EXC-4/CLIC proteins are conserved regulators of Gα-Rac signaling

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Introduction

Biological tube formation (tubulogenesis) is a key process during vasculogenesis and angiogenesis, and is required for the proper exchange of nutrients and waste within tissues.

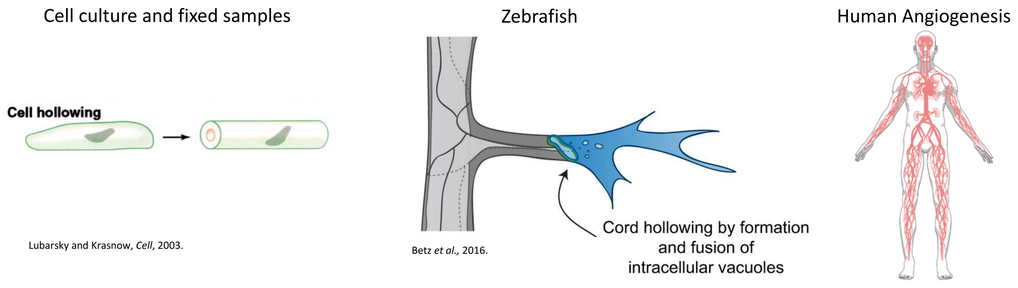

Fig. 1: Modes of cell hollowing tube morphogenesis. Depiction of morphological processes of cell hollowing tube formation and examples of where they occur.

Understanding the mechanism of tubulogenesis and the proteins involved is crucial, because even minor disruptions in this process can lead to disease.

The chloride intracellular channel (CLIC) family was first implicated in tubulogenesis by the discovery that EXC-4, a worm CLIC, is required for development of the *C. elegans* excretory canal[1] (*ExCa*). The *ExCa* is a unicellular cell tube that develops via a cell hollowing mechanism and is a proven model for studying conserved regulators of tubulogenesis[2] (Fig. 2).

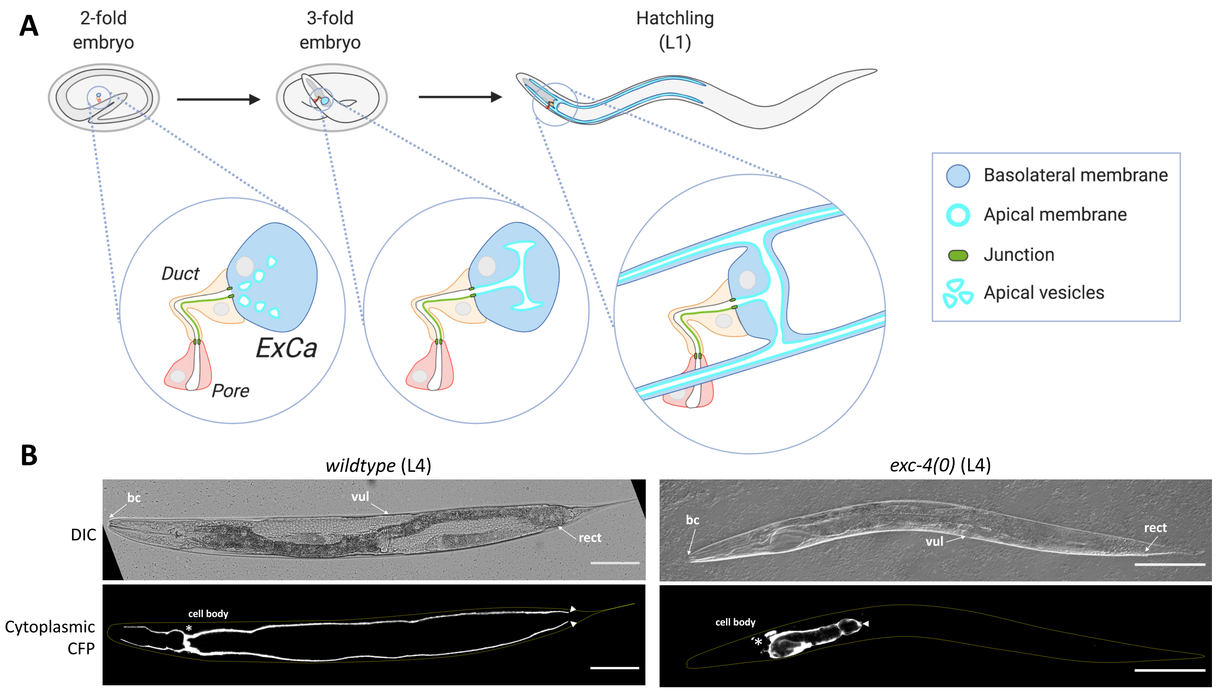

Fig. 2: The *C. elegans* excretory canal (*ExCa*) cell. A) Cartoon of the single-celled *ExCa* development. **C)** The *ExCa* in wildtype and in an *exc-4* null (*0*) showing the cystic (cyst) phenotype caused by loss of EXC-4. The *ExCa* is marked with cytoplasmic CFP, driven by an *ExCa*-specific promoter. *ExCa* tips marked by arrowheads. Cell body marked by asterisk. Yellow dotted line outlines worm body. bc= buccal cavity, vul= vulva, rect=rectum. Scale bar = 100 µm.

EXC-4/CLICs are small globular proteins structurally related to the omega-class of glutathione S-transferases (GSTs) (Fig. 3A, B). CLICs exist in soluble and membrane-associated forms, and in the case of vertebrate CLIC1 and CLIC4, transition between these states appears to be regulated by oxidation[4]. However, the molecular, cellular and physiological function of CLICs is still not well understood.

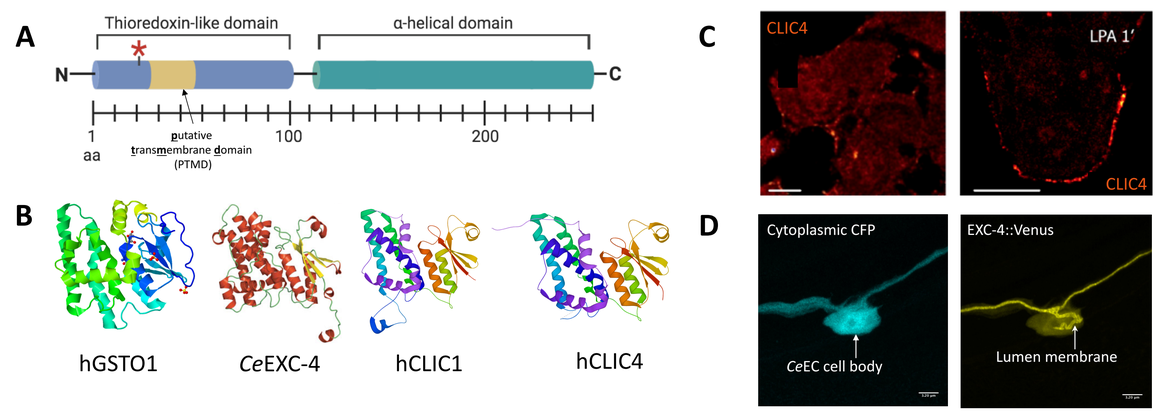

Fig. 3: CLIC structure and localization (adapted from refs. 3-5,17). **A)** CLIC domain structure. N-terminal thioredoxin-like and C-terminal **α**-helical domains are shown in blue and green, respectively. The thioredoxin-like domain has a putative transmembrane domain (PTMD, yellow). Red asterisk indicates a Cys residue found in most CLICs required for thioredoxin-like activity (not conserved in EXC-4). **B)** Tertiary structure of hGSTO1, *Ce*EXC-4, hCLIC1, and hCLIC4, highlighting their similarity. **C)** Membrane translocation of CLIC4 upon GPCR activation by LPA stimulation. **D)** *ExCa* with cytoplasmic CFP, expressed from the transgene *arIs198*, and Venus-tagged EXC-4, expressed from the transgene *arIs191,* showing clear apical membrane accumulation of EXC-4::Venus.

It has been shown that vertebrate CLIC1 and CLIC4 are expressed in vascular endothelial cells and are required for angiogenesis[6-8]. Importantly, CLIC1, when targeted to the apical plasma membrane using the EXC-4 putative transmembrane domain, can rescue *C. elegans exc-4(0)* mutants[9], demonstrating conservation of function, and a critical role for membrane localization in EXC-4/CLIC function during tubulogenesis.

EXC-4/CLIC-mediated Signaling

Human CLIC1 and CLIC4 accumulate in the cytoplasm at steady state, but are recruited to the plasma membrane upon activation of various G-protein-coupled receptors (GPCRs), including the lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) receptors (LPARs and S1PRs)[5]. In contrast, EXC-4 is constitutively enriched at the apical membrane[1,9]. How this localization is regulated in endothelial cells or in *C. elegans* remains unknown.

S1P signaling, via heterotrimeric G proteins and Rho-family effectors, regulates many aspects of endothelial cell physiology, including barrier function, cell migration and lumen formation[10,11]. These behaviors are critical for tube formation during angiogenesis. Work from the Kitajewski Lab suggests that CLIC1 and CLIC4 modulate S1P-signalling in endothelial cells, providing a model for their possible role in angiogenesis. However, the mechanism of CLIC function in this context remains to be defined.

Hypothesis:

EXC-4, like CLICs, are regulated by heterotrimeric G-proteins and modulates Rho-family signaling in the *ExCa*.

G-protein Rho-family signaling in *C. elegans*

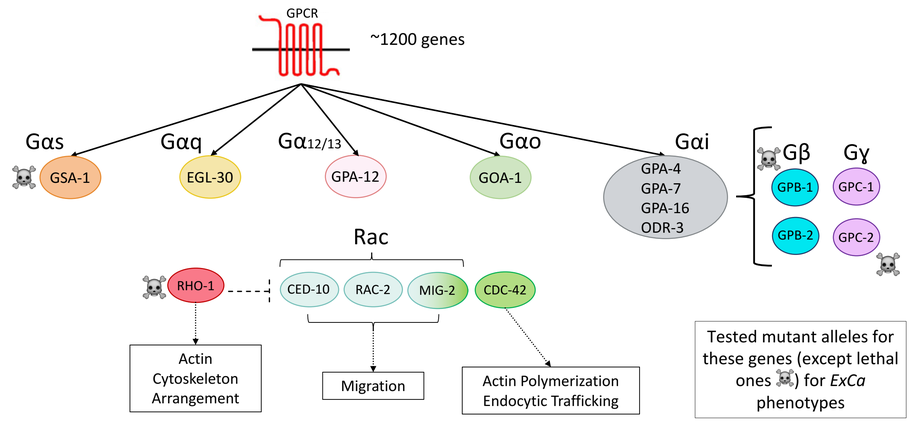

Fig. 4: Simplified GPCR Rho-family signaling. GPCRs stimulate heterotrimeric G proteins and downstream effectors[14, 15]. ☠ = null mutation lethal

ced-10/Rac and *mig-2/Rac* mutants have *ExCa* phenotypes and are redundant for *ExCa* outgrowth.

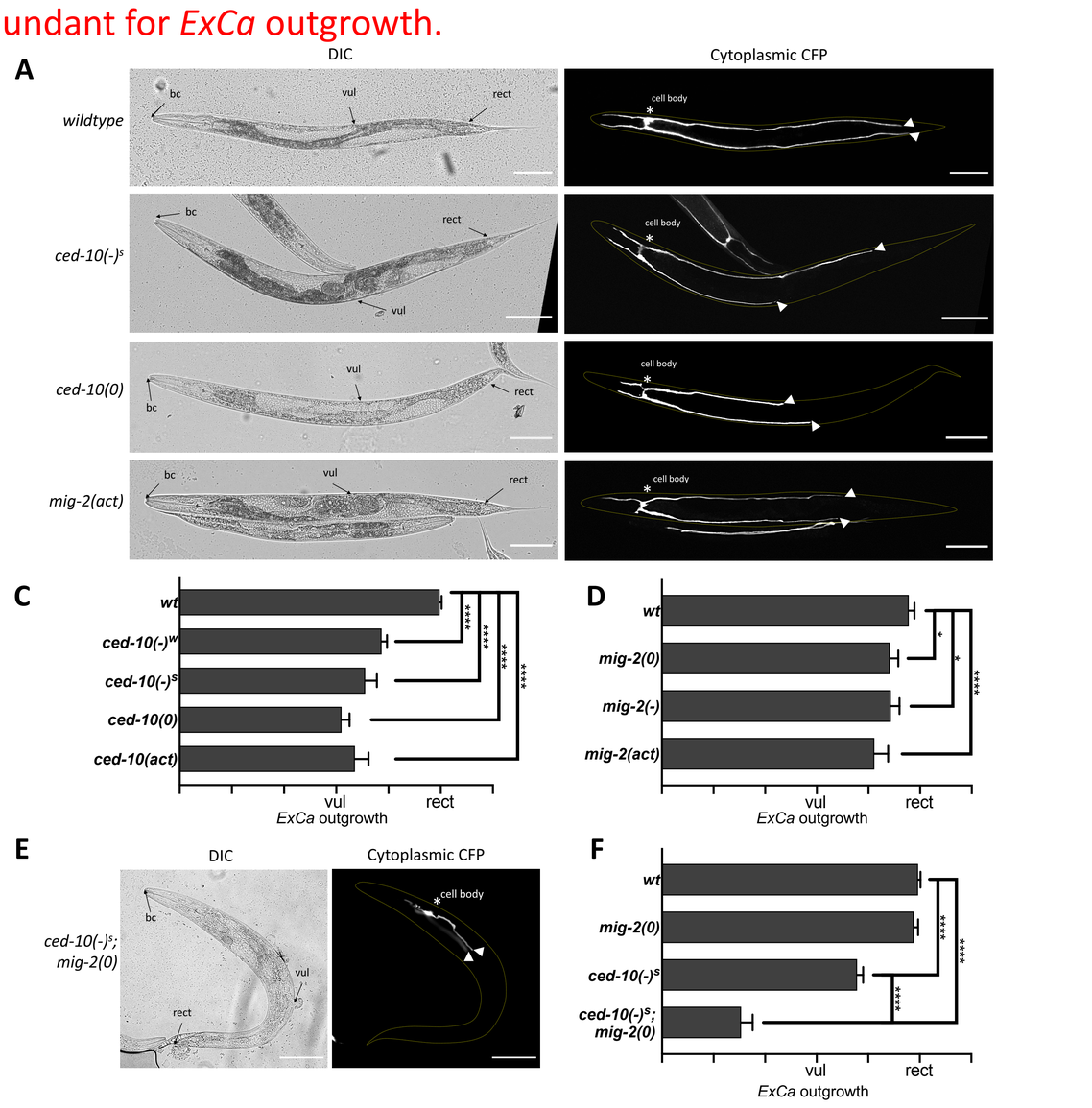

Fig. 5: *ced-10* and *mig-2* mutate analysis in the *ExCa*. A) Image of *wildtype* and mutant alleles of *ced-10* and *mig-2*. **B,C)** Quantification of *ExCa* outgrowth of mutant alleles of *ced-10* and *mig-2*. **E)** Image of ced*-10(-)s; mig-2(0)* double mutant. **F)** Quantification of *ExCa* outgrowth of ced*-10(-)s; mig-2(0)* double mutant. *ExCa* tips marked by arrowheads. Cell body marked by asterisk. Yellow dotted line outlines worm body. bc= buccal cavity, vul= vulva, rect=rectum. Scale bar = 100 µm.

New hypomorphic allele of *exc-4* provides sensitized background for identifying genetic interactions

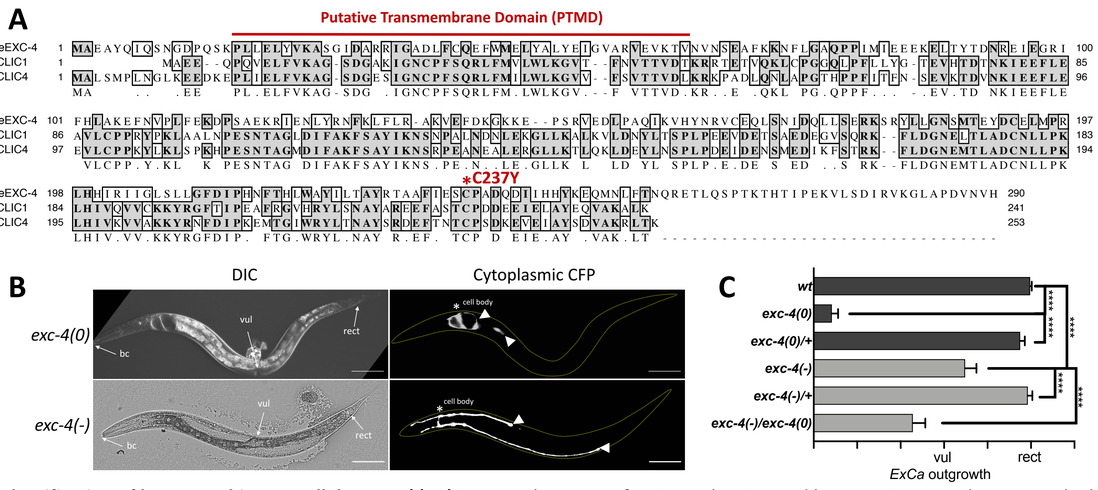

Fig. 6: Identification of hypomorphic *exc-4* allele, *exc-4(-)*. A) Protein alignment of *Ce*EXC-4, hCLIC1, and hCLIC4. Conserved amino acids shaded in dark grey, similar amino acids shaded in light grey. Putative transmembrane domain (PTMD) labeled in red. Cys 237 to Tyr mutation in hypomorphic allele of *exc-4* marked by asterisk. **B)** Image of *exc-4(0)* and *exc-4(-)* worms. *ExCa* tips marked by arrowheads. Cell body marked by asterisk. Yellow dotted line outlines worm body. bc= buccal cavity, vul= vulva, rect=rectum. Scale bar = 100 µm. **C)** *ExCa* outgrowth quantifications of *exc-4(-)* compared to *exc-4(0)*. *exc-4(-)* allele is recessive and failed to complement *exc-4(0)*.

Gαq, Gαs, and Gα12/13 genetically interact with *exc-4* to negatively regulate *ExCa* outgrowth, while Gαi genetically interacts with *exc-4* to positively regulate *ExCa* outgrowth.

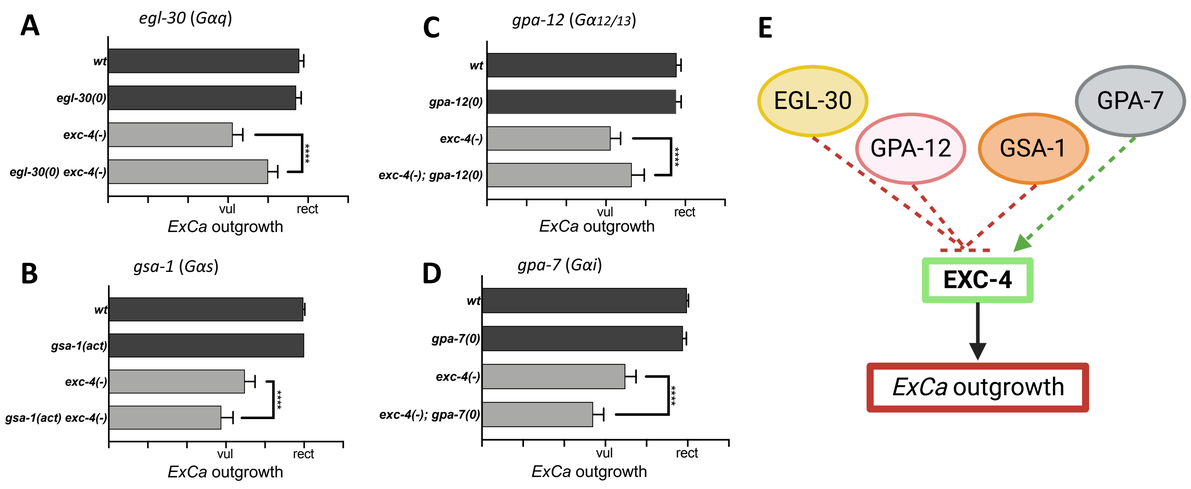

Fig. 7: Quantification of *ExCa* outgrowth from Gα subunits with genetic interactions to *exc-4(-)*. A,B,C) Gαq, Gαs (note activated allele used), and Gα12/13 genetically interact with *exc-4* to negatively regulate *ExCa* outgrowth, while **D)** Gαi genetically interacts with *exc-4* to positively regulate *ExCa* outgrowth. **E)** Proposed model.

mig-2/Rac genetically interacts with *exc-4* in a parallel pathway to promote *ExCa* outgrowth.

ced-10/Rac genetically interacts with *exc-4* in the same pathway, with *exc-4* epistatic to *ced-10*. Mutant analysis suggests that *ced-10* acts as a negative regulator of *exc-4* dependent *ExCa* outgrowth.

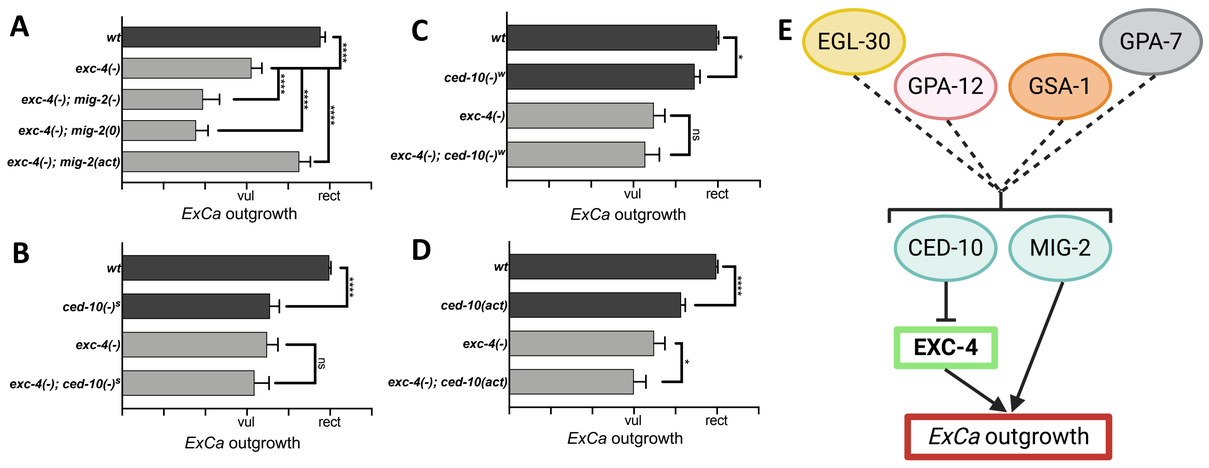

Fig. 8: Quantification of *ExCa* outgrowth from *mig-2* and *ced-10* genetic interactions with *exc-4(-)*. A) *mig-2* mutant alleles reveal parallel genetic interaction with *exc-4* to promote *ExCa* outgrowth. **B,C,D)** *ced-10* genetically interacts with *exc-4* in the same pathway. B,C) *exc-4* epistatic to *ced-10*. D) *ced-10* negatively regulates *exc-4*. **E)** Proposed model.

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