Ral GTPase Controls Cell Polarity Organization during Epithelial Tissue Remodeling

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Abstract

Ral, a Ras-like GTPase, is an essential component of Ras-driven oncogenesis that supports tumor initiation and progression. We explored the role of Ral in the polarity organization of epithelial tissue in Drosophila. We found that Ral is dispensable for the polarization of proliferative epithelial tissue, and that Ral is required for the maintenance of apical-basal polarity of post-mitotic epithelial cells during tissue remodeling. More precisely, lack of Ral activity results in loss of cortical apical aPKC and lateral Lgl, and in apical spreading and accumulation of Armadillo/beta-catenin. Our analysis demonstrates that Ral regulates polarity organization by acting as a negative regulator of JNK and p38 MAPK signaling. Ral controls aPKC apical localization by down-regulating JNK and controls Armadillo localization at adherens junction by down-regulating p38 MAPK signaling, whereas Lgl membrane localization depends on both pathways. Finally, in the absence of Ral function aPKC becomes dispensable for Lgl basolateral localization and Armadillo is recruited to the cell membrane independently of DE-Cadherin and Bazooka localization. This suggests that additional mechanisms control Lgl and Arm distribution in polarized epithelia.

Keywords: Ral GTPase; Epithelium; Cell polarity; Signal transduction; Drosophila melanogaster

Introduction

Ral, a Ras-like GTPase, is an essential component of Ras-driven oncogenesis supporting tumor initiation and progression [1,2]. Moreover, the important role of Ral in many cellular events affected in cancer cells, such as polarized exocytosis, apoptosis, autophagy and migration, was recently demonstrated [3-6]. Apical-basal (AB) polarity is a fundamental to epithelial organization and several human pathologies, including cancers, are associated with defects in epithelial polarity. Yet the role of Ral in epithelial cell polarity is only partially deciphered [7-10]. In particular, the analysis of the Ral polarity function in Drosophila developing tissue has been conducted using overexpression of dominant active or negative Ral forms. This analysis has demonstrated a Ral function in AB polarization of dividing neuroblasts [7].

Epithelial cells comprise at least four distinct cortical domains: an apical domain, an apical-lateral junction (the adherens junction in Drosophila), a lateral domain and a basal domain [11,12]. AB polarity is achieved by the concerted action of several conserved protein complexes. The Crumbs complex (Crumbs/Pals/Par3) and the Par complex (aPKC/Par6/Par3) are associated with apical domain. Adherens Junction (AJ) complex contains DE-Cadherin (DE-Cad), Armadillo/β-catenin (Arm) and γ-catenin. Basolateral domain contains the Scribble complex (Scribble/Dlg/Lgl) and the recently identified Yurt/Coracle group (Yurt/Coracle/NaK-ATPase/Nrx-IV). Maintenance of AB polarity depends in part on antagonistic interactions between the polarity complexes, which define distinct cortical domains and the position of the adherens junctions [13-20].

Follicular epithelium of Drosophila ovary is an important model system to analyze the mechanisms underlying establishment and maintenance of epithelial cell polarity in vivo. Ovarian follicle or egg chamber is the structural and functional unit of insect ovary. When cell divisions end each egg chamber consists of 16 germ line cells (15 nurse cells and oocyte) surrounded by a monolayer of approximately 850-900 somatic epithelial cells called Follicle Cells (FC). Egg chambers are formed in the anterior tip of the ovariole, in a region called germarium. They bud from the germarium (stage 2) and continue to move posterior through the ovariole as oogenesis proceeds. Initial polarization of FC takes place within the germarium and in stage 1 egg chambers [21]. FC stop proliferating at stage 6/7, enter an endocycle and begin to differentiate. By stage 8, they are fully differentiated along the anterior-posterior axis of the oocyte, forming a morphologically uniform cuboidal epithelium, around the germine tissue [22]. Follicular epithelium undergoes tissue remodeling including both extensive cell shape changes and directed cell migration during stage 9. These morphogenetic rearrangements result in the characteristic organization and distribution of cells in egg chambers at stage 10A. Majority of FC cover the posterior-positioned oocyte and take on a distinctive, highly columnar morphology and eventually synthesize the eggshell. Concurrently, the most anterior FC forms a so-called squamous epithelium, which covers the nurse cells [23-25]. Hence, ovarian follicle epithelium development permits to distinguish and analyze gene functions underlying polarity maintenance during either mitotic divisions or epithelial tissue remodeling.

Using a newly generating Ral null loss-of-function allele, we show that Ral is not required for the polarization of dividing epithelial cells, but that it is essential to maintain the polarity of post-mitotic epithelial cells during tissue remodeling. We demonstrate that the absence of Ral activity causes loss of apical aPKC, loss of lateral Lgl and Lgl cytoplasmic mislocalization, and Arm apical spreading and accumulation. Our genetic analysis indicates that Ral controls epithelial polarity by down-
regulation of the JNK and p38 MAPK pathway activities. Finally, our analysis of the Ral loss of function suggests additional mechanisms for the maintenance of polarity in post-mitotic epithelial cells since in the absence of Ral function, aPKC becomes dispensable for Lgl basolateral localization and Arm is recruited to the cell membrane independently of DE-Cad localization.

Materials and Methods

Fly stocks and genetics

Mutations and constructs are described in FlyBase (http://flybase.bio.indiana.edu). All mutant alleles used in the study are either null or strong loss of function mutations. The following mutant alleles were used: msn172; bsk; mpk2 (p38a); 14-3-3e (28B); mekk1; fai; vnd; sec511; Rap1; puc228. The following UAS transgenes were used in this study: msn172; UAS-Flp; FRT101; T155-Gal4, UAS-Flp flies (Ral polarity phenotypes); in Ral FRT101/ Ubi-GFP, FRT101; mutX/+; T155-Gal4, UAS-Flp (2nd chromosome) or Ral, FRT101/Ubi-GFP, FRT101; mutX/T155-Gal4, UAS-Flp (3rd chromosome) (modulations of Ral-induced polarity phenotypes). msn172 and mpk2 control clones were induced using hsFlp and 2 hours 37°C heat shocks on two consecutive days during the second instar. Clones were analyzed in y, w, hs Flp/+; msn172, FRT80B; Ubi-GFP, FRT80B and in y, w, hs Flp/+; FRT82B, mpk2/FRT82B, Ubi-GFP (respectively). Ral germline clones were generated by the FRT/FLP-ovoD system [27] using hs FLP and 2 hours 37°C heat shocks on two consecutive days during the second instar.

Immunohistochemistry

Egg chambers were fixed and stained as previously described [28]. Primary antibodies were: mouse anti-Grk 1D12 (1:10; Developmental Studies Hybridoma Bank, DSHB), goat anti-Steffen (1:50, provided by A. Guichet), rat anti-DE-cadherin DCAD2 (1:20; DSHB), mouse anti-Arm N27A1 (1:50; DSHB), mouse anti-Crumbs Cq4 (1:50; DSHB), rat anti-DE-cadherin DCAD2 (1:20; DSHB), mouse anti-Arm N27A1 (1:50; DSHB), mouse anti-Crumbs Cq4 (1:50; DSHB), rat anti-DE-cadherin DCAD2 (1:20; DSHB), mouse anti-Arm N27A1 (1:50; DSHB), mouse anti-Crumbs Cq4 (1:50; DSHB), rat anti-DE-cadherin DCAD2 (1:20; DSHB), mouse anti-Arm N27A1 (1:50; DSHB), mouse anti-Crumbs Cq4 (1:50; DSHB), rat anti-DE-cadherin DCAD2 (1:20; DSHB), mouse anti-Arm N27A1 (1:50; DSHB), mouse anti-Crumbs Cq4 (1:50; DSHB), rat anti-DE-cadherin DCAD2 (1:20; DSHB), mouse anti-Arm N27A1 (1:50; DSHB), mouse anti-Crumbs Cq4 (1:50; DSHB), rat antidependent on the cell membrane independently of DE-Cad localization.

Biochemistry

Protein extracts from whole embryos and 1st instar larvae were prepared as previously described [4]. 75 μg of proteins/lane were separated in 12%-acrylamide SDS-PAGE gels, transferred on nitrocellulose membranes and incubated overnight at 4°C using primary antibodies: anti-drosophila-Ral (1:500), mouse anti-p-JNK (1:250, Santa Cruz Biotechnology), rabbit anti-ERK1/2 (1:1000, Sigma). A polyclonal antibody against Drosophila Ral protein was raised in guinea pig by injection of the entire Ral protein (produced in bacteria as GST-fusion) (Eurogentec, Belgium).

Results

Ral is implicated in the maintenance of apical-basal polarity in post-mitotic epithelial cells

Two classes of Ral alleles, lethal or viable but displaying a loss-of-..
clones for aPKC, Lgl and Arm, respectively) (Figure 1H). Thus, Ral loss of function altered polarity organization of the FC that have stopped proliferating and come through morphogenetic rearrangement.

**Ral controls polarity organization of FC by regulating JNK and p38 MAPK activation**

Previously we showed that Ral induced apoptosis during sensory organ development by acting as an upstream regulator of the JNK and the p38 MAPK pathways [4]. Accordingly, we observed that lack of Ral activity in Ral 

The strong increasing of phospho-JNK level was also observed in homozygote Ral 

We observed that reduction of the activities of Bsk, the Jun N-terminal Kinase (JNK) or Msn, the MAP4K4 orthologous of vertebrate HGK were sufficient to rescue the aPKC (n=12/15 and n=12/12 clones, respectively) and Lgl (n=10/10 and n=11/12 clones, respectively) polarity defects observed in Ral 

We therefore explored genetic interactions between Ral and the components of JNK and p38 MAP kinase pathways using either loss-of-function mutations and overexpression of Dominant Negative (DN) alleles to reduce signaling or overexpression of Wild-type (WT) forms to promote these pathways (Figures 2C-N and S2A-1).

We observed that the absence of Ral function results in loss of aPKC and Lgl membrane localization (Figures 2C and 2D).

To test whether overactivation of JNK signaling induces polarity defects in follicle epithelium we overexpressed activated form of hepCA hermipterous JNKK (hepCAhermipterous JNKK) under the control of Tj-Gal4 driver in all FC [30]. Polarization of FC as well as FC polarity organization during mitotic divisions was not affected, and both aPKC and Lgl were correctly localized from stage 2 till stage 8 egg chambers (data not shown). Hence, massive apoptosis was induced after stage 8 and no
egg chambers older than early-stage 9 was observed. Although these results confirmed that the JNK overactivation doesn’t affect apical-basal polarity of FC during mitotic divisions the massive cell death of the post-mitotic follicular epithelium precluding any detailed analysis of its role during tissue remodeling.

Neither up- nor down-regulation of JNK pathway modified apical accumulation of Arm in Ral\(^{70a}\) FC (Figures 2K and 2N and data not shown) suggesting that another Ral signaling pathway is implicated in Ral regulation of Arm localization. We therefore tested whether the p38 MAPK signaling modulates the Ral loss of function polarity.

Figure 2: JNK and p38 MAPK signaling cascades in Ral-dependent polarity phenotypes. 
A. Top view of Ral\(^{35d}\) (Ral\(^{35d};\) pucE69/+) and Ral\(^{70a}\) mutant and control (pucE69/+) follicle cells which are stained for phospho-JNK (Promega) (red). Lack of Ral activity in Ral\(^{70a}\) as well as decreasing Ral activity in Ral\(^{35d}\) hypomorphic mutant resulted in elevated level of phosphorylated JNK. Lectin-FITC (green) is used to mark the membranes in Ral\(^{35d}\) hypomorphic and control FC. Ral\(^{70a}\) clones are marked by the absence of GFP (green).

B. Western blot analysis of pJNK protein level in wild-type (w) and in Ral\(^{70a}\) embryos (e-70a) and 1st stage larvae (l-70a). Extracts of proteins from whole embryos or larvae were tested using antibodies against phospho-JNK (Santa Cruz Biotechnology). Increased level of pJNK observed in Ral\(^{70a}\) homozygotes indicates that lack of Ral activity results in JNK activation. No pJNK could be detected in wild type embryo, in spite of the fact that twice more of total protein was loaded (loading ratio 2.0). Erk was used as a loading control.

C-N. Confocal microscopy images of FC stained (red) for aPKC (C, F, I and L), Lgl (D, G, J and M) and Arm (E, H, K and N) present genetic interactions between Ral\(^{70a}\) and the mutants of JNK and p38 MAPK pathways. The Ral-induced polarity phenotypes (C-E) were suppressed by decreasing either p38 MAPK (G, H) or JNK (I, J, L and M) signaling. Yellow line indicates Ral\(^{70a}\) GFP-negative FC.
phenotypes. The decrease of p38 MAPK signaling using mpk2\(^{-}\) (encodes mpk2\(^{\text{p38 MAPK}}\)) (\(n=13/16\) clones) restored wild-type Arm localization at AJ in Ral-deficient FC (Figure 2H) and decreasing the protein level of p38 MAP3K (encoded by mekk1\(^{1,236\text{NE}}\)) resulted in partial rescue of Ral-induced Arm phenotype (\(n=16/23\) clones) (Figure S2I). Furthermore, decreasing of p38 MAPK signaling in Ral\(^{\text{RF}}\) FC restored wild-type membrane localization of Lgl (\(n=16/17\) for mpk2\(^{-}\) and \(n=14/16\) clones for mekk1\(^{1,236\text{NE}}\)) (Figures 2G and S2H). Ral-induced aPKC phenotype was not modified by down-regulation of p38 MAPK signaling (\(n=8/11\) for mpk2\(^{-}\), Figure 2F) and (\(n=11/12\) clones for mekk1\(^{1,236\text{NE}}\), data not shown). The genetic interactions observed between Ral and p38 MAPK mutants indicate that Ral controls Arm and Lgl localizations by negatively regulating the p38 MAPK signaling pathway in FC during tissue remodeling (Figure 3G).

Thus, our genetic analysis reveals Ral function in aPKC apical localization by down-regulating JNK signaling and Ral function in Arm localization at AJ by down-regulating p38 MAPK signaling. Furthermore, Ral controls Lgl lateral localization by down-regulating both JNK and p38 MAPK pathways.

MAP kinase Wnd, ubiquitin hydrolase Faf and E3-ligase Hiw act as effectors of Ral activity in the regulation of epithelial polarity

Our analysis of Ral function in FC polarity organization enables us to further investigate the function of additional components of the JNK and p38 MAPK pathways. Wallenda (wnd\(^{\text{D}}\)) (an ortholog of vertebrate kinases DLK and LZK) is a MAP kinase kinase kinase (MAP3K) in JNK signaling (in Drosophila nervous system, [31] but also proposed to act as MAP3K in p38 MAPK signaling (in C. elegans, [32]). We analyzed whether a decrease of Wnd signaling affects Ral-dependent polarity phenotypes. The decrease of Wnd activity in Ral-deficient FC (Ral\(^{\text{RF}}\) clones induced in wnd\(^{\text{D}}\) or in T155-Gal4>UAS-wnd\(^{\text{D}}\) FC) suppressed Ral-dependent aPKC phenotype (\(n=17/26\) and \(n=8/10\) clones) and Lgl phenotypes (\(n=23/27\) and \(n=7/7\) clones) but did not modify Ral-dependent Arm phenotype (\(n=9/9\) clones for wnd\(^{-}\)) (Figure 3A-3C, S2J and S2K). These results indicate that Wnd acts as MAP3K in the JNK pathway in FC (Figure 3G).

A role for Wnd in JNK signaling was further confirmed by the analysis of two of its upstream regulators, Highwire (hiw) and Fat facet (faf). Wnd has been shown to be down-regulated by the E3-ubiquitin ligase Hiw (mycBP2 in mammals) and up-regulated by the ubiquitin hydrolase Faf (USP9X in mammals) [31]. We observed that overexpression of Hiw or down-regulation of Faf (faf\(^{\text{BX4}}\) context) in Ral\(^{\text{RF}}\) FC restored wild-type localization of Lgl (\(n=14/15\) or \(n=13/13\) clones, respectively) and aPKC (\(n=11/11\) or \(n=20/27\) clones, respectively) but did not affect Arm apical spreading and accumulation (\(n=13/17\) clones for faf\(^{\text{BX4}}\)) (Figures 3D-3F, S2L and S2M). Thus, we obtained the similar rescues as those produced by down-regulation of Wnd or of JNK signaling in Ral-deficient FC.

**Discussion**

**Ral loss of function suggests additional mechanisms for the maintenance of polarity in post-mitotic epithelial cells**

Our analysis of Ral-deficient follicular epithelium demonstrates that Ral GTPase is required for the maintenance of AB polarity in polarized post-mitotic epithelial cells during morphogenetic reorganization of follicular epithelium. Lack of Ral signaling results in polarity defects: loss of cortical apical aPKC, and lateral Lgl, and Arm apical spreading and accumulation. Low fluorescent level of aPKC and increased of Arm observed in Ral mutant FC (Figure 1) indicates that lack of Ral function affect either the aPKC or Arm protein levels or their post-transcriptional modifications.

Ral supports polarity organization of FC by acting as an upstream negative regulator of MSN/JNK and MEKK1/p38 MAPK signaling pathways. Ral controls aPKC apical localization by down-regulating JNK signaling and Arm localization at AJs by down-regulating p38 MAPK signaling, whereas Ral regulation of Lgl membrane localization depends on both JNK and p38 MAPK pathways. While we cannot exclude that polarized secretion is affected in absence of Ral function, the rescue of Ral polarity phenotypes by JNK and MAPK loss of function suggests that over activation of JNK and/or p38 MAPK signaling in the absence of Ral function results in the defects of polarity organization in polarized post-mitotic epithelial cells. Moreover, our data demonstrated that in the absence of Ral signaling in polarized post-mitotic epithelial cells both basolateral anchoring and apical exclusion of Lgl can be maintained in the absence of apical aPKC, and apical
mislocalization and accumulation of Arm takes place independent of Baz and DE-Cad wild-type localization in AJs.

In current models of polarity organization of epithelia, the regulation of Lgl by aPKC phosphorylation is critical in the control of the membrane domains integrity whereas Baz/Par3 determines the AJ domain by playing a key role in positioning the DE-Cad/β-catenin complex [12,15,33]. Baz/PAR-3 apical exclusion and its restriction to the apical side of the lateral domain are also regulated by the apical complex (Crumbs and aPKC) [15,17,34] and the phosphorylation of Baz on Serine 980 (pBazS980) by aPKC is essential for Baz apical exclusion and its localization at AJ [35,36].

Strikingly, our analysis reveals that in the absence of Ral function in polarized post-mitotic epithelial cells, aPKC becomes dispensable for maintaining Lgl basolateral localization. Indeed, in Ral−/− clones both apical pAKC and lateral Lgl membrane localizations are compromised (Figure 1) and the decrease of p38 MAPK signaling in Ral−/− FC can restore Lgl localization at the basolateral domain in the absence of apical aPKC (Figure 2). Thus, in the absence of Ral signaling both Lgl cytoplasmic release and Lgl basolateral membrane anchoring seem independent from aPKC apical localization and aPKC kinase activity. These results indicate the existence of additional mechanisms of Lgl lateral localization in the absence of aPKC polarization that can be achieved by the polarized basolateral delivery of Lgl or by the apical exclusion of Lgl by a yet unknown kinase.

In addition, in the absence of Ral signaling the wild type localization of Baz at AJ was observed whereas apical aPKC membrane localizations was compromised in Ral−/− FC (Figure S1A) as well as in Ral−/−, mpk2−/− FC (data not shown) indicating that Baz distribution is not controlled by apical aPKC and the phosphorylation of Baz by aPKC is not essential for Baz localization during late oogenesis.

Furthermore, in the absence of Ral activity in polarized post-mitotic FC Arm distribution becomes largely independent of DE-Cad and Baz. In fact, Arm apical spreading and accumulation is observed in spite of the fact that both Baz and DE-Cad are correctly localized at AJ (Figure S1). Thus, the interactions DE-Cad/Arm and Baz/Arm seem to be not sufficient to properly localize Arm suggesting that in polarized epithelial cells Arm might be recruited to the cell membrane independently of DE-Cad localization.

Accordingly, analysis of AJ organization and dynamics in polarizing epithelial cells and in polarized epithelial cells demonstrated that in the polarized epithelial cells during tissue remodeling Baz localization was largely independent of aPKC [17] and Arm localization at AJs are less controlled by Baz [34] as well as Arm membrane dynamics could be distinct from the one of DE-Cad [37,38].

Thus, analysis of Ral loss of function during epithelial morphogenesis in Drosophila ovaries reveals that additional mechanisms might control Lgl and Arm distribution in polarized epithelial cells during tissue remodeling.

Conclusions

Our data demonstrate that Ral GTPase is required for the maintenance of AB polarity during tissue remodeling associated with cell shape changes. Lack of Ral activity during morphogenetic reorganization of follicular epithelium causes loss of apical aPKC, loss of lateral Lgl and its cytoplasmic mislocalization, and Arm/β-catenin apical spreading and accumulation. Our analysis demonstrates that Ral regulates polarity organization of FC acting as an upstream negative regulator of MSN/JNK and MEKK1/p38 MAPK signaling pathways. Ral controls aPKC apical localization by down-regulating JNK signaling and Arm/β-catenin localization at adherens junctions by down-regulating p38 MAPK signaling, whereas Lgl membrane localization depends on down-regulation of both JNK and p38 MAPK signaling pathways. Furthermore, analysis of Ral loss of function during epithelial morphogenesis in Drosophila oogenesis demonstrates that additional mechanisms control Lgl and Arm distribution in polarized epithelial cells during tissue remodeling.

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