HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2013 April 25.

Published in final edited form as:

Oncogene. 2012 October 25; 31(43): 4630–4638. doi:10.1038/onc.2011.607.

CHMP6 and VPS4A mediate recycling of Ras to the plasma membrane to promote growth factor signaling

Ze-Yi Zheng, PhD[1,*], **Chiang-Min Cheng, PhD**[1,2,*], **Xin-Rong Fu, PhD**[1], **Liuh-Yow Chen, PhD**[3], **Lizhong Xu, PhD**[4], **Sonia Terrillon, PhD**[4,¶], **Stephen T. Wong, PhD**[5], **Dafna Bar-Sagi, PhD**[4], **Zhou Songyang, PhD**[3], and **Eric C. Chang, PhD**[1,2]

[1]Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, TX, USA

[2]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

[3]Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, USA

[4]Department of Biochemistry, New York University School of Medicine, New York, NY, USA

[5]Department of Systems Medicine and Bioengineering, Methodist Hospital Research Institute, Houston, TX, USA

Abstract

While Ras is well-known to function on the plasma membrane (PM) to mediate growth factor signaling, increasing evidence suggests that Ras has complex roles in the cytoplasm. To uncover these roles, we screened a cDNA library and isolated H-Ras-binding proteins that also influence Ras functions. Many isolated proteins regulate trafficking involving endosomes; CHMP6/VPS20 and VPS4A, which interact with ESCRT-III, were chosen for further study. We showed that the binding is direct and occurs in endosomes. Furthermore, the binding is most efficient when H-Ras has a functional effector-binding-loop and is GTP-bound and ubiquitylated. CHMP6 and VPS4A also bound N-Ras, but not K-Ras. Repressing CHMP6 and VPS4A blocked Ras-induced transformation, which correlated with inefficient Ras localization to the PM as measured by cell fractionation and photobleaching. Moreover, silencing *CHMP6* and *VPS4A* also blocked EGFR recycling. These data suggest that Ras interacts with key ESCRT-III components to promote recycling of itself and EGFR back to the PM to create a positive feedback loop to enhance growth factor signaling.

Keywords

signal transduction; oncogene; tumorigenesis; trafficking; ESCRT-III

Corresponding author: Eric C. Chang, 713-798-3519/ echang1@bcm.tmc.edu.

[*]These authors contribute equally

[¶]Current address: Department of Molecular & Cellular Pharmacology, Abbott Bioresearch Center, Worcester, MA, USA

Conflict of interest

The authors declare no conflict of interest.

Introduction

There are three *RAS* genes in humans, *H-*, *N-*, and *K-RAS*, and the latter encodes two proteins, K-Ras-4A and K-Ras-4B. These Ras proteins, when GTP-bound, signal through many effectors to control a wide range of functions including tumorigenesis (Karnoub and Weinberg, 2008). The importance of Ras localization to the plasma membrane (PM) is well established as PM is where Ras proteins associate with receptors, such as EGFR, to mediate growth factor signaling. However, increasing evidence suggests that Ras proteins can also signal from many cytoplasmic membrane compartments, and some of these cytoplasmic activities are required for cell transformation (Chang and Philips, 2006; Fehrenbacher *et al.*, 2009).

Among these cytoplasmic compartments on which Ras is known to signal, the roles of endosomes are particularly complex and intriguing. RIN1 is a Ras effector (Han *et al.*, 1997) and a guanine nucleotide exchange factor (GEF) for Rab5, which controls membrane docking and fusion during the early stage of endocytosis (Han *et al.*, 1997; Tall *et al.*, 2001). One of the Rab5 effectors is RABGEF1/Rabex-5, which functions as a ubiquitin ligase for H- and N-Ras to facilitate their endosome association (Xu *et al.*, 2010; Yan *et al.*, 2010). This pathway appears to attenuate Ras signaling, as measured by MEK/Erk activation, presumably by internalizing Ras, as well as growth factor receptors such as EGFR (Barbieri *et al.*, 2003; Tall *et al.*, 2001). However, Ras and EGFR on endosomes appear to be active — that is, Ras proteins remain GTP-bound, while EGFR remains phosphorylated (Balbis *et al.*, 2007; Jiang and Sorkin, 2002). These observations raise the important question as to whether endosomal Ras pathways continue to signal or whether the observed activation of its components is only temporary without substantial biological significance. In short, the roles of endosomes in Ras signaling are very complex and poorly understood.

In this study, we attempted to define Ras cytoplasmic functions by screening for Ras-binding proteins that selectively interact with Ras in the cytoplasm. We thus identified 26 proteins that can bind and alter Ras activities. Intriguingly, many of the isolated Ras-binding proteins function at endosomes, two of which, CHMP6/VPS20 and VPS4A, were analyzed in detail. Our results support a model whereby CHMP6 and VPS4A are novel Ras effectors needed for transformation. Ras can interact with CHMP6 and VPS4A to control recycling of itself, as well as EGFR, back to the PM, thus generating a positive feedback loop to enhance Ras signaling.

Results

Screen human Ras-interacting proteins in live mammalian cells using BiFC

To identify Ras-interacting proteins in live human cells, we screened a human cDNA library (hORFeome v1.1) containing 8,100 full-length clones (Rual *et al.*, 2004), using the Biomolecular Fluorescence Complementation (BiFC) system (Kerppola, 2008). This system has been used previously to detect the binding between constitutively active H-Ras (e.g., H-Ras(12V) or H-Ras(61L)) fused to a N-terminus fragment of YFP (Yn) and many Ras effectors, such as Raf, Cdc42, or RIN1, fused to a C-terminus fragment of YFP (Yc, (Cheng *et al.*, 2011)). The Yc-tagged cDNAs were transfected into HT1080 cells stably expressing

Yn-H-Ras(12V), and the cells were sorted by FACS (see Materials and Methods). The Ras binding domain in Raf, RafRBD, was fused to Yc as the positive control (Figure S1), while the Yc alone was analyzed as the negative control. Isolated cDNAs were further tested against Yn-GST, as well as Yn-H-Ras E(12V), in which the effector-binding loop has been mutated. We thus identified 64 proteins that bind H-Ras(12V), but not the GST control. Some of them also bind H-Ras E(12V) with substantially reduced affinity (Figure S2). One of the identified proteins is A-Raf, which validates the effectiveness of the screen to identify Ras effectors.

To determine whether the isolated proteins also control Ras functions, we overexpressed all of them, except A-Raf, in NIH3T3 cells transformed by constitutively active H-Ras(61L) and then measured colony formation in soft-agar. These proteins were also tested for the ability to alter Ras-induced gene transcription by an Elk-1-mediated luciferase reporter assay, which is controlled by the Raf-MAP kinase pathway. Only 26 proteins were able to inhibit both Ras-related activities (Table 1). Intriguingly, only one protein, LEPROTL1, appears to *enhance* a Ras-induced activity when overexpressed in the gene expression assay. It is possible that most of these isolated genes act as dominant-negatives when overexpressed (see below).

Validate the binding between Ras and isolated proteins by BiFC fluorescent microscopy

Nine out of the 26 isolated proteins are known to associate with cellular membrane and/or to regulate protein transportation, suggesting that as a group this screen has identified proteins that may regulate Ras trafficking. In particular, two of them, CHMP6/VPS20 and VPS4A, are well known to interact with ESCRT-III (Endosomal Sorting Complex Required for Transport-III) components to control endosomal protein sorting (Raiborg and Stenmark, 2009). Endosome trafficking has been shown to impact Ras signaling as discussed above; thus, CHMP6 and VPS4A were chosen for detailed analyses.

We determined where in the cells these two proteins can interact with Ras and whether they form a stable complex. HT1080 cells expressing both Yn-tagged H-Ras(12V) and Yc-tagged either CHMP6 or VPS4A were examined by confocal microscopy (Figure 1a). In accordance with the fact that VPS4A and CHMP6 are known to function in endosomes, the reconstituted YFP signals were found only in the cytoplasm, but not at the PM, and co-localized in particular with those of CFP-tagged early endosome marker Rab5A, as well as with the late endosome/MVB (multivesicular bodies) marker Rab7A, but not with recycling endosome marker Rab11A and Golgi marker GalT. These results suggest that these proteins mostly interact in early and late endosomes/MVBs, but not in recycling endosome, Golgi, or PM.

H-Ras binding to CHMP6 and VPS4A is direct and dependent on GTP and ubiquitylation

Next, we analyzed whether the binding between Ras and CHMP6 and VPS4A was GTP-dependent by performing co-immunoprecipitation experiments using ectopically expressed proteins. As shown in Figure 1b, H-Ras(61L), which is expected to be preferentially GTP-bound, efficiently co-immunoprecipitated CHMP6 and VPS4A, while the wild type H-Ras could barely do so; conversely, when CHMP6 was immunoprecipitated, H-Ras(61L) was

readily co-immunoprecipitated, while wild type H-Ras was not. Similar results were obtained with VPS4A. Furthermore, protein cargos that are sorted and/or transported in endosomes (and by the ESCRT-III complex, in particular) are frequently ubiquitylated. H-Ras has been shown to be ubiquitylated; therefore, we investigated whether ubiquitylation of H-Ras is critical for binding to these two proteins. To this end, we first repeated the co-immuoprecipitation experiments using H-Ras-8RK and H-Ras(12V)-8RK, in which all five solvent-exposed lysines, along with the three lysines located in the hyper variable region, were mutated to arginine to block ubiquitylation (Jura *et al.*, 2006). When either H-Ras was immunoprecipitated, no detectable CHMP6 could be seen and *vice versa* (Figure 1b). Furthermore, when VPS4A was immunoprecipitated, very little H-Ras(12V)-8RK or H-Ras-8RK could be co-immunoprecipiated, as compared to a similar experiment when H-Ras(12V) or H-Ras was examined. Likewise when the reverse immunoprecipitation was performed, while considerably more H-Ras-8RK and H-Ras(12V)-8RK than the normal H-Ras(12V) were immunoprecipitated, very little VPS4A was detected.

To more directly show that the binding is direct and requires H-Ras to be ubiquitylated in mammalian cells, we performed FRET analysis using CFP-tagged Ras proteins and YFP-tagged VPS4A. VPS4A was chosen because it binds Ras more strongly (Figure 1b), and its repression blocks Ras-induced transformation more efficiently (see below). Besides H-Ras(61L) and H-Ras(12V)-8RK, we also examined Ub-H-Ras, which is a fusion protein between wild type H-Ras and an ubiquitin moiety (Jura *et al.*, 2006). As shown in Figure 1c, FRET signals were readily detected when YFP-VPS4A was co-expressed with CFP-H-Ras(61L), but not with CFP-H-Ras(12V)-8RK. Furthermore, despite the fact that Ub-H-Ras is not constitutively GTP-bound, it still bound VPS4A more strongly than did H-Ras(61L).

Endogenous Ras isoform-specific binding to VPS4A and CHMP6

To determine whether different endogenous Ras isoforms bind CHMP6 and VPS4A with different efficiencies, we immunoprecipitated Ras proteins using isoform-specific antibodies from DCIS.COM, HT1080, and CAPAN-1 cells, which are known to endogenously express oncogenic *H-RAS*, *N-RAS*, and *K-RAS*, respectively. Figure 1d showed that only H-Ras and N-Ras, but not K-Ras, can be co-immunoprecipitated with CHMP6 or VPS4A. These data suggest that efficient binding of CHMP6 and VPS4A to Ras proteins requires the latter to be ubiquitylated and GTP-bound and that N- and H-Ras proteins may bind them more efficiently than K-Ras does.

Proper CHMP6 and VPS4A levels are important for H-Ras-induced cell transformation

To further elucidate how CHMP6 and VPS4A regulate Ras functions, we knocked down CHMP6 and VPS4A by shRNAs in H-Ras(61L)-transformed NIH3T3 cells, and found that the ability of these cells to form colonies in soft agar was greatly reduced, which was rescued by cDNAs that are refractory to the shRNAs (Figure 2a). These shRNAs did not have any adverse effects on overall H-Ras levels or cell growth under normal conditions (Figure S3). We conducted two experiments to determine whether CHMP6 and VPS4A influence H-Ras-induced transformation with selectivity. In the first, we induced cell transformation by the SV40 T-antigen, and found that silencing either molecule had also no detectable effect on focus formation (Figure 2b). In the second, we silenced *CHMP6* or

VPS4A in HT-1080 and CAPAN-1 cells, which carry oncogenic *N*- and *K-RAS*, respectively, and found that colony formation in soft agar was only inhibited in the former cells (Figure 2c). These data suggest that CHMP6 and VPS4A are selectively required for H-Ras and N-Ras to transform cells.

During the initial phase of the screen, overexpressing Yc-tagged CHMP6 and VPS4A also blocked Ras activities (Table 1). GFP-tagged ESCRT-III components, when overexpressed, have been reported to act in a dominant negative fashion (Howard *et al.*, 2001), possibly due to the bulky GFP tag. To examine whether it is the bulk of tag that was responsible for blocking Ras activities in our study, we replaced the Yc with FLAG, and the resulting proteins still inhibited soft agar colony formation (Figure 2d). Like their down-regulation, overexpressing these two molecules did not affect H-Ras(61L) levels or cell proliferation under normal conditions (data not shown). We conclude from these studies that proper levels of CHMP6 and VPS4A are critical for oncogenic Ras to induce cell transformation.

CHMP6 and VPS4A control Ras localization to the plasma membrane

Since both VPS4A and CHMP6 are involved in endosomal protein sorting and bind Ras in endosomes, we investigated the possibility that they are important for proper Ras localization to various membrane compartments in the cell. We thus examined the distribution of H-Ras in the CHMP6 or VPS4A-silenced cells by sucrose gradient fractionation. H-Ras can be found in both the lighter fractions (marked by caveolin-1, fractions 3–6) containing lipid rafts as well as the heavier fractions (marked by Na^+/K^+ ATPase, fractions 11–13) containing bulk PM (Figure 3a). When H-Ras is GTP-bound, it shifts from the lighter to the heavier fractions (Prior *et al.*, 2001). Our data show that when VPS4A levels were repressed, the levels of wild-type and oncogenic H-Ras proteins in the heavier fractions were dramatically reduced. The levels of H-Ras proteins in heavier fractions of CHMP6 knocked-down cells were also significantly decreased (wild-type H-Ras, 20%; oncogenic H-Ras, 26%). These data support the possibility that VPS4A and CHMP6 are required for H-Ras to properly localize to bulk PM.

To further decipher how CHMP6 and VPS4A impact trafficking of H-Ras to the PM, we performed photobleaching experiments. The aforementioned shRNA vectors contain GFP, which was used to mark cells whose CHMP6 or VPS4A levels were repressed (Figure 3b). To choose another fluorescent tag for photobleaching, we expressed in these cells an H-Ras tagged with a green-to-red photoactivatable fluorescent protein Dendra2. In our lab, the Dendra2 can be most efficiently photoconverted to a red fluorescent protein by UV (see Materials and methods), and we did not detect any difference in photoconversion efficiency in the control *vs.* knock down cells. We then photobleached the photoconverted red H-Ras at the PM by all lasers and measured its fluorescence recovery by scanning the whole field at 543 nm, which is not expected to induce additional photoconversion. Our data showed that when either *CHMP6* or *VPS4A* was silenced, substantial delay in recovery was seen at the PM (Figure 3b). We note that as signal at the PM increased/recovered over time, there was a concurrent decrease of fluorescent signal in the cytoplasm in the same photobleached cells, as one would expect as Dendra2 tagged H-Ras moved from the cytoplasm to the PM. Further, when a neighboring unbleached cell was examined as control, we detected a slight

decrease of fluorescence over time, characteristic of spontaneous photobleaching. These observations rule out the possibility that the method used for detecting photoconverted Dendra2 induced new photoconversion. In summary, together with the biochemical data in Figure 3a, we conclude that CHMP6 and VPS4A play critical roles in the efficient transport of H-Ras to the PM.

Ras is also necessary for efficient EGFR recycling

CHMP6 and VPS4A are components of the ESCRT-III pathway, whose best known function is to sort proteins in endosomes. Intriguingly, it has been shown recently that this complex also controls recycling of EGFR back to the PM (Baldys and Raymond, 2009). We confirmed this in HT1080 cells — EGFR appears to internalize 15 min after EGF addition, and within 30 min, a large portion of the internalized EGFRs was recycled back to the PM to reach equilibrium (Figure 4 and (Baldys and Raymond, 2009)). In contrast, in *CHMP6* or *VPS4A*-silenced cells, there was no detectable EGFR at the PM 30 min after EGF addition, indicating that its recycling was inhibited. To determine whether Ras can also control EGFR recycling, we knocked down endogenous N-Ras expression in these cells, and at 30 min after adding EGF, measured fluorescence levels at the PM vs. that in the whole cell (Figure 4). Our data show that the proportion of labeled EGFR at the PM in *N-RAS*-silenced cells was reduced by 3-fold compared to that in control cells. By contrast, knocking down *K-RAS* in MDA-MB-231 cells, which express oncogenic *K-RAS*, did not affect EGFR recycling (Figure S4). These data indicate that N-Ras, but not K-Ras, can act like components of the ESCRT-III complex to play a role in EGFR recycling.

Discussion

Our screen identified many novel Ras-binding proteins that are known to regulate trafficking and membrane dynamics with a slight enrichment in proteins that function in endosomes. In particular, both VPS4A and CHMP6 are known to interact with ESCRT-III. Our data show that inactivating either of them blocks Ras-induced transformation, as well as localization of Ras and EGFR to the PM. Since the binding to these two proteins is enhanced when Ras is GTP-bound, it seems likely that the Ras-VPS4A/CHMP6 interaction generates a positive-feedback loop to enhance Ras signaling by replenishing important components in the Ras pathway, such as Ras itself and EGFR, on the PM for sustained and prolonged signaling. This conclusion agrees with several studies that also show that Ras localization to endosomes can positively impact Ras-induced transformation. For example, Roy *et al.* have shown that blocking endocytosis inhibits transformation induced by both H- and N-Ras (Roy *et al.*, 2002), and we have shown that H- and N-Ras proteins can act via Cdc42 in endosomes to transform cells (Cheng and Chang, 2011; Cheng *et al.*, 2011). Finally, as discussed earlier, H-Ras internalization by RIN1 appears to attenuate Ras signaling, and internalized K-Ras can be sent to lysosomes for degradation (Lu *et al.*, 2009). Our study suggests that there is an alternative fate for endosomal Ras proteins — they can interact with the ESCRT-III, which act as a sorting station, to recycle back to the PM to enhance Ras-induced activities. A key question for the future is to decipher how the decision is made for a given Ras protein in the endosome to remain internalized and be later degraded or to be sent back to the PM.

Ras localization to endosomes is enhanced by ubiquitylation, and in an earlier study using CHOK1 cells, only H- and N-Ras, but not K-Ras-4B, were found to be ubiquitylated (Jura *et al.*, 2006). This observation agrees with the fact that high levels of H- and N-Ras are frequently found in cytoplasm, but K-Ras-4B is often found exclusively on the PM. In a more recent study, K-Ras-4B was found to be ubiquitylated in HEK293 cells (Sasaki *et al.*, 2011). This new finding suggests that ubiquitylation of H- and N-Ras are more common in a wide range of cells, while ubiquitylation of K-Ras-4B may be more cell-type specific. This difference in how various Ras proteins become ubiquitylated may affect how Ras proteins interact with proteins on endosomes. In the present study, even though we also used HEK293 cells, neither CHMP6 nor VPS4A co-immunoprecipitated proteins that could be detected by the pan-reactive Ras antibody. It is likely that there was only a small fraction of endogenous K-Ras (or H- and N-Ras) that is GTP-bound and/or ubiquitylated. Moreover, using human cancer cell lines that carry different oncogenic Ras proteins, we showed that only H- and N-Ras can bind VPS4A and CHMP6. Based on these results, we conclude that H- and N-Ras, but not K-Ras, can readily bind VPS4A and CHMP6 because H- and N-Ras are more efficiently ubiquitylated than K-Ras in the cell lines we examined.

Our biochemical and microscopy data strongly suggest that the binding between H-Ras and ESCRT-III is direct and requires the former to be ubiquitylated. If Ras ubiquitylation is critical for recycling components of the Ras pathway back to the PM, it may seem counterintuitive at first glance that ectopically expressed H-Ras(12V)-8RK has been shown to still efficiently activate Raf (Jura *et al.*, 2006). Further, in a later follow up study, we found that H-Ras(12V)-8RK can efficiently transform cells (Figure S5). We suggest that these observations are consistent with the fact that H-Ras(12V)-8RK, which has a fully functional effector-binding domain, is not efficiently internalized so it accumulates at high levels at the PM, a key compartment on which it interacts with Raf. Moreover, when a protein is transiently ectopically expressed, it is frequently overexpressed. Thus, besides the PM pool of Raf, H-Ras(12V)-8RK may accumulate at high enough levels in many other cell compartments to activate Raf, as well as additional Ras effectors.

While the PM has long been thought to be the key compartment from which Ras signals to mediate tumor formation, Ras can also signal from many cytoplasmic compartments. H-Ras, when targeted to ER and Golgi, can still transform cells efficiently (Chiu *et al.*, 2002). The important roles of Golgi and ER in Ras-induced transformation are further underscored by the fact that our screen identified many proteins (e.g. RTN3 (Wakana *et al.*, 2005)) that mediate intracellular trafficking involving these two compartments. We note that our screen using the BiFC technology has at least two advantages that are not found with a more conventional approach based on co-immunoprecipitation to allow for efficient identification of new Ras-binding proteins. First, this method can detect transient and weak protein-protein interactions because once a protein complex is formed, it is secured by not only the binding between the two proteins of interest, but also by the strong hydrogen bonds formed between the N-and C-terminal regions of YFP (Huang *et al.*, 2007). Second, our screen was not influenced by abundance of proteins in the cell at steady state since each gene in the ORFeome cDNA library is equally represented. Future screens using cDNA libraries with

even better coverage (Lamesch *et al.*, 2007) may further expand our understanding of how Ras functions in the cytoplasm.

Materials and methods

Cell culture

293FT, HT1080, COS-1, and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 200 mg/ml L-glutamine (Invitrogen). NIH3T3 cells were cultured in the same medium, except that fetal calf serum (Colorado Serum Co.) was used instead of FBS. DCIS.COM cells were cultured in DMEM/F12 (1:1, Invitrogen), supplemented with 5% horse serum, 20 ng/ml EGF, 10 ug/ml insulin, 500 μg/ml hydrocortisone and 100 ng/ml cholera toxin (Sigma). The medium for CAPAN-1 cells was Iscove's Modified Dulbecco's Medium (Invitrogen) plus 20% FBS.

Plasmids construction

The vectors expressing ubiquitylation-deficient H-Ras, and FLAG-tagged H-Ras, H-Ras(12V), Cdc42(17N) were constructed as previously described (Cheng *et al.*, 2011; Jura *et al.*, 2006). To tag Ras with CFP, the cDNAs encoding H-Ras(61L), H-Ras Ras(12V)-8RK, and Ub-H-Ras were first amplified from pCDNA3.1-H-Ras(61L), pCGT-H-Ras(12V)-8RK, and pCGT-Ub48RΔG-H-Ras vectors (Cheng *et al.*, 2011; Jura *et al.*, 2006) by PCR to generate DNA fragments that can be cloned into pENTR by the Directional TOPO cloning system (Invitrogen). These DNAs were finally transferred to pCL/CFP vector by the Gateway cloning system (Invitrogen). The resulting vectors were named pCL-CFP-H-Ras(61L), pCL-CFP-H-Ras(12V)-8RK and pCL-CFP-Ub-H-Ras. To tag H-Ras by a photoactivatable fluorescent protein, Dendra2 (Gurskaya *et al.*, 2006) was first amplified by PCR to add Sal I and Cla I as cloning sites for insertion into pLNCX2 (Clontech) to finally generate pLNCX2-Dendra2. The *H-RAS* cDNA was modified by PCR to include Cla I and Age I sites for cloning into pLNCX2-Dendra2 to generate pLNCX2-Dendra2-H-Ras. To build YFP-VPS4A, FLAG-tagged CHMP6 and VPS4A, cDNAs encoding these proteins from the library used for the screen (see below) were transferred into pCL/YFP or pCL/FLAG vector using the Gateway cloning system. The resulting vectors were named pCL-YFP-VPS4A, pCL-FLAG-CHMP6, and pCL-FLAG-VPS4A. Non-silencing control and shRNAs against *N-RAS*, *CHMP6*, and *VPS4A* in the lentiviral pGIPZ vector were purchased from OpenBiosystems. The sense sequence (5′–3′) for knocking down *N-RAS*, *CHMP6*, and *VPS4A* were: CGCAGATGTTCAAATATTGTAA, CGCGCAATCACTCAGGAACAAA, and ACCAATCAAATTCCCACACTTG. Retroviral vectors expressing CHMP6 and VPS4A refractory to shRNAs were constructed by mutating their cDNAs using the overlap extension PCR method. The four PCR primers (5′–3′) for mutating *CHMP6* were: CACCATGGGTAACCTGTTCGGC, TCTGCTCTTGTGTAATAGCACTCAGCTCCTCCAGGATGGC, AGTGCTATTACACAAGAGCAGATAGAGCTGCCAGAGGTTCC, and TTACGAAGCTGCCACCAG; while those for mutating *VPS4A* were: CACCATGACAACGTCAACCCTCCA, AAGATGAGGAAACTTTATAGGTAAAATGACAGCTTCTTTGAGGG,

ACCTATAAAGTTTCCTCATCTTTTCACAGGCAAGCGCACCC, and TAACTCTCTTGCCCAAAGTCC. pCL-FLAG-CHMP6 and pCL-FLAG-VPS4A were the templates for PCR. The resulting mutated cDNAs were cloned into pENTR, and then transferred to pCL by the Gateway cloning system. All mutations were confirmed by sequencing.

Isolation of Ras-binding proteins by the BiFC system

The human cDNAs were fused either N- or C-terminally with Yc (amino acids 156–239 of Venus YFP) by shuffling the cDNAs into pBabe-n-Yc or pBabe-c-Yc (Chen *et al.*, 2007) by the Gateway system (Invitrogen) to generate the n-Yc and c-Yc libraries. The bait, Yn-H-Ras(12V) was carried by pBabe-n-Yn-H-Ras(12V), which expressed H-Ras(12V) fused N-terminally to an N-terminal fragment (Yn, amino acids 1–155) of Venus YFP. As a control, all the amino acid residues in the effector binding region (residue 33–40) were mutated to alanines in H-Ras(12V) to create H-RasΔE(12V) (Cheng *et al.*, 2011). To generate HT1080 cells stably expressing Yn tagged H-Ras(12V), H-Ras E(12V), or GST, cells were infected and then cultured in growth medium for 36 hours before being seeded into10-cm dishes to be selected by 500 μg/mlG418 for 2 weeks. Protein expression was confirmed by Western blots, and clones with similar expression levels were pooled. HT1080 cells ($\approx 5\times10^5$) stably expressing Yn-H-Ras(12V) were transfected with 4 μg of n-YC or c-YC library. After 24 hours, cells were trypsinized and resuspended in culture medium containing 4 μg/ml of propidium iodide (Sigma) to a density of 1×10^6 cells/ml. Cells were examined for YFP intensity by an EPICS XL-MCL FACS instrument (Beckman Coulter). A total of 4,404 and 17,180 cells were collected from screening the nYC and cYC libraries, respectively. Each group of isolated cells were pooled and their DNA were extracted in an extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS and 0.2 mg/ml protease K) at 55°C for 1 hour, followed by addition of 0.5 mg/ml RNase A at room temperature for another 1 hour. The samples were then phenol-extracted. DNA was precipitated in the presence of 50 μg/ml of glycogen (Ambion) and finally dissolved in standard TE buffer. The isolated DNA was then transformed into high-efficiency 10-Beta bacterial cells (New England Biolabs). The resulting colonies were scraped and cultured in LB^{Amp} medium for two hours, and their plasmid DNAs were finally extracted as sub-libraries. Approximately 2×10^5 HT1080 cells were re-screened by each sub-library by FACS, and 460 and 2,184 cells were collected from the use of the nYC and cYC sub-library, respectively. DNAs were again isolated from each group of these cells. All the DNA from the 460 isolated cells was used to transform *E. coli* cells as above, while half of the DNA extracted from the 2,184 isolated cells was used to transform *E. coli*. From the former transformation, 351 *E. coli* colonies were obtained, while 768 *E. coli* colonies were obtained from the latter. Individual bacterial colony was picked, and its plasmid containing the Yc-tagged cDNA was isolated. The isolated cDNA clone was then transfected into fresh cells stably expressing Yn-H-Ras(12V), Yn-H-RasΔE(12V) or Yn-GST and examined by FACS to validate the positivity of YFP signal.

Transfection and virus production

Transfection was performed by Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Viruses were produced in 293FT cells by cotransfecting

packaging (pAmpho for retrovirus, pMD2.G and psPAX2 for lentivirus) and expression vectors. The transfection media were replaced with growth media 16 hours post-transfection and cells were cultured for another 48 hours. The supernatant containing the virus was harvested and filtered (Nalgene). The cells were finally infected with retrovirus for 16 hours in medium containing 4 μg/ml polybrene (Sigma). For lentivirus expressing shRNAs, cells were infected twice — first for 16 hours followed by another 24 hours — in medium containing 6 μg/ml polybrene.

Luciferase assay

NIH3T3 cells stably expressing H-Ras(61L) (Cheng *et al.*, 2011) were transfected with vectors expressing isolated Yc-tagged proteins, pFA2-Elk1, and pFR-luc (generous gifts from Dr. Mark Philips, New York University School of Medicine, New York, NY). Cells were also co-transfected with pGL4.70/hRluc (Promega) to express Renilla luciferase as an internal transfection efficiency control. Sixteen hours after transfection, cells were seeded onto 24-well plates in duplicates and incubated at 37°C for another 24 hours. The Firefly and Renilla luciferase levels were measured by Dual-Luciferase Reporter Assay Kit (Promega) in a Luminoskan Ascent luminometer (Thermo Scientific).

Cell transformation and proliferation assays

Soft-agar colony formation and the focus formation assay were both performed essentially as described (Cheng *et al.*, 2011). The cell proliferation was measured daily by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega).

Western blot

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). To detect Ras, pan-reactive Ras10 (Calbiochem), anti-H-Ras, anti-N-Ras and anti-K-Ras antibodies (Santa Cruz Biotechnology) were used. Anti-CHMP6 and anti-VPS4A antibodies are generous gifts from Dr. Wesley Sundquist (University of Utah, Salt Lake City, UT, (Morita *et al.*, 2010)). Anti-GAPDH antibody is purchased from Santa Cruz Biotechnology, anti-FLAG clone M2 antibody is from Sigma and anti-GFP antibody is from Fitzgerald. The fluorescein-conjugated secondary antibodies were obtained from Li-COR Biosciences, and the protein levels were quantified by an Odyssey infrared imaging system (Li-COR Biosciences).

Cell fractionation by sucrose gradient centrifugation

The gradient was generated as described previously (Cheng *et al.*, 2011). Samples were centrifuged at 220,000 ×g, 4°C for 16 hours, and 13 fractions of 400 μl each were collected and examined by Western blots.

Immunoprecipitation

Cells were lysed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonylfluoride, 1% Triton X-100, and protease inhibitors (Roche Applied Science). Cell lysates were pre-cleared with mouse IgG-conjugated beads (Sigma) for 3 hours at 4°C. To examine co-precipitation between H-Ras and epitope tagged VPS4A and

CHMP6 in 293 cells, the pre-cleared lysates were incubated with either pan-reactive Ras10 antibody-conjugated agarose beads (Calbiochem) or anti-FLAG clone M2 antibody-conjugated beads (Sigma) overnight at 4°C. Cell lysates were similarly treated with mouse IgG-conjugated beads as controls. To specifically precipitate a given Ras isoform from different cell lines, the pre-cleared cell lysates were incubated with Protein A-agarose beads (Roche Applied Science) together with Ras isoform-specific antibodies (Santa Cruz Biotechnology) or mouse IgG control at 4°C for overnight. All beads were finally washed three times with TBS (20 mM Tris-HCl (pH 7.5) and 150 mM NaCl). The bound proteins were eluted with 2× SDS sample buffer by boiling for 5 minutes, and examined by Western Blots.

Confocal microscopy and photobleaching

All microscopy was performed on a Leica TCS SP5 confocal microscope with a 63×/1.4 oil objective lens, and cells were cultured in glass-bottom plates (MatTek). The Golgi marker GalT and the early endosome marker Rab5A were examined as previously described (Cheng *et al.*, 2011). The late and recycling endosome markers Rab7A and Rab11A were generous gifts from Dr. Juan Bonifacino (NIH, Bethesda, MD, (Leifer *et al.*, 2004)) For all experiments, the diode (405 nm) and He/Ne lasers (543 and 633 nm) were operated at full power, while the argon laser (125 mW) was operated at 30% power. To detect CFP, GFP, and YFP signals, the samples were imaged at 458, 488 and 514 nm respectively with 50% of available argon laser power. Dendra2 and Alexa647 were measured with 80% available He/Ne laser power at 543 and 633 nm respectively. For all imaging, the pinhole was set at 1 airy unit (AU) and scanned at 100 Hz. For photobleaching experiments, the pinhole was also 1 AU, but the scanning frequency was 400 Hz. Dendra2 can be photoconverted by either intense blue light or UV (Gurskaya *et al.*, 2006). However under the conditions we set up for this confocal experiment, we could not efficiently photoconvert Dendra2 at 488 nm. As an alternative, Dendra2 tagged H-Ras was photoconverted by UV from the mercury light source (using the filter set for visualizing DAPI) for 5 seconds. The photobleaching was then performed on the plasma membrane by turning on all lasers (405, 458, 476, 488, 514, 543 and 663 nm) at 100% available power for 2 seconds for 10 iterations. Fluorescence recovery at the plasma membrane was then measured by scanning at 543 nm every 5 seconds for 400 seconds. All images were analyzed using the LAS AF software (Leica). The data were corrected for background fluorescence taken from a region in the same field without cells, and the fluorescence in an un-bleached neighboring cell was also measured to correct for spontaneous photobleaching.

Fluorescence Resonance Energy Transfer (FRET)

CFP was used as the donor and YFP as the acceptor molecule. pCL-YFP-VPS4A was cotransfected into COS-1 cells with equal amount of pCL-CFP-H-Ras(61L), pCL-CFP-H-Ras(12V)-8RK, or pCL-CFP-Ub-H-Ras, and the transfected cells were seeded to glass-bottom plates. Cells with similar fluorescence levels were chosen to be examined by confocal microscopy (see above). For each cell, we selected for FRET measurement the focal plane with the most apparent colocalization between the CFP and YFP-tagged proteins. To measure FRET, we performed the sensitized emission method following Leica's protocol (FRET SE Wizard). The apparent FRET efficiency $E_A(i)$ is defined as: [B −

$(A \times \beta) - (C \times \gamma)]/C$, in which A, B, and C correspond to the signal intensities from the donor, FRET, and acceptor, and the calibration factors β and γ were generated by donor only and acceptor only references (Wouters *et al.*, 2001).

EGFR recycling assay

This experiment was performed largely as described (Raiborg *et al.*, 2008). Briefly, cells were pre-treated with 10 μg/ml cycloheximide for 1 hour at 37°C, and then treated with 50 ng/ml Alexa647-conjugated EGF (Invitrogen) in cycloheximide-containing medium for 5 or 30 minutes. After incubation, cells were washed with PBS, fixed with 4% paraformaldehyde, and imaged by confocal microscopy.

Statistical analysis

All data are shown as mean ± s.e.m (standard error of the mean), and p-values were analyzed by unpaired Student's *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Gary Chamness and Evelin Young for thoughtfully reading the manuscript. We are particularly grateful for the generosity of Wesley Sundquist, Mark Philips, and Juan Bonifacino for providing many reagents and for sharing unpublished results that are critical for this study. ZZ is supported by a postdoctoral fellowship from the Susan G. Komen for the Cure Foundation (PDF0707860). ST was supported by a fellowship from Human Frontier Science Program (LT00147/2005). STW is supported by a grant from NIH (U54CA149169). ZS is a Leukemia and Lymphoma Society Scholar and supported by grants from NIH (CA133249 and GM081627). ECC is supported by grants from NIH (CA90464, CA107187, GM81627, and P50-CA58183).

References

Balbis A, Parmar A, Wang Y, Baquiran G, Posner BI. Compartmentalization of signaling-competent epidermal growth factor receptors in endosomes. Endocrinology. 2007; 148:2944–2954. [PubMed: 17363458]

Baldys A, Raymond JR. Critical role of ESCRT machinery in EGFR recycling. Biochemistry. 2009; 48:9321–9323. [PubMed: 19673488]

Barbieri MA, Kong C, Chen PI, Horazdovsky BF, Stahl PD. The SRC homology 2 domain of Rin1 mediates its binding to the epidermal growth factor receptor and regulates receptor endocytosis. J Biol Chem. 2003; 278:32027–32036. [PubMed: 12783862]

Belgareh-Touze N, Avaro S, Rouille Y, Hoflack B, Haguenauer-Tsapis R. Yeast Vps55p, a functional homolog of human obesity receptor gene-related protein, is involved in late endosome to vacuole trafficking. Mol Biol Cell. 2002; 13:1694–1708. [PubMed: 12006663]

Castle A, Castle D. Ubiquitously expressed secretory carrier membrane proteins (SCAMPs) 1–4 mark different pathways and exhibit limited constitutive trafficking to and from the cell surface. J Cell Sci. 2005; 118:3769–3780. [PubMed: 16105885]

Chang EC, Philips MR. Spatial segregation of Ras signaling: new evidence from fission yeast. Cell Cycle. 2006; 5:1936–1939. [PubMed: 16931912]

Chen LY, Liu D, Songyang Z. Telomere maintenance through spatial control of telomeric proteins. Mol Cell Biol. 2007; 27:5898–5909. [PubMed: 17562870]

Cheng CM, Chang EC. Busy traveling Ras. Cell Cycle. 2011; 10:1180–1181. [PubMed: 21436618]

Cheng CM, Li H, Gasman S, Huang J, Schiff R, Chang EC. Compartmentalized Ras proteins transform NIH 3T3 cells with different efficiencies. Mol Cell Biol. 2011; 31:983–997. [PubMed: 21189290]

Chiu VK, Bivona T, Hach A, Sajous JB, Silletti J, Wiener H, et al. Ras signalling on the endoplasmic reticulum and the Golgi. Nat Cell Biol. 2002; 4:343–350. [PubMed: 11988737]

Cutler ML, Bassin RH, Zanoni L, Talbot N. Isolation of rsp-1, a novel cDNA capable of suppressing v-Ras transformation. Mol Cell Biol. 1992; 12:3750–3756. [PubMed: 1508180]

Fehrenbacher N, Bar-Sagi D, Philips M. Ras/MAPK signaling from endomembranes. Mol Oncol. 2009; 3:297–307. [PubMed: 19615955]

Gurskaya NG, Verkhusha VV, Shcheglov AS, Staroverov DB, Chepurnykh TV, Fradkov AF, et al. Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol. 2006; 24:461–465. [PubMed: 16550175]

Han L, Wong D, Dhaka A, Afar D, White M, Xie W, et al. Protein binding and signaling properties of RIN1 suggest a unique effector function. Proc Natl Acad Sci U S A. 1997; 94:4954–4959. [PubMed: 9144171]

Howard TL, Stauffer DR, Degnin CR, Hollenberg SM. CHMP1 functions as a member of a newly defined family of vesicle trafficking proteins. J Cell Sci. 2001; 114:2395–2404. [PubMed: 11559748]

Huang JR, Craggs TD, Christodoulou J, Jackson SE. Stable intermediate states and high energy barriers in the unfolding of GFP. J Mol Biol. 2007; 370:356–371. [PubMed: 17512539]

Jiang X, Sorkin A. Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. Mol Biol Cell. 2002; 13:1522–1535. [PubMed: 12006650]

Jura N, Scotto-Lavino E, Sobczyk A, Bar-Sagi D. Differential modification of Ras proteins by ubiquitination. Mol Cell. 2006; 21:679–687. [PubMed: 16507365]

Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol. 2008; 9:517–531. [PubMed: 18568040]

Kerppola TK. Bimolecular fluorescence complementation: visualization of molecular interactions in living cells. Methods Cell Biol. 2008; 85:431–470. [PubMed: 18155474]

Lamesch P, Li N, Milstein S, Fan C, Hao T, Szabo G, et al. hORFeome v3.1: a resource of human open reading frames representing over 10,000 human genes. Genomics. 2007; 89:307–315. [PubMed: 17207965]

Leifer CA, Kennedy MN, Mazzoni A, Lee C, Kruhlak MJ, Segal DM. TLR9 is localized in the endoplasmic reticulum prior to stimulation. J Immunol. 2004; 173:1179–1183. [PubMed: 15240708]

Lu A, Tebar F, Alvarez-Moya B, Lopez-Alcala C, Calvo M, Enrich C, et al. A clathrin-dependent pathway leads to KRas signaling on late endosomes en route to lysosomes. J Cell Biol. 2009; 184:863–879. [PubMed: 19289794]

Morita E, Colf LA, Karren MA, Sandrin V, Rodesch CK, Sundquist WI. Human ESCRT-III and VPS4 proteins are required for centrosome and spindle maintenance. Proc Natl Acad Sci U S A. 2010; 107:12889–12894. [PubMed: 20616062]

Nebl G, Fischer S, Penzel R, Samstag Y. Dephosphorylation of cofilin is regulated through Ras and requires the combined activities of the Ras-effectors MEK and PI3K. Cell Signal. 2004; 16:235–243. [PubMed: 14636893]

Prior IA, Harding A, Yan J, Sluimer J, Parton RG, Hancock JF. GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. Nat Cell Biol. 2001; 3:368–375. [PubMed: 11283610]

Raiborg C, Malerod L, Pedersen NM, Stenmark H. Differential functions of Hrs and ESCRT proteins in endocytic membrane trafficking. Exp Cell Res. 2008; 314:801–813. [PubMed: 18031739]

Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature. 2009; 458:445–452. [PubMed: 19325624]

Roy S, Wyse B, Hancock JF. H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. Mol Cell Biol. 2002; 22:5128–5140. [PubMed: 12077341]

Rual JF, Hirozane-Kishikawa T, Hao T, Bertin N, Li S, Dricot A, et al. Human ORFeome version 1.1: a platform for reverse proteomics. Genome Res. 2004; 14:2128–2135. [PubMed: 15489335]

Sasaki AT, Carracedo A, Locasale JW, Anastasiou D, Takeuchi K, Kahoud ER, et al. Ubiquitination of k-ras enhances activation and facilitates binding to select downstream effectors. Sci Signal. 2011; 4:ra13. [PubMed: 21386094]

Su Y, Zhu H, Xiao W, Xu Q, Zhu L, Zhang X, et al. Selectively oncolytic mutant of HSV-1 lyses HeLa cells mediated by Ras/RTN3. Cancer Biol Ther. 2007; 6:202–208. [PubMed: 17218780]

Tall GG, Barbieri MA, Stahl PD, Horazdovsky BF. Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev Cell. 2001; 1:73–82. [PubMed: 11703925]

Wakana Y, Koyama S, Nakajima K, Hatsuzawa K, Nagahama M, Tani K, et al. Reticulon 3 is involved in membrane trafficking between the endoplasmic reticulum and Golgi. Biochem Biophys Res Commun. 2005; 334:1198–1205. [PubMed: 16054885]

Wouters FS, Verveer PJ, Bastiaens PI. Imaging biochemistry inside cells. Trends Cell Biol. 2001; 11:203–211. [PubMed: 11316609]

Xu L, Lubkov V, Taylor LJ, Bar-Sagi D. Feedback regulation of Ras signaling by Rabex-5-mediated ubiquitination. Curr Biol. 2010; 20:1372–1377. [PubMed: 20655225]

Yan H, Jahanshahi M, Horvath EA, Liu HY, Pfleger CM. Rabex-5 ubiquitin ligase activity restricts Ras signaling to establish pathway homeostasis in Drosophila. Curr Biol. 2010; 20:1378–1382. [PubMed: 20655224]

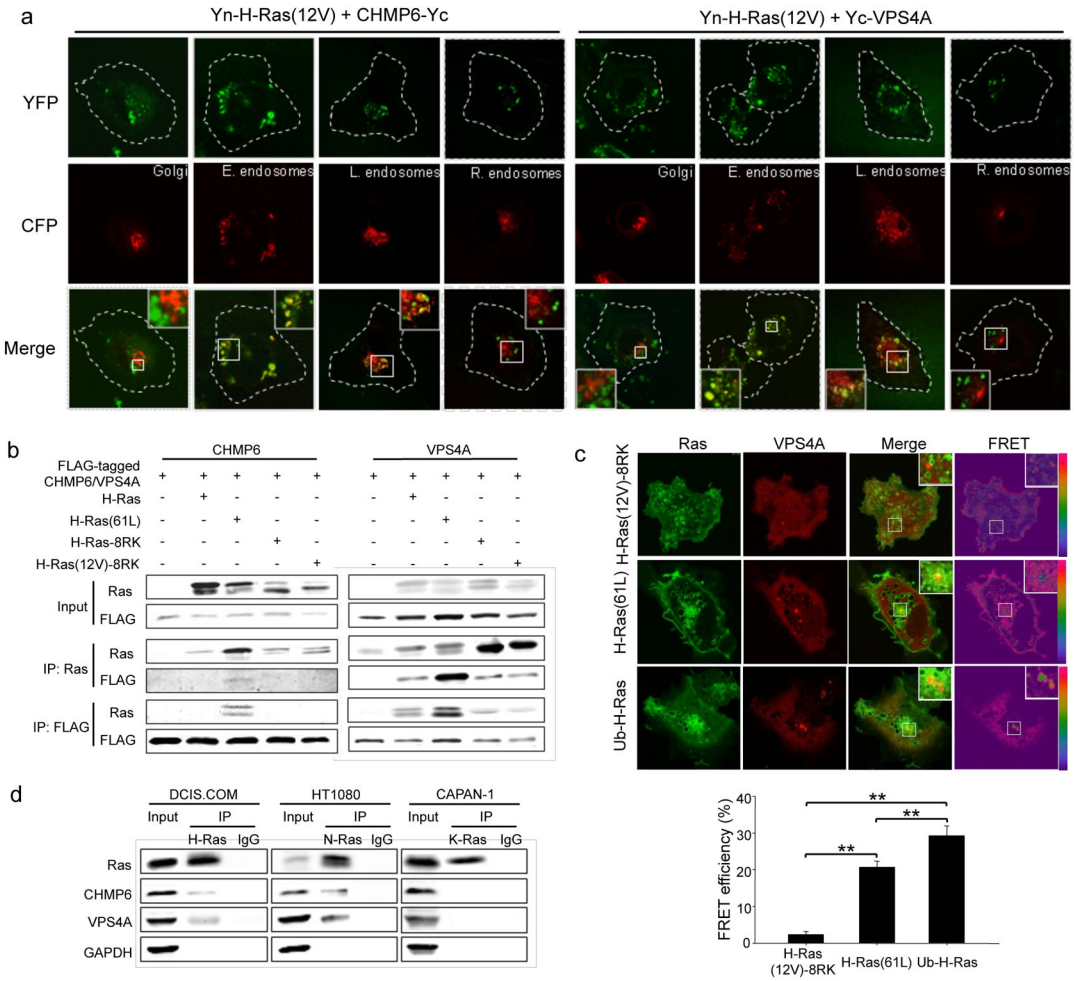

Figure 1.

CHMP6 and VPS4A are novel H-Ras binding proteins. (**a**) HT1080 cells expressing Yn-H-Ras(12V) and either Yc-tagged CHMP6 or VPS4A, together with CFP-tagged GalT, Rab5A, Rab7A, or Rab11A, which mark Golgi, early (E.) endosomes, late (L.) endosomes/MVBs, or recycling (R.) endosomes, respectively, were analyzed by confocal microscopy. CFP and the reconstituted YFP signals were pseudo-colored red and green. Dotted lines mark the cell boundaries. Insets are scaled up images to better show co-localization between the CFP and YFP signals. (**b**) FLAG-tagged CHMP6 or VPS4A were co-expressed with indicated H-Ras proteins in 293 cells. Immunoprecipitation was performed using either a pan-reactive Ras antibody or an antibody against the FLAG tag. Immunoprecipitated proteins as well as the total lysate inputs were analyzed by Western blots using indicated antibodies. (**c**) YFP-VPS4A was coexpressed with CFP-tagged H-Ras(12V)-8RK, H-Ras(61L), or Ub-H-Ras in COS-1 cells. These cells were examined by laser confocal microscopy. Regions of interest are scaled up in the inset to better show co-localization between CFP and YFP signals, as well as FRET. A heat-map is included to show the relative FRET efficiency from low (violet) to high (red). The quantified FRET efficiencies are shown at the bottom. The number of cells (n) examined were: 15 (H-Ras(12V)-8RK), 11 (H-Ras(61L)), and 12 (Ub-H-Ras). **P<0.01. (**d**) Cell lysates were immunoprecipitated with

indicated Ras isoform-specific antibodies, and the resulting samples were similarly analyzed by Western blot using indicated antibodies.

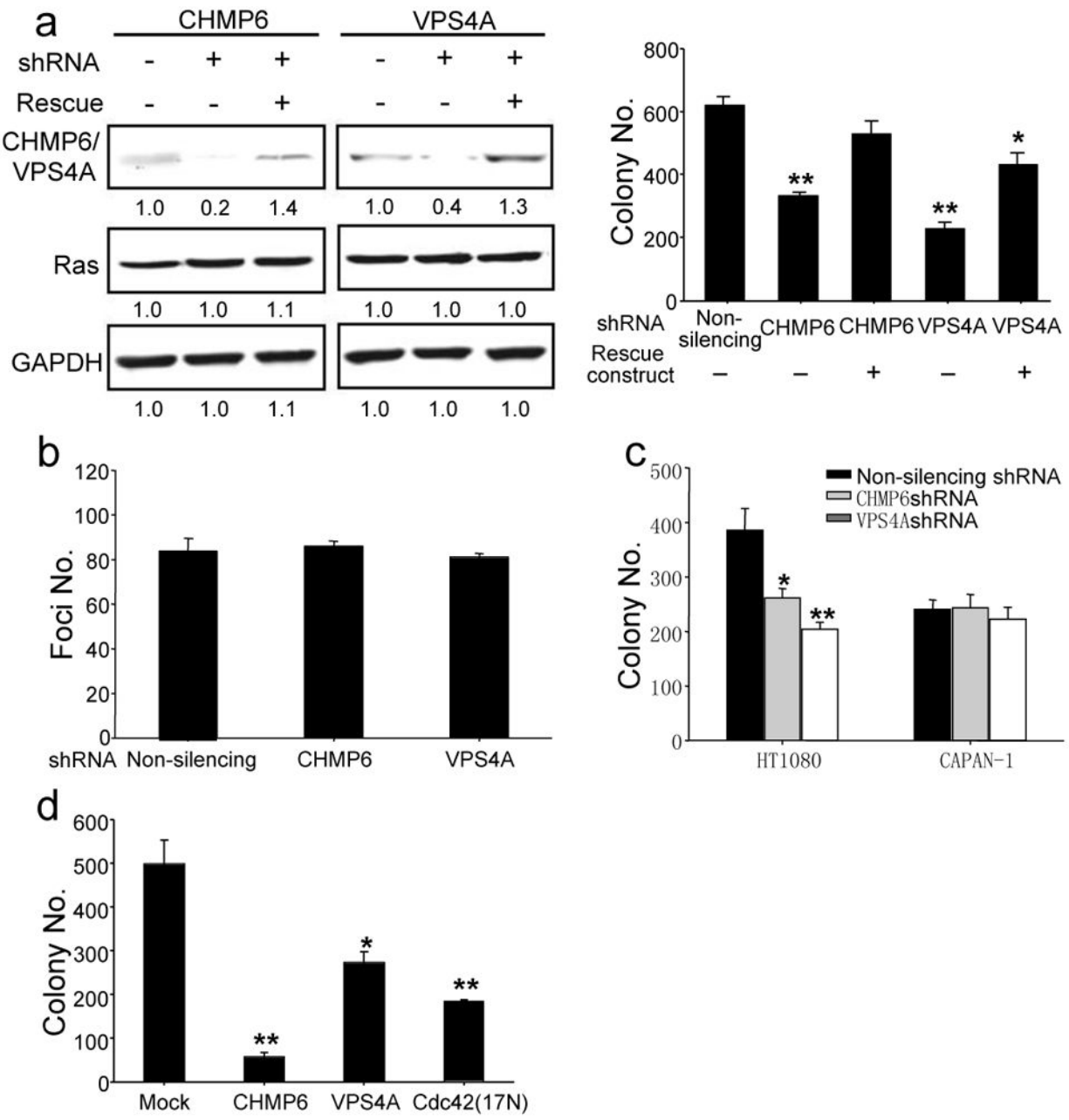

Figure 2.

Proper CHMP6 and VPS4A levels are important for H-Ras-induced cell transformation. (**a**) *CHMP6* or *VPS4A* in NIH3T3 cells stably expressing H-Ras(61L) was silenced by shRNA. cDNA clones of CHMP6 and VPS4A refractory to shRNAs were also co-expressed in some samples. One the left, cell lysates were examined by Western blots using indicated antibodies and GAPDH is the loading control. The numbers below indicate protein levels relative to the non-silenced controls. On the right, the same cells were seeded into soft agar in triplicate and allowed to grow for 21 days before being stained with MTT. (**b**) NIH3T3 cells expressing SV40 T-antigen were infected with indicated shRNAs. Cells were cultured in quadruplicate (n=4) for 14 days before being fixed and stained with crystal violet. (**c**)

CHMP6 or VPS4A in HT1080 and CAPAN-1 cells was silenced by shRNA. The cells were then seeded into soft agar in triplicate (n=3) and stained with MTT after 21 days. (**d**) NIH3T3 cells stably expressing H-Ras(61L) were infected with retroviruses to express CHMP6 or VPS4A before being seeded in soft agar in triplicate (n=3). Dominant negative Cdc42(17N) was used as a positive control for blocking Ras functions (Cheng *et al.*, 2011). Colonies were counted 21 days later after MTT staining. All experiments were repeated at least one more time and similar results were obtained. *P<0.05, **P<0.01.

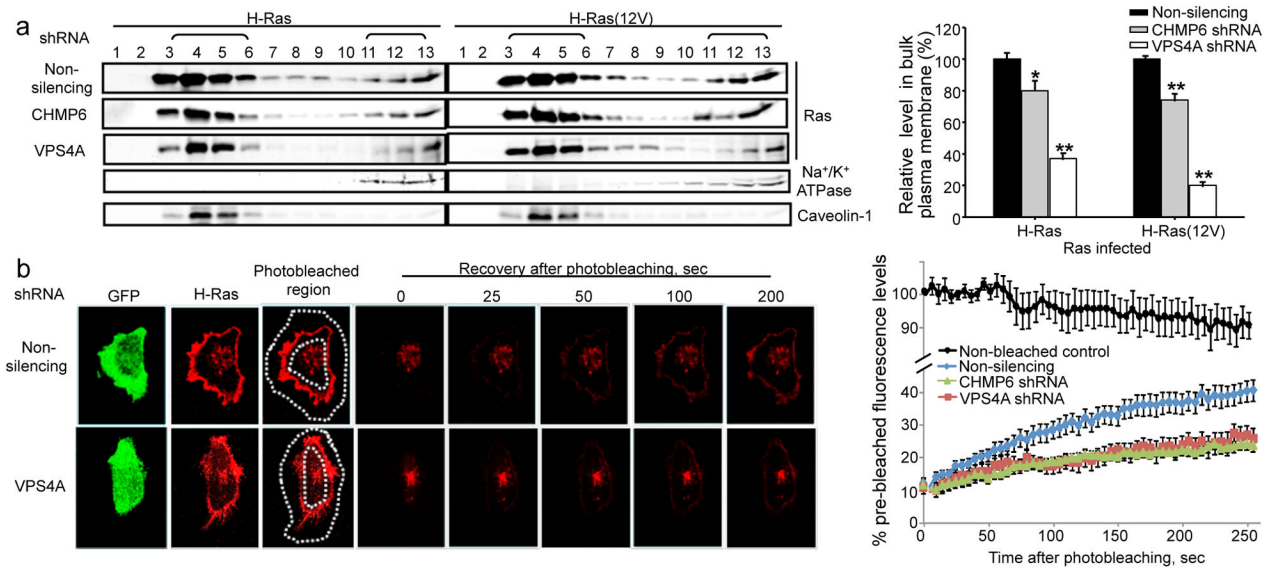

Figure 3.

CHMP6 and VPS4A are important for Ras association and mobility on the membranes. (**a**) HT1080 cells expressing either H-Ras or H-Ras(12V) and the indicated shRNAs were harvested and fractionated by sucrose gradient ultracentrifugation. The fractions were examined for indicated proteins by Western blots (left). Na^+/K^+ ATPase and caveolin-1 are the markers for bulk membrane and lipid rafts, respectively. The sums of Ras proteins in fractions 11–13 over that in all fractions were analyzed and shown on the right. n = 3 separate experiments. The Ras protein levels in the non-silenced cells were set to 100%. *P<0.05, **P<0.01. (**b**) HT1080 cells were infected to express Dendra2 tagged H-Ras, as well as an shRNA against either CHMP6 or VPS4A. UV-activated Dendra2-H-Ras at the PM (dotted lines) was photobleached, and the fluorescence recovery in the same region was recorded over time (left). Recovered fluorescence, corrected for both spontaneous photobleaching of an unbleached cell in the same field and for background fluorescence, relative to that before photobleaching (100%) was plotted over time (right). The number of cells examined (n) were: 8 (non-bleached control), 13 (non-silencing shRNA), 10, (*CHMP6* silenced), and 14 (*VPS4A* silenced).

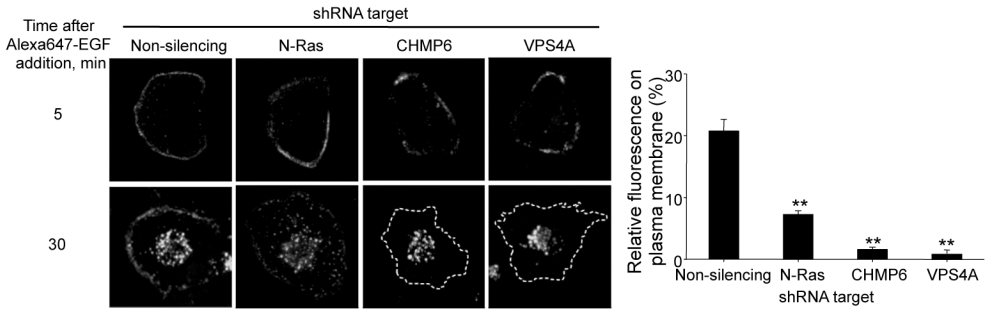

Figure 4.

Ras, CHMP6 and VPS4A control EGFR recycling. HT1080 cells carrying indicated shRNAs were stimulated with Alexa647-conjugated EGF in cycloheximide-containing medium. At indicated time points, cells were fixed and examined by confocal microscopy (left). Dotted lines were placed just *outside* the cell boundaries to show that there was no detectable signal at the PM. The red fluorescence at PM vs. total red fluorescence in the cell 30 min after EGF addition was quantified and shown on the right. The number of cells examined (n) were: 23 (non-silencing shRNA), 20 (*N-RAS* silenced), 7 (*CHMP6* silenced), and 9 (*VPS4A* silenced). **P<0.01.

Oncogene. Author manuscript; available in PMC 2013 April 25.

Table 1

Ras-binding proteins that also alter Ras activities

Gene[a]	Relative binding[b]		Impact on Ras activity (% of control)[c]		Known properties		
	H-Ras(12V)/GST	H-Ras(12V)/H-RasΔE(12V)	Trans formation	Gene Expression	Known to interact w/Ras	Involvement in transport	Localization to endosomes
ARAF	N/D[d]	N/D	N/D	N/D	Known Ras effector for transformation	N/A[d]	
C12orf53	+	++	36	39	N/A	N/A	
C3orf18	++	++	10	6	N/A	N/A	
CFL2/cofilin 2	+	++	81	83	Ras activation induces cofilin 2 dephosphorylation to regulate actin reorganization[e]	N/A	
CHMP6/VPS20	++	++	12	10	N/A	Initiates ESCRT-III assembly	Yes
DIRAS2	+	+/−	36	57	N/A	N/A	
FCAR/CD89	+++	+	40	23	N/A	N/A	
GNAS	++	++	55	23	N/A	N/A	
GNGT2	++	++	35	44	N/A	N/A	
HSPBP1	++	++	27	30	N/A	N/A	
LDHB/LDH-H	+++	+++	29	70	N/A	N/A	
LEPROTL1/VPS 55	++	++	68	166	N/A	Yeast homolog is involved in late endosome to vacuole trafficking[h]	Yes
LMAN2/VIP36	+	+/−	7	49	N/A	Glycoprotein trafficking from endoplasmic reticulum to Golgi	
LXN/latexin	+	+/−	53	39	N/A	N/A	
MAL	++	++	49	31	N/A	Trafficking between Golgi and plasma membrane	Yes
MALL	++	++	3	13	N/A	Raft-mediated trafficking between Golgi and plasma membrane	
MARVELD1	+	+/−	15	10	N/A	N/A	
PARK7/DJ1	++	+/−	32	68	N/A	N/A	
PFN1/profilin 1	+++	++	18	54	N/A	N/A	
RBM3	+	+	28	51	N/A	N/A	

Gene[a]	Relative binding[b]		Impact on Ras activity (% of control)[c]		Known properties		
	H-Ras(12V)/GST	H-Ras(12V)/H-RasΔE(12V)	Trans formation	Gene Expression	Known to interact w/Ras	Involvement in transport	Localization to endosomes
RSU1	+/−	+	71	121	Inhibits Ras-induced cell transformation[f]	N/A	
RTN3/reticulon 3	+	+	55	49	Binds Ras at endoplasmic reticulum[g]	Trafficking between endoplasmic reticulum and Golgi	
SCAMP4	+++	++	22	28	N/A	Located in endosomes; Trans-Golgi network trafficking	Yes[i]
STXBP6/amisyn	+	+/−	22	29	N/A	Inhibits exocytosis	
TRIM44	+	+	32	18	N/A	N/A	
VPS4A	+	++	55	61	N/A	Regulates ESCRT-III disassembly	Yes

[a] Adapted from NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/).

[b] As determined by the data in Fig. S1: +++, >2-fold; ++, 1.5 to 2-fold; +, 1.15 to 1.5-fold; +/−, 1 to 1.15-fold increase in binding, respectively.

[c] As measured by soft-agar colony formation assay and Elk-1-mediated luciferase activity assay, in which cells transfected with the vector control were set to 100%.

[d] N/D, Not determined; N/A, Not available.

[e] Ref. (Nebl *et al.*, 2004)

[f] Ref. (Cutler *et al.*, 1992)

[g] Ref. (Su *et al.*, 2007)

[h] Ref. (Belgareh-Touze *et al.*, 2002)

[i] Ref. (Castle and Castle, 2005)