RaLA and RaLB Proteins Are Ubiquitinated GTPases, and Ubiquitinated RaLA Increases Lipid Raft Exposure at the Plasma Membrane*  

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Background: RaLA GTPases are downstream effectors for Ras oncogenic activity.

Results: RaLA ubiquitination is regulated at lipid rafts, and ubiquitinated RaLA regulates lipid raft exposure at the plasma membrane.

Conclusion: Ubiquitination represents a novel post-translational modification of RaLA GTPases determining RaLA subcellular localization and impacting raft trafficking.

Significance: Exploration of the mechanisms of RaLA GTPase post-translational modification is crucial to our understanding of Ras-driven signaling in tumorigenesis.

Ras GTPases signal by orchestrating a balance among several effector pathways, of which those driven by the GTPases RaLA and RaLB are essential to Ras oncogenic functions. RaLA and RaLB share the same effectors but support different aspects of oncogenesis. One example is the importance of active RaLA in anchorage-independent growth and membrane raft trafficking. This study has shown a new post-translational modification of RaLA GTPases: nondegradative ubiquitination. RaLA (but not RaLB) ubiquitination increases in anchorage-independent conditions in a caveolin-dependent manner and when lipid rafts are endocytosed. Forcing RaLA mono-ubiquitination (by expressing a protein fusion consisting of ubiquitin fused N-terminally to RaLA) leads to RaLA enrichment at the plasma membrane and increases raft exposure. This study suggests the existence of an ubiquitination/de-ubiquitination cycle superimposed on the GDP/GTP cycle of RaLA, involved in the regulation of RaLA activity as well as in membrane raft trafficking.

RaLA and RaLB GTPases are downstream effectors for many Ras-mediated biological functions and are indispensable to Ras oncogenic activity (1–3). Although RaLA and RaLB are 82% identical and share a set of effectors, they contribute in very different ways to cell (patho)physiology. RaLA is required for anchorage-independent proliferation and tumor growth, whereas RaLB participates in cancer cell survival and metastasis formation (1, 4), although these roles may be very context-dependent (5, 6). RaLA is involved in apicobasal cell polarization (7) and RaLB in cell motility (8–10) and autophagy (11). Finally, RaLA and B act during different phases of cytokinesis. This temporal difference is based at least in part on their different spatial distribution, together with the action of their activators, the RaL-GEFs (12). What drives the specific localization of RaLA and B and their relationship to specific functions remains elusive.

Phosphorylation of RaLA and B by different kinases (13–15) is a mechanism that contributes to driving their specific activation and localization. Here, we have explored whether ubiquitination, another major post-translational modification, takes place on RaLA or B, and, if so, under which circumstances and for what purpose. Ubiquitination is important for proteasome-mediated protein degradation in the ubiquitin proteasome system, but also for other processes, including protein activation, localization, and interactions (see examples in Refs. 16–18). It is noteworthy that the proto-oncogenes H-Ras and N-Ras are ubiquitinated (18, 19), and this ubiquitination markedly disrupts the balance between plasma and endomembrane localization of Ras, favoring the latter and disfavoring signal transduction toward ERK1/2 (18, 20, 21). We show here that the endogenous RaLA and RaLB GTPases are ubiquitinated, which contributes to selective membrane localization. RaLA (but not RaLB) de-ubiquitination occurs in lipid raft microdomains and promotes raft endocytosis upon loss of cell-matrix interactions.

**Experimental Procedures**

Cell culture, DNA, and siRNA—HeLa cells were cultured as described previously (22). Plasmid and siRNA transfections were performed using Jet-PEI (Polyplus Transfection) and HiPerfect (Qiagen), according to supplier protocols, respectively. siRNA for caveolin was a pool of four siRNAs from Qiagen.

A vector (pRK5-Ubi)8 to express proteins fused by their N terminus to ubiquitin was generated by inserting an oligonu-
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cleotide encoding ubiquitin in the polylinker of plasmid pRK5. In the resulting plasmid pRK5-Ubi, the ubiquitin coding region is followed by a polylinker containing unique sites for EcoRI, BamHI, XbaI, Sall, PstI, and HindIII (frame gaa ttc gga tct ctt aga gtc gac ctg cag aag ctt). Coding regions for 3×FLAG-RalA and 3×FLAG-RalB (12) were amplified by polymerase chain reaction (PCR) and cloned between the BamHI and PstI sites of pRK5-Ubi.

Inhibitors—Inhibition of the proteasome was performed using 10 mM MG132 or ALLN for 4 h. Inhibition of lysosomes was performed using a combination of the inhibitors E-64 and pepstatin at 10 mg/ml and 50 mM, respectively, for 4 h. Inhibition of ubiquitination was achieved using the E1 inhibitor PYR41 at 50 mM for 6 h. Inhibition of dynamin-1 and -2 was performed with Dynasore (80 mM for 30 min). Destruction of lipid rafts was performed by treating cells with 15 mM methyl-β-cyclodextrin for 30 min. All inhibitors were purchased from Sigma.

Immunoblotting—Cell lysis was performed using a radiolabeled immunoprecipitation assay buffer (150 mM NaCl, 0.1% Nonidet P-40, 0.1% SDS, protease inhibitors, 50 mM NaF, 1 mM NaVO₃, and 50 mM Tris-HCl, pH 8). Lysates were mildly sonicated. Immunoprecipitation of vesicular stomatitidis virus- or HA-tagged ubiquitin was carried out with vesicular stomatitis virus (Abcam) or HA antibody (Roche Applied Science) according to standard procedures. Sepharose G and A beads were purchased from GE Healthcare. The bound proteins and whole-cell extracts were analyzed by immunoblotting with the antibodies detecting the following proteins: RalA (BD 610222), RalB (Upstate 04-037; Cell Signaling 3523), b-catenin (BD 610154), caveolin-1 (BD 610060), and FLAG epitope (Sigma F1804). Secondary antibodies coupled to peroxidase were obtained from Jackson Laboratories and used at a dilution of 1/25,000.

Cobalt Affinity Chromatography—Purification of ubiquitinated proteins was performed according to published procedures (23). In short, HeLa cells were transfected with expression vectors encoding a His₆-tagged-ubiquitin (His₆-Ubi) and/or Ral alleles. Then, 10 × 10⁶ cells were lysed for the purification of ubiquitinated overexpressed proteins, and 20 × 10⁶ cells for the purification of ubiquitinated endogenous ones. Cells were harvested in buffer B (PBS, pH 8, 6 M guanidium HCl, 0.1% Nonidet P-40, 10 mM b-mercaptoethanol), lysed by sonication (cycles of 30 s for 15 min at 200 W; Bioruptor, diagenode) and incubated for 4 h at 4 °C with 100 ml of TALON metal affinity beads (Clontech; 1:1 slurry equilibrated in buffer B). Prior to purification, a 100-ml aliquot of the lysate was precipitated with 10% ethanol to eliminate buffer B and resuspended in 50 ml of sample buffer 2×. The resin was washed once with buffer B, twice with buffer 1B/2C (1 volume of buffer B), 2 volumes of buffer C), once in buffer 1B/3C (1 volume of buffer B, 3 volumes of buffer C), and once in buffer C. Buffer C is 0.1% Nonidet P-40, 5% glycerol, and 20 mM imidazole in PBS, pH 8. Proteins were eluted with 100 ml of sample buffer supplemented with 200 mM DTT.

For those experiments concerning the impact of adhesion on ubiquitination, 10 × 10⁶ transfected cells were plated on a 10-cm dish coated with 1% agarose (cells in suspension) for 90 min and afterward on fibronectin (25 mg/ml), where cells were allowed to adhere for 20 min prior to lysis and purification.

RESULTS

Ral GTPases Are Ubiquitinated, but Not for Degradation—We determined whether RalA and RalB can undergo ubiquitination in vivo (Fig. 1). Plasmids expressing Ral and His-tagged ubiquitin were transfected into HeLa cells together and separately as controls. Cobalt affinity chromatography was used to purify ubiquitin conjugates, followed by Western blotting to detect RalA and RalB, as done previously for Ras GTPases (18). In Fig. 1A, Western blot analysis using specific anti-Ral antibodies revealed two RalA and RalB ubiquitin-conjugated species: a major ubiquitinated band at 42 kDa and a minor ubiquitinated band at 35 kDa (arrowheads). These bands were not detected when His-tagged ubiquitin or Ral GTPases were expressed alone in the cells and disappeared when the experiments were performed in the presence of the E1 ubiquitin-activating enzyme inhibitor PYR41 (24) (supplemental Fig. 1A). Ubiquitination of RalA mutants deleted of the C-terminal domain required for Ral membrane localization (RalA ΔCterm) was strongly decreased (supplemental Fig. 2C). HA-fused ubiquitin was also coexpressed with RalA or RalB, and ubiquitinated proteins were immunoprecipitated with anti-HA antibodies. Both RalA and RalB were detected in these immunoprecipitates and displayed patterns similar to those obtained by affinity chromatography (Fig. 1B). Ral ubiquitination was neither cell type- nor species-specific. It was observed in murine cells (hamster HET-SR fibroblasts (25) and CHO cells) as well as in human ones (HeLa (Fig. 1) and HEK293 (data not shown)). Considering that a single ubiquitin molecule is ~8 kDa, Ral (25 kDa) was ubiquitinated mostly by one (minor band at ~35 kDa) or two ubiquitin moieties (major band at ~42 kDa), but higher molec-
ular mass species were also observed (Fig. 1A). To distinguish between poly-ubiquitination (a second ubiquitin attached to a lysine of the first ubiquitin, attached directly to Ral) and multi-ubiquitination (a second ubiquitin attached directly to Ral), a plasmid expressing a vesicular stomatitis virus G-tagged ubiquitin mutated on all lysines (K0-ubiquitin) was used. Supplementary Fig. 1B shows that the same pattern of Ral ubiquitination was observed with both wild-type and K0-ubiquitin. The second ubiquitin is therefore not attached to the first one. When bi-ubiquitinated, the two ubiquitins are directly attached to Ral on different lysines. Poly-ubiquitination targets proteins for proteosomal degradation. Cells were treated with proteasome inhibitors MG132 or ALLN and lysosomal degradation inhibitors pepstatin /H9252 and E64 or (Fig. 1C) leupeptin (data not shown). Whole cell extracts were analyzed by immunoblotting with anti-RalA or anti-RalB antibodies. No increase in the quantity of RalA or B was detectable under these conditions. The regulation of cellular quantities of Ral GTPases did not rely on proteasome or lysosome degradation.

### FIGURE 1. Ral GTPases are ubiquitinated, but not for degradation.

**A**, exogenously expressed RalA and RalB were ubiquitinated. Lysates from HeLa cells overexpressing His6-Ubi and/or RalA or RalB were subjected to affinity chromatography purification on cobalt beads followed by immunodetection using anti-RalA or RalB antibodies after SDS-PAGE and blotting (IB). Whole lysate contents were analyzed by immunoblotting with the indicated antibodies (cell lysate). Black and gray arrowheads indicate the mono- and bi-ubiquitinated forms of Ral, respectively. **B**, exogenously expressed Ral GTPases immunoprecipitates with HA-ubiquitin. Lysates from HeLa cells overexpressing RalA or RalB and/or HA-ubiquitin were subjected to HA immunoprecipitation (IP) followed by immunoblotting with the indicated antibodies. Whole lysates were analyzed as in **A**. C, ubiquitination of Ral GTPases was not a signal for degradation. HeLa cells were treated with dimethyl sulfoxide (DMSO), MG132 (10 μM), ALLN (10 μM), or E64 and pepstatin (10 μg/ml and 50 μM, respectively) for 6 h. Whole cell extracts were analyzed by immunoblotting with anti-RalA or anti-RalB antibodies. Ratios between β-actin and RalA or RalB are indicated above the lanes. No increase in the quantity of RalA or B was detectable under these conditions. The regulation of cellular quantities of Ral GTPases did not rely on proteasome or lysosome degradation.

**Ral GTPase Ubiquitination Is Dependent on Raft Trafficking**—To ascertain whether the ubiquitination of Ral was dependent on its activation, we tested dominant active RalA mutants (G23V) and two dominant negative mutations (S28N and G26A). RalA G23V was more ubiquitinated than wild-type RalA, but the G26A and S28N mutants showed opposite behaviors, with the former more and the latter less ubiquitinated than wild type (supplementary Fig. 2B). Similar results were obtained for RalB (data not shown). Ral GTPase activity therefore did not modulate their ubiquitination.

Subsequent investigation concerned the link between Ral GTPase ubiquitination and function. RalA has been reported to support anchorage-independent growth (2). In nonadherent cells, RalA promotes membrane raft exocytosis to sustain proliferative signaling (26). Exogenous Ral GTPase ubiquitination status was therefore explored in nonadherent versus adherent cells. First, the ubiquitination of exogenous Ral GTPases was analyzed. Cells overexpressing RalA or RalB together with His6-Ubi were grown in adherent conditions or in suspension for
48 h (Fig. 2A). Nonadherent cells displayed a substantial and reproducible 2-fold increase in RalA ubiquitination (Fig. 2A). No variation in the ubiquitination level of RalB was detected under these conditions (Fig. 2A).

When cells are detached from their substrate, lipid rafts are internalized. Upon reattachment to the matrix, rafts are exocytosed for surface display using the RalA/Sec5 pathway (26). To test whether RalA ubiquitination might be regulated by raft trafficking or adhesion, RalA ubiquitination was investigated within the time frame of raft internalization (90 min of suspension) and re-exposure (20 min of re-adhesion). To avoid any effects of overexpressed RalA in cell adhesion, it was decided to...
follow endogenous RalA ubiquitination (Fig. 2C). Conditions to detect endogenous Ral GTase ubiquitination were previously established (Fig. 2B and supplemental Fig. 2A). By doubling the cell number (20 × 10^6) used for purification (compare experiments in Figs. 1 and 2B), it was possible to detect a major mono-ubiquitinated endogenous RalA band at 35 kDa and a weaker bi-ubiquitinated band at 42 kDa, suggesting that endogenous RalA is mostly mono-ubiquitinated. The bi-ubiquitinated band at 42 kDa increased in highly exposed blots (supplemental Fig. 2A), but high exposure also revealed nonspecific 50-kDa bands. The proportion of mono-ubiquitinated endogenous RalA among RalA proteins increased by a 1.5 factor in cells detached from the substrate for 90 min (Fig. 2C) and returned to steady-state levels after 20 min of cell re-adhesion (Fig. 2C), when rafts are also re-exposed on the plasma membrane. These observations indicated that endogenous RalA ubiquitination was up-regulated upon cell detachment from the substrate and perhaps also upon raft endocytosis. We also confirmed that the activation of endogenous RalA did not regulate its ubiquitination because serum stimulation induced no change in endogenous RalA-ubiquitinated 35- and 42-kDa bands (supplemental Fig. 2A). Lipid raft endocytosis has been reported to be dependent on caveolin-1 and dynamin-2 (27, 28). Caveolin-1 was depleted in His6-Ubi-expressing HeLa cells. The purification of endogenous ubiquitinated RalA was less efficient in RNAi-treated cells, but it was possible to detect the mono-ubiquitinated RalA band increasing 1.5-fold in siControl cells detached from the matrix compared with adherent cells (Fig. 2D). siCaveolin-1-treated cells showed a RalA ubiquitination inhibited by 80% in both adherent and nonadherent cells compared with siControl cells (Fig. 2D). When dynamin-2 was inhibited with the chemical inhibitor dynasore, decrease in endogenous RalA ubiquitination was also observed (Fig. 2E). Because RalA was less ubiquitinated when rafts were at the plasma membrane, one possibility could be that RalA is de-ubiquitinated in rafts at the plasma membrane. This hypothesis is supported by the observation that when raft structures were destroyed (by treating cells for 30 min with methyl-β-cyclodextrin, which extracts cholesterol) (29), endogenous RalA ubiquitination was increased 1.5-fold (Fig. 2F). We conclude that lipid rafts at the plasma membrane are required for RalA de-ubiquitination.

De-ubiquitination of RalA Is Required for Raft Endocytosis—To explore the role of RalA ubiquitination, efforts were devoted to identifying the lysine(s) of Ral that are subjected to ubiquitination. By changing all 21 RalA lysines into arginine one by one, it was found that several lysines on RalA could serve as ubiquitination sites (supplemental Table 1). Ubiquitinated RalA was detected in all combinations with the exception of the variant in which all lysines were mutated (data not shown). These results showed that Ral can be ubiquitinated on several lysines, but precluded any genetic approach to unraveling the functional significance of Ral ubiquitination.

It was therefore decided to use a constitutively ubiquitinated form of Ral in which the ubiquitin moiety was fused via a peptide bond to the N terminus of RalA or RalB. This approach has been successfully employed elsewhere for functional studies of Ras ubiquitination (18). Three FLAG epitopes were inserted between ubiquitin and the Ral protein, leading to a protein with a topology of N-ubiquitin-3×FLAG-Ral-C. The 3×FLAG-RalA and ubiquitin-3×FLAG-RalB fusions served as controls. These Ubi-Ral constructs were consistently highly overexpressed as fusion proteins, suggesting, as expected, that they are not sensitive to de-ubiquitin hydrolases (data not shown). As shown in Fig. 3A and as previously published, RalA was observed at the plasma membrane as well as in endomembranes (30). By contrast, Ubi-RalA was almost totally absent from the cytoplasm and mainly localized at the plasma membrane. However, RalB was observed on endomembranes and at the plasma membrane, whereas Ubi-RalB was absent from the cytoplasm but accumulated in internal punctate structures of 400–900 nm in size (Fig. 3A). The different impacts of ubiquitin fusions on RalA and RalB localization strongly support the notion that ubiquitin fusion itself does not drive nonspecific protein localization and suggest that the ubiquitination of RalA is a signal for plasma membrane targeting or stabilization.

To track the fate of rafts as a function of Ral ubiquitination status, the CTX receptor GM1 was used as a marker of lipid rafts, and transferrin receptor as a marker of clathrin-coated...
Lipid rafts were found scattered in low quantities at the plasma membrane of control cells either not transfected or transfected with plasmids expressing ubiquitin alone (data not shown) or FLAG-RalA (Fig. 3B; upper panel, t = 0). By contrast, lipid rafts were dramatically enriched at the plasma membrane of cells expressing Ubi-RalA (Fig. 3B; t = 0) but not...
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The endocytosis of lipid rafts was then followed upon expression of RalA, RalB, Ubi-RalA, and Ubi-RalB. After binding of CTX-Alexa Fluor 488 to the ganglioside receptor GM1 at 4°C, cells were washed and then shifted at 37°C, and GM1 localization was observed 40 min later. In both nontransfected cells and cells transfected to express Ubi alone (data not shown), RalA (Fig. 3B, upper panel, t = 40), or RalB (supplemental Fig. 3), CTX was found to be predominantly internalized as “normal.” In contrast, cells expressing Ubi-RalA did not internalize GM1: almost all of the CTX-Alexa Fluor 488 fluorescence was detected at the plasma membrane (Fig. 3B, t = 40). Transferrin receptor was tracked in the same cells using transferrin-Alexa Fluor 647. The abundance of transferrin receptor at the plasma membrane and its internalization were not affected by the expression of Ubi-RalA (Fig. 3B), demonstrating the specificity of the effect of ubiquitinated RalA on lipid rafts. When Ubi-RalB was expressed, levels of labeled CTX and transferrin, as well as their localization and dynamics, were also equivalent to control (supplemental Fig. 3), indicating that the effect on lipid rafts was specific to ubiquitinated RalA.

Ubi-RalA was also sufficient to inhibit GM1 internalization in suspension cells (Fig. 3B, lower panel). These results suggest that the de-ubiquitination of RalA is required for the endocytosis of lipid rafts.

Interplay between Ubiquitination and Activation of Ral—Because RalA-GTP is known to promote raft exposure (26), testing was performed to determine whether Ral ubiquitination impacted Ral activity. The quantities of GTP-bound forms of RalA, RalB, Ubi-RalA, and Ubi-RalB were tested using a standard pulldown methodology (32). It was observed that the ubiquitinated form of RalA (Fig. 3C) was extremely enriched in GTP. If RalA G23V is considered 100% bound to GTP, Ubi-RalA was then bound at 96%. Ubiquitination therefore seemed to favor Ral activation. An ubiquitination/de-ubiquitination cycle could be superimposed on the GDP/GTP cycle of RalA for the regulation of membrane raft trafficking.

The Effect of Ubiquitinated RalA in Rafts Is Mediated by Exocyst Complex Components—Effector loop mutants of Ubi-RalA have been generated, which selectively impair interactions of Ral withRalBP1, Sec5, Exo84, and Sec5 plus Exo84 (mutations 49N, 38R, 48W and 49E, respectively) (7, 33–35). These mutants were tested for impact on Ubi-RalA enrichment at the plasma membrane, as opposed to mutants unable to interact with either Sec5 or Exo84. Exocyst complex mediated the Ubi-RalA effect on lipid rafts, and the two Ral effectors Exo84 and Sec5 appeared to compensate for one another. This was supported by the impact of the depletion of Sec5, Exo84, or
both. The silencing of both subunits was required to abolish Ubi-RalA-induced raft exposure. Interestingly, membrane targeting of Ubi-RalA did not require interaction with its effectors involved in endocytosis and exocytosis. Mutants unable to interact with the exocyst complex or RalBP1 successfully reached the plasma membrane. We propose the existence of a molecular choreography in which ubiquitination targets RalA to the plasma membrane, where it is de-ubiquitinated in raft microdomains. In this scenario, RalA de-ubiquitination would be necessary for raft dynamics via Sec5 and Exo84.

Ubi-RalA induced lipid raft plasma membrane accumulation in nonadherent cells as well. RalA ubiquitination may play a role in promoting raft exocytosis of tumoral nonadherent cells, as does active RalA (26). However, because Ral ubiquitination was not influenced by Ral activation, it could be argued that the cycle of ubiquitination/de-ubiquitination is superimposed on the GDP/GTP RalA cycle. The ubiquitination/de-ubiquitination cycle represents a novel post-translational modification of Ral GTPases determining RalA subcellular localization and raft trafficking, and which could have a major impact on tumor growth. One might speculate that the E3 ligase(s) responsible for RalA ubiquitination should support RalA function in raft exocytosis and contribute positively to anchorage-independent growth and tumorigenesis. In return, the de-ubiquitin hydro-lases with ubiquitinated RalA as a target would then be tumor suppressors.

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REFERENCES

1. Chien, Y., Kim, S., Bumeister, R., Loo, Y. M., Kwon, S. W., Johnson, C. L., Balakireva, M. G., Romeo, Y., Kopelovich, L., Gale, M., Jr., Yeaman, C., Camonis, J. H., Zhao, Y., and White, M. A. (2006) RalB GTPase-mediated activation of the IκB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell 127, 157–170

2. Chien, Y., and White, M. A. (2003) RAL GTPases are linchpin modulators of human tumour cell proliferation and survival. EMBO Rep. 4, 800–806

3. Drosten, M., Dhawahir, A., Sum, E. Y., Urosevic J, Lechuga, C. G., Esteban, L. M., Castellano, E., Guerra, C., Santos, E., and Barbacid, M. (2010) Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival. EMBO J. 29, 1091–1104

4. Lim, K. H., O’Hayer, K., Adam, S. J., Kendall, S. D., Campbell, P. M., Der, C. J., and Counter, C. M. (2006) Divergent roles for RalA and RalB in the control of anchorage-independent growth.
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22. Balakireva, M., Rossé, C., Langevin, J., Chien, Y. C., Gho, M., González-Treboul, G., Vogelging-Lemaire, S., Aresta, S., Lepesant, J. A., Bellaiache, Y., White, M., and Camonis, J. (2006) The RaI/exocyst effector complex counters c-Jun N-terminal kinase-dependent apoptosis in Drosophila melanogaster. Mol. Cell. Biol. 26, 8953–8963

23. Laney, J. D., and Hochstrasser, M. (2002) Analysis of protein ubiquitination. Curr. Protoc. Protein Sci. Chapter 14:Unit 14.5

24. Yang, Y., Kitagaki, J., Dai, R. M., Tsai, Y. C., Lorick, K. L., Ludwig, R. L., Pierre, S. A., Jensen, J. P., Davydov, I. V., Oberoi, P., Li, C. C., Kenter, J. H., Beutler, J. A., Vosden, K. H., and Weissman, A. M. (2007) Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. Cancer Res. 67, 9472–9481

25. Deichman, G. I., Kashleva, H. A., Kluchareva, T. E., and Matveeva, V. A. (1989) Clustering of discrete cell properties essential for tumorigenicity and metastasis. II. Studies of Syrian hamster embryo fibroblasts transformed by Rous sarcoma virus. Int. J. Cancer 44, 908–910

26. Balasubramanian, N., Meier, J. A., Scott, D. W., Norambuena, A., White, M. A., and Schwartz, M. A. (2010) RaI-exocyst complex regulates integrin-dependent membrane raft exocytosis and growth signaling. Curr. Biol. 20, 75–79

27. Del Pozo, M. A. (2004) Integrin signaling and lipid rafts. Cell Cycle 3, 725–728

28. del Pozo, M. A., Balasubramanian, N., Alderson, N. B., Kissos, W. B., Grande-García, A., Anderson, R. G., and Schwartz, M. A. (2005) Phosphatase1 mediates integrin-regulated membrane domain internalization. Nat. Cell Biol. 7, 901–908

29. Kildson, E. P., Yancey, P. G., Stoudt, G. W., Bangertler, F. W., Johnson, W. J., Phillips, M. C., and Rothblatt, G. H. (1995) Cellular cholesterol efflux mediated by cycloxdetrins. J. Biol. Chem. 270, 17250–17256

30. Shipitsin, M., and Feig, L. A. (2004) RaI but not RaI enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. Mol. Cell. Biol. 24, 5746–5756

31. Harder, T., Schellfle, P., Verkade, P., and Simons, K. (1998) Lipid domain structure of the plasma membrane revealed by patching of membrane components. J. Cell Biol. 141, 929–942

32. Wolthuis, R. M., Franke, B., van Triest, M., Bauer, B., Cool, R. H., Camonis, J. H., Akkerman, J. W., and Bos, J. L. (1998) Activation of the small GTPase Ral in platelets. Mol. Cell. Biol. 18, 2486–2491

33. Bauer, B., Mirey, G., Vetter, I. R., García-Banea, J. A., Valencia, A., Wittlinger, A., Camonis, J. H., and Cool, R. H. (1999) Effector recognition by the small GTP-binding proteins Ra and Ral. J. Biol. Chem. 274, 17763–17770

34. Jin, R., Junutula, J. R., Matern, H. T., Ervin, K. E., Scheller, R. H., and Brunger, A. T. (2005) E604 and Sec5 are competitive regulatory Sec6/8 effectors to the RaI GTPase. EMBO J. 24, 2064–2074

35. Moskalenko, S., Tong, C., Rosse, C., Mirey, G., Formstecher, E., Daviet, L., Camonis, J., and White, M. A. (2003) RaI GTPases regulate exocyst assembly through dual subunit interactions. J. Biol. Chem. 278, 51743–51748

36. Camonis, J. H., and White, M. A. (2005) RaI GTPases: corrupting the exocyst in cancer cells. Trends Cell Biol. 15, 327–332

37. Bodemann, B. O., and White, M. A. (2008) RaI GTPases and cancer: linchpin support of the tumorigenic platform. Nat. Rev. Cancer 8, 133–140

38. Lim, K. H., Baines, A. T., Fiordalisi, J. J., Shipitsin, M., Feig, L. A., Cox, A. D., Der, C. J., and Counter, C. M. (2005) Activation of RaI is critical for Ras-induced tumorigenesis of human cells. Cancer Cell 7, 533–545