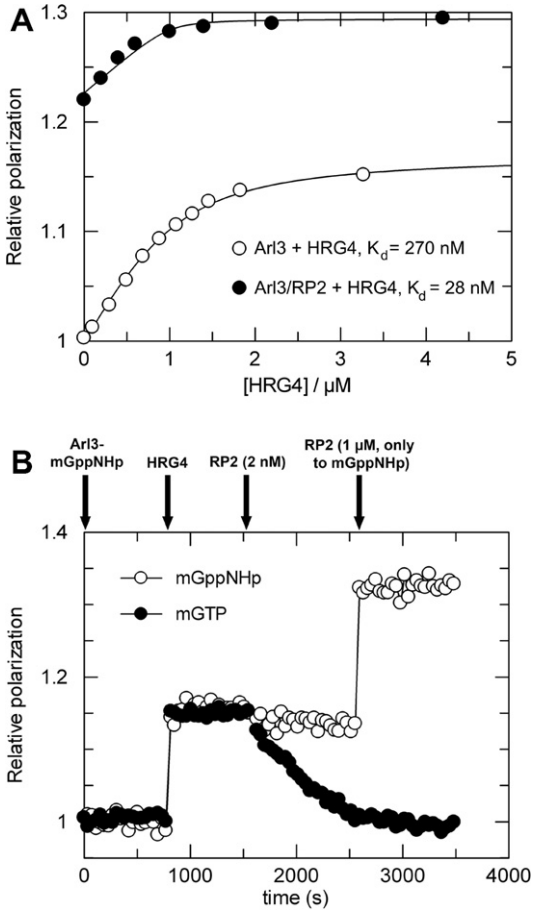


Fig. 5. Functional dissection of the ternary complex: (A) fluorescence polarization obtained after adding HRG4 to 1 μM Arl3mGppNHp or 1 μM Arl3mGppNHp, preincubated with 4 μM RP2. (B) Fluorescence polarization of 1μM mGTP or mGppNHp bound to Arl3, as indicated, before and after addition of the indicated amounts of HRG4 and RP2.

effector complex, the Arl3-HRG4 complex is down-regulated by the transient formation of a ternary complex. This resembles the situation in Arf1 and Sar1, where GAP and effectors can also bind simultaneously in order to induce hydrolysis.

The molecular function of HRG4 in photoreceptors is not known. Its homolog PDEδ possesses a hydrophobic pocket similar to RhoGDI that presumably binds and solubilizes the prenyl anchors of target proteins [26,31]. Residues lining this pocket are also conserved in HRG4 pointing towards a similar function, although no prenylated target proteins of HRG4 have yet been identified. Although the exact molecular function of the HRG4–Arl3–RP2 pathway is unknown, we would predict an involvement in the transport of membrane associated photoreceptor proteins. Since RP2 contains two N-terminal acylation motifs and is membrane-associated, down-regulation may thus involve a specific membrane site.

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