

**Rab GTPases bind at a common site within the angiotensin II type I  
receptor carboxyl-terminal tail: Evidence that Rab4 regulates receptor  
phosphorylation, desensitization and resensitization**

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**Running Title:** Rab4 GTPase regulation of AT<sub>1</sub>R signaling

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**ABBREVIATIONS:** AngII, Angiotensin II; AT<sub>1</sub>R, angiotensin II type 1 receptor; ANOVA, analysis of variance;  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEPES buffered saline solution (HBSS); HEK 293 cells, human embryonic kidney cells, IP, inositol phosphate; PBS, phosphate buffered saline.

## ABSTRACT

The human angiotensin II type 1 receptor (AT<sub>1</sub>R) is a member of the G protein-coupled receptor (GPCR) superfamily and represents an important target for cardiovascular therapeutic intervention. Agonist-activation of the AT<sub>1</sub>R induces  $\beta$ -arrestin-dependent endocytosis to early endosomes where the receptor resides as a protein complex with the Rab GTPase Rab5. In the present study, we examined whether other Rab GTPases that regulate receptor trafficking through endosomal compartments also bind to the AT<sub>1</sub>R. We find that Rab4, Rab7 and Rab11 all bind to the last 10 amino acid residues of the AT<sub>1</sub>R carboxyl-terminal tail. Rab11 binds AT<sub>1</sub>R more effectively than Rab5, whereas Rab4 binds less effectively than Rab5. Alanine scanning mutagenesis reveals that proline 354 and cysteine 355 contribute Rab protein binding and mutation of these residues does not affect G protein coupling. We find that the Rab GTPases each compete with one another for receptor binding and that although Rab4 interacts poorly with the AT<sub>1</sub>R, it effectively displaces Rab11 from the receptor. In contrast, Rab11 overexpression does not prevent Rab4 binding to the AT<sub>1</sub>R. Overexpression of wild-type Rab4, but not Rab11, facilitates AT<sub>1</sub>R dephosphorylation, and a constitutively active Rab4-Q67L mutant reduces AT<sub>1</sub>R desensitization and promotes AT<sub>1</sub>R resensitization. Taken together, our data indicates that multiple RabGTPases bind to a motif localized to the distal end of the AT<sub>1</sub>R tail and that increased Rab4 activity may contribute to the regulation AT<sub>1</sub>R desensitization and dephosphorylation.

## INTRODUCTION

The angiotensin II type 1 receptor (AT<sub>1</sub>R) is a member of the G protein-coupled receptor (GPCR) superfamily, the largest family of integral membrane receptors and represents an important pharmacological target for drug therapy in hypertension (Hoffman and Lefkowitz, 1996). The AT<sub>1</sub>R is coupled through G $\alpha_{q/11}$  to the activation of phospholipase C  $\beta$  resulting in the formation of diacylglycerol and inositol 1, 4,5 trisphosphate leading to the release of intracellular calcium stores and the activation of protein kinase C. Agonist activation of the AT<sub>1</sub>R also results in the attenuation of receptor signalling as the consequence of receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and protein kinase C. Agonist activation and GRK-mediated phosphorylation of the AT<sub>1</sub>R facilitates the recruitment of the cytosolic adaptor protein,  $\beta$ -arrestin, which functions to sterically uncouple the AT<sub>1</sub>R from the heterotrimeric G protein and targets the AT<sub>1</sub>R for clathrin-mediated endocytosis (Benovic et al., 1987; Freedman et al., 1995; Ferguson et al., 1995; Ferguson et al., 1996; Opperman et al., 1996; Krupnick and Benovic, 1998; Ferguson, 2001; Ferguson, 2007). Once internalized, GPCRs may be either sequestered in early endosomes, dephosphorylated and recycled back to the plasma membrane or targeted to lysosomes for degradation (Ferguson, 2001; Gáborik and Hunyady, 2004; Seachrist and Ferguson, 2003). In the case of the AT<sub>1</sub>R, the receptor is internalized as a complex with  $\beta$ -arrestin and is retained in the early endosomal compartment and is not readily dephosphorylated (Anborgh et al., 2000).

The Rab subfamily of small Ras-like GTPases regulate the intracellular trafficking of proteins between intracellular compartments through their ability to regulate vesicular targeting, docking and fusion (Seachrist and Ferguson, 2003; Gáborik and Hunyady, 2004). Rab protein function is in turn tightly regulated at the level of protein expression,

localization, membrane association, and activation. Different Rab isoforms regulate different aspects of intracellular trafficking such as internalization (Rab5), recycling (Rab4 and Rab11) and degradation (Rab7) and different GPCRs are known to preferentially traffic through certain Rab pathways (Seachrist et al., 2000; Hunyady et al., 2002; Seachrist et al., 2002; Dale et al., 2004; Hamelin et al., 2005; Holmes et al., 2006; Wang et al., 2008; Li et al., 2008; Parent et al., 2009). For example, Rab5a has been shown to interact with the AT<sub>1</sub>R carboxyl-terminal tail and retain the receptor in Rab5a-positive early endosomes. Nevertheless, overexpression of either Rab7 or constitutively active Rab11 can redistribute AT<sub>1</sub>R into either Rab7-positive late endosomes or Rab11-positive recycling endosomes, respectively (Seachrist et al., 2000; Dale et al., 2004). Additionally, although AT<sub>1</sub>R is not readily dephosphorylated and efficiently recycled, there is evidence to suggest that the receptor can be recycled via both slow (Rab11-mediated) and rapid (Rab4-mediated) pathways (Hunyady et al., 2002; Li et al., 2008). Rab binding to a GPCR is not unique to the AT<sub>1</sub>R, as Rab11 has been shown to bind to the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR), thromboxane A<sub>2</sub> receptor and prostacyclin receptor (Seachrist et al., 2002; Hamelin et al., 2005; Parent et al., 2009; Reid et al., 2010). Emerging evidence suggests that Rab interactions with these GPCRs are also critical for regulating both the trafficking and activity of these receptors. For example, previous studies with the  $\beta_2$ AR have shown that the transit of the receptor from the Rab5-positive early endosome to the Rab4-positive recycling endosome is required for the dephosphorylation of the receptor (Seachrist et al., 2000).

In the present study, we have investigated whether other Rab GTPases (Rab4, Rab7 and Rab11) can interact with AT<sub>1</sub>R carboxyl-terminal tail and compete with Rab5

for binding. We report here that Rab4, Rab5, Rab7 and Rab11 each compete for an overlapping site in the last 10 amino acid residues of the AT<sub>1</sub>R carboxyl-terminal tail and that proline residue 354 and cysteine residue 355 represent important amino acid residues involved in Rab protein binding. Moreover, we find that overexpression of either wild-type or constitutively active Rab4, but not Rab11, promotes AT<sub>1</sub>R dephosphorylation. The overexpression of a constitutively active Rab4 mutant also results in reduced AT<sub>1</sub>R desensitization and promotes AT<sub>1</sub>R resensitization. Taken together, our data indicate that multiple Rab GTPases are able to associate with their cargo and that the activity of the AT<sub>1</sub>R may be regulated by the interaction of different Rab GTPases at the carboxyl-terminal Rab binding site.

## MATERIALS AND METHODS

### *Materials:*

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*o*-(<sup>3</sup>H)Inositol and (<sup>32</sup>P)orthophosphate were acquired from PerkinElmer Life Sciences (Waltham, MA). Dowex 1-X8 (formate form) resin 200–400 mesh was purchased from BioRad (Mississauga, ON). Rabbit anti-GST, -Rab4 (sc-26562), -Rab5a (sc-312) and -Rab11 (sc-309) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA) and goat anti-GST as well as ECL Western blotting detection reagents were purchased from GE Healthcare (Oakville, Ontario, Canada). Horse radish peroxidase-conjugated anti-rabbit and anti-goat IgG secondary antibody was from BioRad (Mississauga, ON). QuikChange™ site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Rabbit anti-FLAG antibody, M2 anti-FLAG agarose and all other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

*DNA Construction:* An AT<sub>1</sub>R mutants lacking the distal 10 amino acids (AT<sub>1</sub>R-C1) was generated using the QuikChange™ Site-directed mutagenesis kit (Stratagene) to introduce a stop codon after residue 319 in the AT<sub>1</sub>R carboxyl-terminal tail. Subsequently, primers were designed for mutagenesis such that amino acid residues within the last 10 amino acid residues of the AT<sub>1</sub>R tail were mutated in pairs to alanine residues using the QuikChange Site-directed mutagenesis kit.

*Cell Culture:* Human embryonic kidney (HEK) 293 cells were maintained in Eagle's minimal essential medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (Invitrogen, Burlington, ON) and 50 µg/ml gentamicin. Cells seeded in 100 mm

dishes were transfected using a modified calcium phosphate method as described previously (Ferguson and Caron, 2004). Following transfection (18 h), the cells were incubated with fresh medium and allowed to recover for 24 hrs for co-immunoprecipitation studies. Otherwise, they were allowed to recover for 6-8 hrs and reseeded into 24-well dishes and then grown an additional 18 hrs prior to experimentation.

*Co-Immunoprecipitation:* HEK 293 cells were transiently transfected with FLAG-tagged AT<sub>1</sub>R and either GST-tagged Rab4, Rab4-Q67L, Rab4-S22N, Rab5, Rab7, Rab7-Q67L, Rab7-N125I, Rab 11, Rab11-Q70L or Rab11-S25N. Following transfection, the cells were incubated for 20 minutes in Hepes balanced salt solution (HBSS) at 37°C with or without 100 nM AngII. The cells were then placed on ice, washed two times with ice-cold phosphate-buffered saline (PBS) and lysed with cold-lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin, and 5 µg/ml aprotinin). The lysates were placed on a rocking platform for 15 min at 4°C and centrifuged at 15000 x g for 15 min at 4°C to pellet insoluble material. Cleared supernatant containing 250 µg protein were incubated with 25 µL of FLAG M2-affinity beads (Sigma) for 1 h rotating at 4°C to immunoprecipitate FLAG-AT<sub>1</sub>R. Following incubation, the beads were washed twice with lysis buffer and twice with PBS, and proteins were solubilized in a 3X SDS sample buffer containing 2-mercaptoethanol (BME). Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immuno-blotted to identify co-immunoprecipitated GST-tagged Rab proteins using a primary polyclonal rabbit or goat anti-GST antibody (1:1000



dilution, Santa Cruz, GE Healthcare) followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:10000, BioRad) or secondary anti-goat (1:2500, BioRad). Receptor and Rab protein expression was determined by immunoblotting 10  $\mu$ g of protein from each cell lysate used for immunoprecipitation. Proteins were detected using chemiluminescence with the ECL kit from GE Healthcare.

*Whole cell phosphorylation:* AT<sub>1</sub>R phosphorylation was measured as described previously (Anborgh et al., 2000). HEK 293 cells were transiently transfected with FLAG-AT<sub>1</sub>R along with either pEGFP (control), GFP-tagged Rab4, Rab4Q67L, Rab4S22N, Rab5, Rab5-S34N, Rab5-Q79L, Rab11, Rab11Q70L or Rab11S25N. Seventy-two hours post transfection cells were rinsed twice and incubated at 37°C for one hour in phosphate-free HBSS (5 mM NaHCO<sub>3</sub>, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, pH 7.4). Cells were then incubated at 37°C for one hour in 100  $\mu$ Ci/mL [<sup>32</sup>P]orthophosphate, and treated for 10 min with and without 100 nM AngII, rinsed and allowed to recover at 37 °C for 0, 20 or 40 min in phosphate-free HBSS. Cells were placed on ice and lysates were collected in the presence of protease inhibitors (0.1 mM PMSF, 10  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml aprotinin) and phosphatase-inhibitors (10mM NaF and 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>) and incubated with M2 anti-FLAG affinity agarose for 2-3 hours to immunoprecipitate receptor protein. Beads were washed and bound proteins were solubilized in SDS-PAGE sample buffer. Equal amounts of receptor protein, as determined by protein measurement and flow cytometry were separated by SDS-PAGE and receptor phosphorylation was determined via autoradiography at -80°C.

*Measurement of inositol phosphate formation:* Desensitization of AT<sub>1</sub>R signalling of inositol phosphate was measured as described previously (Olivares-Reyes et al., 2001) with some modifications. HEK 293 cells were transiently transfected with the cDNAs as described. Forty-eight hours post-transfection cells were incubated overnight in inositol-free DMEM with 100  $\mu$ Ci/mL *myo*-(<sup>3</sup>H)inositol. Cells were washed twice and incubated for one hour in warm HBSS then preincubated for 3 min at 37°C in either HBSS (lacking LiCl) alone or with 100 nM AngII (desensitizing stimulus). After a brief acid wash (50 mM glycine, 150 mM NaCl, pH 3.0), cells were washed twice and were then incubated with either 10 mM LiCl alone or 10 mM LiCl with 100 nM AngII for 10 min. The resensitization of AT<sub>1</sub>R-mediated IP formation was assessed in the same fashion except that desensitized cells were allowed to recover for 30 min prior to the second incubation with either 10 mM LiCl alone or 10 mM LiCl with 100 nM AngII for 10 min. Cells were placed on ice and the reaction was stopped with 500  $\mu$ L of perchloric acid and was neutralized with 400  $\mu$ l of 0.72 M KOH, 0.6 M KHCO<sub>3</sub>. Total cellular (<sup>3</sup>H)inositol incorporation was determined in 50  $\mu$ l of cell lysate. Total inositol phosphate was purified by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin and (<sup>3</sup>H)inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

*Statistical Analysis:* Densitometric data were normalized first for protein expression and the maximum value was set to 100, with all other values displayed as percentage thereof. One-way analysis of variance test (ANOVA) was performed to determine significance, followed by a post-hoc Tukey multiple comparison test or Bonferroni's

multiple comparisons test to determine which means were significantly different ( $p < 0.05$ ) from one another.

## RESULTS

### *Rab4, Rab5, Rab7 and Rab11 all interact with the AT<sub>1</sub>R*

Previous research showed direct association between Rab5a and AT<sub>1</sub>R, as well as co-localization of the AT<sub>1</sub>R in Rab7- and Rab11-positive endosomes following Rab GTPase overexpression (Seachrist et al., 2002; Dale et al., 2004). Thus, we investigated whether Rab binding to the human AT<sub>1</sub>R C-tail was either exclusive to Rab5 or was also observed for Rab4, Rab7 and Rab11. HEK293 cells were transiently transfected with FLAG-AT<sub>1</sub>R and either GST-tagged Rab4, Rab5, Rab7 or Rab11. We find that similar to what we observed previously for Rab5a, each of the GST-Rab4, GST-Rab7 and GST-Rab11 proteins could be co-immunoprecipitated with the FLAG-AT<sub>1</sub>R from HEK 293 cells (Fig. 1A and 1B). We found that in the absence of agonist treatment significantly more GST-Rab11 and significantly less Rab4 protein could be co-immunoprecipitated with the FLAG-AT<sub>1</sub>R, when compared to GST-Rab5 (Fig. 1A and 1B). Treatment of cells with 100 nM AngII to activate the FLAG-AT<sub>1</sub>R resulted in a small and statistically insignificant increase in GST-Rab5 and GST-Rab7 binding to the receptor, but had no effect on the association of either Rab4 or Rab11 (Fig. 1A and 1B). We also examined whether endogenous Rab4, Rab5 and Rab11 could be co-immunoprecipitated with the FLAG-AT<sub>1</sub>R from HEK 293 cells. We found that Rab4 could be co-immunoprecipitated and that a gonist stimulation increased Rab4 co-immunoprecipitation with the FLAG-AT<sub>1</sub>R by  $1.6 \pm 0.3$  fold ( $P < 0.05$ ) (Fig. 1C). However, agonist treatment had no effect upon the co-immunoprecipitation of either Rab5 or Rab11 with the receptor (Fig. 1D and 1E).

The rat AT<sub>1A</sub>R was previously shown to preferentially bind to the GDP-bound form of Rab5 (Rab5-S34N) and the GDP-bound form of Rab11 interacted specifically

with the thromboxane A<sub>2</sub> receptor (Seachrist et al., 2002; Hamelin et al., 2005). We found that wild-type Rab4, dominant-negative Rab4-S22N, and constitutively active Rab4-Q67L did not exhibit a preference for binding to the FLAG-AT<sub>1</sub>R (Fig. 2A). In contrast, constitutively active Rab7-Q67L mutant exhibited preferential binding to the FLAG-AT<sub>1</sub>R (Fig. 2B). Unlike what was previously observed for the thromboxane A<sub>2</sub> receptor wild-type Rab11 interacted with the FLAG-AT<sub>1</sub>R, but both constitutively active Rab11-Q70L and dominant-negative Rab11-S25N mutants did not effectively interact with FLAG-AT<sub>1</sub>R (Fig. 2C). This observation suggests that GTP hydrolysis is required for Rab11 binding to the AT<sub>1</sub>R. Taken together, the data indicated that Rab4, Rab5, Rab7 and Rab11 each bind to the AT<sub>1</sub>R but that the association of each of the Rab GTPases was mediated by different activation states of the GTPases.

#### *Identification of the AT<sub>1</sub>R Rab GTPase binding site*

Previously, we demonstrated that the deletion of the last 10 amino acid residues of the rat AT<sub>1A</sub>R C-tail (AT<sub>1A</sub>R-C1) resulted in a loss of AT<sub>1A</sub>R colocalization with GFP-Rab5a (Dale et al., 2004). Therefore, we tested whether the deletion of the distal 10 amino acid residues of the human AT<sub>1</sub>R would result in both the loss of Rab5 binding, as well as a loss of Rab4, Rab7 and Rab11 binding to a human FLAG-AT<sub>1</sub>R-C1 construct. We found that the deletion of the last 10 amino acid residues resulted in a significant decrease in Rab4, Rab5, Rab7 and Rab11 protein that was co-immunoprecipitated with the FLAG-AT<sub>1</sub>R-C1 mutant (Fig. 3A-D). Therefore, we examined which amino acid residues localized with the distal AT<sub>1</sub>R C-tail sequence KKPAPCFEVE were required for Rab4, Rab5, Rab7 and Rab11 binding to the receptor by performing alanine scanning mutagenesis of pairs of amino acid residues (Fig. 3A). We found that Rab4, Rab5, Rab7

and Rab11 binding to FLAG-AT<sub>1</sub>R-KK, FLAG-AT<sub>1</sub>R-PA, FLAG-AT<sub>1</sub>R-FE and FLAG-AT<sub>1</sub>R-VE mutant receptors was unaffected by alanine substitutions at the corresponding residues ( Fig. 3A -D). In contrast, Rab4, Rab5, and Rab11 were not co-immunoprecipitated effectively with the FLAG-AT<sub>1</sub>R-PC alanine substitution mutant (Fig. 3 A, 3 B and 3 D). Although Rab7 binding to the FLAG-AT<sub>1</sub>R-PC alanine substitution mutant was reduced, binding was not statistically significantly different from control (Fig. 3C). None of the alanine substitutions to the AT<sub>1</sub>R C-tail affected the coupling of the AT<sub>1</sub>R to the activation of IP formation (Fig. 4). Taken together, the data suggested that proline residue 354 and cysteine residue 355 played an important role in the binding of the Rab4, Rab5, Rab7 and Rab11 GTPases to the AT<sub>1</sub>R and that each of these different Rab GTPases bind to the same site on the receptor.

#### *Rab GTPases compete with each other for association with AT<sub>1</sub>R*

Because Rab4, Rab5, and Rab11 interact with an overlapping site in the AT<sub>1</sub>R C-tail and the overexpression of constitutively active Rab7 and Rab11 was previously shown to alter the intracellular trafficking of the receptor (Dale et al., 2004), we examined whether Rab GTPases compete for binding to the AT<sub>1</sub>R. We found that the co-immunoprecipitation of GST-Rab5 with the FLAG-AT<sub>1</sub>R could be antagonized by the overexpression of increasing amounts of HA-Rab11 protein (Fig. 5A). Moreover, despite the fact that GST-Rab4 was apparently a weak FLAG-AT<sub>1</sub>R-interacting protein, the overexpression of HA-Rab4 effectively prevented GST-Rab11 co-immunoprecipitation with FLAG-AT<sub>1</sub>R in an expression-dependent manner ( Fig. 5 B). Unexpectedly, increasing expression levels of HA-Rab11 did not result in the attenuation of GST-Rab4 binding to FLAG-AT<sub>1</sub>R (Fig. 5C).

*Rab4 but not Rab11 affects the phosphorylation state and desensitization of AT<sub>1</sub>R signalling*

Because Rab 4, Rab5 and Rab11 GTPases appeared to compete for a common binding site on the carboxyl-terminal tail of the AT<sub>1</sub>R, we examined whether the overexpression of wild-type, dominant-negative and constitutively active Rab4, Rab5 and Rab11 mutants might lead to altered AT<sub>1</sub>R phosphorylation and dephosphorylation. Consistent with previous studies (Opperman et al., 1996; Anborgh et al., 2000), agonist-stimulation of the AT<sub>1</sub>R for 10 min effectively promoted the phosphorylation of the AT<sub>1</sub>R (Fig. 6 A-C). However, when an agonist was washed out for 20 and 40 min, no dephosphorylation of the AT<sub>1</sub>R was observed under control conditions (Fig. 6A-C). In contrast, the overexpression of either wild-type Rab4 or constitutively active Rab4-Q67L significantly reduced the extent of a gonist-stimulated AT<sub>1</sub>R phosphorylation (Fig. 6A). Consistent with a role of Rab4 in promoting AT<sub>1</sub>R dephosphorylation, overexpression of a dominant-negative Rab4-S22N mutant resulted in a significant increase in agonist-stimulated AT<sub>1</sub>R phosphorylation, which was reduced to phosphorylation levels observed in control cells following agonist washout (Fig. 6A). The overexpression of wild-type Rab5 had no effect on either AT<sub>1</sub>R phosphorylation or dephosphorylation (Fig. 6B). However, the overexpression of either constitutively active Rab5-Q79L or dominant-negative Rab5-S34N appeared to result in a trend towards increased dephosphorylation of the receptor the results did not reach statistical significance. The extent of agonist-stimulated AT<sub>1</sub>R phosphorylation when compared to control cells was not altered by the overexpression of either wild-type, dominant-negative Rab 11-S25N or constitutively

active Rab11-Q67L and none of the Rab11 proteins resulted in AT<sub>1</sub>R dephosphorylation following agonist washout (Fig. 6C).

Given that wild-type Rab4 and constitutively active Rab4-Q67L lead to decreased AT<sub>1</sub>R phosphorylation, we examined whether the expression of either wild-type or dominant-negative Rab4, Rab5 and Rab11 would alter the desensitization and resensitization of the AT<sub>1</sub>R. To assess AT<sub>1</sub>R desensitization, cells were pretreated with 100 nM AngII for 3 min in HBSS lacking LiCl (desensitizing stimulus), washed and then treated with and without AngII for 10 min in HBSS containing LiCl. Receptor resensitization of AT<sub>1</sub>R-mediated IP responses was measured in the same way except that cells were allowed to recover in the absence of agonist for 30 min prior to being subjected to a second round of agonist treatment. The pretreatment of control cells (desensitizing stimulus) reduced AT<sub>1</sub>R-stimulated IP formation to between  $41 \pm 4\%$  and  $48 \pm 2\%$  of control (naïve) response when cells were exposed to a subsequent 10 min exposure to AngII (Fig. 7A-C). The overexpression of constitutively active Rab4-Q67L significantly reduced the extent of AT<sub>1</sub>R desensitization and increased the extent of AT<sub>1</sub>R resensitization (Fig. 7A). The overexpression of the constitutively active Rab5-Q67L mutant did not alter AT<sub>1</sub>R desensitization, but facilitated the resensitization response (Fig. 7B). None of the other Rab constructs had any effect on AT<sub>1</sub>R desensitization and resensitization. Taken together these results indicate that Rab4 binding, but not Rab11 binding, to the AT<sub>1</sub>R carboxyl-terminal tail alters the phosphorylation status of the AT<sub>1</sub>R leading to reduced AT<sub>1</sub>R desensitization.



## DISCUSSION

In the present study we have investigated whether multiple Rab GTPases might associate with the carboxyl-terminal tail of the AT<sub>1</sub>R tail and influence the activity and function of the receptor. We find that Rab4, Rab5, Rab7 and Rab11 each exhibit the capacity to bind to the distal 10 amino acids of the AT<sub>1</sub>R carboxyl-terminal tail and can compete with one another for binding. Previously, we demonstrated that the AT<sub>1</sub>R preferentially associated with the GDP-bound form of Rab5. We show here that the AT<sub>1</sub>R does not distinguish between GDP- and GTP-bound forms of Rab4, binds preferentially to GTP-bound Rab7 and interacts with wild-type Rab11 and does not associate with either constitutively active or dominant negative Rab11 mutants. We have also identified two amino acid residues (proline 354 and cysteine 355) within the Rab binding domain of the AT<sub>1</sub>R carboxyl-terminal tail that are essential for the association of Rab4, Rab5 and Rab11 but not Rab7. The association of different Rab GTPases with the AT<sub>1</sub>R carboxyl-terminal tail has different functional outcomes, with Rab5 promoting the retention of the AT<sub>1</sub>R in early endosomes (Seachrist et al., 2002), Rab7 facilitating the trafficking of the AT<sub>1</sub>R to lysosomes (Dale et al., 2004) and Rab4 promoting the dephosphorylation and resensitization of the receptor. Taken together, our data indicate that the association of different Rab GTPases with the carboxyl-terminal tail domain of the AT<sub>1</sub>R may regulate different functional outcomes for AT<sub>1</sub>R signalling in tissues that may express differing levels of each of the relevant Rab GTPases as the overexpression of a constitutively active Rab4-Q67L mutant decreases AT<sub>1</sub>R desensitization, while facilitating resensitization.

In the current study, we have demonstrated that the domain required for Rab GTPase interactions with AT<sub>1</sub>R are identical for Rab4, Rab5, and Rab11. Previously, we

identified that the Rab5 binding domain resides within the distal 10 amino acids of AT<sub>1</sub>R carboxyl-terminal tail and that deletion of this motif resulted in altered AT<sub>1</sub>R trafficking to lysosomes as opposed to the retention of the receptor in early endosomes (Dale et al., 2004). We have further defined the critical residues required for Rab GTPase binding to the AT<sub>1</sub>R and show that proline 354 and cysteine 355 are essential for binding Rab4, Rab5, and Rab11. Previously, it has been shown that the dephosphorylation and resensitization of the  $\beta_2$ AR occurs as the receptor transits between the Rab 5-positive early endosome and the Rab4-positive rapid recycling endosome (Seachrist et al., 2000). Moreover, it has been reported that phosphorylated  $\mu$ -opioid receptor is preferentially recycled through Rab4-positive endosomes (Wang et al., 2008). We find here, that the overexpression of a constitutively active Rab4-Q67L mutant decreases both AT<sub>1</sub>R phosphorylation and desensitization, while promoting the resensitization of the receptor. Thus, these data are consistent with the hypothesis that the Rab4-positive recycling endosome functions as the compartment in which GPCR dephosphorylation is mediated by phosphatases.

Several GPCRs have now been reported to associate with Rab GTPases including the  $\beta_2$ AR, thromboxane A<sub>2</sub> receptor and prostacyclin receptor (Hamelin et al., 2005; Parent et al., 2009; Reid et al., 2010). However, the residues that we have identified to be essential for Rab GTPase binding to the AT<sub>1</sub>R are not conserved in any of these GPCRs. Rab11 binding to the thromboxane A<sub>2</sub> receptor is mediated by residues 335-345 that are localized within the central region of the thromboxane A<sub>2</sub> receptor carboxyl-terminal tail and Rab11 binds  $\alpha$ -helix 8 at the proximal end of the prostacyclin receptor. In contrast, Rab11 binding to the  $\beta_2$ AR involves a bipartite binding motif, with arginine 333 and

lysine 348 representing the essential amino acid residues mediating Rab11 binding to the receptor (Parent et al., 2009). Thus, to date there is no clearly defined consensus motif for Rab GTPase association with GPCRs. However, previous work from our laboratory using yeast two hybrid screens suggest that the regional of the AT<sub>1A</sub>R carboxyl-terminal tail that is proximal to the seventh transmembrane spanning domain of the AT<sub>1A</sub>R may also be involved in Rab5 binding (Seachrist et al., 2002). Thus, the fact that we do not observe complete loss of binding of the Rab GTPases to the carboxyl-terminal tail of the receptor suggests that secondary residues within the membrane proximal domain of the receptor likely also contribute in part to Rab protein binding.

Rab GTPases not only influence the intracellular trafficking and recycling of GPCRs by directly interacting with these vesicular cargo proteins, but Rab GTPases also indirectly influence the trafficking of receptors between intracellular compartments as a consequence of their intrinsic activity. Following their internalization, many GPCRs have been shown to either recycle to the cell surface via the Rab4-mediated rapid pathway directly from sorting endosomes or via the Rab11-mediated slow pathway from perinuclear recycling endosomes. The recycling of other GPCRs, including the corticotrophin releasing factor receptor 1, somatostatin-3 receptor, vasopressin V<sub>2</sub> receptor, neurokinin-1 receptor, chemokine CXCR2, m4 muscarinic acetylcholine receptor and protease receptor, are also differentially regulated by Rab4 and Rab11 (Innamorati et al., 2001; Kreuzer et al., 2001; Schmidlin et al., 2001; Signoret et al., 2001; Fan et al., 2002; Volpicelli et al., 2002; Roosterman et al., 2003; Holmes et al., 2006). Thus, potential alterations in individual Rab GTPase protein expression may have profound effects on GPCR activity. This could occur as the consequence of either direct competition for GPCR binding or by increasing the relative efficiency of the intracellular

trafficking and membrane fusion of vesicular compartments within the cell that is regulated by the Rab GTPase. Rab GTPase protein expression and activity has been demonstrated to be regulated by a number of different signals. First, Rab1, Rab4 and Rab6 protein expression is altered in dilated cardiomyopathy model of heart failure and overexpression of Rab4 in the heart leads to altered  $\beta_2$ AR desensitization and resensitization (Wu et al., 2001; O'Leary et al., 2004). Second, parasitic infection of cardiomyocytes *in vitro* with the protozoan *Tyranosoma cruzi* results in the downregulation of both Rab7 and Rab11 protein expression (Batista et al., 2006). Finally, insulin is able to stimulate GTP-loading of Rab11 in cardiomyocytes indicating the potential of Rab GTPases to serve as substrates for GPCR activated kinases such as phosphatidylinositol 3-kinase (Schwenk and Eckel, 2007). Thus, taken together alterations in Rab GTPase expression and activity have the potential to both directly and indirectly influence GPCR signalling under both physiological and pathophysiological conditions suggesting that these proteins may represent targets for the treatment of cardiovascular-related diseases.

In HEK 293 cells, the  $AT_1R$  is internalized to and retained in early endosomes, where it remains phosphorylated and does not recycle to the plasma membrane (Anborgh et al., 2000; Seachrist et al., 2002; Dale et al., 2004). We find that the overexpression of different Rab GTPases can specifically alter the intracellular trafficking fate of the  $AT_1R$  with Rab7 overexpression favouring the trafficking of the receptor to lysosomes and Rab4 overexpression favouring the dephosphorylation of the receptor. In contrast, although Rab11 effectively interacts with the  $AT_1R$ , the interaction of the wild-type Rab11 does not influence the dephosphorylation of the receptor, although it can promote

plasma membrane recycling (Dale et al., 2004). Interestingly, Rab4 is able to effectively displace Rab11 binding to the AT<sub>1</sub>R, despite the observation that Rab11 is more effectively co-immunoprecipitated with the receptor. Therefore, even small differences in Rab4 expression may lead to profound changes in AT<sub>1</sub>R activity. However, Rab binding to the AT<sub>1</sub>R, if competitive, should be reciprocal and Rab11 protein expressed at sufficiently high levels should be able to compete for binding. It is possible that in our experiments we have not achieved Rab11 expression that can displace Rab4 from the receptor at complimentary expression levels. Moreover, the overexpression of one Rab protein may shift the receptor from one cellular compartment to another that is not available to the competing Rab protein. It is also possible that Rab GTPases selectively bind to different receptor sites depending upon their activation state, since wild-type Rab7 does not bind to the receptor as effectively as Rab7-Q70L and wild-type Rab7 binding is not significantly impaired when the AT<sub>1</sub>R C-tail is truncated. This may explain why we previously observed that truncation of the AT<sub>1</sub>R C-tail resulted in the targeting of the receptor to endosomes (Dale et al., 2004). Nevertheless, depending on the complement of Rab GTPases expressed in different tissue and cell types, it is likely that the AT<sub>1</sub>R will exhibit differences in its functional regulation ranging from prolonged desensitization associated with impaired dephosphorylation and resensitization to rapid resensitization associated with receptor dephosphorylation.

To date, few GPCRs, including the AT<sub>1</sub>R,  $\beta_2$ AR, thromboxane A<sub>2</sub> receptor and prostacyclin receptor have been shown to directly associate with members of the Rab family. Emerging evidence suggests that these interactions are critical to proper trafficking and regulation of these receptors. Understanding the role of Rabs in the regulation of GPCR redistribution into different intracellular compartments will serve to

improve our understanding of the molecular and physiological consequences of GPCR signalling. It is now evident that multiple small GTP-binding proteins, including Ras interact with GPCRs and future studies should reveal whether GPCRs either interact with or regulate additional components of the intracellular trafficking machinery.

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## **AUTHOR CONTRIBUTION**

Participated in research design: J. L. Esseltine, L. B. Dale, and S. S. G. Ferguson.

Conducted experiments: J. L. Esseltine, L. B. Dale

Performed data analysis: J. L. Esseltine, and S. S. G. Ferguson.

Wrote or contributed to the writing of the manuscript: J. L. Esseltine, L. B. Dale, and S. S. G. Ferguson.

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## FOOTNOTES

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## FIGURE LEGENDS

### **Figure 1: Rab4, Rab5, Rab7 and Rab11 each co-immunoprecipitate with AT<sub>1</sub>R. (A )**

Representative immunoblots showing the co-immunoprecipitation of GST-Rab4, GST-Rab5, GST-Rab7 and GST-Rab11 with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. (B) Densitometric analysis of GST-Rab4, GST-Rab5, GST-Rab7 and GST-Rab11 co-immunoprecipitated with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Data represents the mean  $\pm$  SD of 5 independent experiments. Data were normalized for both individual Rab protein expression levels and normalized to maximum Rab protein binding to the AT<sub>1</sub>R in each experiment. \* $p < 0.05$  compared to Rab5 co-immunoprecipitated with the AT<sub>1</sub>R and correspondingly treated. (C) Immunoblot demonstrating the co-immunoprecipitation of endogenous Rab4 protein with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Rab4 co-immunoprecipitated with GFP antibody (Con) is used as a control. Data represents the mean  $\pm$  SD of 4 independent experiments. (D) Immunoblot demonstrating the co-immunoprecipitation of endogenous Rab5 protein with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Rab5 co-immunoprecipitated with GFP antibody (Con) is used as a control. Data represents the mean  $\pm$  SD of 4 independent experiments. (E) Immunoblot demonstrating the co-immunoprecipitation of endogenous Rab11 protein with the FLAG-AT<sub>1</sub>R from HEK 293 cells in the absence (-) and presence (+) of 100 nM AngII treatment for 20 min. Rab11 co-immunoprecipitated with GFP antibody (Con) is used as a control. Data represents the mean  $\pm$  SD of 4 independent experiments.



**Figure 2: Co-immunoprecipitation of wild-type, dominant-negative and constitutively active Rab4, Rab7 and Rab11 GTPases with the AT<sub>1</sub>R.** (A) Representative immunoblot and densitometric analysis showing the co-immunoprecipitation of GST-Rab4 (WT), constitutively active GST-Rab4-Q67L (CA) and dominant-negative GST-Rab4-S22N (DN) with FLAG-AT<sub>1</sub>R from HEK 293 cells. (B) Representative immunoblot and densitometric analysis showing the co-immunoprecipitation of GST-Rab7 (WT), constitutively active GST-Rab7-Q67L (CA) and dominant-negative GST-Rab7-N125I (DN) with FLAG-AT<sub>1</sub>R from HEK 293 cells. \**p* < 0.05 compared to wild-type Rab7 co-immunoprecipitated with the AT<sub>1</sub>R. (C) Representative immunoblot and densitometric analysis showing the co-immunoprecipitation of GST-Rab11 (WT), constitutively active GST-Rab11-Q70L (CA) and dominant-negative GST-Rab7-S25N (DN) with FLAG-AT<sub>1</sub>R from HEK 293 cells. \**p* < 0.05 compared to wild-type Rab11 co-immunoprecipitated with the AT<sub>1</sub>R. Data represents the mean ± SD of 3-5 independent experiments. All data were normalized for individual Rab protein expression levels in each experiment.

**Figure 3: Identification of the Rab GTPase binding site within the AT<sub>1</sub>R carboxyl-terminal tail.** (A) Representative immunoblot showing the co-immunoprecipitation of Rab4 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R-C1 (1-349), AT<sub>1</sub>R-K350A/K351A (KK), AT<sub>1</sub>R-P352A/A353G (PA), AT<sub>1</sub>R-P354A/C355A (PC), AT<sub>1</sub>R-F356A/E357A (FE), and AT<sub>1</sub>R-V358A/E359A (VE) mutants from HEK 293 cells. (B) Representative immunoblot showing the co-immunoprecipitation of Rab5 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R mutants from HEK 293 cells. (C) Representative immunoblot showing the co-immunoprecipitation of Rab7 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R mutants from HEK 293 cells. (D) Representative

immunoblots showing the co-immunoprecipitation of Rab11 with either the wild-type AT<sub>1</sub>R (WT) or AT<sub>1</sub>R mutants from HEK 293 cells. Data represents the mean  $\pm$  SD of 3-5 independent experiments. Data were normalized for both individual Rab protein expression levels and wild-type Rab protein binding to the AT<sub>1</sub>R in each experiment. \* $p$  < 0.05 compared to wild-type Rab co-immunoprecipitated with the AT<sub>1</sub>R.

**Figure 4: Agonist-stimulated AT<sub>1</sub>R inositol phosphate formation.** Shown is agonist-stimulated (100 nM AngII, 10 min) inositol phosphate formation mediated by either the wild-type FLAG-AT<sub>1</sub>R (WT) or FLAG-AT<sub>1</sub>R-C1 (1-349), FLAG-AT<sub>1</sub>R-K350A/K351A (KK), FLAG-AT<sub>1</sub>R-P352A/A353G (PA), FLAG-AT<sub>1</sub>R-P354A/C355A (PC), FLAG-AT<sub>1</sub>R-F356A/E357A (FE), and FLAG-AT<sub>1</sub>R-V358A/E359A (VE) mutants from HEK 293 cells. Data represents the mean  $\pm$  SD of 3 independent experiments.

**Figure 5: Competition between RabGTPases for co-immunoprecipitation with FLAG-AT<sub>1</sub>R.** (A) Representative immunoblots and densitometric analysis of the co-immunoprecipitation of GST-Rab5 with FLAG-AT<sub>1</sub>R in the absence or presence of increasing amounts of HA-Rab11. \* $p$  < 0.05 compared GST-Rab5 co-immunoprecipitated with the AT<sub>1</sub>R in the absence of HA-Rab11. (B) Representative immunoblots and densitometric analysis of the co-immunoprecipitation of GST-Rab11 with FLAG-AT<sub>1</sub>R in the absence or presence of increasing amounts of HA-Rab4. \* $p$  < 0.05 compared to GST-Rab11 co-immunoprecipitated with the AT<sub>1</sub>R in the absence of HA-Rab4. (C) Representative immunoblots and densitometric analysis of the co-immunoprecipitation of GST-Rab4 with FLAG-AT<sub>1</sub>R in the absence or presence of increasing amounts of HA-Rab11. \* $p$  < 0.05 compared to GST-Rab4 co-immunoprecipitated

with the AT<sub>1</sub>R in the absence of HA-Rab11. Data represents the mean  $\pm$  SD of 3-5 independent experiments. Data were normalized for both GST-Rab protein expression levels and GST-Rab protein binding to the AT<sub>1</sub>R in absence of HA-Rab.

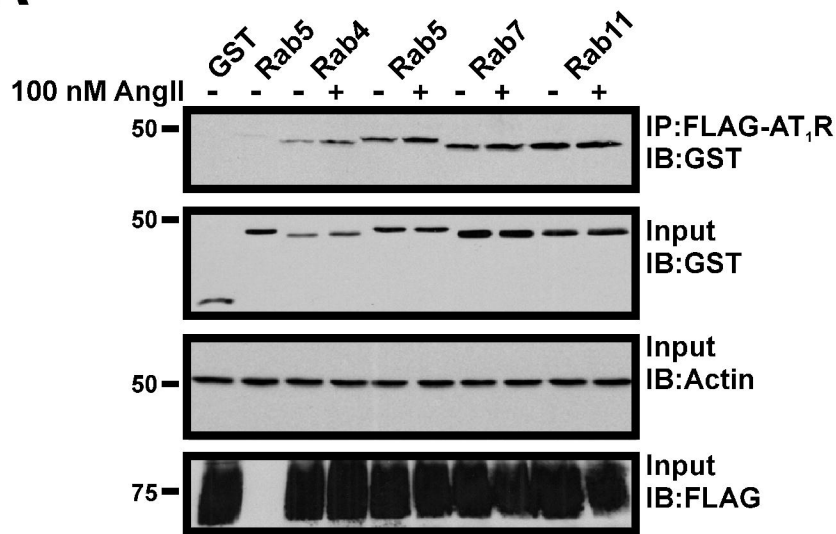
**Figure 6: Whole cell phosphorylation of AT<sub>1</sub>R in the presence and absence of wild-type and mutant Rab4 and Rab11 proteins.** (A) Representative autoradiograph and densitometric analysis of AT<sub>1</sub>R phosphorylation in absence (control) and presence of wild-type Rab4 (WT), constitutively active Rab4-Q67L (CA), and dominant-negative Rab4-S22N (DN) mutants. HEK 293 cells expressing FLAG-AT<sub>1</sub>R were treated with 100 nM AngII for 10 min, washed and allowed to recover for 0 (desensitization), 20 (resensitized) and 40 (resensitized) min. Data represents the mean  $\pm$  SD of 6 independent experiments. \* $p < 0.05$  compared to corresponding control. (B) Representative autoradiograph and densitometric analysis of AT<sub>1</sub>R phosphorylation in absence (control) and presence of wild-type Rab5 (WT), constitutively active Rab5-Q79L (CA), and dominant-negative Rab5-S34N (DN) mutants. Data represents the mean  $\pm$  SD of 4 independent experiments. \* $p < 0.05$  compared to corresponding control. (C) Representative autoradiograph and densitometric analysis of AT<sub>1</sub>R phosphorylation in absence (control) and presence of wild-type Rab11 (WT), constitutively active Rab11-Q70L (CA), and dominant-negative Rab11-S25N (DN) mutants. Data represents the mean  $\pm$  SD of 4 independent experiments. \* $p < 0.05$  compared to corresponding control.

**Figure 7: Desensitization and resensitization of AT<sub>1</sub>R-mediated inositol phosphate formation.** (A) HEK 293 cells transfected with FLAG-AT<sub>1</sub>R with empty pEBG vector (NT), wild-type Rab4 (WT), constitutively active Rab4-Q67L (CA), and dominant-negative Rab4-

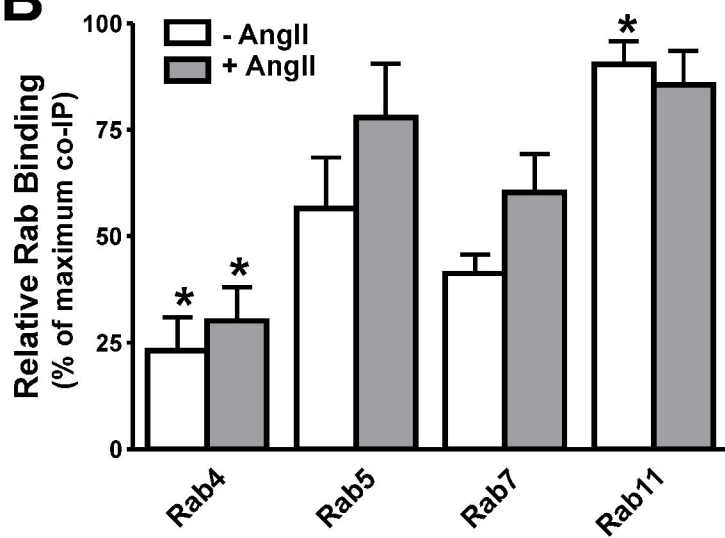
S22N (DN) mutants. (B) HEK 293 cells transfected with FLAG-AT<sub>1</sub>R with empty pEBG vector (NT), wild-type Rab5 (WT), constitutively active Rab5-Q79L (CA), and dominant-negative Rab4-S34N (DN) mutants. (C) HEK 293 cells transfected with FLAG-AT<sub>1</sub>R with empty pEBG vector (NT), wild-type Rab11 (WT), constitutively active Rab11-Q70L (CA), and dominant-negative Rab11-S25N (DN) mutants. Transfected cells were treated either with or without 100 nM AngII for 3 min in the absence of LiCl (desensitizing stimulus) and then either washed and subjected to a second treatment of 100 nM AngII for 10 min in the presence of LiCl (desensitized) or washed and allowed to recover for 30 min prior to a second treatment of 100 nM AngII for 10 min in the presence of LiCl (resensitized). Data were normalized for protein expression and basal IP formation and desensitized and resensitized IP responses compared to naive control cells that were not subjected to desensitizing stimulus. Data are representative of 5 independent experiments. \*p < 0.05 compared to corresponding control.

# Figure 1

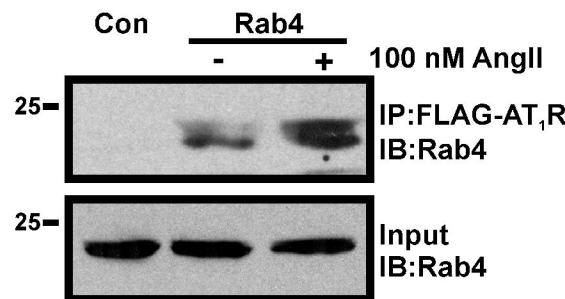
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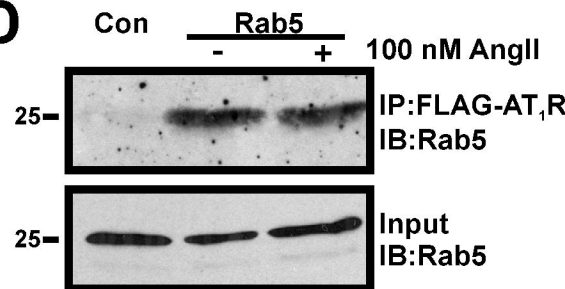
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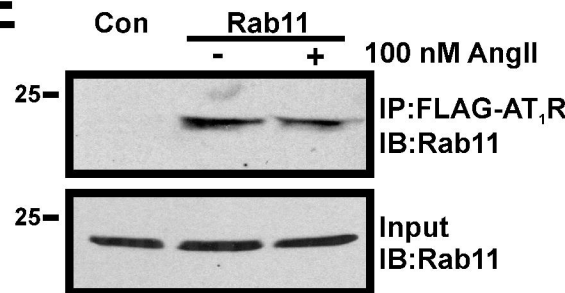
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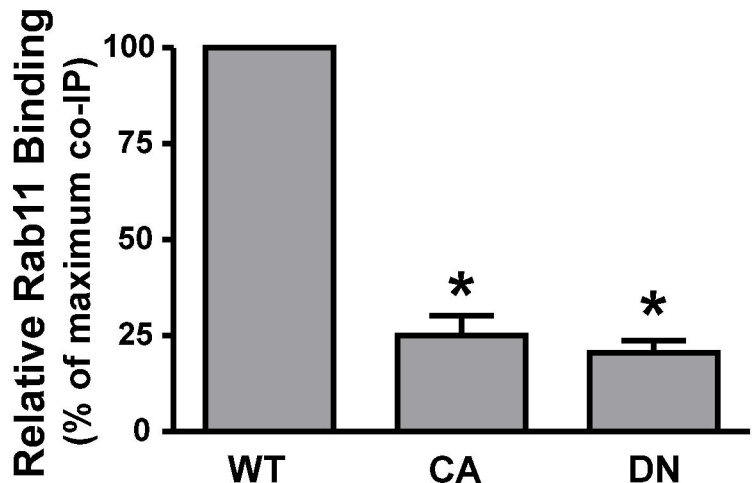
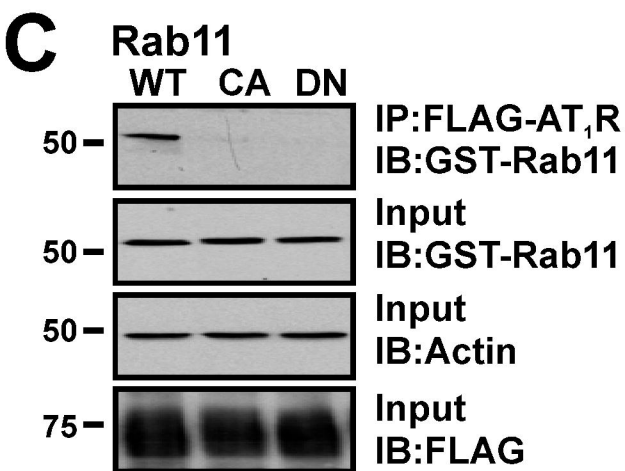
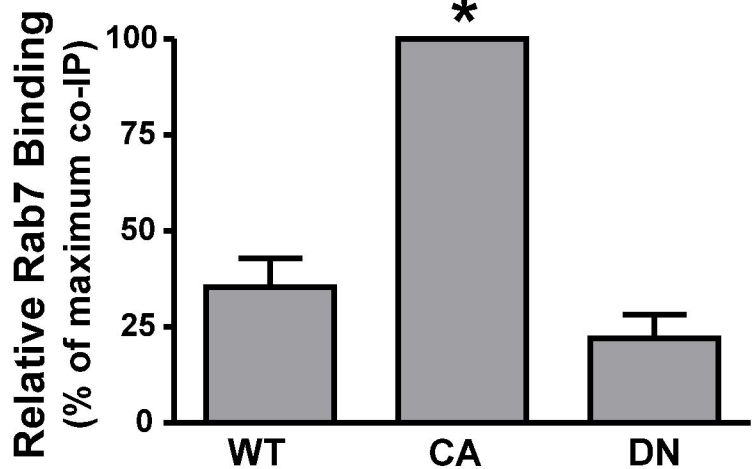
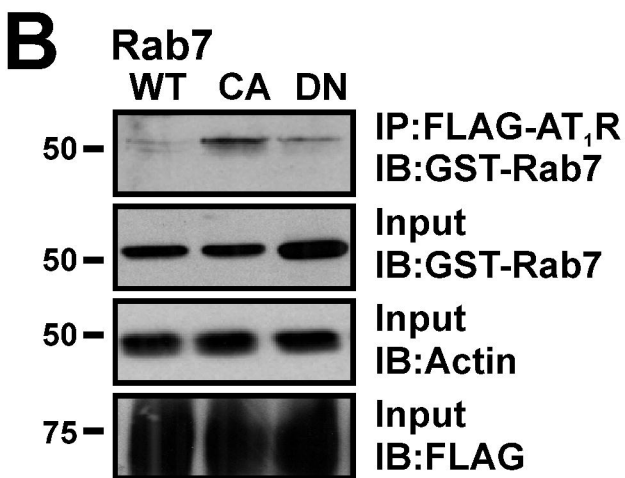
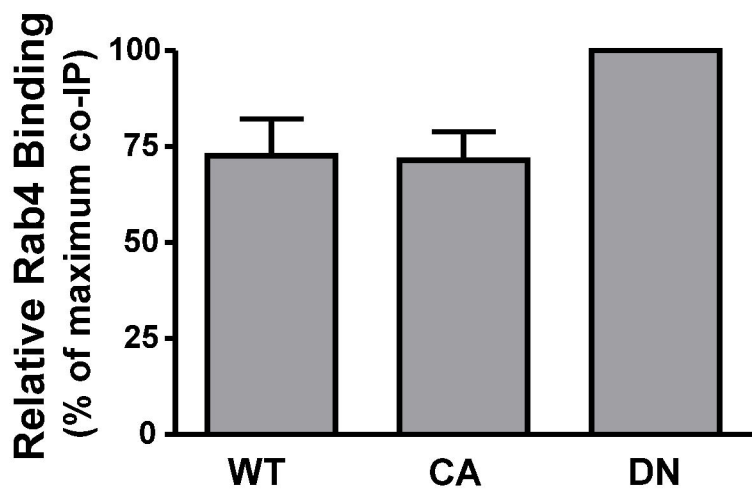
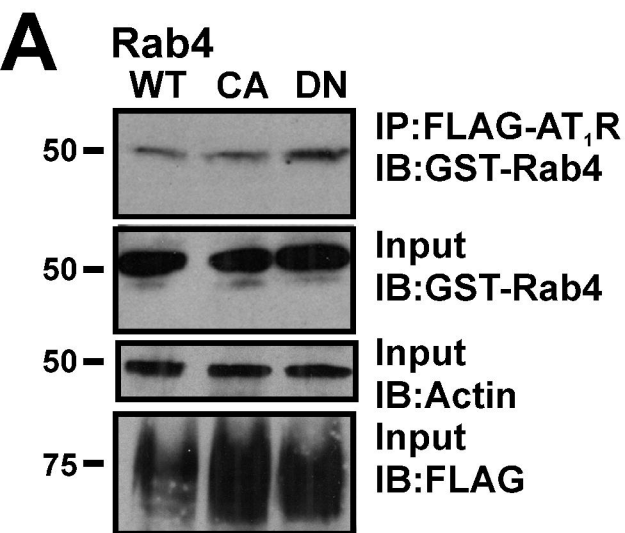
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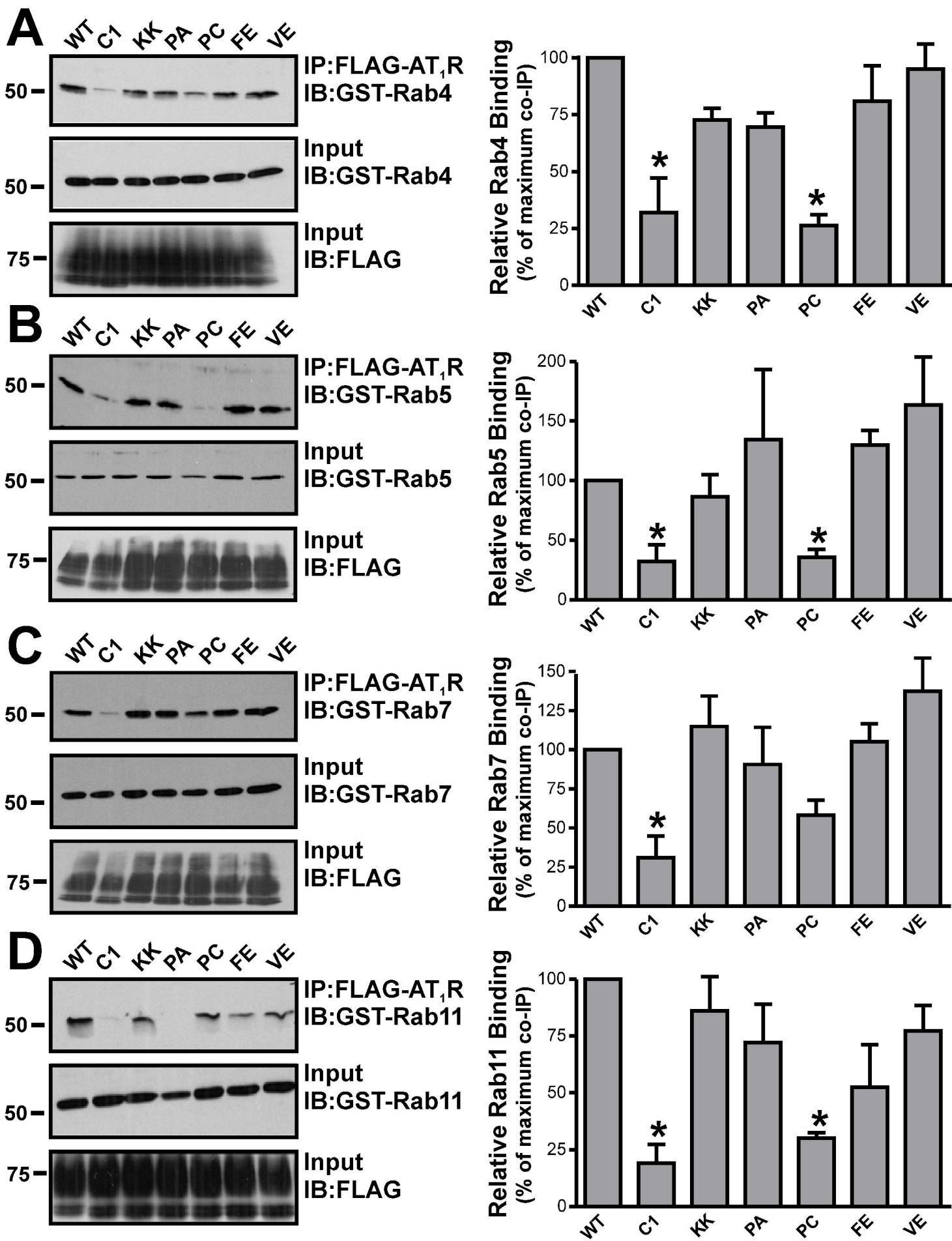
## E



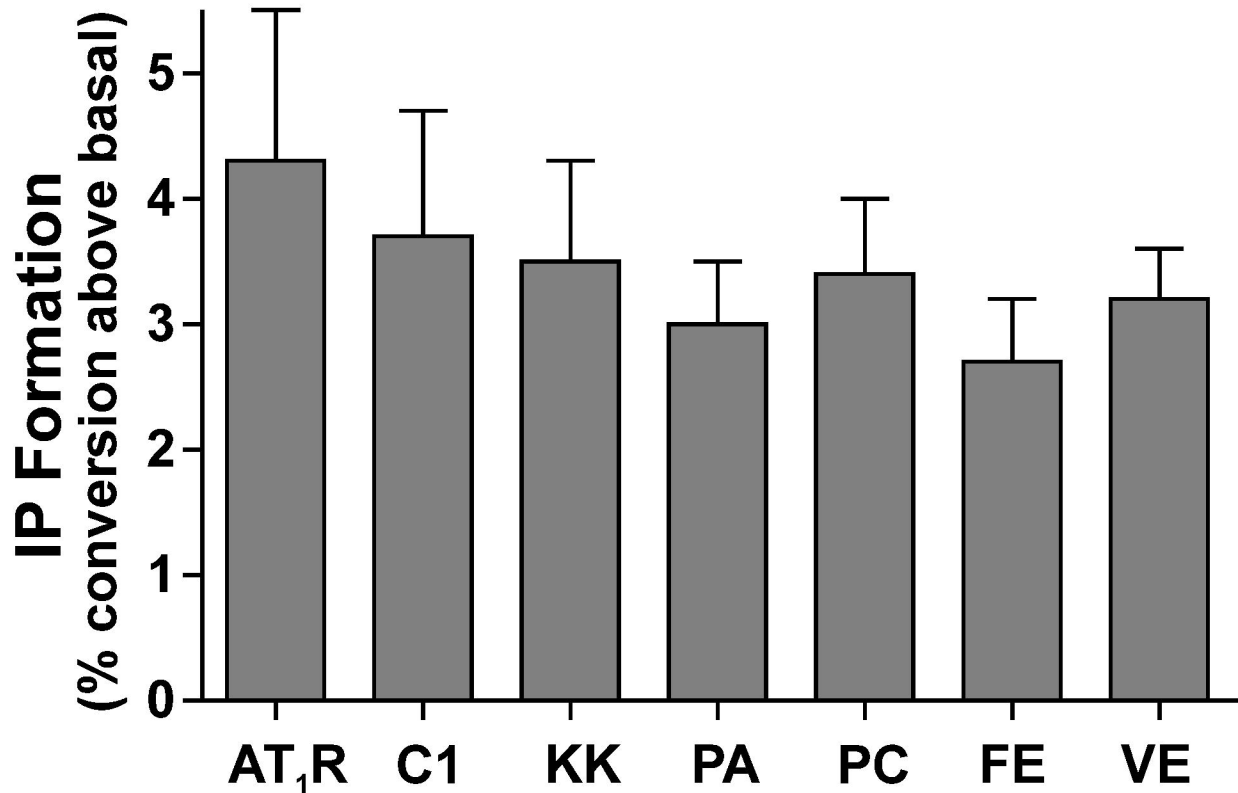
# Figure 2



# Figure 3



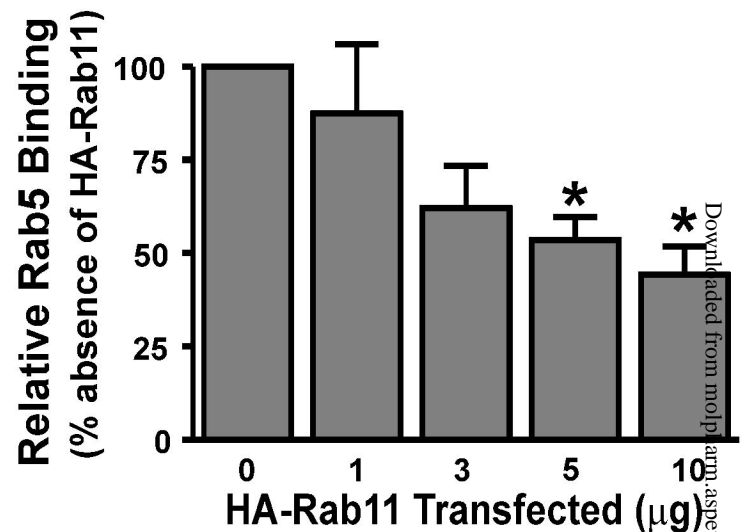
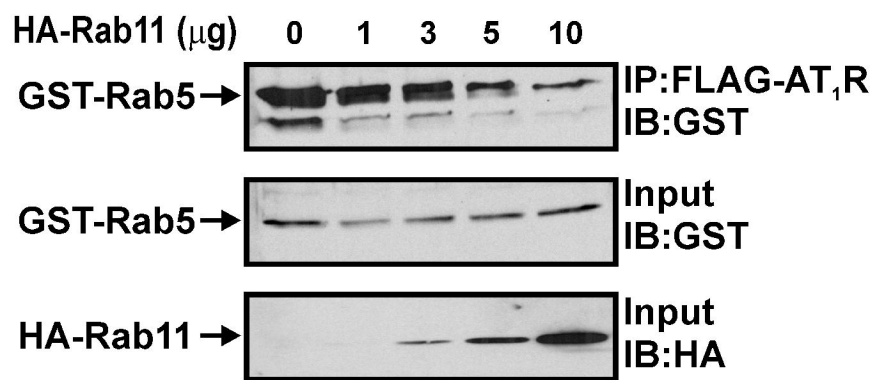
**Figure 4**



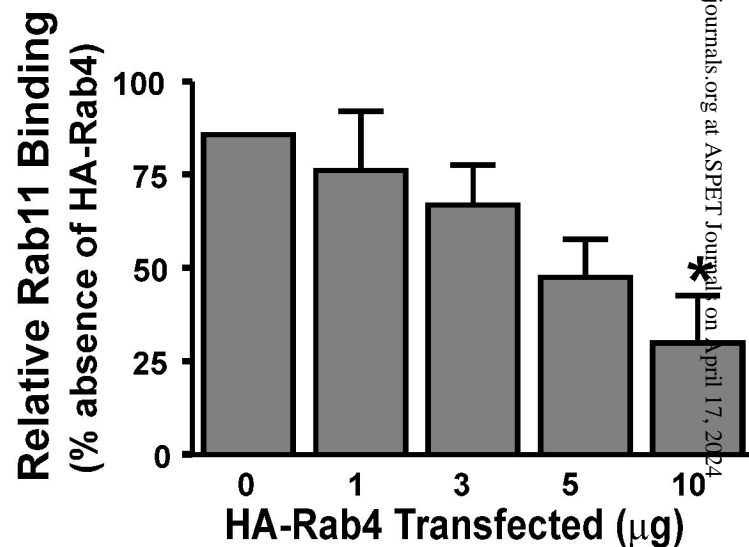
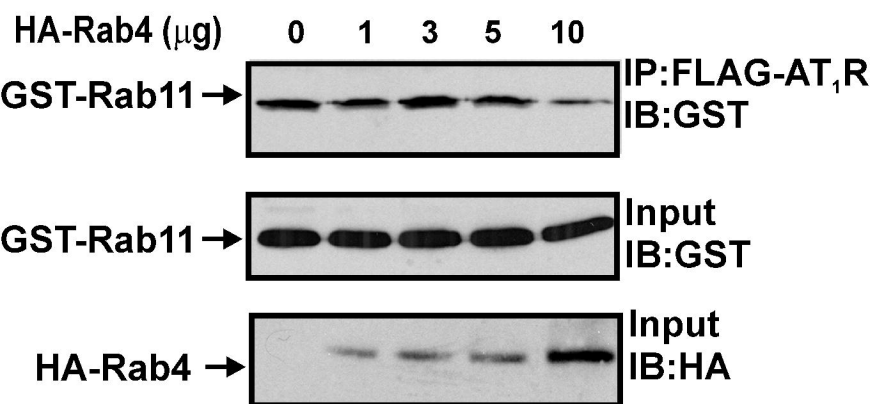


# Figure 5

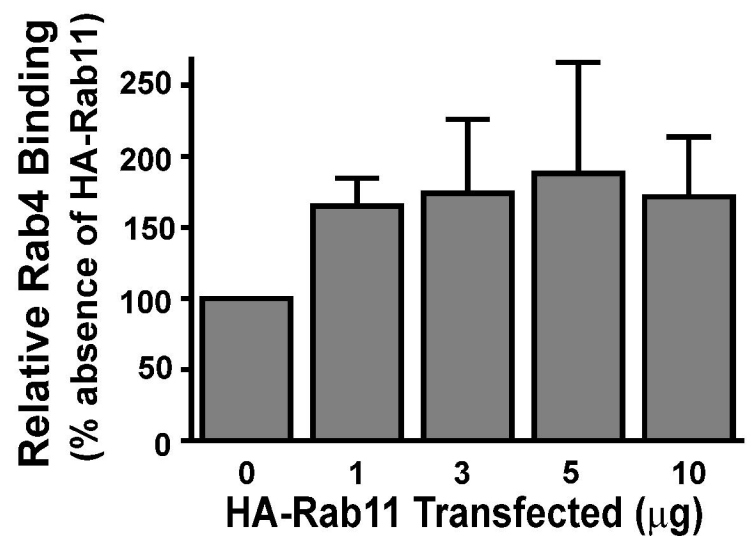
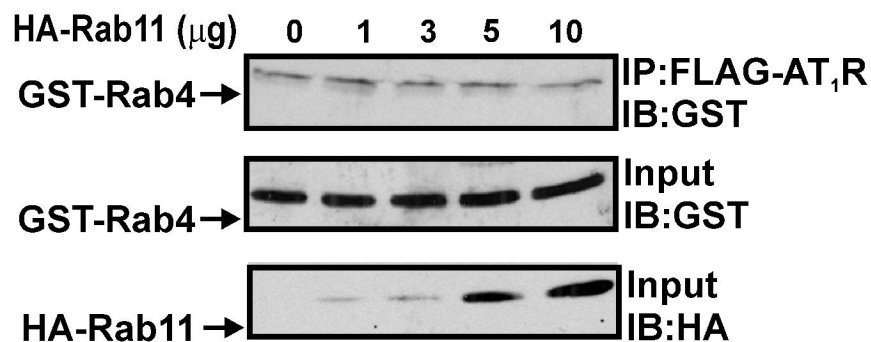
## A



## B



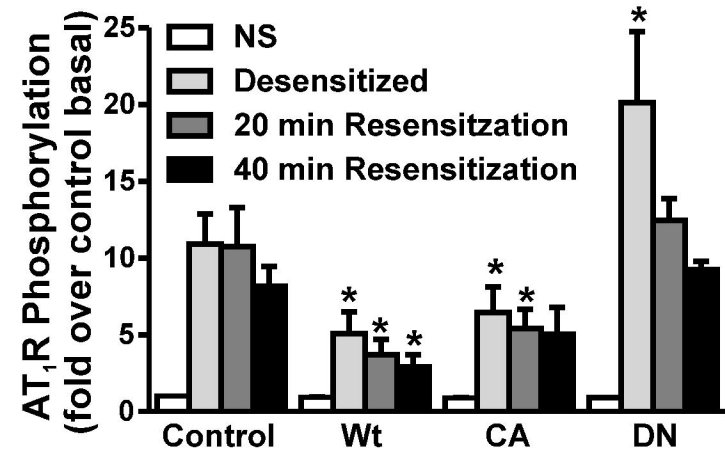
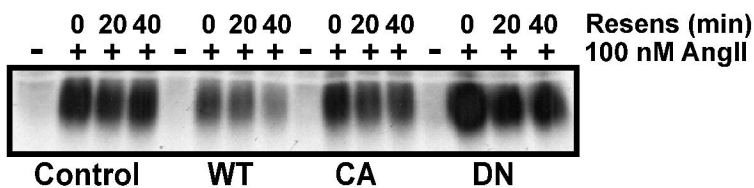
## C



# Figure 6

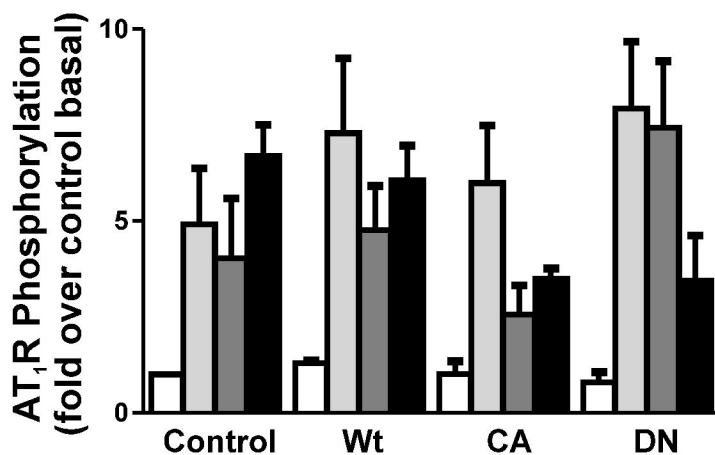
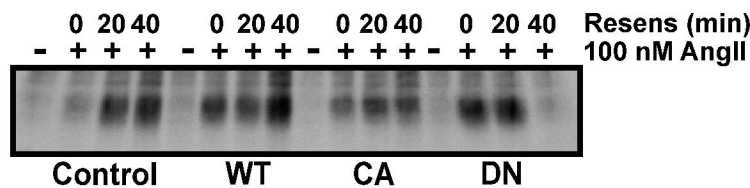
## A

### Rab4



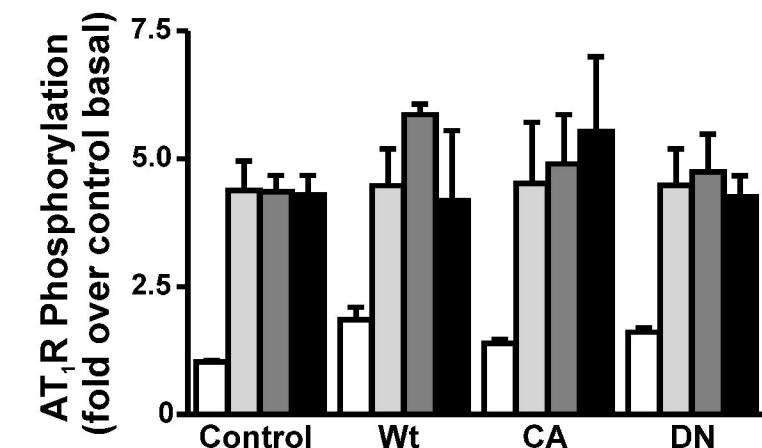
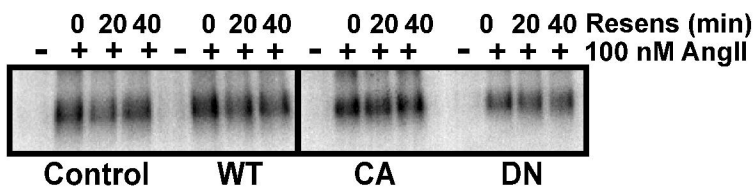
## B

### Rab5



## C

### Rab11



# Figure 7

