Supplementary Materials

Figure S1. The average number of PMA-induced ventral actin structures in Beas-2b cells is enhanced by expression of EFA6 or activated Arfs. Beas-2b cells were either mock transfected ("Control"), or transfected with Flag-EFA6, HA-Arf1 Q71L, or untagged Arf6 Q67L. Cells were treated with PMA, immunostained with antibodies against Flag, HA, or Arf6 and stained with rhodamine-phalloidin to visualize actin. The number of ventral actin structures per cell was counted for a minimum of 10 cells for each condition. Error bars represent standard error. ANOVA test determined that the means of all groups were statistically different ($p<0.005$).

Figure S2. Endogenous Arf1 and Arf6 are present at PMA-induced actin waves.

Beas-2b cells were treated with PMA for 30 min, fixed, and immunostained with antibodies against Arf1 or Arf6 (green) and rhodamine-phalloidin. Arrowheads indicate ventral actin structures. Scale bar, 10 μm.

Figure S3. Ventral ruffle formation is dependent on PKC and Src family kinase activities. HeLa cells expressing EFA6 were treated with 200 nM PMA (A top row), or 200 nM PMA in the presence of 10 μM GF 109203x (A bottom row), 10 μM PP3 (B top row) or 10 μM PP2 (B bottom row) for 30 min prior to fixation and immunofluorescence staining as described. Cells were stained with rhodamine phalloidin and co-stained with an antibody against the epitope tag on EFA6, detected with Alexa 488-conjugated goat anti-mouse. Bars,10 μm. (C) Fraction of EFA6-transfected cells with 1 or more ventral ruffles was quantified and is expressed as the average percentage obtained from three independent experiments. Error bars represent the standard deviation from the mean.

Figure S4. Expression of dominant negative forms of Arf6, Rac and Arf1 inhibit ventral ruffle formation in HeLa cells co-expressing EFA6. HeLa cells were co-transfected with plasmids encoding Flag-EFA6 alone or EFA6 plus Arf6 T27N, Rac1 T31N, myc-tagged p72 PIP 5-phoshatase, or Arf1 T31N-HA. Cells were treated with 200 nM PMA for 30 min prior to fixation and immunostaining with appropriate antibodies as described in the Materials and Methods. Cells were co-stained with Alexa 633-conjugated phallodin. Bars,10 μm.

Figure S5. Cortactin is associated with ventral actin structures in both cell types while focal adhesion protein distribution differs between HeLa and Beas-2b cells. (A) HeLa cells expressing Flag-EFA6 or (B) untransfected Beas-2b cells were treated with PMA and then fixed and labeled with antibodies to cortactin, phospho-paxillin (PY Pax), or PY397 FAK and rhodamine phalloidin. Merged images of untreated cells are shown in the far right column (no PMA). Bars, 20μM.

Figure S6. Depletion of Arf1 or Arf6 in HeLa cells inhibits ventral ruffle formation. (A) Arf1 expression level was depleted by 85% to that of control cells when normalized to actin, as measured by densitometry of western blot (inset). Quantified is the average expression obtained from three independent experiments and error bars represents standard error. (B) Control and Arf1-depleted HeLa expressing Flag-EFA6 cells were treated with 100 nM PMA and percentage of cells exhibiting one or more ventral actin ruffles was quantified. In total, 200 cells were counted from three independent experiments. Error bars represent standard error for proportional data, $P<0.0001$ (Fishers Exact Test) indicating that Arf1 depletion reduced the percentage of cells forming ventral structures. (C) Control (top row), Arf1-depleted cells (middle row), and Arf6-depleted cells expressing EFA6-Flag were treated with PMA, immunostained with an antibody against Flag and stained with rhodamine phalloidin to detect F-actin. Untreated cells ("no PMA") are shown in right panel. Bar, 20 μM. (D) Arf6 expression level was depleted by 70% to that of control cells when normalized to tubulin, as measured by densitometry of western

blot (inset). Quantified is the average expression obtained from three independent experiments and error bars represents standard error. (E) Control and Arf6-depleted HeLa cells were treated with 100 nM PMA and the proportion of cells with ventral actin ruffles were quantified. A minimum of 400 cells in total was counted from 5 independent experiments. Error bars represent standard error for proportional data, $P<0.0001$ (Fishers Exact Test) indicating that Arf6 depletion reduced percentage of cells forming ventral structures.

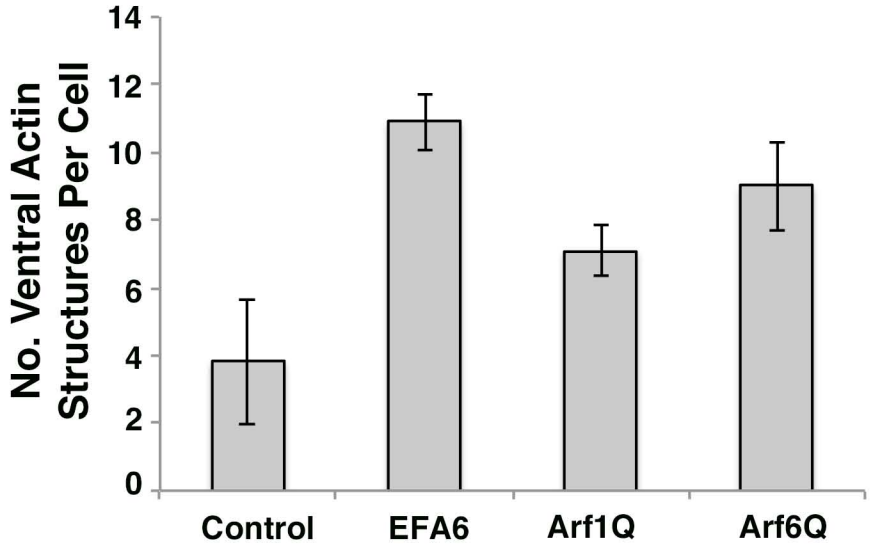
Supplementary Materials - Movies

Movie 1. Actin dynamics upon PMA-treatment of Beas-2b cells. Time-lapse recording of Beas-2b cells expressing Life-Act-RFP and Mem-GFP imaged every 30 sec (total of 20 min), corresponding to Figure 1D. Movie was compiled at a rate of 10 frames/sec (play-back rate of 300X).

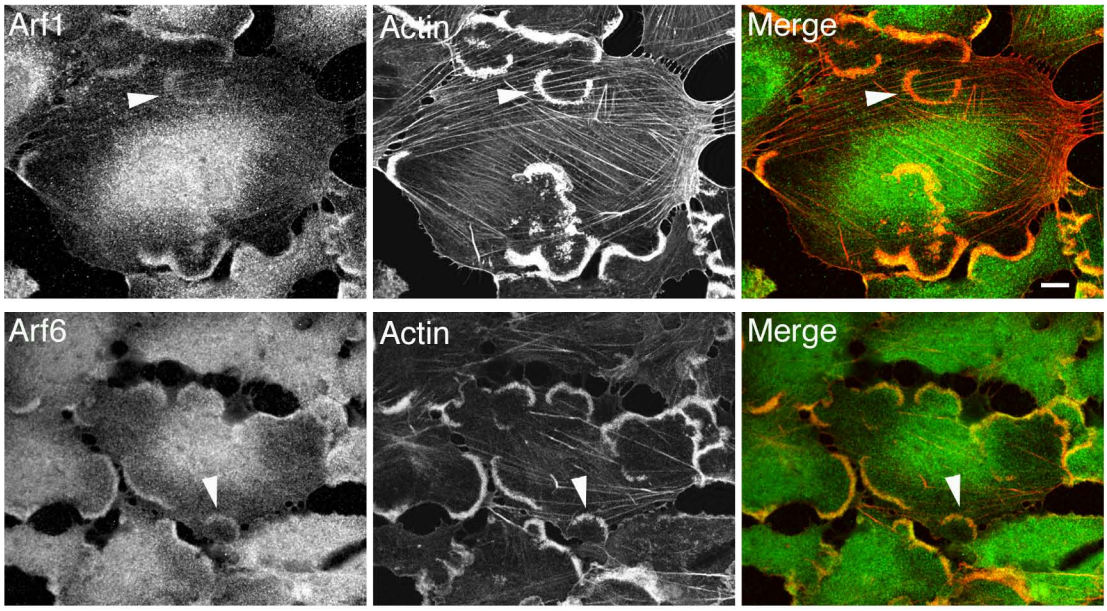
Movie 2. Actin and membrane dynamics in PMA-treated BEAS2B cells expressing active Arf6. Time-lapse recording of BEAS2B cells expressing Lifeact-RFP, Arf6Q67L and Mem-GFP to mark PM and vacuole membranes that were treated with PMA and imaged every 30 sec (total of 20 min), corresponding to Figure 1E.

Movie 3. Arf1 and Actin dynamics in PMA-treated BEAS2B cells expressing active Arf1. Time-lapse recording of BEAS2B cells expressing actin-GFP and Arf1Q71L-RFP treated with PMA and imaged every 30 sec (total of 15 min), corresponding to Figure 1F.

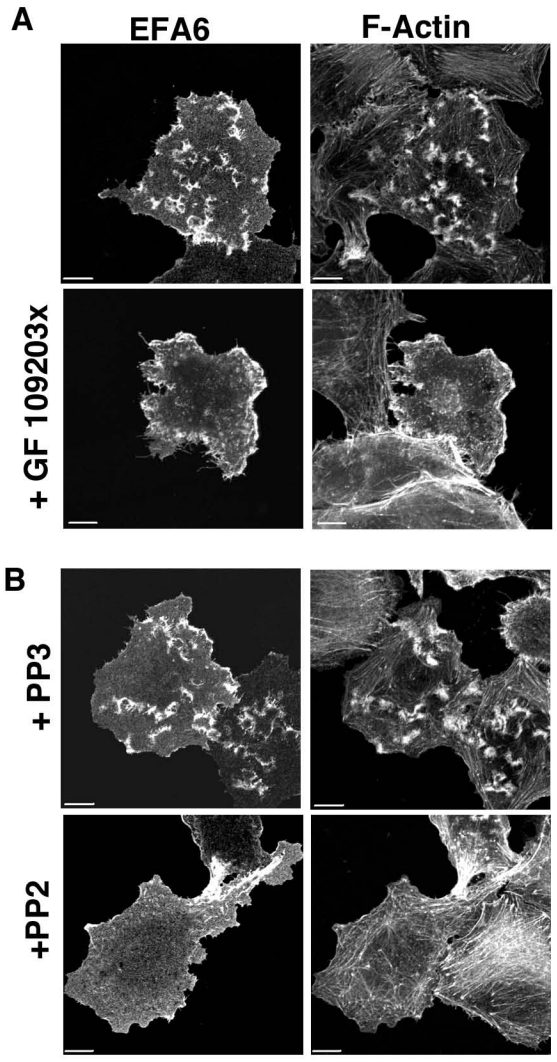
Movie 4. Actin dynamics in HeLa cells expressing EFA6. Time-lapse recording of HeLa cells expressing EFA6, Life-Act-RFP and Mem-GFP taken every 30 sec (total of 30 min) corresponding to Figure 5. Movie was compiled at a rate of 10 frames/sec (play-back rate of 300X).



Supplemental Figure 1



Supplemental Figure S2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B
+ PP3
+PP2
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% transfectants w/ ventral ruffles
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70
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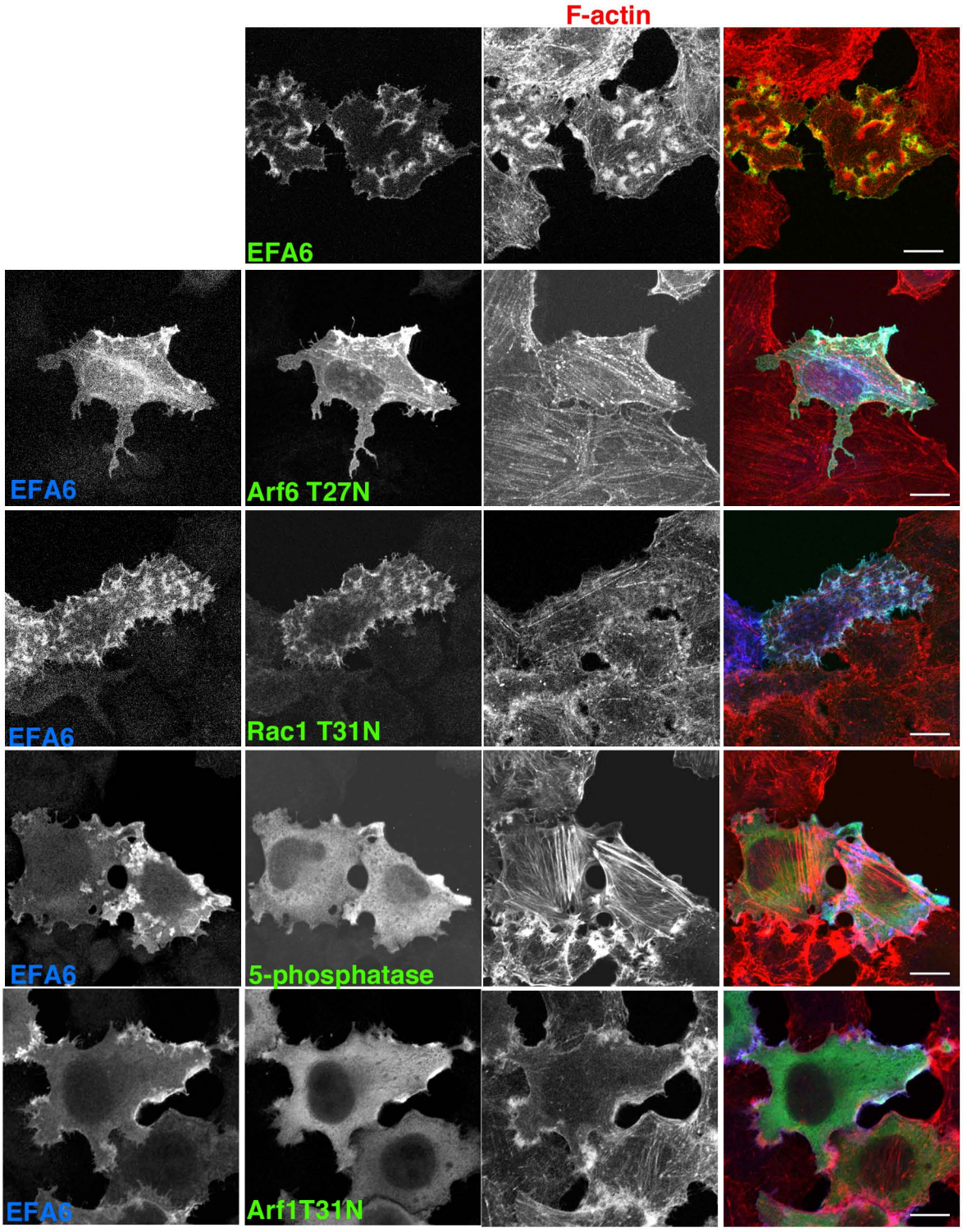
Supp Fig S3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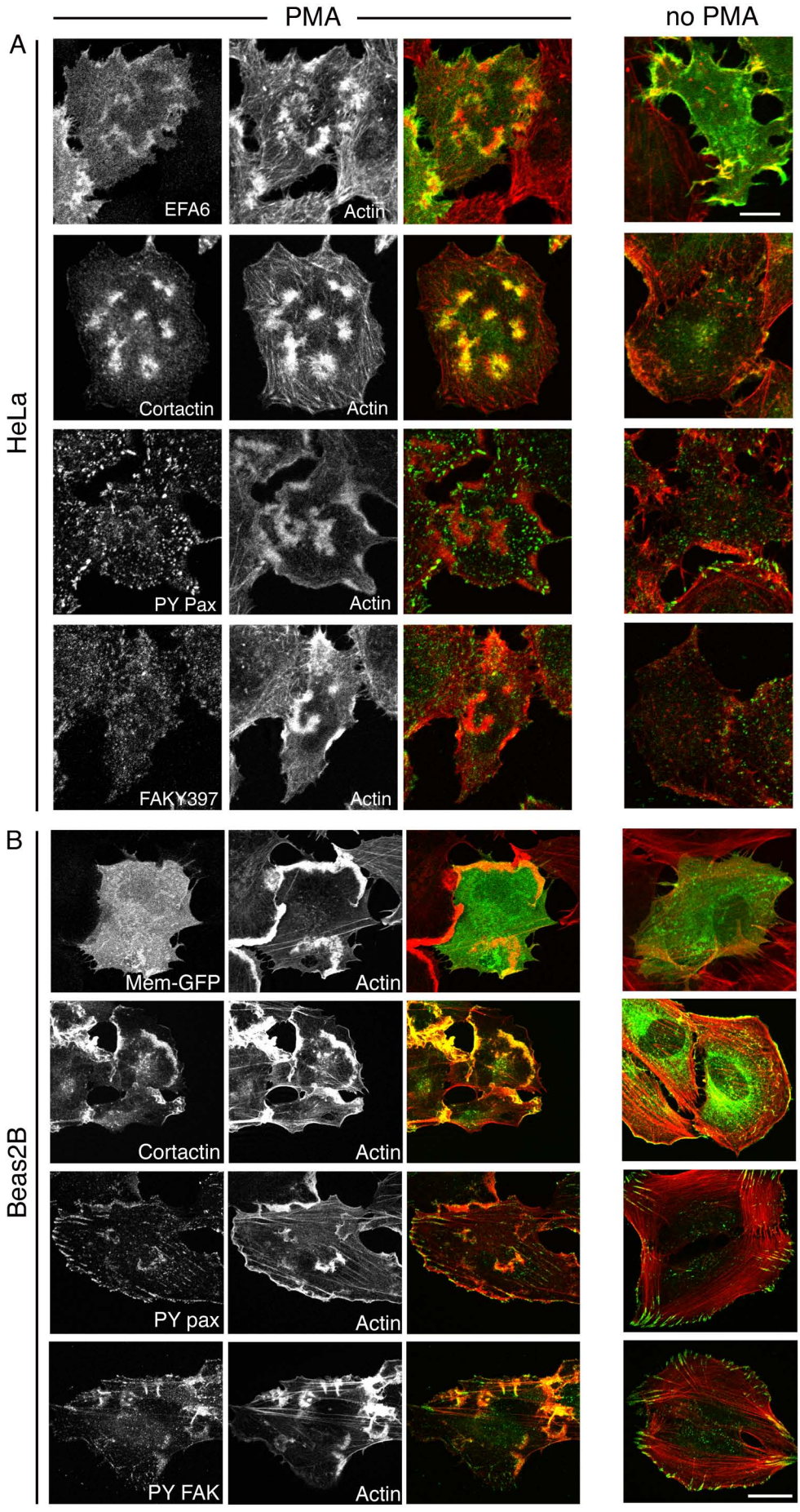
Supp Fig S4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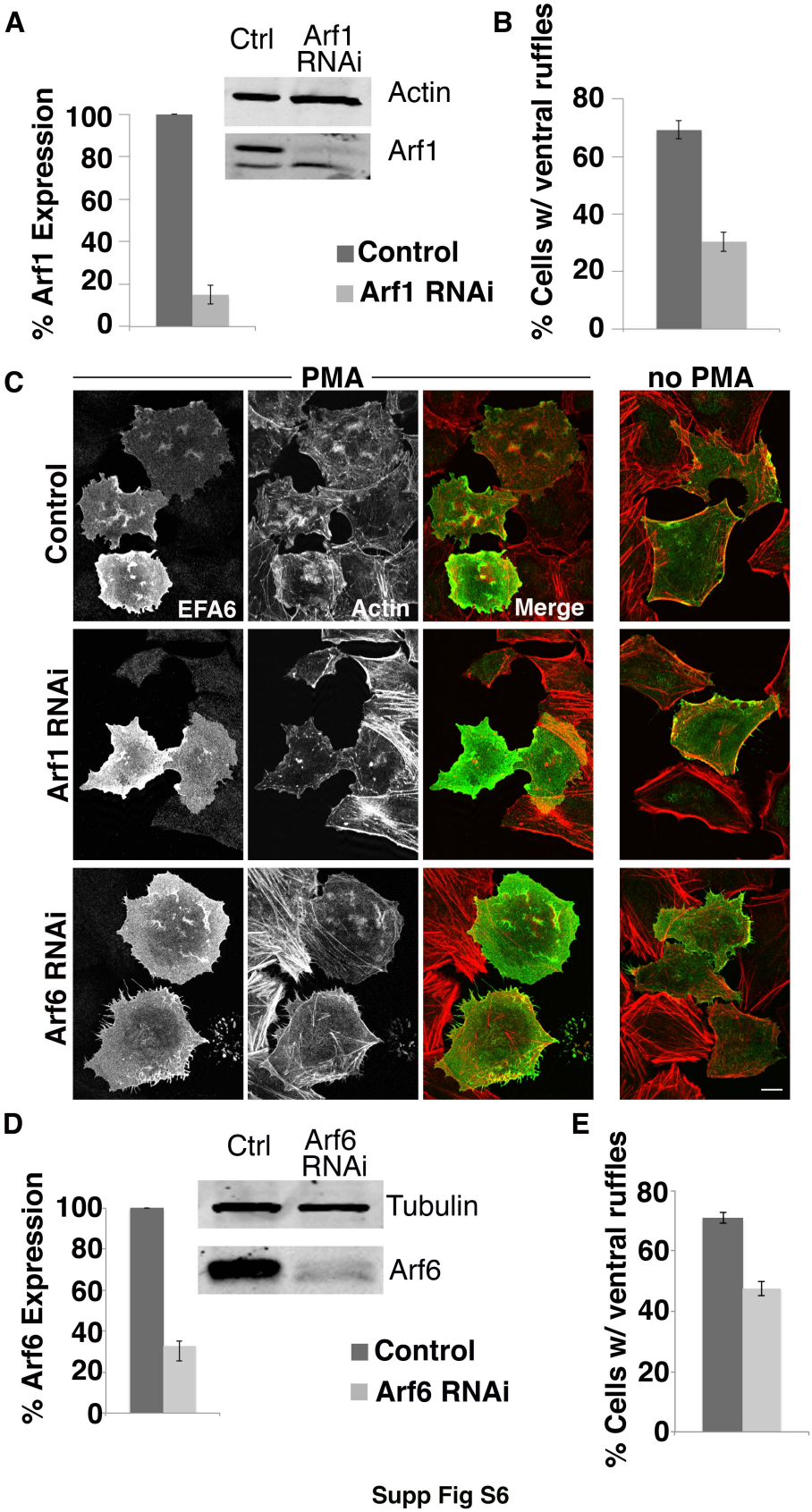
Supp Fig S5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Supp Fig S6