Introduction

The EGFR/Ras GTPase/Mitogen Activated Protein Kinase (MAPK) signal transduction pathway is evolutionarily conserved and is essential for animal development [1,2]. Activating mutations in the EGFR/Ras/MAPK pathway are commonly found in human cancers and mutations in several components of the Ras/MAPK pathway have been shown to cause Noonan syndrome as well as several related developmental disorders [3–5]. The EGFR is activated by ligand binding, which stimulates receptor dimerization, transautophosphorylation of cytoplasmic Tyrosine residues, and recruitment of phospho-Tyrosine binding proteins such as Grb2. The binding of the Grb2 adaptor protein and the associated SOS protein (a Ras Guanine nucleotide Exchange Factor) to the EGFR results in the activation of the membrane associated Ras GTPase, and subsequently activation of the MAPK cascade consisting of Raf, MAPK/ERK Kinase (MEK) and Extracellular Regulated Kinase (ERK) [6]. EGFR and Grb2 also recruit Cbl, an E3-ubiquitin ligase, which ubiquitinates Lysine residues on the EGFR, targeting it for lysosomal degradation [7]. Ubiquitination contributes to EGFR endocytosis and sorting into late endosomes/MVBs. A series of ESCRT complexes (0, I, II, & III) on the MVBs recognize and internalize the ubiquitinated EGFR into intraluminal vesicles (ILVs), sequestering the EGFR away from the cytoplasm [7]. The ultimate fusion of MVBs with the lysosome results in degradation of the EGFR. Since the EGFR can continue to signal from endosomes, regulators of endocytic trafficking are positioned to regulate the duration and strength of signaling.

The Rab5 and Rab7 GTPases are key regulators of early endosome and late endosome trafficking, respectively, and regulate EGFR trafficking to the lysosome [8]. Rab5 regulates EGFR internalization, while EGFR signaling can modulate Rab5 activity by regulating its Guanine nucleotide exchange factor, RIN1, and GTPase Activating Protein, RN-tre [9–13]. In some cases, Rab5 can function as a downstream effector of EGFR signaling [14]. While Rab7 activity promotes EGFR trafficking from late endosomes/MVBs to the lysosome [15–17], it is unclear whether inhibiting EGFR degradation late in the endocytic pathway would impact EGFR signaling. EGFR accumulates in the ILVs of MVBs of Rab7 RNAi treated cells, where it would potentially be sequestered away from the cytoplasm and downstream effectors [17].

The EGFR/Ras/MAPK pathway is highly conserved in the nematode C. elegans where it is required for specifying cell fates during development (Figure 1A) [1]. During C. elegans vulva development, LET-23 EGFR and LIN-12 Notch signaling pathways specify three of six VPCs to adopt the 1* and 2* vulval...
cell fates (Figure 1B) [18]. The six VPCs are polarized epithelial cells, named P3.p-P8.p, that have LET-23 EGFR localized to the basolateral membrane. The Anchor Cell, in the overlying gonad, secretes the LIN-3 EGFR-like ligand, activating LET-23 EGFR signaling cascade most strongly in P6.p, the closest VPC, inducing it to adopt a 1° vulval cell fate. P6.p subsequently activates LIN-12 Notch signaling in the neighboring cells. LIN-12 Notch signaling, along with the graded LIN-3 signal, induce the P5.p and P7.p cells to adopt the 2° vulval cell fate. The remaining P3.p, P4.p, and P8.p cells adopt a 3° non-vulval cell fate, divide once, and fuse with the surrounding hypodermis (roughly 50% of P3.p cells fuse prior to dividing). The induced P3.p-P7.p cells undergo a stereotypic set of cell divisions to give rise to the 22 cells of the mature vulva, 8 cells from the 1° (P6.p) cell and 7 cells from each of the 2° (P5.p and P7.p) cells (Figure 1B). Thus, mutations that reduce LET-23 EGFR signaling result in a Vulvaless (Vul) phenotype in which fewer than three VPCs are induced, and mutations that enhance LET-23 EGFR signaling result in a Multivulva (Muv) phenotype in which greater than three VPCs are induced.

Here we present genetic evidence demonstrating a role for RAB-7 as a negative regulator of LET-23 EGFR signaling during C. elegans vulva development. We show that a rab-7 deletion mutant suppresses the Vul and enhances the Muv phenotypes of let-60 ras hypomorphic and hypermorphic alleles, respectively. Similar to previously characterized negative regulators of EGFR signaling, rab-7(ok511) is synthetic Muv in combination with loss of negative regulators unc-101 Ap-1 and ask-1 Ack, and suppresses the Vul phenotypes of mutations that disrupt the basolateral localization of LET-23 EGFR. We show that rab-7 functions upstream of or in parallel to lin-3 EGF and let-23 EGFR, and that it functions in the VPCs and regulates LET-23:GFP localization. Importantly, our data suggest that Rab7 could negatively regulate EGFR signaling in humans where increased EGFR signaling can contribute to oncogenesis.

Materials and Methods

C. elegans alleles and general methods

General methods for the handling and culturing C. elegans were as previously described [19]. C. elegans var Bristol strain N2 is the wild-type parent for all strains used in this work. E. coli strain HB101 was used as a food source. Specific genes and alleles are described on Wormbase (www.wormbase.org) and are available from the Caenorhabditis Genetics Center. LGI: lin-10(e1439), unc-101(y108), gai-27 [let-23::GFP:nsv-6::unc-0606]. LGII: mbl1(dpy-10(e126) mls14), hh-2(y768), let-23(sy97), let-23(sy97), unc-4(e120), mnd7, rbs-7(ok311), lin-7(e1435). LGIII: lin-119(ed3). LGIV: lip-1(zh15), lin-3(e1417), let-60(ga89gf::ts), let-60(n1046gf), let-60(n1876), let-60(n2021), dpy-20(e1292), unc-22(s7), ask-1(y247). LGX: cbh-1(y143), gap-1(ga333), dpy-23(e840), lin-2(e1309). Linkage unknown: 159F2 [P_gf;::NLS::GFP::Lux-2(+)+;+P_suz-3::GFP::xhs-2501; P_gf;::let-23::gfp] [20]. Extrachromosomal array: zkhEx1 [Plin-3::GFP::rab-7 + P_hcb-6::GFP::Ch-unc-119(+)] (this study).

Plasmid and Transgenic construction

The GFP::rab-7 fusion was amplified from a P_suz-6::GFP::rab-7 vector (a gift from Barth Grant) using Sal I and Not I tagged oligos CRO264 (5’-GTA CGT CAG TGG TCG TAT TGG TAC CGG TAG-3’) and CRO265 (5’-GTA CGG GCC CGG GCC GGG TTA ACA ATT GCA TCC CGG-3’) and subcloned into the lin-31 promoter plasmid, p255 [21] to generate a P_lin-31::GFP::rab-7 plasmid. Transgenic animals were generated by DNA microinjection [22] of the P_lin-31::GFP::rab-7 plasmid and a marker plasmid containing P_suz-6::GFP::Ch-unc-119(+)[23] at a concentration of 50 ng/μl each into unc-119(ed5) animals. Three transgenic lines were generated based on selection of unc-119(ed5) rescue and intestinal GFP expression (P_hcb-6::GFP).

RNA interference

RNAi-feeding was performed essentially as described earlier [24]. RNA production was induced using 1 mM IPTG. The RNAi-feeding clones rab-7 (II-8G17), vps-28 (I-6N04), hgrs-1 (IV-4K17), and W03C9.5 (II-8G17) are from the Ahringer library (www.geneservice.co.uk) and gfp RNAi-feeding clone L4417 (pPD128.110) from the Fire Vector kit (www.addgene.org) [25]. Clones were verified by DNA sequencing.

Microscopy and Phenotype Analysis

General methods for Nomarski differential interference contrast (DIC) microscopy of live animals were as previously described [26]. Animals were analyzed on an Axio Zeiss A1 Imager
RAB-7 antagonizes LET-60 Ras signaling during vulva cell fate specification

To determine if RAB-7 regulates EGFR/Ras signaling during vulva development, we used the *rab-7(ok511)* deletion allele generated by the *C. elegans* Knockout Consortium. The *rab-7(ok511)* allele consists of a 741 base-pair deletion/a 17 base-pair insertion that deletes the first three exons of *rab-7* as well as the 3′ UTR of the upstream gene, *W03C9.5* [Figure 1C; www.wormbase.org] [30]. ok511 likely represents a null allele of *rab-7* as it deletes the sequences encoding the putative switch regions and guanine nucleotide and Mg2+ binding sites essential for GTPase function. Consistent with this prediction, we placed ok511 over the chromosomal deficiency, *mdf87*, and found that *bh-2(e768) rab-7(ok511)/mdf87* animals were indistinguishable from *bh-2(e768) rab-7(ok511)* homozygotes in their small body size, granular intestinal appearance, enlarged yolk platelets and maternal effect embryonic lethality (data not shown). We believe that the effects of ok511 in this study are specifically due to loss of *rab-7* and not due to deletion of the 3′ UTR of *W03C9.5*. As demonstrated later in this paper, *rab-7(RX4i)*, but not *W03C9.5(RX4i) suppressed the *lin-2(e1309)* Vul phenotype, and an extrachromosomal array, expressing *rab-7* in the VPCs, reversed the *rab-7(ok511)*; *lin-2(e1309)* suppressed Vul phenotype.

We assayed whether the *rab-7(ok511)* deletion mutant could modulate the vulval phenotypes of *let-60 ras* gain-of-function and loss-of-function alleles. The *rab-7(ok511)* allele confers a maternal effect embryonic lethal phenotype; therefore *rab-7(ok511)* homozygous animals, which did not display any defects in vulva development (Table 1), were derived from heterozygous mothers. *let-60(n1046gf)* gain-of-function mutants display a moderate Muv phenotype and are sensitive to mutations that either decrease or rescue vulval defects [31].

### Table 1. *rab-7* antagonizes LET-60 Ras-mediated vulval cell fate induction.

| GENOTYPE | Muv, % | Vul, % | AVG. # of VPCs INDUCED | VPCs INDUCED, % | n |
|----------|--------|--------|-------------------------|----------------|---|
| *rab-7(ok511)* | 0 | 0 | 3.0 | 0 | 0 | 100 | 100 | 100 | 0 | 33 |
| *rab-7(ok511); let-60(n1046gf)* | 74 | 0 | 3.82 | 26 | 34 | 100 | 100 | 100 | 22 | 34 |
| *rab-7(ok511); let-60(n1046gf)* | 100** | 0 | 5.09**** | 50 | 74** | 100 | 100 | 100 | 85**** | 34 |
| *rab-7(ok511); let-60(ga89gf,ts)* | 3 | 0 | 3.03 | 0 | 7 | 100 | 100 | 97 | 0 | 29 |
| *rab-7(ok511); let-60(ga89gf,ts)* | 17 | 3 | 3.12 | 0 | 17 | 100 | 100 | 98 | 0 | 29 |
| *rab-7(ok511); let-60(n2021)* | 3 | 0 | 2.52 | 0 | 0 | 92 | 88 | 72 | 0 | 30 |
| *rab-7(ok511); let-60(n2021)* | 0 | 13* | 2.92 | 0 | 7 | 98 | 98 | 87 | 2 | 30 |
| *rab-7(ok511); let-60(n1876)* | 0 | 100 | 0.03 | 0 | 0 | 0 | 3 | 0 | 0 | 20 |
| *rab-7(ok511); let-60(n1876)* | 100 | 0 | 0.05 | 0 | 0 | 0 | 5 | 0 | 0 | 22 |

All experiments were performed at 20°C. Statistical analysis was performed comparing *rab-7/+* heterozygotes with *rab-7(ok511)* homozygotes for each vulval mutant background. *rab-7(ok511)* is balanced in cis by *min1(+), rab-7(ok511)* is marked in cis with *bh-2(e768)* in the strain containing *let-60(n1876)*. Fisher’s exact test (www.graphpad.com/quickcalcs) was used to determine statistical significance. n, number of animals scored.

*P < 0.05,

**P < 0.01,

****P < 0.0001.

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Table 1. *rab-7* antagonizes LET-60 Ras-mediated vulval cell fate induction.
or increase Ras signaling [32–35]. We found that rab-7(ok511); let-60(n1046gf) progeny from rab-7(ok511)/+;let-60(n1046gf) mothers displayed a more penetrant and severe Muv phenotype than that of their rab-7(ok511)/+; let-60(n1046gf) siblings (Figure 2, A and B, and Table 1). However, we found that rab-7(ok511) did not significantly enhance the Muv phenotype of let-60(ga89gf,ts) (Table 1). The let-60(ga89gf,ts) Muv phenotype may be less sensitive to loss of upstream components of the pathway [36], suggesting that RAB-7 might act upstream of LET-60 Ras. To further test if RAB-7 has an inhibitory role in vulva development, we tested whether rab-7(ok511) could suppress the Vul phenotype of let-60 ras loss-of-function mutants. We found that rab-7(ok511) suppressed the Vul phenotype of a weak hypomorphic allele, let-60(n2021) (Figure 2, C and D, and Table 1). However, rab-7(ok511) failed to suppress the Vul phenotype of a severe hypomorphic allele, let-60(n1876) (Table 1). Therefore, RAB-7 negatively regulates LET-60 Ras signaling upstream or in parallel to LET-60 Ras.

**rab-7(ok511) is synthetic Muv with mutations in negative regulators of EGFR/Ras signaling**

Several negative regulators of EGFR/Ras/Mapk signaling have been previously described, including SLI-1, a homolog of the Cbl E3-ubiquitin ligase [37,38]; ARK-1, an Ack-related tyrosine kinase that interacts with SEM-5 Grb2 [32]; DEP-1, a putative protein Tyrosine phosphatase [33]; GAP-1, a homolog of Ras GTPase Activating Protein [39]; LIP-1, a homolog of MAPK phosphatase [40], as well as DPY-23, an AP-2 μ2 subunit [41,42], and UNC-101, an AP-1 μ1 subunit [43], both components of adaptor protein complexes that associate with clathrin coated

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**Figure 2. rab-7(ok511) modulates the vulva phenotypes of mutations affecting components of the EGFR/Ras/MAPK pathway.** Representative DIC images of the vulva phenotypes of rab-7(ok511)/mIn1;let-60(n1046gf) (A), rab-7(ok511);let-60(n1046gf) (B), rab-7(ok511)/mIn1;let-60(n2021) (C), rab-7(ok511);let-60(n2021) (D), rab-7(ok511)/mIn1; dpy-20(e1282) ark-1(sy247) (E), rab-7(ok511); dpy-20(e1282) ark-1(sy247) (F), unc-101(sy108); rab-7(ok511)/mIn1 (G), unc-101(sy108); rab-7(ok511) (H), rab-7(ok511)/mIn1; lin-2(e1309) (I), and rab-7(ok511); lin-2(e1309) (J). Open circles mark the normal site of vulva cell invagination, stars mark vulva invaginations due to ectopic induction, vertical lines mark failed vulva cell inductions, and horizontal lines mark ectopic inductions that fail to invaginate. Bar, 10 μm (A).

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vesicles derived from the plasma membrane and internal membranes, respectively [44] (Figure 1A). Similar to rab-7, mutations affecting these negative regulators do not cause vulval phenotypes alone, but various double mutant combinations can result in a synthetic Muv phenotype. To further characterize the requirements for rab-7 in EGFR/Ras signaling, we made double mutants between rab-7(ok511) and mutations in these other negative regulators. We found that rab-7(ok511) caused a significant Muv phenotype in combination with mutations in ark-1; dep-1, gap-1, or lip-1 MKP (Figure 2, E-H, and Table 2). We observed ectopic vulval cells that are visible under the dissecting microscope (data not shown). Furthermore, the ectopically induced vulval cells of either P5.p or P7.p (Figure 2H), and thus behaved like vulva induced cells in wild-type L2-L3 stage larvae, egf-17::CFP, a sensitive marker of LET-23 EGFR receptor activation [41], was detected in egl-17::CFP animals (Figure 1A and Discussion). Despite the strong Muv phenotype of unc-101(sy108); rab-7(ok511) animals as assayed by DIC microscopy, they rarely displayed ectopic vulval cells that are visible under the dissecting microscope (data not shown). Furthermore, the ectopically induced mutants in unc-101(sy108); rab-7(ok511) animals always did not lift from the ventral cuticle and/or clustered with the progeny of either P3.p or P7.p (Figure 2H), and thus behaved like vulva cells of the 2 lineage rather than those of the 1 lineage. To further explore the fate of the ectopically induced cells, we looked at expression of egf-17::CFP, a sensitive marker of LET-23 EGFR signaling [41], in the VPCs of both rab-7(ok511); ark-1(sy247) and unc-101(sy108); rab-7(ok511) animals. In wild-type L2-L3 stage larvae, egf-17 is normally expressed at highest levels in P6.p and lower levels in P3.p and P7.p [45,41]. We found that egf-17::CFP was ectopically expressed in either P4.p or P8.p in 3 out of 21 rab-7(ok511); ark-1(sy247) animals (Figure 3A and B), consistent with the 20% penetrant Muv phenotype in these animals being caused by ectopic activation of EGFR/Ras signaling. In contrast, no ectopic egf-17::CFP was detected in unc-101(sy108); rab-7(ok511) animals (n = 21), despite their having a more penetrant Muv phenotype (Figure 3C and D). A subset of the 2 vulval cells of wild-type animals express egf-17 during the late L4 stage, but the ectopically induced vulval cells of unc-101(sy108); rab-7(ok511) animals did not express egf-17::CFP (n = 12) (Figure 3E-G). Therefore, the ectopically induced vulval cells in unc-101(sy108); rab-7(ok511) animals might adopt an incomplete or mixed vulval fate [33] (see Discussion).

**rab-7(ok511)** suppresses the Vul phenotypes of mutations that disrupt the basolateral membrane localization of LET-23

The LIN-2/LIN-7/LIN-10 (CASK/Veli/Mint) complex is required for localizing the LET-23 receptor to the basolateral membrane of the VPCs [46,47]. Mutations in lin-2, lin-7, lin-10, or the let-23(sy1) mutation that specifically abrogates LET-23 binding to LIN-7, result in the apical mislocalization of the LET-23 receptor and the failure to induce the VPCs [31,46–50]. Mutations in negative regulators dep-1 Protein Tyrosine Phosphatase and lip-1 MKP have been shown to partially suppress the Vul phenotype of lin-2, lin-7, lin-10, and let-23(sy1) mutants [33,40]. Mutations in negative regulators unc-101 AP-1, gap-1, and sl-1 Cbl not only suppressed the Vul phenotype, but have been shown to induce a Muv phenotype in combination with lin-2, lin-7, lin-10, and let-23(sy1) mutants [38,39,43], while an ark-1 Ack mutant has been shown not to suppress the Vul phenotype of let-23(sy1) [32]. We found that rab-7(ok511) robustly suppressed the Vul phenotypes and induced a Muv phenotype in combination with let-23(sy1) and mutations in lin-2, lin-7, lin-10 (Figure 2I and J, and Table 3). Therefore, rab-7(ok511) behaved similarly to mutations in unc-101 AP-1, apk-1, and sl-1 Cbl with respect to its genetic interactions with let-23(sy1) and mutations in lin-2, lin-7, and lin-10.

**Table 2.** rab-7(ok511) is synthetic Multivulva with unc-101 and ark-1 mutants.

| GENOTYPE                      | Muv, % | Vul, % | AVG. # of VPCs INDUCED | VPCs INDUCED, % | n  |
|-------------------------------|--------|--------|------------------------|-----------------|----|
|                               |        |        | P3.p       | P4.p       | P5.p  | P6.p  | P7.p  | P8.p  |
| rab-7(ok511);+/; ark-1(sy247) | 0      | 0      | 3.0        | 0           | 0     | 100   | 100   | 100   | 0     | 31   |
| rab-7(ok511);+/; ark-1(sy247) | 20*    | 0      | 3.18       | 2           | 10    | 100   | 100   | 100   | 7     | 30   |
| rab-7(ok511);+/; sl-1(sy143)  | 0      | 0      | 3.0        | 0           | 0     | 97    | 100   | 100   | 3     | 31   |
| rab-7(ok511);+/; sl-1(sy143)  | 13     | 0      | 3.10       | 0           | 0     | 100   | 100   | 100   | 10    | 31   |
| unc-101(sy108); rab-7(ok511);+ | 0      | 3      | 2.98       | 0           | 3     | 100   | 100   | 95    | 0     | 30   |
| unc-101(sy108); rab-7(ok511);+ | 57**** | 0      | 3.57       | 2           | 12    | 98    | 100   | 100   | 45****| 30   |
| dep-1(hc34)                   | 0      | 0      | 3.0        | 0           | 0     | 100   | 100   | 100   | 0     | 24   |
| dep-1(hc34)                   | 4      | 4      | 3.02       | 0           | 0     | 100   | 100   | 100   | 2     | 24   |
| dep-1(hc34)                   | 0      | 0      | 3.0        | 0           | 0     | 100   | 100   | 100   | 0     | 30   |
| dep-1(hc34)                   | 7      | 0      | 3.03       | 0           | 2     | 100   | 100   | 100   | 2     | 30   |
| dep-1(hc34)                   | 0      | 0      | 3.0        | 0           | 0     | 100   | 100   | 100   | 22    | 29   |

Statistical analysis was performed as in Table 1, comparing rab-7+/+ heterozygotes with rab-7(ok511) homozygotes for each vulval mutant background, except for in the dep-1(hc34) background where rab-7(ok511) is compared to rab-7(+). rab-7(ok511) is balanced in cis to dpy-20[e1282], dep-1(hc34) control is marked in cis to unc-4(e120). n, number of animals scored.

*P<0.05,

**P<0.0001.

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RAB-7 Antagonizes LET-23 EGFR Signaling

RAB-7 functions in the VPCs to regulate LET-23 EGFR signaling

To determine if RAB-7 regulates LET-23 EGFR signaling in the VPCs, we expressed a GFP:RAB-7 fusion under the control of the lin-31 VPC specific promoter as an extrachromosomal array, vhaEx1. Animals carrying vhaEx1 were wild-type for vulval cell fate specification (Table 3). To determine if vhaEx1 is sufficient to rescue rab-7(ok511) deletion, we tested the ability of vhaEx1 to restore the Vul phenotype of rab-7(ok511); lin-2(e1309) animals. Although vhaEx1 is stochastically lost from some cell lineages, we find that rab-7(ok511); lin-2(e1309); vhaEx1 animals are significantly more Vul than rab-7(ok511); lin-2(e1309) animals (Table 3). These data indicate that RAB-7 can antagonize LET-23 EGFR signaling in the VPCs.

Components of the ESCRT complexes antagonize LET-23 EGFR signaling

Components of the ESCRT complexes are required for the sorting of ubiquitinated cargo into the MVBs and have been shown to be negative regulators of RTK signaling in Drosophila and mammalian cells [53–55]. RNAi knockdown of Rab7 in Hela cells resulted in the accumulation of EGFR in the ILVs of MVBs [17], where the EGFR would presumably not be able to signal to downstream effectors in the cytoplasm. To determine if LET-23 EGFR might transit through MVBs en route to the lysosome in the VPCs, we tested if components of the ESCRT-0, and -I, complexes could antagonize LET-23 EGFR signaling. Similar to rab-7(RNAi), we found that RNAi of hgrs-1 (ESCRT-0), and eps-28 (ESCRT-I) suppressed the severity of the lin-2(e1309) Vul phenotype, while control RNAi, gfp and W03C9.5, did not suppress lin-2(e1309) phenotype (Table 3). Thus, LET-23 EGFR signaling in the vulva is antagonized by the activity of the ESCRT machinery suggesting that LET-23 EGFR does transit through MVBs in the VPCs.

RAB-7 regulates LET-23::GFP EGFR localization in the VPCs and Hyp7

To determine if RAB-7 regulates the trafficking or localization of LET-23 EGFR, we tested if rab-7(ok511) altered the localization of LET-23 tagged with GFP (LET-23::GFP) in the VPCs. LET-23::GFP, expressed from the gsk27 transgene, has previously been shown to localize to cell junctions in the VPCs [50]. While this localization differs from endogenous LET-23 localization based on antibody staining [46,47], gsk27 is sufficient to rescue let-23 loss and LET-23::GFP is apically mislocalized in lin-2 and lin-7 mutants [50]. We found that LET-23::GFP expression was too low to detect in the VPCs of live animals, despite partially rescuing the Vul phenotype of lin-2(e1309) animals (data not shown). To detect LET-23::GFP, we co-immunostained animals carrying gsk27 with anti-GFP antibody and the MH27 antibody, which recognizes AJM-1 (Figure 4A–C). In most animals (21/24), LET-23::GFP was also present in the cytoplasm and in occasional foci in the basal

rab-7(ok511) does not suppress a strong hypomorphic alleles of lin-3 or let-23

To determine if LET-23 EGFR is required for the enhanced signaling in rab-7(ok511) animals, we tested if rab-7(ok511) could suppress the Vul phenotype of let-23(sy97), a strong hypomorphic allele that is defective in Ras/MAPK signaling in multiple tissues [49,51]. We found that rab-7(ok511) failed to suppress the Vul phenotype of let-23(sy97) animals (Table 3), indicating that LET-23 EGFR is required for the enhanced signaling in rab-7(ok511) mutants.

To determine if LIN-3, EGF-like ligand, is required for the enhanced signaling in rab-7(ok511) animals, we tested if rab-7(ok511) could suppress the Vul phenotype of lin-3(e1417), a strong hypomorphic allele that specifically disrupts expression in the Anchor cell and hence vulva cell fate specification [52]. We found that rab-7(ok511) failed to suppress the Vul phenotype of lin-3(e1417) animals (Table 3), indicating that the LIN-3 ligand is required for the enhanced signaling in rab-7(ok511) mutants. Therefore, RAB-7 acts upstream or in parallel to LIN-3 and LET-23.
region of the P6.p cells (Figure 4A–C). As previously described, we found LET-23::GFP to be predominantly localized in the apical region of lin-2(e1309) animals with a few animals having weak fluorescence in the cytoplasm of the basal region (3/21) (Figure 4D–F). In rab-7(ok511) animals, LET-23::GFP is similar to wild-type, except 50% of rab-7(ok511) animals have prominent LET-23::GFP positive foci consistent with LET-23::GFP accumulation in endocytic vesicles or MVBs (Figure 4G–I). This is more apparent in rab-7(ok511); lin-2(e1309) animals, where LET-23::GFP positive foci can be seen in the basal cytoplasm (22/31), suggesting that RAB-7 regulates LET-23 EGFR trafficking in the VPCs (Figure 4J–L).

The small size of the VPCs make it difficult to quantify a difference in the number of LET-23::GFP vesicles in rab-7(ok511) versus wild-type larvae. To better assess the effect of rab-7 on LET-23::GFP trafficking, we used a transgenic strain carrying xhIs2501, which expresses a LET-23::GFP under the control of the dpy-7 promoter in the large hypodermal syncytium, Hyp7 (but not the VPCs), where it can be seen in endosomes [20,57]. We find that rab-7(ok511) mutants have a two-fold increase in the number of LET-23::GFP endosomal foci as compared to both wild-type and

### Table 3. **rab-7(ok511)** suppresses the Vul phenotypes of mutations that mislocalize LET-23 but not strong alleles of lin-3 and let-23.

| GENOTYPE | Muv, % | Vul, % | AVG. # of VPCS INDUCED | VPCS INDUCED, % |
|----------|-------|-------|-------------------------|-----------------|
|          |       |       | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | n    |
| let-23(sy1) | 4    | 84   | 1.06 | 0    | 2    | 42   | 40   | 22   | 0    | 25   |
| let-23(sy1); rab-7(ok511) | 8    | 50*  | 2.42**** | 0    | 8    | 75*  | 92** | 67** | 0    | 24   |
| let-23(sy97) | 0    | 100  | 0.08 | 0    | 0    | 0    | 8    | 0    | 0    | 20   |
| rab-7(ok511); let-23(sy1) | 0    | 100  | 0.0  | 0    | 0    | 0    | 0    | 0    | 0    | 20   |
| rab-7(ok511); lin-2(e1309) | 28** | 16**** | 3.06**** | 2    | 18   | 98**** | 98**** | 88**** | 2    | 25   |
| rab-7(ok511); lin-2(e1309); vheX1 | 7    | 67**** | 2.0** | 2    | 13   | 65** | 76*  | 43*** | 2    | 27   |
| vheX1 | 0    | 0    | 3.0   | 0    | 0    | 100  | 100  | 100  | 0    | 19   |
| rab-7(ok511); lin-7(e1413);/+ lin-7(e1413) | 5    | 95   | 0.93 | 0    | 14   | 33   | 36   | 10   | 0    | 21   |
| rab-7(ok511); lin-7(e1413) | 43** | 20**** | 2.90 **** | 5    | 33   | 88** | 90** | 71**** | 2    | 21   |
| lin-7(e1439); rab-7(ok511);/+ | 4    | 92   | 0.72 | 0    | 6    | 28   | 24   | 14   | 0    | 25   |
| lin-7(e1439); rab-7(ok511) | 12   | 90   | 1.18 | 0    | 33   | 68   | 18   | 0    | 0    | 20   |
| rab-7(ok511); lin-3(e1417) | 0    | 90   | 1.28 | 0    | 0    | 35   | 63   | 30   | 0    | 20   |

Statistical analysis was performed as in Table 1 comparing rab-7/+ heterozygotes with rab-7(ok511) homozygotes for each vulval mutant background, except for in the let-23(sy1) and let-23(sy97) backgrounds where rab-7(ok511) is compared to rab-7(+). rab-7(ok511); lin-2(e1309); vheX1 is compared to rab-7(ok511); lin-2(e1309). rab-7(ok511) is balanced in trans by mIn1(+), rab-7(ok511) is marked in cis with bli-2(e768) in the strain containing lin-3(e1417), let-23(sy1) and let-23(sy97) are marked in cis with unc-4(e120), and lin-7(e1413) is linked in cis to mIn1 on the rab-7(+) chromosome. n, number of animals scored.

*P<0.05,
**P<0.01,
***P<0.001,
****P<0.0001.

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### Table 4. **RNAi of rab-7, hgrs-1 and vps-28 suppresses the lin-2(e1309) Vul phenotype.**

| GENOTYPE | Muv, % | Vul, % | AVG. # of VPCS INDUCED | VPCS INDUCED, % |
|----------|-------|-------|-------------------------|-----------------|
|          |       |       | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | n    |
| gfp(RNAi); lin-2(e1309) | 0    | 100  | 0.32 | 0    | 2    | 10   | 16   | 5    | 0    | 31   |
| rab-7(RNAi); lin-2(e1309) | 1    | 84*  | 1.07**** | 0    | 2    | 32*  | 43** | 30** | 0    | 82   |
| hgrs-1(RNAi); lin-2(e1309) | 0    | 88   | 1.08**** | 0    | 5    | 44** | 34   | 25   | 0    | 40   |
| vps-28(RNAi); lin-2(e1309) | 0    | 93   | 0.98**** | 0    | 0    | 28   | 43*  | 28*  | 0    | 40   |
| W03C9.5(RNAi); lin-2(e1309) | 0    | 100  | 0.25 | 0    | 1    | 6    | 11   | 6    | 0    | 40   |

Statistical analysis was performed as in Table 1 comparing each RNAi experiment to the gfp RNAi control. n, number of animals scored.

*P<0.05,
**P<0.01,
***P<0.001,
****P<0.0001.

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region of the P6.p cells (Figure 4A–C). As previously described, we found LET-23::GFP to be predominantly localized in the apical region of lin-2(e1309) animals with a few animals having weak fluorescence in the cytoplasm of the basal region (3/21) (Figure 4D–F). In rab-7(ok511) animals, LET-23::GFP is similar to wild-type, except ~50% of rab-7(ok511) animals have prominent LET-23::GFP positive foci consistent with LET-23::GFP accumulation in endocytic vesicles or MVBs (Figure 4G–I). This is more apparent in rab-7(ok511); lin-2(e1309) animals, where LET-23::GFP positive foci can be seen in the basal cytoplasm (22/31), suggesting that RAB-7 regulates LET-23 EGFR trafficking in the VPCs (Figure 4J–L).
RAB-7 Antagonizes LET-23 EGFR Signaling

Discussion

Here we show that RAB-7 antagonizes LET-23 EGFR-mediated vulval cell induction in C. elegans. Our genetic analysis using the \textit{rab-7(ok511)} deletion mutant is consistent with RAB-7 acting as a negative regulator of LET-23 EGFR signaling to a similar extent as previously described negative regulators [32,33,38,59,60]. Similar to previously described negative regulators, \textit{rab-7(ok511)} mutant animals display no defects in vulval cell fate specification. We found that \textit{rab-7(ok511)} enhanced the Muv phenotype of a \textit{let-60 ras} gain-of-function mutation and suppressed the Vul phenotype of a \textit{let-60 ras} weak loss-of-function mutant. \textit{rab-7(ok511)} strongly suppressed the Vul phenotype of mutations in \textit{lin-2}, \textit{lin-7}, and \textit{lin-10}, as well as the \textit{let-23(gf)} allele. Furthermore, \textit{rab-7(ok511)} was synthetic Muv with mutations in the negative regulators \textit{ark-1} Ack, and \textit{unc-101} AP-1 µ. Our genetic data indicate that \textit{rab-7} is a potent negative regulator of LET-23 EGFR signaling.

Genetic epistasis suggests that RAB-7 antagonizes LET-23 EGFR signaling in a manner that is distinct from previously described negative regulators. We show that \textit{rab-7(ok511)} cannot suppress the Vul phenotypes of strong loss-of-function mutations in \textit{let-60 ras}, \textit{let-23 EGFR}, and \textit{lin-3 EGF} suggesting that \textit{rab-7} is required upstream or in parallel to these genes. The fact that expression of \textit{GFP::RAB-7} in the VPCs can rescue the suppression of the \textit{lin-2(e1309)} Vul phenotype by \textit{rab-7(ok511)} indicates that RAB-7 functions in parallel in the VPCs to regulate signaling. The requirement for \textit{LIN-3} and \textit{LET-23} would be consistent with a known role of mammalian Rab7 in trafficking activated EGFR to the lysosome for degradation [16,17]. Also consistent with RAB-7 functioning at the level of LET-23 EGFR, \textit{rab-7(ok511)} has stronger genetic interactions with negative regulators, \textit{ark-1} and \textit{unc-101}, that act at the level of LET-23 EGFR, than with downstream negative regulators \textit{gap-1} and \textit{lip-1}. While the weak interaction between \textit{rab-7(ok511)} and \textit{sli-1 Cbl} would be consistent with both acting in the same pathway to target LET-23 EGFR for lysosomal degradation, \textit{sli-1} mutations do suppress \textit{let-23(gf97)} [38] while \textit{rab-7(ok511)} does not, suggesting that \textit{SLI-1} and \textit{RAB-7} are antagonizing LET-23 EGFR signaling via different mechanisms. In fact, \textit{rab-7} is distinct from other negative regulators in that loss of \textit{rab-7} fails to suppress \textit{lin-3(e1417)} Vul phenotype, while mutations in \textit{unc-101}, \textit{ark-1}, \textit{sli-1}, \textit{gap-1}, and \textit{sli-3} can suppress the Vul phenotype of \textit{lin-3} alleles \textit{e1417} and/or \textit{n378} [32,38,39,43,60].

The synthetic Muv phenotypes seen in \textit{unc-101}; \textit{rab-7} and \textit{rab-7}; \textit{ark-1} double mutants suggests that RAB-7 functions in parallel to \textit{UNC-101} and \textit{ARK-1} to antagonize LET-23 EGFR signaling at a common point of the pathway. \textit{ARK-1} is a non-receptor tyrosine kinase related to Ack that can interact with the SEM-5 Grb2 adaptor [32]. Although neither the kinase substrate(s) for \textit{ARK-1} nor the mechanism by which it inhibits signaling are known, the genetic data point to LET-23 as the likely target. \textit{ARK-1} might function to inactivate LET-23 through phosphorylation, while RAB-7 mediates LET-23 trafficking to the lysosome, thus functioning in parallel to reduce the amount of active LET-23.

Figure 4. \textit{rab-7(ok511)} alters LET-23::GFP localization in the VPCs of \textit{lin-2(-)} animals. (A–L) Single section confocal images of the VPCs (lateral view) of mid-L3 stage larvae following the first round of VPC division (Pn.p.x stage) immunostained with anti-GFP to detect LET-23::GFP (A, D, G and J) and the MH27 monoclonal antibody to detect the AJM-1 junctional protein (B, E, H, and K) demarcating the apical/basal boundary, and (C, F, I and L) are merged images with P6.pa and P6.pp cells underlined. (A–C) wild-type larva carrying \textit{gaIs27(let-23::GFP)} larva with basal cytoplasmic and apical LET-23::GFP localization. (G–I) \textit{rab-7(ok511)}; \textit{gaIs27(let-23::GFP)} larva with basal cytoplasmic and apical LET-23::GFP expression in P6.pa and P6.pp with LET-23::GFP in cytoplasmic foci. (J–L) \textit{rab-7(ok511)}; \textit{lin-2(e1309)}; \textit{gaIs27(let-23::GFP)} larva with LET-23::GFP localization similar to that in \textit{rab-7(ok511)}; \textit{gaIs27(let-23::GFP)} larvae. Bar, 10 µm (C). doi:10.1371/journal.pone.0036489.g004

ok511/+ heterozygotes (Figure 5), consistent with reduced LET-23::GFP degradation. We tested if LET-23::GFP accumulated in early endosomes, a potential platform for signaling, by co-immunostaining with anti-GFP and anti-EEA-1, a marker for early endosomes [58]. However, we detected almost no colocalization in either \textit{rab-7(+) or rab-7(ok511)} animals (data not shown), suggesting that LET-23::GFP accumulates in a later endosomal compartment. Taken together, these data suggest that RAB-7 may exert antagonistic effects on EGFR/Ras/MAPK signaling through regulation of LET-23 EGFR trafficking.
The adaptor complex AP-1 mediates trafficking between the Trans-Golgi, endosomes, and the plasma membrane, where it functions to sort and cluster cargo into clathrin coated vesicles [44]. Although the mechanism is not understood, two partially redundant AP-1 subunits, UNC-101 and APM-1, negatively regulate LET-23 EGFR signaling [43,61]. Despite the fact that RAB-7 and AP-1 regulate distinct steps in the vesicular trafficking network, the strong Muv phenotype of unc-101(gy108); rab-7(ok511) double mutants might represent compounded defects in the sorting and trafficking of LET-23 and/or other transmembrane regulators of the LET-23 signaling, such as the DEP-1 protein tyrosine phosphatase [33,62]. The fact that the induced cells in unc-101(gy108); rab-7(ok511) failed to invaginate or express the egl-17::GFP marker suggested that they did not fully adopt a 1° or 2° vulval cell fate implies that AP-1 and RAB-7 might regulate additional inductive pathways such as LIN-12 Notch signaling. In Hela cells, the overexpression of a dominant-negative Rab7[N125I] or Rab7 RNAi inhibited EGFR-induced EGFR degradation, and the EGFR accumulated in ILVs of MVBs [16,17], where the EGFR would be unable to engage downstream signaling molecules. However, our findings suggest that LET-23 EGFR is still competent to signal in the VPCs when rab-7 activity is inhibited, leading us to question whether LET-23 also transits through MVBs. Four ESCRT complexes (0, I, II, and III) are required for MVB formation and sort ubiquitinated EGFR (and other cargos) into ILVs [63]. Components of the ESCRT-0 and ESCRT-I complexes antagonized RTK signaling in mammalian cells and in Drosophila [53–55]. While the ESCRT components have been shown to regulate LET-23:GFP localization in the embryonic hypodermis, they have not been shown to modulate LET-23 EGFR signaling [20]. Here we demonstrated that RNAi of hgs-1, and sps-28, components of the ESCRT-0, and ESCRT-I complexes, respectively, suppressed the severity of the lin-2(e1309) Vul phenotype suggesting that the ESCRT complexes negatively regulate LET-23 EGFR signaling and that in the VPCs, LET-23 EGFR is targeted to the lysosome via MVBs. Therefore, the enhanced LET-23 EGFR signaling in rab-7(ok511) mutants is not for a lack of trafficking through MVBs. Consistent with RAB-7 regulating LET-23 EGFR trafficking, we demonstrated that RAB-7 acts within the VPCs and influences LET-23:GFP localization. LET-23:GFP localizes to foci in the VPCs of rab-7(ok511) animals, that are more apparent in the lin-2(e1309); rab-7(ok511) background. We further explored this using a LET-23:GFP that is expressed in the hypodermal syncytium where is can be seen in endosomal foci [20]. We found that there is nearly a two-fold increase in the number LET-23:GFP foci in rab-7(ok511) animals consistent with LET-23:GFP accumulating in an endosomal compartment. Since Rab7 regulates early to late endosome maturation [64–67], in addition to transition of cargo between late endosomes/MVBs and lysosomes [17,68–70], some LET-23 EGFR could become trapped on early endosomes prior to entry into MVBs. However, we failed to detect any significant colocalization between LET-23:GFP and the early endosome marker, EEA-1, in either wild-type or rab-7(−) animals. Alternatively, LET-23 EGFR could enter into MVBs, but in the absence of lysosomal degradation might exit the MVB via back-fusion. Viral nucleocapsids can escape the MVB via back-fusion of ILVs [71], however it is not known whether the EGFR or other cell surface receptors can exit MVBs by this manner. In either case, the LET-23 EGFR could conceivably signal from these internal membranes or be recycled back to the plasma membrane to reengage LIN-3 EGF. Consistent with LET-23 EGFR recycling, several regulators of endosome recycling can promote LET-23 EGFR signaling during vulva development (A. Holmes and G. Michaux, personal communication).

Like mutations in the negative regulators unc-101, shi-1, and gap-1, the rab-7(ok511) mutant strongly suppresses the Vul phenotypes of lin-2, lin-7, and lin-10 mutants as well as the let-23(sy1) allele, often resulting in a Muv phenotype. LET-23 EGFR is mislocalized to the apical domain of the VPCs in lin-2, lin-7, lin-10, and let-23(sy1) mutants [46,47,50]. These mutations could restore VPC induction in lin-2, lin-7, lin-10, and let-23(sy1) mutants by restoring LET-23 localization to the basolateral membrane, or alternatively might simply lower the threshold for VPC induction as has been suggested for gap-1 mutants [39]. The accumulation of endosomal LET-23:GFP in rab-7(ok511) mutants would be most consistent with suppressing lin-2, lin-7, lin-10, and let-23(sy1) mutants by lowering the threshold for activation.

We previously identified rab-7 in an RNAi screen for regulators of LET-23 EGFR signaling during embryogenesis for specification of the excretory duct cell [72]. In that process, rab-7 appears to promote LET-23 EGFR signaling. However, many of the genes identified in the screen appear to act indirectly to promote LET-23 EGFR signaling, and we cannot rule out that rab-7 might also indirectly promote excretory duct cell fate specification. Alternatively, it might be possible that RAB-7 has cell type specific effects.

**Figure 5.** rab-7(−) animals accumulate LET-23::GFP-positive puncta in the hypodermis. (A and B) Representative epifluorescence images of LET-23:GFP positive foci in the hypodermis of the mid-body of L3 stage rab-7(ok511); xhs2501 (A) and rab-7(ok511); xhs2501 (B) larvae. (C) A scatter dot plot of the number of LET-23:GFP positive foci within a fixed area of the hypodermis of xhs2501, rab-7(ok511); xhs2501, and rab7(ok511); xhs2501 L3 larvae. Error bars represent the mean +/- SEM. In an unpaired t test there is a significant difference (P value=0.0001) between the number of LET-23:GFP positive foci in rab-7(ok511) animals as compared to both rab-7(+) and rab-7(ok511)/+ animals. n=number of animals scored. Bar, 10 μm (A).

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on LET-23 EGFR signaling. In contrast to what is seen in Hela cells, Rab7 has recently been suggested to promote EGFR stability in A431 and MCF7 cancer cells by protecting EGFR from proteosomal degradation [73]. We have not found a role for Rab-7 in modulating LET-23 EGFR signaling during the specification of the P12.p-ab hypodermal cell (data not shown). However, the P12.p-ab cell fate is specified at an earlier developmental stage than the vulval cells and could be maternally rescued in rab-7 homozygous progeny of a heterozygous parent.

In summary, we show that Rab-7 antagonizes LET-23 EGFR signaling during C. elegans vulva development. The requirements for Rab-7 in LET-23 EGFR signaling are similar to, but distinct from those of previously described negative regulators. Because the EGFR, as well as many of its downstream effectors, can have oncogenic properties in humans [5], our findings that Rab-7 antagonizes LET-23 EGFR signaling, suggest the possibility of Rab7 having tumor suppressor activities in humans like that of c-Cbl through promoting downregulation of activated RTKs.

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Author Contributions

Conceived and designed the experiments: CER. Performed the experiments: OS CER. Analyzed the data: OS CER. Wrote the paper: OS CER.

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