Role of RalGPS2, a new possible oncogene, in transformed and cancer cells

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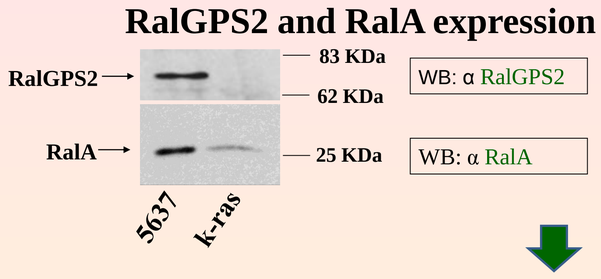
ABSTRACT: Ral GTPases have been implicated in tumorigenesis and metastasis *in vitro* and animal models; furthermore Ral pathway appears to play a prominent role in transformation of human cells (Smith et al. 2007 *Clin. Cancer Res.* 13, 3803-3813). RalGPS2 is a GEF for RalA belonging to RalGPS family that contains a well conserved Ras-GEF domain, a PxxP motif and a PH domain. Previous experiments demonstrated that RalGPS2 activates RalA *in vivo*, while its PH-PxxP domain acts as a dominant negative for RalA activation in NIH3T3 and PC12 cells. These data suggest that RalGPS2 PH-PxxP domain could inhibit RalA activation influencing cytoskeleton rearrangements. The aim of this project is to analyse the role of RalGPS2 and of its PH-PxxP domain in human bladder cancer cells (5637) and rodent transformed cells (NIH3T3-k-Ras). RalA and RalGPS2 were highly expressed in 5637 cells and the overexpression of PH-PxxP domain reduced the level of RalA-GTP. Besides the overexpression of PH and PH-PxxP domains in both cell lines induced a marked cytoskeleton re-organization: in particular the PH domain caused formation of thin vesiculating protrusion while the PH-PxxP domain caused formation of long inter-cellular structure probably involved in the exchange of signals and cellular components between cells.

AIM of the project: the Aim of the project is to analyze the role of RalGPS2 and of its PH-PxxP domain in human and rodent cells transformation using 5637 and NIH-3T3 k-ras cells

RalGPS2 and RalA expression in 5637 and NIH3T3 k-ras cells



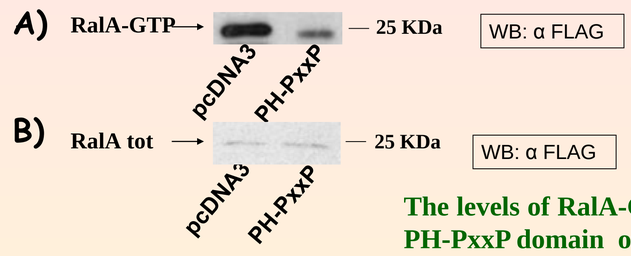
Western blot assays were performed on total protein extracts of 5637 and NIH3T3 k-ras cells to analyze endogenous RalGPS2 and RalA expression. Equal amount of total proteins for each sample was loaded on a 10% SDS-polyacrilamide gel. Membranes were probed with anti-RalGPS2 and anti-RalA antibodies.

RalA and RalGPS2 are highly expressed in 5637 human bladder cancer cells.

RalGPS2 seems to be not expressed in NIH3T3 k-ras and Ral A is expressed at low level

The PH-PxxP domain of RalGPS2 inhibits RalA activation behaving as a dominant negative in 5637 cells

To analyze RalA activation a pull down assay was performed on 5637 transfected with pcDNA3 or PH-PxxP domain of RalGPS2. An equal amount of total protein extracts was loaded on SDS-polyacrilamide gel for normalization. RalA protein was visualized using anti-FLAG antibodies.

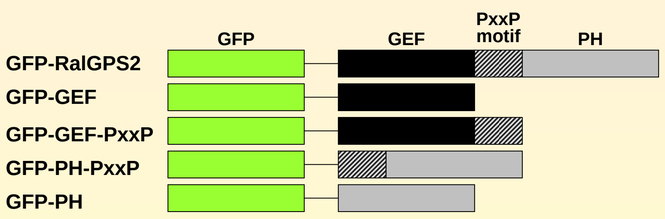


Panel A: RalA-GTP protein immunoprobed with anti-FLAG antibodies

Panel B: total extracts immunoprobed with anti-FLAG antibodies

The levels of RalA-GTP decrease in 5637 cells that overexpress PH-PxxP domain of RalGPS2.

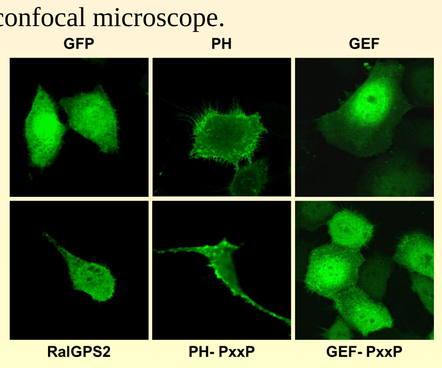
GFP-fusion constructs of RalGPS2

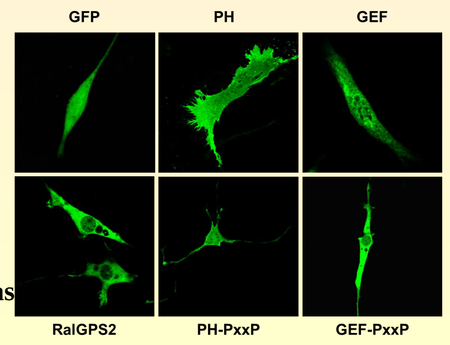


To identify the localization of RalGPS2 full length protein and of its single domain in 5637 and in NIH3T3 k-ras cells were used GFP fusion constructs previously realized in pEGFP-C1 vector (Ceriani M. et al 2007 Exp. Cell Res. 313, 2293-2307).

Localization of RalGPS2 and of its domains in NIH3T3 k-ras and 5637 cells

To identify the localization of RalGPS2 full length protein and of its single domain, 5637 and NIH3T3 k-ras cells were transfected with pEGFP-C1 vector or with different RalGPS2 GFP fusion constructs. Two days after transfection cells were fixed with paraformaldehyde and photographed at confocal microscope.



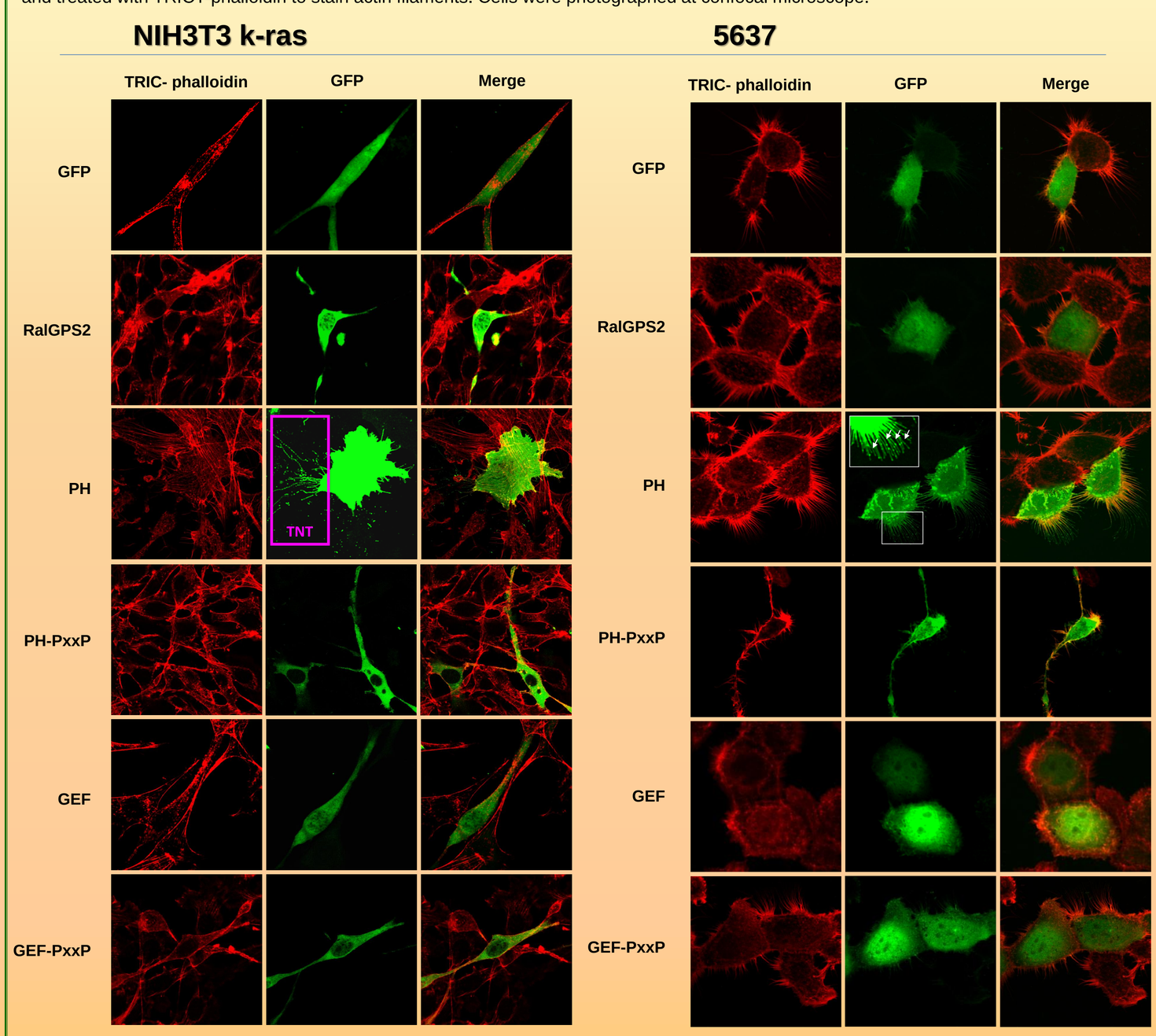


- **RalGPS2 protein is diffused in the whole cytoplasm in both cell lines and shows a partial membrane localization in 5637 cells**
- **PH domain alone is strongly localized in membranes and in cytoplasmatic regions near by the "hairs" structures**
- **The PH-PxxP region localizes in membranes and cytoplasm**

- **GEF domain alone shows a prevalent nuclear localization in 5637, while is expressed also in cytoplasm in NIH3T3 k-ras**
- **The GEF-PxxP region localizes both in cytoplasm and nucleus in 5637; in NIH3T3 k-ras only in cytoplasm.**

Effects of the overexpression of RalGPS2 different constructs on NIH3T3 k-ras and 5637 cells cytoskeleton

To test the possible role of RalGPS2 on cytoskeleton, 5637 cells, plated on polylysine pre-treated glasses, were transfected with pEGFP-C1 vector or with different RalGPS2 GFP fusion constructs. Two days after transfection cells were fixed and treated with TRICT-phalloidin to stain actin filaments. Cells were photographed at confocal microscope.



NIH3T3 k-ras:

- **RalGPS2** overexpressing NIH3T3 k-ras cells show a different morphology in respect of GFP control
- The overexpression of **PH domain** alone in NIH3T3 k-ras causes formation of thin long membranous protrusions structures that could be **tunnelling nanotubes (TNTs)**. TNTs have been observed in a variety of cell types and are believed to **connect cells** of the same or different types. TNT-like close-ended long membrane protrusions are often called cytonemes or filopodial bridges. (Zhao Y. et al. 2009 *Nat. Cell Biol.* 11: 1396- 1397*)*
- Overexpression of **PH-PxxP domain** causes formation of inter-cellular protrusions made of actin in NIH3T3 k-ras cells
- In cells overexpressing **GEF** and **GEF-PxxP regions** there are no evident morphological changes of NIH3T3 k-ras cells

5637:

- **RalGPS2** overexpression doesn't show any remarkable morphological changes in 5637 cytoskeleton.
- 5637 cells overexpressing **PH domain** alone have an increased number of "hairs" structure in respect of GFP control
- Overexpression of **PH-PxxP domain** causes formation of inter-cellular protrusions made of actin in 5637
- In 5637 cells overexpressing **GEF** and **GEF-PxxP domain** the number of "hairs" structure is decreased in respect of GFP control

Conclusions

The data obtained till now have shown that the overexpression of PH and PH-PxxP domain of RalGPS2 acts on trasformed murine fibroblasts and human bladder cancer cells modifying cytoskeletal structures. In particular PH domain causes formation of thin protrusions with the presence of vesicles which could be Tunneling Nanotubes (TNT). PH-PxxP domain causes formation of long inter-cellular structure, absent when the full length protein is expressed, that are probably involved in the exchange of signals and cellular components between different cells. It will be interesting in the future to understand the molecular nature of these structures, that could be nanotubes (Hase et al., 2009 *Nature Cell Bio*. 11:1427-32**). Furthermore overexpression of PH-PxxP domain causes a decrease in RalA-GTP expression levels in 5637 cell line.**

These preliminary data let us suppose that the PH-PxxP domain could acts as dominant negative for RalA activation in these cell lines. In the future stable clone of 5637 expressing the different RalGPS2 constructs will be generated and tested for migration, invasion and anchorage-independent growth to confirm this hypothesis.

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