



**Figure 2-figure supplement 1. Cytohesins interact with INAVA to Promote GEF Independent Actin Assembly.**

- (A) Protein interaction Platform (PIP) Assay in Yeast. ARNO full length, CC-SEC7 and CC domain constructs were fused to YFP, and INAVA was fused to  $\mu$ NS, and transformed in yeast. Yeast were mated and induced with galactose for 4 hours and imaged. Empty vector  $\mu$ NS was used as negative control.
- (B) As in (A) but with b, PIP assay with INAVA and full length Cytohesin 1, or GRP1.
- (C) GST pulldown control with recombinant GST and His-MBP tagged coiled-coil (CC) region of Cytohesin 1, ARNO, or GRP1.
- (D) Recombinant ARNO CC-SEC7 alone or complexed with GST-INAVA-CUPID (1  $\mu$ M) were incubated with MANT-GTP (25  $\mu$ M) and  $\Delta$ N17ARF1-GDP (40 $\mu$ M) for 15 minutes at 25°C in duplicate. Guanine exchange reactions were measured by fluorescence at ex/em 355/445 nm. Background from control samples with MANT-GTP and  $\Delta$ N17ARF1-GDP was subtracted. GEF assays with Cytohesin 1, GRP1, or Cytohesin 4 CC-SEC7 with or without GST-INAVA-CUPID. Data representative of 3 independent experiments. Error bars  $\pm$  SD.
- (E) Immunoblot of endogenous ARNO and actin in Caco2BBE wildtype and cells expressing INAVA (anti-GFP). ImageJ was used to quantify relative band intensity.
- (F) As in (E) but with INAVA knockout lines.
- (G) MDMs (n=6) were transfected with empty vector (EV) or WT HA-INAVA, and Myc-ARNO at the indicated concentrations. Summary graph with mean fluorescent intensity (MFI) values of ARNO expression (as detected by Myc antibody using intracellular flow cytometry) + SEM (similar results seen in an additional n=6). Tx, treatment.  $\dagger\dagger$ ,  $p < 1 \times 10^{-5}$ .
- (H) Confocal images of Caco2BBE expressing INAVA-GFP-CUPID (aa 100-261) with ARNO. Cells were stained with anti-myc (ARNO) and TRITC-phalloidin (F-actin).
- (I) As in (H) but with MCF7 cells stably expressing INAVA-GFP.
- (J) As in (I) but with MCF7 cells expressing doxycycline induced ARNO.
- (K) As in (I) but with MCF7 cells stably coexpressing INAVA-GFP with ARNO wild type, GEF mutant (E156K), ARF6-GTP mutant (K336A), or PIP2 mutant (K268A). Scalebar = 10  $\mu$ M.