Role of Rac GTPase activating proteins in regulation of NADPH oxidase in human neutrophils

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Abstract

Precise spatiotemporal regulation of $O_2^{\bullet-}$-generating NADPH oxidases (Nox) is a vital requirement. In the case of Nox1-3, which depend on the small GTPase Rac, acceleration of GTP hydrolysis by GTPase activating protein (GAP) could represent a feasible temporal control mechanism. Our goal was to investigate the molecular interactions between RacGAPs and phagocytic Nox2 in neutrophilic granulocytes. In structural studies we revealed that simultaneous interaction of Rac with its effector protein $p67^{phox}$ and regulatory protein RacGAP was sterically possible. The effect of RacGAPs was experimentally investigated in a cell-free $O_2^{\bullet-}$-generating system consisting of isolated membranes and recombinant $p47^{phox}$ and $p67^{phox}$ proteins. Addition of soluble RacGAPs decreased $O_2^{\bullet-}$-production and there was no difference in the effect of four RacGAPs previously identified in neutrophils. Depletion of membrane-associated RacGAPs had selective effect: a decrease in ARHGAP1 or ARHGAP25 level increased $O_2^{\bullet-}$-production but a depletion of ARHGAP35 had no effect. Only membrane-localized RacGAPs seem to be able to interact with Rac when it is assembled in the Nox2 complex. Thus, in neutrophils multiple RacGAPs are involved in the control of $O_2^{\bullet-}$-production by Nox2, allowing selective regulation via different signaling pathways.

Keywords

NADPH oxidase; superoxide production; GTPase activating protein (GAP); molecular modeling; Rac small GTPase;

List of abbreviations

AA: Arachidonic acide

Ab: Anti-body

BLAST: Basic Local Alignment Search Tool

BSA: Bovine serum albumin

DFP: Di-isopropyl fluorophosphate

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

GAP: GTPase activating Protein

GEF: Guanine nucleotide exchange factors

GST: Glutathione *S*-transferase

HBSS: Hank's balanced salt solution

NOX: NADPH oxidase

PBS: Phosphate buffered saline

PDB: Protein data bank

PMN: Polymorphonuclear cell

PMSF: Phenylmethanesulfonylfluoride

RMSD: Root-mean-square deviation

SDS: Sodium Dodecyl Sulfate

SEM: Standard error of mean

Smg: Small GTPase

SOD: Superoxide dismutase

TPR: Tetratricopeptide repeat motif

Introduction

NADPH oxidases (Nox) are transmembrane electron carriers that transfer one electron from cytosolic NADPH to molecular oxygen and form superoxide ($O_2^{\bullet -}$) and other reactive oxygen species (ROS) [1-3]. In humans Nox family proteins are involved in vital physiological functions such as antimicrobial defense [4], vestibular function [5], thyroid hormone synthesis [6], or cellular signaling [7]. Defective function of some of these proteins leads to serious pathologic conditions in humans [8, 9]. However, numerous reports show that inappropriate activation of Nox proteins results in serious damage, mainly in the cardiovascular system [7, 10]. Thus, strict spatiotemporal control of the activity of Nox family proteins is vitally important.

Nox2, highly expressed in phagocytic cells, is the best- and longest-known member of the family. The active Nox2 enzyme complex assembles from several subunits which reside in different compartments in resting cells. Upon activation, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ translocate from the cytosol to the (plasma or phagosomal) membrane, where they bind to the complex of $gp91^{phox}$ and $p22^{phox}$, which together form cytochrome b_{558}. Details of the molecular interactions within the complex have been investigated and reviewed extensively [1, 11, 12]. The essential role of the small GTPase Rac (Rac1 in macrophages, Rac2 in neutrophilic granulocytes) was discovered soon after the identification of the Nox-related subunits [13, 14] and was confirmed later in both human pathology [15] and in genetically modified mice [16]. In the Nox2 complex, $p67^{phox}$ has been identified as the effector protein of Rac [17]. The molecular interaction between the tetratricopeptide repeat (TPR) motif of $p67^{phox}$ and RacGTP was suggested [18] and later verified on the basis of crystal structure [19, 20]. A direct interaction between Rac and $gp91^{phox}$ has also been suggested [21] although not accepted in all published models [22, 23]. Nox1 and Nox3 are the closest homologs of Nox2. Both require cytosolic subunits for activity [24-26] and Rac regulates both [27].

Rac is a member of the Rho subfamily of small GTPases (smg) [28]. Similar to other smgs, Rac's activity depends on GTP binding, and declines slowly due to the slow GTP-hydrolytic activity of the protein. GTPase activating proteins (GAPs) accelerate GTP-hydrolysis by several orders of magnitude, and thus serve as important temporal regulators of smg-signaling [29, 30]. The human genome contains approx 70 potential Rho-family GAPs [31, 32] from which each cell type expresses its own „GAP-repertoire" [33]. The specific or overlapping function of these proteins needs further investigation [30, 34].

RacGAP(s) limit the prevalence of Rac in the active, GTP-bound state, and thus are potentially important negative regulators of Rac-dependent Nox proteins, that would curb the intensity of $O_2^{\cdot -}$ -production. Due to their complex domain structure [32, 34] and extensive regulation [30, 35], RacGAPs could fine tune Nox activity. In fact, a role for RacGAPs in the regulation of assembly and function of the Nox2 complex has been indicated in previous *in vitro* studies [36-40] and in genetically modified animals [41-43]. These were all isolated studies and comparative aspects have not been raised.

In human neutrophils the RacGAPs Bcr, ARHGAP35 and ARHGAP1 have been identified by immunoblotting [40] and all three proteins decreased $O_2^{\bullet -}$ -production when they were added to a cell-free system [39, 40]. However, the contribution of endogenous, membrane-bound RacGAPs has not been investigated. Recently, ARHGAP25 has been cloned and shown to be a RacGAP preferentially expressed in hemopoetic cells. In neutrophilic granulocytes it functions as a negative regulator of phagocytosis [44]. Interestingly, ARHGAP1, that was expressed to a similar extent and had similar RacGAP activity as ARHGAP25, did not affect phagocytosis [44]. These data raised the question of specificity of different RacGAPs in the regulation of the phagocytic NADPH oxidase.

In the present study we i.) investigate – based on existing crystal structures - the theoretical possibility of involvement of RacGAPs in the regulation of the phagocytic Nox2

complex and ii.) perform a comparative analysis on the contribution of the RacGAPs identified hitherto in human neutrophils.

Materials and Methods

Materials

Non-prenylated Rac1, full length ARHGAP1 and the GAP domain of BCR (residues 871–1271) were produced in *E. coli* as GST fusion proteins and purified as described in ref. [38]. The *E. coli* clones producing Rac1, BCR and ARHGAP1 were a gift from A. Hall. GST-tagged full length ARHGAP25 was produced as previously decribed in ref. [44]. The GAP domain of ARHGAP35 (residues 1191-1499) was produced in the form of GST fusion protein and purified as described in [45]. Antibodies against ARHGAP1 and ARHGAP25 were raised in rabbits and characterized as described in ref. [40, 44]. Isotype control polyclonal rabbit-Ab was from Santa Cruz (Dallas, USA). Mouse mAb against ARHGAP35 and isotype control monoclonal IgG_1 was purchased from BD Transduction Laboratories (Franklin Lakes, NJ USA). Horseradish-peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from GE Healthcare (Uppsala, Sweden). Protein G–Sepharose beads, nitrocellulose membranes and Ficoll were from GE Healthcare. Protein A–Sepharose beads were from Life Technologies (Carlsbad, Calif. USA). Arachidonic acid (AA), octylglucoside, ferricytochrome *c* (horse heart, type VI), DFP, aprotinin, pepstatin, leupeptin, PMSF, superoxide dismutase (SOD), guanosine 5´-[γ-thio]triphosphate (GTP-γ-S) and GTP were from Sigma-Aldrich (St. Louis, MO, USA). NADPH was from SERVA (Heidelberg, Germany). Sterile endotoxin-free HBSS was from ThermoScientific (Waltham, MA, USA). [γ-^{32}P]GTP was from Izotóp Intézet, Hungary. Spin-X Centrifuge Tube Filter with 450nm pore size was from Corning (Amsterdam, The Netherlands). All other reagents were of the

highest available quality. Incubations were performed in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH7.4). Arachidonic acid was dissolved in ethanol at 20mM and added to the reaction mixture in small volumes (less than 3% of the final volume).

Preparation of neutrophils and subcellular fractions

Human neutrophils were prepared from buffy coats of healthy volunteers as described [46] and isolated PMN cells were suspended in PBS and treated with 1mM DFP for 10 min at room temperature. After being washed in PBS, cells were resuspended in PBS supplemented with 1mM EGTA, 10μg/ml aprotinin, 2μM pepstatin, 10μM leupeptin, 0.1mM PMSF. Thereafter cells were broken by ultrasonic treatment, and membrane and cytosolic fractions were prepared on a discontinuous sucrose gradient as described in [38] .

Measurement of $O_2^{\bullet-}$-production in semi-recombinant cell-free system

The rate of $O_2^{\bullet-}$-production was determined as the superoxide-dismutase-sensitive portion of ferricytochrome *c* reduction measured at 550nm in a Labsystem IeMS microplate reader [47, 48]. A two-step activation system was used. Membrane fraction (10μg of protein) and recombinant $p47^{phox}$, $p67^{phox}$ (optimal amounts (0.3-1μg) were determined before use) were pre-incubated for 10 min in the presence of arachidonic acid (AA), and 125μM cytochrome *c* in a final volume of 150μL of PBS containing 1mM $MgCl_2$. GAP proteins were added either 10 min before or 10 min after AA. Membrane fractions isolated from human PMN contained sufficient Rac for NADPH oxidase assembly [38, 49]. For each condition the optimal concentration of AA was determined and used in the activation mixture. Superoxide production was initiated by addition of NADPH (250 μM final cc.) and followed for 10 min. The initial linear portion of the absorption curves (lasting for 2-4 min) was used for calculation of the rate of $O_2^{\bullet-}$-production. Parallel samples were run in the presence of 100μg

of superoxide dismutase (SOD). For calculation of the $O_2^{\bullet-}$ production, the absorption coefficient of ferricytochrome *c* of 21000$M^{-1} \cdot cm^{-1}$ was corrected to the properties of the plate reader.

Measurement of the GTP-ase Activity of Rac

The GTP-ase activity was measured by the nitrocellulose filter-binding assay as described in [50]. Loading of recombinant Rac1 (1μg of protein) was performed for 5 minutes at room temperature with a high specific radioactivity of [γ-^{32}P]GTP (more than 5000Ci/mM) in low magnesium buffer (16mM Tris-HCl, pH 7.5, 20mM NaCl, 0.1mM dithiothreitol [DTT], 5 mM EDTA, and 100nM [γ-^{32}P]GTP [5 μCi]). Thereafter, $MgCl_2$ was added at 20 mM to diminish further nucleotide exchange. The solution was kept on ice to decrease nucleotide hydrolysis. The GTPase reaction was initiated by the addition of 3μL of small G protein loaded with [γ-^{32}P]GTP to 27μL of a warmed (20°C) buffer (16mM Tris-HCl, pH 7.5, 0.1mM DTT, 1mg/mL bovine serum albumin, and 1mM unlabeled GTP) containing the different GAPs. Aliquots (5μL) were taken at regular intervals and filtered through nitrocellulose membranes, followed by washing 3 times with 2 mL of cold buffer consisting of 50mM Tris-HCl and 5mM $MgCl_2$; pH 7.7. The filters were dried and the radioactivity was measured by the Cerenkov-effect in a Beckman LS 5000TD liquid-scintillation spectrometer. GAP activity is presented as the decrease in protein-bound radioactivity retained on the filters in time.

Immunoprecipitation experiments

Protein G–Sepharose beads (in the case of ARHGAP35 and isotype control IgG_1 Ab) or Protein A–Sepharose beads (in the case of ARHGAP1, ARHGAP25 and isotype control rabbit-Ab) were incubated with the indicated Ab in the presence of 20mg/mL albumin for 40

min and washed five times in PBS to remove traces of unbound immunoglobulins. Membranes from human PMN corresponding to approx. 1 mg of protein were solubilized for 10 min in PBS containing 40mM octylglucoside at room temperature. Immunodepletion was performed in three successive steps, incubating the solubilized material each time for 15 min with approx. 30μL of antibody-loaded Sepharose beads. For the separation of the beads Spin-X Centrifuge Tube Filter with 450nm pore was used. The separated beads were washed three times in PBS supplemented with 0.1% (v/v) Triton X-100. The immunodepleted probes were tested for cell-free *in vitro* superoxide production described above. Except of the solubilization, all steps of immunodepletion were performed at 4°C.

Protein determination

The protein content was determined as described by Bradford [51], with BSA as standard.

Immunoblotting

Identical amount of protein (50μg) of the indicated samples was lysed in 4x Laemmli sample buffer containing β-mercaptoethanol (300μM), boiled and run on 10% (w/v) SDS polyacrylamide gels and transferred to nitrocellulose membranes. After blocking for 1 h in PBS containing 5% defatted milk powder and 0.1% (w/v) Tween 20, blots were incubated with indicated Ab (anti-ARHGAP25 and anti-ARHGAP1 Ab in 1:1000 dilution; anti-ARHGAP35 in 1:1000 dilution in PBS containing 5% defatted milk powder). Bound Ab was detected with enhanced chemiluminescence using horseradish peroxidase-conjugated anti-rabbit-Ig (from donkey) or anti-mouse-Ig (from sheep) secondary antibodies used in 1:5000 dilution in PBS containing 5% defatted milk powder.

Structure analysis

Homologs of Rac and GAP were found in the Protein Database (PDB) via BLAST searches and the subset that contain both Rac and GAP homologs were identified. Crystal structures were visualized and analyses performed in the UCSF Chimera package [52]. The Rho homolog of each of several crystallized Rho-family smg-GAP complexes [1GRN[53], 1TX4[54], 1OW3[55], 1AM4[56], 3MSX (Utepbergenov, D., Cooper, D.R., Derewenda, U., Somlyo, A.V., Derewenda, Z.S. Mechanism of molecular specificity of RhoGAP domains towards small GTPases of RhoA family. Unpublished)] was superimposed onto the Rac protein in a crystal structure of the Rac-p67^{phox} complex (PDB 1E96) using the default settings of Chimera's Matchmaker program [57], allowing circular permutation and iteration of superposition/alignment; RMSD values for the superimposed Rho homolog and Rac were obtained.

Results

Structural considerations

ARHGAP1 interacts equally with RhoA and Rac1 [58]. Thus, as a first step to investigation of the interaction of RacGAPs with the Nox2 complex, we inspected superpositions of 5 crystal structures (PDB ids 1GRN, 1TX4, 10W3, 1AM4, 3MSX) of Rho- or Cdc42/GAP complexes (hereafter referred to as Rho/GAP complexes) onto the crystal structure of the Rac/p67^{phox} complex (PDB 1E96), using the Rac protein as the reference structure. Several of the Rho/GAP complexes were obtained in the presence of fluoride compounds, thought to simulate the GTP hydrolysis transition state [59]. Consistent with their high sequence similarity, Rho or Cdc42 superpositions onto Rac exhibited small RMSDs, ranging from 0.74 to 0.86 Angstroms, indicating very close structural similarity. The

superposition of Cdc42 and Rac reveals that Rac has identical residues in positions corresponding to those identified as participating in binding of Cdc42 to ARHGAP1[56], identifying Rac's presumed interaction surface with GAP. Inspections of the superimposed crystal structures shows that in every case GAP and $p67^{phox}$ TPR bind to distinct, non-overlapping faces of the Rho or Rac protein; one example illustrating the positions of $p67^{phox}$ and ARHGAP1 when RhoA and Rac are superimposed is shown in Figure 1. All of the superpositions of these static structures contain a small area of steric clash (circled, Figure 1A) between the extreme N-terminus of $p67^{phox}$ and the GAP protein, but given the usual mobility of protein termini in the dynamic aqueous environment we concluded that this would not necessarily represent a significant hindrance to simultaneous binding of GAP proteins and $p67^{phox}$ to Rac. The C-terminus of the TPR structure connects to the remainder of the $p67^{phox}$ polypeptide, most of which has not been crystallized, but inspection of the structure reveals a large area away from Rac and GAP available for occupation by the remainder of $p67^{phox}$. Similarly, there is space available for the N-terminal domain of the GAP proteins.

These observations indicate that simultaneous binding of GAP and $p67^{phox}$ to Rac may be sterically possible. Furthermore, as illustrated in Figure 1B, these superpositions indicate that several faces of Rac/$p67^{phox}$ would remain available for interaction with Nox2 if GAP and $p67^{phox}$ were to bind Rac simultaneously. One of these faces includes the insert domain of Rac, shown to be critical for the interaction of Rac and Nox2 [21].

Effect of added RacGAPs on Nox2 activity.

Immunoblots revealed the presence of all identified RacGAPs both in the cytosolic and in the membrane fractions [40, 44]. To mimic the potential effect of cytosolic RacGAPs, we first added these proteins to the semirecombinant cell-free $O_2^{\bullet-}$-generation system. In previous studies it has been shown that addition of Bcr, ARHGAP1 or ARHGAP35 decreased

$O_2^{\bullet-}$-production in crude cell-free systems [39, 40]. However, these studies were carried out under different experimental conditions so that a direct comparison of individual RacGAP effects was not possible. Therefore we carried out a comparative study including the recently discovered hemopoetic cell specific ARHGAP25. In parallel radioactive GTPase measurements we controlled the RacGAP activity of each protein preparation (data not shown). The amount of RacGAP added to the semi-recombinant cell-free system was varied in order to achieve equal GTP hydrolytic activity in all samples.

As shown in Figure 2, when added before arachidonic acid-triggered assembly of the Nox2 complex, all four RacGAPs reduced the rate of $O_2^{\bullet-}$-production. In 5 separate experiments we observed no significant difference between the effect of the applied RacGAPs, ARHGAP25 was as effective as the other three previously studied proteins. Similar to previous observations [40], reduction of $O_2^{\bullet-}$-production changed in parallel with the amount of added GAPs. In contrast to these results, when the same amount of the various RacGAPs was added to the reaction mixture 10 min *after* initiation of the activation process, the rate of $O_2^{\bullet-}$-production was the same as that in the absence of any RacGAP. This result agrees with previous observations [38]. Identical results were obtained when membranes were solubilized in octylglucoside.

Apparently, soluble RacGAPs – representing proteins localized in the cytosol – have similar access to RacGTP under resting conditions, but none of them has access to RacGTP embedded in the assembled Nox2 complex.

Immunodepletion of the membrane fraction

Next we turned our attention to endogenous RacGAPs localized to the isolated membrane fraction of neutrophilic granulocytes. In order to keep the NADPH oxidase activity high, membranes were solubilized in octylglucoside [60]. Immunodepletion was carried out

from the solubilized membranes with repeated short exposure to specific antibodies, taking care to preserve the activity of both Nox2 and the remaining GAPs. One typical experiment and the average of more than 10 separate measurements are represented in Figure 3. On the average we succeeded in depleting 70% of ARHGAP1, 65% of ARHGAP25 and 65% of ARHGAP35 from the solubilized membranes. Combining the two polyclonal antibodies, we also carried out double-depletion, achieving 60% decrease in ARHGAP1 and 50% decrease in ARHGAP25.

Nox2 activity in immunodepleted membranes

We tested the capacity of $O_2^{\bullet-}$-production of intact membranes and membranes treated with isotype control serum or specific antibodies against one or two membrane-associated RacGAPs (Figure 4). Depletion of ARHGAP1 or ARHGAP25 alone resulted in a greater than two-fold increase in the rate of $O_2^{\bullet-}$-generation. Moreover, the effect of the two RacGAPs was additive: in double-depleted membranes $O_2^{\bullet-}$-production was more than 3 times higher than in the isotype treated membranes. In contrast, in 6 separate experiments, depletion of ARHGAP35 from the membranes did not affect $O_2^{\bullet-}$-production at all.

Apparently, some but not all of the membrane-localized RacGAPs do interact with Rac in the Nox2 complex and constitutively diminish $O_2^{\bullet-}$-production.

Effect of membrane-associated RacGAPs on assembly or activity of the Nox2 complex

Finally we tested whether membrane-associated RacGAPs can only interact with uncomplexed Rac, thus inhibiting the assembly/activation of the Nox2 complex; or alternatively if they can interact with Rac in the already-assembled complex. NaF was previously shown to stabilize the smg-GAP complex [59]. In our earlier experiments, addition of NaF reduced the RacGAP effect on $O_2^{\bullet-}$-production of neutrophil membranes [36-38, 40],

but it did not affect the activity of purified cytochrome b_{558} [38, 40]. These findings suggested that NaF acted by sequestering the GAP(s) – present in neutrophil membranes but absent in the cytochrome b_{558} preparation - into a stable complex. To strengthen the specificity of the effect of NaF, we tested its effect on $O_2^{\bullet-}$-production in the different GAP-depleted membranes (Fig. 5A). Both in ARHGAP35-depleted membranes and in control membranes treated with isotype serum, NaF brought about an approx. 4-fold increase of $O_2^{\bullet-}$-production. In contrast, in double-depleted membranes, the increase was only about 1.3-fold.

Finally, we compared the effect of NaF before and after assembly of the catalytically active enzyme complex (Fig. 5B). Addition of NaF before assembly of Nox2 complex increased $O_2^{\bullet-}$-production approximately threefold, but when NaF was added to the assembled complex, $O_2^{\bullet-}$-generation still increased more than twofold (Figure 5). This observation indicates that RacGAP(s) may have access to Rac also in the assembled Nox2 complex.

Discussion

Physiological functioning of NADPH oxidases requires strict spatiotemporal control. For Rac-dependent oxidases (Nox1-3) acceleration of GTP hydrolysis by GAPs provides a reasonable control mechanism. In case of Nox2 it was shown that both the activation and the sustained turn-over of the enzyme requires the active, GTP-bound form of Rac [37, 38], hence regulatable RacGAPs could ensure the efficient fine-tuning of both phases. In this study we provide molecular data supporting this hypothesis.

We report here the first structural analysis to explore the possible interaction of GAP and Nox2 subunits. Our investigations using static superposition of existing crystal structures of Rac and Rho complexes indicate that simultanous interaction of Rac, $p67^{phox}$, RacGAP and Nox2 is plausible.

In human neutrophils RacGAPs are present both in the cytosol and in the membrane fraction. We investigated the potential contribution of RacGAPs at both locations. The effect of cytosolic GAPs was mimicked by addition of soluble RacGAPs to the cell-free activation system. When different proteins were added at equal GAP-activity, all four RacGAPs identified hitherto in PMN similarly decreased $O_2^{\bullet-}$-production. Interestingly, the same proteins had no effect on $O_2^{\bullet-}$-production when they were added to the already assembled enzyme complex. Apparently soluble RacGAPs were able to interact with free RacGTP, but had no access to their target embedded in the Nox2 complex.

In contrast, RacGAPs associated with the membrane fraction seem capable of interacting with Rac even after its incorporation into the assembled Nox2 complex. We show that addition of NaF after the assembly of the enzyme complex still increases $O_2^{\bullet-}$-production. As small GTPases can form a stable complex with fluoride compounds only in the presence of their GAP [59] our finding indicates that membrane-localized RacGAP(s) have access to the assembled enzyme complex. Our structural analysis supports this possibility.

We observed, however, differences in the interaction of membrane-associated RacGAPs to the Nox2 complex. Depletion of more than 50% of the amount of ARHGAP1 or ARHGAP25 resulted in a clear increase of $O_2^{\bullet-}$-production, indicating a constitutive downregulation of Nox2 activity by these proteins. The effect of ARHGAP1 and ARHGAP25 was additive, increasing the rate of $O_2^{\bullet-}$-production approx. threefold. However, maximal $O_2^{\bullet-}$-production (detected in the presence of GTPγS or NaF) was not achieved. Thus, involvement of further, not yet identified RacGAP(s) can not be excluded at this point.

In contrast to ARHGAP1 and ARHGAP25, depletion of ARHGAP35 to a similar extent had no effect on the enzyme activity of Nox2, although the RacGAP activity of ARHGAP35 has been confirmed also in cellular context [61] . The lack of effect upon immunodepletion of ARHGAP35 agrees with data obtained in mice with ARHGAP35-

deficient hemopoetic system [62]. Apparently, membrane-associated RacGAPs exhibit selectivity in their interactions with the Nox2 complex. The basis of this selectivity may simply be the size, as ARHGAP35 (190 kD) is significantly larger than either ARHGAP1 (50kD) or ARHGAP25 (73kD). On the other hand, the structural investigations indicate the possibility of an interacting surface between RacGAP and the Rac-effector $p67^{phox}$. Such a surface presents one possible basis of discrimination between different RacGAPs. In the case of guanine nucleotide exchange factors (GEFs) examples of a trimolecular interaction between the regulatory protein, the small GTPase and the effector protein have been described [63-65]. Simultanous interaction of Rac with the effector protein $p67^{phox}$ and the regulatory protein RacGAP may represent a similar mechanism resulting in specific regulation of the NADPH oxidase.

Our data clearly indicate that in neutrophilic granulocytes more than one RacGAP is involved in the regulation of the NADPH oxidase. Both the activity and the localization of GAPs was shown to be modulated by diverse mechanisms such as phosphorylation, lipid or protein interactions, autoinhibition, etc.[30]. Regulation of one Rac-dependent process by multiple GAPs could allow selective modulation by various signaling pathways and contribute to the fine-tuning of the generation of the toxic superoxide anion.

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References

[1] Bedard, K.; Krause, K. H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* **87:**245-313; 2007.
[2] Lambeth, J. D. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* **4:**181-189; 2004.
[3] Geiszt, M. NADPH oxidases: new kids on the block. *Cardiovasc Res* **71:**289-299; 2006.
[4] Leto, T. L.; Geiszt, M. Role of Nox family NADPH oxidases in host defense. *Antioxid Redox Signal* **8:**1549-1561; 2006.
[5] Banfi, B.; Malgrange, B.; Knisz, J.; Steger, K.; Dubois-Dauphin, M.; Krause, K. H. NOX3, a superoxide-generating NADPH oxidase of the inner ear. *J Biol Chem* **279:**46065-46072; 2004.
[6] Dupuy, C.; Ohayon, R.; Valent, A.; Noel-Hudson, M. S.; Deme, D.; Virion, A. Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cdnas. *J Biol Chem* **274:**37265-37269; 1999.
[7] Takac, I.; Schroder, K.; Brandes, R. P. The Nox family of NADPH oxidases: friend or foe of the vascular system? *Curr Hypertens Rep* **14:**70-78; 2012.
[8] Stasia, M. J.; Li, X. J. Genetics and immunopathology of chronic granulomatous disease. *Semin Immunopathol* **30:**209-235; 2008.
[9] Moreno, J. C.; Bikker, H.; Kempers, M. J.; van Trotsenburg, A. S.; Baas, F.; de Vijlder, J. J.; Vulsma, T.; Ris-Stalpers, C. Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. *The New England journal of medicine* **347:**95-102; 2002.
[10] Nauseef, W. M. Biological roles for the NOX family NADPH oxidases. *J Biol Chem* **283:**16961-16965; 2008.
[11] Vignais, P. V. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol Life Sci* **59:**1428-1459; 2002.
[12] Sumimoto, H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *FEBS J* **275:**3249-3277; 2008.
[13] Abo, A.; Pick, E.; Hall, A.; Totty, N.; Teahan, C. G.; Segal, A. W. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* **353:**668-670; 1991.
[14] Knaus, U. G.; Heyworth, P. G.; Evans, T.; Curnutte, J. T.; Bokoch, G. M. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. *Science* **254:**1512-1515; 1991.
[15] Ambruso, D. R.; Knall, C.; Abell, A. N.; Panepinto, J.; Kurkchubasche, A.; Thurman, G.; Gonzalez-Aller, C.; Hiester, A.; deBoer, M.; Harbeck, R. J.; Oyer, R.; Johnson, G. L.; Roos, D. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proceedings of the National Academy of Sciences of the United States of America* **97:**4654-4659; 2000.
[16] Roberts, A. W.; Kim, C.; Zhen, L.; Lowe, J. B.; Kapur, R.; Petryniak, B.; Spaetti, A.; Pollock, J. D.; Borneo, J. B.; Bradford, G. B.; Atkinson, S. J.; Dinauer, M. C.; Williams, D. A. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity* **10:**183-196; 1999.
[17] Diekmann, D.; Abo, A.; Johnston, C.; Segal, A. W.; Hall, A. Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. *Science* **265:**531-533; 1994.
[18] Koga, H.; Terasawa, H.; Nunoi, H.; Takeshige, K.; Inagaki, F.; Sumimoto, H. Tetratricopeptide repeat (TPR) motifs of p67(phox) participate in interaction with the small GTPase Rac and activation of the phagocyte NADPH oxidase. *J Biol Chem* **274:**25051-25060; 1999.

[19] Lapouge, K.; Smith, S. J.; Walker, P. A.; Gamblin, S. J.; Smerdon, S. J.; Rittinger, K. Structure of the TPR domain of p67phox in complex with Rac.GTP. *Mol Cell* **6:**899-907; 2000.
[20] Grizot, S.; Fieschi, F.; Dagher, M. C.; Pebay-Peyroula, E. The active N-terminal region of p67phox. Structure at 1.8 A resolution and biochemical characterizations of the A128V mutant implicated in chronic granulomatous disease. *J Biol Chem* **276:**21627-21631; 2001.
[21] Diebold, B. A.; Bokoch, G. M. Molecular basis for Rac2 regulation of phagocyte NADPH oxidase. *Nature immunology* **2:**211-215; 2001.
[22] Gorzalczany, Y.; Alloul, N.; Sigal, N.; Weinbaum, C.; Pick, E. A prenylated p67phox-Rac1 chimera elicits NADPH-dependent superoxide production by phagocyte membranes in the absence of an activator and of p47phox: conversion of a pagan NADPH oxidase to monotheism. *J Biol Chem* **277:**18605-18610; 2002.
[23] Bokoch, G. M.; Diebold, B. A. Current molecular models for NADPH oxidase regulation by Rac GTPase. *Blood* **100:**2692-2696; 2002.
[24] Geiszt, M.; Lekstrom, K.; Witta, J.; Leto, T. L. Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *J Biol Chem* **278:**20006-20012; 2003.
[25] Banfi, B.; Clark, R. A.; Steger, K.; Krause, K. H. Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. *J Biol Chem* **278:**3510-3513; 2003.
[26] Kiss, P. J.; Knisz, J.; Zhang, Y.; Baltrusaitis, J.; Sigmund, C. D.; Thalmann, R.; Smith, R. J.; Verpy, E.; Banfi, B. Inactivation of NADPH oxidase organizer 1 results in severe imbalance. *Current biology : CB* **16:**208-213; 2006.
[27] Ueyama, T.; Geiszt, M.; Leto, T. L. Involvement of Rac1 in activation of multicomponent Nox1- and Nox3-based NADPH oxidases. *Molecular and cellular biology* **26:**2160-2174; 2006.
[28] Takai, Y.; Sasaki, T.; Matozaki, T. Small GTP-binding proteins. *Physiol Rev* **81:**153-208; 2001.
[29] Scheffzek, K.; Ahmadian, M. R. GTPase activating proteins: structural and functional insights 18 years after discovery. *Cell Mol Life Sci* **62:**3014-3038; 2005.
[30] Ligeti, E.; Welti, S.; Scheffzek, K. Inhibition and termination of physiological responses by GTPase activating proteins. *Physiol Rev* **92:**237-272; 2012.
[31] Bernards, A. GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. *Biochim Biophys Acta* **1603:**47-82; 2003.
[32] Tcherkezian, J.; Lamarche-Vane, N. Current knowledge of the large RhoGAP family of proteins. *Biol Cell* **99:**67-86; 2007.
[33] Csepanyi-Komi, R.; Safar, D.; Grosz, V.; Tarjan, Z. L.; Ligeti, E. In silico tissue-distribution of human Rho family GTPase activating proteins. *Small GTPases* **4**; 2013.
[34] Csepanyi-Komi, R.; Levay, M.; Ligeti, E. Rho/RacGAPs: embarras de richesse? *Small GTPases* **3:**178-182; 2012.
[35] Bernards, A.; Settleman, J. GAP control: regulating the regulators of small GTPases. *Trends Cell Biol* **14:**377-385; 2004.
[36] Wolfl, J.; Dagher, M. C.; Fuchs, A.; Geiszt, M.; Ligeti, E. In vitro activation of the NADPH oxidase by fluoride. Possible involvement of a factor activating GTP hydrolysis on Rac (Rac-GAP). *Eur J Biochem* **239:**369-375; 1996.
[37] Szaszi, K.; Korda, A.; Wolfl, J.; Paclet, M. H.; Morel, F.; Ligeti, E. Possible role of RAC-GTPase-activating protein in the termination of superoxide production in phagocytic cells. *Free Radic Biol Med* **27:**764-772; 1999.

[38] Moskwa, P.; Dagher, M. C.; Paclet, M. H.; Morel, F.; Ligeti, E. Participation of Rac GTPase activating proteins in the deactivation of the phagocytic NADPH oxidase. *Biochemistry* **41:**10710-10716; 2002.
[39] Heyworth, P. G.; Knaus, U. G.; Xu, X.; Uhlinger, D. J.; Conroy, L.; Bokoch, G. M.; Curnutte, J. T. Requirement for posttranslational processing of Rac GTP-binding proteins for activation of human neutrophil NADPH oxidase. *Mol Biol Cell* **4:**261-269; 1993.
[40] Geiszt, M.; Dagher, M. C.; Molnar, G.; Havasi, A.; Faure, J.; Paclet, M. H.; Morel, F.; Ligeti, E. Characterization of membrane-localized and cytosolic Rac-GTPase-activating proteins in human neutrophil granulocytes: contribution to the regulation of NADPH oxidase. *The Biochemical journal* **355:**851-858; 2001.
[41] Voncken, J. W.; van Schaick, H.; Kaartinen, V.; Deemer, K.; Coates, T.; Landing, B.; Pattengale, P.; Dorseuil, O.; Bokoch, G. M.; Groffen, J.; et al. Increased neutrophil respiratory burst in bcr-null mutants. *Cell* **80:**719-728; 1995.
[42] Cunnick, J. M.; Schmidhuber, S.; Chen, G.; Yu, M.; Yi, S. J.; Cho, Y. J.; Kaartinen, V.; Minoo, P.; Warburton, D.; Groffen, J.; Heisterkamp, N. Bcr and Abr cooperate in negatively regulating acute inflammatory responses. *Molecular and cellular biology* **29:**5742-5750; 2009.
[43] Costa, C.; Germena, G.; Martin-Conte, E. L.; Molineris, I.; Bosco, E.; Marengo, S.; Azzolino, O.; Altruda, F.; Ranieri, V. M.; Hirsch, E. The RacGAP ArhGAP15 is a master negative regulator of neutrophil functions. *Blood* **118:**1099-1108; 2011.
[44] Csepanyi-Komi, R.; Sirokmany, G.; Geiszt, M.; Ligeti, E. ARHGAP25, a novel Rac GTPase-activating protein, regulates phagocytosis in human neutrophilic granulocytes. *Blood* **119:**573-582; 2012.
[45] Levay, M.; Settleman, J.; Ligeti, E. Regulation of the substrate preference of p190RhoGAP by protein kinase C-mediated phosphorylation of a phospholipid binding site. *Biochemistry* **48:**8615-8623; 2009.
[46] Hjorth, R.; Jonsson, A. K.; Vretblad, P. A rapid method for purification of human granulocytes using percoll. A comparison with dextran sedimentation. *J Immunol Methods* **43:**95-101; 1981.
[47] Rada, B. K.; Geiszt, M.; Kaldi, K.; Timar, C.; Ligeti, E. Dual role of phagocytic NADPH oxidase in bacterial killing. *Blood* **104:**2947-2953; 2004.
[48] Ligeti, E.; Tardif, M.; Vignais, P. V. Activation of O2.- generating oxidase of bovine neutrophils in a cell-free system. Interaction of a cytosolic factor with the plasma membrane and control by G nucleotides. *Biochemistry* **28:**7116-7123; 1989.
[49] Fuchs, A.; Dagher, M. C.; Jouan, A.; Vignais, P. V. Activation of the O2(-)-generating NADPH oxidase in a semi-recombinant cell-free system. Assessment of the function of Rac in the activation process. *Eur J Biochem* **226:**587-595; 1994.
[50] Self, A. J.; Hall, A. Measurement of intrinsic nucleotide exchange and GTP hydrolysis rates. *Methods in enzymology* **256:**67-76; 1995.
[51] Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248-254; 1976.
[52] Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of computational chemistry* **25:**1605-1612; 2004.
[53] Nassar, N.; Hoffman, G. R.; Manor, D.; Clardy, J. C.; Cerione, R. A. Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP. *Nature structural biology* **5:**1047-1052; 1998.

[54] Rittinger, K.; Walker, P. A.; Eccleston, J. F.; Smerdon, S. J.; Gamblin, S. J. Structure at 1.65 A of RhoA and its GTPase-activating protein in complex with a transition-state analogue. *Nature* **389:**758-762; 1997.
[55] Graham, D. L.; Lowe, P. N.; Grime, G. W.; Marsh, M.; Rittinger, K.; Smerdon, S. J.; Gamblin, S. J.; Eccleston, J. F. MgF(3)(-) as a transition state analog of phosphoryl transfer. *Chemistry & biology* **9:**375-381; 2002.
[56] Rittinger, K.; Walker, P. A.; Eccleston, J. F.; Nurmahomed, K.; Owen, D.; Laue, E.; Gamblin, S. J.; Smerdon, S. J. Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. *Nature* **388:**693-697; 1997.
[57] Meng, E. C.; Pettersen, E. F.; Couch, G. S.; Huang, C. C.; Ferrin, T. E. Tools for integrated sequence-structure analysis with UCSF Chimera. *BMC bioinformatics* **7:**339; 2006.
[58] Ligeti, E.; Settleman, J. Regulation of RhoGAP specificity by phospholipids and prenylation. *Methods in enzymology* **406:**104-117; 2006.
[59] Mittal, R.; Ahmadian, M. R.; Goody, R. S.; Wittinghofer, A. Formation of a transition-state analog of the Ras GTPase reaction by Ras-GDP, tetrafluoroaluminate, and GTPase-activating proteins. *Science* **273:**115-117; 1996.
[60] Pick, E.; Kroizman, T.; Abo, A. Activation of the superoxide-forming NADPH oxidase of macrophages requires two cytosolic components--one of them is also present in certain nonphagocytic cells. *J Immunol* **143:**4180-4187; 1989.
[61] Levay, M.; Bartos, B.; Ligeti, E. p190RhoGAP has cellular RacGAP activity regulated by a polybasic region. *Cellular signalling* **25:**1388-1394; 2013.
[62] Nemeth, T.; Futosi, K.; Hably, C.; Brouns, M. R.; Jakob, S. M.; Kovacs, M.; Kertesz, Z.; Walzog, B.; Settleman, J.; Mocsai, A. Neutrophil functions and autoimmune arthritis in the absence of p190RhoGAP: generation and analysis of a novel null mutation in mice. *J Immunol* **185:**3064-3075; 2010.
[63] Jaffe, A. B.; Aspenstrom, P.; Hall, A. Human CNK1 acts as a scaffold protein, linking Rho and Ras signal transduction pathways. *Molecular and cellular biology* **24:**1736-1746; 2004.
[64] Jaffe, A. B.; Hall, A.; Schmidt, A. Association of CNK1 with Rho guanine nucleotide exchange factors controls signaling specificity downstream of Rho. *Current biology : CB* **15:**405-412; 2005.
[65] Connolly, B. A.; Rice, J.; Feig, L. A.; Buchsbaum, R. J. Tiam1-IRSp53 complex formation directs specificity of rac-mediated actin cytoskeleton regulation. *Molecular and cellular biology* **25:**4602-4614; 2005.

Figure legends

***Figure 1*:** Example of superposition of crystal structures of rho/GAP complexes onto Rac/p67^{phox} complex. A. Cdc42 (cyan) in complex with ARHGAP1 (pink) (PDB 1AM4) superimposed onto Rac (green) in complex with the tetraticopeptide repeat domain of p67phox (gray) (PDB 1E96) reveals distinct faces for Rac interaction with p67^{phox} and (potentially) ARHGAP1. The arginine (blue) critical for GAP-activated GTP hydrolysis, and

GTP (pink from Cdc42 structure, gray from Rac), are shown in stick representation. Blue circle indicates a small region of steric clash between the extreme N-terminus of p67phox and ARHGAP1. B. Surface view of the same superimposed structures (Cdc42 hidden for clarity) shows interaction surfaces on Rac and $p67^{phox}$ that remain available to other parts of the Nox assembly assuming that ARHGAP1 binds to Rac. Surface colors correspond to ribbon colors in A, except that cyan indicates insert region of Rac.

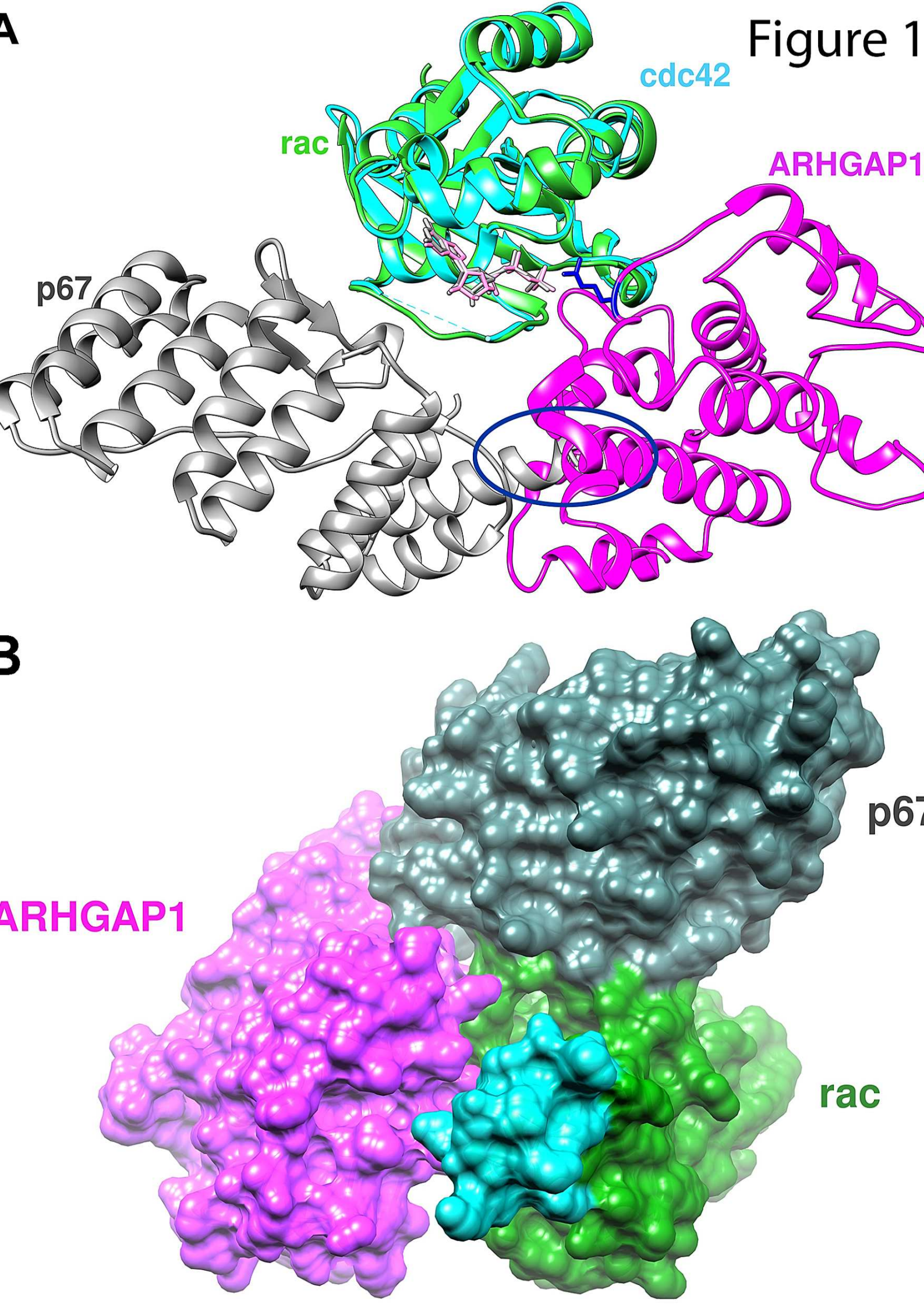
Figure 2: Effect of soluble GAPs on the activation and catalytic activity of NADPH oxidase. PBS control represents the rate of $O_2^{\bullet-}$-production obtained in the semi-recombinant system and is taken as 100% activity (mean for rate in 5 separate experiments was 72.6 nmol $O_2^{\bullet-}$/min/mg membrane protein). Amounts of GAPs added were adjusted to produce equal GTPase activity (as determined separately in a nitrocellulose filter-binding assay). GAPs were added 10 min before (black bars) or 10 minutes after (gray bars) induction of assembly of the Nox2 complex by AA. Mean + SEM of 5 (pre-activation) or 3 (post-activation) separate experiments are shown (*$P < .05$ compared with control).

Figure 3: Western blot of the immunodepletion of the membrane fractions. Intact sample shows the amount of ARHGAP35, ARHGAP25 and ARHGAP1 in the non-treated membrane. Isotype depleted membrane serves as a control for the specificity of the depletion process. Equal amounts of membrane protein (50μg) were used for analysis (A). Densitometric analysis of ARHGAP25 (black bar) and ARHGAP1 (gray bar) signal relative to total protein content of the samples. Mean + SEM of 15 (isotype control and ARHGAP1), 14 (intact and ARHGAP25) or 8 (ARHGAP1+ARHGAP25) separate experiments are shown (B). Densitometric analysis of ARHGAP35 signal relative to total protein content of the samples. Mean + SEM of 4 separate experiments are shown (C). ARHGAP35 serves as

loading control for ARHGAP1 and ARHGAP25 and the two latter serve as loading control for ARHGAP35.

Figure 4**:** Effect of immunodepletion of membrane-localized GAPs on the activity of NADPH oxidase. Isotype control Ab depleted membrane (black bar) was taken as 100% $O_2^{\bullet-}$-production activity (mean of 15 separate experiments was 39.7 nmol $O_2^{\bullet-}$/min/mg membrane protein). White bar: $O_2^{\bullet-}$-production of the intact membrane (mean + SEM of 14 separate experiments) relative to the isotype depleted control. Gray bars: $O_2^{\bullet-}$-production of specific immunodepleted membrane fractions relative to isotype control depleted membrane. Mean + SEM of 15 (ARHGAP1), 14 (ARHGAP25), 8 (ARHGAP1+ARHGAP25) or 4 (ARHGAP35) separate experiments are shown (*$P < .05$ compared with isotype control).

Figure 5: Effect of membrane-localized GAPs on the activity of NADPH oxidase. Activation of the enzyme was carried out in the presence of 30 μM GTP. NaF (40mM) was added to the GAP-depleted (A) or to non-depleted (B) membranes either 10 min before the activation phase or 10 min later, after completion (→) of the activation of the enzyme. Mean + SEM of 8 (Isotype control, ARHGAP1+ARHGAP25) or 4 (ARHGAP35, part B) separate experiments are shown.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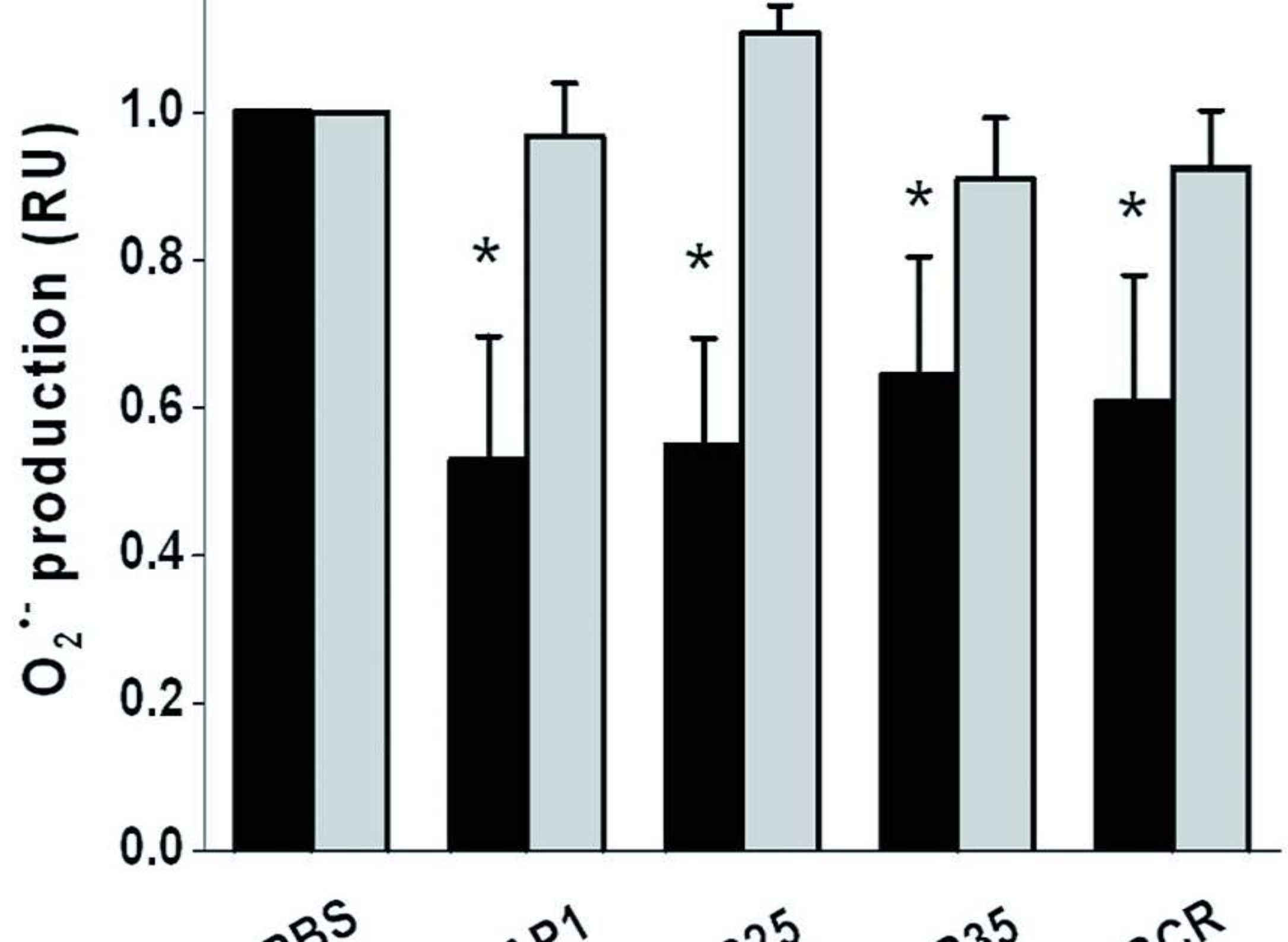
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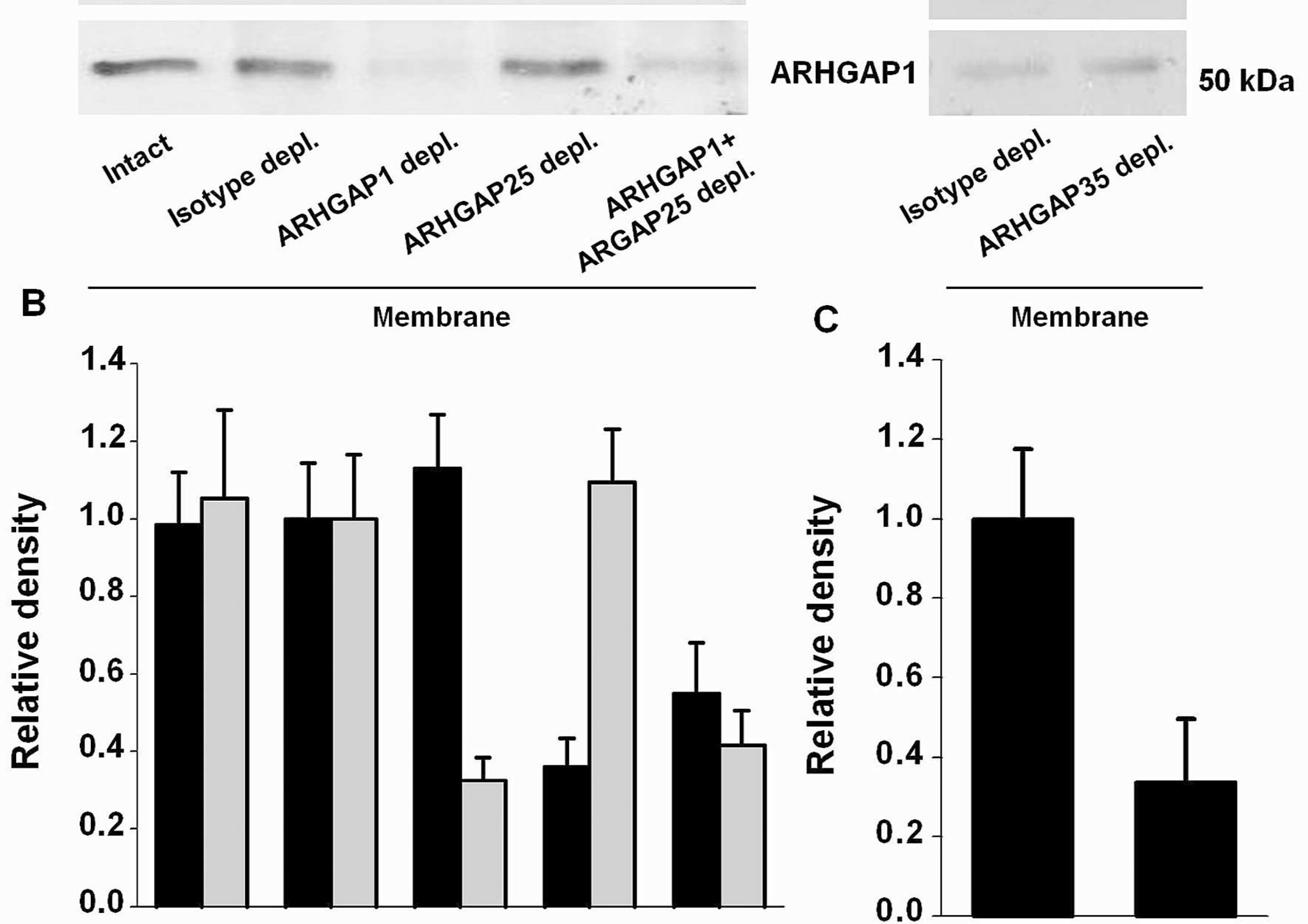
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B
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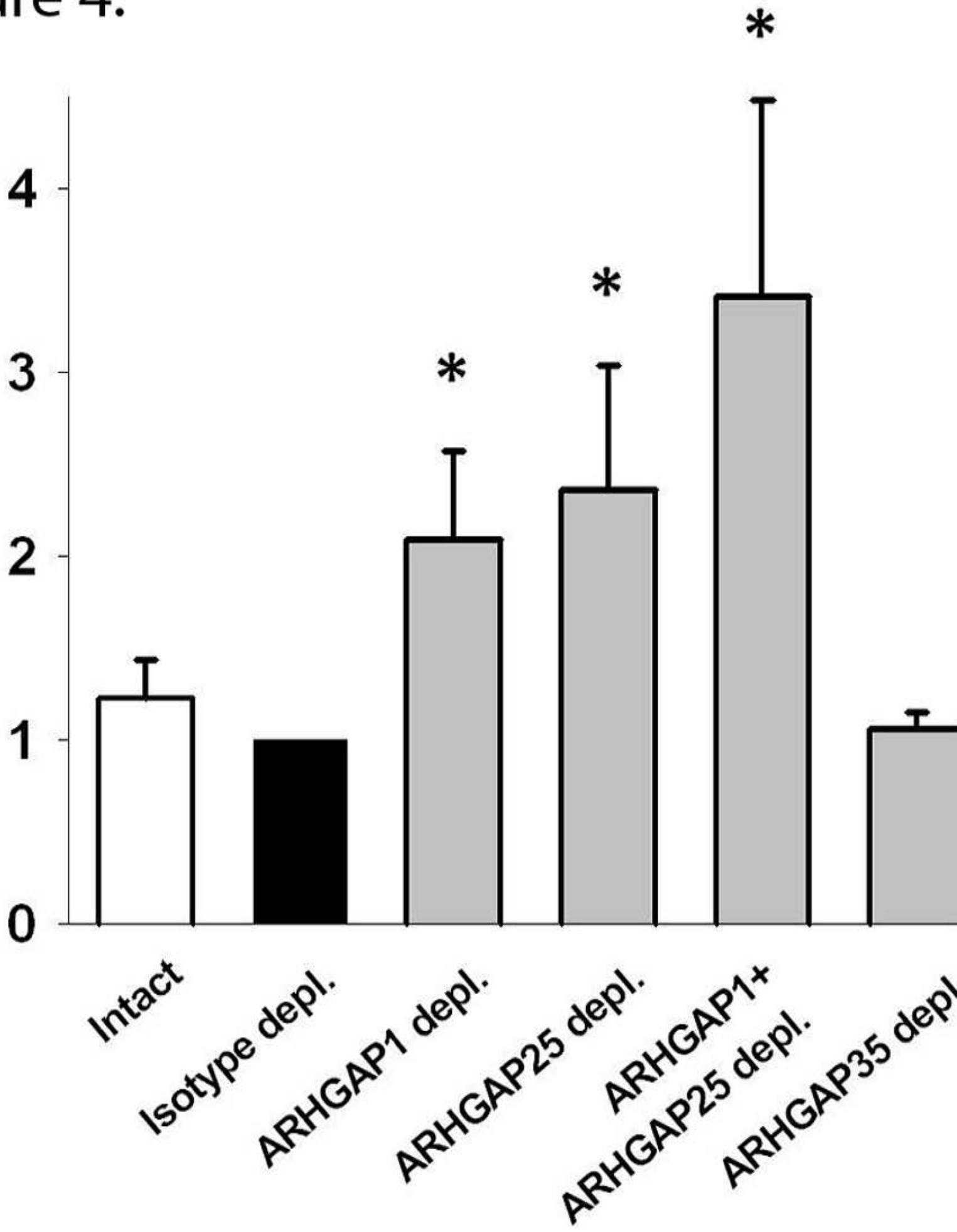
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Figure