Figure Legends of Supplemental Data

ARF6 and GASP-1 Are Post-endocytic Sorting Proteins Selectively Involved in GRK- and PKC-mediated Intracellular Trafficking of Dopamine D_2 Receptor

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Figure S1. Characterization of PMA-induced desensitization of D_2 receptor.

(A) Dose-response curve of D_2 receptor for the inhibition of cAMP production. Cyclic AMP was measured by column chromatography as described in *Materials and methods*. Receptor expression levels were maintained around 1.4 pmol/mg protein.

(B) Effects of PKC inhibitor Gö6976 on the PMA-induced desensitization of D_2 receptor. Cells were pre-treated with 1 μM Gö6976 for 20 min and then treated with 1 μM PMA for 15 min, followed by determination of dose-response curves. ***:p<0.001 when Veh/PMA group was compared to Veh/Veh group.

(C) Effects of PKC inhibitor Gö6983 on the PMA-induced desensitization of D_2 receptor. Cells were pre-treated with 1 μM Gö6983 for 20 min and then treated with 1 μM PMA for 15 min. **:p<0.01 when 'Veh/PMA' group was compared to 'Veh/Veh' group.

(D) Involvement of PKC in the desensitization of D_2 receptor. Cells expressing D_2 receptor were treated with 1 μM PMA or 4α-PMA for 15 min, followed by determination of dose-response curves. ***:p<0.001 when PMA group was compared to vehicle or 4α-PMA group.

Figure S2. Characterization of mutants of D_2 receptor at S/T residues in the PMA-induced desensitization of D_2 receptor.

(A) Effects of mutations of T144/S147/S148 (#3) on the PMA-induced desensitization of D_2 receptor. *:$p<0.05$ when PMA group was compared to each vehicle group. Receptor expression levels were maintained around 1.8 pmol/mg protein.

(B) Effects of mutation of S147 and S148 on the PMA-induced desensitization of D_2 receptor. *:$p<0.05$ when PMA group was compared to each vehicle group.

(C) Effects of mutations of T322 and T324 on the PMA-induced desensitization of D_2 receptor. *:$p<0.05$ when PMA group was compared to each vehicle group.

(D) Effects of mutations of S/T residues involved in the PKC-mediated desensitization on the PMA-induced internalization of D_2 receptor. ***:$p<0.001$ compared with WT group.

Figure S3. Functional analysis of S/T residues located within the endocytic motif of the 3rd intracellular loop, and associated plasma membrane microdomain in which PKC-mediated internalization of D_2 receptor occurs.

Effects of point mutation of T225, S228, and S229 on the PKC-mediated desensitization of D_2 receptor. Cells expressing wild-type or each S/T mutant of D_2 receptor were treated with 1 μM PMA for 15 min, and the dose-response curves were determined. *:$p<0.05$ when PMA-treated group was compared to each vehicle group.

Figure S4. Roles of β-arrestins and Rab5/Rab23 in the recycling of homologously internalized D_2 receptor.

(A) Preparation of double knockout cell lines of β-arrestin1/2. Double knockdown of β-arrestins was conducted as described in *Materials and methods*.

(B) Roles of β-arrestins in the recycling of D_2 receptor. Cells expressing D_2 receptor (around 0.9 pmol/mg protein) were treated with 50 μg/ml cyclohexamide, followed by 10 μM DA for 60 min. After washing with serum-free medium, cells were incubated at 37 °C for the indicated period of time.

(C) Roles of Rab5 in the recycling of D_2 receptor. Cells stably expressing D_2 receptor (~1.1 pmol/mg protein) were transfected with Mock, WR-Rab5, or S34N-Rab5.

(D) Roles of Rab23 in the recycling of D_2 receptor. Cells stably expressing D_2 receptor (~1.1 pmol/mg protein) were transfected with Mock, WR-Rab23, or S23V-Rab23.

Figure S5. Roles of ARF6 in the intracellular trafficking of D_2 receptor.

(A) Roles of ARF6-induced downregulation of D_2 receptor. HEK-293 cells stably expressing D_2 receptor were co-expressed with empty vector, ARF6-WT, ARF6-T27N, or ARF-Q67L, treated with 1 μM quinpirole for 12 hr and 24 hr. Receptor binding was determined as in Figure 1D.

(B) Effects of fast cycling ARF6 mutant on the recycling of homologously internalized D_2 receptor. Cells were transfected with wild-type, T157N-, or Q67L-ARF6. The recycling of homologously internalized D_2 receptor was determined as in Figure 5A. ***:p<0.001 when Q67L group was compared to WT or T157N group.

(C) Effects of ARF6 on the down-regulation of D_2 receptor in response to long-term PMA stimulation. HEK-293 cells expressing ARF6 constructs were treated with vehicle or 1 μM PMA for 12h or 24h, and receptor binding was conducted as in Figure 1D.

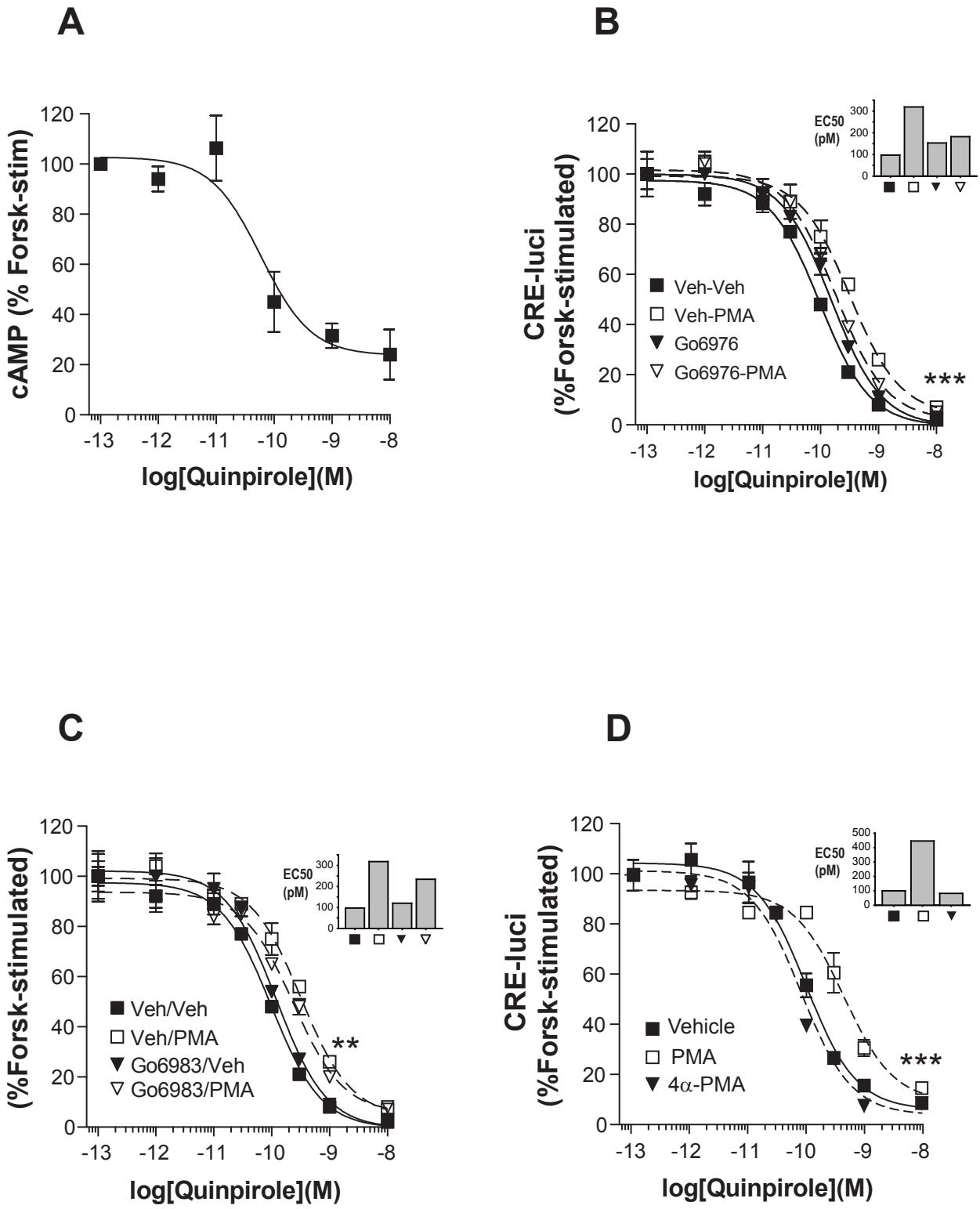
(D) Effects of knockdown of endogenous ARF6 on the recycling of internalized D_2 receptor. HEK-293 cells stably expressing scrambled shRNA or ARF6 shRNA were transfected with D_2 receptor. Receptor recycling was determined as in Figure 1C.

Figure S6. Roles of GASP-1 in the recycling of D_2 receptor internalized in response to PMA stimulation.

(**A**) Effects of PKC inhibitors on the interaction between D_2 receptor and GASP-1. HEK-293 cells expressing FLAG-D_2R and GFP-GASP-1 were pretreated with 1 μM Gö6976 or Gö6983 for 20 min, followed by 1 μM PMA for 5 min. Immunoprecipitation was conducted as in Figure 6D. **: p<0.01, ***:p<0.001 when PMA group was compared with corresponding Veh group.

(B) Roles of GASP-1 in the recycling of D_2 receptor in SH-SY5Y dopaminergic

neuroblastoma cells. (Upper two panels) Roles of GASP-1 in the recycling of D_2 receptor. Cells transfected with FLAG-D_2R and GFP-GASP-1 were stimulated with 1 μM PMA for 60 min (middle panel), followed by washing and incubation at 37 ^{o}C for 60 min (right panel). Immunocytochemistry was conducted as in Figure 5B. (Lower two panels) Cells were transfected either with D_2R-GFP or D_2R-PKCX-GFP, and processed as in upper two panels. The horizontal bars represent 10 μm.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Go6976-PMA
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Veh/PMA
Go6983/Veh
Go6983/PMA
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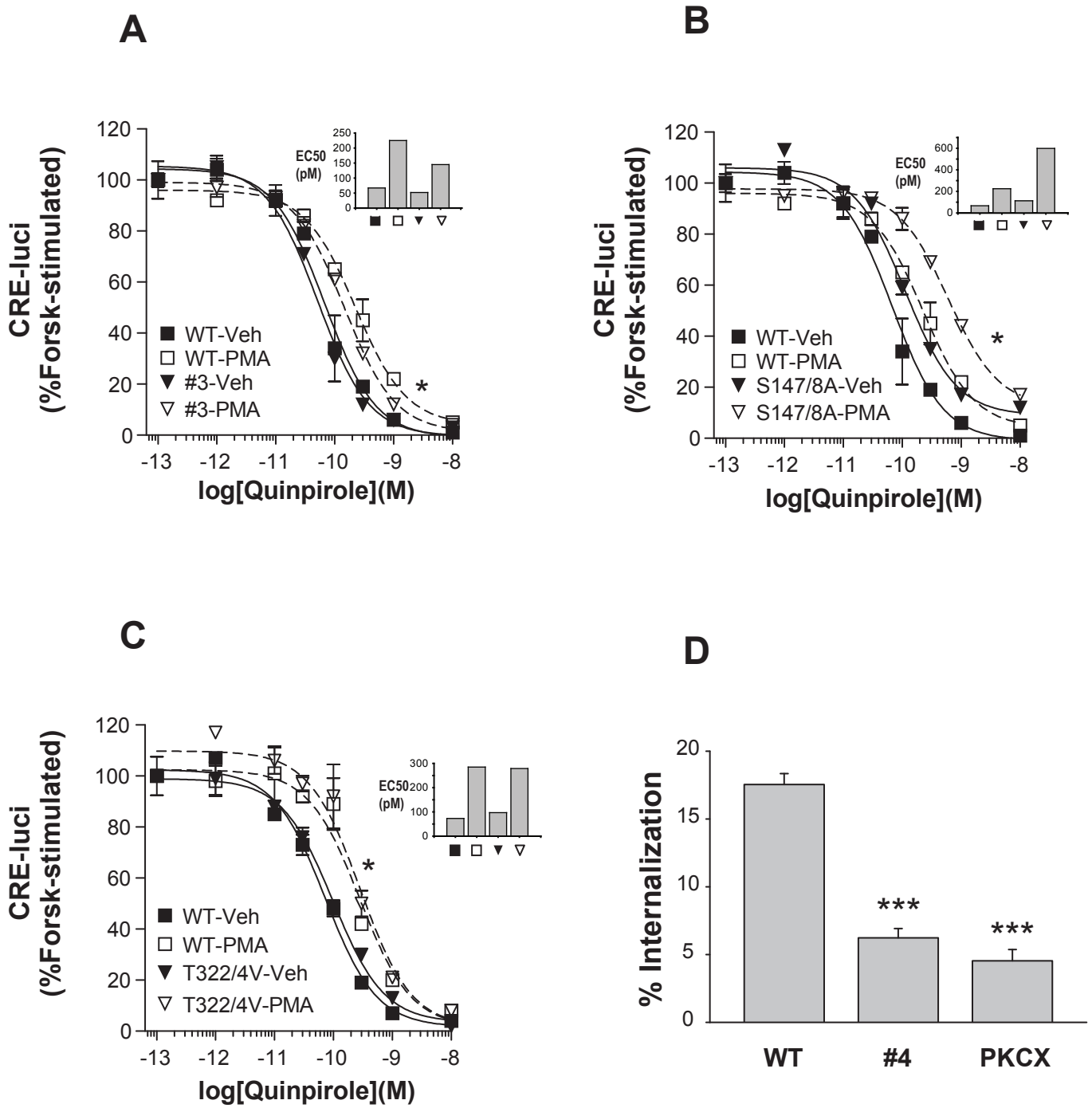
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WT-PMA
#3-Veh
#3-PMA
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T322/4V-PMA
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***
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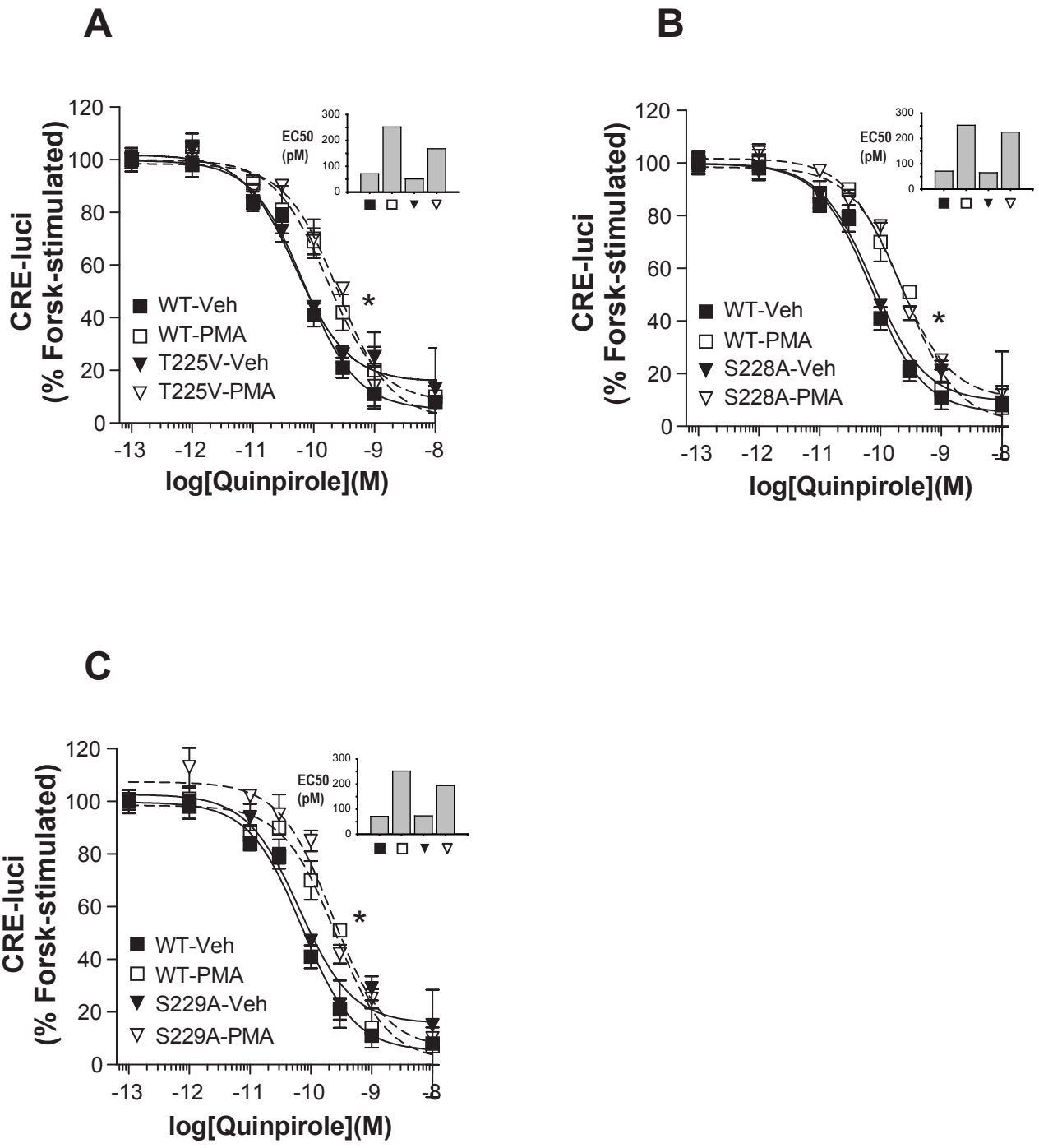
#4
PKCX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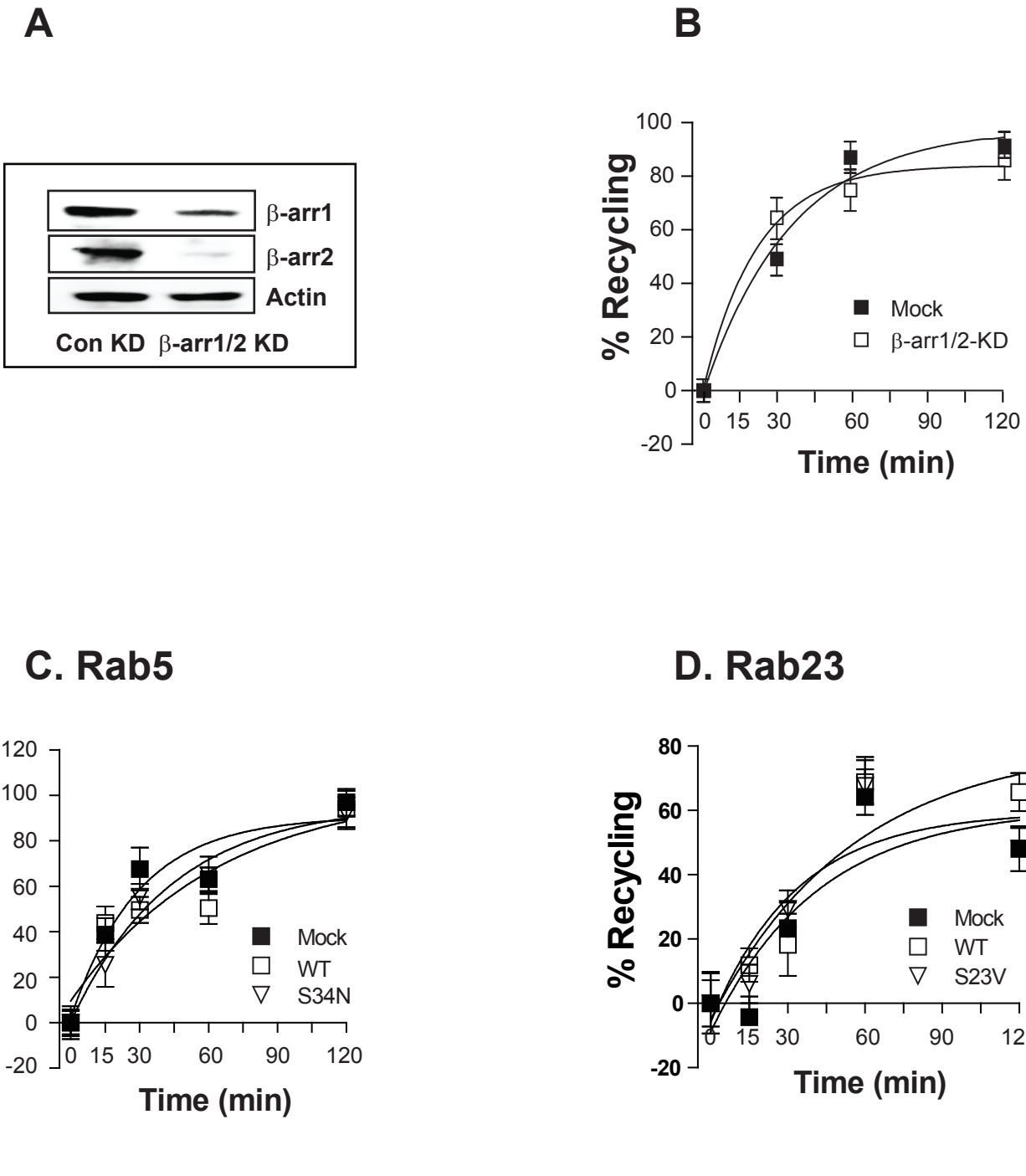
*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Actin
Con KD β-arr1/2 KD
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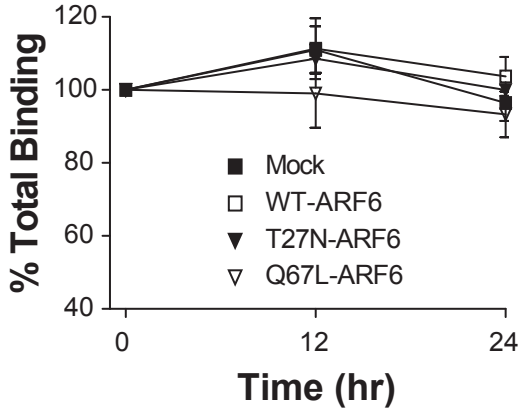
Mock
β-arr1/2-KD
C. Rab5
% Recycling
Time (min)
Mock
WT
S34N
D. Rab23
% Recycling
Time (min)
Mock
WT
S23V

A. Quin-treated



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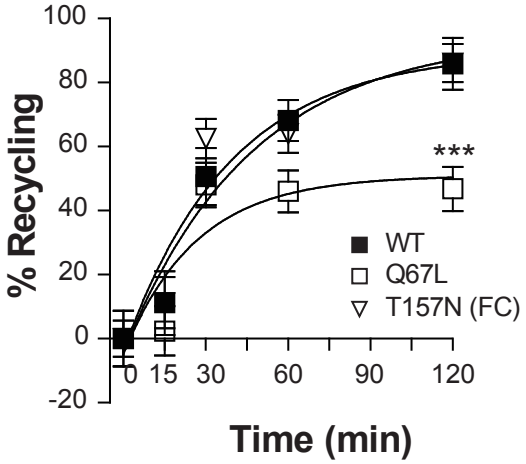

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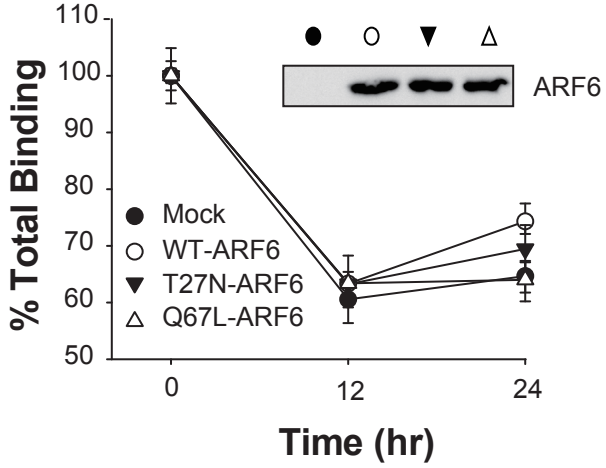
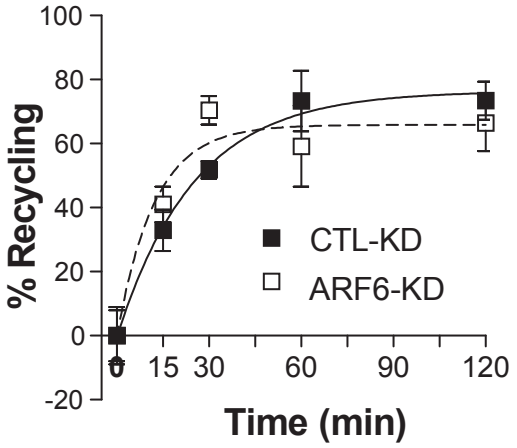
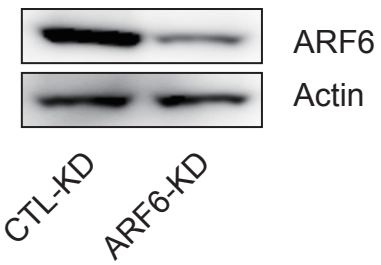
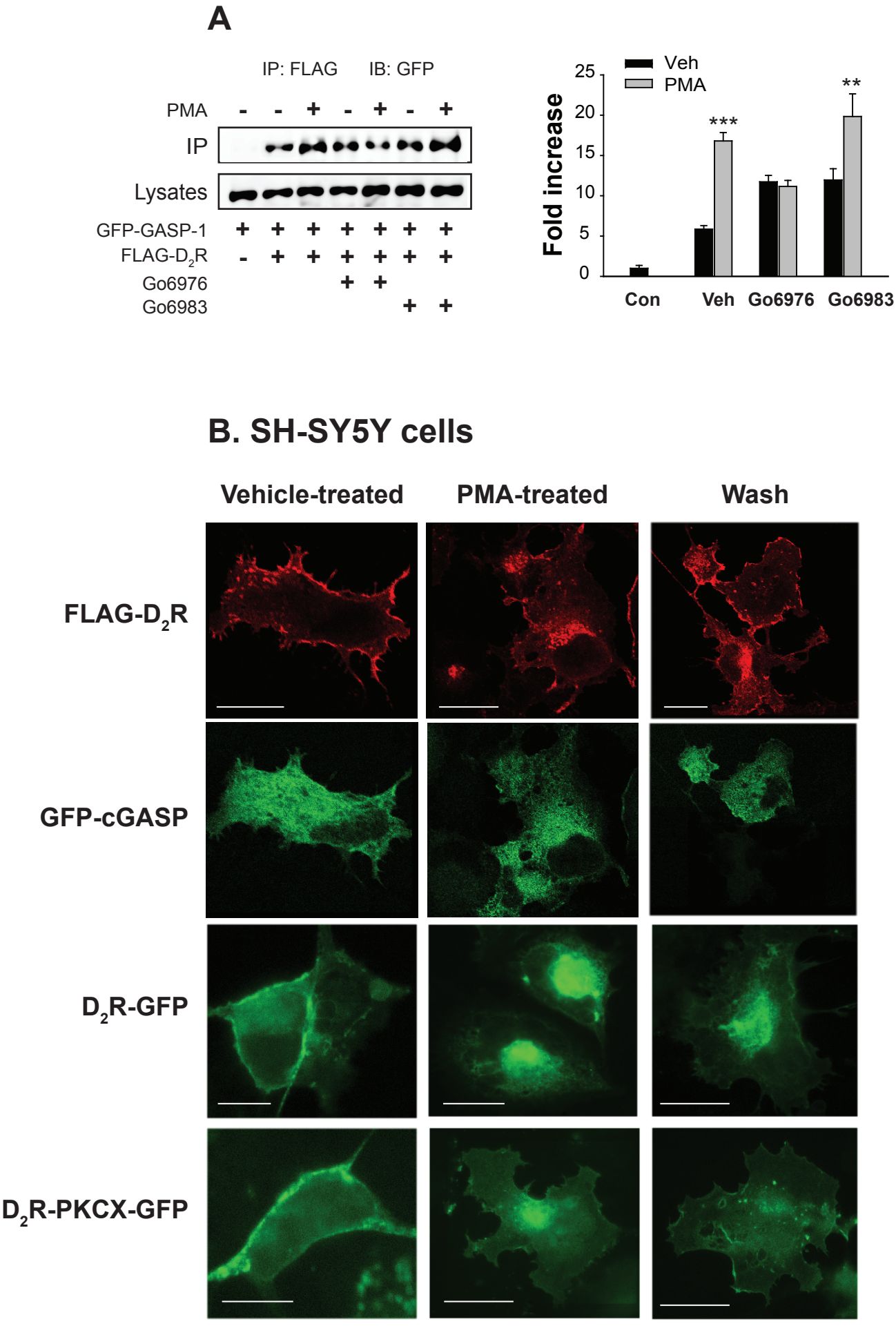


D. ARF6-Knockdown



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IB: GFP
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FLAG-D2R
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Go6983
Veh
PMA
Fold increase
Con
Veh
Go6976
Go6983
B. SH-SY5Y cells
Vehicle-treated
PMA-treated
Wash
FLAG-D2R
GFP-cGASP
D2R-GFP
D2R-PKCX-GFP