Cellular/Molecular

Conditional Deletion of *Ric-8b* in Olfactory Sensory Neurons Leads to Olfactory Impairment

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The olfactory system can discriminate a vast number of odorants. This ability derives from the existence of a large family of odorant receptors expressed in the cilia of the olfactory sensory neurons. Odorant receptors signal through the olfactory-specific G-protein subunit, Gαolf. Ric-8b, a guanine nucleotide exchange factor, interacts with Gαolf and can amplify odorant receptor signal transduction *in vitro*. To explore the function of Ric-8b *in vivo*, we generated a tissue specific knock-out mouse by crossing OMP-Cre transgenic mice to Ric-8b floxed mice. We found that olfactory-specific Ric-8b knock-out mice of mixed sex do not express the Gαolf protein in the olfactory epithelium. We also found that in these mice, the mature olfactory sensory neuron layer is reduced, and that olfactory sensory neurons show increased rate of cell death compared with wild-type mice. Finally, behavioral tests showed that the olfactory-specific Ric-8b knock-out mice show an impaired sense of smell, even though their motivation and mobility behaviors remain normal.

Key words: **G-protein; GEF; knock-out mouse; odorant receptor; olfaction; olfactory sensory neuron**

Significance Statement

Ric-8b is a guanine nucleotide exchange factor (GEF) expressed in the olfactory epithelium and in the striatum. Ric-8b interacts with the olfactory Gαolf subunit, and can amplify odorant signaling through odorant receptors *in vitro*. However, the functional significance of this GEF in the olfactory neurons *in vivo* remains unknown. We report that deletion of Ric-8b in olfactory sensory neurons prevents stable expression of Gαolf. In addition, we demonstrate that olfactory neurons lacking Ric-8b (and consequently Gαolf) are more susceptible to cell death. Ric-8b conditional knock-out mice display impaired olfactory guided behavior. Our results reveal that Ric-8b is essential for olfactory function, and suggest that it may also be essential for Gαolf-dependent functions in the brain.

Introduction

Odorants are detected by a large family of G-protein-coupled receptors expressed in the olfactory sensory neurons of the nose, the odorant receptors (ORs; Buck and Axel, 1991; Malnic et al., 2010). ORs signal through an olfactory-specific G-protein, termed Golf. The Gαolf subunit is restrictedly expressed in the olfactory neurons and also in a few regions of the brain, such as the striatum (Jones and Reed, 1989; Belluscio et al., 1998; Zhuang et al., 2000; Kaupp, 2010).

Mice that are knock-out for Gαolf are anosmic (they lack the sense of smell; Belluscio et al., 1998), which is consistent with a fundamental and central role for this G-protein subunit in odorant signal transduction. In addition, these mice are deficient in striatal dopamine 1 (D1) receptor-mediated behavior, such as a hyper locomotor response to cocaine or other dopamine receptor agonists (Zhuang et al., 2000). Gαs, which shares ~80% amino acid sequence identity with Gαolf, is expressed at low levels in the striatum, where Gαolf is highly expressed (Belluscio et al., 1998). Activation of the neurotransmitter adenosine receptor A2A in the striatum also requires Gαolf (Hervé et al., 2001). Altogether, these results show that Gαolf is also required for normal striatal functions (Hervé, 2011; Xie et al., 2015).

We have previously found that Ric-8b, a guanine nucleotide exchange factor (GEF) interacts with Gαolf (Von Dannecker et al., 2005) and can amplify odorant receptor signal transduction

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through Gαolf *in vitro* (Von Dannecker et al., 2006). Ric-8b can also interact with Gγ13, a Gγ subunit that is found in the cilia of olfactory sensory neurons (Kulaga et al., 2004; Kerr et al., 2008; Sathyanesan et al., 2013), and that has been implicated in odorant signaling (Li et al., 2013). Ric-8b expression in adult mice is predominant in the same tissues where Gαolf is expressed, the olfactory epithelium and the striatum (Zhuang et al., 2000; Von Dannecker et al., 2005). This strict colocalization pattern of expression suggests that Ric-8b plays a role in regulating Gαolf function, in both tissues.

Ric-8b interacts with the Gαolf subunit and catalyzes the exchange of GDP for GTP (Chan et al., 2011a) and therefore has been considered to work as a positive regulator for G-protein signaling (Klattenhoff et al., 2003; Von Dannecker et al., 2005, 2006; Malnic and Gonzalez-Kristeller, 2009; Hinrichs et al., 2012). In this case, Ric-8b would act on the monomeric Gαolf subunit, after odorant receptor activation, to further amplify odorant signaling (Malnic and Gonzalez-Kristeller, 2009). However, there is no direct evidence that Ric-8b plays such a role *in vivo*. Several studies indicate that Ric-8b can promote plasma membrane localization of Gα subunits, as well as G-protein abundance in the cell (Von Dannecker et al., 2006; Kerr et al., 2008; Nagai et al., 2010; Chan et al., 2011b; Gabay et al., 2011). Increased functional expression of the G-protein would also indirectly potentiate signaling (Papasergi et al., 2015).

To investigate the role played by Ric-8b in odorant signal transduction we generated conditional knock-out (cKO) mice where Ric-8b was specifically deleted in the olfactory epithelium. We found that even though the cKO mice are grossly normal, their olfactory sensory neurons do not express the Gαolf protein and show no response to the tested odorant mix. In the cKO mice the mature olfactory sensory neuron layer in the olfactory epithelium is reduced, and the size of the olfactory bulb is smaller compared with those of wild-type mice. We also show that the olfactory function in the cKO mice is severely impaired. Our results show that Ric-8b plays an essential role in olfaction.

Materials and Methods

Animal procedures. All animal procedures in this study were approved by the University of São Paulo Chemistry Institute's Animal Care and Use Committee, under the protocol number 01/2013. Mice bearing the Ric8btmt1a(EUCOMM)Hmgu targeted allele where first crossed to transgenic mice expressing the FlpE recombinase (enhanced FLP1 recombinase) under the control of the human β-actin promoter [B6.Cg-Tg(ACTFLPe)9205Dym/J; stock #005703, The Jackson Laboratory; RRID:MMRRC_005703-UCD] to generate the "floxed" mice, where two *loxP* sites flank exon 4 of the *Ric-8b* gene (Skarnes et al., 2011). Homozygous floxed mice were then bred to heterozygous transgenic OMP-Cre mice (B6; 129P2-Omp $^{tm4(cre)Mom}$/MomJ; The Jackson Laboratory, stock #006668; RRID:IMSR_JAX:006668) to generate homozygous tissue-specific Ric-8b knock-out mice.

Genotyping. Genotypes were determined by PCR using ear DNA with separate pairs of primers for detecting the presence of each transgenic allele. Genotyping of the OMP-Cre alleles was performed according to the protocol provided by The Jackson Laboratory for this specific strain (https://www.jax.org/strain/006668). Genotyping of the floxed Ric-8b allele was performed using the following pair of primers: TGC AGT ATA CTG TGC CAG TGT AC and TGA AAT GGT AGG CAT CCA TGG TG.

In situ *hybridization. In situ* hybridization was performed according to Schaeren-Wiemers and Gerfin-Moser (1993) as previously described (Von Dannecker et al., 2005). Noses or brains were dissected, respectively, from 3-week-old mice and freshly embedded in Tissue-Tek OCT compound (Sakura Finetek). Sequential 16 μm sections were prepared with a cryostat and hybridized with digoxigenin-labeled cRNA probes prepared from OMP, GAP43, Ngn1, Ric-8b, Gαolf, or Olfr1507. Control sections were incubated in an identical concentration with the control sense probe. Color reactions were performed overnight. Images were obtained on a Nikon TE300 microscope.

Western blot. Standard Western blotting procedures were performed using a Bio-Rad electrophoresis and transfer apparatus. Proteins were separated by SDS-PAGE gradient gel and transferred onto nitrocellulose membrane (GE Healthcare). Membrane was blocked with 5% nonfat dry milk and then incubated with Ric-8b (Atlas Antibodies, catalog #HPA042746; RRID:AB_2678143), Gαolf (Santa Cruz Biotechnology, catalog #sc-385; RRID:AB_10160576), and OMP (Wako, catalog #544-10001; RRID: AB_2315007) antibodies. Immunoreactivity was revealed by enhanced chemiluminescence (ECL Plus; GE Healthcare).

Immunofluorescence. Immunofluorescence detection (see Fig. 4*B*) was performed as previously described (Kerr et al., 2008). Antibodies used were anti-OMP (Wako, catalog #544-10001; RRID:AB_2315007) and anti-GAP43 (Abcam, catalog #ab16053; RRID:AB_443303). As a secondary antibody, we used antibodies against goat or chicken IgG conjugated to AlexaFluor 488 (Invitrogen: catalog #A21467; RRID:AB_141893 and catalog #A11039; RRID:AB_142924). Nuclei were stained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI).

For experiments shown in Figure 4*A*, 8-week-old mice were deeply anesthetized, and perfused transcardially with ice-cold 0.9% saline followed by 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The nose was incubated overnight at 4°C in 4% (w/v) PFA, and decalcified for 4 d in 0.45 M EDTA in 0.1 M phosphate buffer, pH 7.4, at 4°C. Cryoprotection was performed in 30% sucrose overnight at 4°C. The tissue was embedded in Tissue Tek OCT (Sakura Finetek), and coronal sections (14 μm thickness) of the main olfactory epithelium were frozen at −80°C until further use. Cryo sections were first rinsed in 0.1 M PBS, and antigens were unmasked by exposure to microwave radiation in 10 mM sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). After blocking nonspecific binding with 4% (v/v) normal goat serum, 3% BSA, and 0.4% Triton X-100 for 1 h, sections were incubated overnight at 4°C with anti-Ric-8B primary antibody (Atlas Antibodies, catalog #HPA042746; RRID:AB_2678143; 1:50) and anti-gamma-tubulin (Abcam, catalog #ab11316; RRID:AB_297920; 1:200) and diluted in the blocking solution. Slides were washed 3× for 10 min with PBS containing 0.2% Triton X-100, and then incubated for 1.5 h at room temperature with anti-rabbit IgG secondary antibody conjugated with AlexaFluor 488 (Invitrogen, catalog #A21206; RRID:AB_141708) and mouse IgG conjugated to AlexaFluor 546 (Invitrogen, catalog #A11003; RRID:AB_141370) and diluted in the same blocking solution. Slides were washed 2× 10 min with PBS containing 0.2% Triton X-100, and counterstained with DAPI for 10 min. To reduce background fluorescence staining, slides were treated with 1% Sudan Black B (Sigma-Aldrich) dissolved in 70% ethanol for 10 min, then washed 3× 5 min with PBS. Slides were mounted with FluorSave reagent (Millipore).

TUNEL assay. Noses were dissected from 3-week-old mice and freshly embedded in Tissue-Tek OCT compound (Sakura Finetek). Sequential 20 μm sections were prepared with a cryostat. Slides were then subjected to the Click-iT Plus TUNEL Assay for *in situ* cell death detection with AlexaFluor dyes (Life Technologies) according to the manufacturer's instructions.

Calcium imaging. Calcium imaging of the dissociated olfactory sensory neurons was performed using 10 μM intracellular Ca^{2+} indicator Fluo3 AM (Invitrogen) for 30 min at 37°C in DMEM F12 (Invitrogen) supplemented with 2 mM $CaCl_2$. Odorants (100 mM stocks in DMSO) were freshly diluted in HBSS containing 2 mM $CaCl_2$. The following odorants (100 μM each) were used to prepare the mix that was applied to the cells: hexanal, heptanal, eugenol, 1-heptanol, (±) citronellal, from Sigma-Aldrich, and 1-nonanol, S(-) limonene, acetophenone and (±) carvone, from Fluka. After exposure to the odorant mix, cells were exposed to 100 mM KCl to assess their viability. The images were captured using a confocal microscope (Zeiss LSM 510 Meta).

Immunohistochemistry. For experiments shown in Figure 6*D*, P60 mice were deeply anesthetized, and rapidly perfused transcardially with ice-cold 0.9% saline followed by 4% (w/v) PFA in 0.1 M phosphate buffer, pH 7.4 (PBS). The brain was incubated for 6 h at 4°C in 4% (w/v) PFA and cryoprotection was performed sequentially in 10%/20%/30% su-

crose overnight at 4°C. The tissue was embedded in Tissue Tek OCT (Sakura Finetek), and coronal sections (20 μm thickness) of the olfactory bulb were frozen at −80°C until further use. Sections were then washed in PBS and treated with 3% H_2O_2 to block endogenous peroxidases. After washing in PBS, sections were incubated at room temperature (RT) in blocking solution (PBS containing 4% donkey serum, 1% BSA, and 0.4% Triton X-100) for 90 min. Using the same buffer solution composition, the sections were incubated overnight at 4°C with primary antibody anti-TH (Bioscience, catalog #MAB318; RRID:AB_2313764; 1:100). After incubation with the primary antibody, OB sections were rinsed in PBS and incubated with compatible biotinylated secondary antibody (Vector Laboratories, catalog #BA-2001; RRID:AB_2336180; 1:250) for 60 min diluted in PBS containing 0,2% Triton X-100. The sections were then washed again with PBS and incubated with ABC reagent (Vector Laboratories, catalog #PK-6100; RRID:AB_2336819) for 60 min at RT. The visualization of peroxidase was performed using 3,3-diaminobenzidine (DAB) method; specimens were incubated for 3–10 min at RT in a freshly made solution containing 0.05% DAB (Sigma-Aldrich, D1,238-4), 0.015% H_2O_2, 0.1 M PBS, pH 7.2 and the reaction was stopped in distilled water. The sections were counterstained with DAPI for nuclear labeling and coverslipped with gelvatol mounting medium. The sections were examined and photographed with a Carl-Zeiss research microscope. To compose several photos to one figure, we used Hugin Panorama Creator (http://hugin.sourceforge.net/). The images were processed to enhance contrast and image quality using GIMP (http://www.gimp.org/) and were assembled using Inkscape.

Behavioral tests. To evaluate odor motivated behavior the buried food-seeking test was performed following the previously published procedures with minor modifications (Luo et al., 2002). Briefly, mice ≥8 weeks old were deprived of food for 24 h. On day 2, a 2 g pellet of regular chow was buried 8 cm beneath the surface of the fresh bedding in one end of a clean test cage of a regular size (24 length × 14 width × 13 cm height). The mouse was transferred from its home cage and released at the opposite end of the test cage. The time from its introduction to the test cage until it found the food pellet and retrieved it to the surface was recorded as the latency. If an animal could not find the food pellet within 10 min, the test was terminated and the latency was recorded as 600 s. For the innate olfactory preference test, mice were habituated to the test conditions before odor exposure. Mice were individually placed in an empty cage for 30 min. Following habituation, mice were transferred to a test cage, and a filter paper scented with a test odorant was introduced. Investigation times of the filter paper during the 3 min test period were recorded. Odor stimuli were peanut butter (10% w/v, 15 μl), milk (15 μl), and water (15 μl). For the innate olfactory avoidance test, following habituation (see innate preference test), a filter paper scented with 5 μl butyric acid (W222119, Sigma-Aldrich) was placed in one corner of the test cage. Mouse behavior was recorded for 30 min. The test cage was subdivided into two equally sized areas. Time spent in Area 1 of the cage (farthest distance from the butyric acid source) was evaluated as avoidance; whereas time spent in Area 2 (the butyric acid source) was evaluated as attraction. Animal movements were tracked using EthovisionXT-7 (Noldus Information Technology).

Experimental design and statistical analysis. For all *in vivo* experiments, at least three animals (of either sex) of each genotype [wild-type (WT), heterozygous (Het), or cKO] were used. The statistical analyses were performed using GraphPad Prism 5. Results are represented as the mean ± SE. Data were compared by Student's *t* test (two-tailed), one-way ANOVA and Tukey or Dunnett post-test. Differences were considered significant at $p \leq 0.05$.

Results

Deletion of *Ric-8b* in the olfactory epithelium

To investigate the roles of Ric-8b in olfaction we generated conditional Ric-8b knock-out in the olfactory epithelium by crossing Ric-8b floxP mice with OMP-Cre mice (Li et al., 2004; Fig. 1*A–D*). Because the OMP (olfactory marker protein) gene is only expressed in mature olfactory neurons, the deletion is restricted to these cells, and does not occur in the striatum or other tissues. In the resulting offspring, the number of detected cKO mice was lower than the expected Mendelian ratio (25% expected, 11% observed; Fig. 1*B*,*E*). In addition, cKO mice were more frequently found in smaller litters than in larger litters (Fig. 1*F*). In the larger litters, deceased mice were found on the day after birth, and genotyping showed that they were from cKO mice (data not shown). These observations indicate that the cKO mice die after they are born, and not *in utero*, possibly due the critical role of the olfactory system in mediating suckling. Newborn knock-out mice that are anosmic and show difficulty in suckling were previously shown to have a better chance to survive when they were born in smaller litters (Brunet et al., 1996; Belluscio et al., 1998; Wong et al., 2000; Li et al., 2013). These results indicate that the *Ric-8b* cKO could show olfactory deficits.

Ric-8b-deficient olfactory sensory neurons do not express Gαolf

Ric-8b deletion in the olfactory epithelium was confirmed by comparing the expression levels of the Ric-8b protein in olfactory epithelium from WT (Ric-8b$^{flox/flox}$/OMP$^{w/w}$), Het (Ric-8b$^{flox/wt}$/OMP$^{Cre/wt}$), and cKO (Ric-8b$^{flox/flox}$/OMP$^{Cre/wt}$) littermates. As shown in Figure 2*A*, Ric-8b protein levels are reduced by ~50% in Het mice and are completely abolished in the cKO mice. Interestingly, Gαolf protein levels are not significantly altered in Het mice, but are completely abolished in the cKO mice (Fig. 2*A*). The Ric-8b gene is normally transcribed in the striatum of cKO mice, confirming that the deletion of *Ric-8b* was specific to the olfactory epithelium (Fig. 2*B*). Accordingly, analysis of protein extracts prepared from the striatum show that, differently from what is observed in the olfactory epithelium, Gαolf protein expression is preserved in the striatum of cKO mice (Fig. 2*C*).

The number of mature olfactory sensory neurons is reduced in Ric-8b cKO mice

The Western blot experiments also showed that the expression levels of the OMP protein, a marker for mature olfactory sensory neurons, are reduced in the olfactory epithelium from cKO mice (Fig. 2*A*). We therefore analyzed whether the structure of the olfactory epithelium in these mice is altered. Markers for different neuronal layers present in the olfactory epithelium were used in *in situ* hybridization experiments on sections cut through the olfactory epithelium from WT and cKO mice. The results show that in cKO animals the basic organization of the olfactory epithelium is preserved, however, the mature (OMP-positive) olfactory sensory neuron layer is thinner compared with that of the WT epithelium (Fig. 3*A*). Quantification of the results shown in Figure 3*A* showed that the total thickness of the olfactory epithelium is reduced in the cKO mice (WT 124.8 ± 4.5 μm; cKO 108.8 ± 3.1 μm; $p = 0.01$, $t_{(23)} = 2.61$, unpaired *t* test), and that whereas the thickness of the OMP layer relative to the total olfactory epithelium is reduced in the cKO mice (WT 80.7 ± 1.3%; cKO 40.9 ± 1.8%; $p = 0.0001$, $t_{(22)} = 18.48$, unpaired *t* test), the thickness of the GAP43 layer is not (WT 49.6 ± 1.2%; cKO 50.4 ± 1.5%; $p = 0.66$, $t_{(23)} = 0.45$, unpaired *t* test).

Ric-8b is expressed throughout the olfactory epithelium in the mature neuronal layers in WT mice, and as expected its expression is negative in the cKO (Fig. 3*B*). Even though no Gαolf protein is detected in the olfactory epithelium from cKO mice (Fig. 2*A*), transcripts corresponding to the Gαolf gene were detected (Fig. 3*B*), indicating that Ric-8b's role as a positive regulator for Gαolf protein expression occurs at the post-transcriptional level.

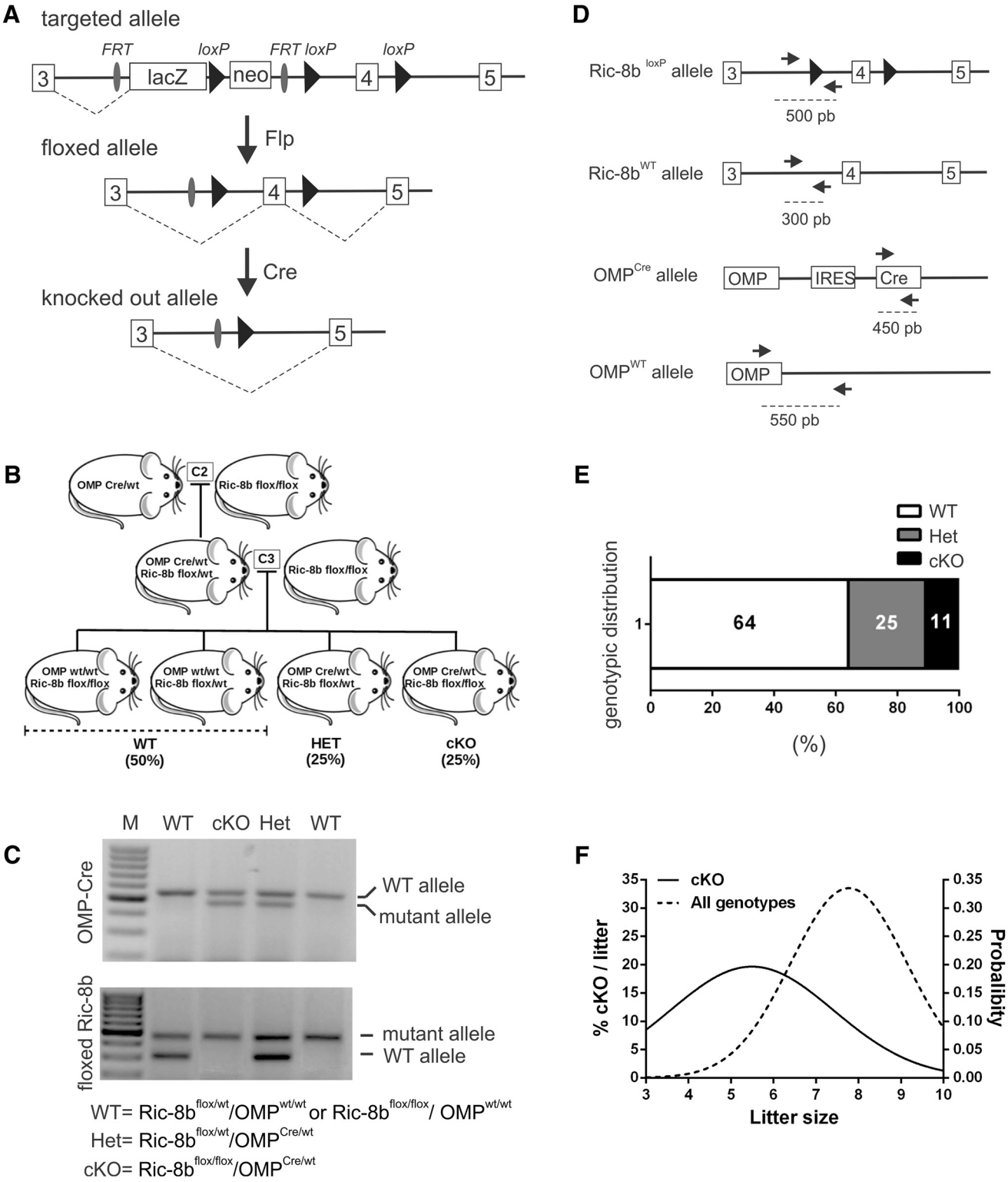

Figure 1. Tissue-specific deletion of the Ric8-b gene. ***A***, Schematic representation of the deletion of the Ric-8b gene in the olfactory epithelium. Mice bearing the Ric8b $^{tmt1a(EUCOMM)Hmgu}$ targeted allele were first crossed to transgenic mice expressing the FlpE recombinase under the control of the human β-actin promoter to generate the floxed mice, where two *loxP* sites flank exon 4 of the Ric-8b gene. Homozygous floxed mice were then bred to transgenic OMP-Cre mice to generate tissue specific Ric-8b knock-out mice (cKO), because deletion of exon 4 leads to a frameshift. ***B***, To obtain the Ric-8b cKO mice, two kinds of crosses were performed (denominated C2 and C3). First, OMP ^{cre} heterozygote animals were crossed with Ric-8b ^{flox} homozygotes (C2). OMP ^{cre} heterozygotes were used because the OMP ^{cre} homozygotes are sub fertile. C2 crosses generate animals which are mutant for both genes. These animals are then crossed with another Ric-8b ^{flox} homozygote (C3) to finally obtain the Ric-8b cKO. ***C***, PCR-based genotyping of mice obtained in C3 crosses. OMP-CRE transgenic mice were crossed to floxed Ric-8b mice and the resulting offspring was genotyped for the presence of the OMP-Cre and floxed Ric-8b alleles. ***D***, The positions of the primers used for genotyping the different WT and mutant alleles and the expected sizes of the amplified products are shown. The agarose gel (***C***) shows examples of mice that were genotyped as WT, Het, or cKO. (M = 100 bp ladder, the stronger band corresponds to 500 bp). ***E***, Ric-8b cKO mice exhibit distorted Mendelian ratios. ***F***, The survival of cKO newborns is reduced in larger litters (total of 403 animals in 66 litters from 12 couples). The continuous line shows the observed percentage of cKO mice according to litter size. The dashed line indicates the probability distribution that a pup, regardless of genotype, comes from a given litter size.

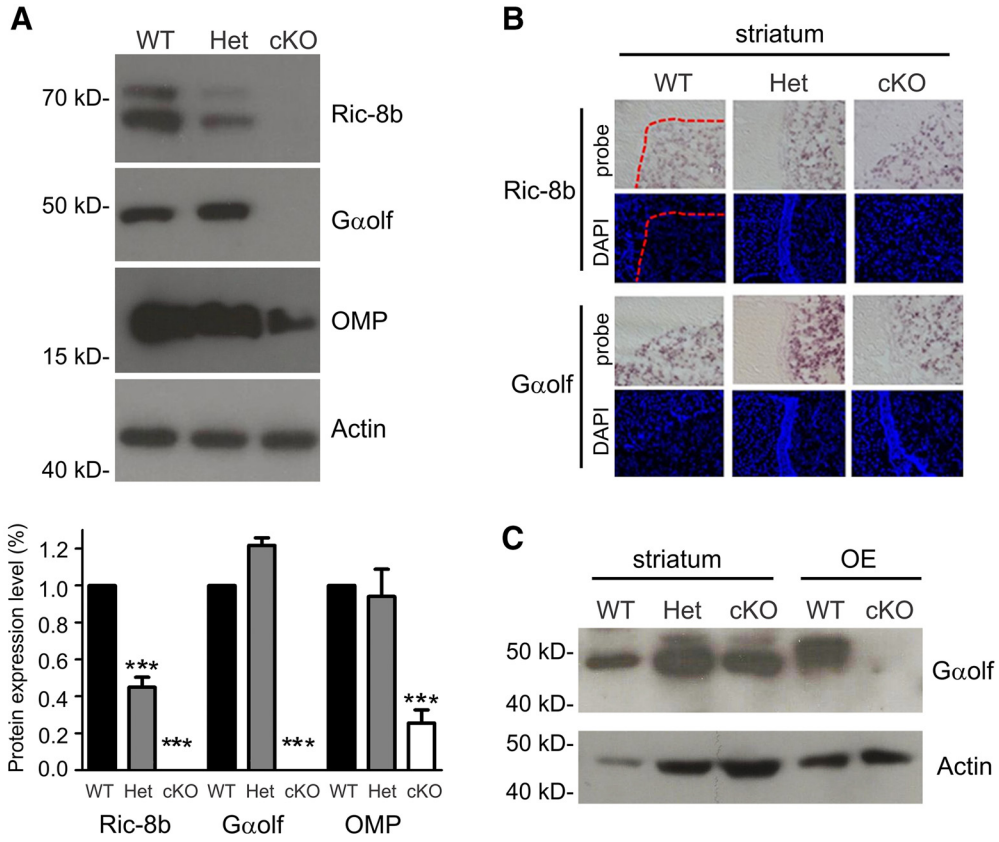

Figure 2. Expression levels of olfactory proteins in the Ric-8b-deficient olfactory epithelium. ***A***, Ric-8b, Gαolf, and OMP protein levels were evaluated by Western blot analysis of total protein extract prepared from the olfactory epithelium from WT (Ric-8b $^{flox/flox}$/OMP $^{w/w}$), Het (Ric-8b $^{flox/wt}$/OMP $^{Cre/wt}$), and cKO (Ric-8b $^{flox/flox}$/OMP $^{Cre/wt}$) mice. β-Actin was used as control to ensure equivalent loading. The bar graph, obtained by densitometric analysis of the Western blot data, represents the mean ± SEM of three independent experiments. The one-way ANOVA and Tukey post-test determined statistically significant differences. ***$p < 0.0001$; $F = 18.99$. ***B***, Coronal sections through the striatum from WT, Het, and cKO mice were hybridized with antisense digoxigenin-labeled probes specific for Ric-8b or Gαolf. Nucleus staining with DAPI is shown in blue. The dashed line delineates the striatum border. ***C***, Gαolf protein levels evaluated by Western blot analysis of total protein extract from the striatum or olfactory epithelium of mice with the indicated genotypes.

Transcription of the odorant receptor gene *Olfr1507* (MOR28) is not altered in the cKO epithelium (Fig. 3*B*). Because the number of OMP-positive olfactory neurons is decreased in the Ric-8b cKO epithelium, one would expect that the number of Olfr1507-positive neurons, should also be reduced in these mice. However, we did not observe this reduction, at least in the P21 mice that we analyzed in the *in situ* hybridization experiments shown in Figure 3*B*. One possible explanation is that in olfactory sensory neurons, the odorant receptors are expressed before OMP (Rodriguez-Gil et al., 2015). Therefore, a fraction of the neurons could express Olfr1507 before the onset of OMP expression, and subsequent deletion of Ric-8b. It is possible that in older cKO mice, where the number of immature neurons is usually smaller and the number of mature olfactory neurons is higher, we would observe a reduced number of the Olfr1507-positive neurons.

To further evaluate the organization of the olfactory epithelium from Ric-8b cKO mice, we performed immunostaining experiments. As expected, Ric-8b immunoreactivity is absent from the cKO epithelium (Fig. 4*A*). In the WT epithelium, strong Ric-8b protein staining is observed in the cell bodies of the olfactory sensory neurons. Double immunostaining with gamma-tubulin, a specific marker for ciliary basal bodies, reveals Ric-8b staining in the dendritic knobs of the olfactory sensory neurons (Fig. 4*A*). The immunostaining experiments also showed that, whereas the immature olfactory neuron layer, stained for GAP43, is not grossly altered in the cKO olfactory epithelium, the mature olfactory sensory neuron layer, revealed by OMP staining, is markedly reduced (Fig. 4*B*).

The sizes of the olfactory bulbs from Ric-8b cKO mice are also reduced compared with the ones of WT mice (Fig. 4*C*). In addition, quantitative analysis indicated that in the olfactory bulbs from cKO mice the number of glomeruli is reduced (WT 88.7 ± 2.5; cKO 53.5 ± 1.9; $p = 0.0001$, $t_{(34)} = 9.22$, unpaired *t* test), and the average sizes of the glomeruli are smaller (WT 50 ± 1.4; cKO 27 ± 1.0; $p = 0.0001$, $t_{(553)} = 11.75$, unpaired *t* test; Fig. 4*D*,*E*). These effects are probably a consequence of the reduced olfactory sensory innervation to the olfactory bulbs. It has been shown, for example, that there is a direct correlation between the number of olfactory sensory neurons expressing a given OR and the volume of the corresponding glomeruli in the olfactory bulb (Bressel et al., 2016).

Ric-8b-deficient olfactory sensory neurons show increased cell death

The results shown above indicate that deletion of Ric-8b in the olfactory epithelium leads to a reduction in the number of mature olfactory sensory neurons. One possibility is that this effect is a result of increased neuronal death. To address this point, we performed TUNEL staining to assess cell death in the olfactory epithelium from Ric-8b cKO and WT mice (Fig. 5*A*). We found that the number of dying cells in the epithelium from the cKO mice is ~ 5× higher than in the epithelium of WT mice (WT

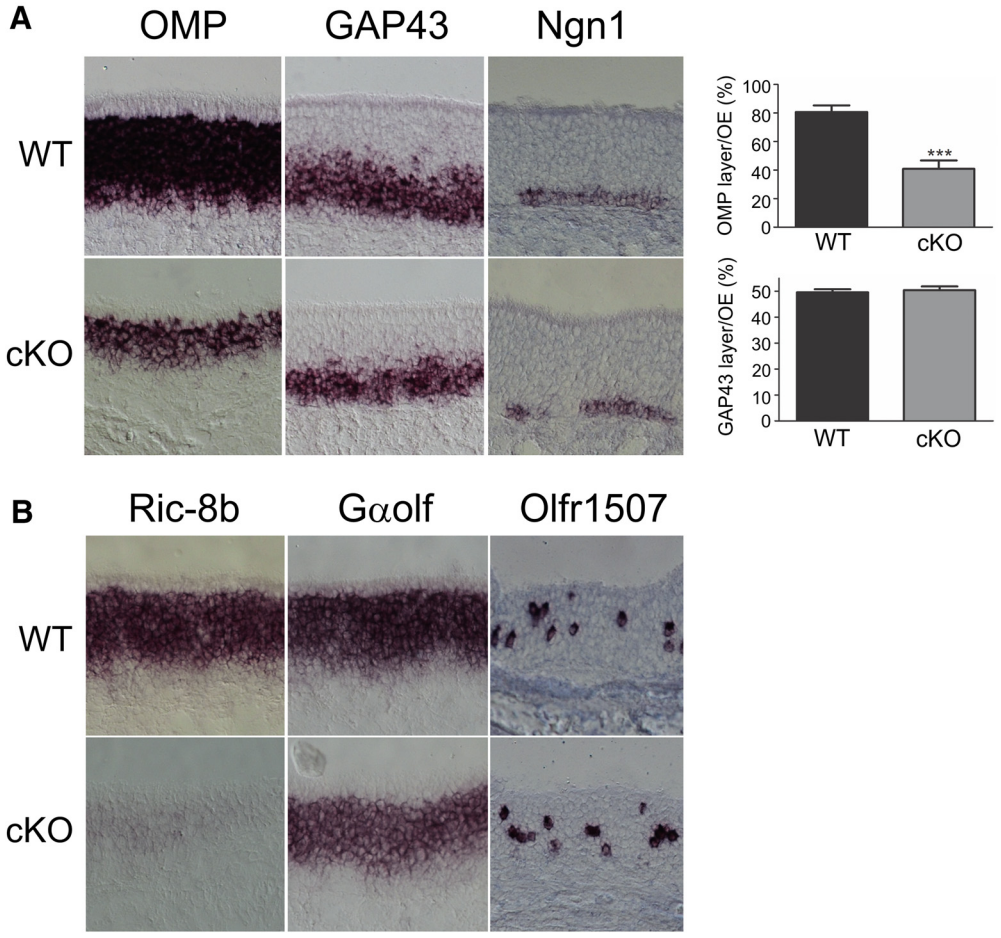

Figure 3. Organization of the Ric-8b-deficient olfactory epithelium. Coronal sections through the olfactory epithelium of WT and cKO 3-week-old mice were hybridized with antisense digoxigenin-labeled probes specific for (***A***) OMP, GAP43, Ngn1, and (***B***) Ric-8b, Gαolf, or Olfr1507, as indicated. ***A***, OMP, GAP43, and Ngn1 are respectively expressed in mature olfactory neurons, immature olfactory neurons, and neuronal progenitors. The thickness of the OMP and GAP43 layers relative to the whole OE thickness were quantified and are shown to the right. Values are mean ± SEM. ***$p < 0.0001$ in unpaired *t* test analysis.

$1.2 \times 10^{-5}/\mu m^2 \pm 0.61$; cKO $5.1 \times 10^{-5}/\mu m^2 \pm 0.91$; $p = 0.02$, $t_{(4)} = 3.49$, unpaired *t* test; Fig. 5*B*). These results indicate that the olfactory sensory neurons in the cKO olfactory epithelium are more susceptible to cell death.

Despite the significant reduction of the mature olfactory sensory neuron layer, Ric-8b cKO mice still have a residual number of OMP-positive neurons (Fig. 4*B*). Therefore, we next asked whether odorant-evoked Ca^{2+} responses occur in the olfactory sensory neurons from Ric-8b cKO mice by performing Ca^{2+} imaging of single dissociated cells. In these experiments, ~30% of the olfactory sensory neurons from WT mice, analyzed as a control, responded to an odorant mix containing nine different odorants at a 100 μM concentration (Fig. 6*A*,*B*). On the other hand, although olfactory neurons from cKO mice showed normal Ca^{2+} transients to KCl like the WT olfactory neurons, no analyzed cell was responsive to the odorant mix (Fig. 6*A*,*C*), demonstrating that odorant signaling is deficient in these neurons. These results are consistent with the finding that Gαolf protein, which is required for odorant signal transduction, is not expressed in the cKO olfactory epithelium.

Tyrosine hydroxylase (TH) expression in the periglomerular cells of the olfactory bulb is dependent on the afferent input from olfactory sensory neurons (Baker et al., 1983; Guthrie et al., 1990; Puche and Shipley, 1999). To evaluate to what extent odorant activation of olfactory sensory neurons is impaired in the cKO mice, we compared TH expression in the olfactory bulbs from WT and cKO mice. We found that in the mutant mice, TH expression is markedly reduced, similar to what is observed in odor-deprived or anosmic mice (Baker et al., 1999; Weiss et al., 2011; Fig. 6*D*; $n = 3$). The reduction is uniform in the olfactory bulb, with only a few scattered periglomerular cells retaining TH staining (Fig. 6*D*, inset), which is consistent with the inability of the mutant olfactory sensory neurons to respond to odorants.

Ric-8b cKO mice show altered behavior in olfactory tests

We first used the buried food-seeking test to check whether the cKO mice can detect odorants. In this test, a pellet of food is hidden beneath the bedding in the animal's cage and the time until the mouse retrieves the pellet is recorded. Mice that retrieve the pellet faster are considered to have a better sense of smell. As shown in Figure 7*A*, cKO mice took significantly longer to retrieve the pellet (WT 143.1 ± 30.2 s; Het 188.3 ± 24.5 s; cKO 378.5 ± 76.3 s; $p = 0.02$, $F_{(2,15)} = 4.54$, one-way ANOVA and Dunnett post-test).

To further analyze the olfactory function in the cKO mice we performed additional olfactory guided behavior tests. The ability to recognize food odorants (peanut butter and milk; Weiss et al., 2011) was evaluated by using an odorant preference test. These odorants were presented to the mice on a piece of filter paper and the investigation times were recorded. In these experiments, the

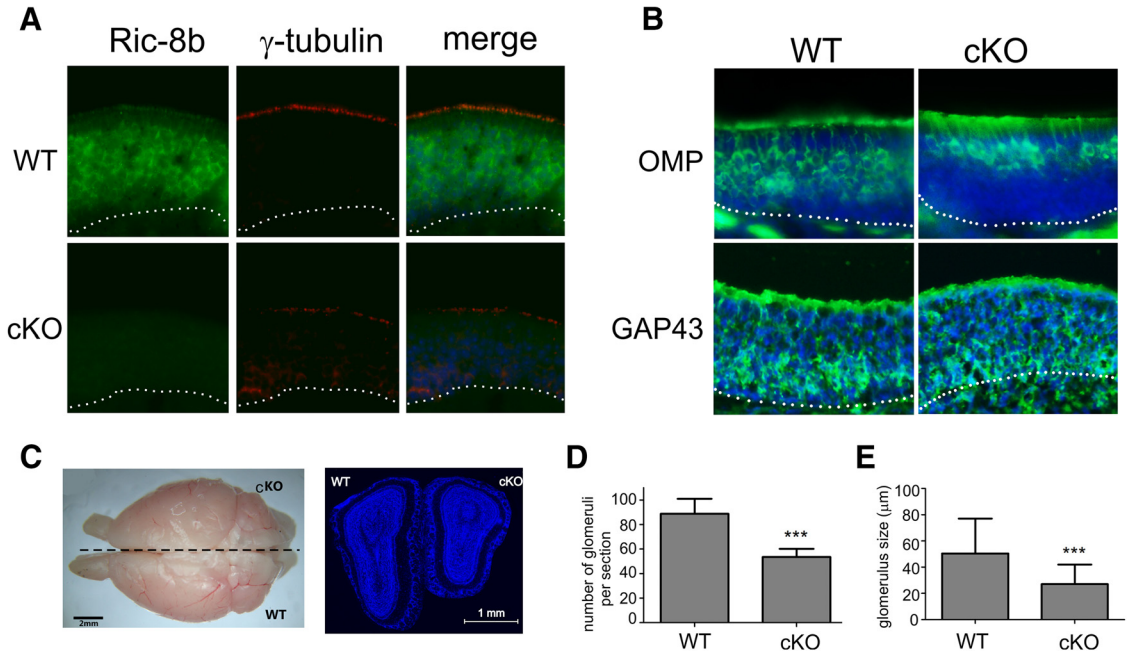

Figure 4. The number of mature olfactory neurons is reduced in the Ric-8b-deficient olfactory epithelium. ***A***, Double-immunostaining of the olfactory epithelium from WT and cKO mice for Ric-8b (green) and gamma-tubulin (red). Nucleus staining with DAPI is shown in blue. ***B***, Immunostaining (green) of the olfactory epithelium from WT and cKO mice for OMP and GAP43. Nucleus staining with DAPI is shown in blue. ***C***, Left, Dorsal view of the brain of WT (left half-brain) and of cKO (right half-brain) from 70-d-old (P70) mice. Right, DAPI stained olfactory bulb coronal sections images from WT and cKO mice. ***D***, The graph shows the number of glomeruli count per section of WT and cKO olfactory bulbs. Error bars represent the mean ± SEM of the number of glomeruli per bulb section. ***$p < 0.0001$, unpaired *t* test. ***E***, The graph shows the medium sizes of glomeruli in WT and cKO mice. Error bars represent the mean ± SEM of the sizes of glomeruli. The analysis of unpaired *t* test determined statistically significant differences. ***$p < 0.0001$.

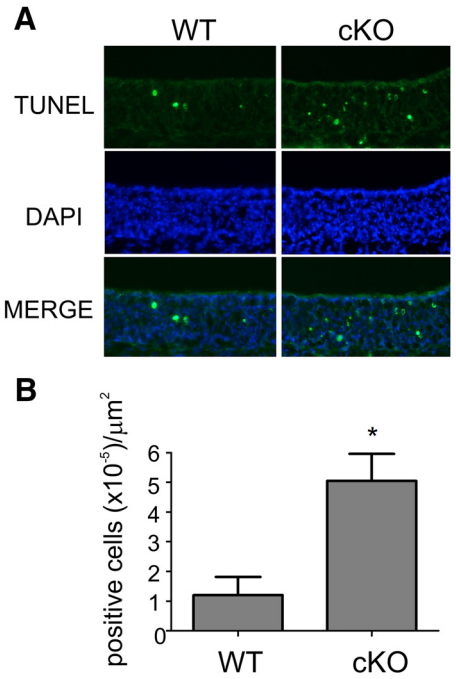

Figure 5. Increased cell death in the Ric-8b cKO olfactory epithelium. ***A***, Representative images of WT and cKO olfactory epithelium sections processed for TUNEL assay. ***B***, Error bars represent the mean ± SEM of three independent experiments from TUNEL in ***A***. The unpaired *t* test determined statistically significant differences. *$p < 0.05$. Mice were 21 d old (P21).

cKO mice showed reduced preference to milk and peanut butter, relative to water, a neutral stimulus, compared with WT and Het mice (peanut butter; WT 128 ± 13 s; Het 122 ± 10 s; cKO 99 ± 1 s; and milk; WT 142 ± 19 s; Het 155 ± 13 s; cKO 100 ± 2 s; $p = 0.01$, $F_{(14,56)} = 12.56$, one-way ANOVA and Dunnett post-test; Fig. 7 *B–D*).

The odorant butyric acid is known to induce avoidance behavior in rodents (Endres and Fendt, 2009). We next tested whether this behavioral response is altered in cKO mice. The experiments showed that although WT and Het mice displayed a significant avoidance response (WT 5.03 ± 3.13 min in the positive area; Het 3.25 ± 2.12 min), the cKO mice lacked this avoidance behavior (11.49 ± 1.7 min in the positive area; $p = 0.0001$, $F_{(5,22)} = 65.31$, one-way ANOVA and Dunnett post-test; Fig. 7*E–H*). Altogether, these experiments indicate that the sense of smell in the Ric-8b cKO mice is greatly impaired.

Because compromised olfactory function has been associated with anxiety and depression-like behaviors (Brunjes, 1992; Song and Leonard, 2005; Glinka et al., 2012; Chen et al., 2014), we performed a series of tests to explore whether the cKO mice display these types of behaviors. We used the open- field, light/dark, and elevated plus-maze tests as measurement for anxiety, and the tail suspension test as a model for depression. The results showed however that, even though the cKO mice show olfactory deficits, they have no evident anxiety, depression, or mobility deficits (data not shown).

Discussion

To examine the role played by Ric-8b in olfaction, we generated mice with a conditional deletion of the Ric-8b gene in the olfactory epithelium.

We found that Ric-8b- deficient olfactory sensory neurons do not express the Gαolf protein (Fig. 2*A*). This result agrees with the previous observation that in a *Ric-8b*$^{-/-}$ embryonic stem (ES) cell line the abundance of the Gαs protein, which can also interact with Ric-8b, was highly reduced (Gabay et al., 2011).

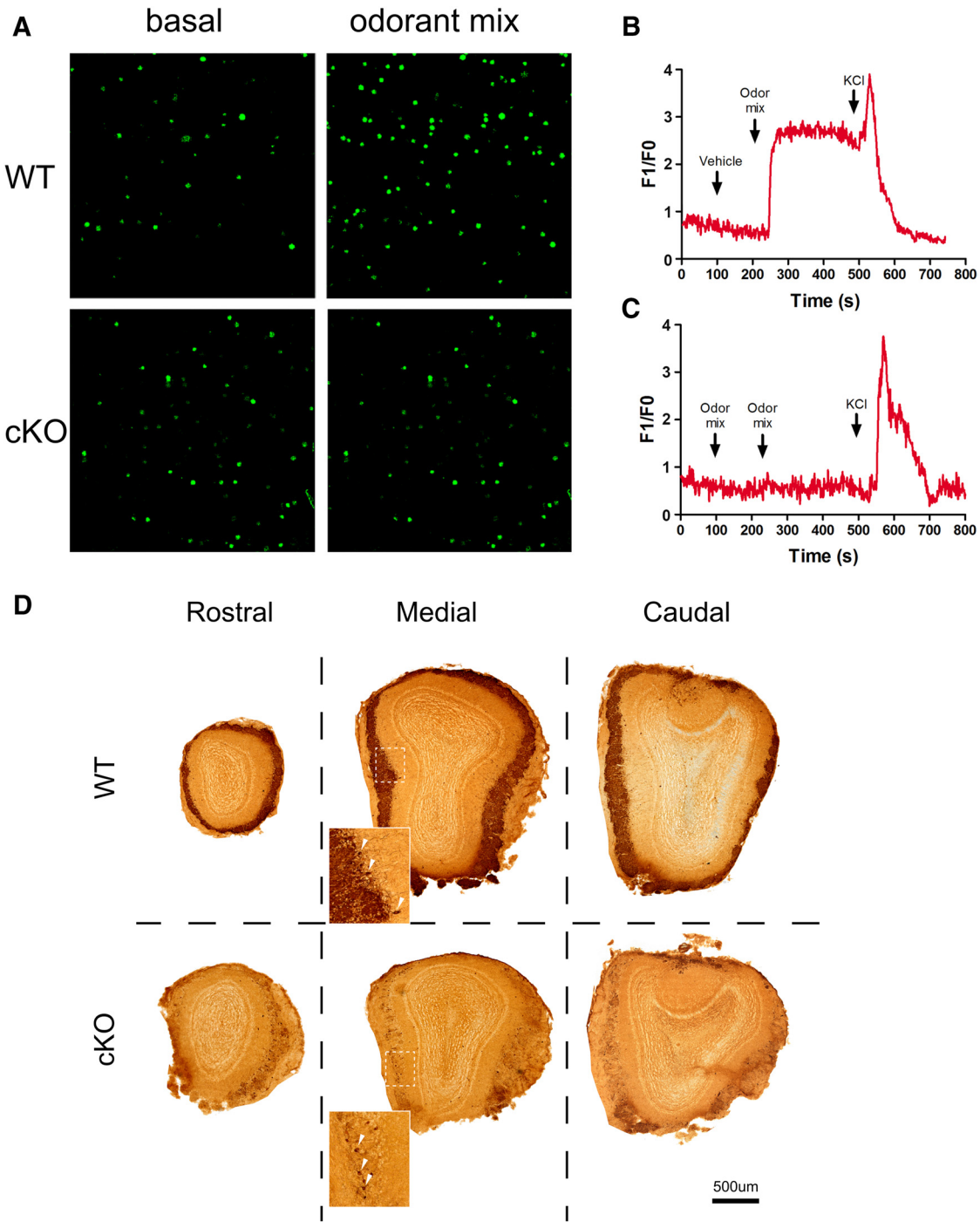

Figure 6. Olfactory sensory neurons from Ric-8b-deficient olfactory epithelium are not responsive to odorants. Ca^{2+} imaging of single olfactory sensory neurons dissociated from WT or cKO olfactory epithelia. ***A***, Images of fluorescence increase in WT or cKO olfactory sensory neurons when exposed to vehicle (basal) or an odorant mix containing nine different odorants at 100 μM concentration. ***B***, ***C***, Graphs show the fluorescent signal normalized as F1/F0 (F1, maximal fluorescence after addition of the odorant mix; F0, basal fluorescence before addition of the odorant mix) in imaged neurons from WT (***B***) and cKO (***C***) mice. In three different experiments, a total of 196 WT and 220 cKO olfactory sensory neurons responsive to KCl were analyzed. Of those, 59 (30%) of the WT neurons responded to the odorant mix, whereas none of the cKO neurons did. ***D***, TH immunostaining of the olfactory bulbs from WT and cKO mice. In the WT mice ($n = 3$), periglomerular cells and fibers within glomeruli are heavily stained. In the cKO mice ($n = 3$), glomeruli have only a few stained periglomerular cells and the fiber staining within the glomeruli is markedly reduced. Sections cut through the rostral, medial, and caudal regions of the olfactory bulbs are shown. Insets, Magnified regions as indicated; arrows denote individual periglomerular cells.

Also, knockdown of Ric-8b in NIH3T3 cells resulted in reduced Gαs protein levels (Nagai et al., 2010). Similar results were observed for an ES cell line that is knock-out for Ric-8a, a related GEF that interacts with different Gα subunits (Tall et al., 2003). In this case, the amounts of $G\alpha i_{1/2}$; Gαo; Gαq, and Gα13 proteins were reduced, but not the amount of Gαs, because Ric-8a does not bind to this last subunit (Gabay et al., 2011). In addition, overexpression of Ric-8b in heterologous cells enhanced Gαs or Gαolf protein levels (Kerr et al., 2008; Nagai et al., 2010; Chan et al., 2011b). Together with the fact that the Gαs and Gαolf genes are normally transcribed in the knock-out experiments (Fig. 3*B*; Nagai et al., 2010; Gabay et al., 2011), these results indicate that

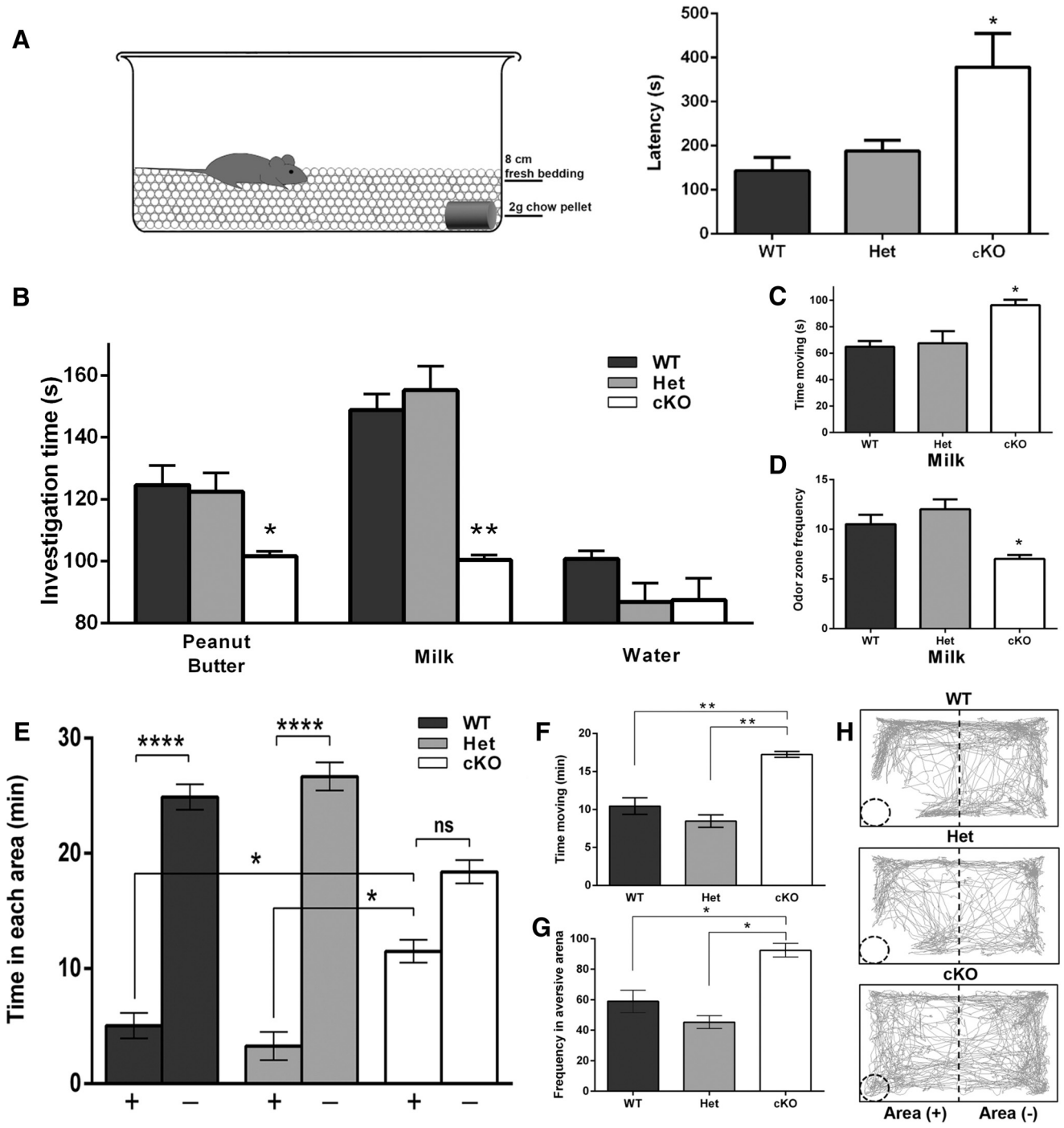

Figure 7. Mice with Ric-8b-deficient olfactory epithelium show impaired olfactory guided behavior. ***A***, Mice were submitted to the buried food-seeking test. Ric-8b cKO mice required significantly longer times to find the food. Mice were 50-d-old (P50). WT ($n = 9$), Het ($n = 6$), and cKO ($n = 9$). ***B–D***, Innate olfactory preference test. Mean investigation times were quantified in WT, Het, and cKO mice during a 3 min test period (***B***). In these experiments, even though cKO mice explored more of the cage than WT and Het mice (***C***), they showed significantly reduced preference for peanut butter and milk compared with water (***B***, ***D***). ***E–H***, Innate olfactory avoidance to butyric acid. Quantification of time spent in the positive area (+) and the negative area (−), during exposure to butyric acid indicates that cKO mice lack avoidance behavior to butyric acid (***E***). In addition, cKO mice explored more of the cage than WT and Het mice (***F***, ***G***). ***H***, Examples of the trajectory plots of a WT, Het, and cKO mouse (30 min video-tracking). The location of butyric acid is indicated by the dashed circle in the lower left corner. For the innate olfactory preference and innate olfactory avoidance test, mice were 60-d-old (P60). WT ($n = 9$), Het ($n = 4$), and cKO ($n = 4$). Values are mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.001$, and ****$p < 0.00001$; one-way ANOVA and Dunnett post-test. n.s., Not significant.

Ric-8b regulates the abundances of these Gα subunits at the post-transcriptional level.

Newly synthesized Gα subunits are first folded in the cytosol, then they are targeted to intracellular membranes, such as the endoplasmic reticulum or Golgi, where they bind to the G$\beta\gamma$ subunits (Marrari et al., 2007). Gα subunit association with G$\beta\gamma$ is required for trafficking of the heterotrimer to the plasma membrane. Previous studies have shown that in *Ric-8a*$^{-/-}$ ES cells the Gα subunits remain unfolded in the cytoplasm, do not associate with the membrane and are rapidly degraded, suggesting that

Ric-8a acts as a chaperone to fold nascent Gα subunits (Gabay et al., 2011; Chan et al., 2013). Consistent with this role, is the demonstration that interaction between Ric-8b and Gαs inhibits ubiquitination (and consequently degradation) of Gαs (Nagai et al., 2010). Altogether these results indicate that Ric-8b plays a similar role, and acts as a chaperone for Gαolf in the olfactory sensory neurons.

Although Ric-8b can act as a GEF *in vitro*, it is not clear whether it acts as a GEF to activate Gαolf *in vivo*. It is possible that the GEF activity may be solely required for the chaperoning activity of Gαolf (Papasergi et al., 2015). Alternatively, Ric-8b may have multiple functions within the cell (Hinrichs et al., 2012). Our immunostaining results show that the Ric-8b protein is abundantly located in the cytosol, which is not consistent with a role in signaling. If low levels of Ric-8b protein in the cilia can still bind to Gαolf and amplify odorant signal transduction in the olfactory sensory neurons, one would expect that in the cKO mice, signaling would be reduced compared with that of WT mice. However, because there is no Gαolf in the Ric-8b cKO olfactory neurons, we cannot examine whether signaling through Gαolf is altered in the absence of Ric-8b. Ric-8b could also play a role in the dendritic knobs, where the basal bodies serve as the organizing center from which the microtubules of the cilia emerge (Jenkins et al., 2009; Williams et al., 2014). It is also important to notice, that two major different Ric-8b isoforms are expressed in the olfactory neurons, the full-length Ric-8b, which displays GEF activity, and Ric-8bΔ9, which does not (Von Dannecker et al., 2005). These two forms of Ric-8b may play different roles, and may be localized to different subcellular compartments. Because in the *Ric-8b* cKO mice both forms are deleted, we cannot discriminate the roles played by each one of them. Thus, whether Ric-8b plays additional functions in the olfactory sensory neurons remains to be determined.

We found that the layer of mature olfactory sensory neurons in the olfactory epithelium of cKO mice is thinner than in WT mice, indicating that there is a significant loss of mature olfactory sensory neurons (Fig. 4*B*). In the cKO mice, deletion of the Ric-8b gene occurs after expression of OMP, so that differentiation of progenitors to mature olfactory sensory neurons must proceed normally (Rodriguez-Gil et al., 2015). Therefore, incomplete development cannot explain the reduced number of olfactory sensory neurons. The loss in olfactory sensory neurons may however be explained by the observation that the olfactory epithelium from cKO mice contain a larger number of dying cells (Fig. 5).

The number of olfactory neurons in the olfactory epithelium is normally controlled through a continuous process of turnover involving neurogenesis and cell death (Cowan and Roskams, 2002; Schwob, 2002). Previous studies have shown that odorant activation and neuronal activity promote survival of olfactory sensory neurons (Farbman et al., 1988; Watt et al., 2004; Suh et al., 2006; Zhao et al., 2013). The *Ric-8b* cKO olfactory sensory neurons do not express Gαolf, which is required for odorant signaling (Belluscio et al., 1998), which, in agreement with the studies mentioned above, would lead to decreased neuronal survival rates. It cannot be excluded though that additional Ric-8b functions contribute to neuronal survival, because it is not clear yet whether deletion of Gαolf alone also leads to neuronal cell death (Belluscio et al., 1998). However, the fact that the vomeronasal neurons also depend on the Gαi2 and Gαo subunits (which are, respectively, coexpressed with the V1R and V2R pheromone receptors) for cell survival, strongly indicates that G-protein signaling is required for survival of these highly specialized types of sensory neurons (Tanaka et al., 1999; Norlin et al., 2003; Chamero et al., 2011; Oboti et al., 2014). In addition, the observation that olfactory neurons that do not express a functional OR show a reduced half-life also supports a role for G-protein signaling in survival (Wang et al., 1998).

Behavioral tests indicated that the *Ric-8b* cKO mice display olfactory deficits (Fig. 7), which is consistent with the observation that the olfactory neurons from these animals were unresponsive to a set of commonly tested odorants (Fig. 6). Previous experiments have shown that genetically modified mice that have olfactory deficits display anxiety-like and depressive-like behaviors (Glinka et al., 2012; Chen et al., 2014). We performed a battery of tests to evaluate whether the *Ric-8b* cKO mice show the same type of behaviors, but the experiments showed no evidence of abnormal motivational and mobility behaviors compared with their WT or Het siblings. Several factors could account for the observed divergence. Because the behavioral tests used involve multiple sensory cues, it is possible that the *Ric-8b* cKO mice have used additional nonolfactory cues to perform the experimental tasks. It is also possible that the genetically modified mice used in the previous studies show additional modifications in the nervous system. For example, the *Cnga2* gene, which is deleted in the anosmic mice used in these experiments, is also expressed in some brain regions (Kingston et al., 1999; Mandiyan et al., 2005). Within this context, the Ric-8b cKO mice may constitute a well suited animal model to evaluate the role played by olfaction in different types of behaviors, because the gene deletion occurs specifically in the olfactory epithelium. Nevertheless, appropriate controls would need to be performed, to exclude the possibility that a few neurons in the brain coexpress OMP and Ric-8b, leading to the loss of the Ric-8b gene in additional nonolfactory neurons (Oboti et al., 2014).

Our results show that some OMP-expressing neurons persist in the Ric-8b-deficient olfactory epithelium. It is unclear whether these are neurons that expressed OMP (and Cre) more recently, and are destined to die, or whether these neurons represent a subpopulation of neurons that express different types of receptors that do not signal through Gαolf, and therefore survive in the cKO mice. Further characterization of these neurons and identification of the receptors that are expressed by these cells should clarify this point.

In summary, by using conditional gene ablation in the olfactory epithelium, we demonstrated that Ric-8b is essential for the normal function of the olfactory system in mice. We show that Ric-8b is required for expression of the Gαolf protein. The Ric-8b-deficient olfactory sensory neurons were not activated by odorants and showed increased rate of cell death. Further studies of the *Ric-8b* cKO mice may provide valuable insights into the mechanisms through which Ric-8b regulates Gαolf protein abundance and neuronal death.

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