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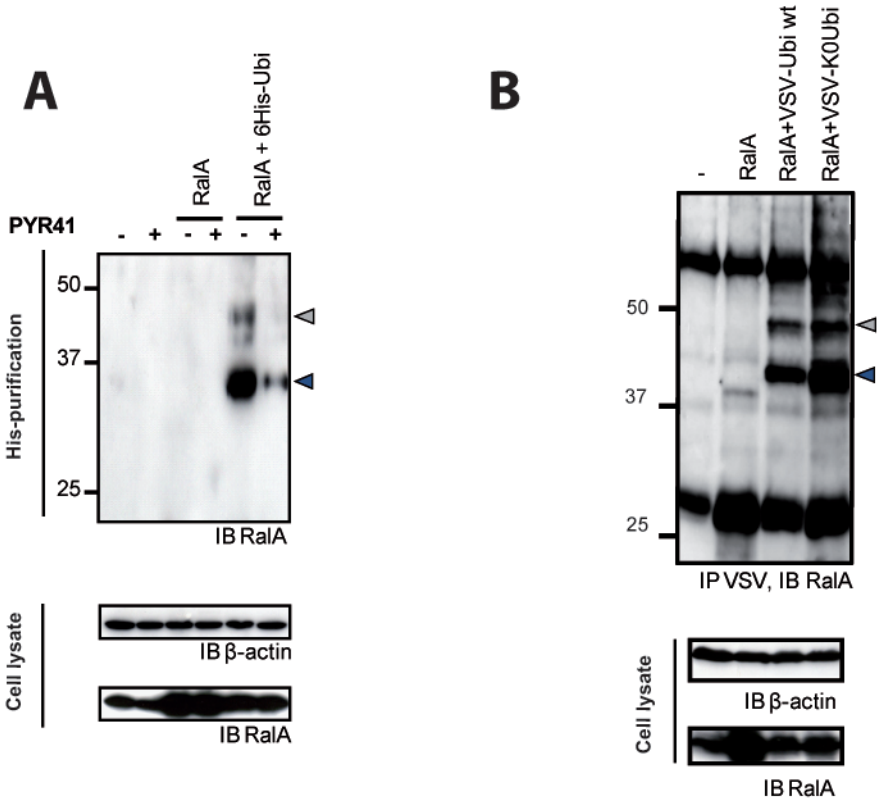
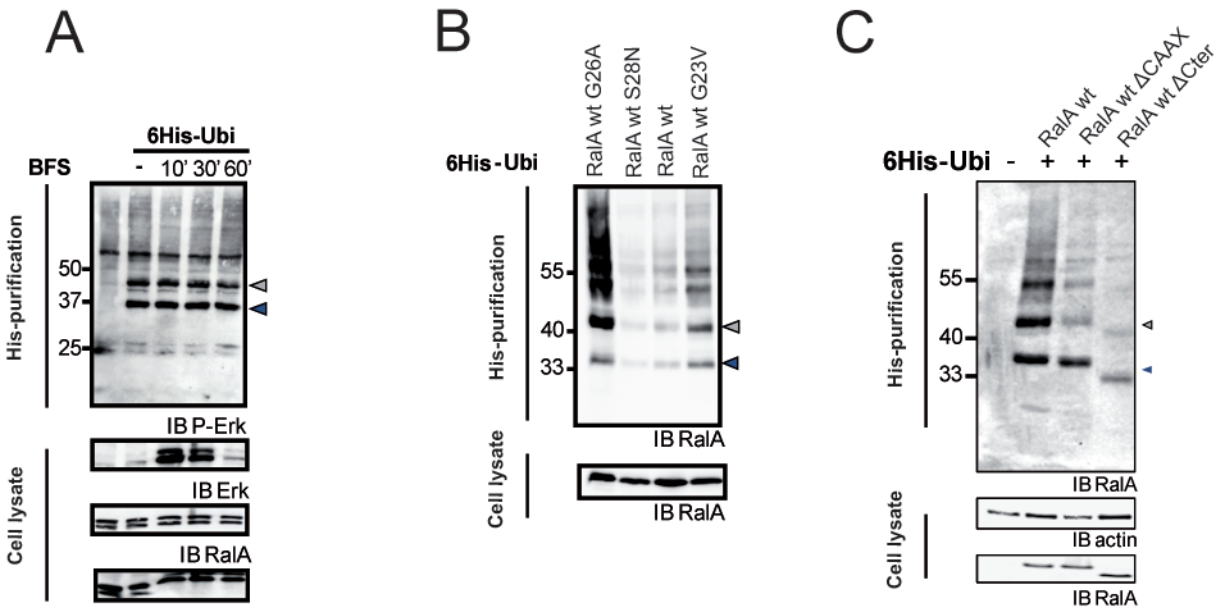
Supplemental Information contains Supplemental Figures 1 to 3, and Supplemental Table 1



Supplemental Figure 1. Ubiquitilation of Ral GTPases: further characterization.
A. **Inhibition of E1 Ubiquitin Activating enzyme decreases the ubiquitilation of RalA in vivo.** HeLa cells transfected by emtpy vector or RalA or RalA and 6His-Ubi were treated with DMSO (-) or with the E1 specific inhibitor PYR41 (+) at 50 μM for 6 h. Lysates were analysed for Ral ubiquitilation as in Figure 1 and whole lysate contents were analyzed by immunoblotting. Black and grey arrowheads indicate the mono and bi-ubiquitilated forms of Ral, respectively.
B. **RalA is multi-ubiquitilated.** Lysates from Hela cells transfected with empty vector (-) or expressing RalA alone or in combination with VSV-wild type ubiquitin (VSV-Ubi wt) or lysine-free VSV ubiquitin (VSV-Ubi K0) were subjected to VSV-immunoprecipitation (IP VSV) followed by immunobloting (IB) with the indicated antibodies. Whole lysate contents were analyzed by immunoblotting. Black and grey arrowheads indicate the mono and bi-ubiquitilated forms of Ral, respectively.



Supplemental Figure 2. Regulation of Ral ubiquitilation.

A. Ubiquitilation does not change under stimulation.

HeLa cells expressing 6His-Ubi were starved overnight and stimulated 10, 30 or 60 minutes with bovine fetal serum (BFS) before RalA and B ubiquitilation was analyzed. The first lane represents not stimulated Hela cells transfected by empty vector.

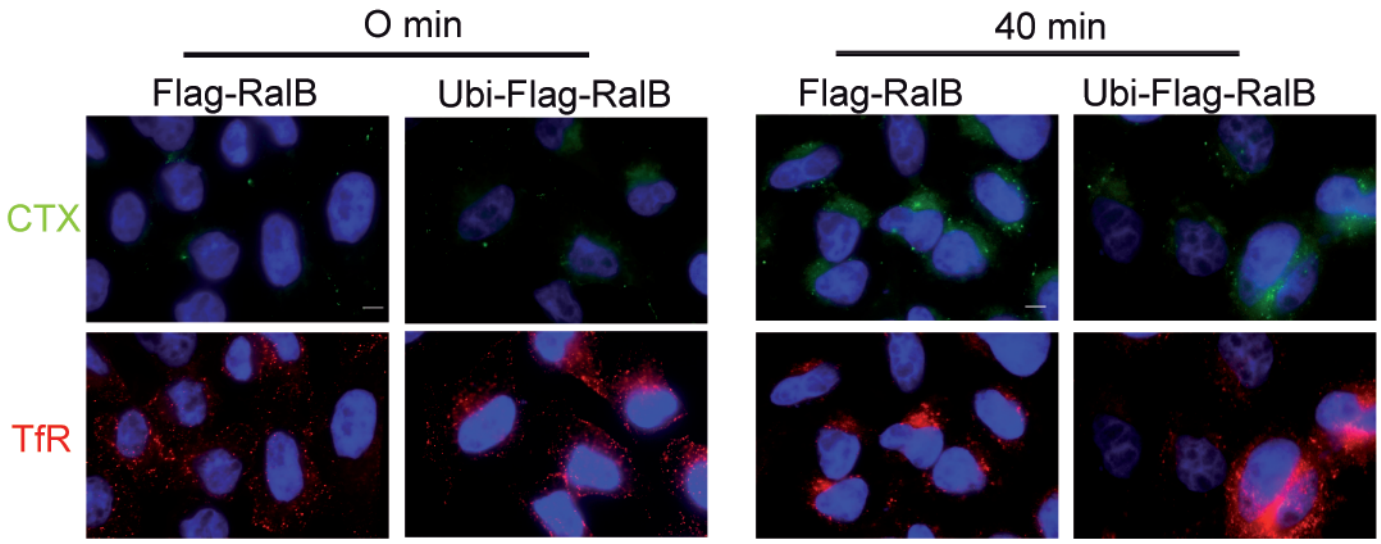
Black and grey arrowheads indicate the mono and bi-ubiquitilated forms of Ral, respectively.

B. **Ubiquitilation pattern of dominan active and dominant negative Ral mutants.**

Lysates from HeLa cells expressing dominant active (G23V) and dominant negative (S28N, G26A) in combination with 6His-Ubi were analyzed for Ral ubiquitilation.

C. **Membrane localization of RalA is required for its ubiquitilation.**

Lysates from HeLa cells expressing RalA wt, RalAΔCAAX (with the last four amino acids deleted) or RalAΔCter mutants (with the last four amino acids and preceding polybasic track deleted) in combination with 6His-Ubi were analyzed for RalA ubiquitilation. Black and grey arrowheads indicate the mono and bi-ubiquitilated forms of Ral, respectively.



Supplemental Figure 3. Ubi-RalB does not induce inhibition of lipid raft endocytosis. HeLa cells expressing Flag-RalB or Ubi-Flag-RalB were analyzed for cholera-toxin (green) or transferrin (red) trafficking such as in Fgiure 4.

	Position in RalA>	5	7	16	27	47	53	54	115	128	134	143	159	166	179	184	186	189	190	191	193	197
Name of mutants	RalA 1K>R-a			R																		
	RalA 1K>R-b				R																	
	RalA 1K>R-c								R													
	RalA 1K>R-d										R											
	RalA 1K>R-e											R										
	RalA 1K>R-f																			R		
	RalA 1K>R-g									R												
	RalA 1K>R-h														R							
	RalA 2K>R-a	R	R																			
	RalA 2K>R-b			R	R																	
	RalA 2K>R-c										R	R										
	RalA 2K>R-d											R								R		
	RalA 2K>R-e												R	R								
	RalA 3K>R-a					R	R	R														
	RalA 3K>R-b										R	R								R		
	RalA 3K>R-c									R			R	R								
	RalA 3K>R-d														R	R	R					
	RalA 4K>R-a								R		R	R								R		
	RalA 4K>R-b	R	R	R	R																	
	RalA 4K>R-c									R			R	R	R							
	RalA 5K>R-a										R	R	R	R						R		
	RalA 5K>R-b																	R	R	R	R	R
	RalA 8K>R														R	R	R	R	R	R	R	R
	RalA 11K>R	R	R	R	R	R	R	R		R			R	R	R							
	RalA 18K>R	R	R	R	R	R	R	R		R			R	R	R	R	R	R	R	R	R	R
	RalA 19K>R	R	R	R	R	R	R	R		R		R	R	R	R	R	R	R	R	R	R	R
	RalA 20K>R	R	R	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R	R	R	R
	RalA 21K>R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Supplemental Table 1. Ubiquitilation of single and combined lysine-to-arginine mutants of RalA.
RalA mutants were made by PCR and all constructs were verified by sequencing the full length of inserts. Lysates from Hela cells were tested for RalA ubiquitilation using the same protocol as used previously in this study. Each mutant was tested at least twice. We could not find any mutant that exhibited a lower ratio of ubiquitilation than wild-type, except when all lysines were mutated into arginines (mutant RalA 21K>R). Note that although we cannot rule out the possibility that Ral undergoes N-terminal ubiquitilation this is unlikely. Firstly, addition of a lysine-free Myc tag to the N-terminus of Ral did not prevent its ubiquitilation (data not shown). Secondly, the first two amino acids of mammalian and nematode Ral are methionine and alanine, and in the fly are methionine and serine. N-amino peptidase and N-acetyl transferase rules suggest that all Ral will have their methionine clipped off and the next amino acid will be acetylated, which will antagonize N-ubiquitilation. However we cannot rule out competition between amino-peptidase and ubiquitilation of the first methionine.