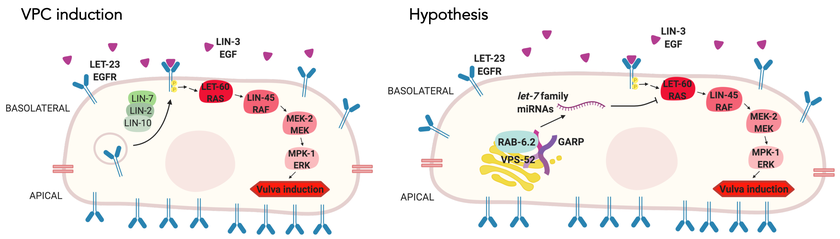
The RAB-6.2 GTPase is a potential regulator of small RNAs and Ras in *C. elegans*

Sarah Gagnon[1,2], Kimberley D. Gauthier[1,2], and Christian E. Rocheleau[1,2,3]

[1]Research Institute of the McGill University Health Center; [2]Department of Anatomy and Cell Biology, McGill University; [3]Department of Medicine, McGill University

INTRODUCTION

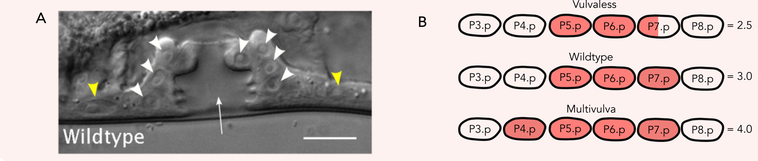
- Overactivation of epidermal growth factor receptor (EGFR)/Ras/MAPK signaling underlies many cancers
- *Caenorhabditis elegans* vulval development can model EGFR/Ras/MAPK as this pathway specifies the vulval cell fate in vulval precursor cells (VPCs)
- The LIN-2/LIN-7/LIN-10 protein complex is essential for basolateral localization and activation of LET-23 EGFR in VPCs. Mutants show severe vulvaless phenotypes
- We found an antagonistic relationship between RAB-6.2 and EGFR/Ras/MAPK-mediated vulva induction but the mechanism behind this remains unclear, although out previous worked ruled out a role for RAB-6.2 in trafficking of EGFR
- The RAB-6.2 effector VPS-52 promotes the activity of microRNAs in *C. elegans*, and the *let-7* family of microRNAs represses *let-60* Ras
- This raises the possibility that RAB-6.2 negatively regulates Ras signaling through the *let-7* family of miRNAs



METHOD: VPC induction scoring

Upon activation of LET-23 EGFR/LET-60 Ras signaling in wildtype animals, 3 VPCs divide to generate 22 vulval cells. VPC progeny (Fig. 1A, white arrowheads) can be identified by their position around the developing lumen (Fig. 1A, arrow) to determine which VPC was or was not induced. VPC induction scores thus provide a quantitative readout of signaling. Signaling overactivation generates a score greater than 3 associated with a multivulva phenotype, while underactivation yields a score lower than 3 associated with a vulvaless phenotype (Fig. 1B).

Figure 1

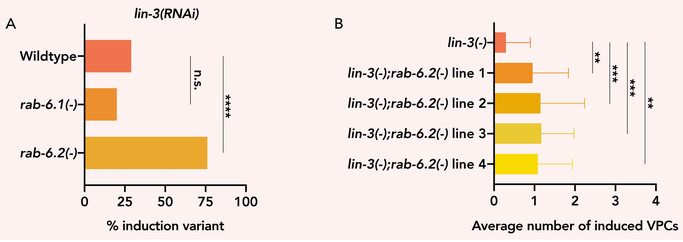


RESULTS

rab-6.2(ok2254) enhances the vulvaless phenotype of *lin-3(RNAi)*, but suppresses the *lin-3(-)* mutant vulvaless phenotype

Loss of the EGF ligand *lin-3* causes insufficient signaling and a vulvaless phenotype. The *lin-3(RNAi)* phenotype is enhanced in *rab-6.2(ok2254)* but not *rab-6.1(tm2124)* mutants (Fig. 2A), suggesting a positive regulatory role for RAB-6.2 in Ras signaling. However, the vulvaless phenotype of *lin-3(-)* mutants is suppressed by *rab-6.2(ok2254)* (Fig. 2B), suggesting instead an antagonistic function for RAB-6.2 in signaling.

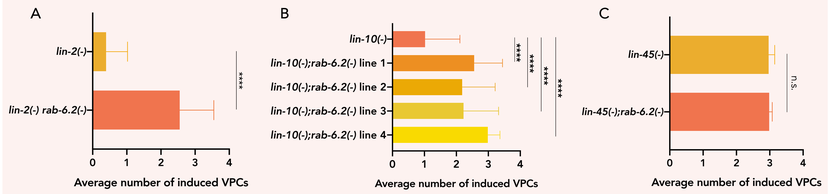
Figure 2



RAB-6.2 antagonizes EGFR/Ras/MAPK signaling and vulva induction

rab-6.2(ok2254) suppresses the vulvaless phenotype of *lin-2(-)* (Fig. 3A) and *lin-10(-)* (Fig. 3B) mutants, indicating restored signaling and supporting a negative regulatory role for RAB-6.2 in Ras signaling. In line with this, *rab-6.2(ok2254)* does not enhance the vulvaless phenotype of *lin-45(-)* Raf hypomorphic mutants (Fig. 3C).

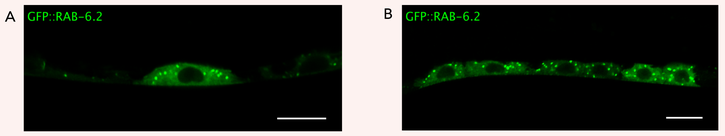
Figure 3



RAB-6.2 localizes to punctate structures in wildtype VPCs

A transgene expressing GFP-tagged RAB-6.2 in the VPCs of wildtype animals revealed that RAB-6.2 localizes to punctate structures (Fig. 4A,B). These may represent Golgi ministacks or Golgi-derived vesicles, as mammalian Rab6 and *C. elegans* RAB-6.2 localize to these structures in other cell types.

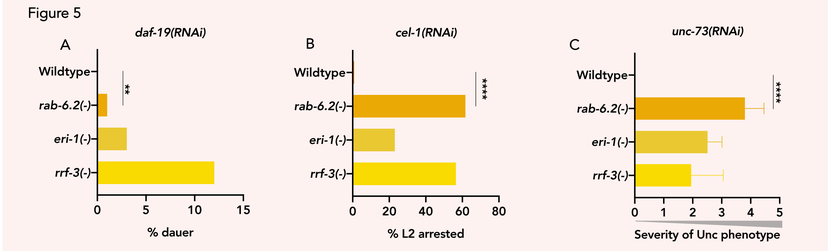
Figure 4



rab-6.2(ok2254) mutants are hypersensitive to RNAi

The enhanced phenotype of *lin-3(RNAi)* in *rab-6.2(ok2254)* mutants (Fig. 2A) could result from altered activity of small RNAs rather than signaling defects. In line with this, *rab-6.2(ok2254)* mutants show an enhanced response to RNAi (Eri phenotype). *daf-19*, *cel-1*, and *unc-73* RNAi only produce their respective phenotypes in RNAi hypersensitive backgrounds such as *rrf-3(-)* and *eri-1(-)* (Fig. 5A-C). Similarly, the phenotypes can be recovered in *rab-6.2(ok2254)* mutants (Fig. 5A-C), indicating dysregulation of small RNAs in the absence of RAB-6.2.

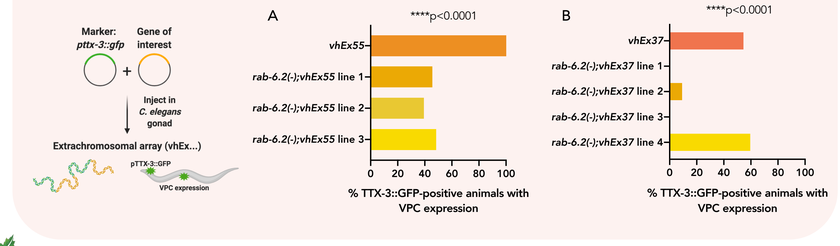
Figure 5



Transgene silencing is enhanced in *rab-6.2(ok2254)* mutants

Mosaic expression of extrachromosomal arrays is reduced in the VPCs of *rab-6.2(ok2254)* mutants (Fig. 6A,B), indicating enhanced transgene silencing as is seen in many Eri mutants. As transgene silencing is mediated by small RNAs, this further hints at a role for RAB-6.2 in the regulation of small RNAs.

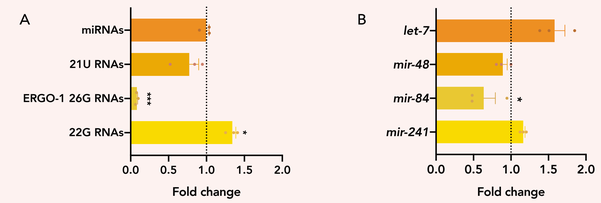
Figure 6



The levels of some endogenous siRNAs and *mir-84* are altered in *rab-6.2(ok2254)* mutants

To test the possibility that RAB-6.2 regulates small RNAs, we performed RNA sequencing of *rab-6.2(ok2254)* mutants. Despite overall miRNAs levels being unchanged (Fig. 7A), levels of the *let-7* family member *mir-84*, but not other family members, are significantly decreased (Fig. 7B). ERGO-1 26G RNAs are required for the production of secondary 22G RNAs, which are the main effectors of endogenous RNAi. ERGO-1 26G RNAs are significantly depleted in *rab-6.2(ok2254)* mutants, while 22G RNAs are mildly increased (Figure 7A). Loss of ERGO-1 26G RNAs is known to cause Eri phenotypes.

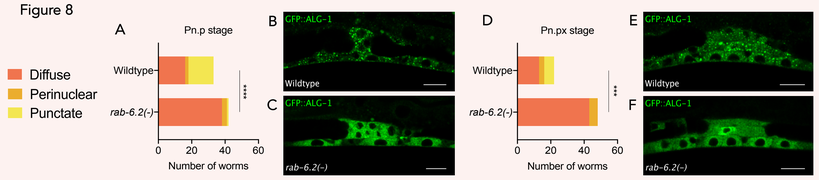
Figure 7



The miRNA-specific Argonaute ALG-1 is mislocalized in *rab-6.2(ok2254)* mutants

ALG-1 is one of the two miRNA-specific Argonaute proteins, and a main effector of miRNA-mediated silencing. Punctate localization of endogenous ALG-1 tagged with GFP (Figure 8A,B,D,E) is lost in the VPCs of *rab-6.2(ok2254)* mutants (Figure 8A,C,D,F). RAB-6.2 may thus regulate the activity of miRNAs through the recruitment of components required for silencing.

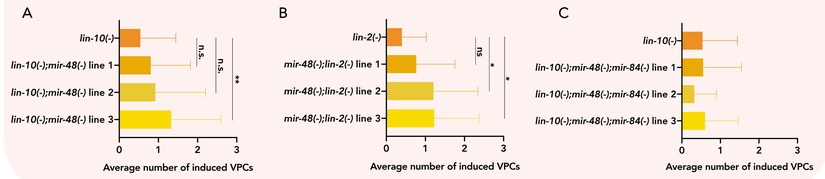
Figure 8



mir-48 plays a minor role in EGFR/Ras/MAPK signaling during vulva induction

Deletion of *mir-48*, a *let-7* family member, partially suppresses the vulvaless phenotype of *lin-2(-)* and *lin-10(-)* mutants (Fig. 9A,B), suggesting that *mir-48* also antagonizes EGFR/Ras/MAPK. Additional loss of *mir-84* does not suppress the *lin-10(-)* vulvaless phenotype (Figure 9C), raising the possibility that not all members of the *let-7* family modulate EGFR/Ras signaling in the context of vulva induction.

Figure 9



CONCLUSION AND FUTURE DIRECTIONS

Our results suggest a role for RAB-6.2 in antagonizing Ras signaling and regulating small RNAs. Confirmation of phenotypes with the *rab-6.2(tm1924)* allele and a rescue transgene is under way to validate our findings. While ALG-1 is mislocalized and levels of *mir-84* are decreased in *rab-6.2(ok2254)* mutants, the *let-7* family members appear to only play a minor role in vulva induction, suggesting that RAB-6.2 acts through additional mechanisms to antagonize EGFR/Ras/MAPK. Depletion of ERGO-1 26G RNAs in *rab-6.2(ok2254)* mutants, along with the Eri phenotypes observed, suggest that additional endogenous small RNAs may contribute to RAB-6.2-mediated regulation of vulva induction. To explore this, we will test for mislocalization of ERGO-1 and other Argonautes of the endogenous RNAi pathways in *rab-6.2(-)* mutants, and for suppression of *lin-2(-)* and *lin-10(-)* vulvaless phenotypes by these Argonautes.

Work funded by: CIHR IRSC, NSERC CRSNG, Fonds de recherche Santé Québec

Illustrations created with BioRender