# Adhesion Dependent Regulatory Crosstalk Of Small GTPases Ral and Arf6 and its Role in Anchorage Dependent Signaling

A thesis submitted in partial fulfilment of requirements

Of the degree of

## Doctor of Philosophy

**By**

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DEDICATION

This work is dedicated to my parents and my sisters.

CERTIFICATE


This is to certify that the project entitled "Adhesion dependent regulatory crosstalk of small GTPases, Ral and Arf6 and its role in anchorage dependent signalling" has been carried out by Ms. Archana Pratap Pawar for partial fulfillment of the requirements for the degree of PhD at Indian Institute of Science Education and Research (IISER), Pune.

This work has been carried out under my guidance between 2nd September 2010 and 31st December 2015. This work has not been submitted for the award of any other degree in this institute or in any other institute.


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## DECLARATION

I declare that this written submission represents my ideas in my own words and where others ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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# ABBREVIATIONS

| | |
|---|---|
| Arf | ADP Ribosylation Factor |
| BFA | BrefeldinA |
| CDC | Cell Division cycle factor |
| Cav1 | Caveolin1 |
| cDNA | Complementary Deoxyribonucleic acid |
| CTxB | Cholera toxin subunit B |
| DAG | Diacylglycerol |
| DAPI | (4',6-diamidino-2-phenylindole) |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| DSHB | Developmental studies hybridoma bank |
| ECM | Extracellular Matrix |
| EDTA | Ethylene di-amine tetra-acetic acid |
| FAK | Focal adhesion kinase |
| FBS | Fetal Bovine Serum |
| GCA | Golgicide-A |
| GDP | Guanine nucleotide diphosphate |
| GFP | Green fluorescent protein |
| GM1 | Ganglioside mannoside1 |
| GTP | Guanine nucleotide triphosphate |
| HEPES | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ) |
| HRP | Horseradish peroxidase |
| IgG | Immunoglobulin G |
| IP | Immunoprecipitation |
| IP3 | Inositol (3,4,5) phosphate |
| kb | Kilobase |
| KCl | Potassium chloride |
| KD | knockdown |
| kDa | Kilo Dalton |
| M | Molar |
| mg | milligram |
| ml | Milliliter |

| | |
|---|---|
| mM | Millimolar |
| ng | Nanogram |
| NSCLC | Non-small cell lung cancer |
| PBS | Phosphate Buffered Saline |
| PIC | protease inhibitor cocktail |
| PI3K | Phosphoinositide 3-kinase |
| PIP-5-K | Phosphatidyl inositol phosphate-5 kinase |
| PI(4,5)P2 | Phosphatidyl inositol-4,5-phosphate |
| PI(3,4,5)P3 | Phosphatidyl inositol-3,4,5-phosphate |
| PKC | Protein kinase C |
| PLD | Phospholipase D |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| Ral | Ras-like GTPase |
| Ras | Rat Sarcoma Viral Oncogene Homolog |
| RNA | Ribonucleic acid |
| RPMI-1640 | Rosewell Park Memorial Institute-1640 |
| SCID | Severe combined immunodeficiency |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate- polyacrylamide gel electrophoresis |
| siRNA | Small interference RNA |
| shRNA | Short hairpin RNA |
| WB | Western blot |
| μg | microgram |

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# ABSTRACT

Anchorage independent growth of cancer cells is a key component of cancer invasion and metastasis. Cancer metastasis is a major cause of mortality, making the understanding of the basis for anchorage dependence and how it is overcome in cancers an important problem. Oncogenic Ras induced transformation in cancers drives anchorage independence through activation of small GTPases of the Ral family, RalA and RalB. Our previous studies in mouse embryonic fibroblasts (MEFs) have shown integrin mediated cell adhesion to the extra cellular matrix activates RalA (not RalB). Active RalA mediates the exocyst-dependent trafficking of membrane raft microdomains to the plasma membrane, to stimulate adhesion dependent signaling. Constitutive activation of RalA downstream of oncogenic Ras in cancers uses this pathway to support anchorage independent signaling. Like RalA, the small GTPase Arf6 is also activated by integrin mediated adhesion and regulates this raft trafficking pathway. Unlike RalA however Arf6 is necessary but not sufficient for this trafficking. Interestingly, RalA and Arf6 are reported to regulate many common cellular functions including GLUT4 receptor recycling, insulin secretion and cytokinesis, sharing signaling partners such as exocyst complex, RalBP1 and Phospholipase D1.

This study identifies a novel regulatory crosstalk between Ral and Arf6 that controls Ral function in cells. In re-adherent mouse fibroblasts (MEFs) integrin dependent activation of RalA drives Arf6 activation. Independent of adhesion constitutively active RalA and RalB could both however activate Arf6. This is further conserved in oncogenic H-Ras containing bladder cancer T-24 cells, which express anchorage independent active Ral that supports Arf6 activation. Arf6 mediates active Ral-exocyst dependent delivery of raft microdomains to the plasma membrane that supports anchorage independent growth signalling. Accordingly in T-24 cells the Ral-Arf6 crosstalk is seen to preferentially regulate anchorage independent Erk signalling. Ral and Arf6 co-precipitate with each other in a functional complex that could mediate this crosstalk. We hence investigated the role of Ral effector proteins, Sec5, Sec10, Exo84, RalBP1 and Phospholipase D1, in mediating Arf6 activation and found them to differentially regulate Arf6. Active Ral uses Ral-RalBP1-ARNO-Arf6 pathway in T-24 cells and MEFs to mediate Arf6 activation. We also looked at the role of GEF proteins in differential activation of RalA/RalB in normal and cancer cells. Our studies identify a novel role for the Ral GEF, RGL1, in mediating the differential activation of RalA downstream of integrins in MEFs and oncogenic Ras in colorectal cancer cell line SW620.

In summary, this study has identified and evaluated a novel regulatory crosstalk between Ral and Arf6, testing its functional significance in mediating anchorage independent signaling and growth. It has also identified a novel role for the RalGEF RGL1 in mediating the integrin dependent differential activation of RalA. In doing so it has revealed a new understanding of how Ral isoforms (RalA and RalB) are differentially regulated and in turn regulate downstream signaling in normal and cancers.

# Synopsis

Anchorage independent growth is a major hallmark of cancer cells that confers cells with abilities of unrestricted growth potential and survival in fluids during metastasis. Cancer cells deploy various oncogenic strategies to overcome anchorage dependence which is inhibition of growth signalling observed upon loss of cell- extra cellular matrix (ECM) adhesion. Heterodimeric integrin receptors are major cell surface receptors governing cell- ECM adhesion, hence critical for anchorage dependent growth signalling.

Small GTPases are key players regulated by integrin signalling. Integrins regulate localization and activation of Rac1 GTPase at the plasma membrane and downstream signalling (Del Pozo et al., 2004). Rac1 GTPase and other signalling proteins are anchored at the plasma membrane within specialized cholesterol and sphingolipid enriched membrane raft microdomains (Golub and Pico, 2005; Palazzo et al., 2004; Simons and Toomre, 2000). Trafficking of these microdomains is regulated by integrin mediated cell adhesion. Upon loss of cell- ECM adhesion, these raft microdomains are internalized via caveolar endocytosis resulting in loss of anchoring sites from plasma membrane and inactivation of growth signalling pathways (del Pozo et al., 2005). Upon re-adhesion of cells to ECM, these microdomains are recycled back and targeted to cell membrane via the RalA-Exocyst complex restoring anchorage dependent growth signalling (Balasubramanian et al., 2010). This exocytic pathway is regulated by integrin dependent activation of two small GTPases, RalA and Arf6 (Balasubramanian et al., 2007, 2010).

Upon loss of adhesion RalA and Arf6 activities are downregulated leading to inhibition of the exocytic trafficking pathway and retention of lipid rafts microdomains in the recycling endosomal pool. When cells are replated back on the ECM, integrins activate RalA and Arf6 to trigger the exocytosis of these raft microdomains to restore integrin dependent signalling. Further, constitutively active RalA and Arf6 were observed to regulate integrin independent raft trafficking and growth signalling. These GTPases hence are critical factors regulated by integrins to mediate anchorage dependent growth signalling (Balasubramanian et al., 2007, 2010). In agreement with this, hyperactivation of RalA is observed to regulate anchorage independent growth in several cancers, including pancreatic and colorectal cancer (Lim et al., 2006; Martin et al., 2011). Oncogenic Ras mutation (Ras-G12V) in these cancers is responsible for hyperactivation of RalA that is shown to be critical to Ras induced tumorigenesis (Lim et al., 2005). Arf6, on the other hand, is reported to be critical to migration and metastatic potential of breast cancer cells owing to its regulatory effect on E-cadherin trafficking (Hashimoto et al., 2004; Palacios et al., 2001; Sabe, 2003). Arf6 also

regulates Erk signalling downstream of EGFR in hepatoma and melanoma cancer cell lines (Hu et al., 2012; Tague et al., 2004). Though Arf6 promotes proliferation of adherent cells through regulation of PLD-mTORC (Knizhnik et al., 2012), a direct correlation of Arf6 and anchorage independent growth of cancer cells is not known so far.

Interestingly Ral and Arf6 GTPases share several signalling intermediates. These include the exocyst complex, phospholipase D1 (PLD1) and RalBP1. Apart from their functional associations with common effector proteins, Ral GTPases and Arf6 also participate in several common pathways. These include dense core granule exocytosis from neuroendocrine cells (Vitale et al., 2002, 2005), insulin stimulated GLUT4 receptor trafficking in adipocytes (Chen et al., 2007; Millar et al., 1999), FCγR receptor mediated phagocytosis by macrophages (Corrotte et al., 2010; Melendez et al., 2001) and integrin mediated membrane raft trafficking in MEFs (Balasubramanian et al., 2007, 2010).

An important consideration while studying RalA GTPase functions in cells is its isoform RalB. RalB shares 89% sequence similarity with RalA and binds the same effector proteins, but mediates distinct functions than RalA in different cellular and spatiotemporal contexts (Shipitsin and Feig, 2004, Rossé et al., 2006, Cascone et al., 2008, Lim et al., 2006; Martin et al., 2011, Chien and White, 2003; Chien et al., 2006). Both Ral isoforms bind the same activators (GEFs)/ deactivators (GAPs) and downstream effectors, but exhibit differential roles in various cellular functions, thus highlighting differences in regulation of their activation and effector engagement. For example, integrin mediated adhesion regulates activation of only RalA, but not that of RalB. Consequently only RalA, and not RalB, regulates adhesion dependent cell spreading (Balasubramanian et al., 2010). However, the mechanism(s) mediating the differential activation of Ral isoforms downstream of integrins remain unknown. This could be mediated by Ral GEFs and / or GAPs. RalGEFs are categorized as Ras-dependent (RalGDS, RaGL1, RGL2, and RGL3) or Ras-independent (RalGPS-1, RalGPS-2). During cytokinesis, differential localization of RalGEFs has been correlated to differential regulation and function of RalA and RalB (Cascone et al., 2008). Whether such a differential regulation by RalGEFs contributes to differential activation of RalA vs RalB downstream of integrins and their function in anchorage dependence remains unknown.

The overlap in Ral and Arf6 mediated cellular processes and their shared effector pathways led us to ask could a regulatory crosstalk exist between Ral and Arf6 downstream of

integrins? Could this crosstalk be conserved in active Ral expressing cancers? Could differential regulation by integrins of RalA and RalB affect this crosstalk and Ral isoform function in normal and anchorage independent cancer cells? To address these questions, the present study was undertaken with the following objectives:

I.   Test and validate the existence of a Ral-Arf6 crosstalk downstream of integrins
II.  Test the presence of Ral-Arf6 crosstalk in cancers and its effect on anchorage independent signalling and growth in cancer cells.
III. Elucidate the mechanism of this Ral-Arf6 crosstalk
IV.  Elucidate role of RalGEFs in mediating differential adhesion dependent activation of RalA vs RalB

## I. Adhesion dependent Ral-Arf6 crosstalk:

Integrin dependent activation of RalA and Arf6 is essential for regulating membrane raft microdomain exocytosis in mouse embryonic fibroblasts (MEFs). Knockdown of either RalA or Arf6 abrogates exocytosis of membrane raft domains in re- adherent cells (Balasubramanian et al., 2007, 2010). Expression of active RalA mutant supports complete delivery of raft domains in non-adherent cells, overriding the effect of diminished integrin signaling (Balasubramanian et al., 2010). However, active Arf6 mutant can support delivery of raft microdomains only up to cortical actin region and fails to deliver them to plasma membrane (Balasubramanian et al., 2007). Hence RalA is both necessary and sufficient to drive this exocytic pathway; while Arf6 is necessary but not sufficient. One possible explanation for this difference could be the existence of a regulatory crosstalk between RalA and Arf6. To understand this relationship, we first tested the effect knockdown (KD) of one GTPase has on the activation status of the other. Depletion of RalA in MEFs did not affect the drop in Arf6 activation on loss of adhesion but disrupted the activation of Arf6 in re-adherent cells. This regulation was further confirmed when expression of siRNA resistant RalA mutant in RalA KD cells rescued Arf6 activation in re- adherent cells. When simultaneously observed knockdown of RalB did not disrupt the drop or recovery of Arf6 activation, suggesting this regulation to be specific to RalA. The differential activation of RalA by integrins could mediate this isoform specificity. Interestingly, Arf6 KD in MEFs did not disrupt adhesion dependent activation of RalA. This suggests the presence of a **linear integrin-RalA-Arf6 pathway** in cells.

To further evaluate if this crosstalk works only downstream of integrins, we asked if Ral could regulate Arf6 in the absence of adhesion. To test this we ectopically expressed the constitutively active mutant of RalA, RalA-G23V (RalA-V23) in MEFs. This mutant is constitutively GTP bound and is active independent of upstream regulatory events, in this case cell-ECM adhesion. Expression of RalA-V23 in MEFs increased Arf6 activity in non-adherent cells to the levels equivalent to those in stable adherent and re-adherent cells. Thus RalA can activate Arf6 independent of the context of cell-ECM adhesion. Since we speculated that Ral isoform specificity for Arf6 regulation could be context dependent, we tested if constitutively active RalB mutant RalB-G23V (RalB-V23) in MEFs can regulate Arf6. Overexpression of RalB-V23 also mimicked the effect of RalA-V23 on Arf6 activation in MEFs. This data indicates that **both Ral isoforms carry the capability to regulate Arf6 depending upon their activation status**, which in turn is dependent on the stimulus. In non-adherent cells we find active RalA/B can hence support anchorage independent Arf6 activation. Such an anchorage independent activation of Ral is also seen downstream of oncogenic Ras and might similarly regulate Arf6, a hypothesis that has been explored in objective II. The immediate question, however, was if Arf6 activated downstream of Ral GTPases is required for Ral function.

To test this we decided to look at the Ral-exocyst dependent regulation of membrane raft trafficking known to be dependent on RalA and Arf6 (Balasubramanian et al., 2007, 2010). Active RalA was necessary and sufficient while active Arf6 was necessary but not sufficient for this trafficking. Knowing the presence of Ral-Arf6 crosstalk in these cells, we asked whether Arf6 activated downstream of active RalA is required for Ral dependent exocytosis. To test this hypothesis we established a stable Arf6 KD cell line, shArf6-MEF. We confirmed their surface GM1 levels and RalA expression were comparable to control MEFs and shRAf6 MEFs. Expression of active RalA-79L showed a 1.5 fold increase in surface GM1 levels relative to control, this increase not seen in active RalA-79L expressing shArf6 MEFs. This data hence revealed an **essential role for Arf6 downstream of active RalA in regulating integrin independent membrane raft exocytosis**. Also since active Arf6 by itself is defective in the delivery of raft components, active RalA and Arf6 could work together in mediating this delivery at the plasma membrane.

We hence enquired if RalA and Arf6 associate with each other as part of such a regulatory complex. MEFs expressing constitutively active RalA-V23 mutant and fast cycling active mutant of Arf6, Arf6-T157A, were replated on fibronectin coated coverslips and fixed when

cells are actively spreading. Immunostaining of these cells for RalA and Arf6 using specific antibodies showed they co-localized extensively at membrane ruffles which are the sites of enhanced membrane delivery in FN-replated MEFs. Since active RalB also regulates Arf6 activation, we tested co-localization of both active RalA and active RalB with WT and active Arf6. Both HA-RalA-V23 and HA-RalB-V23 co-localized to similar extent with FLAG-Arf6-WT and FLAG-Arf6-T157A in rapidly spreading cells at membrane ruffles. Interaction of Ral-Arf6 was further confirmed by immunoprecipitation of FLAG-RalA/RalB-WT or FLAG RalA/RalB-V23 from cell lysates of 293T cells co-expressing HA-Arf6-T157A using anti-FLAG antibody. Active Arf6-T157A specifically and equivalently co-immunoprecipitated with WT and active RalA/B. However, only a small fraction of the expressed Arf6 bound RalA/B suggesting this association to be transient at best. Interestingly, almost similar levels of active Arf6 were pulled down with both WT as well as active Ral suggesting **Ral-Arf6 association to not be a direct effector interaction**. Since active Ral mediates Arf6 activation, a role for a Ral effector as a possible intermediate in mediating this crosstalk was envisaged and explored in objective III.

## II. Role of the Ral-Arf6 crosstalk in mediating anchorage independent signalling and growth:

MEFs expressing active RalA (RalA-79L) drive the adhesion independent targeting of membrane rafts to plasma membrane to support adhesion independent activation of Akt and Erk kinases (Balasubramanian et al., 2010). This corroborates with the well-established role for active RalA in supporting anchorage independent growth across multiple cancer types such as pancreatic, colorectal and bladder cancer. This Ral activation happens downstream of mutated Ras (Ras-V12) through Ral GEFs and is seen to be essential for Ras-V12 driven transformation. Our data in MEFS has shown that constitutively active Ral can support anchorage independent Arf6 activation and this Ral-Arf6 crosstalk is necessary for anchorage dependent raft trafficking which in turn could support anchorage independent growth signalling. Hence we asked whether such Ral- Arf6 crosstalk is detected in Ras-Ral expressing human cancer cells and the role it could have in supporting anchorage independent signalling and growth.

We hence tested the Ral-Arf6 crosstalk in pancreatic cancer cell line MIA-PaCa-2 (K-RAS G12V), fibrosarcoma cell line HT-1080 (N-RAS G12V) and bladder cancer cell lines T-24 (H-RAS G12V) and UM-UC-3 (K-RAS G12V). We observed adhesion independent

activation of RalA and Arf6 in all the tested cancer cell lines. However upon knockdown of RalA and RalB in MIA-PaCa-2, HT-1080, and UM-UC-3, adhesion independent Arf6 activation was unaffected relative to control cells. In T-24 cell line, loss of RalA and RalB did however affect Arf6 activation. Earlier report by Xu et al has shown that in H-Ras-V12 transformed NIH3T3 cells, RalA and Arf6 immunoprecipitated better than in K-Ras-V12 expressing cells (Xu et al., 2003). Hence the Ral-Arf6 crosstalk that is observed in T-24 cells (H-Ras-V12) and not in MIA-PaCa-2, HT-1080, and UM-UC-3 cell lines (K-Ras-V12) could be due to the specificity of upstream Ras isoform stimulus. This regulation of Arf6 by RalA and RalB in non-adherent T-24 cells was also seen to be regulated by the overlap present between integrin and growth factor signalling pathways. Thus **in low serum conditions, both Ral isoforms (RalA and RalB) comparably regulated Arf6, while in the presence of serum this regulation was altered with RalB, more than RalA, mediating this crosstalk.** This means that in the presence of serum, conditions normally used for growth and signalling studies in T-24 cells, RalB is the major regulator of Arf6 and possibly signalling downstream. This is in agreement with earlier observations showing RalB is more active than RalA in T-24 cells cultured in serum containing media (Saito et al., 2013) and hence would have a greater impact on Arf6 activation. Consequently, effector engagement and signalling in T-24 cells are expected to be more RalB dependent. This differential regulation of Arf6 could also contribute to Ral isoform specific functions in these cells which we next explored.

Ral GTPases are known to be vital for anchorage independent growth of bladder cancer cells. Whether Arf6 can regulate anchorage independent signalling and growth of bladder cancer cells has not been tested. Our data so far demonstrates the Ral GTPases regulate anchorage independent activation of Arf6 in the T-24 cell line. Hence we evaluated **contribution of Ral- Arf6 crosstalk to anchorage independent Erk signalling in T-24 cells**. We compared the ratio of phosphorylated Erk (Thr202/Tyr204) to total Erk (pErk/Erk) in non-adherent control cells to RalA KD, RalB KD, Arf6 KD as well as combined RalA+Arf6 KD and RalB+Arf6 KD cells in presence of serum growth factors. The efficiency of knockdown of each of these GTPases in combined knockdown was verified to be equivalent to their individual knockdown. Erk activation in non-adherent T-24 cells was marginally affected by loss of RalA. Its combined knockdown with Arf6 however significantly reduced Erk activation in T-24 cells. This suggested the marginal decrease in Erk activation by RalA KD alone could be a result of its modest effect on

Arf6 activity in non-adherent T-24 cells. This was substantiated by the distinct reduction in Erk activation seen upon loss of RalB, that significantly affects Arf6 distinctly, comparable to the joint RalB and Arf6 knockdown. In bladder cancers RalB is known to be more active than RalA in some studies (Saito et al., 2013) with a reported role in migration, proliferation and anchorage independence as well (Oxford et al., 2005; Smith et al., 2012; Wang et al., 2010). We tested and found that **in non-adherent T-24 cells RalB activity was indeed significantly higher than RalA and hence could mediate differential utilization of Ral effectors and downstream Arf6 activation and signalling**. Erk activation was significantly reduced by the depletion of Arf6, reflecting its role at the end of the linear H-Ras-Ral-Arf6 pathway.

Finally, we explored the contribution of Arf6 to Ral regulated anchorage independent growth of T-24 cells in soft agar colony assays. We observed that **depletion of Arf6 affected growth of cells in soft agar the most followed by RalB KD and RalA KD**. Hence Erk signalling pathway is probably the major mediator of the anchorage independent growth of bladder cancer cells.

The next question we asked was how the Ral-Arf6 crosstalk in T-24 cells regulates anchorage independent Erk signalling. In MEFs, active RalA regulates anchorage independent membrane raft trafficking which in turn regulates growth signalling (Balasubramanian et al., 2010). Our studies have now shown essential role of the Ral-Arf6 crosstalk in the raft trafficking pathway in MEFs and seen to affect anchorage independent Erk signalling in T-24 cells. This led us to test the role Ral and Arf6 GTPases could have in membrane raft trafficking in T-24 cells. We confirmed the expression of Caveolin-1 and its phosphorylation on tyrosine-14 residue in T-24 cells and that membrane raft microdomains were endocytosed upon loss of adhesion as observed in MEFs. Knowing this we tested and found depletion of RalA, RalB or Arf6 GTPases did not significantly affect the surface GM1 levels in non- adherent T24 cells, detected by their immunofluorescence labeling. GM1 is one of the markers localized to raft microdomains whose levels and trafficking can be regulated in a cell type specific manner. Further studies to look at other membrane raft markers (GPI- Aerolysin, Cholesterol levels) will help confirm if Ral and Arf6 can indeed not affect surface raft levels in T-24 cells. Results also suggest that the Ral and Arf6 crosstalk could also use alternate mechanisms to regulate anchorage independent Erk signalling. This hence suggests the role and regulation of the Ral-Arf6 crosstalk could indeed be more complex and may involve

additional regulators in T-24 cancer cells. Anchorage independently active Arf6 detected in MIA-PaCa2, UM-UC-3 and HT1080, though not regulated by Ral, can collaborate with active Ral in mediating anchorage independent signalling and growth. Hence the significance of this crosstalk across other cancer cell lines is worth exploring.

### III. Mechanism of Ral-Arf6 crosstalk:

Arf6 does not possess a Ral binding domain and does not show a differential binding to active Ral vs WT Ral in our immunoprecipitation studies, suggesting the Ral-Arf6 association could not be a simple effector interaction. Hence we speculated Ral effector protein(s) could help mediate the Ral-Arf6 crosstalk. To evaluate this, effect of knockdown of Ral effector protein(s) on Arf6 activation was tested. Since Sec5 has been earlier shown to regulate the membrane raft exocytic pathway (Balasubramanian et al., 2010), it was selected and tested as a likely mediator. **Depletion of Sec5 did not affect adhesion dependent Arf6 activation** suggesting its role in adhesion dependent membrane raft trafficking and cell spreading to be limited as part of the exocyst complex. Other exocyst complex components known to interact with Ral and Arf6- Exo84 and Sec10 were also tested and found to not affect adhesion dependent Arf6 activation in MEFs. Interestingly, **Sec10 was seen to affect basal active Arf6 levels in stable adherent cells**, the mechanism and significance of this regulation is being explored.

Another major Ral effector that is vital for its function in cells is RalBP1 (RLIP76) know to be involved in Ral dependent mitochondrial fission (Kashatus et al., 2011) and invadopodia formation (Neel et al., 2012a). Interestingly, RalBP1 interacts with Arf6 GEF ARNO (Cytohesin-2) to regulate Arf6 activation downstream of R-Ras and cell-adhesion (but independent of Ral) (Lee et al., 2014). In our studies in MEFs, depletion of RalBP1 did not disrupt Arf6 activation in re-adherent cells indicating that RalBP1 does not mediate the Ral-Arf6 crosstalk in re-adherent cells. However, **loss of RalBP1 marginally, but consistently reduced Arf6 activity (~35% decrease) in non-adherent cells** as compared to control cells. Expression of siRNA resistant RalBP1 in the knockdown cells reversed this to support anchorage independent Arf6 activation. This led us to evaluate the role of RalBP1 could have in anchorage independent Arf6 activation in cancer cells.

In bladder cancer cell line UM-UC-3 depletion of RalBP1 decreased the proliferative and neoplastic potential of these cells for lung colonization (Oxford et al., 2005; Wang et al.,

2010). RalBP1 is also over expressed in bladder cancers (Smith et al., 2007). Knowing this and that the Ral-Arf6 crosstalk is detected in bladder cancer T-24 cells, we tested the effect loss of RalBP1 has in regulating Arf6 activity in these cells. **RalBP1 knockdown (tested with two different siRNA sequences) reduced anchorage independent Arf6 activation in suspended T-24 cells** in low (0.2% FBS) as well as high serum (5% FBS) conditions. This effect was comparable to the effect loss of RalB has in these cells. Joint knockdown of RalB and RalBP1 also did not show any additive effects on Arf6 activation suggesting they are likely to work along a linear pathway. Though RalBP1 depletion affected anchorage independent Erk activation comparable to RalB depletion in T-24 cells, the effect on anchorage independent growth in soft agar was much greater than that observed for loss of RalB. Thus RalBP1 along with mediating of Ral-Arf6 crosstalk could also possibly have an independent role in regulating anchorage independent growth in these cancer cells.

Since RalBP1 regulates Arf6 activation in both normal and cancer cells, we further explored the role RalBP1 binding partner ARNO – a known Arf6 GEF, has in mediating the Ral-Arf6 crosstalk (Lee et al., 2014). **Loss of ARNO by two different siRNA significantly decreased Arf6 activation in suspended T-24 cells** supporting its role in this crosstalk. In MEFs expressing active RalA, depletion of RalBP1 and ARNO reduced active RalA supported Arf6 activation in non-adherent cells confirming their role downstream of Ral. We also tested that loss of RalBP1 and ARNO did not affect RalA and RalB activation in T-24 cells. This suggests the presence of a linear **Ral- RalBP1-ARNO-Arf6** regulatory pathway in cells. Loss of RalBP1 and ARNO, like loss of Arf6, reduced active RalA supported membrane raft trafficking non-adherent WTMEFS. This emphasized the functionality of Ral-RalBP1-ARNO-Arf6 pathway in non-adherent T-24 and WTMEFS.

Along with ARNO we simultaneously tested the contribution by other Arf6 GEFs in this crosstalk. We hence compared the expression levels of fourteen ArfGEFs from cytohesin family (*CYTH1-4*), EFA6 family (*EFA6A-D*), BRAG family (*BRAG1-3*), BIG family (*BIG1-2*) and GBF1 (Casanova, 2007) in T24 cells. BRAG3, EFA6A and EFA6C were expressed poorly while CYTH2 (ARNO) and GBF1 were better expressed in T-24 cells. Inhibition of BIG1 and BIG2 by BrefeldinA (BFA) and inhibition of GBF1 by Golgicide-A (GCA) also did not affect Arf6 activity in T-24 cells. Functionality of these inhibitors was confirmed by their effect on Arf1 activity in T-24 cells (Sáenz et al., 2009a) confirming that BIG1, BIG2 and GBF1 were not involved in mediating anchorage independent Arf6 activation in these cells. Depletion of BRAG2 (GEP100), CYTH3 and EFA6B which are implicated in

tumorigenesis (Fu et al., 2014; Li et al., 2006; Morishige et al., 2008), when tested did not affect anchorage independent Arf6 activation.

While RalBP1 regulates the Ral-Arf6 crosstalk in non-adherent active Ral expressing cells (WTMEFs and T-24 cells), it did not mediate RalA dependent Arf6 activation downstream of Integrins. We hence looked at other Ral effectors focusing first on Phospholipase D- a Ral effector that is required for Ral driven secretory functions (Vitale et al., 2005). Phospholipase D (PLD) is a membrane anchored protein that catalyses hydrolysis of phosphatidylcholine into phosphatidic acid (PA) which gets further converted to diacylglycerol (DAG)- an important secondary messenger in cells (Selvy et al., 2011). The two mammalian PLD isoforms- PLD1 and PLD2 though possessing the same enzymatic activity are shown to be differentially localized and hence catalyze different functions (Cockcroft, 2001; Selvy et al., 2011). We hence explored the role both PLD1 and PLD2 could have in regulating integrin dependent Arf6 and Ral activation. **Depletion of PLD1 we found promotes anchorage independent Arf6 activation without affecting Arf6 activity in re-adherent cells.** This effect was not seen on loss of PLD2, suggesting this regulation to be unique to PLD1. Since active Ral in MEFs also activates Arf6 as did PLD1 knockdown, we tested effect PLD1 knockdown on Ral activation. **PLD1 depletion was seen to support anchorage independent RalA activation**, which could support downstream Arf6 activation. Since integrins promote RalA and Arf6 activation while PLD1 inhibits the same, we tested whether integrins could regulate PLD to drive Ral-Arf6 activation. Knowing that integrins activate PKC-α which in turn phosphorylates PLD1 (at Thr147) and activates it (Hornia et al., 1999; Melendez et al., 2001), we compared phospho-PLD1 (Thr147) levels in adherent and suspended cell lysates and found **PLD1 activation to be reduced on loss of adhesion and recovered upon re-adhesion**. To confirm this we tested the total enzymatic activity of PLD using an amplex red based fluorescence assay and found integrins to positively regulate total PLD activation corroborating with the change in phospho-PLD1 levels. **We can hence speculate that RalA-Arf6 activities may be regulated by PLD1 independent of its activation status, possibly dependent on its role as a scaffold.** PLD1 could bind and recruit a GEF and/or GAP protein to the Ral-Arf6-PLD1 complex (Xu et al., 2003). Future studies in the lab aim to address these questions by testing the effect PLD1 specific inhibitors on RalA and Arf6 activities and the ability PLD1 of act as a scaffolding complex.

## IV. Role of RalGEFs in differential adhesion dependent activation of RalA and RalB

Integrins specifically regulate RalA but not RalB activation in MEFs to support adhesion dependent membrane raft trafficking and cell spreading (Balasubramanian et al., 2010). We tested the role RalGEFs could have in mediating the adhesion dependent activation of RalA. We analyzed the relative expression of RalGEFs, Ras dependent (RalGDS, RGL1, RGL2, RGL3) and Ras independent (RalGPS1, RalGPS2), in WTMEFs by quantitative PCR. **These results indicated the expression of GEFs to be RalGSP2<RalGPS1<RalGDS, RGL1, RGL2 and RGL3.** We hence targeted each of these RalGEFs to test their role in adhesion dependent cell spreading (known to be regulated by RalA). **Depletion of RGL1 affected cell spreading of re-adherent cells more than RalGPS1 and RalGDS, while depletion of other GEFs had no effect.** Their combined knockdowns, however, did not show any additive effect on cell spreading suggesting their possible regulation of common downstream mediator, RalA. To confirm the specificity of these results, the effect of RGL1 knockdown on the spreading of caveolin-1 (Cav1) -/- MEFs was tested. In these cells, cell spreading is known to be independent of RalA mediated exocytosis (Balasubramanian et al., 2010). Ral GEF expression profile and knockdown efficiencies in Cav1 -/- MEFs was comparable to WT MEFs. The joint KD of RGL1, RalGPS1 and RalGDS did not affect the spreading of Cav1 -/- MEFs, suggesting the effect of RalGEFs KD in WT MEFs to be along a RalA dependent pathway. We further tested and found this joint knockdown to effect RalA activation in re-adherent cells, but not RalB. We hence tested if one of these GEFs more prominently regulated RalA. **RGL1 depletion we found affected RalA activation profoundly and only a modest effect of RalGDS or RalGPS1 depletion was seen.** RGL1 dependent regulation of RalA activation was strongest at 10 minutes after replating and reduced at 20 minutes, corroborating the role for RalA in early cell spreading of re-adherent MEFs (Balasubramanian et al., 2010). Further knockdown of RGL1 affected cell spreading in WT MEFs and not in Cav1-/- MEFs indicating specificity of RGL1 mediated effect along the RalA pathway.

To further establish the Ral isoform specificity of this integrin-RGL1-RalA pathway in MEFs, we tested the ability of constitutively active RalA and RalB to rescue the defect in cell spreading in RGL1 knockdown MEFs. **Active RalA restored cell spreading, but not active RalB further emphasizing the isoform specificity of this pathway.** This may be result of differential localization of RalA and RalB due to their highly dissimilar C-terminal membrane anchoring domain as seen earlier for Ras isoforms- H-Ras and K-Ras (Karnoub

and Weinberg, 2008; Shipitsin and Feig, 2004). Differential localization is in turn thought to mediate their interaction with a unique set of upstream and downstream binding partners, including RalGEF proteins. The ability of RGL1 to specifically activate RalA and not RalB could hence be a result of their differential localization and interaction in re-adherent MEFs. To test this we compared the ability of an active RalA/RalB tail switch mutant (Lim et al., 2005) to restore cells spreading in RGL1 knockdown cells. These mutants with their C-terminus tails (residues 176 to 206) switched are reported to switch their subcellular localization and isoform specific function such as basolateral membrane delivery (Shipitsin and Feig, 2004) and anchorage independence of transformed cells (Lim et al., 2005). **As speculated expression of active RalB/A mutant (with the tail of RalA) in RGL1 depleted cells rescued the cell spreading defect whereas active RalA/B mutant did not. This hence suggests that the localization of Ral isoforms is critical for their differential activation by RGL1 in re-adherent cells.** We observed endogenous RalA, detected using an anti-RalA antibody to co-localize with Myc-RGL1 in rapidly spreading re-adherent MEFs. RGL1 also localized with focal adhesion protein paxillin as did RalA with focal adhesion kinase (FAK) indicating the possibility that RGL1-RalA pathway could influence focal adhesions to affect adhesion dependent cell spreading, which is being actively tested.

Integrin driven RalA activation controls membrane raft trafficking pathway which in turn regulates anchorage dependent growth signalling in MEFs (Balasubramanian et al., 2010). To corroborate these results, hyperactivation of RalA (not RalB) downstream of oncogenic H-Ras (V12) drives anchorage independence in HEK-HT cells (Lim et al., 2005). Our data so far indicates the Ras dependent RalGEF RGL1 specifically activates RalA and not RalB downstream of integrins. We hence asked if RGL1 could regulate anchorage independent RalA activation in cancers driven by oncogenic Ras. Since RalGDS is previously known to drive Ral activation downstream of Ras-V12 (White et al., 1996b) we analyzed the role of both RalGDS and RGL1 simultaneously. We compared the relative expressions of RGL1 and RalGDS in multiple Ras-V12 expressing cancer cell lines by quantitative PCR. MIA-PaCa-2, HCT116, PC3, UMUC3, DU145 and SKOV3 showed lower expression of RGL1 than that of RalGDS whereas Calu-1, SW620, T-24, HT1080, MDA-MB-231 and U87MG showed comparable or marginally higher RGL1 expression than RalGDS expression. Upon siRNA mediated knockdown of RalGDS and RGL1 **in K-Ras-V12 expressing pancreatic cancer cell line MIA-PaCa-2**, **we observed adhesion independent RalA as well as RalB activation to be regulated by RalGDS but not RGL1**. **In colorectal**

**adenocarcinoma cell line SW620 (K-Ras-V12), only RGL1 depletion affected anchorage independent RalA activation but not that of RalB.** Interestingly, Studies by Martin *et al* have demonstrated that in SW620 cells, loss of RalA, not RalB, inhibits anchorage independent growth in soft agar colony assays (Martin et al., 2011). Hence immediate future experiments would include testing the role of RGL1 in anchorage independent growth of SW620 cells indicating its significance in RalA v/s RalB regulation observed in these cells.

In summary, we have identified a novel Ral-Arf6 crosstalk downstream of integrins. Since integrins activate RalA and not RalB, RalA specifically activates Arf6 in adherent cells, even though both active RalA and RalB can regulate Arf6. Ral mediated activation of Arf6 is essential for its regulation of Ral-exocyst function in fibroblasts. The Ral-Arf6 crosstalk is also detected in H-Ras-V12 expressing bladder cancer T-24 cells. In these cells RalB being more active (than RalA) regulates Arf6 to differentially regulate anchorage independent Erk signalling. The Ral effector RalBP1 and ArfGEF ARNO mediate this crosstalk in both normal and cancer cells downstream of active Ral. We also investigated the cause of differential activation of Ral isoforms downstream of integrins and found their differential localization and regulation by RalGEF RGL1 to mediate the same. RGL1 was also seen to differentially regulate RalA in colorectal cancer cell line SW-620 which could contribute to its role in anchorage independent growth of these cells.

# CHAPTER 1

# Review of Literature and Objectives

## 1.1 SMALL GTPASES:

Small GTPases are low molecular weight (20-40 kDa) G proteins that are conserved from yeast to mammals and essential for components of cellular signalling pathways. These proteins are regulators of cell-cycle progression, cytokinesis, transcription, membrane and protein cargo transport (Manser, 2002a). They respond to a wide variety of external and intracellular stimuli. The distant relatives of this family include the G-proteins, septins, dynamins, transcription initiation and elongation factors and the beta-tubulin subunit which are all regulated by the Guanosine triphosphate (GTP) binding (Wennerberg et al., 2005).

Small GTPases act as molecular switches turning ON and OFF several cellular processes depending upon their GTP/GDP binding status. Transition between nucleotide binding states is tightly regulated by two kinds of regulatory elements: Guanine nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs) (Cherfils and Zeghouf, 2013). Other forms of regulation, such as, phosphorylation and ubiquitination, have also been investigated and shown to be essential for activation of few small GTPases (Lim et al., 2010; Martin et al., 2012; Neyraud et al., 2012).

The GTP bound form of small GTPases is referred to as the 'Active' form and the GDP-bound as 'Inactive' exhibiting two distinct structural conformations that specify differential binding of their regulators and effectors. GEF proteins bind in the GDP bound state and replace the bound GDP with a GTP, resulting in GTPase activation (Zheng and Quilliam, 2003). The peculiar structural conformation of GTP bound form enables interaction with effectors that relay signals downstream in these signalling cascades. Effector proteins may vary from kinases, phosphatases, transcription factors, scaffolding proteins and even GEFs or GAPs for other GTPases (van Dam and Robinson, 2006; Manser, 2002a). The type of effector that interacts with active GTPase could depend on the subcellular location of the active GTPase. Thus GTPase activation recruits its effectors at sites of its activity, for example active Ras recruits RalGDS to plasma membrane from cytosol allowing is interaction with its substrate RalA localized therein (Matsubara et al., 1999). Depending upon which effector is bound a distinct pathway is initiated. GAP proteins function to enhance the intrinsically very low GTP hydrolyzing activity of GTPase and can be terminators of

signalling or initiators of a new activation/deactivation cycle (Figure 1.1). At every cycle more and more effector molecules are engaged leading to an enhancement of the upstream signal. Both GEF and GAP proteins are acted upon by upstream stimuli and help determine what fraction of the total GTPase in cells is active at a point of time. Typically less than ten per cent of any GTPase is found to be active in steady state conditions (Manser, 2002b).
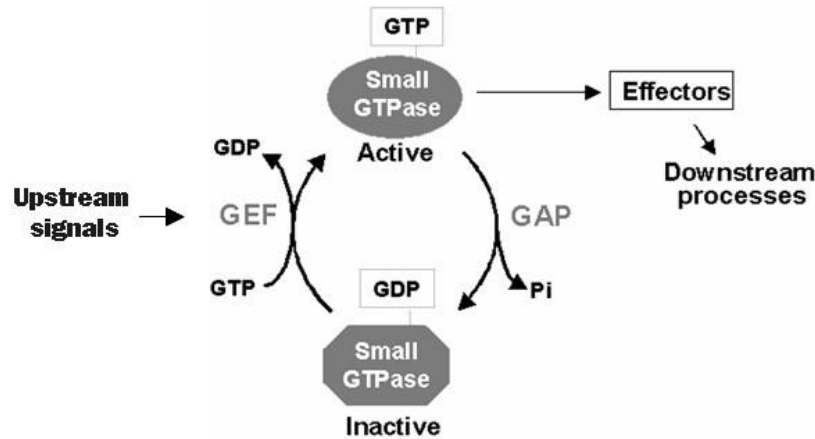


**Figure 1.1: Role and regulation of small GTPases in signal transduction cascade.** Different shapes of small GTPase indicate conformational change in GTPase structure depending on whether GDP or GTP is bound. GEFs can bind the GDP bound form and respond to upstream stimuli to exchange GDP to GTP. Activated GTPase then associates with effectors to relay the signal downstream. GAPs deactivate GTPase by assisting in GTP hydrolysis to terminate signalling. Like GEFs, GAPs can also respond to upstream stimuli. (Adapted from (Nielsen et al., 2008)).

Multiple evidences suggest that not only the active GTPases are functionally relevant in cellular pathways; but also their cycling between the GDP and GTP bound states. For example Rac1 is activated at leading edge of migrating cells by several Rac GEFs such as Tiam1 (Palamidessi et al., 2008), DOCK3 (Sanz-Moreno et al., 2008) and beta-Pix (ten Klooster et al., 2006). However not only this activation, but its local deactivation by Rho family GAP SH3BP1 at the front edge is essential for directed cell migration (Parrini et al., 2011). Similar flux between active and inactive states is shown to be essential for RhoA mediated cytokinesis in *Xenopus* embryos (Miller and Bement, 2009) and Cdc42p dependent yeast cell fusion (Barale et al., 2006). Alternatively with Ran GTPases both GTP bound and GDP bound forms bind different cargos to mediate their nuclear protein import-export (Hutchins et al., 2009).

### 1.1.1 Classification of Small GTPases:

All small GTPases are members of the Ras superfamily, named after its prototype member Ras. Depending on the structural and functional features this superfamily is divided into five major families: Ras, Rho, Rab, Ran and Arf (Wennerberg et al., 2005). Each of these families has several subfamilies that in turn share a subset of structural/ functional features (Figure 1.2). These proteins are conserved across eukaryotes with at least one ortholog from each subfamily being present in metazoans (Colicelli, 2004).

| Family | Examples | Functions |
|---|---|---|
| Ras | H-Ras, K-Ras4A, K-Ras4B, N-Ras, M-Ras, TC21 | Gene expression, cell transformation |
| | Rap1A, Rap1B, R-Ras | Integrin augmentation |
| | RalA, RalB | Vesicular transport and apoptosis |
| Rho | RhoA, Rac1, Cdc42, Rnd1 | Cytoskeletal reorganization, cell polarity maintenance, gene expression |
| | TC10 (RhoQ) | Vesicular transport |
| Arf/Sar1 | Arf1-6 | Vesicle budding, remodeling of actin cytoskeleton |
| Rab | Rab1-60 | Vesicular transport |
| Ran | Ran | Nucleocytoplasmic transport of RNAs and proteins |

**Figure 1.2: Small GTPase families within the Ras superfamily – Ras, Rho, Arf, Rab and Ran**. Examples from each family are mentioned and their broad cell biological functions. (Reproduced from (Kiyokawa et al., 2011))

### 1.1.2 Structural features and their relevance to regulation:

The hallmark feature of Ras superfamily is the G domain which is responsible for both the GTP binding and hydrolysis. This domain has five characteristic GTP/GDP binding motifs called G box 1-5, featuring certain invariant residues within consensus sequences (Figure 1.3A). Two structural elements of the G domain- Switch I and Switch II change conformation upon GTP/GDP binding. In the GTP bound state these switch regions assume a 'loaded spring conformation' wherein the key residues, Thr 35 and Gly 60 of Ras, make co-ordinate bonds with the Gamma-phosphate group of GTP and a magnesium ion in the active site (Figure 1.3B). A fifth co-ordinate bond of phosphate is achieved through the residues of phosphate binding loop (P-Loop). In this conformation switch I region protrudes out allowing effector interaction. Upon GTP hydrolysis, the co-ordinate bonds are broken and the switch

regions attain a 'relaxed conformation' losing interaction with effector (Wennerberg et al., 2005).
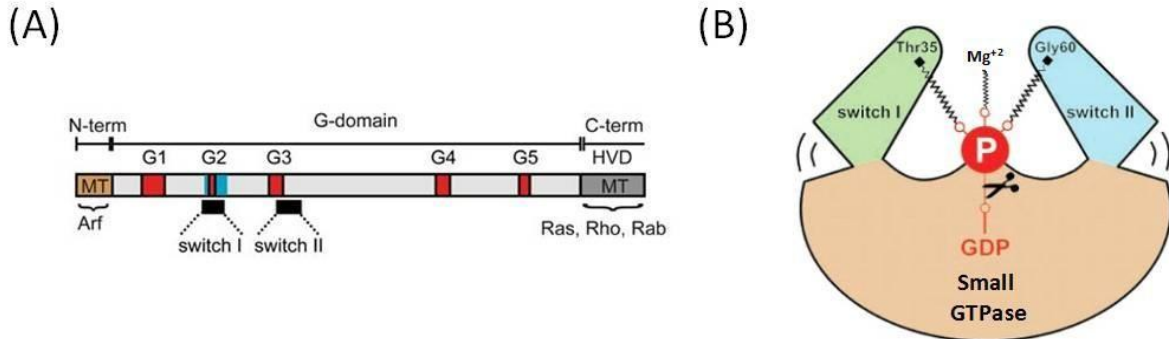


**Figure 1.3: Structural features of the small GTPase. (A)** Schematic depicting G box motifs G1-G5 in G domain. Membrane targeting (MT) domain either is located at C-terminus in the hypervariable domain (HVD) for Ras, Rho and Rab family members or at N-terminus for Arf family members (Adapted from (Neely and Hidalgo, 2014)) **(B)** Schematic for GTP bound  loaded spring conformation of Ras. The gamma phosphate of GTP (in red circle) interacts with invariant residues from Switch I (Thr35 for Ras) and Switch II (Gly60 for Ras) and the magnesium ion in the active site (Adapted from (Vetter and Wittinghofer, 2001)).

Except for Ran family GTPases, all small GTPases are anchored in the lipid bilayer of plasma membrane or organellar/endosomal membrane via post translational lipid modifications- either prenylation or acylation on the terminal residues (Figure 1.3A). The signals for prenyl anchor attachment are encoded by the C-terminal –CAAX motif for Ras and Rho family GTPases where AAX motif is cleaved by a carboxypeptidase attaching lipid moiety at terminal cysteine. Acylation occurs at an internal C-terminal residue near the - CAAX motif for some of the Ras and Rho GTPases (Colicelli J., 2004). Arf family GTPases possess N-terminal myristoyl anchor and an amphipathic helix that is exposed in the GTP bound conformation favoring membrane insertion (Gillingham AK. And Munro S., 2007).

Additional regulatory elements called Guanine nucleotide Dissociation Inhibitors (GDI) bind to the lipid moiety of Rho and Rab GTPases and sequester them into the cytosol, barring their interaction with plasma membrane as well as with their GEFs and effectors (Keep NH. et al., 1997). In doing so GDIs are also now seen to be important regulators of GTPase function though their presence for all GTPases is yet to be defined.

### 1.1.3 Tools for the study of small GTPases:

Considering the fact that GTPases actively cycle between the GDP and GTP bound forms the most useful techniques to decipher their function in cells has been the use of mutants locked in either GTP or GDP bound conformation. Alteration of key residues essential for nucleotide binding provides ways to achieve altered activation status. Constitutively active GTPase mutants are permanently locked in the GTP bound state due to change in a residue that abolishes the intrinsic or GAP mediated GTPase activity; for example Ras G12V and Q61L mutants (Prior et al., 2012). Use of these mutants though very productive for understanding active Ras dependent cell processes, could be misleading for functions of other GTPases that depend on their flux as discussed before. For example a constitutively active Rac1 mutant expression leads to slower migration of cells because a spatial flux of in Rac1 GDP/GTP bound forms is essential at leading edge for directed cell migration (Parrini et al., 2011). Hence fast cycling mutants were identified that rapidly cycle between GDP/GTP forms due to high instability towards nucleotides. Hence these mutants are independent of GEFs and GAPs for their activation and deactivation. Considering the GTP-GDP cycling of GTPases is important for their function these mutants are physiologically more relevant forms of hyperactivation rather that the constitutively active mutants. For example fast cycling mutant of Cdc42 can rescue the yeast cell mating defect induced by constitutively active cdc42 mutant (Barale et al., 2006). Constitutively inactive mutants are permanently locked in GDP bound state (S17N mutation in Ras) and act as dominant negative as they bind GEFs unproductively and dominantly block the activation of endogenous GTPase (Prior et al., 2012). Since GEFs can activate more than one GTPase, such sequestration of GEFs by a dominant negative mutant can dominantly inhibit other GTPases also making these mutants non-specific inhibitors. Effector loop mutants favor binding of the GTPase to only one effector barring interactions with other effectors. These mutants enable functions of a small GTPase to be attributed to a particular effector pathway. For example, Ras T37S mutation allows only Raf1 binding and Ras Y40C mutant binds only PI3K (Vojtek and Der, 1998).

GTPase binding domains of effectors cloned with GST tags are used to pull down only the GTP bound form of the GTPase and analyze relative active GTPase levels under different conditions (Ren and Schwartz, 2000). Single molecule FRET sensors are new advancement

that indicates specific intracellular sites of GTPase activation/ deactivation in live cells (Hall et al., 2008). These techniques represent most significant tools in analyzing GTPases as they enable analysis of endogenous activation status of GTPases rather than overexpression of their mutants that may give misleading results.

## 1.1.4 Role of small GTPases in cancer:

The wide array of cellular functions mediated by small GTPases implicates their importance in various disease conditions. Deregulated expression and activation of small GTPases has been implicated in several cancers and even in certain neurological disorders such as Neurofibromatosis Type I (Rauen, 2013). Owing to their regulation of specific pathways such as role in apoptosis or role in migration, different small GTPases have been demonstrated to support distinct hallmarks of cancer cells such as infinite survival, gain of anchorage independence and invasiveness. Hence the study of regulation of small GTPases is an active area of research.

## 1.2 REGULATORS OF SMALL GTPASES - GEFs and GAPs:

GEFs and GAPs are essential regulators of all small GTPases and are elaborated in further sections. For Rho and Rab family GTPases another set of regulators called Guanine nucleotide dissociation inhibitors (GDIs) exist that bind to GDP-bound forms and retrieve them actively from the plasma membrane and endosomal membranes (Garcia-Mata et al., 2011). An oxidoreductase ERp57 is shown act as GDI for Ral GTPases only in its reduced state (Brymora et al., 2012). Apart from that other GDI proteins are not identified for Ral and Arf families and hence these are not discussed in detail here.

## 1.2.1 Guanine nucleotide Exchange Factors (GEFs) - Classification, function and regulation:

GEFs are the activators of small GTPases and are highly conserved across eukaryotes. GEFs act downstream of a variety of extracellular as well as intracellular stimuli such as ligand-receptor binding and/or dimerization, formation of intercellular junctions or increased

intracellular calcium ion concentration (Bos et al., 2007; Zheng and Quilliam, 2003). Each of the families within the Ras superfamily has its own characteristic GEF proteins forming distinct GEF families. These are distinguished from each other due to the presence of essential structural domains required for the GEF activity towards a specific GTPase. For example the Ras GEFs possess a CDC25 domain, Arf-GEFs a Sec7 domain and Rho GEFs contain tandem DH (dibble homology) and PH (pleckstrin homology) domains (Cherfils and Zeghouf, 2013). Some GEFs such as Rgr possess more than one GEF domain and can act on several GTPases (Osei-Sarfo et al., 2011).

The kinetically unfavorable GDP to GTP exchange on small GTPases makes their activation dependent on GEF proteins. GEFs provide important interactions with residues in switch region that destabilize the GDP association. This is achieved by displacing the magnesium ion from the active site slightly altering the relative positions of switch regions such that the bound GDP is excluded from the active site. This leads to formation of A GEF-GTPase intermediate which is a high affinity complex attacked by GTP that displaces the GEF. The substitution of GTP for GEF on the active site of the GTPase is a result of relatively higher intracellular concentration of GTP than GDP (~ 10 fold) and not a preferential selection of GTP by the GTPase or the GEF (Schmidt and Hall, 2002). Thus a GTPase active site is never free; it's either occupied by a guanine nucleotide or very transiently by a GEF that also functions to stabilize the nucleotide free form of GTPase in addition to the foremost GDP displacing function.

Extracellular signals can further govern the enhancement of GEF activity by several mechanisms. One of the mechanisms is signal induced change in subcellular localization, activating the GTPase molecules at that location. For example active Ras induces translocation of RalGDS leading to activation of RalA localized at plasma membrane (Matsubara et al., 1999). Also conformational changes in the GEF may be induced upon interaction with scaffolding proteins relieving auto-inhibitions or allosterically enhanced the catalytic activity. Post translational modifications such as phosphorylation at key residues may alter GEF activity and specificity; for example the Rho GEF ECT2 exhibits a phosphorylation dependent GEF activity towards Rho, Rac and Cdc42 (Tatsumoto et al., 1999). Additional regulatory elements that dictate the GEF activity are secondary messenger

metabolites such as phorbol esters and DAG. For example PI3-kinase generates PI(3,4,5)P3 that binds to PH domains in Rac GEFs activating them (Welch et al., 2003).

## 1.2.2 GTPase Activating Proteins (GAPs) – Structure, function and regulation:

Similar to GEF proteins, different GAPs have been reported for different families of small GTPases. But the boundaries between different GAP families are diffuse due to less conserved structural similarities. GAP proteins are classified according to their preferential biochemical activities towards a certain class of GTPases, rather than structural homology or motif conservation. These proteins are usually multidomain with SH3 or PH domains that bind other proteins, lipids or secondary messenger molecules (Bos et al., 2007).

The Ras and Rho family GAPS possess a shared feature of a conserved arginine residue called as the 'Arginine finger', that is important for the catalytic activity. In case of Rab GTPases the essential glutamine residue is also provided by the Rab GAPs. Ran GAPs, Rap GAPs utilize an aspargine residue instead of the Arginine residue (Bernards and Settleman, 2004).

Small GTPases possess very weak intrinsic GTPase activity inefficient to achieve rapid responses required during signal transduction necessitating GAP action. Elucidation of Ras-Ras GAP complex structure proposed a mechanism for GAP action. The conserved arginine finger of Ras GAPs could provide a stabilizing interaction with a conserved glutamine 61 (Q61) residue of Ras GTPases and a water molecule in the active site that participates in the hydrolytic reaction. GAPs are proposed to restrict the freedom of this water molecule, orient it for the necessary nucleophilic attack on gamma phosphate of GTP and reduce the activation energy barrier via stabilization of the transition state (Gamblin and Smerdon, 1998). These structural studies have been supported by mutational and biochemical studies for example the Ras Q61L mutant in its GTP-bound locked state was seen to be insensitive to RasGAP.

Multiple domains in GAPs avail interaction with other binding proteins, lipids or secondary messenger molecules or may be sites for post-translational modification. For example the C2

domain of RasGAP1 family members RASA1 and CAPRI responds to intracellular calcium ion concentration (Dai et al., 2011; Sot et al., 2013). A Rho family GAP, RA-RHO is activated upon occupancy of its RA domain by Rap1 GTPase (Bernards and Settleman, 2004). Rac1 GAP is activated via its phosphorylation by Aurora Kinase B during cytokinesis (Minoshima Y. et al., 2003). The lipid binding of ArfGAPs modified their GAP activity via binding of PI(3,4,5)P3 to the PH domains (Vekanteswarlu K. et al., 2003). Similar to some GEF proteins, GAPs can exhibit cross-specificity for different GTPases. ARAP family of GAPs possesses functional ArfGAP and RhoGAP domains along with additional PH and RA domains. The ArfGAP activity is PI(3,4,5)P3 dependent whereas the RhoGAP activity is independent of it (Krugmann et al., 2002; Miura et al., 2002).

Collectively, a small GTPase functions in co-ordination to its binding partners – regulators (GEFs, GAPs, GDIs) and effectors, that constitute components of signalling pathway, with external stimuli influencing the regulators to determine the extent and kinetics of activation. Apart from these components, small GTPase signalling is influenced by other signalling of other small GTPases from the same family or another. This takes place due to the 'crosstalk' of their components including GTPases by themselves or their GEFs, GAPs and effectors, discussed in more detail in the following section.


## 1.3 CROSSTALK BETWEEN SMALL GTPASES:

Small GTPases work as part of multiple signalling networks, regulating at times common overlapping pathways and functions. This in part is mediated by their ability to be regulated by common GEFs /GAPs (as is the case with Arf and Rho GTPases being regulated by GAP protein ARAP3) and/or their ability to regulate common downstream effectors (e.g. Rab GTPases). A regulatory crosstalk between GTPases could further add to this regulation and has been seen to be the case in some GTPases like Ras GTPases. These points of crosstalk allow for the co-ordination of multiple GTPase signalling pathways and generation of a more comprehensive response to an external stimulus. Such a crosstalk can be mediated by the direct association between GTPases and /or indirectly through their downstream effectors.

Ras effector proteins act as GEFs for several small GTPases and regulate their function in correspondence to Ras activation leading to integration of Ras family with other families. These Ras effectors include RalGEFs (GEF for RalA/B), Rin1 (GEF for Rab5), Tiam 1 (GEF for Rac) and PLC- epsilon (GEF for Rap1) (Figure 1.4) (Mitin et al., 2005).
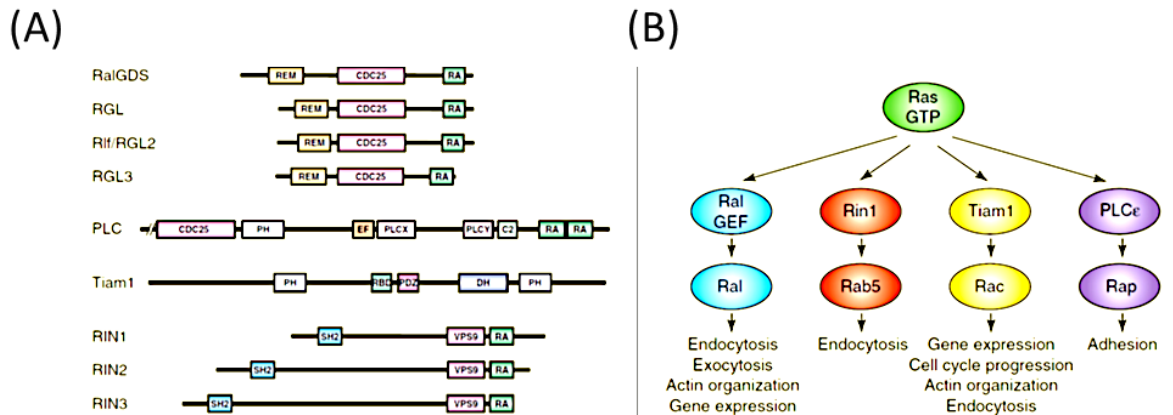


**Figure 1.4: Ras effectors act as GEFs for other Ras superfamily GTPases. (A)** Schematic showing domain architecture of the Ras effectors with Ras association (RA) domain (green box) and GEF domain for others GTPases; CDC25 homology domain for Ral and PLC, tandem DH/PH domains for Rho and VP59 for Rab GTPases **(B)** Schematic depicting translation of Ras activation into signalling pathways and functions downstream of Ral, Rab5, Rac and Rap GTPases via Ras effectors RalGEF, Rin1, Tiam1, PLC-epsilon respectively that act as GEFs for these GTPases (Adapted from Mitin et al., 2005).

The GEF and PH domains of ARNO allow for crosstalk between Arf1 and Arf6 during Salmonella invasion. Salmonella virulence protein activated Arf6 activates its effector PIP-5-Kinase that generates PI(4,5)P2 at plasma membrane. Increased PI(4,5)P2 levels attract ARNO that possesses a PH domain which in turn recruits and activates ARF1 at plasma membrane (Cohen et al., 2007; Humphreys et al., 2013).

Discoveries of crosstalks between GTPases have originated from interaction based studies such as yeast two hybrid screens where binding partners of other pathways attracted attention; for example identification of Rho GAP protein RalBP1 as effector of R-Ras (Goldfinger et al., 2006). Alternatively crosstalks were envisaged based on observation of two GTPases mediating similar signalling pathways leading to the hypothesis that they could work together to do so. Our earlier studies from the lab have observed two small GTPases

from different families, RalA (Balasubramanian et al., 2010) and Arf6 (Balasubramanian et al., 2007) to be mediators of integrin dependent exocytosis and delivery of raft microdomains. This is mediated by the regulation of RalA and Arf6 by integrins (Balasubramanian et al., 2007, 2010). These GTPases have also been seen to work along multiple cellular pathways such as insulin secretion, cytokinesis, phagocytosis etc. (Cascone et al., 2008; Corrotte et al., 2010; Lopez et al., 2008; Millar et al., 1999; Niedergang et al., 2003; Schweitzer and D'Souza-Schorey, 2005) and even seen to associate with each other (Xu et al., 2003). Together this led us to speculate the possibility of a regulatory crosstalk between RalA and Arf6, which this study tests. Before leading into discussing in detail the reasons for speculating such a RalA-Arf6 crosstalk, a brief understanding of the general features, functions and regulation of these GTPases would be very useful.

## 1.4 SMALL GTPASE - Arf6:

Arf6 (ADP ribosylation factor 6) belongs to Arf subfamily Class III. It differs from its other family members in being localized at the plasma membrane versus other members mostly localized to endosomal membranes, especially the Golgi apparatus (Cavenagh et al., 1996; Yang et al., 1998). Arf6 is primarily involved in vesicle trafficking in both endocytic as well as exocytic processes (Schweitzer et al., 2011). In endocytosis Arf6 is essential in clathrin as well as clathrin and caveolae independent pathways (Heikkilä et al., 2010; Karnik et al., 2013; Tanabe et al., 2005). Exocytic functions controlled by Arf6 are mediated via its effectors phospholipase D and exocyst complex (Prigent et al., 2003b; Vitale et al., 2002). Arf6 plays important role in actin remodelling in several processes including cell adhesion (Balasubramanian et al., 2007; Song et al., 1998) and neurite branching (Cheung et al., 2014; Choi et al., 2006; Kobayashi and Fukuda, 2012) by regulating Rac1 GTPase downstream (Koo et al., 2007; Radhakrishna et al., 1999; Santy and Casanova, 2001).

## 1.4.1 Arf Family:

Arf protein was first isolated from bovine brain as co-factor for cholera toxin in adenylate cyclase activation (Kahn and Gilman, 1984) and later Arf isoforms were characterized as small GTPases (Tsuchiya et al., 1991). The Arf subfamily GTPases are unique in their lipid modification from the Ras, Ral and Rho families (Pasqualato et al., 2001). They are

myristoylated at their N-terminus and also possess an amphipathic helix at N-terminus that assists in their membrane anchoring (Goldberg, 1998; Pasqualato et al., 2002). Mammalian Arf GTPases are grouped into three classes: **Class I** (Arf1, Arf2, Arf3), **Class II** (Arf4, Arf5) and **Class III** (Arf6), based on their homology, gene structure and localization in the cell (Gillingham and Munro, 2007). Class I and Class II Arf GTPases function in maintenance of Golgi integrity and endosomal trafficking between endocytic reticulum and Golgi (Gaynor et al., 1998). Recently few studies have demonstrated redundancy, co-operation and negative feedback loops between Arf isoforms in mediating their activation and function (Padovani et al., 2014; Stalder et al., 2011; Volpicelli-Daley et al., 2005).

### 1.4.2 ArfGEFs and ArfGAPs:

ArfGEFs are characterized with a Sec7 domain that is essential for their GEF activity (Casanova, 2007). There are 4 major families of ArfGEFs: Cytohesin (CYTH1-4), EFA6 (EFA6A-D), BRAG (BRAG1-3) and large Sec7 GEFs (BIG1, BIG2 and GBF1) (Donaldson and Jackson, 2000). F-Box protein Fbx8 also carries a Sec7 domain but functions to ubiquitinate Arf6 instead of as a GEF (Yano et al., 2008) (Figure 1.5A). Most GEFs can act on all Arf isoforms in vitro and their localization is determinant of their activity seen on specific Arf isoforms. For example, BIG1, BIG2, GBF1 localize to the Golgi and hence act on Golgi localized Arfs, such as Arf1 (Anders and Jürgens, 2008; Wright et al., 2014). Cytohesin2 (ARNO) localized predominantly at plasma membrane however acts on Arf6, though it has recently been shown to act on pool of Arf1 that translocates to the plasma membrane in response to certain stimuli (Cohen et al., 2007; Humphreys et al., 2012, 2013).

ArfGAP proteins are characterized by presence of an ARF GAP domain and carry a characteristic four cysteine residue containing motif that binds a zinc ion and participates in GTP hydrolysis. A conserved arginine residue is also present and essential for GAP activity (Gillingham and Munro, 2007).  Based on the presence of additional domains ArfGAPs are divided into ten subfamilies: Centaurin-alpha, Centaurin beta (ACAPs), Centaurin gamma (AGAPs), Centaurin-delta (ARAPs), DDEF (ASAPs), ArfGAP1, ArfGAP3, SMAP, Hrb and GIT family. Additional domains include PH domain, Rho GAP domain, Bar domain, Ankyrin repeats, SH3 domain, paxillin binding domain and C-terminal motifs (Figure 1.5B). These domains contribute to the localization and activation of Arf GAPs as well as the crosstalk of

Arf6 with other signalling pathways (Donaldson and Jackson, 2000). The A'Z'AP type
GAPs- ACAP/ARAP/ASAP/AGAP regulate Arf6 for invadopodia and podosome formation
while non-AZAP type GAPs- GIT1, Arf1GAP1 and ArfGAP3 have been shown to be
important for focal adhesion and actin remodelling functions of Arf6 (Randazzo and Hirsch,
2004;Randazzo et al.,2007).
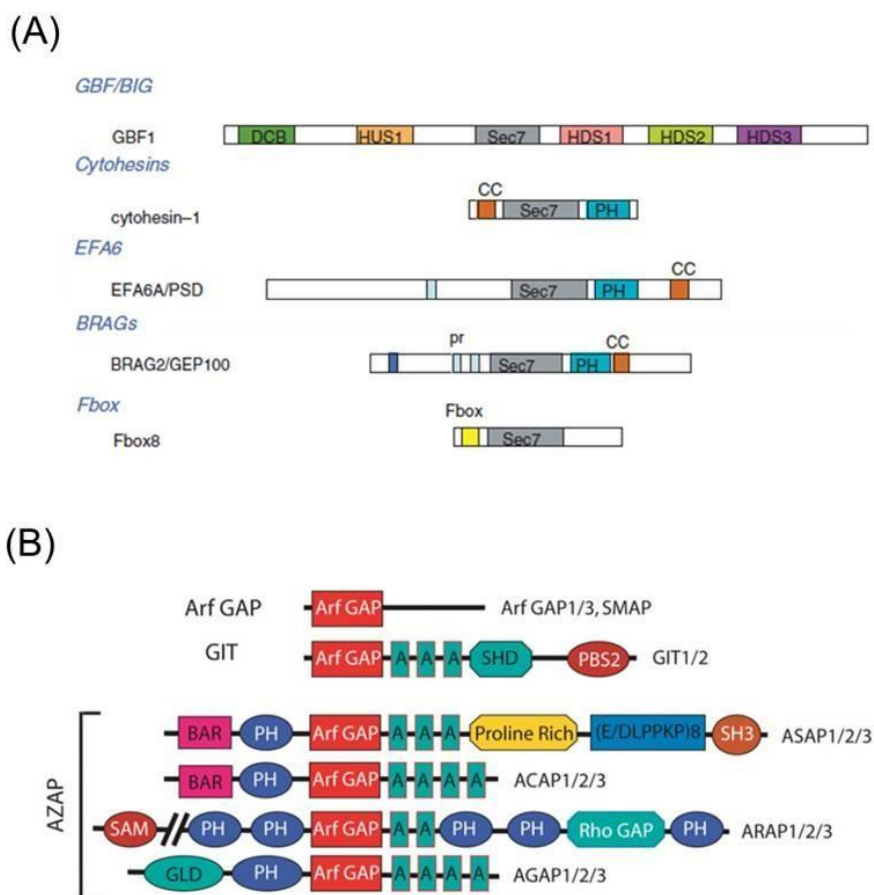


**Figure 1.5: ArfGEFs and ArfGAPs.** Schematics showing general domain architecture of
the **(A)** ArfGEF families with an example of each family and **(B)** ArfGAP families- non
AZAP type (ARFGAP1, ArfGAP3, SMAP and GIT families) and AZAP type (ASAP/DDEF,
ACAP/Centaurin-beta, ARAP/Centaurin-delta , AGAP/Centaurin gamma families).
(Adapted from (Casanova, 2007; Donaldson and Jackson, 2011)).

## 1.4.3 Role of Arf6 in normal and cancer cells:

Arf6 is distinguished from the other Arf family members owing to its role in cells
attributed by its distinct localization. A regulatory role for Arf6 has been reported in cell

migration (Palacios et al., 2001), cytokinesis (Chesneau et al., 2012), endocytosis (Palacios et al., 2002) and endocytic recycling of membrane, integrins, cholesterol, E-cadherin and several receptors (Radhakrishna et al., 1999;Powelka et al., 2004;Schweitzer et al., 2011), actin remodelling (Song et al., 1998), myoblast fusion (Chen et al., 2003; Dudek et al., 2010) and adherence junction turnover (Palacios et al., 2001). At the molecular level, Arf6 regulates activation of Rac1 (Koo et al., 2007), PLD1 and PLD2 (Oude Weernink et al., 2007b), PIP-5 kinase (Aikawa and Martin, 2003; Honda et al., 1999) and functionally collaborates with Rab family GTPases (Schweitzer et al., 2011) in vesicle trafficking.

In cancers the deregulation of Arf6 activity is responsible for mesenchymal transition of cells with enhanced migration and invasiveness as studied for breast cancer cell lines (Hashimoto et al., 2004). The Arf6 GDP/GTP cycling is central to E-cadherin trafficking in epithelial cells and hence the stability of E-cadherin dependent cell-cell junctions (Palacios et al., 2002). The Arf6 GAP protein AMAP1 is over-expressed in tumor tissues and found to be necessary for the invasiveness of highly metastatic breast cancer cell lines (Onodera et al., 2005). Arf6 also supported such invasiveness by regulating the release of protease loaded micro vesicles in a melanoma cancer cell line (Muralidharan-Chari et al., 2009). Recent evidence by Knizhnik et al., has also suggested a pro-proliferative role for Arf6 via the PLD-mTORC1-p38 pathway in melanoma cells (Knizhnik et al., 2012). Overexpression of several Arf GEF proteins such as cytohesin3, BRAG2 and EFA6A, in hepatocellular, melanoma and breast cancer cells is detected and their oncogenic role demonstrated through their effect on Arf6 activity (Fu et al., 2014; Li et al., 2006; Morishige et al., 2008). Similarly expression of ArfGAP proteins AMAP1 and Git1 are also deregulated in breast cancer cells (Onodera et al., 2005; Sabe et al., 2006) that are shown to be important to Arf6 mediated invasion potential of these cells. Arf6 by itself is seen to be upregulated in triple negative breast cancers (Eades et al., 2015). Collectively Arf6 has been strongly implicated in invasion of several cancer types. However a prominent role for Arf6 in mediating anchorage independence of cancer cells has not been directly probed. This is of interest owing to the fact that RalA GTPase, that we speculate shares a regulatory crosstalk with Arf6, is a well-known mediator of anchorage independence in multiple cancers (Bodemann and White, 2008; Lim et al., 2006; Martin et al., 2011)

## 1.5 SMALL GTPASE – Ral:

The Ral (<u>Ra</u>s-<u>l</u>ike) subfamily of small GTPases was first identified through domain structure homology shared with Ras family GTPases (van Dam and Robinson, 2006). The two mammalian Ral isoforms, RalA and RalB are 82 identical in their protein sequence. Despite their similarity, RalA and RalB are found to be differentially activated in response to variety of upstream stimuli and also regulate distinct downstream pathways and cellular processes (Gentry et al., 2014). The list of known Ral effectors include transcriptional factor ZONAB, actin modulator Filamin-A, phosphoinositide signalling components- phospholipase D and phospholipase Cδ1, cell secretory machinery exocyst complex, and a multidomain, multifunctional protein RalBP1/ RLIP76 (Bodemann and White, 2008). Ral proteins are key players in cytokinesis (Chen et al., 2006), cell migration (Hatzoglou et al., 2006), exocytosis (Moskalenko et al., 2002), transcriptional regulation (Frankel et al., 2005), phagocytosis (Corrotte et al., 2010), apoptosis (Chien and White, 2003), autophagy (Bodemann et al., 2011) and mitochondrial fission dynamics (Kashatus et al., 2011). The capability to regulate exocyst complex and the multifunctional protein RalBP1 are central to Ral function.

### 1.5.1 RalA and RalB:

The two Ral isoforms share 82% sequence identity with the highest similarity in the N-terminus and least in the C-terminal hypervariable region (Martin and Der, 2012) (Figure 1.6A). Despite their similarities the striking distinction in their regulation and functions has been of particular interest. Our earlier studies have shown RalA, not RalB activation to be regulated by integrin dependent cell adhesion to control membrane raft microdomain exocytosis and cell spreading (Balasubramanian et al., 2010). RalA, not RalB is essential for basolateral delivery of membrane in epithelial cells (Shipitsin and Feig, 2004) and is also specifically seen to regulate Ras induced tumorigenesis (Lim et al., 2005). RalB is further seen to be uniquely involved in regulation of cell survival (Chien and White, 2003; Chien et al., 2006), development of tight junctions (Hazelett et al., 2011) and migration of bladder cancer cells (Oxford et al., 2005). Stable shRNA mediated knockdown of RalA and RalB in the pancreatic cancer cell lines showed RalA to be vital for anchorage independent proliferation and RalB for the survival of tumor derived cell lines (Lim et al., 2006).  A similar role for RalA is reported in colorectal cancer cell lines (Martin et al., 2011). RalA and

RalB mediate distinct steps in cytokinesis employing the effector exocyst complex at spatially and temporally separated stages (Cascone et al., 2008).

The basis of the functional distinction of Ral isoforms is only now being analyzed and does remain a mystery particularly since they share many common effectors like RalBP1 and the exocyst complex (Bodemann and White, 2008; Camonis and White, 2005; Kashatus et al., 2011; Neel et al., 2012a). Their differential subcellular localization is speculated and demonstrated in few cases to be responsible for their differential function (Shipitsin and Feig, 2004). The C-terminal hypervariable regions of RalA and RalB are shown direct them to different subcellular locations thus facilitating their interaction with different regulators (GEFs/GAPs) and/or effectors. Differences in the CAAX motif (CCIL for RalA and CCLL for RalB) and differential dependence on enzymes for post translational modification are recently reported to affect Ral isoform localization, activation and stability (Gentry et al., 2015). Thus when the C-terminal tails were switched between Ral isoforms, it is seen to affect their isoform specific functions in membrane delivery and anchorage independence (Lim et al., 2005; Shipitsin and Feig, 2004). RalA and RalB are also phosphorylated at unique residues in their C terminal hypervariable region by AuroraA (RalA S194) and protein kinase C-alpha (RalB S198) and this phosphorylation plays important role in their localization and cellular functions (Lim et al., 2010; Martin et al., 2012). Monoubiquitination of Ral isoforms was detected and seen to regulate their localization and association with lipid raft microdomains and their activation at plasma membrane and intracellular vesicles (Neyraud et al., 2012).

## 1.5.2 RalGEFs and RalGAPs:

RalGEFs are divided into two groups as Ras-dependent GEFs and Ras-independent GEFs based on their ability to interact be controlled by Ras GTPases. Ras-dependent GEFs possess Ras exchange motif (REM) and CDC25 homology domain that is essential for their RalGEF activity and Ras association (RA) domain that mediates their ability to talk to Ras and regulate Ral downstream (Figure 1.7). The first RalGEF to be identified was a Ral Guanine nucleotide Dissociation Stimulator (RalGDS) though its sequence similarity to RalGEFs. This was followed by the identification of RalGDS like GEFs, RGL1, RGL2 (Rlf in mouse) and RGL3 though yeast two hybrid screens of active Ras and Rap GTPases

(Neel et al., 2011). These GEFs are conserved with orthologs in *C. elegans* and *Drosophila*. RalGPS1 and RalGPS2 represent a second group of Ras independent RalGEFs that contain PH and SH3 domains instead of a RA domain I addition to CDC25 homology domain (Rebhun et al., 2000a). They are suggested to work in phosphoinositide dependent manner and may be regulators of calcium induced Ral activation (Figure 1.6B). Rgr while identified as a RalGEF is also capable of acting as a GEF for other small GTPases (Osei-Sarfo et al., 2011).
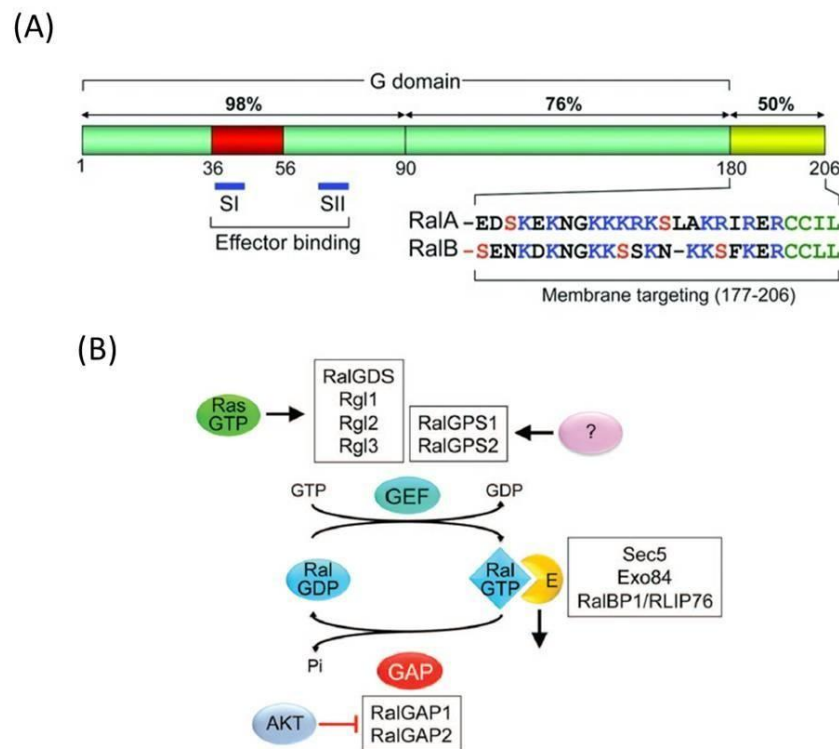


**Figure 1.6: Ral isoforms and their regulators.** **(A)** Schematic showing amino acid sequence similarity between different regions of RalA and RalB with the C-terminal hypervariable region sequence elaborated to highlight differences (blue letters indicate basic residues and red letters are phosphorylation sites). **(B)** Ral GTP/GDP cycle and its regulators-Ras-dependent GEFs (RalGDS, RGL1, RGL2, RGL3), Ras independent GEFs (RalGPS1/2), RalGAP complexes (RalGAP1/2) and effectors Exo84, Sec5 and RalBP1. (Adapted from (Martin and Der, 2012)).

Ral GAPs are similar to Rheb GAPs rather than Ras GAPs. There are two RalGAP complexes RalGAP1 and RalGAP2 (also called RGC1 and RGC2) that contain either of the two catalytic subunits alpha1 or alpha2 respectively and a common beta subunit (Shirakawa et al., 2009). These have been identified to be regulated by PI3K/Akt mediated phosphorylation and binding to 14-3-3 proteins (Chen et al., 2014, 2011b; Leto et al., 2013) (Figure 1.6B). The Rheb GAPs, TSC1 and TSC2 also exhibit GAP activity towards Ral

isoforms and RalGAP1 is observed to modulate the Rheb target mTORC1 leading to a Ral-mTOR crosstalk (Martin et al., 2014).
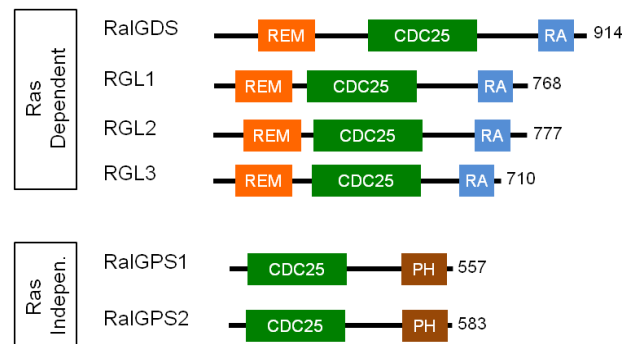


**Figure 1.7: Ral guanine exchange factors (RalGEFs).** Schematic depicts domain organization of six known Ral-GEFs. CDC25 homology domain is essential for binding and GEF activity towards Ral GTPases. Presence of Ras Exchange Motif (REM) and Ras Association domain (RA) is seen in Ras dependent RalGEFs (in red box) while Ras independent GEFs lack these domains. They possess a C-terminal PH domain (binds phospholipids), SH3 binding motif (binds SH3 domain proteins) and a proline rich region containing PXXP motifs (Reproduced from (Gentry et al., 2014))

## 1.5.3 Ral GTPases and cancer:

The first evidence of involvement of Ral GTPases in Ras mediated oncogenesis emerged from investigations of a RalGEF, RalGDS as a molecule that supports transformation (Urano et al., 1996; White et al., 1996a). In 2003, a pioneering study by Chien and White revealed the distinct roles of RalA and RalB in tumor cell proliferation and survival respectively in HeLa, colorectal cancer cell line SW620 and breast cancer cell line MCF-7 (Chien and White, 2003). Further RalA was shown to be essential for tumorigenesis and metastasis of Ras transformed human cells (Lim et al., 2005; Tchevkina et al., 2005). The differential contribution that RalA and RalB make to anchorage independent growth and invasion of pancreatic, bladder and colorectal cancer cell lines was demonstrated in a series of studies that further suggest they could have distinct functional roles in cancers (Lim et al., 2006; Martin et al., 2011; Oxford et al., 2005). Subsequently the oncogenic nature of Ral GTPases has been described in a variety of cancer types including- prostate cancer (Yin et al., 2007), breast cancer (Li et al., 2009), melanoma (Zipfel et al., 2010), malignant nerve sheath tumors(Bodempudi et al., 2009), T-cell neoplasms (Osei-Sarfo et al., 2011), oral squamous cell carcinoma (Hamada et al., 2011) and lung cancer (Male et al., 2012).
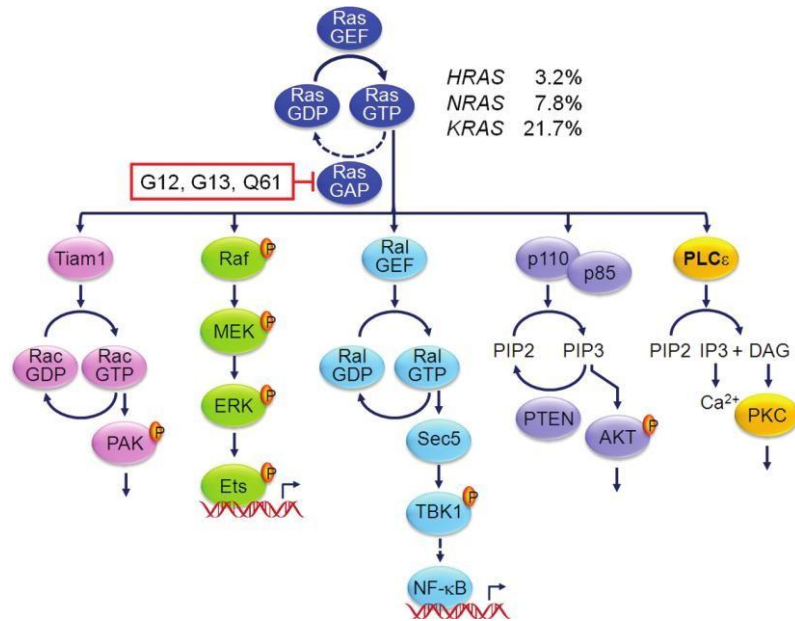
**Figure 1.8: Ras effector pathways supporting tumorigenesis.** Activating mutations (G12, G13, Q61) have been detected in Ras isoforms H-Ras, N-Ras and K-Ras together in approximately 33% of cancer cases. This hyper-activated Ras drives tumorigenesis via perpetual signalling to its downstream effector pathways that include Tiam1, Raf kinase, RalGEFs, p110-p85 and PLC-ε. These effectors in turn mediate deregulated proliferation, cell survival and invasion. (Adapted from (Neel et al., 2011)).

Recent studies have identified deregulated expression of RalGEFs and RalGAPs to promote tumorigenesis via aberrant Ral activation (Saito et al., 2013; Vigil et al., 2010a). RalGDS is over-expressed in hepatocellular carcinoma and ovarian cancer and RGL2 is over-expressed in T-cell malignancies (Ezzeldin et al., 2014; Vigil et al., 2010b; Wang et al., 2013). Rgr is also identified as a proto-oncogene in T cell malignancies (Osei-Sarfo et al., 2011). RalGAPs that function to terminate Ral signalling could hence be potential tumor suppressors, which has now been reported for the α2 subunit of RalGAP2 in bladder cancer cell lines and patient tissue samples (Saito et al., 2013).

The role of Ral GTPases in tumorigenesis and metastasis has led to the design and testing of anti-Ral cancer therapies. These include GGT-I (Geranyl-geranyl transferase I) inhibitors that target lipid modification of Ral to affect their localization (Falsetti et al., 2007; Hamada et al., 2011). Inhibitors against Aurora A kinase have also been tested for their ability to affect localization of RalA (Ren et al., 2007- unpublished data). Recently specific Ral inhibitors have been developed that bind specifically to GDP-bound conformation of RalA and RalB

GTPases in a Ral specific pocket. These inhibitors regress tumors in mice induced upon subcutaneous injection of Ral dependent cancer cells, emphasizing their clinical potential (Yan et al., 2014).

## 1.6 COMMON EFFECTORS OF RAL AND ARF6 GTPASES:

Amongst several Arf6 and Ral effector proteins, Phospholipase D, exocyst complex and RalBP1 are of special interest to us due to their direct and/or functional interaction with both GTPases (Figure 1.9). They could hence contribute to the cross talk between Ral and Arf6 and are hence selected for discussion here.



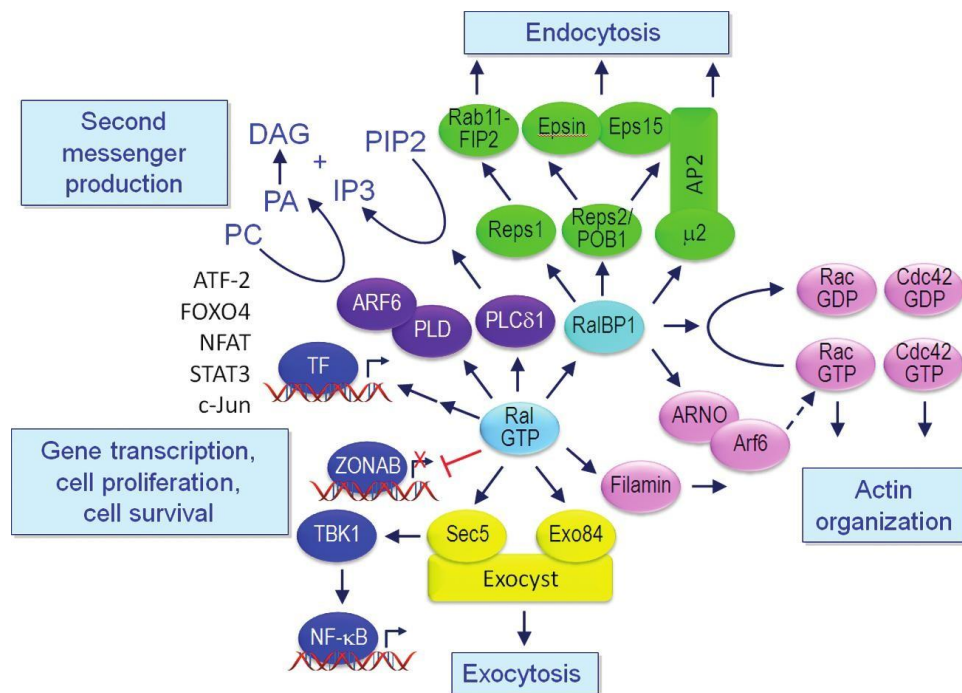**Figure 1.9: Ral effectors and their interaction with Arf6.** Active Ral GTPase binds can interact with several effectors including PLD, RalBP1, Exocyst complex components, PLC-delta-1, Filamin and ZONAB. Amongst these, RalBP1 and PLD have been shown to directly or functionally interact with Arf6. (Adapted from (Neel et al., 2011)).

### 1.6.1 Arf6 effector- Phospholipase D:

Phospholipase D (PLD) is a lipid modification enzyme that hydrolyses phosphatidylcholine to phosphatidic acid (PA) and choline. The different cellular functions of PLD such as endocytic recycling, actin cytoskeletal reorganization, activation of mTOR pathway, are mediated by its secondary messenger PA (Jang et al., 2012). PLD activity is upregulated in response to Ras induced mitogenic and tumorigenic stimuli and is involved in cancer cell proliferation and migration (Lu et al., 2000; Santy and Casanova, 2001). All Arf GTPases are capable of regulating activation of PLD1 and PLD2. Arf6-GTP binds to PLD1 in its effector domain and activates it to mediate functions such as endosomal membrane recycling (Melendez et al., 2001). Arf6 also binds PLD2 and regulates its activity (Hiroyama and Exton, 2005b). N-terminal motif of Ral GTPases binds PLD in GTP independent manner. Ral regulation of PLD1 is Arf6 dependent in regulation of secretory granule release from neuroendocrine cells (Vitale et al., 2005). Synergistic action of RalA and Arf6 is reported to activate PLD1 in H-Ras transformed cells (Xu et al., 2003).

### 1.6.2 Ral effector- RalBP1:

RalBP1 (RLIP76) functions as a multifunctional protein acting as an ATP dependent transporter of glutathione conjugates and other drugs (Awasthi et al., 2003), as an effector for RalA/B and R-Ras (Cantor et al., 1995; Goldfinger et al., 2006) and as GAP protein for Rho family GTPases (Dorseuil, 1995) RalBP1 is an important signalling intermediate across multiple signalling pathways. Both RalA and RalB GTPases utilize RalBP1 to mediate their cellular functions of mitochondrial fission (Kashatus et al., 2011) and invadopodia formation (Neel et al., 2012a). Downstream of Ral isoforms RalBP1 is seen to promote anchorage independent growth and invasiveness in colon and pancreatic cancers (Martin et al., 2011; Neel et al., 2012b). Recent studies have also reported a Ral independent oncogenic role of RalBP1 is in prostate cancer, bladder cancer, melanoma and colorectal cancer (Mollberg et al., 2012; Singhal et al., 2006; Wu et al., 2010).

### 1.6.3 Ral effector- exocyst complex:

Exocyst complex is a conserved octameric complex that mediates polarized delivery of endosomal vesicles to specific plasma membrane sites (Heider and Munson, 2012). Few evidences suggest existence of two sub-complexes, one vesicle bound (Sec15, Sec10, Exo84) and one plasma membrane bound (Sec 3, Sec5, Sec6, Sec8, Exo70) (Jin et al., 2005; Moskalenko et al., 2003). Ral GTPases are called as the master regulator of exocyst complex since they bind Sec5 (plasma membrane localized) and Exo84 (vesicle localized) directly in GTP dependent manner facilitating fusion of sub-complexes and delivery of vesicles (Moskalenko et al., 2002) (Figure 1.10). Ral driven exocyst complex regulates a multitude of cellular functions including maintenance of cell polarity (Lalli and Hall, 2005), autophagosome assembly (Bodemann et al., 2011), secretory granule exocytosis (Chen et al., 2011a) and cytokinesis (Chen et al., 2006). The exocyst complex components are also found to mediate the oncogenic function of Ral GTPases (Issaq et al., 2010; Martin et al., 2011).
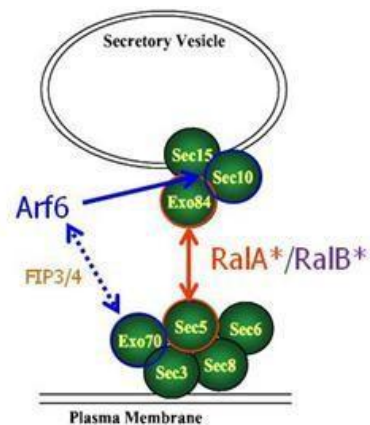


**Figure 1.10: RalA/B and Arf6 bind and regulate exocyst complex**. Active RalA and RalB directly bind to their effectors Exo84 and Sec5 and this dual subunit interaction regulates exocyst function. Active Arf6 binds its effector Sec10 directly and Exo70 indirectly (broken blue line) via its interaction with effectors FIP3/4 proteins (Adapted from (Moskalenko et al., 2003)).

Arf6 is functionally seen to be involved with both these Ral effectors. Downstream of R-Ras, RalBP1 is seen to bind the Arf6 GEF ARNO to regulate Arf6 and control cell spreading (Goldfinger et al., 2006; Lee et al., 2014). Arf6 also binds the exocyst subunit Sec10 in a GTP dependent manner (Prigent et al., 2003a) and Exo70 indirectly via effectors FIP3/4 (Fielding et al., 2005) to regulate endosomal recycling and cytokinesis (Figure 1.10).

## 1.7 RAL AND ARF6 REGULATE INTEGRIN MEDIATED MEMBRANE RAFT TRAFFICKING

### 1.7.1 Integrin mediated membrane raft microdomain trafficking and implications to anchorage dependent growth:

Membrane rafts are dynamic cholesterol and sphingolipid enriched plasma membrane microdomains (Simons and Gerl, 2010). These microdomains function as anchoring sites on plasma membrane for proteins involved in growth factor signalling. Lipid Raft microdomains are mediators of integrin mediated adhesion and growth factor signalling crosstalk (Del Pozo et al., 2004). Integrin mediated adhesion of cells to extracellular matrix (ECM) regulates trafficking and plasma membrane localization of raft microdomains (Del Pozo et al., 2004). Upon loss of cell-ECM adhesion raft microdomains are internalized through caveolar endocytosis (del Pozo et al., 2005) and held in recycling endosomal pool in non-adherent cells (Balasubramanian et al., 2007). When cells are replated back on fibronectin (FN), raft microdomains return back to the plasma membrane, via a RalA-exocyst complex and Arf6 dependent pathway (Balasubramanian et al., 2010) (Figure 1.11).
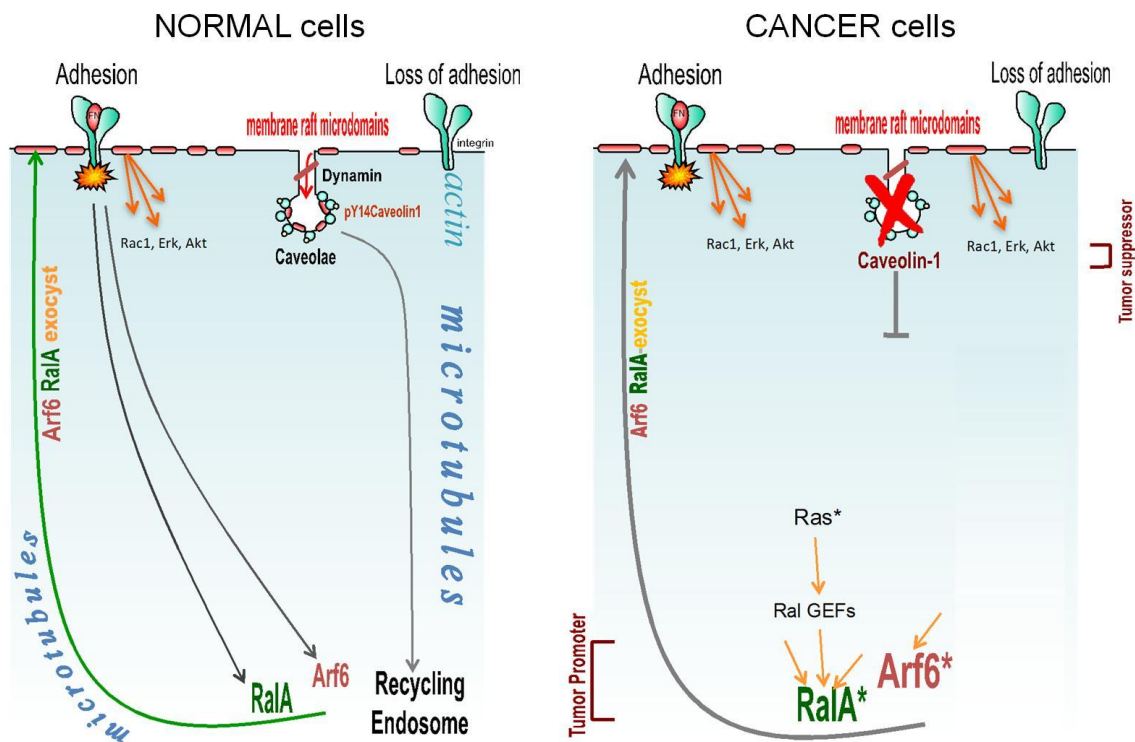


**Figure 1.11: Integrin dependent membrane raft trafficking pathway in normal and cancer cells**. Schematic representing regulation of integrin dependent membrane raft trafficking pathway in non-transformed 'normal' cells and cancer cells. Caveolar endocytosis and RalA-Arf6-exocyst dependent exocytosis pathways mediate this trafficking pathway which can be deregulated in cancer cells (Courtesy Dr. Nagaraj Balasubramanian).

In the absence of cell adhesion, clearance of these signalling platforms from the plasma membrane downregulates anchorage dependent growth signalling, conferring anchorage dependence in 'normal' cells (Del Pozo et al., 2005). Hence deregulation of the membrane raft trafficking pathways can help drive anchorage independent growth signalling in cancer cells. This can be mediated by the deregulation of the endocytic pathway through Caveolin1 and/or its phosphorylation or the deregulation of the exocytic pathway through RalA and/or Arf6. Indeed Caveolin-1 is a known tumor suppressor that is downregulated in cancers (Fiucci et al., 2002; Han et al., 2009a) and the expression and/or activation of RalA and Arf6 has been reported in several cancers (Eades et al., 2015; Knizhnik et al., 2011; Smith et al., 2007) (Figure 1.11).

## 1.7.2 Role of RalA in integrin dependent membrane raft trafficking:

Integrin dependent RalA activation regulates the exocytic recycling of membrane raft microdomain and adhesion dependence in normal cells. Active RalA uses its effector exocyst component, Sec5, in this pathway (Balasubramanian et al., 2010). A fast cycling RalA mutant (RalA79L) was found to mediate anchorage independent membrane raft exocytosis and anchorage independent Akt and Erk signalling (Balasubramanian et al., 2010). Interestingly, integrins regulated activation of only RalA and not that of RalB resulting in these pathways being unresponsive to RalB depletion (Figure 1.12).

Loss of RalA and not RalB in a pancreatic cancer MIA-PaCa-2 cells reduced the plasma membrane targeting of raft microdomains to affect anchorage independent growth signalling in these cells (Balasubramanian et al., 2010). This corroborates earlier reports describing RalA and not RalB to be essential for anchorage independent growth of MIA-PaCa-2 cells (Lim et al., 2006). Hence this could constitute a major pathway by which RalA activated downstream of oncogenic Ras could drive anchorage independent growth of cancer cells.
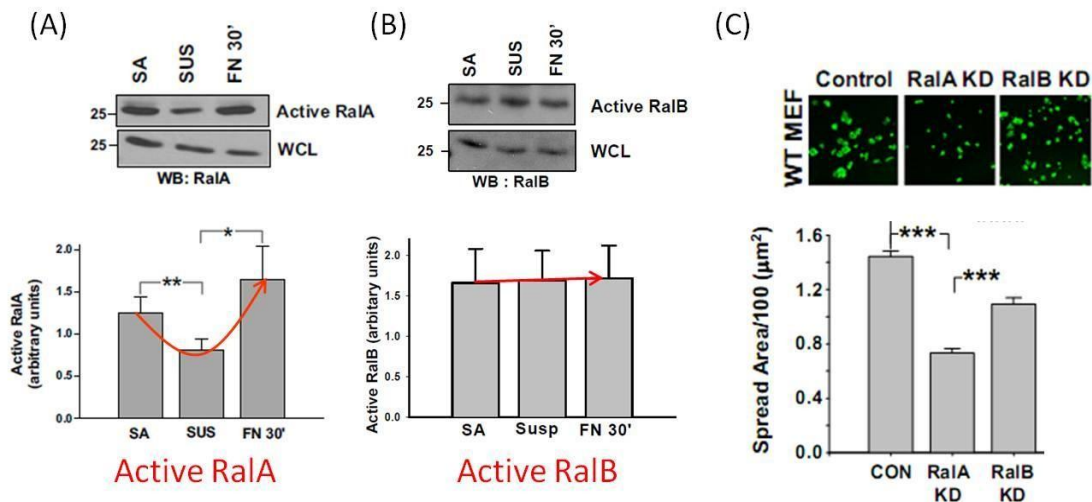
**Figure 1.12: Role of Ral GTPases in adhesion dependent membrane raft trafficking.** Activation of **(A)** RalA, not **(B)** RalB is regulated by integrin dependent cell adhesion to ECM. SA: stable adherent cells, SUS: suspended/ non-adherent cells, FN 30': cells re-adherent on fibronectin for 30 minutes. Graphs represent ratio of active Ral fraction to total Ral fraction in whole cell lysate (WCL). **(C)** Loss of RalA, not RalB affects adhesion dependent cell spreading. Graph represents average cell spread area of control (CON), RalA knockdown (RalA KD), RalB knockdown (RalB KD) cells. (Adapted from (Balasubramanian et al., 2010))

## 1.7.3 Role of Arf6 in integrin dependent membrane raft trafficking:

Arf6 regulates only the integrin dependent exocytosis and not endocytosis raft microdomains in fibroblasts (Balasubramanian et al., 2007). Arf6, like RalA, is activated in integrin dependent manner and regulated adhesion dependent cell spreading (Figure 1.13).
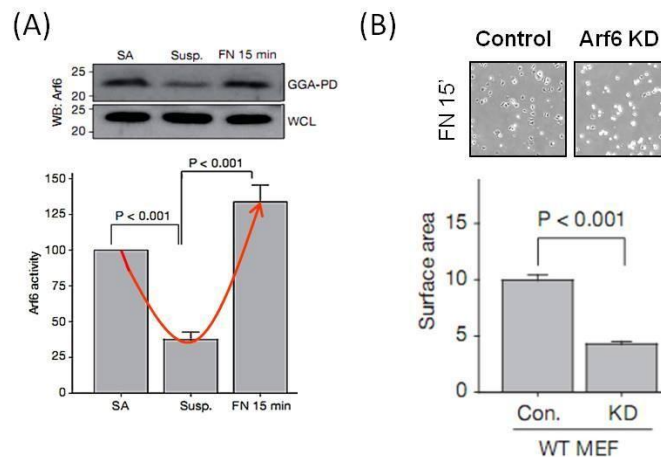
**Figure 1.13: Role of Arf6 in adhesion dependent membrane raft trafficking. (A)** Activation of Arf6 is regulated by integrin dependent cell adhesion to ECM. SA: stable adherent cells, Susp: suspended/ non-adherent cells, FN 15': cells re-adherent on fibronectin for 15 minutes. Graphs represent ratio of active Arf6 fraction enriched by GST-GGA3 pulldown (GGA-PD) to total Arf6 fraction in whole cell lysate (WCL). **(B)** Loss of Arf6 affects adhesion dependent cell spreading. Graph represents average cell spread area of control and Arf6 knockdown (Arf6 KD) cells. (Adapted from (Balasubramanian et al., 2007))

Activation of Arf6 while found to be necessary for the exocytosis of membrane raft microdomains was not sufficient to mediate the complete delivery of raft microdomains. These microdomains were found to accumulate in the cortical actin region, suggesting the likely presence of a collaborative partner for Arf6 mediated delivery (Balasubramanian et al., 2007). Knowing RalA was both necessary and sufficient for this pathway lead us to speculate the presence of a RalA-Arf6 crosstalk which could account for the role of both these proteins in exocyst mediated membrane raft trafficking and delivery (Figure 1.14).

Several cellular processes involving secretory pathways are regulated by RalA and Arf6 including release of exosomes or micro-vesicles from tumor cells (Hyenne et al., 2015; Muralidharan-Chari et al., 2009), secretion of dense core granules from neuronal endocrine cells (Vitale et al., 2002, 2005), secretion of insulin from pancreatic beta cells (Lawrence and Birnbaum, 2003a; Ljubicic et al., 2009) and recycling of GLUT4 receptor in adipocytes (Chen et al., 2007; Millar et al., 1999). Many cellular processes requiring active membrane delivery at particular subcellular sites are also known to be regulated by Ral and Arf6
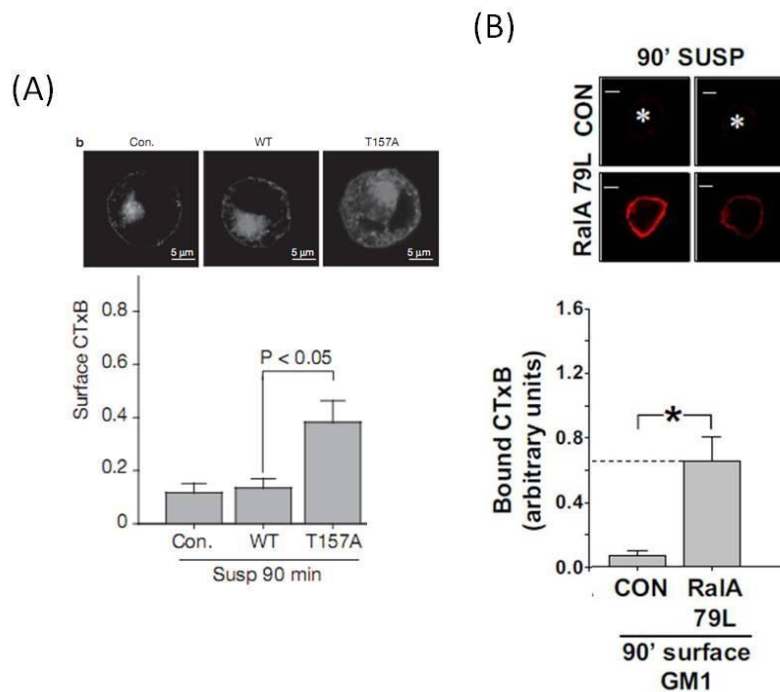
**Figure 1.14: Active RalA is sufficient for membrane raft exocytosis, active Arf6 is not sufficient. (A)** Fast cycling active Arf6 mutant-Arf6 T157A inefficiently delivers membrane rafts (marked by CTxB) to plasma membrane relative to CON and WT Arf6 in non-adherent cells with most trapped in cortical actin region. **(B)** Expression of fast cycling active RalA mutant- RalA79L completely restores membrane rafts to plasma membrane to the levels in adherent cells marked by dotted line. RalA79L shows approximately seven fold enrichment over control cells as compared to only two fold increase by Arf6T157A. (Adapted from (Balasubramanian et al., 2007, 2010))

including cytokinesis (Cascone et al., 2008; Schweitzer and D'Souza-Schorey, 2005), phagocytosis (Corrotte et al., 2010; Melendez et al., 2001), *Salmonella* invasion (Davidson et al., 2015; Humphreys et al., 2012, 2013; Nichols and Casanova, 2010), autophagy (Farré and Subramani, 2011; Martin et al., 2014; Moreau et al., 2012; Tracy et al., 2016), invadopodia formation (Hashimoto et al., 2004; Neel et al., 2012a; Onodera et al., 2005) and neurite branching (Cheung et al., 2014; Kobayashi and Fukuda, 2012; Lalli and Hall, 2005) are also shown to be regulated by both RalA and Arf6 GTPases.

The joint role Ral and Arf6 have along many of these cellular processes and the presence of shared effector pathways (RalBP1, Exocyst complex and PLD1) lead us to ask - Could a regulatory crosstalk exist between RalA and Arf6? Could this crosstalk be relevant to RalA and Arf6 mediated functions in normal and cancer cells? Would this crosstalk be specific to RalA vs RalB? And finally how integrins differentially activate RalA v/s RalB? To address these questions my study will test the following aims.

## 1.8 AIMS OF THE STUDY:

1. To test and validate the existence of a integrin dependent Ral-Arf6 crosstalk

2. To test the contribution this Ral-Arf6 crosstalk makes to anchorage independent signalling in cancers

3. To elucidate the mechanism of this Ral-Arf6 crosstalk

4. To elucidate the role of RalGEFs in mediating integrin dependent differential activation of Ral isoforms

# CHAPTER 2:

# Characterization of integrin dependent Ral-Arf6 crosstalk in MEFs

## 2.1 RATIONALE:

The activation and deactivation cycle of small GTPases is critical to their functioning as molecular switches in the cell. Upstream signalling events employ small GTPases to turn ON or OFF cellular processes. Likewise, integrin mediated cell-ECM (Extra Cellular Matrix) adhesions engage two small GTPases- RalA and Arf6 to regulate exocytosis of membrane raft microdomains (Balasubramanian et al., 2007, 2010) to the plasma membrane to regulate anchorage dependent growth signalling (Del Pozo et al., 2004; del Pozo et al., 2005). Thus loss of adhesion decreases RalA and Arf6 activity ensuring minimal exocytosis of raft microdomains and decreased growth signalling. Conversely re-adhesion of cells activates RalA and Arf6 triggering membrane raft exocytosis and plasma membrane delivery to restore growth signalling. This is mediated by the exocyst complex, that binds RalA and Arf6, in a microtubule and actin dependent manner (Balasubramanian et al., 2007, 2010).

A major difference between how these GTPases regulate this trafficking is that RalA is sufficient for this pathway whereas Arf6 is necessary but not sufficient (Balasubramanian et al., 2007, 2010). Ral and Arf6 work along several other common cellular pathways (Chen et al., 2006; Corrotte et al., 2010; Jayaram et al., 2011; Melendez et al., 2001; Vitale et al., 2002, 2005) with shared effectors (Corrotte et al., 2010; Fielding et al., 2005; Melendez et al., 2001; Moskalenko et al., 2003; Prigent et al., 2003b) leading us to ask the primary question- Could there exist a regulatory crosstalk between RalA and Arf6 that could mediate their functions along this pathway in cells? This chapter hence evaluates this regulation in context of integrin mediated adhesion. The differential regulation of RalA and RalB isoforms by integrins and its implications for this crosstalk and integrin dependent trafficking are also explored in this part of the study.

## 2.2 MATERIALS AND METHODS:


### 2.2.1 Reagents:

Human plasma fibronectin used in these studies was from Sigma. Cholera toxin subunit B (CTxB) labeled with Alexa 594 (C22843) was procured from Molecular Probes. Primary antibodies used in this study were anti RalA (BD Transduction), anti RalB (R&D Biosystems), anti-beta tubulin E7 (DSHB), anti-beta actin (Abcam), anti-FLAG(M2) (Sigma) and anti-FLAG(M2)-HRP (Sigma). Anti-HA antibody for western blot was from Covance and Anti-HA antibody for immunofluorescence was from Roche. Anti Arf6 antibody was a kind gift from Dr. James Casanova (University of Virginia, Virginia). Primary antibodies were detected with the following HRP conjugated secondary antibodies: anti-mouse IgG, anti- rabbit IgG and anti-goat IgG (Jackson Immuno Research Laboratories). 3X FLAG peptide was from Sigma. Alexa-488/ Alexa-568 conjugated anti-mouse, anti-rabbit and anti-rat secondary antibodies for immunofluorescence were from Molecular Probes. Fluoromount-G was from Southern Biotech. Protease inhibitor cocktail II (100X) was from Sigma. Protein-G-Dynabeads was from Invitrogen. RNA interference sequences used for knockdown studies were procured from Sigma and as listed in appendix table 4.


### 2.2.2 Tissue Culture:

Mouse embryonic fibroblasts (from the lab of Dr. Richard Anderson, University of Texas Health Sciences Center, Dallas TX) were cultured in high glucose DMEM medium with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). 293T cells, a kind gift from Dr. Sanjeev Galande's lab were cultured in high glucose DMEM medium with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). For plasmid transfections, cells were seeded in either in 6 well plate or 60 mm dishes at 50 per cent confluency and transfected with 2 µg or 5 µg of plasmid DNA using the Lipofectamine LTX (Invitrogen) reagent on the same day for MEFs or the next day for 293T cells. For siRNA mediated knockdowns in MEFs, 0.5X $10^5$ cells seeded in 60 mm dishes (about 30 per cent confluency) were transfected first with a standardized amount of duplex siRNA oligo using the RNAiMax transfection reagent (Invitrogen) followed by a second transfection the following day with the same amount of siRNA oligo. Cells were used 48 hours after the second transfection. For reconstitutions, cells were electroporated 24 hours after second siRNA transfection with the rescue vectors (30ug

plasmid +10ug salmon sperm DNA), allowed to recover for 24 hours and then used. Stable shArf6 MEFs were made from a single cell population selected after transfection of pSuper-shArf6-Neo-GFP vector using G418 (Roche).

### 2.2.3 Cell suspension assay:

Cells were serum starved for at least 12 hours with DMEM supplemented with 0.2% FBS and PenStrep (low serum DMEM). Cells were detached with 1X Trypsin-EDTA, which was diluted with low serum medium and cells were held in suspension for 120 minutes with 0.75% methylcellulose in low serum DMEM. Following the incubation cells were washed twice with low serum DMEM and were replated on dishes or coverslips coated overnight with fibronectin at 4°C (2µg/ml or 10µg/ml as indicated in figure legends).

### 2.2.4 Plasmids and site directed mutagenesis:

FLAG-WT-RalA, FLAG-G23V-RalA, untagged G23V-RalA, FLAG-G23V-RalB were kind gifts from Dr. Michael White (UT South-western Medical Center, Texas). pSuper-shArf6-Neo-GFP construct was a kind gift from Dr. Eunjoon Kim (Choi et al., 2006). CFP-RalA-WT, CFP-RalA-V23, HA-RalB-WT and YFP-RalB-V23 plasmids were kind gifts from Dr. Dan Theodorescu. FLAG-Arf6-WT and FLAG-Arf6-T157A were kind gifts from Dr. James Casanova (University of Virginia, School of Medicine, Virginia). FLAG R79L RalA mutant was developed by site-directed mutagenesis using FLAG WT RalA as template. Primers were designed using QuickChange primer design tool from Agilent Technologies as listed in Appendix table 2. hRalA* (siRNA insensitive mutant) is described earlier (Balasubramanian et al., 2010). Briefly, HA-hRalA plasmid was used as template and primers used for site directed mutagenesis were as listed in Appendix Table 2.

### 2.2.5 Cell surface labeling with CTxB:

Serum starved MEFs held in suspension for 120 minutes were washed and re-suspended in 2 ml low serum DMEM medium. Cells were then held on ice for 10 minutes, incubated with 50ng/ml of CTxB-Alexa 594 for 15 minutes on ice, washed twice with cold PBS, spun down and fixed with 3.5% paraformaldehyde for 15 minutes at room temperature. Cells were mounted with Fluoromount-G (Southern Biotech) allowed to dry overnight at room temperature and imaged within the 48 hours using a Zeiss LSM 710 laser confocal-

Anisotropy microscope with a 40X objective. Samples to be compared were imaged at identical conditions and analyzed using the Image J software (NIH). Thresholds were set to define the cell edge that was then used to create a mask for each cell. The total integrated density within the mask was compared between samples.

## 2.2.6 Arf6 and RalA activity assay:

Serum starved cells were detached, counted using hemocytometer and 0.6 million cells per time point (1.2 million for three time points) were held in suspension as described in 2.2.3. Following the incubation cells were washed and cell pellet was suspended in 3 ml of low serum medium. 1 ml of this suspension was spun down in a microcentrifuge tube (SUSP – suspension) and pellet frozen at -80°C. The remaining cell suspension was distributed in two 10 µg/ml FN coated 60 mm dishes, one dish to be frozen at 15 minutes (15' FN-re-adherent) and other at 4 hours (SA- stable adherent) post replating cells. Absence of any clumping was confirmed by observing the cells under a microscope. Cells were immediately frozen at -80°C at their respective time points and were together revived on ice for lysis with the Arf6 or RalA activity assay buffer as required (Arf6 activity assay buffer: 50mM Tris, 150mM NaCl, 2 mM MgCl2, 0.1% TritonX-100, 1 mM NaF, 0.1 mM Na-orthovanadate, 1X PIC; RalA activity assay buffer: 50mM Tris, 150mM NaCl, 10mM MgCl2, 0.1% NP-40, 1 mM NaF, 0.1 mM Na-orthovanadate, 1X PIC). Cells plated on dishes were lysed with the help of a cell scraper in lysis buffer. For Arf6 activity assay 0.6 million cells of each time point were lysed in 500 µl buffer whereas for RalA activity assay the same number of cells was lysed in 1 ml buffer. 90 µl of the cell lysate was added to 30 µl of 4X Laemmli buffer to make whole cell lysate (WCL). 400 µl of lysate were incubated with Glutathione Sepharose beads (GE-Amersham) bound to 60 µg GST-Sec5-RBD (for active RalA pulldown) or 60 µg GST-GGA3-VHS-GAT (for active Arf6 pulldown) for 35 minutes at 4°C on a rotary mixer. Beads were washed thrice with the respective activity buffer, eluted with 2X Laemmli buffer to make pulldown lysate. All of the pulldown lysate and 30 µl / 120 µl of WCL were resolved by SDS PAGE followed by western blotting. RalA and Arf6 were detected by western blotting with anti RalA and anti Arf6 antibodies and chemiluminescent substrates from Pierce. Images were recorded using the LAS4000 detection system (Fujifilm-GE) and band intensities calculated by densitometric analysis using Image J software (NIH). Per cent active levels of Arf6 or RalA were determined by using the following calculation.

Percentage Activity = Pulldown Band Intensity / (Corresponding WCL band intensity x Dilution Factor)

The dilution factor was calculated as the ratio of the amount of total cell lysate used for the pulldown (400 µl) and the amount of this lysate run on SDS PAGE for western blot detection (22.5 µl/ 30 µl of WCL) (Dilution factor: 400 /22.5 = 17.77). Active Arf6 or active RalA levels under different treatment conditions were normalized to stable adherent (SA) or control (CON).

## 2.2.7 Co-immunoprecipitation:

Co-immunoprecipitation (CO-IP) protocol as originally described (Adorno et al., 2009) was modified as follows. 293T cells were transfected with 7 µg of FLAG/ FLAG-WT-RalA/ FLAG-G23V-RalA/ FLAG-WT-RalB/ FLAG-G23V-RalB and 7 µg of HA-T157A-Arf6 with Lipofectamine LTX reagent. 48 hours post transfection, cells were lysed in CO-IP lysis buffer (20 mM HEPES pH 7.5, 400 mM KCl, 5% Glycerol, 5 mM EDTA, 0.4% NP-40 + 1X Protease inhibitor cocktail (PIC) + phosphatase inhibitors) for 30 minutes on ice. Lysates were sonicated using Diagenode Bioruptor for 2 pulses of 60% amplitude in 5 seconds ON/ 5 seconds OFF cycle and cleared by centrifugation at 15000 rpm for 30 minutes. Cell lysate (700 µg equivalent) was incubated with Anti-FLAG (M2) antibody bound Protein G-Dyna beads (Invitrogen) for 2 hours at 4°C in binding buffer (20 mM HEPES pH 7.5, 50 mM KCl, 5% Glycerol, 5 mM EDTA, 0.05% NP-40 + 1X PIC + phosphatase inhibitors) for 30 minutes at 4°C on a rotary mixer. The immune-complexes were washed with binding buffer and eluted using 200 µg/ml of 3X FLAG peptide for 15 minutes in thermomixer at 25°C. Elutes with 3% of whole cell lysate (WCL) used for IP were western blotted and probed with anti-HA and anti-FLAG-HRP antibody and developed using chemiluminescent substrates from Pierce and the LAS4000 detection system (Fujifilm-GE).

## 2.2.8 Immunofluorescence:

### (A) Immunofluorescence with FLAG and HA antibodies:

MEFs transfected with 2ug of CFP-WT-RalA, CFP-G23V-RalA, and HA-WT-RalB, YFP-V23-RalB and FLAG-T157A-Arf6 / FLAG-WT-Arf6 using Lipofectamine LTX reagent were trypsinized and replated on fibronectin coated coverslips (10 µg/ml) for 15 minutes and

fixed with 3.5% paraformaldehyde. Permeabilization was carried out using PBS containing 0.1% Triton-X-100 for 5 minutes. Blocking was performed with 10% BSA for 30 minutes at 37°C followed by incubation with 1:2000 mouse anti-FLAG (M2) and rat anti-HA antibody (Roche) in 3% BSA for 1 hour at 37°C. Bound primary antibodies were detected using fluorescently conjugated anti-mouse and anti-rat secondary antibodies (1:1000). Anti-mouse Alexa-568 was used to stain all coverslips. By contrast, HA-RalB WT+ FLAG-Arf6 (WT/T157A) coverslips were stained with anti-mouse Alexa-568 + anti-rat Alexa-488 for 1 hour at room temperature. All incubations were done in a humidified chamber. Washes were done with 1X PBS at room temperature. Stained and washed coverslips were mounted with Fluoromount-G (Southern Biotech) and imaged using a Zeiss LSM 710 laser confocal-Anisotropy microscope with a 63X objective.

### (B) Immunofluorescence with anti-RalA and anti-Arf6 antibodies:

MEFs transfected with 2μg of FLAG-RalA-V23 and HA-Arf6-T157A using Lipofectamine LTX reagent were trypsinized and replated on fibronectin coated coverslips (10μg/ml) for 15 minutes and fixed with 3.5% paraformaldehyde. Cells were permeabilized with PBS containing 5% BSA and 0.05% Triton-X-100 for 15 minutes and blocked with 5% BSA for 1 hour at room temperature followed by incubation with 1:500 rabbit anti-Arf6 (Abcam) and 1:500 mouse anti-RalA (BD Transduction laboratories) antibodies in 5% BSA for 3 hours. Cells were finally stained with 1:1000 diluted secondary antibodies (anti-mouse Alexa-488 and anti-rabbit-Alexa-568) for 1 hour at room temperature. All incubations were done in a humidified chamber. Washes were done with 1X PBS at room temperature. Stained and washed coverslips were mounted with Fluoromount-G (Southern Biotech) and imaged using a Zeiss LSM 710 laser confocal-Anisotropy microscope with a 63x objective.

### 2.2.9 Statistical analysis:

Statistical analysis of data was done using the two tailed unpaired Student's T test and when normalized to respective controls using the two tailed single sample T test. All analysis was done using Prism Graphpad analysis software. Statistical significance was considered at $P<0.05$.

## 2.3 RESULTS:

### 2.3.1 RalA regulates adhesion dependent activation of Arf6 in MEFs

To evaluate the possible regulatory association between RalA and Arf6, the inter-dependence of their activities was first tested. Since activities of RalA and Arf6 activities (but not RalB) were regulated by integrins, we tested the effect knockdown of RalA and RalB has on the integrin dependent activation status of Arf6. As reported earlier (Balasubramanian et al., 2007) we saw that in control cells (CON) Arf6 activity decreased upon 120 minutes of holding cells in suspension (SUSP) relative to stable adherent cells (SA) and recovered after 15 minutes of re-adhesion to fibronectin (FN) (Figure 2.1C). siRNA mediated specific depletion of RalA (RalAi) in MEFs (Figure 2.1A) did not affect the drop in Arf6 activity upon loss of adhesion but prevented its recovery on re-adhesion (Figure 2.1C). The specificity of this regulation was confirmed when expression of siRNA resistant RalA mutant (hRalA*) in RalAi cells (Figure 2.1D) rescued Arf6 activation in re-adherent cells (Figure 2.1E). RalB knockdown (RalBi) (Figure 2.1B) did not disrupt integrin dependent Arf6 activation (Figure 2.1C) suggesting this regulation to be specifically mediated by RalA. This effect could be attributed the differential regulation of Ral isoforms by integrins (Balasubramanian et al., 2010). Depletion of Arf6 (Arf6i) (Figure 2.2A) did not affect RalA activation (Figure 2.2B) indicating the presence of a **linear integrin-RalA-Arf6 pathway** in MEFs.

### 2.3.2 Active Ral supports anchorage independent Arf6 activation

To further evaluate if this crosstalk works only downstream of integrins, we wanted to determine if these proteins are capable of regulating each other in the absence of adhesion. To test this we ectopically expressed constitutively active mutant of RalA, RalA-G23V (RalA-V23) in MEFs. This mutant is constitutively GTP bound and active independent of upstream regulatory events. Expression of RalA-V23 in MEFs (Figure 2.3A) prevented the drop in Arf6 activity in non-adherent cells (SUSP) relative to stable adherent (SA) cells effectively making it anchorage independent (Figure 2.3C). Thus RalA can activate Arf6 independent of integrin mediated adhesion to the extracellular matrix (ECM). Since we speculated that Ral

isoform specificity for Arf6 regulation could be context dependent, we also tested if constitutively active RalB mutant RalB-G23V (RalB-V23) can regulate Arf6. Overexpression of RalB-V23 (Figure 2.3B) mimicked the effect of RalA-V23 has on Arf6 activation in MEFs (Figure 2.3D). **This data indicated both Ral isoforms are capable of regulating Arf6 depending upon their activation status, which in turn is dependent on the stimulus.** Ral GTPases are activated by various stimuli including growth factors and oncogenic Ras which are explored in detail as part of chapter 2.
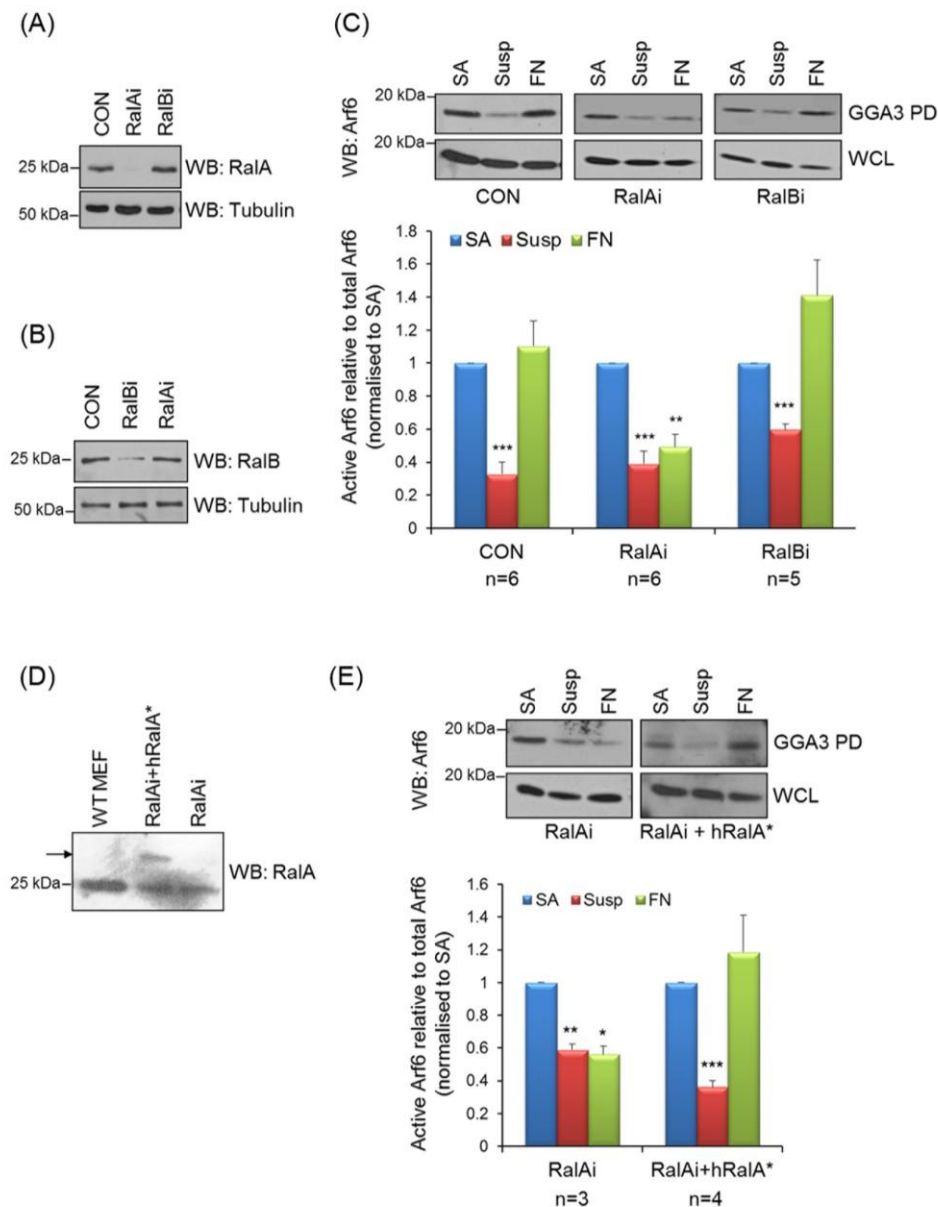
**Figure 2.1: RalA, but not RalB regulates adhesion dependent activation of Arf6 in MEFs. (A)**, **(B)** Representative western blots showing knockdown of **(A)** RalA and **(B)** RalB with respect to Tubulin as loading control **(C)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) and total Arf6 in the respective whole cell lysate (WCL) from control (CON), RalA knockdown (RalAi) and RalB knockdown (RalBi) MEFs. Serum starved cells held in suspension for 120 minutes (SUSP) were replated on fibronectin (10 μg/ml) for 15min (FN) and 4 hours (stable adherent- SA). **(D)** Representative western blot showing the detection of RalA, endogenous and expressed HA tagged hRalA* mutant (resistant to siRNA), in RalA knockdown MEFs **(E)** Western blot and quantitation for active Arf6 relative to total Arf6 under SA, SUSP and FN conditions in RalA knockdown (RalAi) MEFs and those reconstituted with HA-hRalA* (RalAi+hRalA*). Percentage active Arf6 levels were calculated as described in methods and normalized to respective SA. Graphs represent mean ± SEM from a minimum of three and maximum of six independent experiments (as indicated below each graph). Statistical analysis of all the above data was done using the one sample two tailed T-test and significance represented (* p value <0.05, ** p value <0.01, *** p value <0.001).
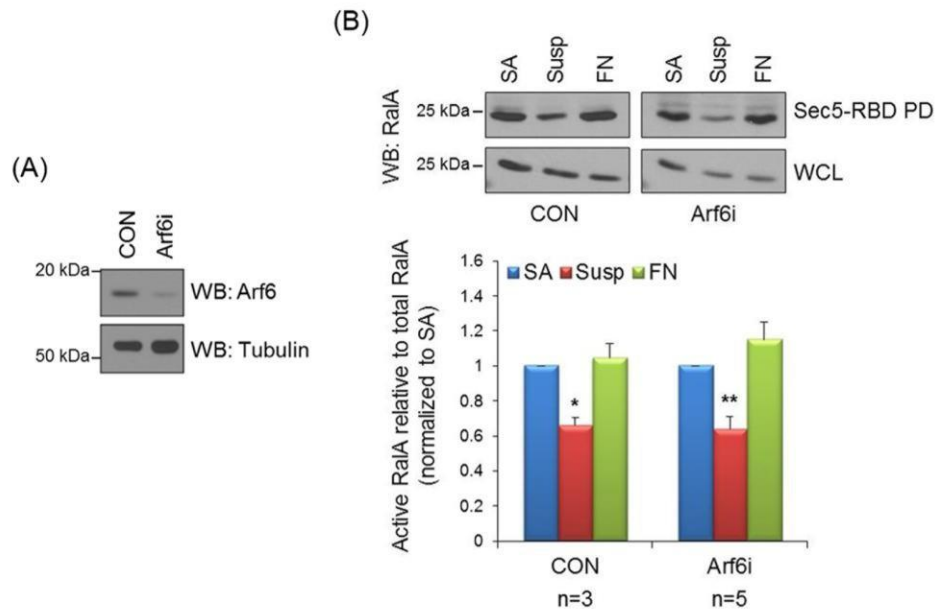
**Figure 2.2: Arf6 does not regulate adhesion dependent activation of RalA in MEFs.**
**(A)** Representative blots showing knockdown of Arf6 with respect to Tubulin as loading control **(B)** Western blot detection and quantitation of active RalA pulled down by GST-Sec5-RBD (Sec5-RBD-PD) and total RalA in the respective whole cell lysate (WCL) under SA, SUSP and FN conditions in control (CON) and Arf6 knockdown (Arf6i) MEFs. Percentage active RalA levels were calculated as described in methods and normalized to respective SA. Graph represents mean ± SEM from a minimum of three and maximum of five independent experiments (as indicated below each graph). Statistical analysis of all the above data was done using the one sample two tailed T-test and significance represented (* p value <0.05 and ** p value <0.01).
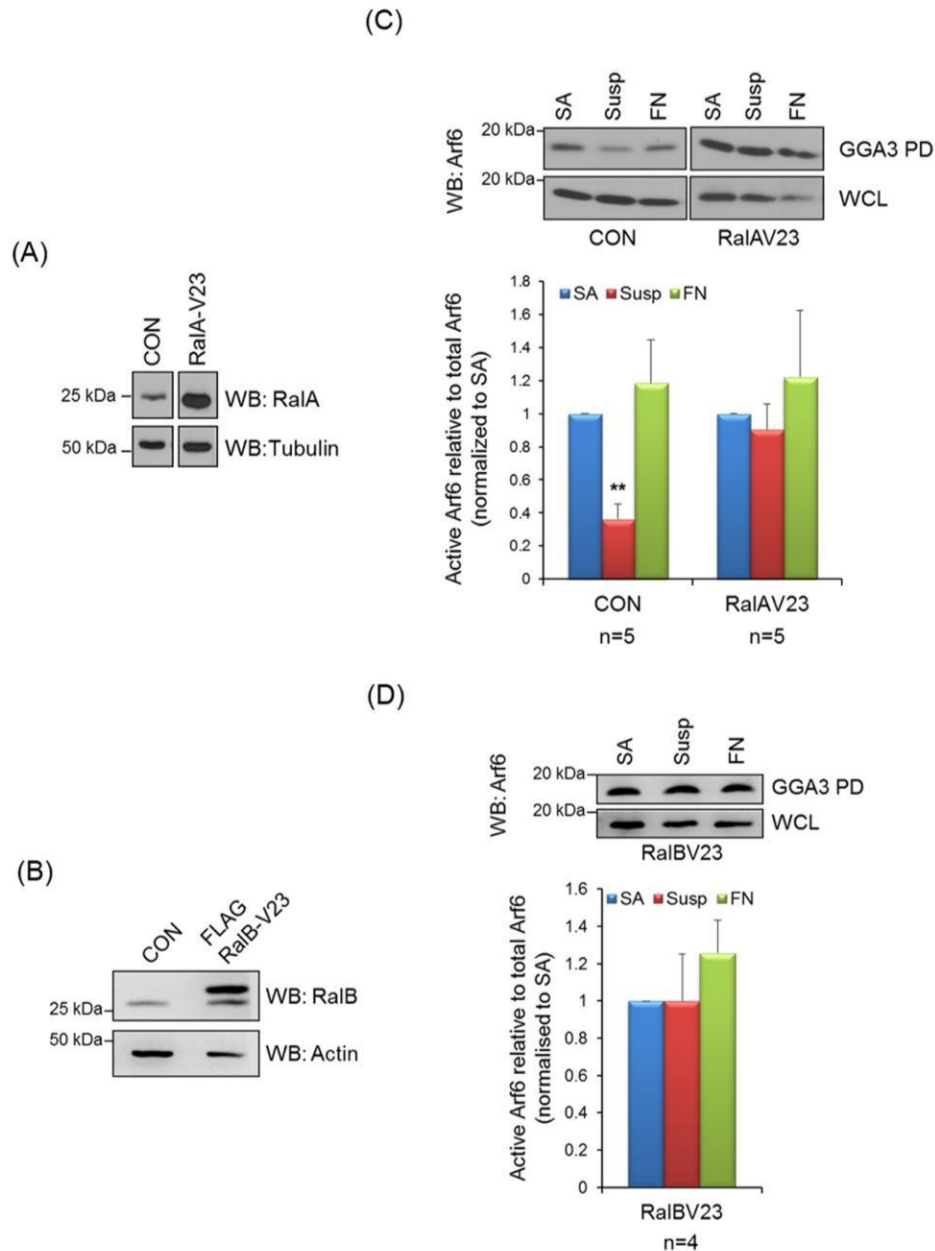
**Figure 2.3: Constitutively active RalA and RalB support anchorage independent Arf6 activation in MEFs. (A), (B)** Representative western blots showing expression of **(A)** untagged RalA-V23 and **(B)** FLAG-RalBV23 in MEFs with respect to Tubulin or Actin as loading controls respectively. **(C), (D)** Western blot and quantitation of active Arf6 relative to total Arf6 under SA, SUSP and FN conditions from control (CON) and **(C)** active RalA (RalA-V23) and **(D)** active RalB (RalB-V23) expressing MEFs. Percentage active Arf6 levels were calculated as described in methods and normalized to respective SA. Graph represents mean ± SEM from a minimum of four and maximum of five independent experiments (as indicated below each graph). Statistical analysis was done using the one sample two tailed T-test and significance represented (** p value <0.01).

### 2.3.3 Ral-Arf6 crosstalk is essential for active Ral function in membrane raft exocytosis

To understand the Ral-Arf6 crosstalk we also wanted to investigate if this crosstalk is relevant for Ral function. To test this we decided to look at the regulation of membrane raft trafficking which is known to be dependent on RalA and Arf6, through the exocyst complex. Depletion of RalA and Arf6 has shown that both of them are independently required for adhesion dependent exocytosis of membrane raft microdomains (Balasubramanian et al., 2007, 2010). Expression of an active fast cycling Arf6-T157A mutant in non-adherent MEFs triggered exit of raft microdomains from the recycling endosomes and their trafficking to the cell cortex, but could not efficiently deliver them to the plasma membrane (as was seen in re-adherent cells)(Balasubramanian et al., 2007). This suggests the presence of an additional integrin dependent regulator for this pathway that was found to be RalA. Unlike active Arf6, expression of active fast cycling RalA-79L mutant supported the exocytosis and complete delivery of raft microdomains to the plasma membrane (Balasubramanian et al., 2010). Thus active RalA was sufficient while active Arf6 was necessary but not sufficient for this trafficking. This can now be explained by the linear Ral-Arf6 crosstalk provided RalA requires Arf6 to mediate exocytosis. To test this hypothesis we established a stable Arf6 KD cell line, shArf6-MEF (Figure 2.4A). We confirmed that MEFs and shArf6-MEFs have equivalent basal RalA and caveolin-1 expression (Figure 2.4B) and surface GM1 levels (Figure 2.4C). Loss of Arf6 does not affect caveolar endocytic pathway for internalization of raft microdomains upon loss of adhesion (Figure 2.4D). We expressed empty FLAG vector and FLAG-RalA-R79L (RalA-79L, fast cycling mutant of RalA) in MEFs and shArf6 MEFs and compared surface GM1 levels in non-adherent cells (Figure 2.5A, 2.5B). As expected, RalA-79L expressing MEFs showed about 1.5 fold increase in surface GM1 levels as compared to control MEFs (Figure 2.5C). However equivalent expression of RalA-79L in shArf6 MEF cell line did not show any increase in surface GM1 as compared to control shArf6 cells (Figure 2.5C). **This suggests an essential role for Arf6 downstream of active RalA in regulating exocyst mediated membrane raft exocytosis.** Since active Arf6-T157A mutant was defective only in the fusion of raft components to plasma membrane, interaction of active RalA and active Arf6 could be essential at the plasma membrane, a possibility we then tested.
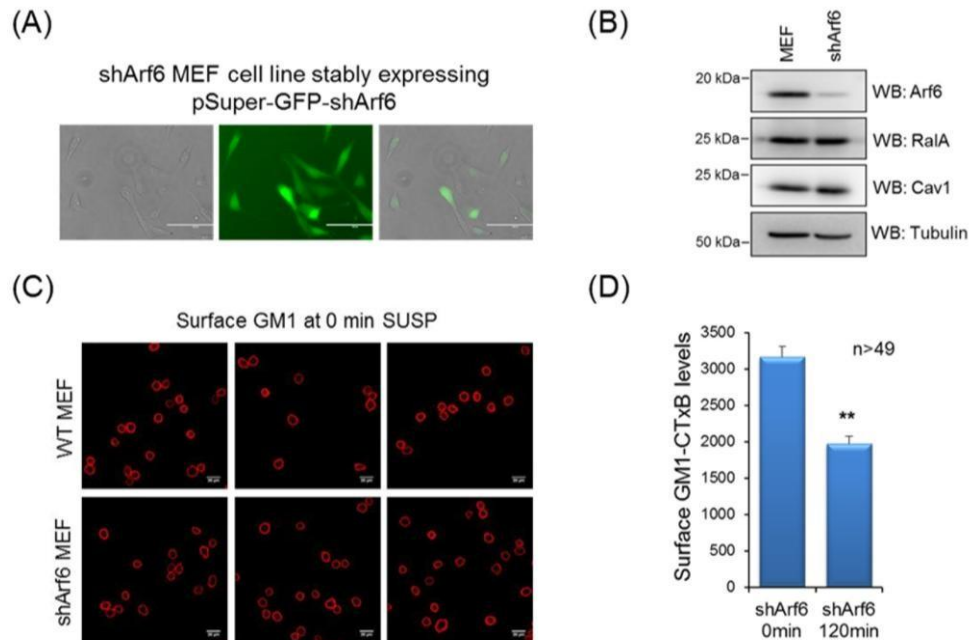
**Figure 2.4: Establishment and validation of stable shArf6 MEF cell line. (A)** Expression of pSuper-Neo-GFP-shArf6 plasmid in stable Arf6 knockdown MEF cell line detected by fluorescence (GFP) and phase contrast imaging **(B)** Western blot for Arf6 (WB: Arf6) , RalA (WB: RalA), Caveolin-1 (WB: Cav-1) and Tubulin (WB: Tubulin) in control MEFs (MEF) and shArf6 MEFs (shArf6) showing Arf6 knockdown   and unaltered expression of RalA and Caveolin-1. **(C)** Representative confocal images of surface CTxB-Alexa-594 stained control MEFs and shArf6 MEFs at 0' SUSP; confirming comparable basal surface GM1 levels. **(D)** Graph represents quantification of surface CTxB-GM1 levels of shArf6 MEFs at 0 minutes and 120 minutes in suspension. Data plotted as mean ± SEM for 49 or more cells imaged in this experiment.
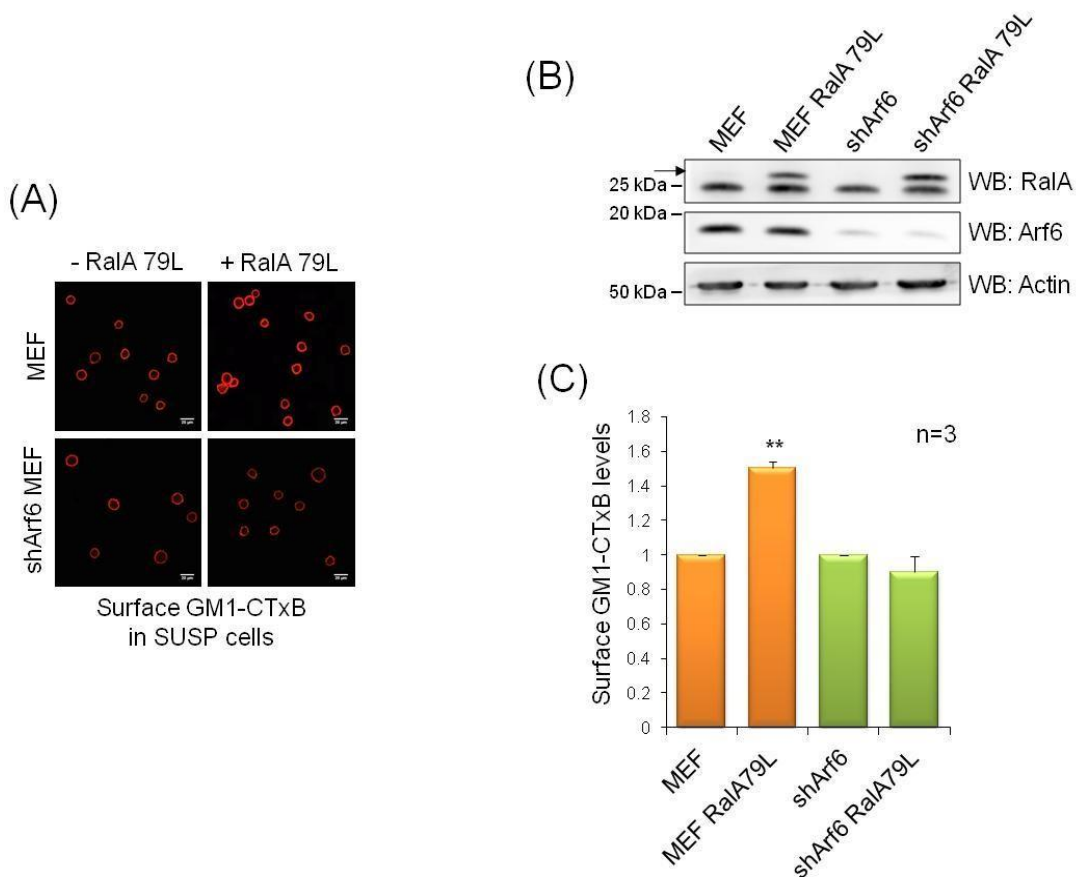
**Figure 2.5: Arf6 is essential for active RalA induced anchorage independent membrane raft trafficking. (B)** Expression of FLAG tagged fast cycling active RalA mutant (RalA 79L) (WB: RalA) (marked by arrow) in control MEFs and shArf6 MEFs. Loss of Arf6 (WB: Arf6) relative to actin (WB: Actin) was confirmed. These cells were held in suspension for 120 minutes, then surface labeled with CTxB-Alexa 594 and imaged as described in methods **(A)** Representative images and **(C)** Quantitation of surface GM1 levels of RalA79L expressing MEFs and shArf6 MEFs represented in the graph relative to their respective control. Data plotted as mean ± SEM from three independent experiments. Statistical analysis was done using the one sample two tailed T-test. (** p value<0.01).

## 2.3.4 Ral and Arf6 interact as a part of regulatory complex

With the evidence that active RalA activates Arf6 and earlier reports that both these proteins regulate adhesion dependent raft exocytic pathway, we wished to determine whether RalA and Arf6 are part of a regulatory complex that drives the exocytic pathway. We hence tested if they co-localize in re-adherent actively spreading cells. MEFs expressing constitutively active RalA-V23 mutant and fast cycling active mutant of Arf6, Arf6-T157A (Figure 2.6A), were held in suspension for 120 minutes followed by re-plating on fibronectin coated coverslips. Cells were fixed 15 minutes after replating when cells are actively spreading. These cells were immunostained with anti RalA and Arf6 antibodies that detected their **joint localization at membrane ruffles, active sites of membrane delivery** (Figure 2.6B). Since active RalB also regulates Arf6 activation, we expressed and tested the localization of both active RalA and active RalB with WT and active Arf6 (Figure 2.6C, 2.6E). We ensured expression of these constructs was comparable or less than endogenous levels. Both HA-RalA-V23 and HA-RalB-V23 co-localised to similar extent with FLAG-Arf6- T157A (Figure 2.6D) and FLAG-Arf6-WT (Figure 2.6E) in rapidly spreading cells at membrane ruffles. Under physiological conditions since integrins activate RalA (and not RalB) it's likely this association could be mediated by RalA more than RalB at these ruffles.  Interaction of Ral-Arf6 was confirmed by immunoprecipitation of FLAG-RalA/RalB-WT or FLAG RalA/RalB-V23 from cell lysates of 293T cells co-expressing HA-Arf6-T157A using FLAG antibody. Active Arf6-T157A was comparably co-immunoprecipitated with WT and active RalA/B (Figure 2.7A, 2.7B). However, only **a small fraction of the total Arf6 was seen to be pulled down suggesting its association with RalA/B could be weak or transient at best**. Interestingly, almost similar levels of active Arf6 were pulled down with both WT as well as active Ral (Figure 2.7A, 2.7B), suggesting that **Arf6 is not a direct effector of Ral.**  This does make the role Ral effectors could have in this crosstalk also of much interest.

(A)

(B)

| | RalA | Arf6 | Merged | Merged |

MEFs15'FN

(C) FLAG-Arf6-T157A

WB: RalA

WB: Arf6

(D) FLAG-Arf6-T157A

WB: RalB

WB: Arf6

(E) FLAG-Arf6-T157A

WB: RalB

WB: Arf6

(F)

CFP-RalA WT    CFP-RalA V23    HA-RalB WT    YFP-RalB V23

MEFs15'FN
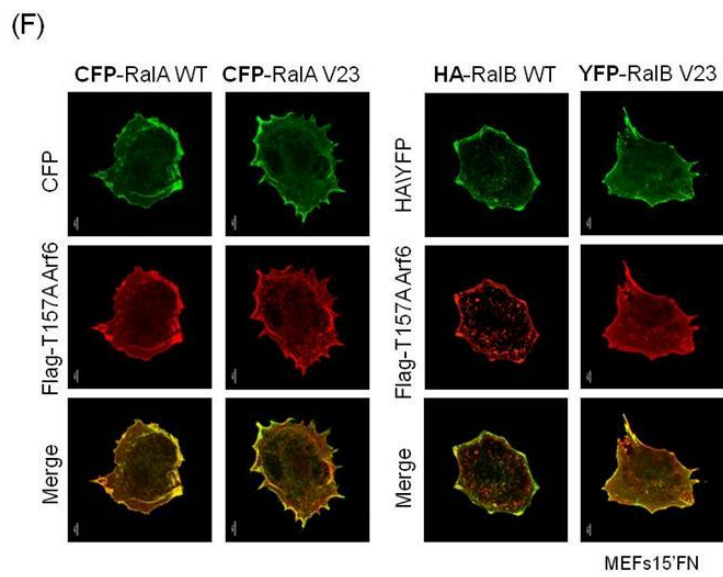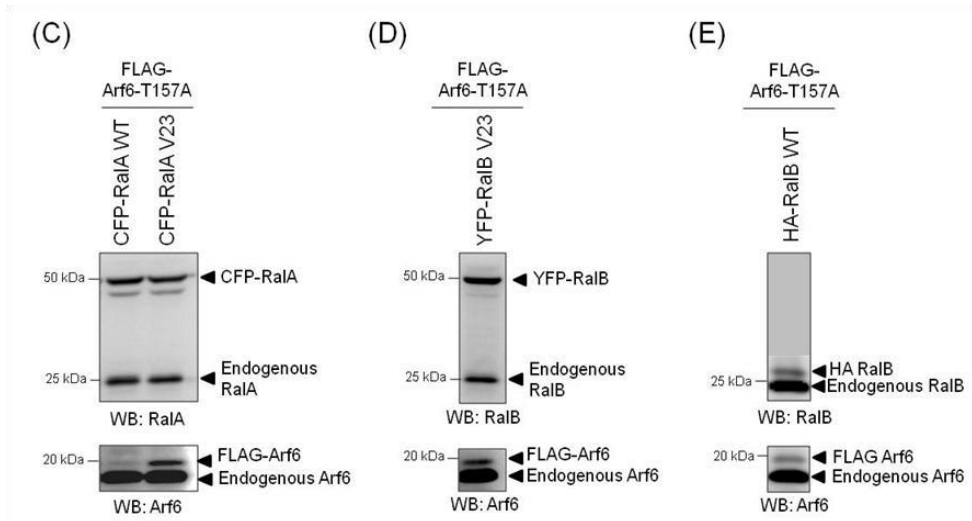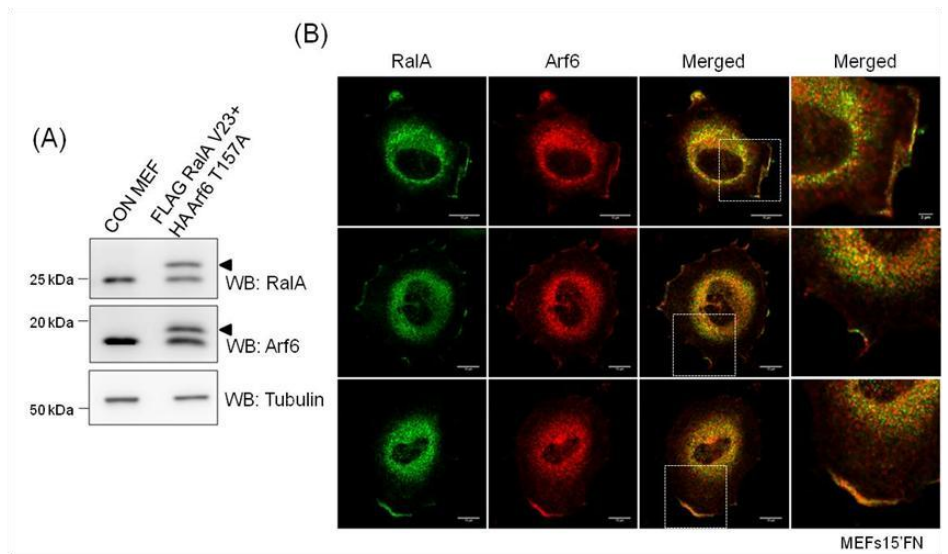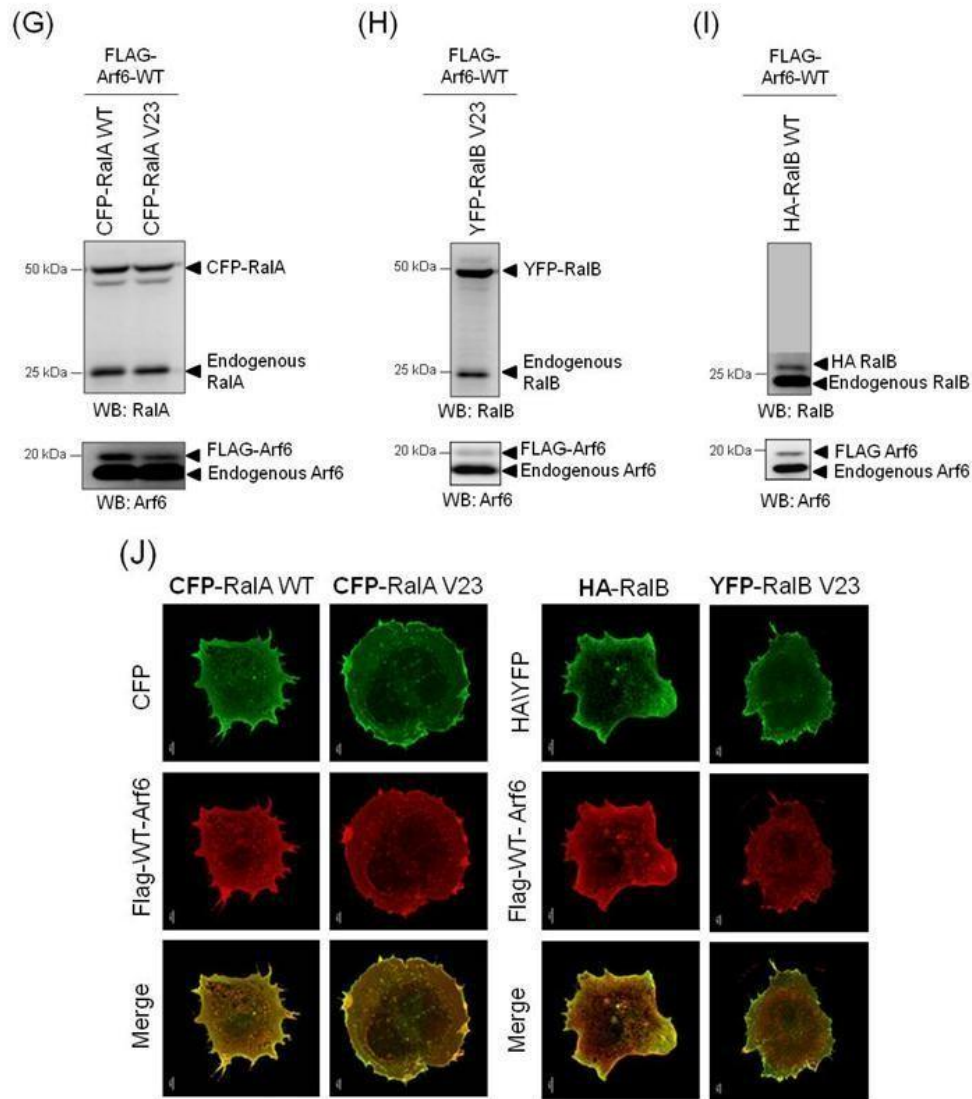
**Figure 2.6: Active RalA and Active Arf6 co-localize at membrane ruffles.**
**(A)** Representative western blots showing expression of FLAG RalA-V23 and HA-Arf6 T157A in MEFs. Tubulin serves as loading control. **(B)** Representative confocal images showing co-localization of active RalA and active Arf6 at membrane ruffles in rapidly spreading MEFs expressing FLAG RalA-V23 and HA-Arf6-T157A. Cells were fixed at FN 15' followed by immunofluorescence assay with anti-RalA (mouse) and anti-Arf6 (rabbit) antibodies. Data is representative of two independent experiments. **(C-E), (G-I)** Representative western blots showing co-expression of CFP-RalA-WT, CFP-RalA-V23, HA-RalB-WT, YFP-RalB-V23 with **(C-E)** FLAG-Arf6-T157A or **(G-I)** FLAG-Arf6-WT in MEFs. **(F), (J)** Representative confocal images showing co-localization of WT/active RalA or RalB with **(F)** T157A Arf6 or **(J)** WT Arf6 at membrane ruffles in rapidly spreading MEFs co-expressing the respective constructs. Cells were fixed at FN 15' followed by immunofluorescence assay with anti-FLAG-M2 (mouse) antibody. For cells expressing HA-RalB-WT, immunofluorescence was done with anti-FLAG-M2 (mouse) and anti-HA (rat) antibodies. Data is representative of two independent experiments.
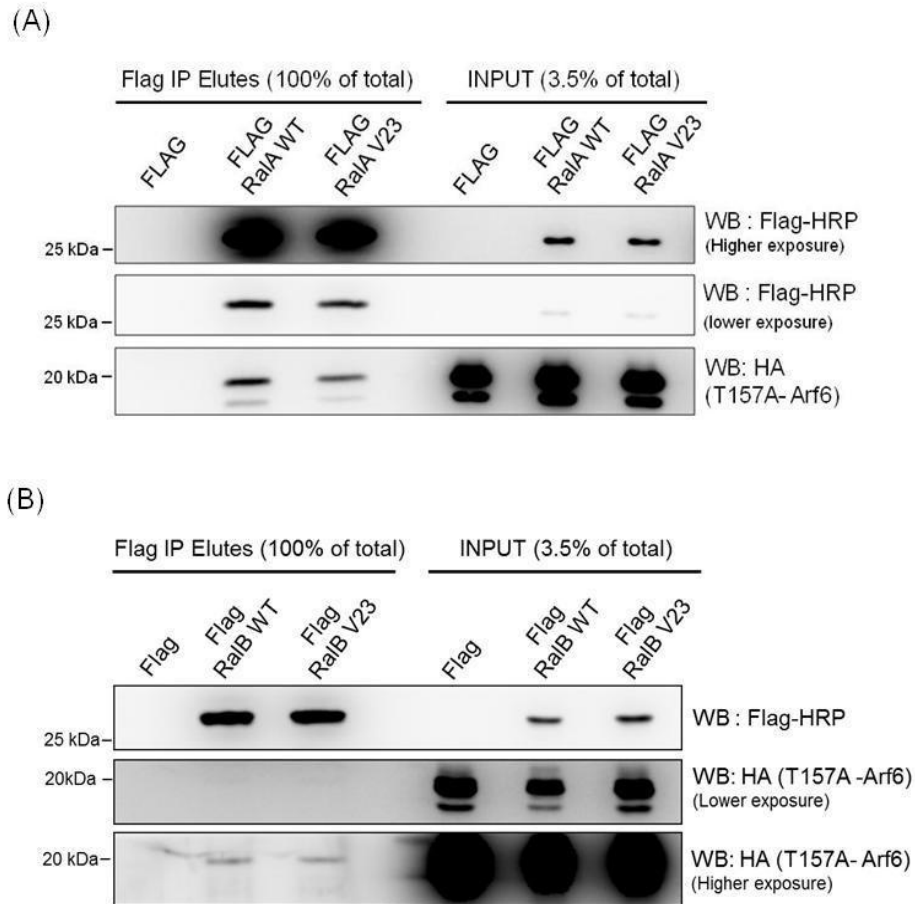
**Figure 2.7: RalA and RalB interact with active Arf6. (A)**, **(B)** Western blots probed with FLAG-HRP antibody showing immunoprecipitation of **(A)** FLAG-RalA-WT/V23 and **(B)** FLAG-RalB-WT/V23 in HEK-293T cells with empty FLAG vector as control. HA-Arf6-T157A co-expressed in these cells co-immunoprecipitates with FLAG-RalA/RalB as seen in the western blot probed with anti-HA antibody. Data is representative of at least two independent experiments.
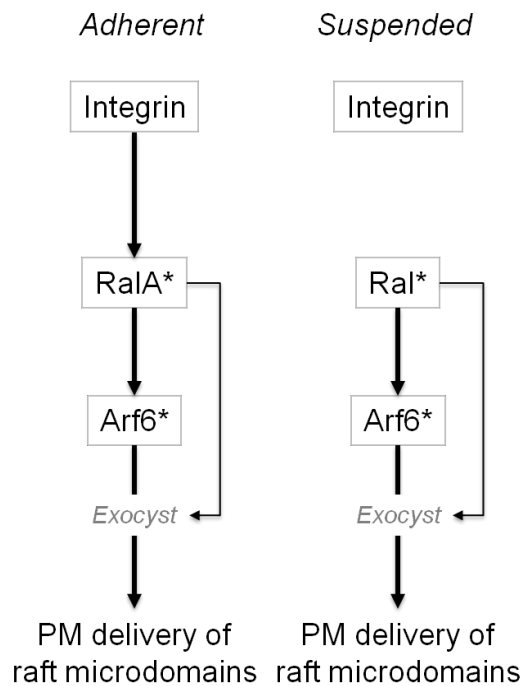
**Figure 2.8: Ral-Arf6 crosstalk and its function in MEFs.** Schematic depicts the linear Integrin-RalA-Arf6 crosstalk identified in this study. Since integrins activate RalA and not RalB, RalA (RalA*) specifically activates Arf6 in adherent cells. However, both active RalA and RalB (Ral*) are capable of inducing integrin independent Arf6 activation in suspended cells. RalA activated Arf6 along with RalA regulates exocyst function to mediate plasma membrane (PM) delivery of raft microdomains.

## 2.4 SUMMARY:

The role of integrins in mediating the activation of RalA and Arf6 in context of membrane raft exocytic recycling is already well known. The goal of this study is to further explore whether there is any regulatory crosstalk between these GTPases. We found RalA regulated adhesion dependent Arf6 activation but not vice versa (Figure 2.8). This was further confirmed by the expression of siRNA resistant RalA in RalA knockdown cells which restored Arf6 activation in re-adherent cells. This suggests the presence of a linear **integrin-RalA-Arf6 pathway** in cells. RalB did not regulate this crosstalk downstream of integrins. However, expression of constitutively active RalA or RalB mutants supported anchorage independent Arf6 activation indicating a Ral-Arf6 crosstalk that could be differentially mediated by individual or both Ral isoforms depending on the stimuli. In addition to mediating Arf6 activation, we also found the crosstalk to be essential for active RalA-exocyst driven membrane raft exocytosis and delivery which has been shown to support anchorage dependent growth signalling (Figure 2.8) (Balasubramanian et al., 2010; del Pozo et al., 2005). This crosstalk is further facilitated by the fact that active RalA and RalB both bind Arf6 in an immunoprecipitable complex and localize with active Arf6 at membrane ruffles. **Arf6 bound WT and active Ral comparably suggesting it is not a direct effector and this crosstalk is possibly dependent on additional mediators (likely Ral effectors).**

## 2.5 CONCLUSION:

**RalA regulates integrin dependent Arf6 activation to control exocyst dependent membrane raft exocytosis. Independent of integrins, RalA and RalB can both activate Arf6 likely mediated through a transient regulatory complex in cells.**

# CHAPTER 3:

# Characterization of oncogenic Ras driven Ral-Arf6 crosstalk in cancer cells

## 3.1 RATIONALE:

Plasma membrane levels of membrane raft microdomains have been shown to regulate anchorage dependent growth signalling in MEFs (Del Pozo et al., 2004). Their membrane localization is regulated by their endocytosis through caveolae (Del Pozo et al., 2005) and exocytosis through the RalA-Arf6 dependent exocyst complex (Balasubramanian et al., 2007, 2010). MEFs expressing constitutively active RalA drive the adhesion independent targeting of membrane rafts to plasma membrane to support adhesion independent activation of Akt and Erk (Balasubramanian et al., 2010). This corroborates the well-established role active RalA has in supporting anchorage independent growth across cancers such as pancreatic cancer, colorectal cancer, nerve sheath tumors, breast cancer and lung cancer (Bodempudi et al., 2009; Li et al., 2009; Lim et al., 2006; Male et al., 2012; Martin et al., 2011). Ral activation downstream of oncogenic Ras through RalGEFs (Ferro and Trabalzini, 2010; Vigil et al., 2010b) is seen to be essential for Ras mediated transformation of HEK-HT cells.

Our studies in MEFs show that constitutively active Ral can support anchorage independent Arf6 activation and this Ral-Arf6 crosstalk is necessary for membrane raft trafficking which in turn could support anchorage independent growth signalling. We hence wanted to investigate if the active Ral-Arf6 crosstalk exists downstream of oncogenic Ras in human cancer cells. In addition, our studies were also aimed to determine the role such a Ral-Arf6 crosstalk could have in supporting anchorage independent signalling and growth in these cells. Finally, we also studied the differential role RalA and RalB could have in mediating this crosstalk and how that could contribute, if at all, to their function in cancer cells.

## 3.2 MATERIALS AND METHODS:

### 3.2.1 Reagents:

Human Plasma fibronectin used in these studies was from Sigma. Cholera toxin subunit B (CTxB) labeled with Alexa-594 (C22843) was procured from Molecular Probes. Primary antibodies used in this study were anti RalA (BD Transduction), anti RalB (R&D Biosystems), anti-beta tubulin E7 (DSHB), anti-beta actin (Abcam), anti pErk1/2 (P-p44/42 MAPK- Thr202/Tyr204) (Cell Signalling Technology) and anti Erk1/2 (p44/42-MAPK) (Cell Signalling Technology). Anti Arf6 antibody was a kind gift from Dr. James Casanova (University of Virginia, Virginia). Primary antibodies were detected with the following HRP conjugated secondary antibodies: anti-mouse IgG, anti- rabbit IgG and anti-goat IgG (Jackson Immuno Research Laboratories). Protease inhibitor cocktail II (100X) was from sigma. Amplex red phospholipase D assay kit was from Invitrogen. RNA interference sequences used for knockdown studies were procured from Sigma and are indicated in appendix table 4.

### 3.2.2 Tissue Culture:

MIA-PaCa2 cells were procured from ECACC and cultured in RPMI1640 with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). T-24, HT-1080 and UM-UC-3 cells were procured from ECACC and cultured in high glucose DMEM, respectively, with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). For siRNA mediated knockdowns, cells seeded in 60 mm dishes at 30 per cent confluency were transfected first with a standardized amount of duplex siRNA oligo using the RNAiMax transfection reagent (Invitrogen) followed by a second transfection the following day with the same amount of siRNA oligo. Cells were used 48 hours after the second transfection.

### 3.2.3 Cell suspension assay:

Assays of MIA-PaCa-2 and UM-UC-3 cells were done with low serum media. Assays of HT1080 cells were done with 5% serum FBS containing medium. Assays of T-24 cells were done with both low serum and 5% serum media. Assays were performed as described in 2.2.3.

### 3.2.4 Cell surface labeling with CTxB:

As described in 2.2.5.

### 3.2.5 Ral and Arf6 activity assay:

As described in 2.2.6.

### 3.2.6 Anchorage independent Erk signalling assay:

T-24 cells seeded in 60 mm dishes were transfected with duplex siRNA oligo (hRalA/hRalB/hmArf6-1+hmArf6-2) or a combination of siRNA oligos (hRalA+hmArf6-1+hmArf6-2/ hRalB+hmArf6-1+hmArf6-2) as described above. Cells were detached with Trypsin-EDTA, washed and held in suspension for 120 minutes with 0.75% methylcellulose. Following this incubation cells were washed and lysed in Laemmli buffer and cell equivalent volumes of lysates were resolved by SDS PAGE, transferred to PVDF and blocked with 5% non-fat dry milk in TBS+0.5% Tween-20 (TBS-T). Blots were probed with anti-phospho-Erk1/2 (Thr202, Tyr204), anti-Actin, anti-RalA, anti-Arf6 and anti-Erk, anti-Actin and anti-RalB antibodies overnight at 4°C. Following the respective secondary antibody incubations blots were developed using chemiluminescent substrates from Pierce using the LAS 4000 developing system (Fujifilm-GE). Densitometric analyses of blots were done using Image J software (NIH) to calculate the pErk/total Erk ratio.

### 3.2.7 Soft agar colony assay:

T24 cells transfected with siRNA as mentioned above were trypsinized after 48 hours of second transfection and counted using hemocytometer. 5000 cells per well were mixed with warm 0.3% agar containing DMEM and layered on top of 0.5% base agar layer in wells of 6-well plate. Each of the control and siRNA treated knockdown cells was plated in duplicates. The agar was allowed to solidify and then 2 ml of DMEM containing 5% FBS and antibiotics was added slowly from the edges of the dish. The dishes were maintained for three weeks with change of the medium after every three days. Colonies formed in the agar were then stained with 0.05% crystal violet dissolved in 20% ethanol containing distilled water for one hour at room temperature followed by repeated washing with distilled water to destain the agar layers until a visible contrast between the agar and stained colonies was achieved. The colonies were then imaged on Olympus MVXC10 microscope at 0.63X zoom in HDR mode. Colonies were counted using particle analysis tool of Image J software.

### 3.2.8 Statistical analysis:

Statistical analysis of data was done using the two tailed unpaired Student's T test and when normalized to respective controls using the two tailed single sample T test. All analysis was done using Prism Graphpad analysis software. Statistical significance was considered at $P<0.05$.

## 3.3 RESULTS:

### 3.3.1 Ral-Arf6 crosstalk in Ras-driven cancer cell lines

The Ral-Arf6 crosstalk- identified in MEFs- was tested in several cancer cell lines including pancreatic cancer cell line MIA-PaCa-2 (K-RAS G12V), fibrosarcoma cell line HT-1080 (N-RAS G12V) and bladder cancer cell lines UM-UC-3 (K-RAS G12V) and T-24 (H-RAS G12V). We observed adhesion independent activation of Ral and Arf6 in all the tested cancer cell lines except in HT-1080 where RalA and RalB activation was anchorage dependent but Arf6 activation was anchorage independent (Figure 3.1A, 3.1B; Figure 3.2A; Figure 3.3A, 3.3B, 3.3C and Figure 3.4A, 3.4B). However, upon knockdown of RalA and RalB in MIA-PaCa-2 (Figure 3.1C, 3.1E) and UM-UC-3 (Figure 3.2B) adhesion independent Arf6 activation was unaffected (MIA-PaCa-2 Figure 3.1D, 3.1F; UM-UC-3 Figure 3.2C). In T-24 cell line, loss of RalA or RalB (Figure 3.4D) did however comparably affect Arf6 activation (3.4E). Earlier report by Xu et al has shown that in H-Ras-V12 transformed NIH3T3 cells, RalA and Arf6 co-immunoprecipitated better than in K-Ras-V12 expressing cells (Xu et al., 2003). **Hence the Ral-Arf6 crosstalk while observed in T-24 cells expressing oncogenic H-Ras is not seen in MIA-PaCa-2, HT-1080, and UM-UC-3 cell lines expressing oncogenic K-Ras or N-Ras**. This in part could be mediated by differential interaction between Ral andArf6 in these cancer cells as has been observed in NIH3T3 cells transformed by active H-Ras v/s active K-Ras expression (Xu et al., 2003).

The above studies looking at Ral-Arf6 crosstalk in T-24 cells were done in low serum conditions so that integrin mediated adhesion could be observed as the primary mediator of signalling. Knowing the existence of integrin-growth factor synergies (Schwartz et al., 1995) we further tested this crosstalk in the presence of 5% serum, aware that anchorage independent growth studies implicating Ral in cancers were also done with serum. In the presence of serum, Arf6 activation in suspended cells was modestly increased relative to stable adherent cells (Figure 3.4C). In the presence of serum growth factors, conditions normally used for growth and signalling studies in T-24 cells, RalB is the major regulator of Arf6 and possibly signalling downstream (Figure 3.4F, 3.4G). This is in agreement with earlier observations showing RalB is more active than RalA in T-24 cells in the presence of serum growth factors (Saito et al., 2013) and reported in our studies as well (Figure 3.5F). Consequently effector engagement and signalling in T-24 cells is expected to also be more

dependent on RalB than RalA. This differential regulation of Arf6 activation could hence contribute to Ral isoform specific function in these cells.

**Figure 3.1: RalA and RalB do not regulate adhesion independent Arf6 activation in pancreatic cancer cell line MIA-PaCa-2. (A)** Western blot (upper panel) and quantitation (lower panel) of active RalA pulled down by GST-Sec5-RBD (GST-Sec5) relative to total RalA in the respective whole cell lysate (WCL) from stable adherent (SA) and 120min suspended (SUSP) serum starved MIA-PaCa-2 cells. **(B)** Similar western blot detection and quantitation of active Arf6 pulled down by GST-GGA3 (GST-GGA3) relative to total Arf6. **(C), (E)** Western blot (upper panel) and quantitation (lower panel) of **(C)** RalA knockdown (WB: RalA) and **(E)** RalB knockdown (WB: RalB) in serum starved MIA-PaCa-2 cells relative to the Tubulin (WB: Tubulin) as loading control **(D), (F)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GST-GGA3) relative to total Arf6 in respective WCL from CON, **(D)** RalA knockdown (RalAi) or **(E)** RalB knockdown (RalBi) MIA-PaCa-2 cells suspended for 120 minutes. Percentage active Ral and Arf6 levels were calculated as described in methods and normalized to SA or CON. Graphs represent mean ± standard error data from minimum of three and maximum of four independent experiments.
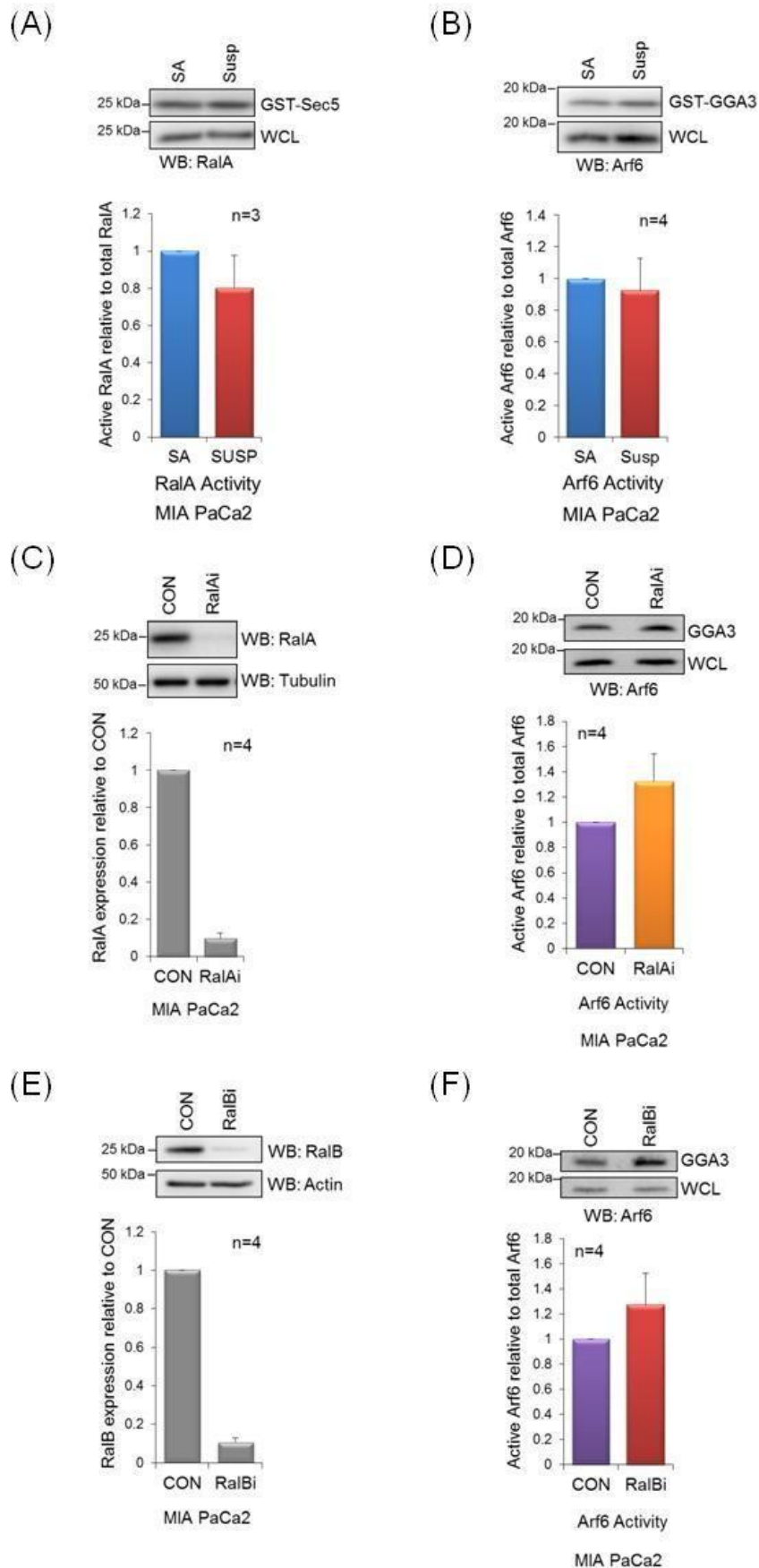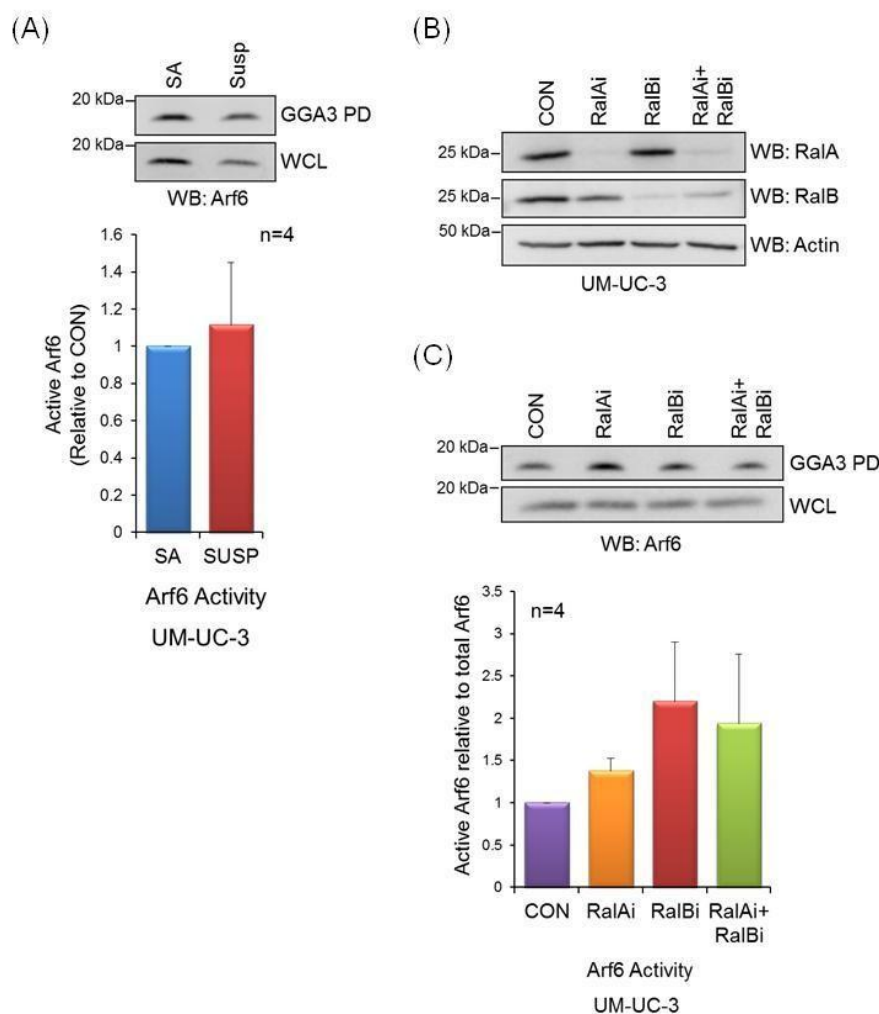
**Figure 3.2: RalA/RalB do not regulate adhesion independent Arf6 activation in bladder cancer cell line UM-UC-3. (A)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GST-GGA3) relative to total Arf6 in respective WCL from SA and SUSP conditions of serum starved UM-UC-3 cells. **(B)** Representative western blots for RalA knockdown (WB: RalA) and RalB knockdown (WB: RalB) in serum starved UM-UC-3 cells relative to the Actin (WB: Actin) as loading control **(C)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GST-GGA3) relative to total Arf6 in respective WCL from control (CON), RalA knockdown (RalAi), RalB knockdown (RalBi) and RalA+RalB knockdown (RalAi+RalBi) UM-UC-3 cells that were serum starved and suspended for 120 minutes. Percentage active Arf6 levels were calculated as described in methods and normalized to SA or CON. Graphs represent mean ± standard error data from four independent experiments.
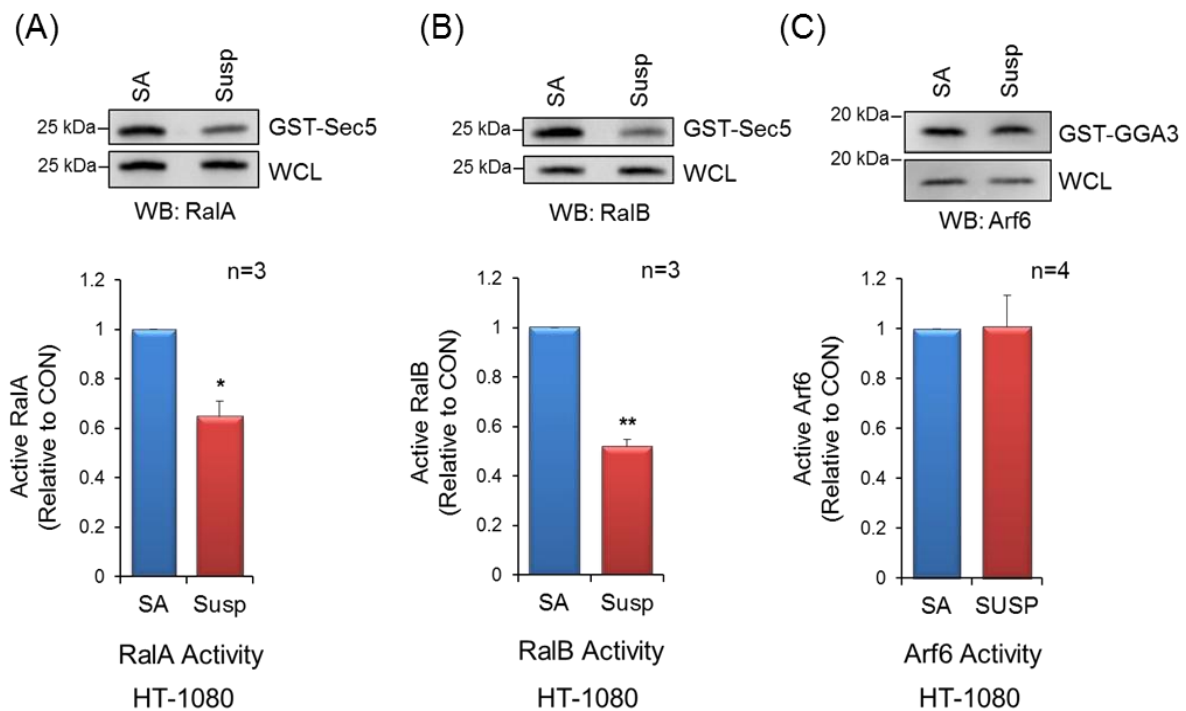
**Figure 3.3: Ral activation is anchorage dependent whereas Arf6 activation is adhesion independent in fibrosarcoma cell line HT-1080.** **(A), (B), (C)** Western blot (upper panel) and quantitation (lower panel) of **(A)** active RalA **(B)** active RalB pulled down by GST-Sec5-RBD (GST-Sec5) and **(C)** active Arf6 pulled down by GST-GGA3 (GST-GGA3) relative to total protein in respective WCL from SA and SUSP conditions of HT-1080 cells. Ral GTPase activation is anchorage dependent whereas Arf6 activity is anchorage independent.

(A)

25 kDa— GST-Sec5
25 kDa— WCL
WB: RalA

(B)

20 kDa— GST-GGA3
20 kDa— WCL
WB: Arf6

(C)

20 kDa— GST-GGA3
20 kDa— WCL
WB: Arf6

T-24- without serum

T-24- With serum

(D)

25 kDa— WB: RalA
25 kDa— WB: RalB
50 kDa— WB: Tubulin

T-24

T-24

(E)

20 kDa— GGA3 PD
20 kDa— WCL
WB: Arf6

Arf6 Activity

T-24- without serum
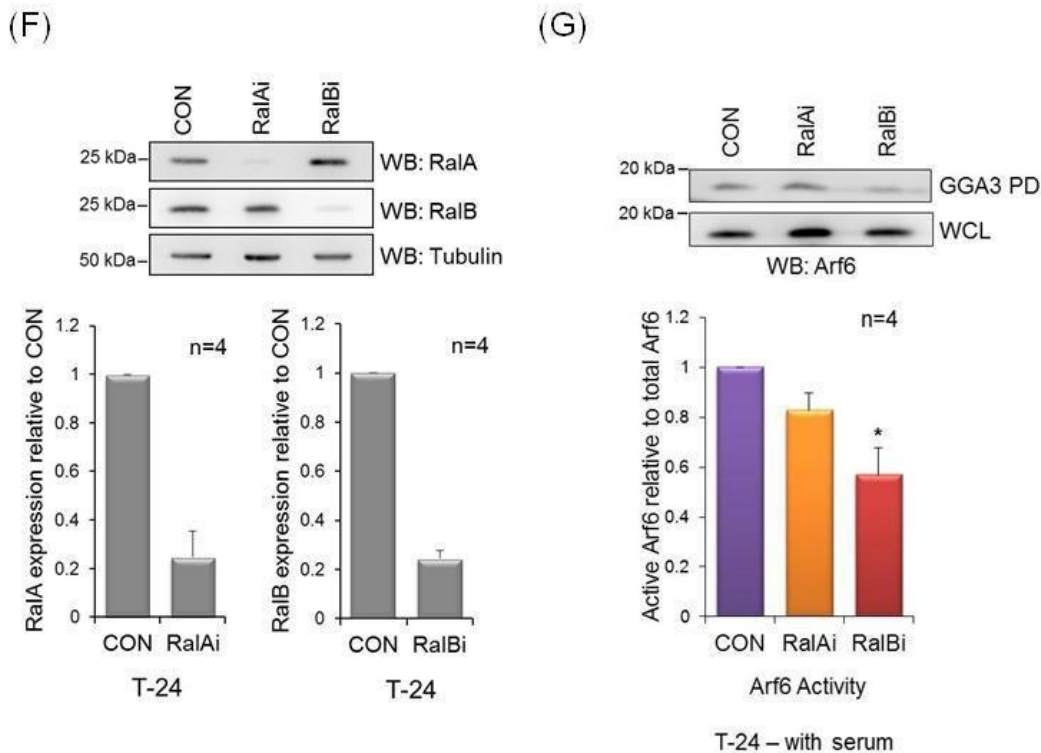
**Figure 3.4: RalA and RalB regulate adhesion independent Arf6 activation in bladder cancer cell line T-24. (A), (B)** Western blot (upper panel) and quantitation (lower panel) of **(A)** active RalA pulled down by GST-Sec5-RBD (GST-Sec5) and **(B)** active Arf6 pulled down by GST-GGA3 (GST-GGA3) relative to total protein in respective WCL from SA and SUSP conditions of serum starved T-24 cells (low serum). **(C)** Adhesion independent Arf6 activation similarly tested in T-24 cells in the presence of 5% serum (with serum). **(D), (F)** Western blots (upper panel) and quantitation (lower panel) for RalA knockdown (WB: RalA) and RalB knockdown (WB: RalB) in **(D)** serum starved T-24 cells or **(F)** with serum T-24 cells relative to the Tubulin (WB: Tubulin) as loading control. **(E), (G)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GST-GGA3) relative to total Arf6 in respective WCL from control (CON), RalA knockdown (RalAi) and RalB knockdown (RalBi) T-24 cells suspended for 120 minutes either **(E)** with low serum and **(G)** with 5% serum. Percentage active RalA and Arf6 levels were calculated as described in methods and normalized to SA or CON. Graphs represent mean ± standard error data from m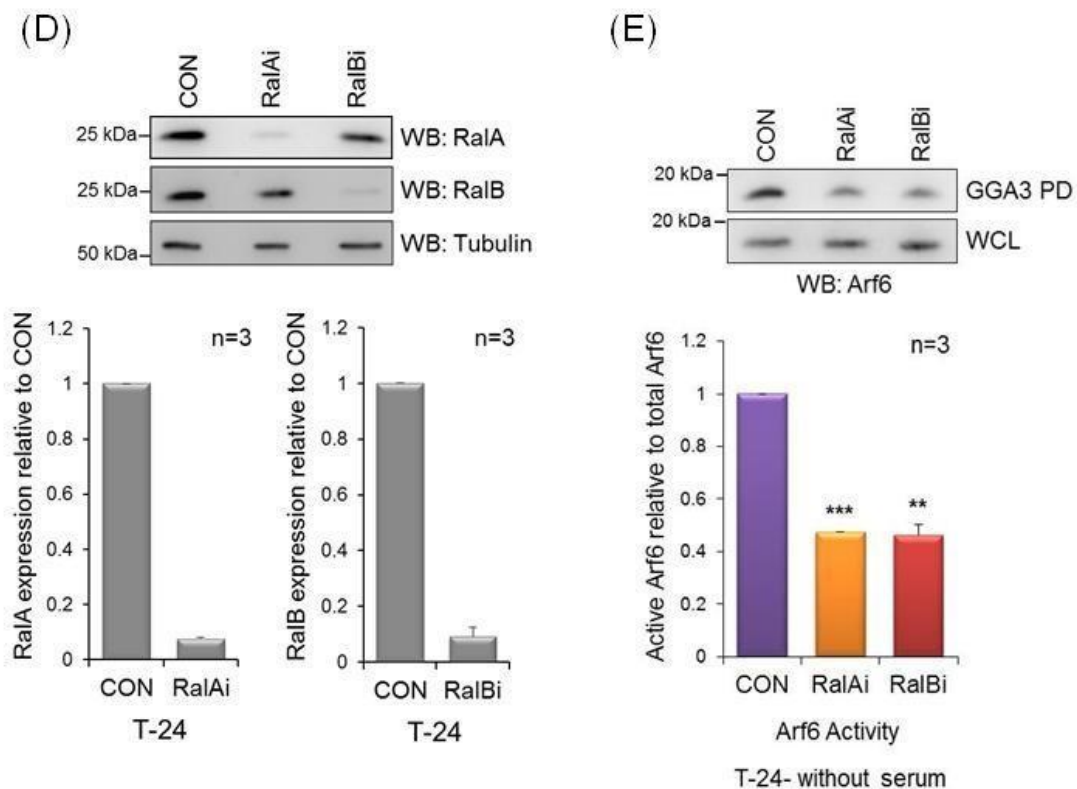inimum of three and maximum of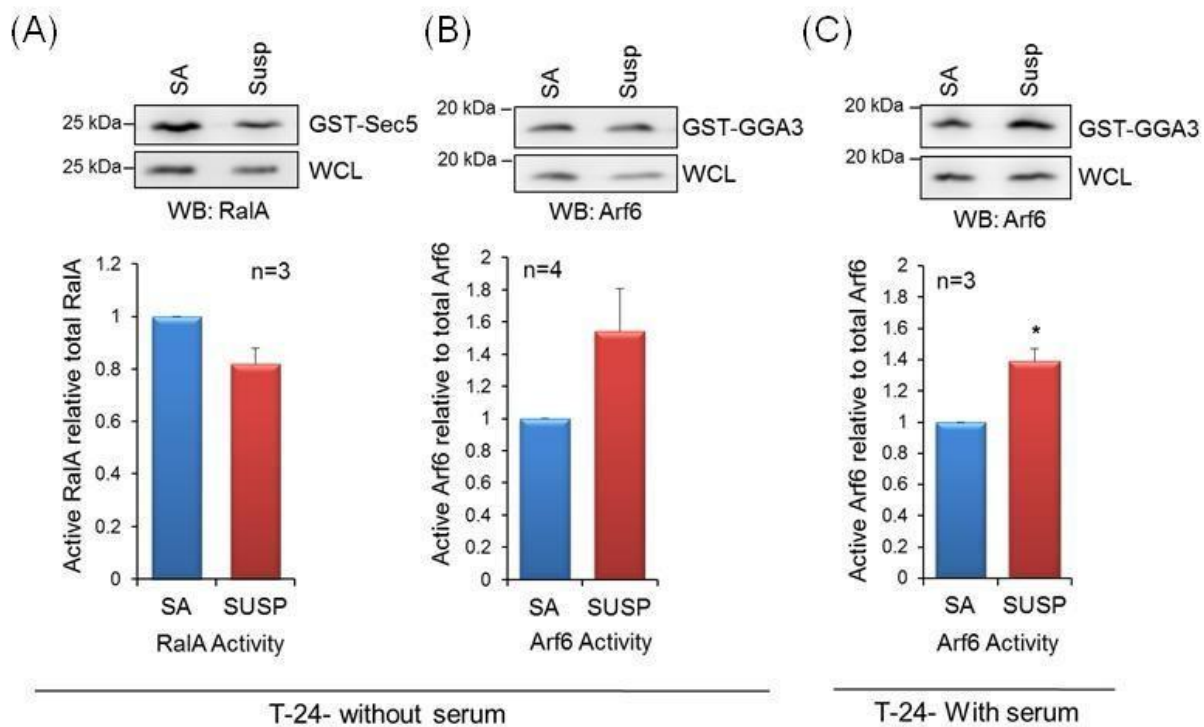 four independent experiments. Statistical analysis of all the above data was done using the one sample two tailed T-test and significance represented (* p value <0.05 and ** p value <0.01, *** p value <0.001).

**3.3.2 Role of Ral-Arf6 crosstalk in anchorage independent signalling and growth of T-24 cells.**

Ral GTPases are known to be vital for anchorage independent growth of bladder cancer cells. Our data so far demonstrates that Ral GTPases regulate anchorage independent activation of Arf6 in the T-24 cell line. Hence we evaluated the contribution Ral activated Arf6 makes to anchorage independent growth signalling in T-24 cells. We compared the activation status of Erk by comparing its phosphorylation on the Thr 202 and Tyr 204 residues of Erk1/2 in non-adherent control vs RalA KD vs RalB KD vs Arf6 KD vs RalA+Arf6 KD vs RalB+Arf6 KD cells in the presence of serum growth factors. The efficiency of knockdown of each of these GTPases in their combined knockdown was verified to be equivalent to their individual knockdown (Figure 3.5A, 3.5B, 3.5C). Erk activation in non-adherent T-24 cells was marginally affected by loss of RalA (Figure 3.5D). Its combined knockdown with Arf6 however significantly reduced Erk activation in T-24 cells suggesting the marginal decrease in Erk activation by RalA KD alone could be result of its modest effect on Arf6 activity in non-adherent T-24 cells. This was substantiated by the distinct reduction in Erk activation seen upon loss of RalB that significantly affected Arf6 distinctly, comparable to the joint RalB and Arf6 knockdown (Figure 3.5D, 3.5F). In bladder cancers RalB is known to be more active than RalA in some studies (Saito et al., 2013) with a reported role in migration, proliferation and anchorage independence as well (Oxford et al., 2005; Smith et al., 2012; Wang et al., 2010). In non-adherent T-24 cells, we tested and found RalB activity to indeed be significantly higher than RalA (Figure 3.5F). This could hence mediate their differential utilization of Ral effectors and downstream Arf6 activation and signalling. Notably, Erk activation was significantly reduced by the depletion of Arf6, reflecting its role at the end of the linear H-Ras-Ral-Arf6 pathway. This could be unique to T-24 cells or could be conserved across cancers, which our ongoing studies are exploring. Finally we explored the contribution Arf6 makes to Ral dependent anchorage independent growth of T-24 cells in soft agar colony assays. We observed depletion of Arf6 affected the number of cells recovered at the end of the knockdown experiment, suggesting a likely effect on cell survival and/or proliferation in T-24 cells. Interestingly our attempts to stably knockdown Arf6 in T-24 cells using a shRNA construct also resulted in extensive loss of cells preventing the selection of stable clones. The exact cause of this effect Arf6 has on T-24 is something we are evaluating. Transient siRNA mediated Arf6 knockdown also significantly affected growth of T-24 cells in soft agar assays as did loss of RalB and RalA (Figure 3.6A, 3.6B). Since Erk activation most prominently

affected by loss of Arf6, one speculation could be that Erk pathway could be a major mediator of the anchorage independent growth of T-24 bladder cancer cells as has been seen in K-Ras expressing colorectal cancer cells (Ebi et al., 2011).

In summary our data provides evidence for existence of RalA, RalB and Arf6 dependent pathway(s) that govern anchorage independent signalling in the bladder cancer cell line T-24. The contribution of Ral-Arf6 crosstalk is seen in its regulation of anchorage independent Erk activation. In addition to confirming a role for Ral GTPases, we also detected a role for Arf6 in regulating Erk signalling possibly regulating their anchorage independent growth.

The next question we asked was how the Ral-Arf6 crosstalk in T-24 cells could be regulating anchorage independent Erk signalling. In MEFs, active RalA regulates anchorage independent membrane raft trafficking which in turn regulates growth signalling (Balasubramanian et al., 2010). Our studies have now shown an essential role for the Ral-Arf6 crosstalk in the raft trafficking pathway in MEFs and in anchorage independent Erk signalling in T-24 cells. This led us to test the role Ral and Arf6 GTPases could have in membrane raft trafficking in T-24 cells. We first confirmed presence of Caveolin-1 and its phosphorylation at tyrosine 14 in T-24 cells, since these are critical for endocytosis of membrane rafts upon loss of cell adhesion (Del Pozo et al., 2004; del Pozo et al., 2005), ensuring that endocytosis of rafts would not be defective in T-24 cells (Figure 3.7A). Indeed we found that GM1 labeled membrane raft microdomains were endocytosed upon loss of adhesion in T-24 cells (Figure 3.7B) as has been observed in MEFs (Del Pozo et al., 2004; del Pozo et al., 2005). Knowing this we tested and found depletion of RalA, RalB or Arf6 GTPases (Figure 3.7C, 3.7D) did not significantly affect the surface GM1 levels in non-adherent T24 cells, detected by Alexa-CTxB-594 labeling (Figure 3.7E). GM1 is one of the markers localized to raft microdomains whose levels and its trafficking can be regulated in a cell type specific manner. Further studies to look at other membrane raft markers (GPI-linked proteins, cholesterol levels) will help ascertain the true regulation of raft microdomains in T-24 cells and the role Ral and Arf6 could have in mediating their trafficking.

(A)

CNT RalAi RalBi Arf6i RalAi+Arf6i RalBi+Arf6i

25 kDa — WB: RalA
50 kDa — WB:Actin

n=5

RalA expression relative to control

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

CNT RalAi RalBi Arf6i RalAi+Arf6i RalBi+Arf6i

(B)

CNT RalAi RalBi Arf6i RalAi+Arf6i RalBi+Arf6i

25 kDa — WB: RalB
50 kDa — WB:Actin

n=5

RalB expression relative to control

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

CNT RalAi RalBi Arf6i RalAi+Arf6i RalBi+Arf6i

(C)

CNT RalAi RalBi Arf6i RalAi+Arf6i RalB+Arf6i

20 kDa — WB:Arf6
50 kDa — WB:Actin

n=5

Arf6 expression relative to control

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

CNT RalAi RalBi Arf6i RalAi+Arf6i RalB+Arf6i

(D)

CON RalAi RalBi Arf6i RalAi+Arf6i RalBi+Arf6i

50 kDa —
37 kDa — WB: pErk
50 kDa —
37 kDa — WB: Erk

n=4

Ratio of pErk to total Erk

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

CON RalAi RalBi* Arf6i** RalAi +Arf6i** RalBi +Arf6i**

(E)

T-24 cells in suspension

25 kDa — Sec5-RBD
25 kDa — WCL
WB: RalA WB: RalB

n=4

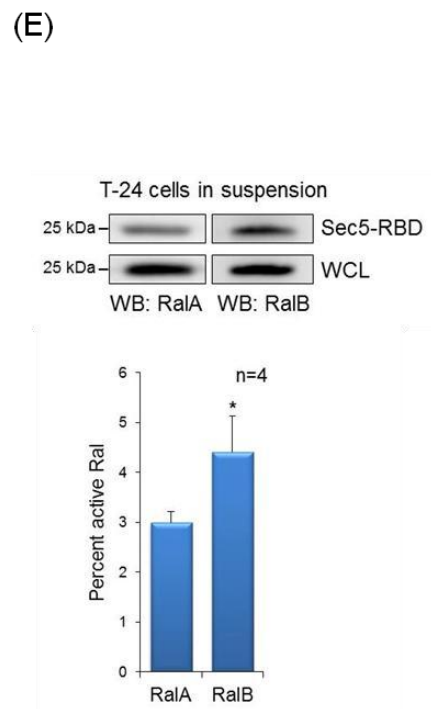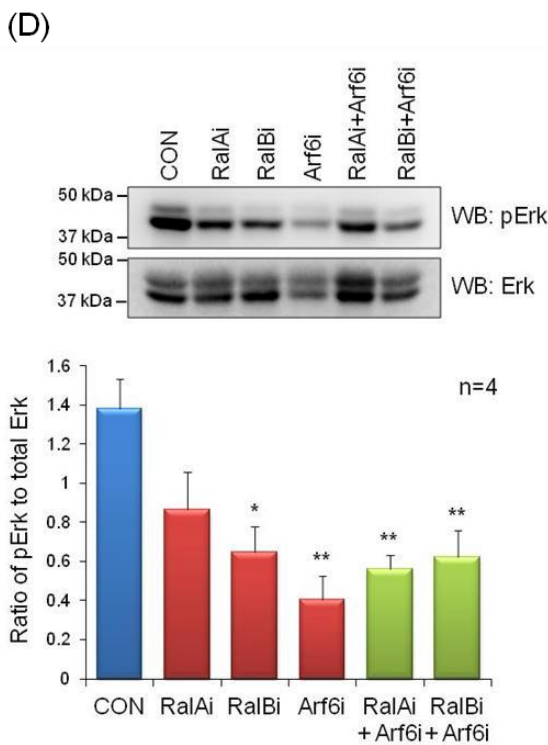Percent active Ral

6
5
4
3
2
1
0

RalA RalB*

**Figure 3.5: Ral-Arf6 crosstalk regulates anchorage independent Akt and Erk signalling in T24 cells. (A)**, **(B)**, **(C)** Representative western blots (upper panel) and quantitation of **(A)** RalA, **(B)** RalB and **(C)** Arf6 expression levels in control (CNT), RalA knockdown (RalAi), RalB knockdown (RalBi), Arf6 knockdown (Arf6i), combined RalA and Arf6 knockdown (RalAi+Arf6i) and combined RalB and Arf6 knockdown (RalBi+Arf6i) T-24 cells. Expression of RalA (WB: RalA), RalB (WB: RalB) and Arf6 (WB: Arf6) relative to actin levels (WB: Actin) as loading control plotted normalized to CNT. Graphs represent mean ± standard error data from five independent experiments. **(D)** Western blot (upper panel) and quantitation (lower panel) of Erk1 phosphorylation at Thr202/Tyr204 residues (WB: pErk) relative to total Erk1 (WB: Erk) in whole cell lysates from CON, RalAi, RalBi, Arf6i, RalAi+Arf6i and RalBi+Arf6i T-24 cells that were held in suspension for 120 minutes with serum. For (D) and (F), graphs represent mean ± standard error of the band intensity ratio of pAkt/total Akt or pErk/total Erk from five independent experiments. **(E)** Percentage active RalA and RalB levels in T-24 cells suspended with serum. Graphs represent mean ± standard error from 4 independent experiments, analyzed using the unpaired Student's T-test (* p-value <0.05).
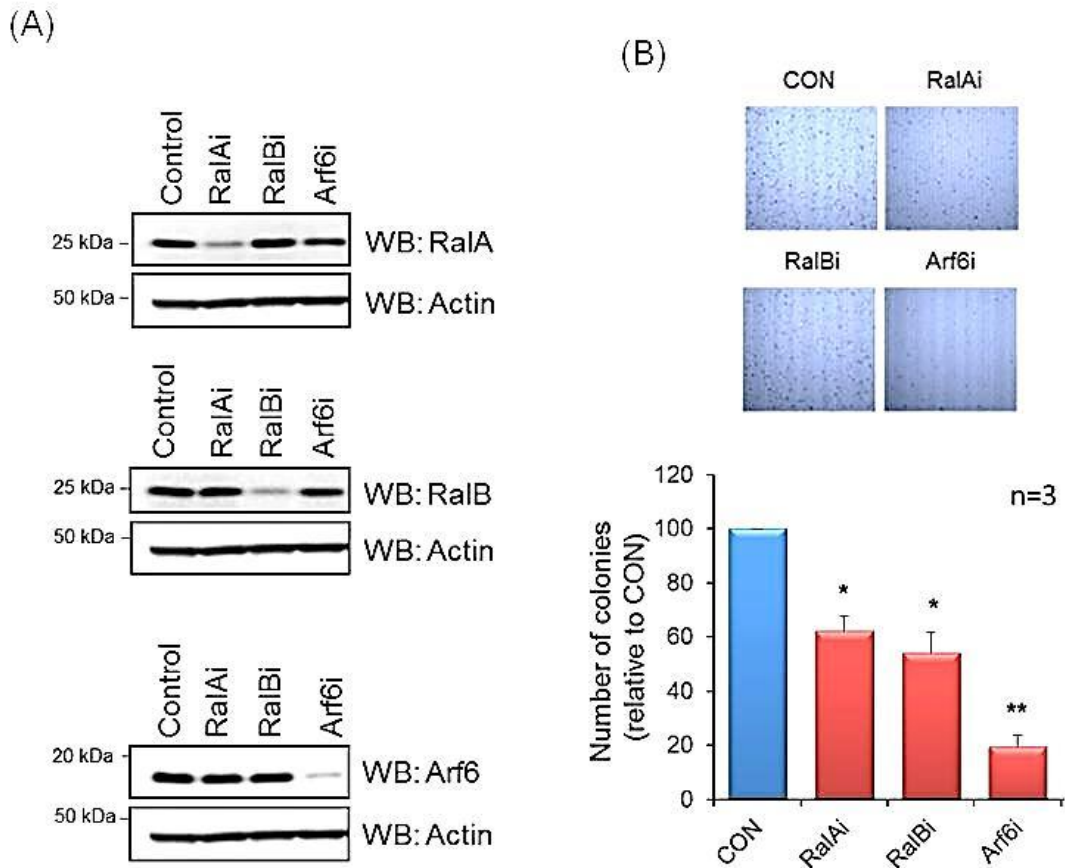
**Figure 3.6: Ral-Arf6 crosstalk regulates anchorage independent growth in T-24 cells.**
**(A)** Representative western blots of RalA (WB: RalA), RalB (WB: RalB) and Arf6 (WB: Arf6) levels in control (CON), RalA knockdown (RalAi), RalB knockdown (RalBi) and Arf6 knockdown (Arf6i) T-24 cells. Actin levels (WB: Actin) serves as loading control. Data is representative of at least two independent experiments. **(B)** Representative phase contrast images (upper panel) and quantitation (lower panel) of number of colonies of CON, RalAi, RalBi and Arf6i T-24 cells subjected to soft agar colony assay. Colonies were fixed, stained and imaged after three weeks of growth in 0.3% agarose as described in methods. Number of colonies counted using Image J software is plotted as relative to CON. Graph represents mean ± standard error of four replicates from two independent experiments. Statistical analysis was done using the One sample two tailed T-test (* p-value <0.05, ** p-value <0.01).
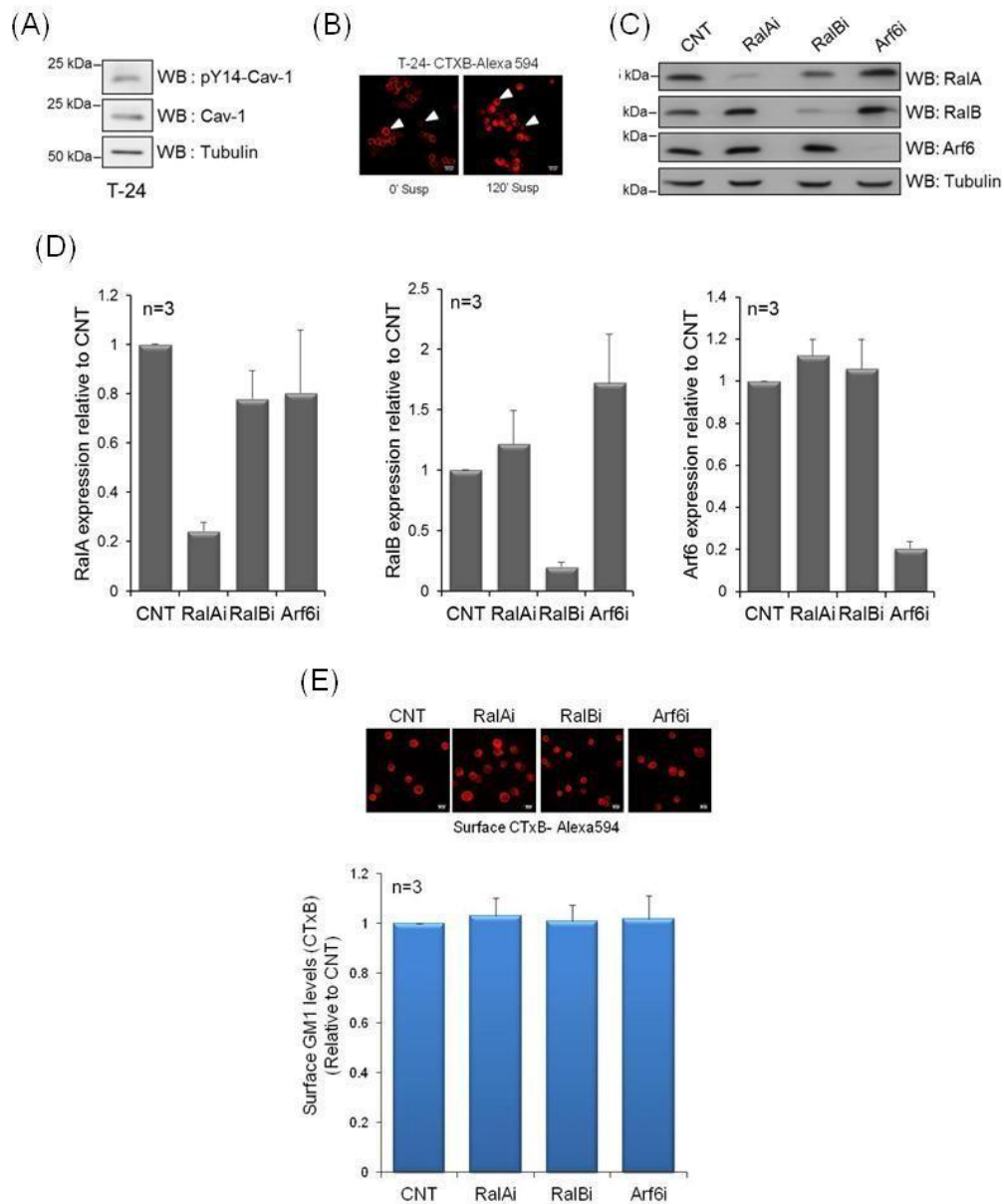
**Figure 3.7: RalA, RalB and Arf6 do not regulate anchorage independent membrane raft microdomain exocytosis in T-24 cells.** **(A)** Western blots for detection of Tyr-14 phosphorylation of Caveolin-1 (WB: pY14-Cav1) relative to total Caveolin-1 (WB: Cav1) and Tubulin (WB: Tubulin). **(B)** Representative confocal images of CTxB-Alexa-594 signal from T-24 cells suspended for 0 minutes (0' SUSP) and 120 minutes (120' SUSP) showing internalized GM1 domains at 120' SUSP. Data indicates presence of functional caveolar endocytic pathway for adhesion dependent endocytosis of membrane raft microdomains in T-24 cells. Arrowheads in (B) indicate variability in GM1 levels in T-24 cell population. **(C)** Western blot and **(D)** quantitation for RalA (WB: RalA), RalB (WB: RalB) and Arf6 (WB: Arf6) levels in control (CNT), RalA knockdown (RalAi), RalB knockdown (RalBi) and Arf6 knockdown (Arf6i) T-24 cells relative to Tubulin (WB: Tubulin) as loading control. **(E)** Representative confocal images (upper panel) and quantitation (lower panel) of surface CTxB-Alexa-594 signal from CNT, RalAi, RalBi and Arf6i T-24 cells suspended for 120 minutes. Surface CTxB signal quantified as described in methods and graph plotted as normalized to CNT. Graphs represent mean ± standard error data from three independent experiments.

**Non-adherent T-24**

H-Ras*

↓

RalB*

↓

Arf6*

↓

Anchorage independent
Erk Activation

**Figure 3.8: Ral-Arf6 crosstalk and its function in cancer cells.** In oncogenic H-RasV12 (H-Ras*) expressing bladder cancer T-24 cell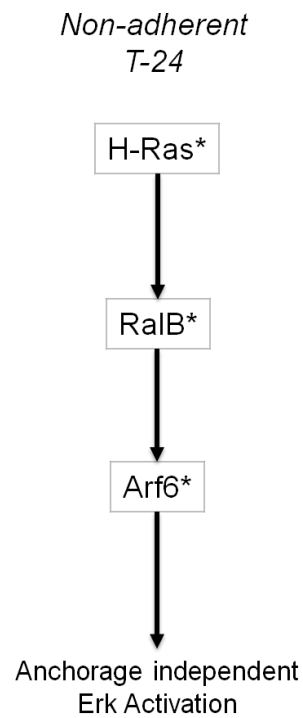 line Arf6 activation was found to be anchorage independent (Arf6*) and regulated by active RalB (RalB*). This RalB-Arf6 crosstalk regulates anchorage independent Erk signalling in these cells.

## 3.4 SUMMARY:

Knowing the presence of the integrin-Ral-Arf6 pathway in mouse embryonic fibroblasts and the role Ral has downstream of oncogenic Ras in several cancers, we asked if this crosstalk is present in cancer cells and whether it is functionally important for Ral mediated anchorage independent signalling and growth. We observed anchorage independent Arf6 activation across active Ral expressing MIA-PaCa-2 (K-Ras*), UM-UC-3 (K-Ras*), HT-1080 (N-Ras*) and T-24 (H-Ras*) cancer cells. In T-24 bladder cancer cells, both RalA and RalB comparably regulate anchorage independent Arf6 activation in low serum conditions. However in these cells, serum growth factors preferentially activate RalB (more than RalA) supporting its regulation of Arf6 activation in the presence of serum. This is further translated into a prominent role for RalB in regulating anchorage independent Erk signalling, that we find is dependent on RalB-Arf6 crosstalk in T-24 cells. Erk signalling could contribute to Ral-Arf6 dependent anchorage independent growth in T-24 cells. Ral independent activation of Arf6 in MIA-PaCa2, UM-UC-3 and HT1080 cells indicate the presence of alternate mechanisms of Arf6 activation downstream of oncogenic Ras (Figure 3.8). The role Ral and Arf6 have in these cancers versus those where their regulatory crosstalk is observed remains to be explored.


## 3.5 CONCLUSION:

**The Ral-Arf6 crosstalk was detected downstream of active Ras in bladder cancer cell line T-24 (expressing H-Ras) and found to regulate anchorage independent Akt signalling in these cells. RalB being more active than RalA in these cells differentially regulated Arf6 activation and hence Erk signalling; indicating possible role of Arf6 in mediating isoform specific Ral function in cellular pathways.**

# CHAPTER 4:

# Role of Ral effectors in mediating

# Ral-Arf6 crosstalk

**4.1 RATIONALE:**

Several known Ral effector proteins are implicated in regulating Ral driven pathways in cells. These include the exocyst complex, RalBP1, PhospholipaseD1, Filamin-A, PKC-η and ZONAB that bind RalA and RalB in a stimulus, cell type dependent and spatiotemporal manner (Cantor et al., 1995; Frankel et al., 2005; Han et al., 2009b; Jiang et al., 1995; Kashatus et al., 2011; Ljubicic et al., 2009; Moskalenko et al., 2002; Ohta et al., 1999; Shirai et al., 2011). Arf6 does not show a differential binding to active Ral in our immunoprecipitation studies, suggesting the Ral-Arf6 association to not be an effector interaction. The fact that active Ral can regulate Arf6 activation leads us to speculate that Ral effector protein(s) could hence mediate this Ral-Arf6 crosstalk in cells.

RalA mediated regulation of the exocyst complex drives integrin dependent membrane raft exocytosis and signalling (Balasubramanian et al., 2010). RalA interacts with exocyst components Sec5 and Exo84 to control exocyst function (Moskalenko et al., 2002, 2003). Sec5 in earlier studies is seen to regulate Ral-exocyst dependent membrane raft trafficking, that Arf6 also regulates, making it an attractive candidate mediator of the Ral-Arf6 crosstalk (Balasubramanian et al., 2007, 2010). Since RalA interacts with EXO84 (Jin et al., 2005; Moskalenko et al., 2003) and Arf6 binds exocyst component Sec10 in a GTP dependent manner (Prigent et al., 2003b), we also tested their role in mediating this crosstalk.

Another major Ral effector RalBP1 (RLIP76) is known to be involved in Ral dependent mitochondrial fission and invadopodia formation (Kashatus et al., 2011; Neel et al., 2012a). RalBP1 also has a Rho GTPase GAP domain and R-Ras association domain in addition to the Ral binding domain (Goldfinger et al., 2006; Jullien-Flores et al., 1995). It hence is a key R-Ras effector mediating its role in cell spreading (Goldfinger et al., 2006). Interestingly, RalBP1 interacts with Arf6 GEF ARNO (Cytohesin-2) to regulate Arf6 activation downstream of R-Ras (Lee et al., 2014). RalBP1 is interestingly over-expressed and implicated in bladder and colorectal cancers (Mollberg et al., 2012; Smith et al., 2007; Wang et al., 2010), leading us to evaluate its role in the Ral-Arf6 crosstalk in normal and cancer cells.

Like the exocyst complex, Phospholipase D1 (PLD1) also binds both RalA and Arf6 (Luo et al., 1998). A membrane anchored protein, PLD1 catalyses hydrolysis of phosphatidylcholine into phosphatidic acid (PA) which gets further converted to diacylglycerol (DAG)- an

important secondary messenger in cells (Selvy et al., 2011). Ral GTPases directly bind PLD1 in GTP independent manner and (Jiang et al., 1995; Luo et al., 1998) while Arf6 binds it in GTP-dependent manner (Hiroyama and Exton, 2005a). This RalA-Arf6 association with PLD1 actively and synergistically regulates its activity in H-Ras transformed cells (Xu et al., 2003). They also work with PLD to mediate exocytosis of dense core granules (Vitale et al., 2005). Though PLD appears to be downstream of Ral and Arf6, a feedback loop of involving PA driven PIP-5-kinase, its product PI(4,5)-P2, and Arf6GAPs could also regulate Arf6 in turn (Oude Weernink et al., 2007b). We hence evaluated the role PLD1 could have in mediating Ral and Arf6 crosstalk in cells.

## 4.2 MATERIALS AND METHODS:

### 4.2.1 Reagents:

Human Plasma fibronectin used in these studies was from Sigma. Primary antibodies used in this study were anti RalA (BD Transduction), anti RalB (R&D Biosystems), anti-beta tubulin E7 (DSHB), anti-beta actin (Abcam), Anti-Sec5 (N15) (Santa Cruz Biotechnology), Anti-RalBP1(I33), Anti-phospho PLD1 (Thr147), anti-PLD1, anti pErk1/2 (P-p44/42 MAPK-Thr202/Tyr204) and anti Erk1/2 (p44/42-MAPK) (Cell Signalling Technology). Anti Arf6 antibody was a kind gift from Dr. James Casanova (University of Virginia, Virginia). Primary antibodies were detected with the following HRP conjugated secondary antibodies: anti-mouse IgG, anti- rabbit IgG and anti-goat IgG (Jackson Immuno Research Laboratories). Protease inhibitor cocktail II (100X) was from sigma. siRNA sequences for knockdown of Sec5, Sec10, Exo84, ARNO (CYTH2), PLD1 and PLD2 were On-Target Plus SMARTpool from Dharmacon and are listed in appendix table 5. RNA interference sequences used for knockdown studies of RalA, RalBP1, CYTH1, CYTH3, EFA6B, BRAG1 and BRAG2 were procured from Sigma and are listed in appendix table 4.

### 4.2.2 Tissue Culture:

Mouse embryonic fibroblasts (from the lab of Dr. Richard Anderson, University of Texas Health Sciences Center, Dallas TX) were cultured in high glucose DMEM medium with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). MIA PaCa2 cells were procured from ECACC and cultured in RPMI1640 with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). T-24 cells were procured from ECACC and cultured in high glucose DMEM with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). For siRNA mediated knockdowns, cells seeded in 60 mm dishes at 30 per cent confluency were transfected first with a standardized amount of duplex siRNA oligo using the RNAiMax transfection reagent (Invitrogen) followed by a second transfection the following day with the same amount of siRNA oligo. Cells were used 48 hours after the second transfection. For reconstitutions, cells were transfected first with siRNA followed by transfection with plasmid DNA transfection after 24 hours and cells were used post 48 hours after plasmid transfection.

**4.2.3 Cell suspension assay:**

Assays of MEFs were done with low serum media. Assays of T-24 cells were done with both low serum and 5% serum media. Assays were performed as described in 2.2.3.

**4.2.4 Plasmids and site directed mutagenesis:**

Untagged G23V-RalA was a kind gift from Dr. Michael White's lab. FLAG-RalBP1 was a kind gift from Dr. Lawrence Goldfinger's lab. hRalBP1* (siRNA insensitive mutant) was developed by site-directed mutagenesis using FLAG-RalBP1 as template. Primers were designed using QuickChange primer design tool from Agilent Technologies and are summarized in Appendix Table 1.

**4.2.5 Arf6 and RalA activity assay:**

As described in 2.2.6.

**4.2.6 Anchorage independent Erk signalling assays:**

T24 cells seeded in 60 mm dishes were transfected with duplex siRNA oligo (hRalBP1/hmArf6-1+hmArf6-2) or a combination of siRNA oligos (hRalBP1+hmArf6-1+hmArf6-2) as described above. Assays were performed as described in 3.2.7.

**4.2.7 Soft agar colony assays:**

As described in 3.2.7.

**4.2.8 Quantitative PCR:**

Total RNA was isolated from samples using Trizol reagent (Invitrogen) followed by cDNA preparation using Reverse Transcriptase from Promega. Quantitative PCR reactions were set

up using SYBR FAST qPCR master mix reagent from Kapa Biosystems. Primers used for measuring transcript levels in qPCR are summarized in Appendix Table 2.

### 4.2.9 Estimation of PLD activity by amplex red assay kit:

Serum starved cells were held in suspension for 120 minutes and washed and suspended in 3 ml media. Fractions of cell suspension (0.4 million cells per time point) were replated on dishes coated overnight with fibronectin at 4°C (10µg/ml) to serve as either stable adherent (SA) or cells re-adherent for 15 minutes (15'FN) or collected as pellet in micro-centrifuge tube to serve as suspension (SUSP) time point. Cells were immediately frozen at -80°C at their respective time points and were together revived on ice for lysis. Cells were lysed in 200 µl of lysis buffer (50mM Tris pH 8.0+ 0.1% TritonX100). 50 µl of lysate was then added per well of black 96 well plate and diluted with 50 µl of 1X reaction buffer from the kit. Each sample was represented in triplicates. 100 µl of reaction mix containing Lecithin, amplex red reagent and choline oxidase and horse radish peroxidase from the kit was then added to each well and mixed well. $H_2O_2$ was used instead of lysate as a positive control. The reaction was incubated for 90 minutes at 37°C and fluorescence estimated using Tecan spectrophotometer with excitation at 560 nm and emission at 590 nm. The same cell lysate was used to estimate total protein levels using BCA assay kit from Pierce and fluorescence values were normalized to total protein to plot graph of PLD activity levels. One suspension assay was estimated twice for PLD activity, once with non-frozen FN and SUSP time points and once with SA and SUSP time points that were frozen at -80C. The data from these two sets was plotted together after normalization of SUSP time point. Raw PLD activity levels did not vary significantly between frozen and non-frozen SUSP time points.

### 4.2.10 Inhibition of ArfGEFs using ArfGEF inhibitors:

Cells seeded in 60 mm dishes were pre-treated with DMSO (Control), 10uM of Golgicide-A or 10 µg/ml BrefeldinA-A for 2 hours at 37°C. Cells were detached with Trypsin-EDTA, washed and held in suspension for 120 minutes with 0.75% methylcellulose DMEM with the required concentration of inhibitor. Cells were then washed twice with media containing the inhibitor, pelleted in microcentrifuge tube, frozen at -80C and processed for Arf activity assay as described above.

**4.2.11 Statistical analysis:**

Statistical analysis of data was done using the two tailed unpaired Student's T test and when normalized to respective controls using the two tailed single sample T test. All analysis was done using Prism Graphpad analysis software. Statistical significance was considered at $P<0.05$.

**4.3 RESULTS:**

**4.3.1 Role of exocyst complex proteins in mediating Ral-Arf6 crosstalk:**

Knowing that Ral is upstream to Arf6, we sought to evaluate whether a Ral-effector can mediate the Ral-Arf6 crosstalk in MEFs, we tested the effect knockdown of Ral effector proteins have on adhesion dependent Arf6 activation.

Since Sec5 has been earlier shown to regulate the membrane raft exocytic pathway (Balasubramanian et al., 2010), it was the first to be tested as a likely mediator. **Depletion of Sec5 did not affect adhesion dependent Arf6 activation** (Figure 4.1A, 4.1B). This suggested **Sec5 could work with RalA independent of Arf6.** Active RalA and Sec5 as part of the exocyst complex could mediate the delivery of raft microdomains from recycling endosomes to plasma membrane whereas Arf6 could have a role in mediating fusion of these microdomains with the plasma membrane. This again highlights the possible existence of parallel pathways downstream of Ral and Arf6 involved in mediating the delivery of raft microdomains. Other exocyst complex components known to interact with Ral and Arf6- **Exo84 and Sec10 were then tested and found not to affect Arf6 activation in re-adherent MEFs** (Figure 4.1C, 4.1D, 4.1E and 4.1F). Interestingly, loss of Sec10 was seen to affect basal active Arf6 levels in stable adherent cells (Figure 4.1F) which suggests Arf6 activation and its regulation in these cells could be distinctly different from that seen in re-adherent cells.

**4.3.2 Role of RalBP1 in mediating Ral-Arf6 crosstalk in MEFs:**

We next tested RalBP1 as a candidate mediator of this crosstalk and found its depletion did not disrupt Arf6 activation in re-adherent cells indicating that it does not mediate the Ral dependent Arf6 activation in re-adherent cells (Figure 4.2A, 4.2B). However, loss of RalBP1 marginally, but consistently, reduced Arf6 activity (~35% decrease) in non-adherent cells as compared to control cells. To evaluate this further we tested the effect expression of a siRNA resistant RalBP1 mutant, made by the insertion of five non-sense mutations in the siRNA binding site (Figure 4.3A, 4.3B), has on Arf6 activation. Expression of FLAG-RalBP1* in the RalBP1 knockdown cells reversed the drop in Arf6 activation effectively causing anchorage independent Arf6 activation (Figure 4.4A, 4.4B), as was seen earlier with active Ral in MEFs (Figure 2.3). Loss of RalBP1 significantly decreased active RalA (RalAV23)

mediated anchorage independent Arf6 activation in MEFs (Figure 4.4C, 4.4D, 4.4E), confirming its role downstream of Ral. This coupled with the fact that RalBP1 is known to be over-expressed in bladder cancer cells (Smith et al., 2007) led us to evaluate the role it could have in Ral regulated anchorage independent Arf6 activation in T-24 cells.
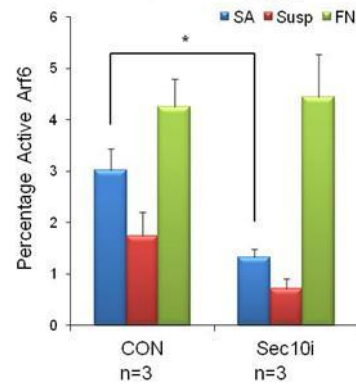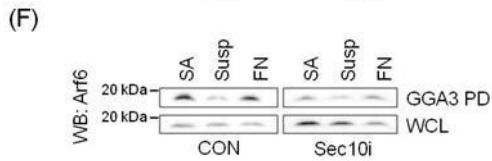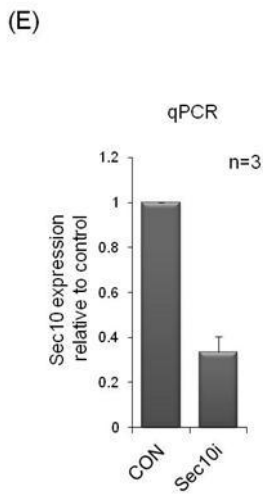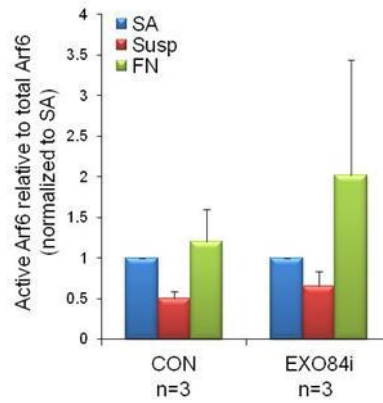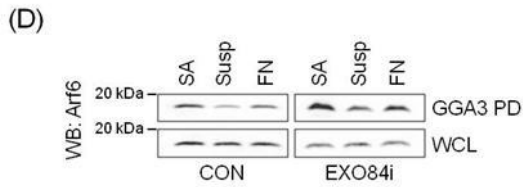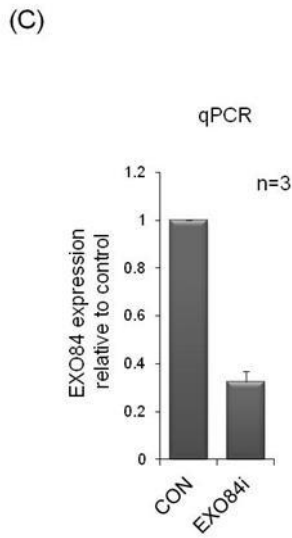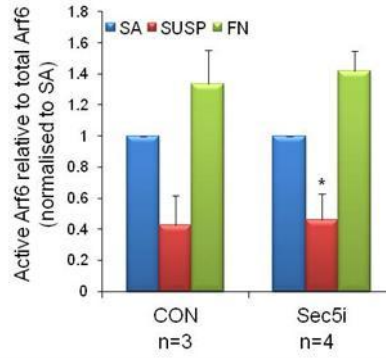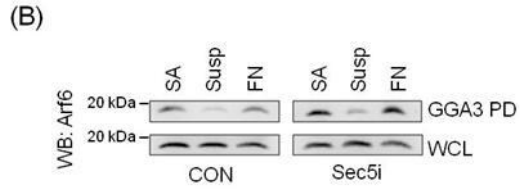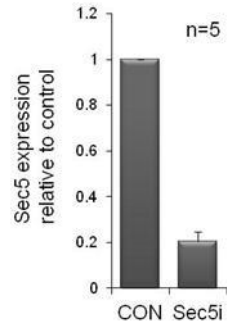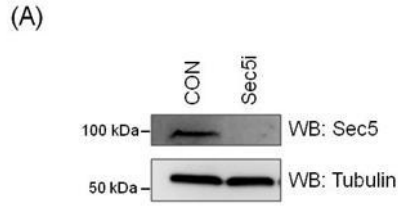
**Figure 4.1: Role of exocyst complex components Sec5, Exo84 and Sec10 in regulating adhesion dependent activation of Arf6 in MEFs. (A)** Representative western blots (upper panel) and quantitation (lower panel) for knockdown of Sec5 (WB: Sec5) in MEFs relative to Tubulin (WB: Tubulin) as loading control. **(C), (E)** Quantitative PCR (qPCR) showing relative expression of **(C)** Exo84 and **(E)** Sec10 in control (CON) and Exo84 knockdown (EXO84i) or Sec10 knockdown (Sec10i) MEFs plotted as normalized to CON. **(B), (D), (F)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) and total Arf6 in the respective whole cell lysate (WCL) from control (CON) and **(B)** Sec5 knockdown (Sec5i), **(D)** Exo84 knockdown (Exo84i) or **(F)** Sec10 knockdown (Sec10i) MEFs in SA, SUSP and FN conditions. Percentage active Arf6 levels were calculated as described in methods and normalized to respective SA and plotted in **(B)** and **(D)**. In **(F)** percentage active Arf6 levels are plotted. Graphs represent mean ± standard error data from a minimum of three and maximum of four independent experiments (as indicated below each graph). Statistical analysis of was done using One sample two tailed T-Test for **(B)** and **(D)** and done using unpaired Student's T-test for **(F)** and significance represented (* p value <0.05).
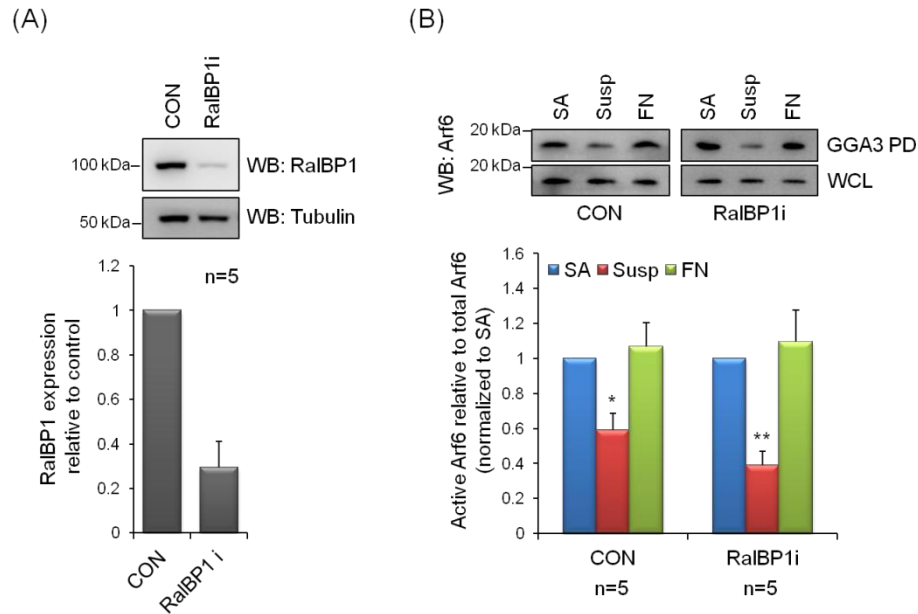
**Figure 4.2: Role of RalBP1 in regulating adhesion dependent activation of Arf6 in MEFs. (A)** Representative western blots (upper panel) and quantitation (lower panel) for knockdown of RalBP1 (WB: RalBP1) in MEFs relative to Tubulin (WB: Tubulin) as loading control. **(B)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) and total Arf6 in the respective whole cell lysate (WCL) from control (CON) and RalBP1 knockdown (RalBP1i) MEFs in SA, SUSP and FN conditions. Percentage active Arf6 levels were calculated as described in methods and normalized to respective SA. Graphs represent mean ± standard error data from five independent experiments (as indicated below each graph). Statistical analysis of was done using One sample two tailed T-Test and significance represented (* p value <0.05, ** p value <0.01).

(A)

WT RalBP1 --- GAT GCA **GTA GAG AGG ACC ATG ATG** TAT GAT ---

                 D    A    V    E    R    T    M    M    Y    D

RalBP1*     --- GAT GCA **GTC GAA CGA ACT ATG ATG** TAT GAT ---

                 D    A    V    E    R    T    M    M    Y    D

RalBP1*: siRNA resistant RalBP1 mutant (5 muations)
Bold letters: siRNA binding site
Red letters: Non-sense mutations introduced by SDM
Blue letters: corresponding amino acids

(B)
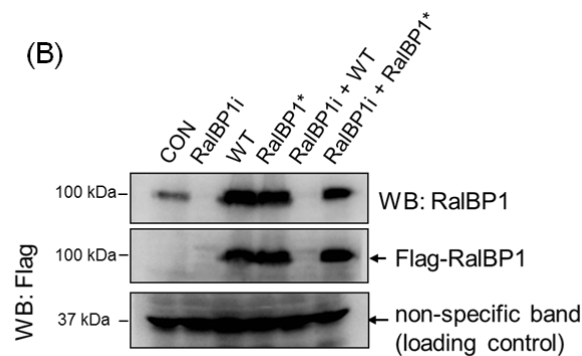
**Figure 4.3: Making siRNA resistant mutant of RalBP1. (A)** Strategy for making siRNA resistant mutant of RalBP1 by introduction of five silent mutations (RalBP1*) using site directed mutagenesis approach for FLAG-WT-RalBP1 (WT-RalBP1) construct. Mutations introduced do not alter amino acid sequence as indicated in blue letters. **(B)** Western blots for expression of endogenous and exogenously expressed FLAG-WT-RalBP1 and FLAG-RalBP1* (WB: RalBP1 and WB: FLAG) from whole cell lysates of control MEFs (CON), RalBP1 knockdown MEFs (RalBP1i), MEFs expressing either FLAG-WT-RalBP1 (WT) or RalBP1* (RalBP1*) or expressing these constructs in RalBP1 knockdown cells (RalBP1i+WT/ RalBP1i+RalBP1*). A non-specific band from FLAG blot serves as loading control.
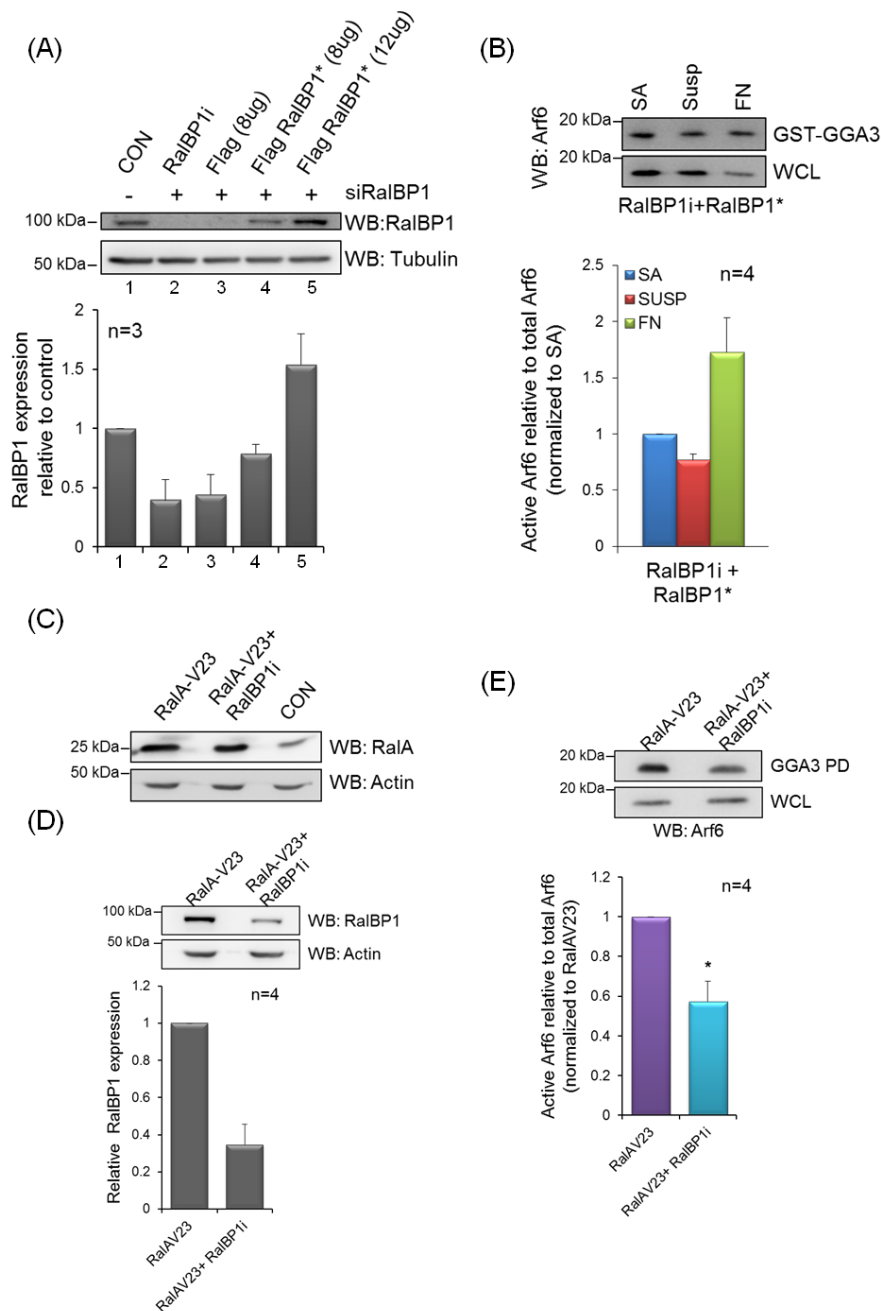
**Figure 4.4: RalBP1 supports anchorage independent Arf6 activation downstream of active RalA in MEFs. (A)**, **(D)** Representative western blots (upper panel) and quantitation (lower panel) for RalBP1 expression (WB: RalBP1) in **(A)** RalBP1 knockdown MEFs and **(D)** in MEFs expressing untagged RalAV23 relative to Tubulin/Actin (WB: Tubulin/ WB: Actin) as loading control. **(C)** Representative western blots for expression of RalA (WB: RalA) in CON and untagged RalA-V23 expressing serum starved MEFs (RalAV23, RalAv23 + RalBP1i) relative to Actin (WB: Actin) as loading control. **(B)**, **(E)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) and total Arf6 in the respective whole cell lysate (WCL) from **(B)** RalBP1 knockdown MEFs expressing FLAG-RalBP1*(RalBP1i+RalBP1*) in SA, SUSP and FN conditions or **(E)** MEFs expressing untagged RalAV23 without RalBP1 knockdown (RalAV23) or with RalBP1 knockdown (RalAV23+RalBP1i). Percentage active Arf6 levels were calculated as described in methods and normalized to **(B)** SA or **(E)** RalAV23. Graph represents mean ± standard error data from four independent experiments. Statistical analysis of was done using One sample two tailed T-Test and significance represented (* p value <0.05).

### 4.3.3 Role of RalBP1 in mediating Ral-Arf6 crosstalk in T-24 cells:

RalBP1 knockdown (RalBP1i#1) significantly reduced Arf6 activation in suspended T-24 cells in the low serum (Figure 4.5A, 4.5B) as well as with serum conditions (Figure 4.5C, 4.5E). This was confirmed by RalBP1 knocked down by another siRNA sequence (RalBP1i#2) (Figure 4.5C, 4.5F). This effect was comparable to the effect loss of RalB has in these cells (Figure 3.4E). Also, joint knockdown of RalB and RalBP1 did not show any additive effects on Arf6 activation suggesting they are likely to work along a linear pathway (Figure 4.5G, 4.5H). RalBP1 depletion also significantly affected anchorage independent Erk activation and anchorage independent growth of T-24 cells (Figure 4.6).

### 4.3.4 Role of ArfGEFs in mediating Ral-Arf6 crosstalk:

With the detection of a Ral-RalBP1-Arf6 pathway in cancer cells, understanding how RalBP1 could support Arf6 activation remained an important question to address. We hence envisaged a role for Arf6 GEF/GAP in this pathway. We first explored the role of Arf6 GEFs and looked at the RalBP1 binding Arf6 GEF ARNO (Lee et al., 2014) in mediating the Ral-Arf6 crosstalk. Loss of ARNO, by two siRNA sequences (ARNOi#1 and ARNOi#2) (Figure 4.7A, 4.7B), significantly decreased Arf6 activation in non-adherent T-24 cells with serum, supporting its role in this crosstalk (Figure 4.7C, 4.7D). This suggests a role for ARNO in mediating anchorage independent Arf6 activation. In MEFs expressing active RalA (RalAV23) where anchorage independent Arf6 activation is also seen (Figure 2.3), ARNO knockdown was again able to reduce Arf6 activation confirming its role downstream of active RalA (Figure 4.7E, 4.7F, 4.7G). This for the first time puts ARNO downstream of Ral along a Ral-RalBP1-ARNO-Arf6 pathway in cells. Functionally this pathway was confirmed when depletion of ARNO and RalBP1 in active RalA (RalA79L) expressing MEFs decreased active RalA supported membrane raft microdomain delivery to plasma membrane (Figure 4.8A, 4.8B), as observed before upon loss of Arf6 (Figure 2.5). Further loss of ARNO and RalBP1 (with either siRNA#1 and siRNA#2 sequences) in T-24 cells did not affect anchorage independent RalA and RalB activation (Figure 4.9) indicating linearity of the Ral-RalBP1-ARNO-Arf6 pathway in T-24 cells.

We simultaneously explored the contribution by other Arf6 GEFs in mediating Ral dependent Arf6 activation in T-24 cells. We hence checked the expression levels of all known 14 Arf

GEFs from the cytohesin family (CYTH1-4), EFA6 family (EFA6A-D), BRAG family (BRAG1-3) and BIG family (BIG1-2, GBF1) (Casanova, 2007) in T-24 cells. BRAG3, EFA6A and EFA6C were expressed poorly while CYTH2 (ARNO) and GBF1 were better expressed in T-24 cells (Figure 4.10A, 4.10B). Depletion of CYTH1, CYTH3, BRAG1, BRAG2 (GEP100) and EFA6B (Figure 4.10C), implicated in mediating Arf6 activation in other cancers (Fu et al., 2014; Morishige et al., 2008; Weizhong et al., 2011; Zangari et al., 2014) did not affect anchorage independent Arf6 activation in T-24 cell line (Figure 4.10D). Inhibition of BIG1 and BIG2 by BrefeldinA-A (BFA) and inhibition of GBF1 by Golgicide-A also did not affect Arf6 activity in T-24 cells (Figure 4.10F). Functionality of these inhibitors was confirmed by their inhibition of Arf1 activity (Figure 4.10E) as reported earlier (Sáenz et al., 2009b). These results hence suggest Arf GEFs BIG1, BIG2 and GBF1 to not be involved in mediating anchorage independent Arf6 activation in these cells. The role of other lesser expressed Arf6 GEFs or Arf6 GAPs in further mediating the Ral-RalBP1-Arf6 crosstalk is being actively explored.
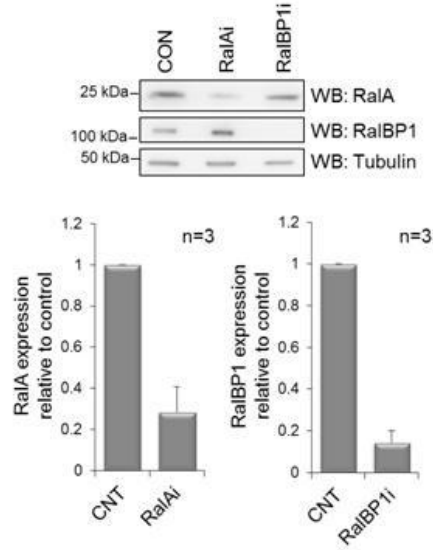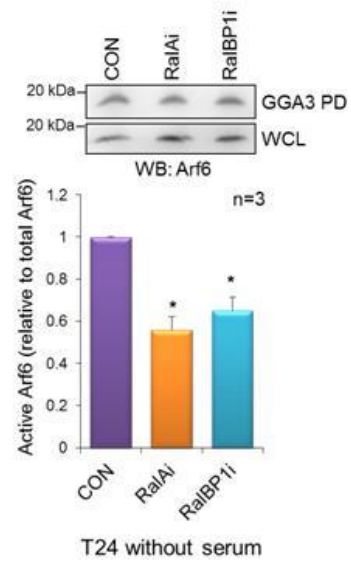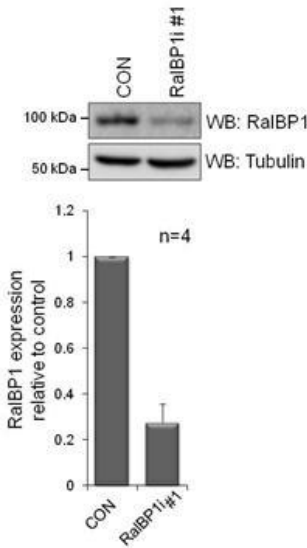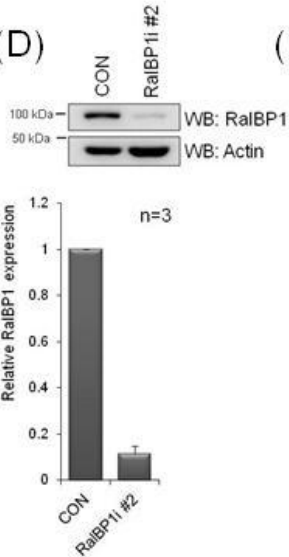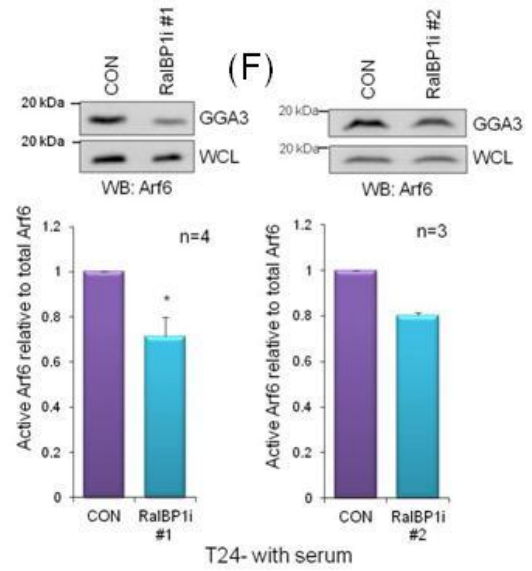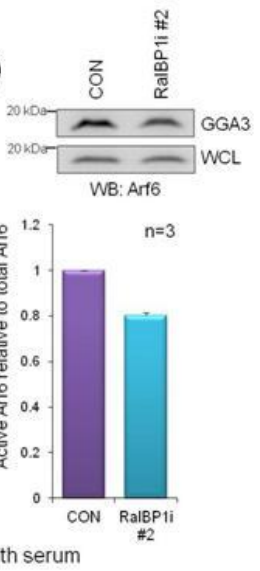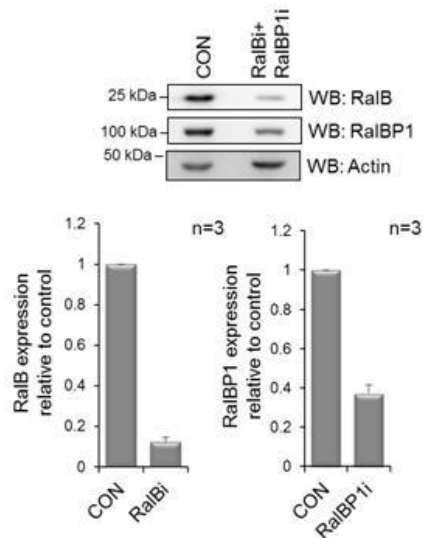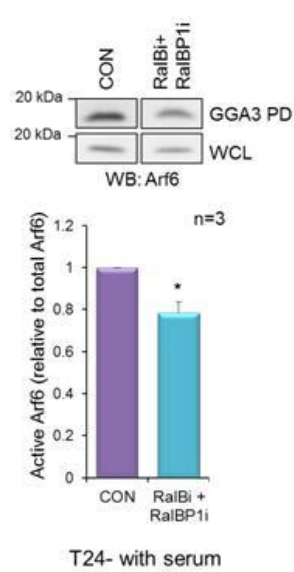
**Figure 4.5: Role of RalBP1 in regulating anchorage independent Arf6 activation in T-24 cells (A)**, **(C)**, **(D)**, **(G)** Representative western blots (upper panel) and quantitation (lower panel) for knockdown of RalA (WB: RalA), RalB (WB: RalB) and RalBP1 (WB: RalBP1) in **(A)** serum starved T-24 cells or **(C)**, **(D), (G)** with serum T-24 cells relative to Tubulin (WB: Tubulin) or actin (WB: Actin) as loading control. **(B)**, **(E)**, **(F), (H)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) and total Arf6 in the respective whole cell lysate (WCL) from **(B)** control (CON), RalA knockdown (RalAi) and RalBP1 knockdown (RalBP1i) serum starved T-24 cells, **(E), (F)** CON and RalBP1 knockdown  T-24 cells with RalBP1 siRNA sequence #1 (RalBP1i#1) or RalBP1 siRNA sequence #2 (RalBP1i#2) with serum or **(H)** CON and RalBi+RalBP1i T-24 cells with serum. Percentage active Arf6 levels were calculated as described in methods and normalized to CON. Graph represents mean ± standard error data from minimum of three and maximum of four independent experiments. Statistical analysis of all the above data was done using two tailed one sample T-test and significance is represented (* P-value<0.05, ** P-value<0.01).
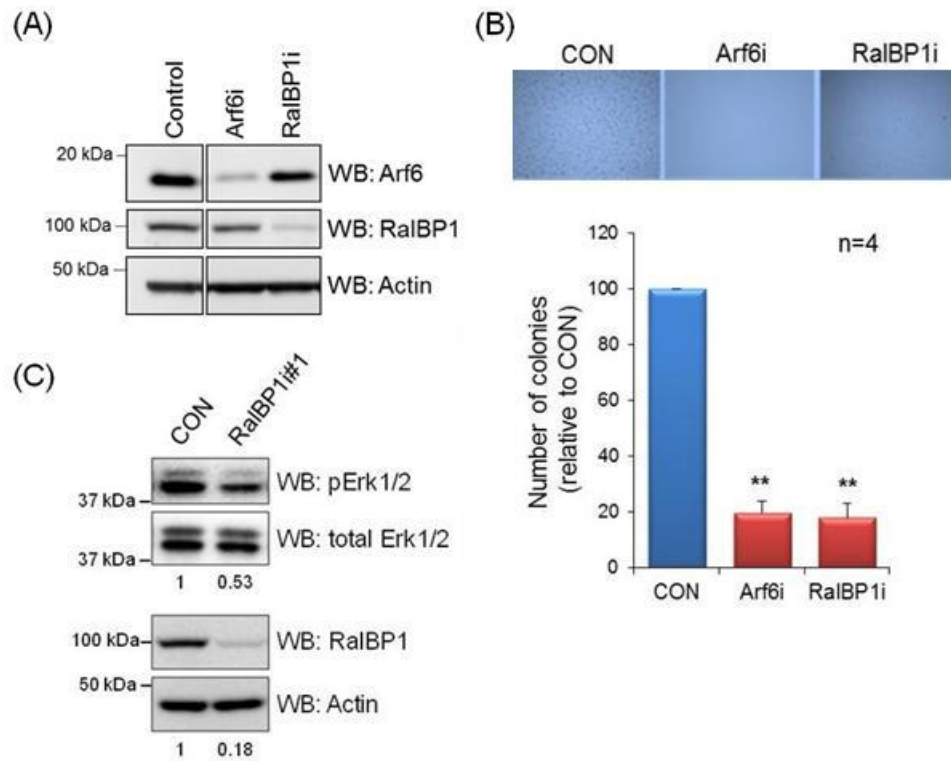
**Figure 4.6: RalBP1 regulates anchorage independent Erk activation and anchorage independent growth of T-24 cells.** **(A)** Representative western blots for Arf6 knockdown (WB: Arf6) and RalBP1 knockdown (WB: RalBP1) in T-24 cells relative to actin (WB: Actin) as loading control. **(B)** Representative phase contrast images (upper panel) and quantitation (lower panel) of number of colonies of CON, Arf6i, RalBP1i, RalBP1i+Arf6i T-24 cells subjected to soft agar colony assay. Colonies were fixed, stained and imaged after three weeks of growth in 0.3% agarose as described in methods. Number of colonies were counted using Image J software and plotted as relative to CON. Graph represents mean ± standard error of four replicates from two independent experiments. Statistical analysis was done using two tailed one sample T-test (* p-value <0.05, ** p-value <0.01). **(C)** Representative western blot of Erk1/2 phosphorylation at Thr 202/Tyr204 (WB: pErk1/2) relative to total Erk1/2 (WB: total Erk1/2) in whole cell lysates from control (CON), RalBP1 knockdown (RalBP1i) in T-24 cells that were held in suspension for 120 minutes with serum. Following densitometric scanning band intensity ratios of pErk/total Erk and RalBP1/Actin were calculated and normalized to control (CON). Blots are representative of two independent experiments that gave similar results.
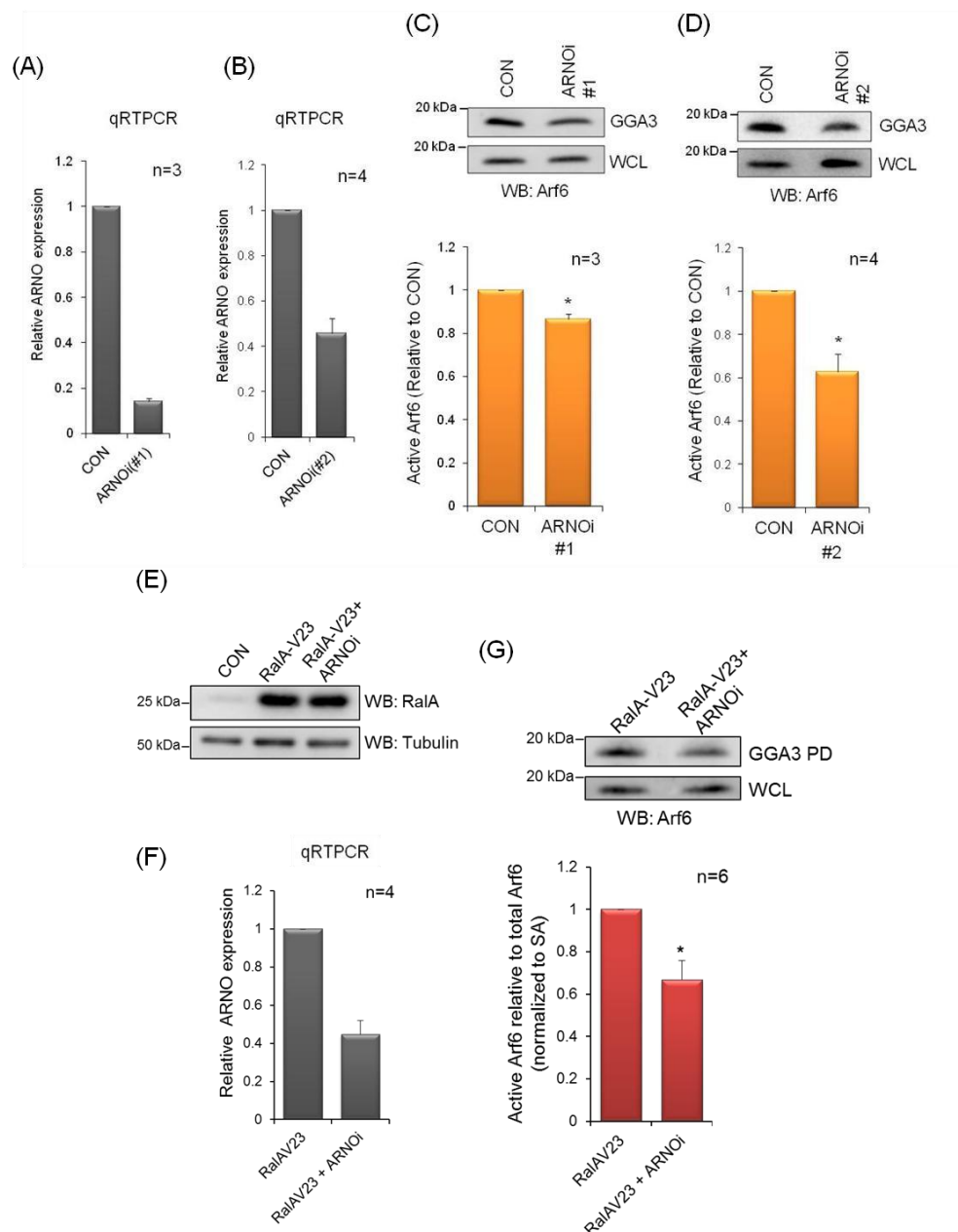
**Figure 4.7: Arf6 GEF ARNO regulates RalA-Ar6 crosstalk in T-24 cells and MEFs.**
**(A),(B), (F)** Quantitative PCR (qPCR) showing relative expression of ARNO in **(A)** control (CON) and ARNO knockdown with siRNA sequence #1 (ARNOi#1) and **(B)** with siRNA sequence #2 (ARNOi#2) in T-24 cells and **(F)** MEFs expressing untagged RalA-V23 (RalA-V23) and ARNO knockdown MEFs expressing untagged RalA-V23 (RalA-V23+ARNOi) plotted as normalized to respective controls. **(E)** Representative western blots for expression of RalA (WB: RalA) in CON and untagged RalA-V23 expressing serum starved MEFs (RalAV23, RalA-V23 + ARNOi) relative to Tubulin (WB: Tubulin) as loading control. **(C)**, **(D)**, **(G)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) and total Arf6 in the respective whole cell lysate (WCL) from **(C)** CON and ARNOi#1, **(D)** CON and ARNOi#2 T-24 cells and **(G)** RalA-V23 and RalA-V23+ARNOi MEFs at 120' SUSP. Percentage active Arf6 levels were calculated as described in methods and normalized to **(C), (D)** CON or **(E)** RalAV23. Graphs represent mean ± standard error data from minimum of three and maximum of six independent experiments. Statistical analysis of all the above data was done using two tailed one sample T-test and significance is represented (* p-value <0.05).
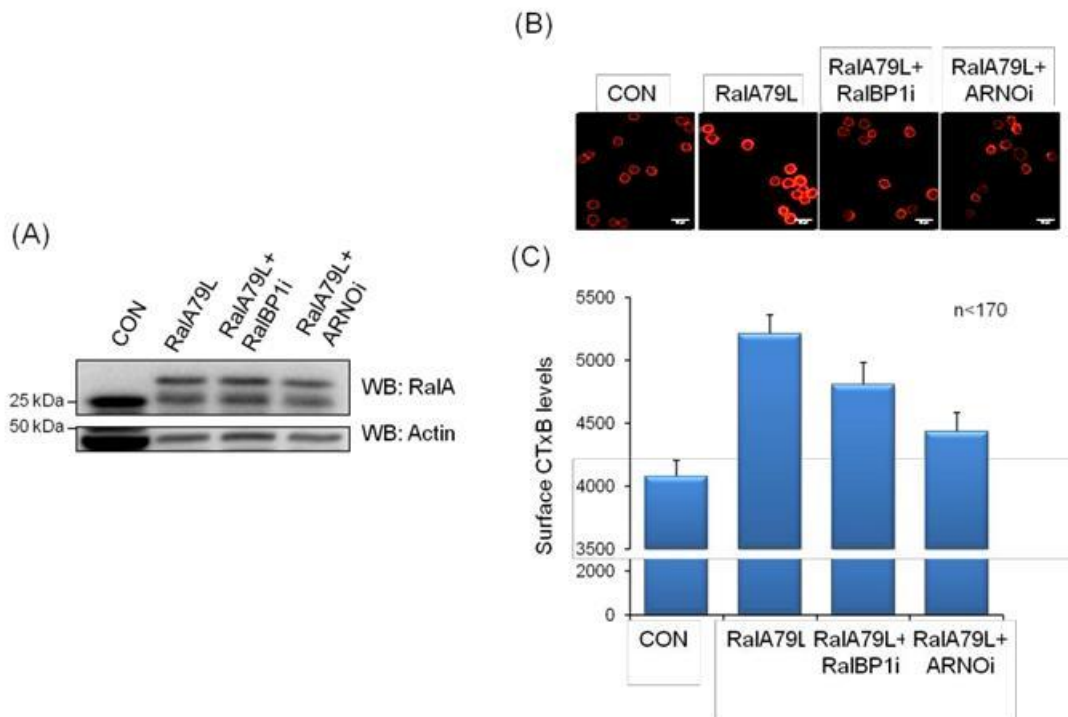
**Figure 4.8:    RalBP1 and ARNO are required for active RalA induced anchorage independent membrane raft trafficking. (A)** Expression of FLAG tagged fast cycling active RalA mutant (RalA 79L) (WB: RalA) in MEFs (RalA79L) either lacking RalBP1 (RalA79L+RalBP1i) or ARNO (RalA79L+ARNOi) relative to actin as loading control. These cells were held in suspension for 120 minutes, then surface labeled with CTxB-Alexa 594 and imaged as described in methods. **(B)** Representative images and **(C)** Quantitation of surface GM1 levels in control (CON), RalA79L expressing MEFs (RalA79L), and RalA79L expressing MEFs lacking either RalBP1 (RalA79L+RalBP1i) or ARNO (RalA79L+ARNOi). Surface labeling intensity quantitated by measuring integrated density for a minimum of 170 cells and mean ± standard error represented in the graph.   Graph is representative of two independent experiments that gave similar results. Statistical analysis was done using the two tailed unpaired T Test and their significance represented (* p value <0.05, ** p value <0.01, *** p value <0.001).

(A)

WB : RalA

WB : RalA

WB : RalB

WB : RalB

(B)

WB : RalA

WB : RalA

WB : RalB

WB : RalB

(C)

WB: RalBPI

WB: Actin

N=4

(D)

qRTPCR

N=4
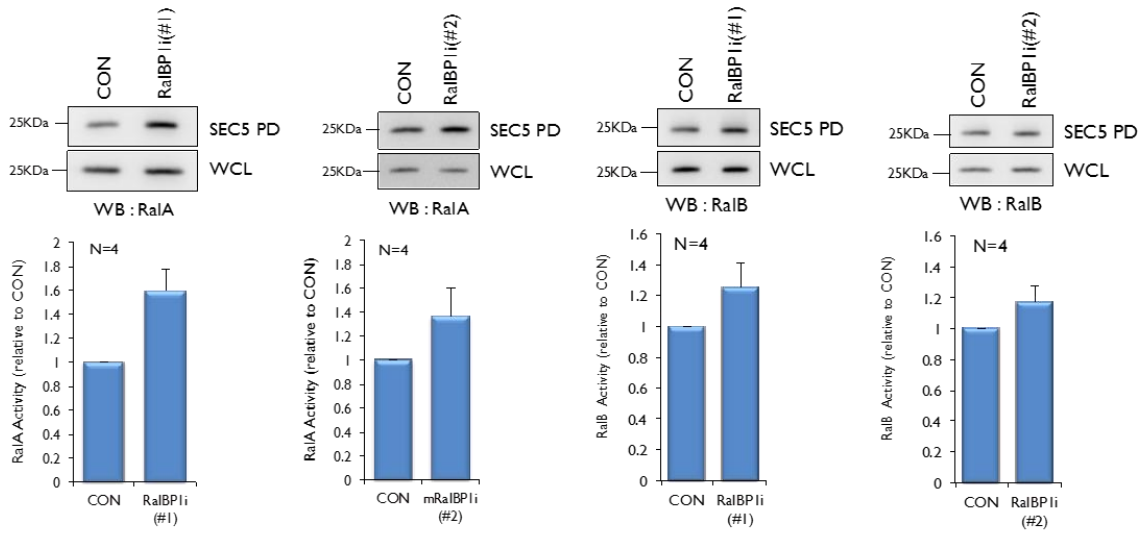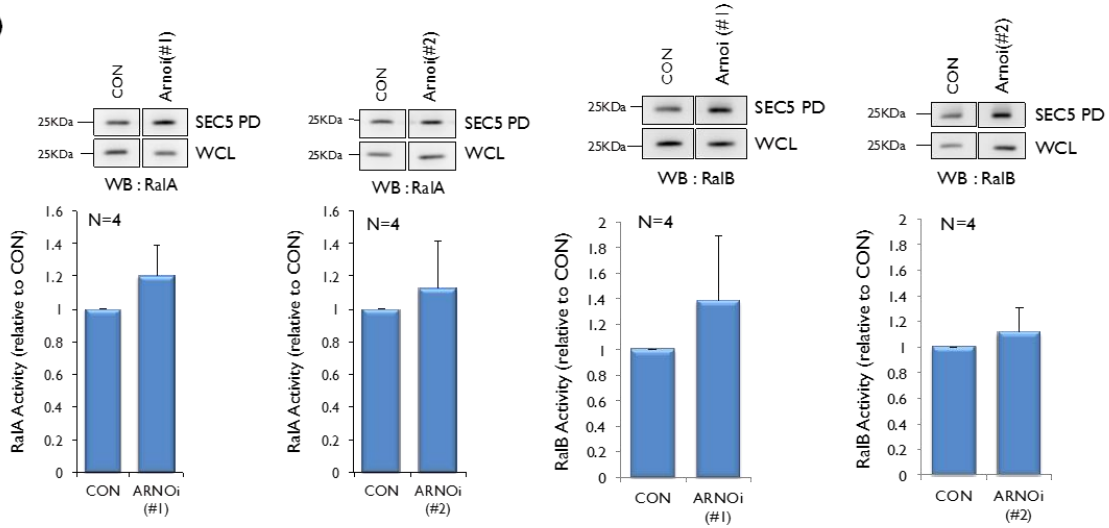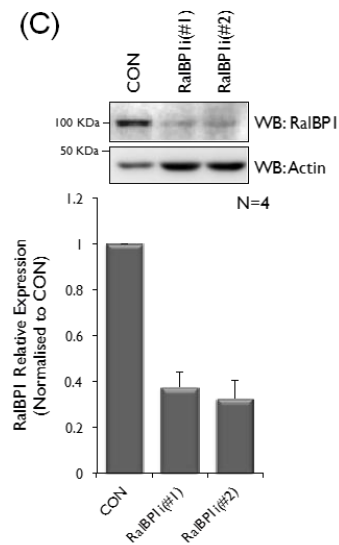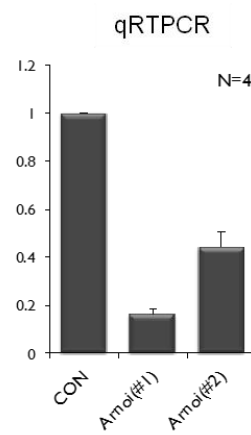
**Figure 4.9: RalBP1 and ARNO do not regulate RalA/ RalB activation in T-24 cells.** **(A), (B)** Western blot detection and quantitation of active RalA/RalB pulled down by GST-Sec5 (Sec5 PD) and total RalA/RalB in respective whole cell lysates (WCL) from control and **(A)** T24 cells with serum where RalBP1 is knocked down with siRNA sequence #1 (RalBP1i #1) and siRNA sequence #2 (RalBP1i #2) or **(B)** ARNO is knocked down with siRNA sequence #1 (ARNOi #1) and siRNA sequence #2 (ARNOi #2) of T-24 cells held in suspension for 120 minutes with serum. Calculated percentage active RalA/RalB levels in knockdown cells were normalized to their respective control (CON). Graph represents mean ± standard error from four independent experiments. **(C)** Representative western blots and quantitation for knockdown of RalBP1 with siRNA sequence #1 (RalBP1i #1) and siRNA sequence #2 (RalBP1i #2) in T-24 cells. **(D)** Quantitative RTPCR detection and quantitation of the knockdown of ARNO with siRNA sequence #1 (ARNOi #1) and siRNA sequence #2 (ARNOi #2) in T-24 cells. Graphs represent mean ± standard error relative to respective control (CON) from four independent experiments.

(A)

(B)

(C)

(D)

(E)

(F)
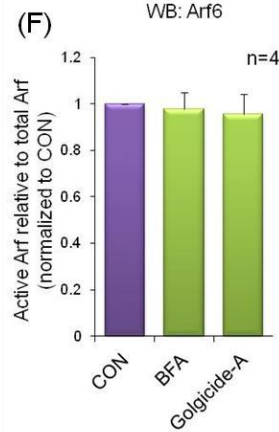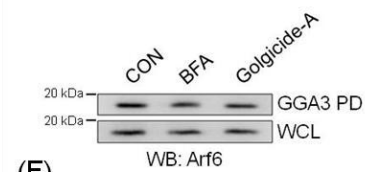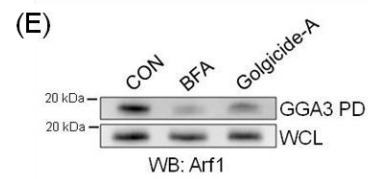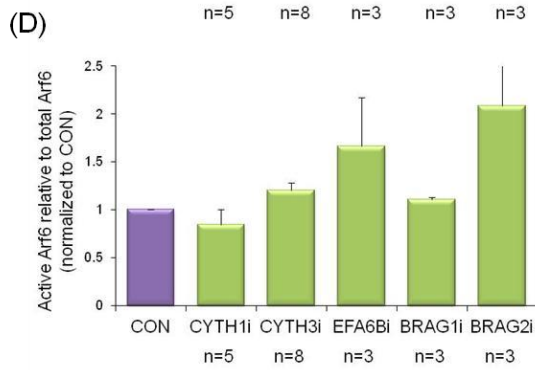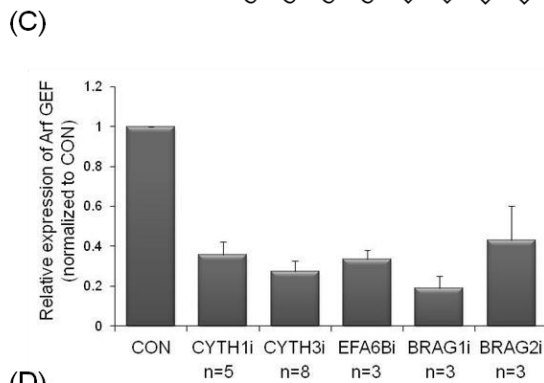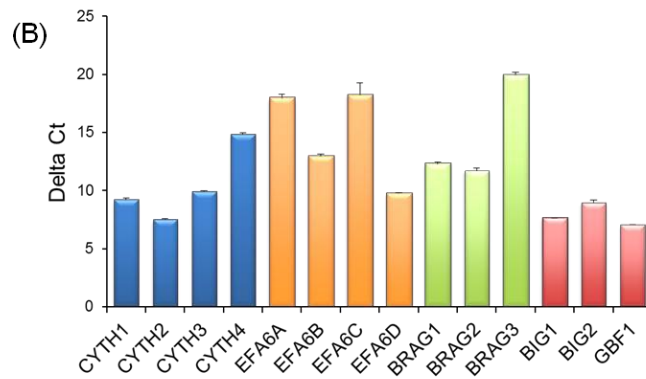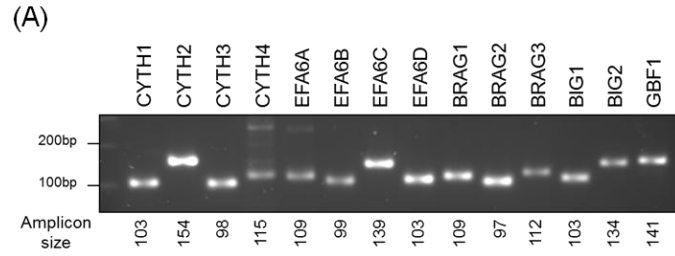
**Figure 4.10: ArfGEFs expression in T-24 cells and effect of their loss or inhibition on anchorage independent Arf6 activation** (A) Representative agarose gel image showing amplified products of Arf GEFs CYTH1-4, EFA6A-D, BRAG1-3, BIG1-2, GBF1 from quantitative PCR run for forty cycles of cDNA from T-24 cells **(B)** Quantitative PCR (qPCR) showing expression profile of Arf GEFs CYTH1-4, EFA6A-D, BRAG1-3, BIG1-2, GBF1 relative to actin as internal control. Graph represents mean delta Ct ± standard error from three independent experiments. **(C)** Quantitative PCR (qPCR) showing relative expression of CYTH1, CYTH3, BRAG1, BRAG2 and EFA6B in control and knockdown T-24 cells plotted as normalized to respective controls. **(D)** Quantitation of active Arf6 pulled down by GST-GGA3 and total Arf6 in the respective whole cell lysate from control (CON) and CYTH1 knockdown (CYTH1i), CYTH3 knockdown (CYTH3i), BRAG1 knockdown (BRAG1i) BRAG2 knockdown (BRAG2i) and EFA6B knockdown (EFA6Bi) T-24 cells held in suspension for 120 minutes with serum. Percentage active Arf6 levels were calculated as described in methods and normalized to respective CON. Graphs represent mean ± standard error data from minimum of three and maximum of eight independent experiments. **(E)** Western blot for active Arf1 pulled down by GST-GGA3 (GGA3 PD) relative to total Arf1 in the respective whole cell lysate (WCL) and **(F)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) relative to total Arf6 in the respective whole cell lysate (WCL) from control (CON) and BrefeldinA-A (BFA) and Golgicide-A treated T-24 cells held in suspension for 120 minutes with serum. Percentage active Arf6 levels were calculated as described in methods and normalized to respective CON. Graph represents mean ± standard error data from four independent experiments.
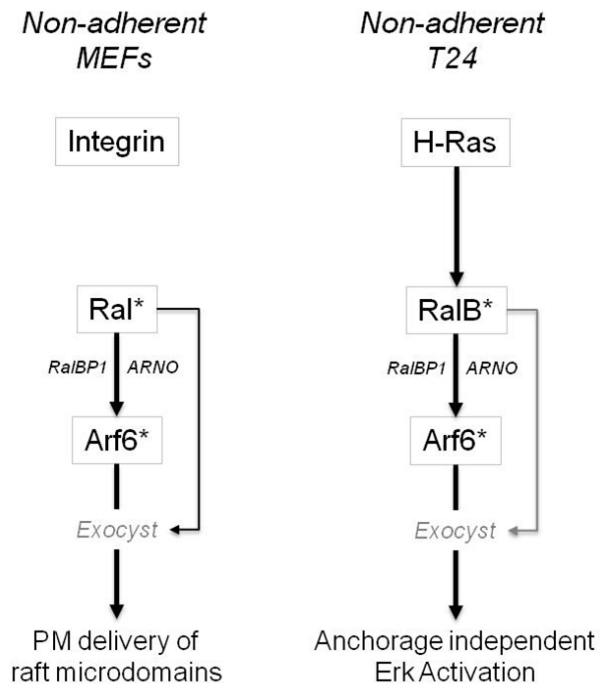
**Figure 4.11: Role of RalBP1 and Arf GEFs in regulating Ral-Arf6 crosstalk in MEFs and T-24 cells.** Schematic depicts role of by RalBP1 and its binding partner ArfGEF-ARNO in regulation of anchorage independent Ral-Arf6 crosstalk in mediating plasma membrane (PM) delivery of membrane raft microdomains and anchorage independent Erk signalling in non –adherent MEFs and T-24 cells respectively.

**4.3.5 Role of phospholipase D in mediating Ral-Arf6 crosstalk:**

While RalBP1 regulates the Ral-Arf6 crosstalk in active Ral expressing non-adherent cells (WTMEFs and T-24 cells), it does not mediate RalA dependent Arf6 activation downstream of integrins in re-adherent cells. We hence looked at the role other Ral effectors could have downstream of integrins. We chose to first look at phospholipase D (PLD)- an effector of both RalA and Arf6 that is required for Ral and Arf6 dependent secretory functions (Vitale et al., 2005). Mammalian PLD has two isoforms PLD1 and PLD2 that both catalyze breakdown of phosphatidyl choline to phosphatidic acid and choline, but do so in different cellular processes (Choi et al., 2002; Cockcroft, 2001). Both isoforms are known to be regulated by Arf6 (Hiroyama and Exton, 2005b; Kim et al., 1998). Depletion of PLD1 in MEFs promoted anchorage independent Arf6 activation without affecting its activity in re-adherent cells (Figure 4.12A, 4.12B). This suggests that PLD1 could help mediate the drop in Arf6 activity on loss of adhesion, which in turn is reversed on its knockdown. This effect interestingly was not seen on loss of PLD2, suggesting this regulation to be unique to PLD1 (Figure 4.12C, 4.12D). We tested and confirmed that knockdown of one PLD isoform did not alter levels of the other isoform (Figure 4.12E, 4.12F). Since active Ral in MEFs regulates Arf6 activation (Figure 2.2A, 2.2B, 2.3A), we also tested possibility that loss of PLD1 could regulate Ral activation upstream of Arf6. PLD1 depletion was seen to support anchorage independent RalA activation (Figure 4.13A, 4.13B), which could in turn support downstream activation of Arf6. Knockdown of PLD1 is expected to regulate its protein levels and hence activation status in MEFs. To evaluate both these possibilities we first tested if integrins can regulate PLD1 activity. Knowing that integrins activate PKC-α which in turn phosphorylates PLD1 at Thr147 to drive its activation (Hornia et al., 1999; Melendez et al., 2001), we compared phospho-PLD1 levels (relative to total PLD1 levels) in adherent and suspended cells. This revealed the phosphorylation and hence activation of PLD1 to be reduced on loss of adhesion and restored on re-adhesion (Figure 4.14A). To confirm this we also tested total enzymatic PLD activity (PLD1+PLD2) using an amplex red based fluorescence assay and found total PLD activity to show a modest drop on loss of adhesion and recovery on re-adhesion (Figure 4.14B). These results suggest that knockdown of PLD1 causing activation of RalA/Arf6 in non-adherent cells is unlikely to be mediated by a change in the activation status of PLD1, as it is seen to drop on loss of adhesion. This suggests one of two possibilities a) knockdown of PLD1 reduces its activation significantly more than loss of adhesion and this in turn promotes RalA/Arf6 activation or b) loss of PLD1 protein, independent of its activation status,

regulates RalA/Arf6 activation. This could possibly mean a role for PLD1 as a scaffold and/or binding partner in this regulation. In this context we are evaluating the differential effect PLD1 specific inhibitors could have on RalA and Arf6 activities to further understand the regulation of Ral and Arf6.
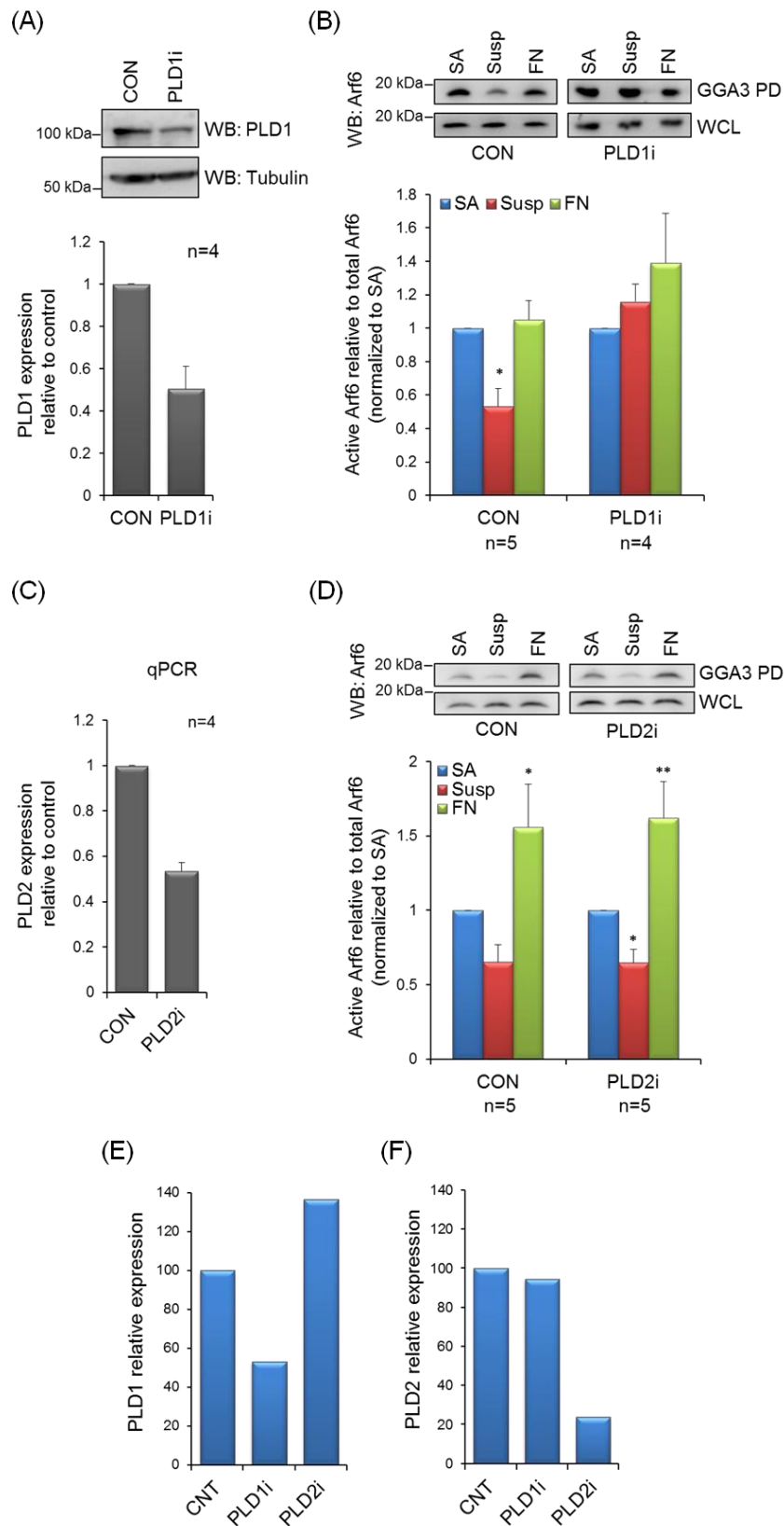
**Figure 4.12: Role of PLD1 in regulating adhesion dependent activation of Arf6 in MEFs. (A)** Representative western blots (upper panel) and quantitation (lower panel) for knockdown of PLD1 (WB: PLD1) in control (CON) and PLD1 knockdown (PLD1i) MEFs relative to Tubulin (WB: Tubulin) as loading control. **(C)** Quantitative PCR (qPCR) showing relative expression of PLD2 in control (CON) and PLD2 knockdown (PLD2i) MEFs plotted as normalized to CON. **(B), (D)** Western blot (upper panel) and quantitation (lower panel) of active Arf6 pulled down by GST-GGA3 (GGA3 PD) and total Arf6 in the respective whole cell lysate (WCL) from control (CON) and **(B)** PLD1 knockdown (PLD1i) or **(D)** PLD2 knockdown (PLD2i) MEFs in SA, SUSP and FN conditions. Percentage active Arf6 levels were calculated as described in methods and normalized to respective SA. Graphs represent mean ± standard error data from a minimum of four and maximum of five independent experiments (as indicated below each graph). Statistical analysis was done using the two tailed one sample T-test (* P-value <0.05, ** P-value <0.01). **(E), (F)** Quantitative PCR (qPCR) showing relative expression of **(E)** PLD1 and **(F)** PLD2 in control (CNT), PLD1 knockdown (PLD1i) and PLD2 knockdown (PLD2i) MEFs plotted as normalized to CNT.
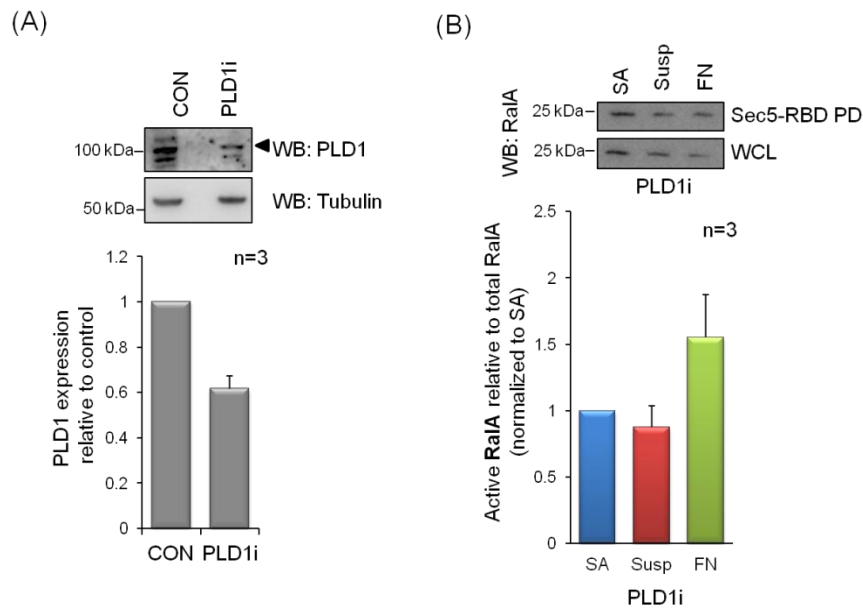
**Figure 4.13: Role of PLD1 in regulating adhesion dependent activation of RalA in MEFs. (A)** Representative western blots (upper panel) and quantitation (lower panel) for knockdown of PLD1 (WB: PLD1) in control (CON) and PLD1 knockdown (PLD1i) MEFs relative to Tubulin (WB: Tubulin) as loading control. **(B)** Western blot (upper panel) and quantitation (lower panel) of active RalA pulled down by GST-Sec5-RBD (Sec5-RBD PD) and total RalA in the respective whole cell lysate (WCL) from PLD1 knockdown (PLD1i) MEFs in SA, SUSP and FN conditions. Percentage active RalA levels were calculated as described in methods and normalized to respective SA. Graph represents mean ± standard error data from three independent experiments.
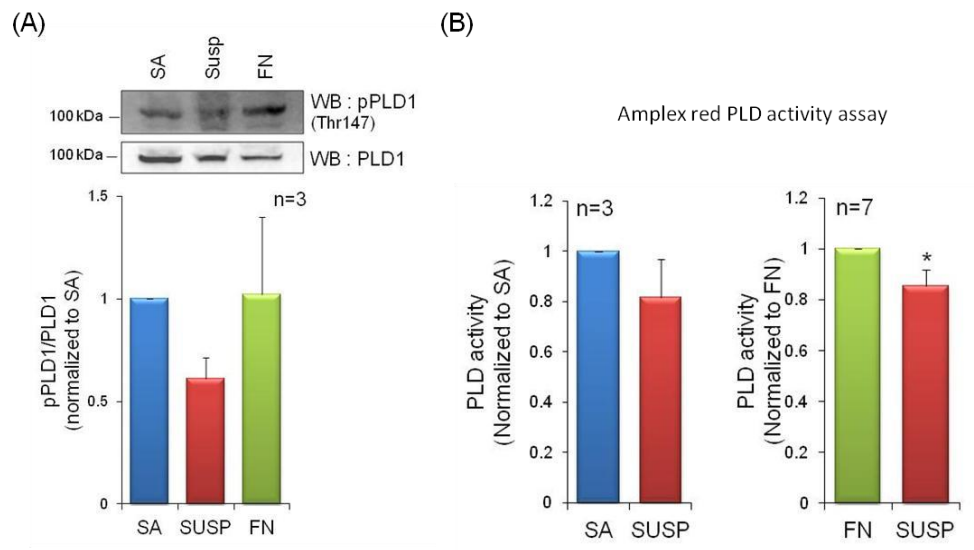
**Figure 4.14: Adhesion dependent regulation of PLD activity. (A)** Western blots (upper panel) and quantitation (lower panel) for PLD1 phosphorylation at Threonine 147 residue (WB: pPLD1-Thr147) relative to total PLD1 (WB: PLD1) in whole cell lysates SA, SUSP and FN conditions of MEFs. Graph represents mean of ratio of band intensities of pPLD1 and PLD1 (pPLD1/PLD1) normalized to SA ± standard error data from three independent experiments. **(B)** PLD activation in SA and SUSP and FN and SUSP conditions of MEFs measured by amplex red PLD activity assay kit as described in methods plotted as normalized to SA or FN. Graphs represent mean ± standard error data from a minimum of three and maximum of seven independent experiments (as indicated for each graph). Statistical analysis was done using two tailed one sample T-test (* p-value <0.05).

## 4.4 SUMMARY:

While active Ral is able to regulate Arf6 activation their association does not reflect Arf6 to be a direct effector of Ral. This hence suggests a possible role for Ral effectors in mediating this crosstalk, leading us to investigate role of Sec5, Exo84, Sec10 and RalBP1. Exocyst component Sec5 though essential for mediating integrin dependent membrane raft exocytosis did not mediate adhesion dependent Arf6 activation in MEFs. This suggests that it likely works with Ral but not to mediate the Ral-Arf6 crosstalk. Exo84, Sec10 and RalBP1 also did not regulate adhesion dependent Arf6 activation. RalBP1 we however found did regulate Arf6 activation downstream of active Ral in non-adherent MEFs and T-24 cells. RalBP1 mediates this through the Arf6 GEF ARNO suggesting the presence of a **Ral-RalBP1-ARNO-Arf6** pathway. RalBP1 and ARNO were found be required along the Ral-Arf6 pathway in mediating active Ral supported membrane raft microdomain delivery in non-adherent MEFs and anchorage independent Erk signalling in T-24 cells (Figure 4.11). Along with ARNO, the role of other Arf GEFs expressed in T-24 cells was tested and found CYTH1, CYTH3, BRAG1, BRAG2 (GEP100), EFA6B (known to be deregulated in other cancers) and BIG1, BIG2 and GBF-1 (seen to be better expressed in T-24 cells) to not regulate anchorage independent Arf6 activation.

Phospholipase D1 (PLD1), a common effector of Ral and Arf6 was seen to regulate anchorage independent activation of both GTPases. PLD2 did not mediate this suggesting that the regulation to be isoform specific. Such a feedback regulation between PLD, Ral and Arf6 and its significance in anchorage independent signalling and growth that Ral and Arf6 are involved in remains to be studied. Understanding how PLD1 mediates the same is also of interest and something our ongoing studies are actively exploring. Similarly the role Sec10 has in mediating the basal Arf6 activation, its regulation and role in cells is being actively explored.

## 4.5 CONCLUSION:

**Ral effector RalBP1 and its binding partner ArfGEF ARNO mediate Ral-Arf6 crosstalk in non-adherent MEFs and T-24 cells to mediate membrane raft exocytosis in MEFs and anchorage independent Erk signalling in T-24 cells.**

# CHAPTER 5:

# Role of RalGEFs in regulating differential activation of Ral isoforms by integrins

**5.1 RATIONALE:**

The role Ral GTPases have in cellular function is governed by their activation-deactivation cycle that in turn is controlled by Ral specific Guanine Nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs) (Bos et al., 2007). GEFs exchange bound GDP with GTP activating Ral while GAPs assist the hydrolysis of bound GTP to GDP supporting its inactivation. Altered expression of GEFs and GAPs is known to support the deregulation of the Ral pathway in disease conditions (Neel et al., 2011; Saito et al., 2013; Vigil et al., 2010b, 2010c). Integrin mediated adhesion specifically regulates RalA and not RalB, to control adhesion dependent membrane raft exocytosis and anchorage dependence in MEFs (Balasubramanian et al., 2010).   In identifying the presence of an **integrin-RalA-Arf6** pathway in MEFs, these studies have revealed a role for the differential activation of Ral in mediating this crosstalk and Ral function. Thus understanding how adhesion differentially regulates RalA versus RalB is of much interest. As a first step towards understanding this phenomenon we have evaluated the role RalGEFs could have in mediating differential Ral activation downstream of integrins. RalGEFs that were studied (Figure 5.1) include Ras dependent GEFs (RalGDS, RGL1, RGL2, RGL3) with a C-terminal Ras Association (RA) domain (Isomura et al., 1996; Spaargaren and Bischoff, 1994) and Ras independent GEFs (RalGPS-1, RalGPS-2) that mediate Ral activation downstream of other stimuli (Rebhun et al., 2000b).   Another major contributor of differential regulation of Ral isoforms is their subcellular localization. Both active RalA and RalB localize to plasma membrane, active RalA additionally present in the recycling endosomal pool (Shipitsin and Feig, 2004). They localize differentially owing to the difference in their C- terminal hypervariable region which could mediate their differential interaction with distinct GEF/GAP proteins. Such a localization dependent spatiotemporal activation of RalA and RalB by GEFs is seen to mediate their isoform specific roles in early and late cytokinesis (Cascone et al., 2008). Hence in evaluating RalGEFs, we have also explored the role differential localization of RalA and RalB could have in their specific activation and function in MEFs. Knowing that Ral is also activated downstream of Ras and regulates anchorage independence in cancers we wanted to evaluate the role GEFs identified in this study might have downstream of Ras in regulating Ral in cancers.
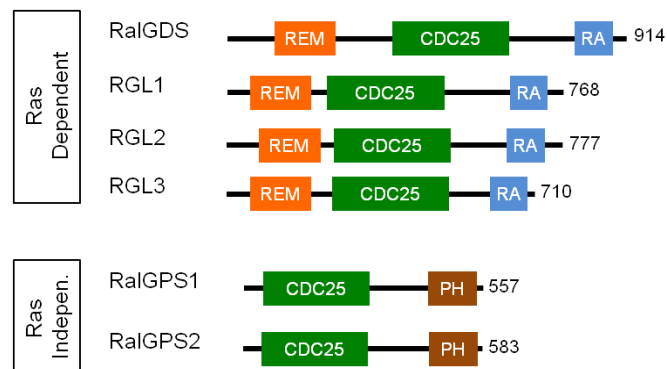
**Figure 5.1: Ral guanine exchange factors (RalGEFs).** Schematic depicts domain organization of six known Ral-GEFs. CDC25 homology domain is essential for binding and GEF activity towards Ral GTPases. Presence of Ras Exchange Motif (REM) and Ras Association domain (RA) is seen in Ras dependent RalGEFs (in red box) while Ras independent GEFs lack these domains. They possess a C-terminal PH domain (binds phospholipids), SH3 binding motif (binds SH3 domain proteins) and a proline rich region containing PXXP motifs (Reproduced from (Gentry et al., 2014)).

## 5.2 MATERIALS AND METHODS:

### 5.2.1 Reagents:

Human plasma fibronectin used in these studies was from Sigma. Primary antibodies used in this study were anti RalA (BD Transduction), anti RalB (R&D Biosystems), anti-beta tubulin E7 (DSHB) and anti-beta actin (Abcam), anti-Myc (Santa Cruz), anti-paxillin (BD transduction), and anti-FAK (Cell signalling technology). Primary antibodies were detected with the following HRP conjugated secondary antibodies: anti-mouse IgG, anti- rabbit IgG and anti-goat IgG (Jackson Immuno Research Laboratories). Phalloidin-Alexa488 was from Molecular Probes. Fluoromount-G was from Southern Biotech. Protease inhibitor cocktail II (100X) was from Sigma. Myc-RGL1 plasmid was kind gift from Prof Michael White's lab (Tzuling Cheng, 2008). FLAG-RalAQ72L/B (ID: 11120) and Myc-RalBQ72L/A (ID: 11121) were from Prof. Christopher Counter's lab (Lim et al., 2005) and obtained from Addgene. siRNA sequences for knockdown of RalGPS1, RalGPS2, RalGDS, RGL1, RGL2 and RGL3 in MEFs and RGL1 in cancer cell lines were On-Target Plus SMARTpool from Dharmacon as listed in appendix table 4. Additional RNA interference sequences of single duplex used for knockdown studies were procured from Sigma and are listed in appendix table 4.

### 5.2.2 Tissue Culture:

Mouse embryonic fibroblasts (from the lab of Dr. Richard Anderson, University of Texas Health Sciences Center, Dallas TX) were cultured in high glucose DMEM medium with 5% fetal bovine serum, penicillin and streptomycin (Invitrogen). SW620 (obtained from National Centre for Cell Science, Pune, India) were cultured in L-15 with 5% fetal bovine serum and antibiotics. Calu-1, MiaPaCa2, HCT116, PC3, UM-UC-3, T-24, DU145, MDA-MB-231, 101080, CFPAC-1, U87MG, SKOV3 were from ECACC and cultured in either RPMI1650 or high glucose DMEM (as per ECACC guidelines) with 5% fetal bovine serum and antibiotics. For plasmid transfections, cells were seeded in either in 6 well plate or 60 mm dishes at 50 per cent confluency and transfected using Lipofectamine LTX reagent (Invitrogen). For siRNA mediated knockdowns, $0.5 \times 10^5$ cells seeded in 60 mm dishes were transfected first with a standardized amount of SMARTpool siRNA or single siRNA oligo using the Lipofectamine RNAiMax transfection reagent (Invitrogen) followed by a second transfection

the following day with the same amount of siRNA. For rescue experiments, 0.5 X 10^5 cells seeded in 6- well plate were co-transfected with 4 μg plasmid and 120 pmols of siRNA using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were used 48 hours after transfection.

### 5.2.3 Cell suspension assay:

Assays of MEFs were done with low serum media. Assays of SW620 and MiaPaCa2 cells were done with 5% serum media. Assays were performed as described in 2.2.3.

### 5.2.4 Cell spread assay:

Cells were held in suspension for 120 minutes as described. Following the incubation and washes cell pellet was suspended in low serum medium. Cell suspension equivalent to 0.5 million cells was pelleted separately to be used for Trizol extraction followed by RNA isolation. Cell suspension equivalent to 0.1 million cells was plated on FN coated coverslips (coated with 2 μg/ml FN overnight at 4°C). Cells were fixed at two time-points between 10 minutes and 25 minutes after re-plating. These time points were chosen to capture early cell spreading events. Fixation was done using 3.5% paraformaldehyde at room temperature for 15 minutes followed by three washes with PBS. Fixed cells were then stained with 1:500 phalloidin-Alexa-488 (1 mg/ ml) for 30 minutes at room temperature followed by washes and DAPI staining for 2 minutes. Coverslips were then mounted on slides using Fluoromount-G (Southern Biotech) and imaged using a Zeiss LSM 710 laser confocal-Anisotropy microscope with a 40X objective. Samples to be compared were imaged at identical settings and analyzed using the Image J software (NIH). Thresholds were set to define the cell edge that was then used to create a mask for each cell. The total area within the mask was then measured and values obtained compared between samples after normalization to control.

### 5.2.5 Ral activity assay:

Assays of MEFs were done with low serum media. Assays of SW620 and MiaPaCa2 cells were done with 5% serum media. Assays were performed as described in 2.2.3.

### 5.2.6 Quantitative PCR:

As described in 4.2.8. Primers used for measuring transcript levels in qPCR are summarized in Appendix Table 3.

### 5.2.7 Statistical analysis:

Statistical analysis of data was done using the two tailed unpaired Student's T test and when normalized to respective controls using the two tailed single sample T test. All analysis was done using Prism Graphpad analysis software. Statistical significance was considered at $P<0.05$.

**5.3 RESULTS:**

**5.3.1 Role of RalGEFs in regulating adhesion dependent cell spreading and Ral activation**

Integrins specifically regulate RalA, but not RalB activation in MEFs to support adhesion dependent membrane raft trafficking and cell spreading (Balasubramanian et al., 2010). Ral activation downstream of multiple stimuli is mediated by their regulation of RalGEFs and Ral GAPs (Gentry et al., 2014). We hence tested the role RalGEF(s) could have in mediating RalA activation and cell spreading in re-adherent MEFs. We first analyzed the relative expression of Ras dependent RalGEFs (RalGDS, RGL1, RGL2, RGL3) and Ras independent RalGEFs (RalGPS1, RalGPS2), in WTMEFs by quantitative PCR. These results indicated the expression of Ras dependent RalGEFs (RalGDS, RGL1, RGL2, RGL3) to be better than Ras independent GEFs (Figure 5.2A, 5.2B). We hence targeted each of these RalGEFs with On-target plus siRNA SMARTpool (mix of 4 siRNA oligos) (Dharmacon) (Figure 5.3A, 5.3B) and tested their role in adhesion dependent cell spreading (known to be regulated by RalA) (Balasubramanian et al., 2010). Of all the GEFs tested, depletion of RGL1 most affected re-adherent cell spreading, better than loss of RalGPS1 or RalGDS (Figure 5.3C, 5.3D). These effects seen with the SMARTpool siRNAs were further confirmed using single siRNA duplexes against RGL1, RalGPS1 and RalGDS (Figure 5.4A, 5.4B). RGL1 knockdown (using single siRNA duplex) also showed it to be the major regulator of re-adherent cell spreading significantly more so than RalGDS and RalGPS1 (Figure 5.4C, 5.4D). The combined knockdown of these three GEFs did not show any additive effect on cell spreading (Figure 5.5C, 5.5D- WTMEFs) suggesting their possible regulation of common downstream mediator, likely RalA. To confirm the specificity of these effects on cell spreading we also compared the effect RGL1 knockdown has on the spreading of caveolin-1 (Cav1) -/- MEFs whose spreading was shown to be independent of RalA mediated exocytosis (Balasubramanian et al., 2010). RalGEF expression profile and knockdown efficiencies in Cav1 -/- MEFs were tested and seen to be comparable to WTMEFs (Figure 5.5A, 5.5B). The joint knockdown of RGL1, RalGPS1 and RalGDS that affected cell spreading of WTMEFs (Figure 5.5C, 5.5D- WTMEFs) did not affect the spreading of Cav1 -/- MEFs (Figure 5.5C, 5.5D- Cav1-/- MEFs) suggesting their regulation of cell spreading to be mediated through a RalA-exocyst dependent pathway in WTMEFs. We hence next evaluated the role these RalGEFs have in mediating adhesion dependent Ral activation. We first tested and found the combined knockdown of RalGEFs- RGL1, RalGPS1 and RalGDS (Figure 5.6A) specifically

disrupted adhesion dependent RalA (but not RalB) activation (Figure 5.6B. 5.6C). We extended this study to ask if one of these GEFs more prominently regulated RalA activation, as is seen in cell spreading. Our experiments revealed that loss of RGL1 (Figure 5.7A, 5.7D) had the most prominent and significant effect on RalA activation (Figure 5.7B, 57C, 5.7E, 5.7F) unlike loss of RalGDS and RalGPS1, agreeing with its distinctly pronounced effect on cell spreading. The effect of RGL1 knockdown on RalA activation was strongest at 10 minutes (Figure 5.8A, 58B) after replating and reduced at 20 minutes (Figure 5.8C); corroborating the role RalA is known to have in early re-adherent cell spreading (Balasubramanian et al., 2010). RGL1 interestingly did not regulate RalB activation (Figure 5.8A, 5.8B, 5.8C), which agrees with it not being involved in re-adherent cell spreading (Balasubramanian et al., 2010). Further we found RGL1 knockdown to affect cell spreading only in WT MEFs and not in Caveolin1 -/- MEFs (Figure 5.9A, 5.9B, 5.9C) indicating RGL1 working along the integrin-RGL1-RalA pathway.
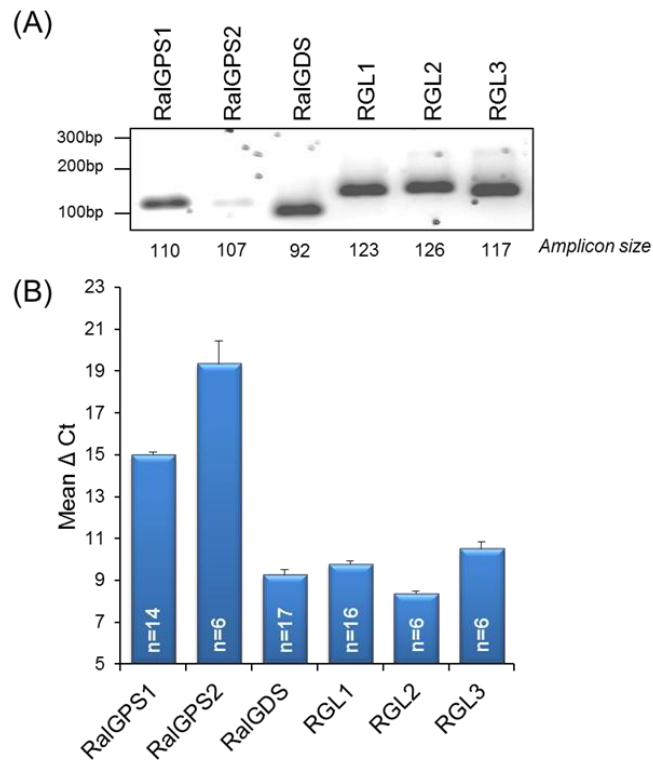
**Figure 5.2: Ral Guanine nucleotide exchange factors (RalGEFs) mRNA expression profile in mouse embryonic fibroblasts (MEFs). (A)** Agarose gel for PCR products from MEFs cDNA amplified using specific RalGEF primers showing relative expression and single specific amplicons of RalGEFs (amplicon size mentioned below each band). **(B)** Relative expression of RalGEFs in MEFs from quantitative PCR reaction using the same primers. Graph represents mean delta Ct values ± standard error data from minimum of six and maximum of seventeen independent experiments as indicated on each bar.
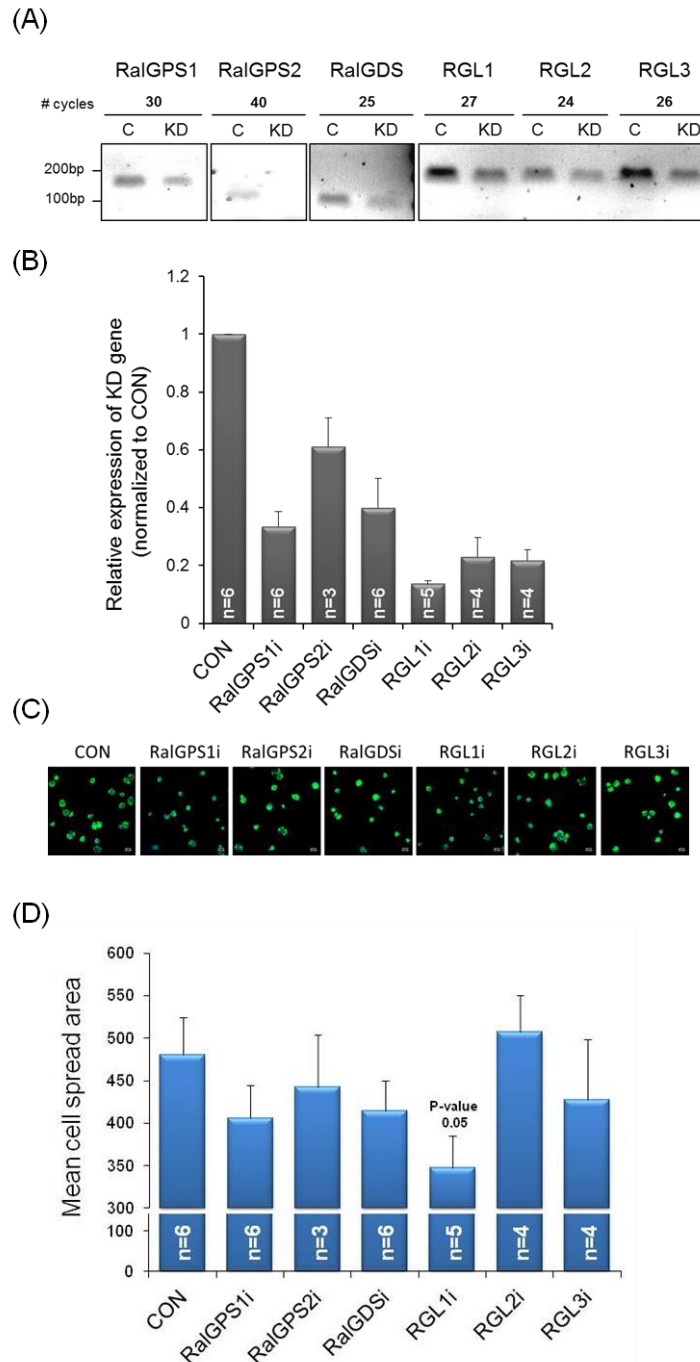
**Figure 5.3: Effect of SMARTpool siRNA mediated knockdown of individual RalGEFs on adhesion dependent cell spreading in MEFs. (A)** Agarose gel for semi-quantitative PCR for knockdown (KD) of individual RalGEFs using SMARTpool siRNA in MEFs relative to control MEFs- C. Numbers of amplification cycles used for semi-quantitative PCR for each RalGEF gene are indicated. **(B)** Relative transcript levels of individual RalGEFs upon knockdown in MEFs determined by quantitative PCR. Actin was used as endogenous control. Error bars represents standard error data from minimum of three and maximum of six independent experiments. **(C)** Representative images and **(D)** quantitation from cell spreading assay in control MEFs (CON) and upon knockdown of RalGPS1 (RalGPS1i), RalGPS2 (RalGPS2i), RalGDS (RalGDSi), RGL1 (RGL1i), RGL2 (RGL2i) and RGL3 (RGL3i) fixed at early spreading time point upon re-adhesion on 2 µg/ml FN coated coverslips and stained with phalloidin-Alexa-488 and DAPI. Graph represents mean cell spread area ± standard error data from minimum of three and maximum of six independent experiments. Statistical analysis was done using the two tailed one sample T-test and significance represented (* p- value <0.05, ** p-value <0.01).
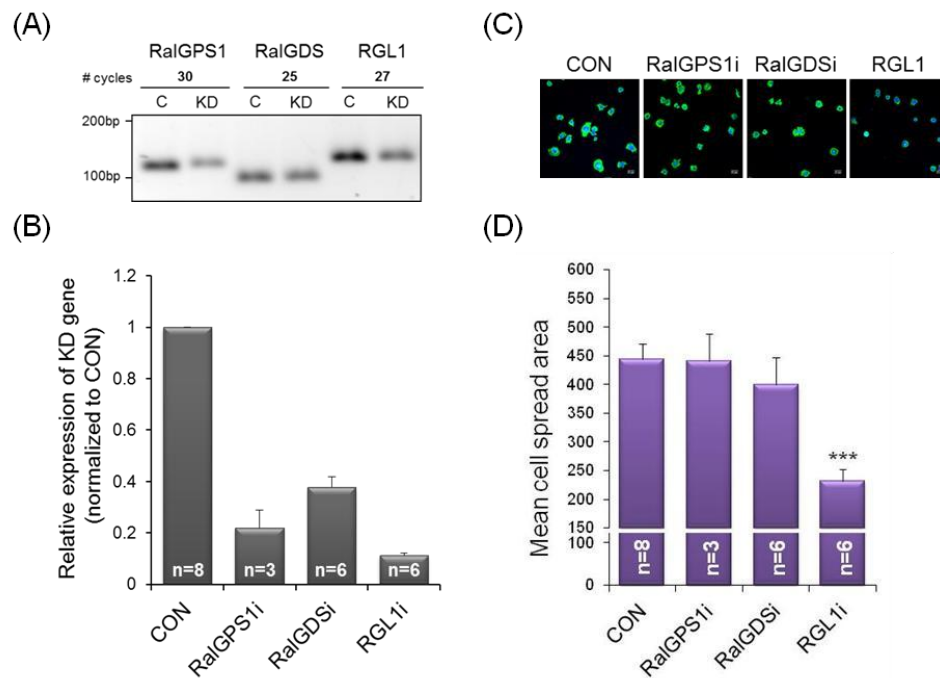
**Figure 5.4: Effect of single siRNA duplex mediated knockdown of RalGPS1, RalGDS and RGL1 on adhesion dependent cell spreading in MEFs. (A)** Agarose gel for semi-quantitative PCR for knockdown (KD) of RalGPS1, RalGDS and RGL1 in MEFs using single siRNA duplex against each gene relative to control MEFs- C. Numbers of amplification cycles used for semi-quantitative PCR for each RalGEF gene are indicated. **(B)** Relative transcript levels of RalGPS1, RalGDS and RGL1 upon knockdown in MEFs determined by quantitative PCR relative to control MEFs (CON). Actin was used as endogenous control. Error bars represents standard error data from minimum of three and maximum of eight independent experiments. **(C)** Representative images and **(D)** quantitation from cell spreading assay in control MEFs (CON) and upon knockdown of RalGPS1 (RalGPS1i), RalGDS (RalGDSi) and RGL1 (RGL1i) fixed at early spreading time point and stained with phalloidin-Alexa-488 and DAPI. Graph represents mean cell spread area ± standard error data from minimum of three and maximum of eight independent experiments. Statistical analysis was done using the two tailed one sample T-test and significance represented (* p value <0.05).
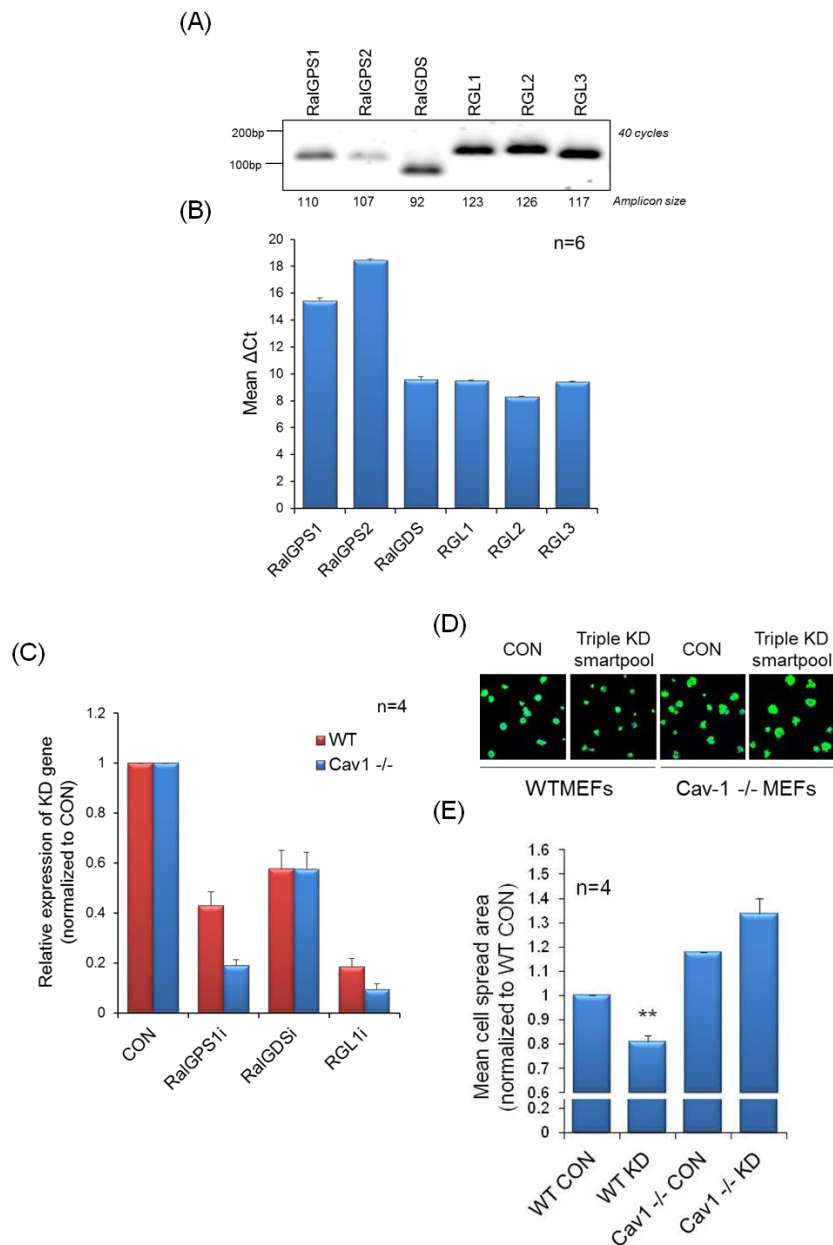
**Figure 5.5: Effect of combined SMARTpool siRNA mediated knockdown of RalGPS1 + RalGDS + RGL1 on adhesion dependent cell spreading in WT MEFs and Cav1 -/- MEFs.** **(A)** Agarose gel for PCR products from Cav1 -/- MEFs cDNA amplified using specific RalGEF primers showing relative expression and single specific of RalGEFs amplicons (amplicon sizes mentioned below each band). (B) Relative expression of RalGEFs in Cav1 -/- MEFs from quantitative PCR reaction using the same primers. Graph represents mean delta Ct values ± standard error data from six independent experiments **(C)** Relative transcript levels of RalGPS1, RalGDS and RGL1 upon their combined knockdown using SMARTpool siRNA determined by quantitative PCR in WT MEFS (red bars) and Cav1 -/- MEFs (blue bars). Actin was used as endogenous control. Error bars represents standard error data from four independent experiments. **(D)** Representative images and **(E)** quantitation from cell spreading assay in control WT MEFs (WT CON), control Cav1 -/- MEFs (Cav1 -/- CON) and upon combined knockdown of RalGPS1 + RalGDS + RGL1 in WT MEFs (WT KD) and Cav1 -/- MEFs (Cav1 -/- KD) fixed at early spreading time point and stained with phalloidin-Alexa-488 and DAPI. Graph represents mean cell spread area ± standard error data from four independent experiments. Statistical analysis was done using the two tailed one sample T-test and significance represented (** p-value <0.01).
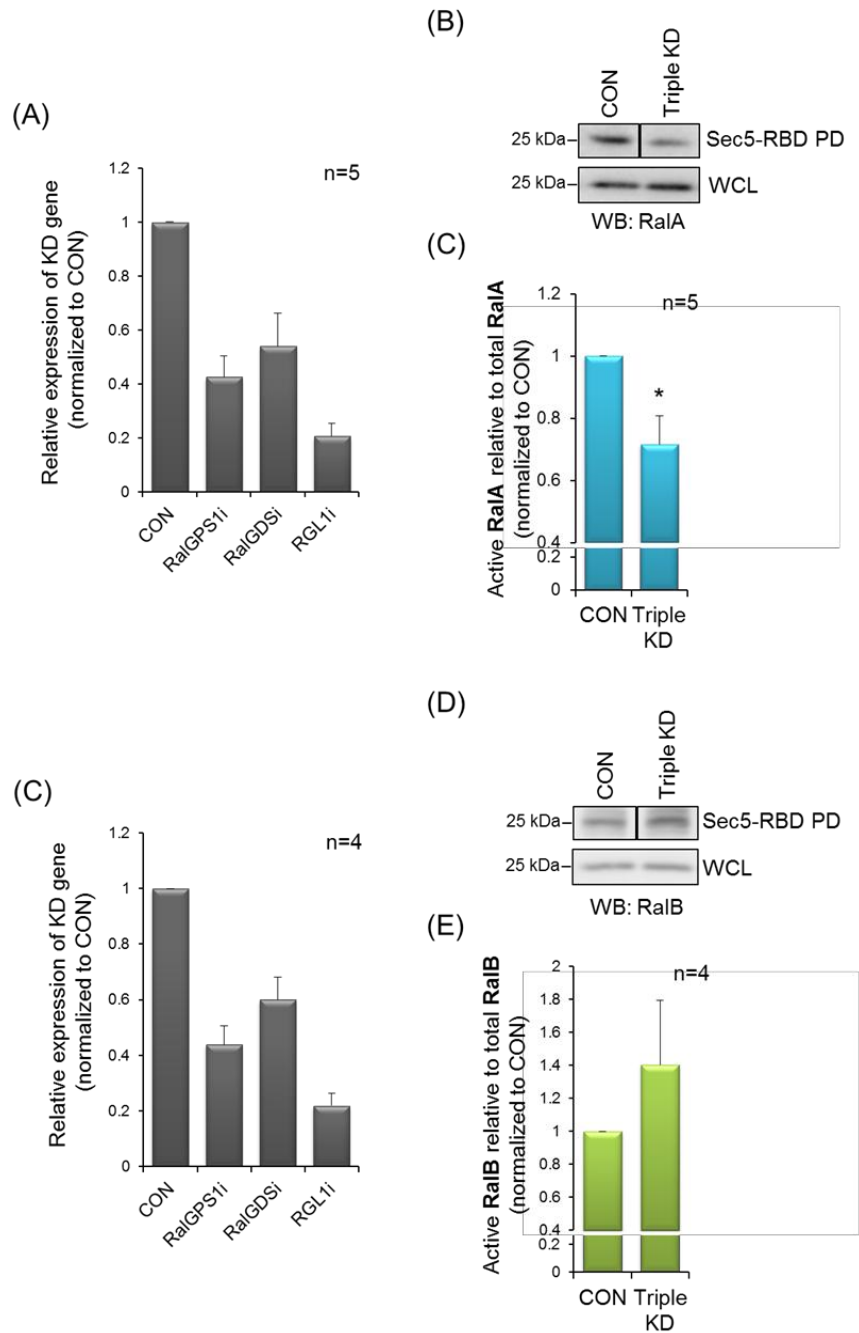
**Figure 5.6: Effect of combined SMARTpool siRNA mediated knockdown of RalGPS1 + RalGDS + RGL1 on adhesion dependent RalA and RalB activation in MEFs. (A)** Relative transcript levels of RalGPS1, RalGDS and RGL1 upon their combined knockdown in MEFs using SMARTpool siRNA determined by quantitative PCR. Actin was used as endogenous control. Error bars represent standard error data from six independent experiments. **(B)** Western blot and **(C)** quantitation of active RalA (cyan bars) and RalB ( green bars) pulled down by GST-Sec5-RBD (Sec5-RBD PD) and total RalA/RalB in the respective whole cell lysate (WCL) from re-adherent control MEFs (CON) and upon combined knockdown of RalGPS1 + RalGDS + RGL1 (Triple KD). Percentage active RalA and RalB levels were calculated as described in methods and normalized to respective CON. Graph represents mean ± standard error data from minimum of four and maximum of six independent experiments. Statistical analysis was done using two tailed one sample T-test and significance represented as p-value above the bar.
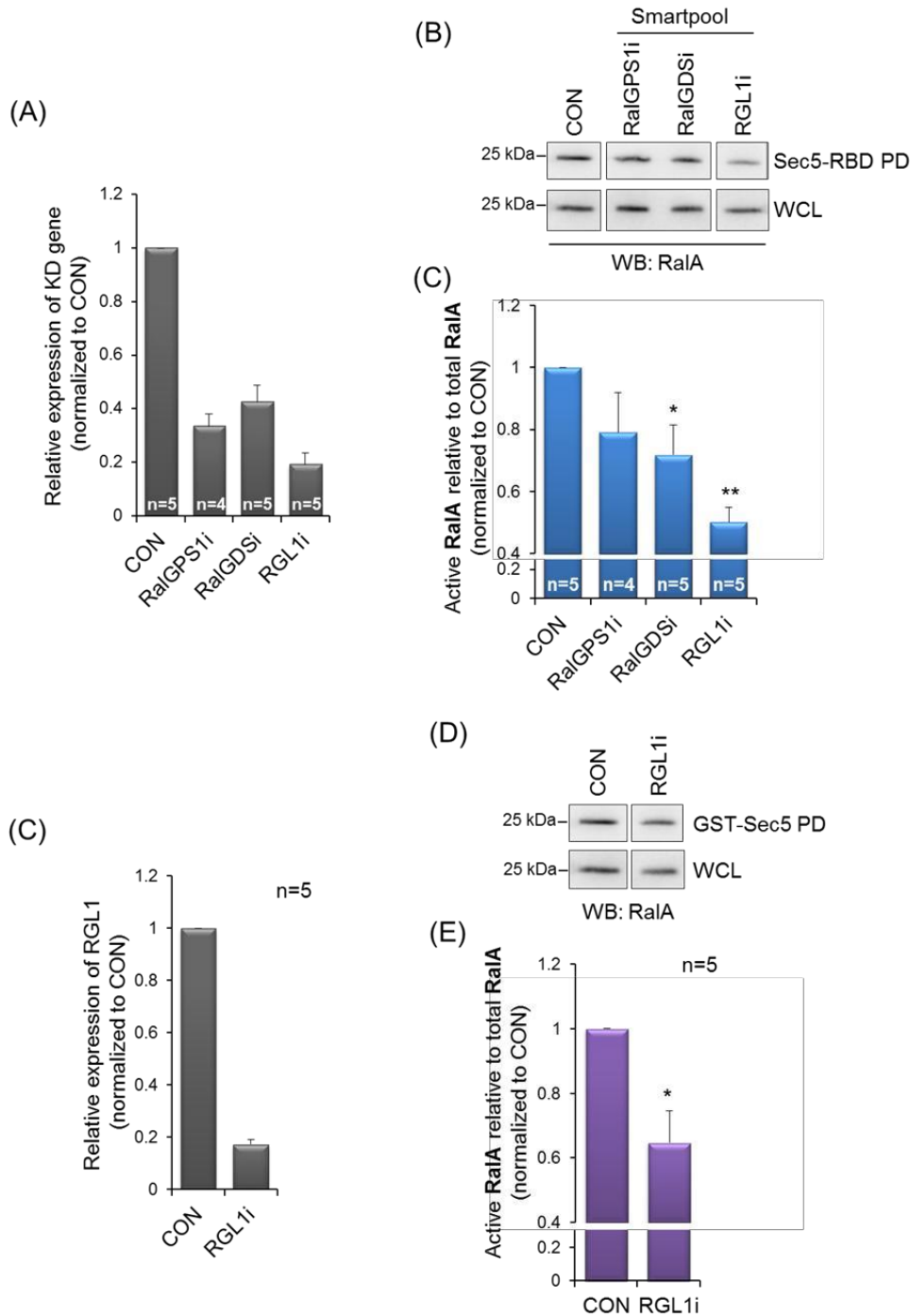
**Figure 5.7: Effect of individual knockdown of RalGPS1, RalGDS and RGL1 on adhesion dependent RalA activation in MEFs. (A)**, **(D)** Relative transcript levels of RalGPS1, RalGDS and RGL1 upon their individual knockdown in MEFs using **(A)** SMARTpool siRNA or **(D)** single siRNA oligo against RGL1, determined by quantitative PCR. Actin was used as endogenous control. Error bars represent standard error data from five independent experiments. **(B)**,**(E)** Western blot and **(C)**,**(F)** quantitation of active RalA pulled down by GST-Sec5-RBD (Sec5-RBD PD) and total RalA in the respective whole cell lysate (WCL) from re-adherent control MEFs (CON) and upon **(B,C)** individual SMARTpool siRNA knockdown of RalGPS1 (RalGPS1i), RalGDS (RalGDSi) and RGL1 (RGL1i) or **(E,F)** single siRNA knockdown of RGL1 (RGL1i). Percentage active RalA levels were calculated as described in methods and normalized to respective CON. Graphs represent mean ± standard error data from minimum of four and maximum of six independent experiments. Statistical analysis was done using two tailed one sample T-test and significance represented (* p-value <0.05, ** p-value <0.01 or p-value as indicated above the bar.).
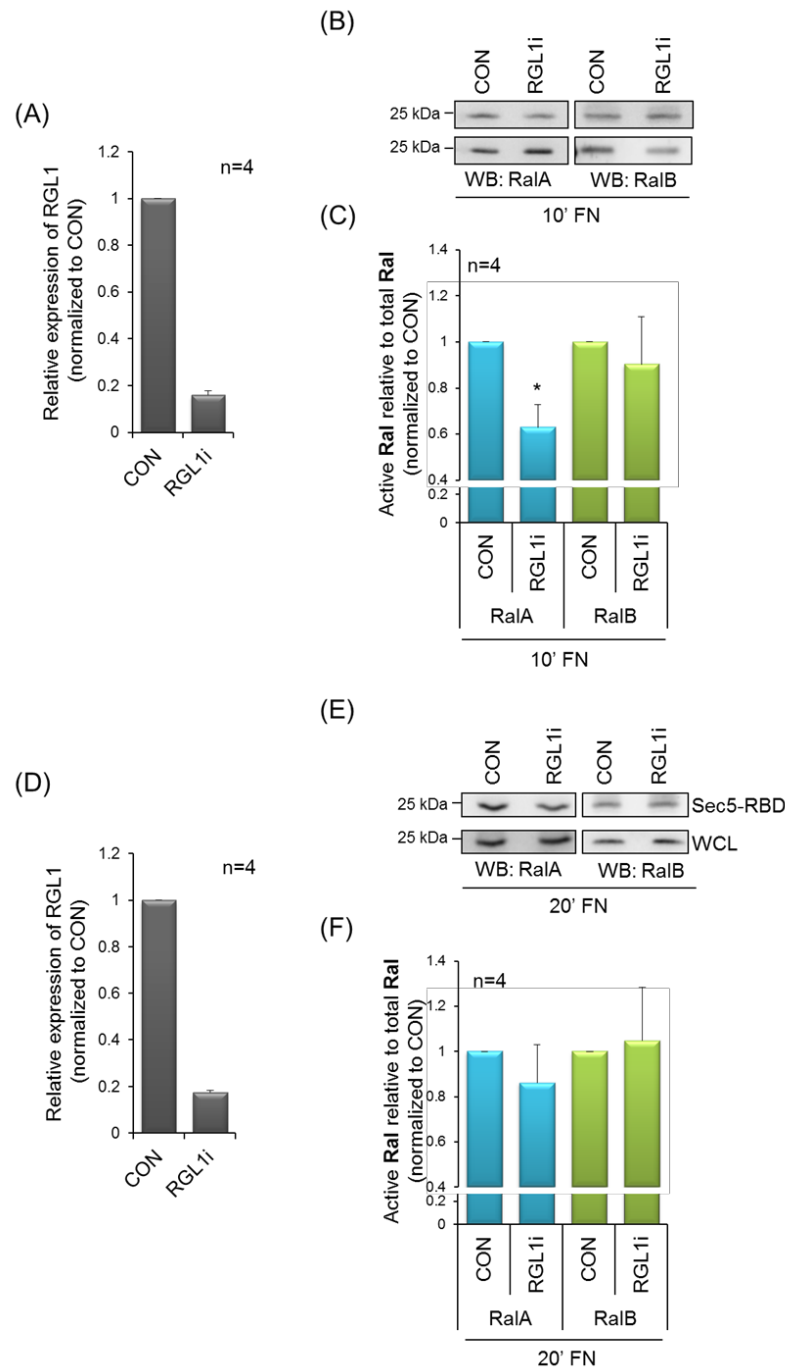
**Figure 5.8: Effect of SMARTpool siRNA mediated knockdown of RGL1 on adhesion dependent RalA and RalB activation in MEFs. (A)** Relative transcript levels of RGL1 upon knockdown in MEFs using SMARTpool siRNA determined by quantitative PCR. Actin was used as endogenous control. Error bars represent standard error data from seven independent experiments. **(B), (C)** Western blot (upper panel) and quantitation (lower panel) of active RalA/RalB pulled down by GST-Sec5-RBD (Sec5-RBD PD) and total RalA/RalB in the respective whole cell lysate (WCL) from control MEFs (CON) and upon knockdown of RGL1 (RGL1i RalA/ RGL1i RalB) in cells re-adhered on FN for **(B)** 10 minutes (10'FN) or **(C)** 20 minutes **(**20'FN**)**. Percentage active Ral levels were calculated as described in methods and normalized to respective CON. Graphs represent mean ± standard error data from minimum of three and maximum of six independent experiments. Statistical analysis was done using two tailed one sample T-test and significance represented as p-value above the bar.
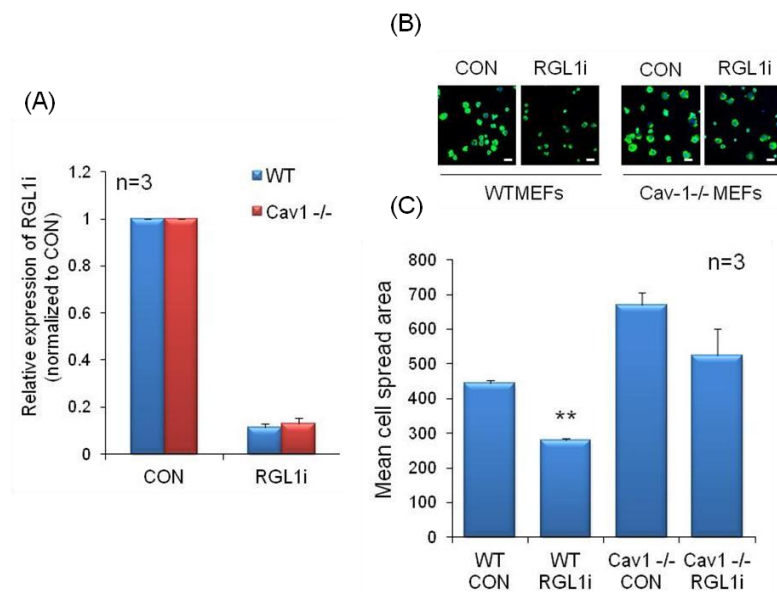
**Figure 5.9: Effect of SMARTpool siRNA mediated knockdown of RGL1 cell spreading in WT MEFs and Cav1 -/- MEFs. (A)** Relative transcript levels of RGL1 upon its knockdown using individual siRNA determined by quantitative PCR in WT MEFS (red bars) and Cav1 -/- MEFs (blue bars). Actin was used as endogenous control. Error bars represents standard error data from three independent experiments. **(B)** Representative images and **(C)** quantitation from cell spreading assay in control WT MEFs (WT CON), control Cav1 -/- MEFs (Cav1 -/- CON) and upon RGL1 knockdown in WT MEFs (WT KD) and Cav1 -/- MEFs (Cav1 -/- KD) fixed at early spreading time point and stained with phalloidin-Alexa-488 and DAPI. Graph represents mean cell spread area ± standard error data from three independent experiments. Statistical analysis was done using the two tailed one sample T-test and significance represented (* p-value <0.05).

## 5.3.2 Differential localization of Ral isoforms mediates their differential activation by RGL1 and role in cell spreading

To further confirm the role of each Ral isoform in mediating the integrin-RGL1-Ral pathway in MEFs, we tested the ability of constitutively active RalA and RalB to rescue the defective spreading of RGL1 knockdown MEFs. When expressed in RGL1 knockdown cells active RalA (RalA-V23) restored cell spreading, but not active RalB (RalB-V23), further emphasizing this regulatory pathway to be isoform specific (Figure 5.10). This could be the result of the differential localization of RalA and RalB mediated by their hypervariable C-terminal tail region (residues 176 to 206) as has been seen for Ras isoforms- H-Ras and K-Ras (Karnoub and Weinberg, 2008; Shipitsin and Feig, 2004). Localization of Ral is in turn thought to mediate its interaction with a unique set of upstream and downstream binding partners, including RalGEF proteins (Lim et al., 2005). The ability of RGL1 to specifically activate RalA and not RalB could hence also be a result of such a localization mediated association in re-adherent MEFs. To test this we made use of known active RalA/RalB tail switch mutants (Lim et al., 2005) where their C-terminus tails are exchanged and reported to switch their subcellular localization (Shipitsin and Feig, 2004) and isoform specific function in basolateral membrane delivery (Shipitsin and Feig, 2004) and anchorage independence of transformed cells (Lim et al., 2005). Expression of active RalB72L/A mutant (RalB with the tail of RalA) in RGL1 depleted MEFs rescued the cell spreading defect (unlike active RalB itself) whereas active RalA72L/B mutant did not (unlike active RalA) (Figure 5.11). These results suggest that the localization of Ral isoforms is critical for their differential activation by RGL1 and function in re-adherent cells. We hence tested and found endogenous RalA, detected using an anti-RalA antibody, to co-localize with Myc tagged RGL1 in plasma membrane ruffles and filopodial extensions in actively spreading re-adherent MEFs (Figure 5.12A, 5.12B). Interestingly RGL1 also localizes with focal adhesions markers, as does RalA (Kuo et al., 2011) which are known sites for integrin clustering and signalling (Figure 5.11C, 5.11D). When stained along with actin and observation closely, RGL1 localized to both membrane ruffling edges and to tips of focal adhesions (Figure 5.12E, 5.12F).
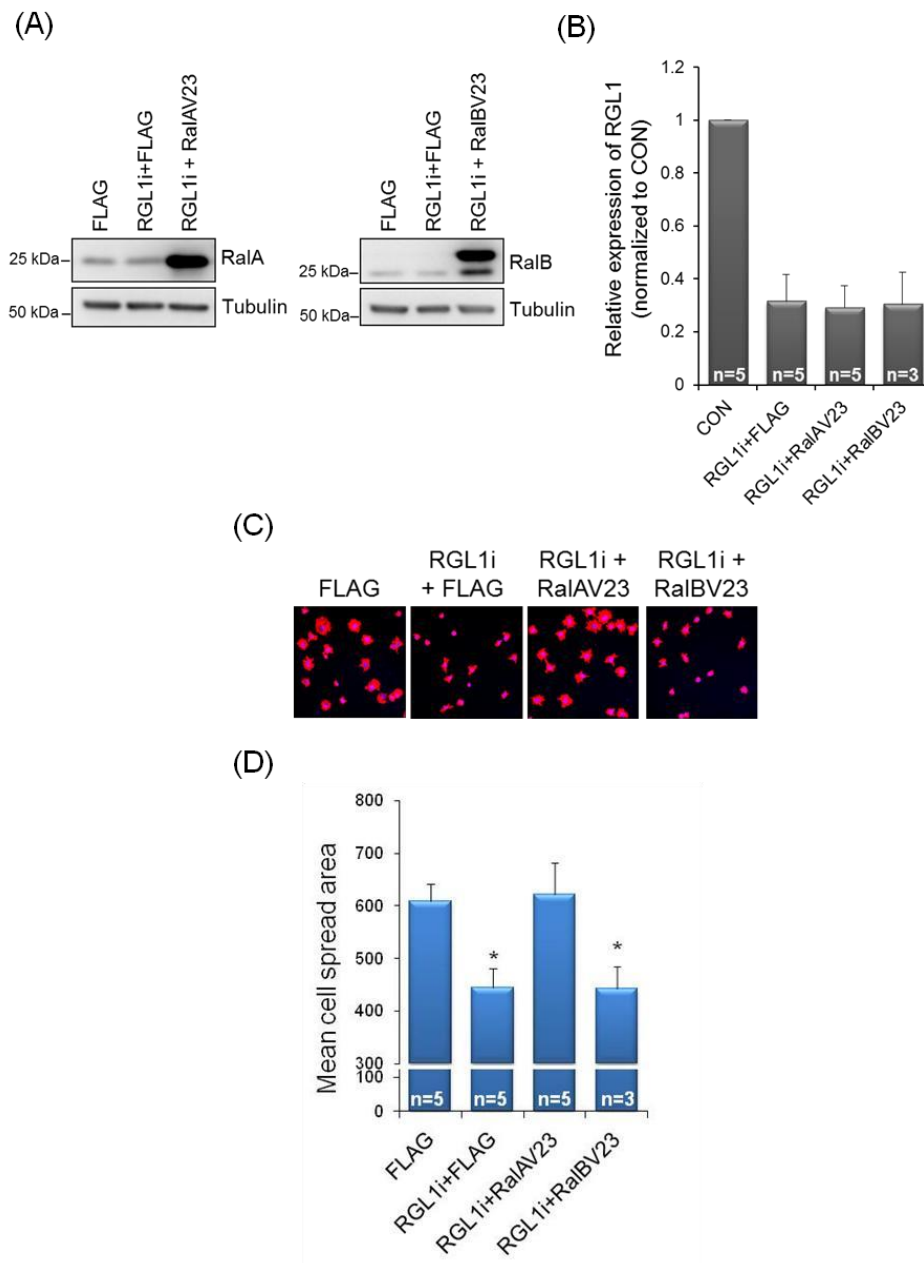
**Figure 5.10: Expression of active RalA, and not active RalB, rescues cell spreading defect induced upon RGL1 knockdown in MEFs.** **(A)** Representative western blots for expression of untagged active RalA (RalAV23) and FLAG-tagged active RalB (RalBV23) in RGL1 knockdown MEFs. Tubulin was used as loading control. **(B)** Relative transcript levels of RGL1 upon knockdown in MEFs using SMARTpool siRNA determined by quantitative PCR. Actin was used as endogenous control. Error bars represent standard error data from minimum of three and maximum of five independent experiments. **(C)** Representative images and **(D)** quantitation from cell spreading assay in control MEFs (CON), RGL1 knockdown MEFs (RGL1i) and RGL1 knockdown MEFs expressing active RalA (RGL1i + RalAV23) or active RalB (RGL1i + RalBV23) fixed at early cell spreading time point and stained with phalloidin-Alexa-488 and DAPI. Graph represents mean cell spread area $\pm$ standard error data from minimum of three and maximum of five independent experiments. Statistical analysis was done using two tailed one sample T-test and significance represented (** p-value<0.01).
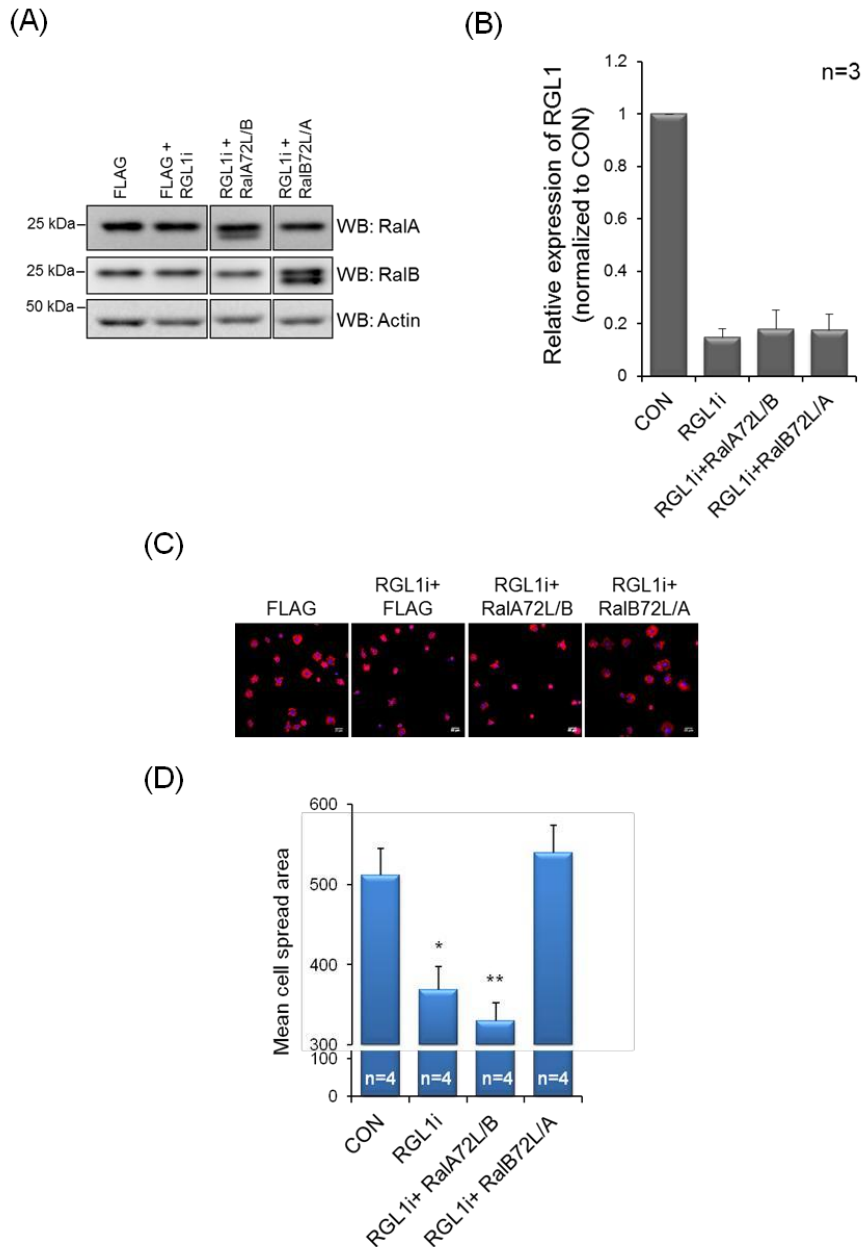
**Figure 5.11: Expression of tail switch mutant of active RalB, and not active RalA, rescues cell spreading defect induced upon RGL1 knockdown in MEFs.** **(A)** Representative western blots for expression of tail switch mutants of active RalA (RalA72L/B) and active RalB (RalB72L/A) in RGL1 knockdown MEFs. Actin was used as loading control. **(B)** Relative transcript levels of RGL1 upon knockdown in MEFs using a single siRNA duplex are determined by quantitative PCR. Actin was used as endogenous control. Error bars represent standard error for data from three independent experiments. **(C)** Representative images and **(D)** quantitation from cell spreading assay in control MEFs (CON), RGL1 knockdown MEFs (RGL1i) and RGL1 knockdown MEFs expressing RalA72L/B (RGL1i + RalA72L/B) or RalB72L/A (RGL1i + RalB72L/A) fixed at early spreading time point and stained with phalloidin-Alexa-488 and DAPI. Graph represents mean cell spread area ± standard error for data from four independent experiments. Statistical analysis was done using two tailed one sample T-test and significance represented (** p-value<0.01).
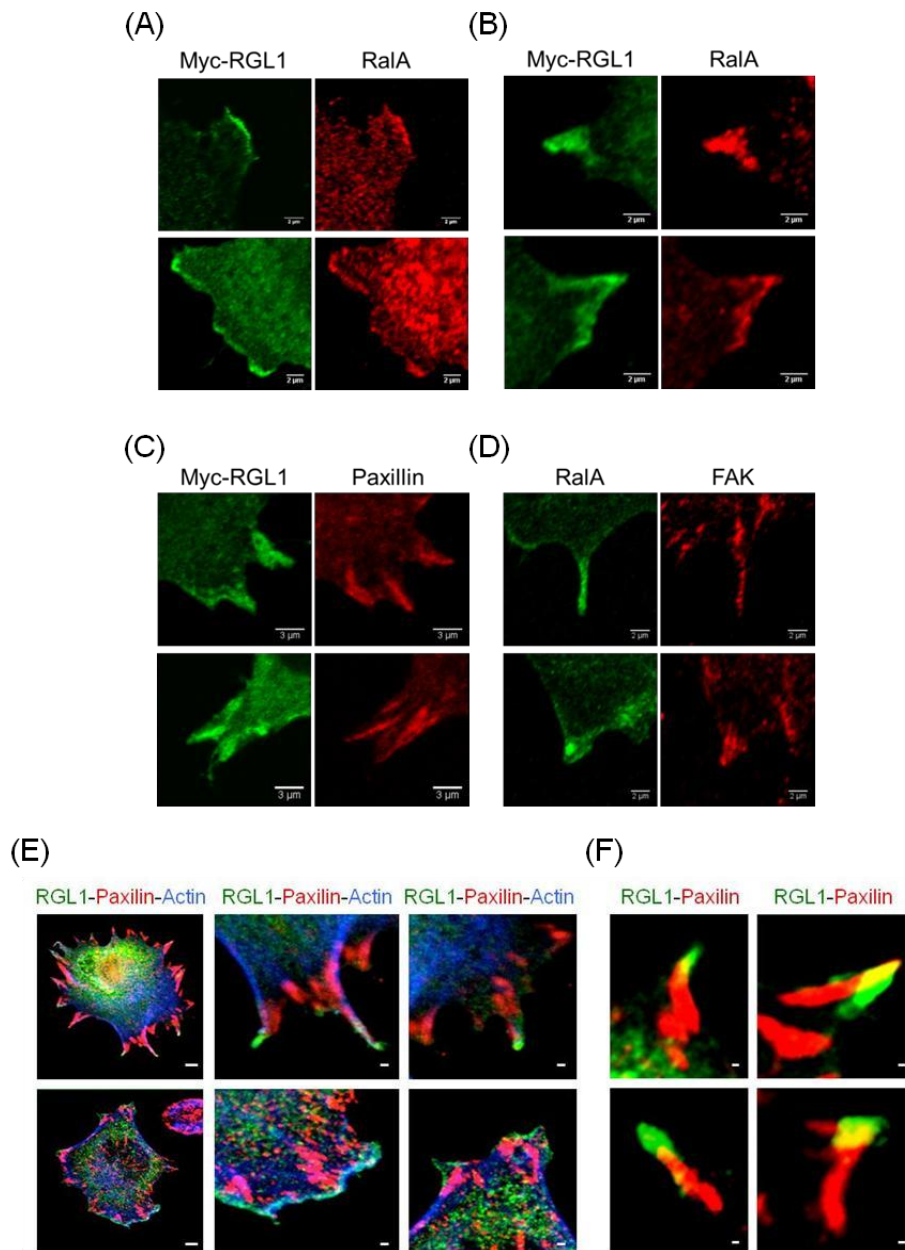
**Figure 5.12: RGL1 co-localizes with RalA and focal adhesion markers in actively spreading re-adherent MEFs. (A)**, **(B)** Representative images of MEFs expressing Myc-RGL1 re-adherent on FN for 15mins and stained with anti Myc and anti RalA antibody. RGL1 is seen to co-localize with endogenous RalA **(A)** at plasma membrane ruffling and **(B)** protrusive edges. **(C)**, **(D)** Representative images of coverslips from the above experiment stained with **(C)**, **(F)** Myc and paxillin antibodies or **(D)** RalA and FAK antibodies or **(E)** Myc and paxillin antibodies along with Phalloidin to satin actin. Images show RGL1 and RalA localize to regions enriched in focal adhesion markers and RGL1 to localize to both plasma membrane ruffles and focal adhesions but with only a partial overlap with focal adhesions. Data is representative of two independent experiments.

### 5.3.3 Role of RGL1 in regulation of oncogenic Ras dependent RalA activation

Integrin driven RalA activation controls membrane raft trafficking which in turn regulates anchorage dependent growth signalling in MEFs (Balasubramanian et al., 2010; del Pozo et al., 2005). Downstream of oncogenic H-Ras (V12), activation of RalA (not RalB) drives anchorage independence in Ras-transformed HEK-HT cells (Lim et al., 2005). Our data so far indicates that Ras dependent RalGEF- RGL1 specifically activates RalA and not RalB downstream of integrins. We hence asked if RGL1 could have a role in regulating anchorage independent RalA activation in cancers downstream of oncogenic Ras.  Such a role for RGL1 in cancers has not been reported earlier making this of particular interest. Since RalGDS is the known major mediator of Ral activation downstream of Ras-V12 in transformed cells (White et al., 1996) and oncogenic Ras expressing hepatocellular carcinoma and ovarian cancer cells (Ezzeldin et al., 2014; Wang et al., 2013), we analyzed compared the relative roles of RalGDS and RGL1 in these cells. We first compared the relative expressions of RGL1 and RalGDS in multiple oncogenic Ras expressing cancer cell lines by quantitative PCR. Of the cancer cells studied MIA-PaCa-2, HCT116, PC3, UM-UC-3, DU145 and SKOV3 expressed more RalGDS than RGL1 whereas Calu-1, SW620, T-24, HT1080, MDA-MB-231 and U87MG showed comparable levels of RalGDS and RGL1 (Figure 5.13A). We chose one representative cell line from either group (MIA-PaCa-2 and SW620) to further evaluate the relative role of RGL1 in context of RalGDS (Figure 5.13B, 5.13C). In oncogenic K-Ras expressing pancreatic cancer MIA-PaCa-2 cells, we observed adhesion independent Ral activation to not be regulated by RGL1 (Figure 5.14A, 5.14B, 5.14C).  Considering this might be mediated by the prominent expression of RalGDS in these cells we tested and found RalGDS regulates RalA and RalB activation in these cells (Figure 5.13A, 5.13B, 5.13C). In colorectal adenocarcinoma SW620 cells however RGL1 regulated anchorage independent activation of RalA (but not that of RalB) (Figure 5.14D, 5.14E, 5.14F). Interestingly, studies by Martin et al have demonstrated that in SW620 cells, loss of RalA, not RalB, inhibits anchorage independent growth in soft agar colony assays (Martin et al., 2011) which we can speculate could be driven by RGL1 dependent RalA (not RalB) activation. Despite multiple attempts we could only get a 30% knockdown of RalGDS with multiple siRNAs in SW620 cells making it difficult to interpret the effect on Ral activation in RalGDS knockdown SW620 cells. Functionally we found loss of RGL1 to affect anchorage independent Erk signalling in SW620 cells as did loss of RalA, but not that of RalB (Figure 5.15). This further emphasized the role of RGL1 in differential activation of RalA and RalB leading to their

differential function in cells. Our ongoing studies aim to establish stable RGL1 knockdown cells and evaluate the role it has in anchorage independent growth in SW620 cells.
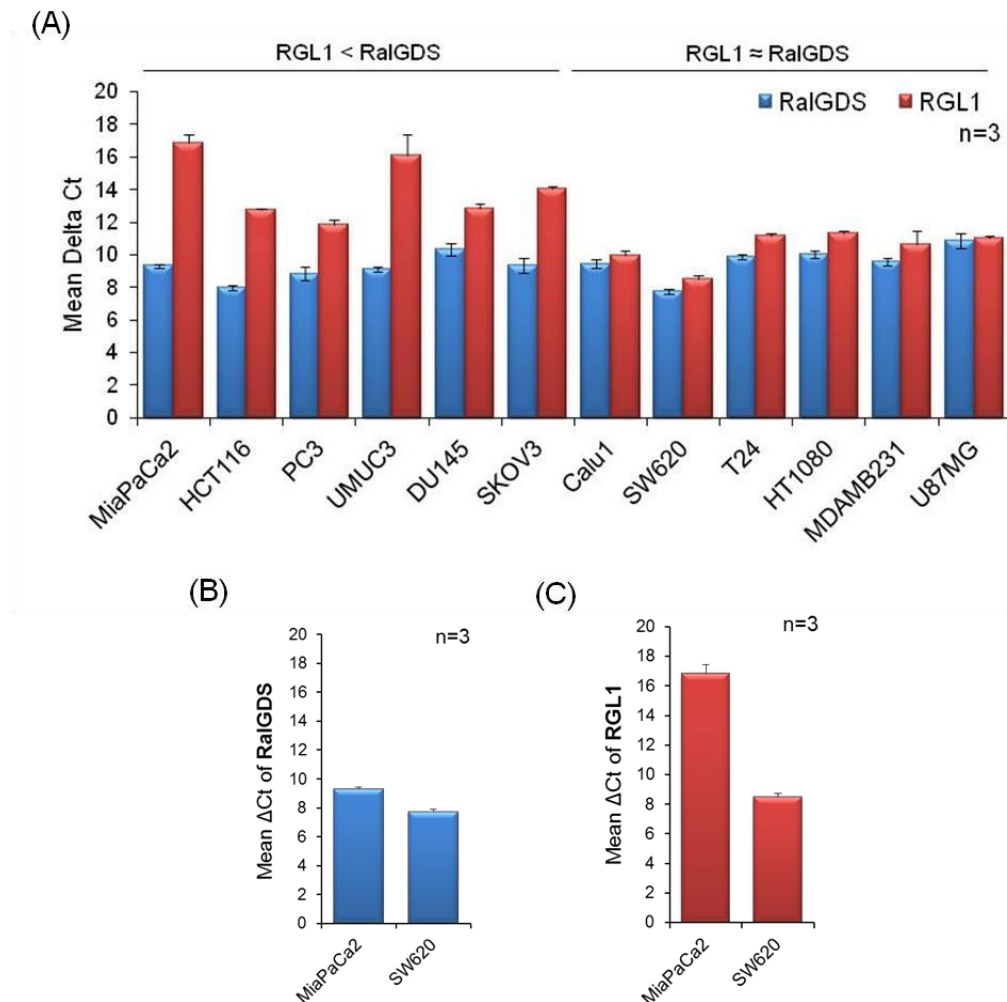


**Figure 5.13: Relative expressions of RGL1 and RalGDS in cancer cells. (A)** Relative transcript levels of RalGDS (blue bars) and RGL1 (red bars) in cancer cell lines MiaPaCa2, HCT116, PC3, UM-UC-3, DU145, SKOV3, Calu1, SW620, T-24, HT1080, MDA-MB-231, U87MG from quantitative PCR reaction using the specific RalGDS and RGL1 primers. Actin was used as endogenous control. Graph represents mean delta Ct values ± standard error data from three independent experiments. Based on expression profile cell lines are arranged in two groups- one with RGL1 expression lesser than RalGDS (RGL1 < RalGDS) and other RGL1 ≈ RalGDS. **(B), (C)** Relative transcript levels of **(B)** RalGDS (blue bars) and **(C)** RGL1 (red bars) in MiaPaCa2 and SW620 cancer cells from **(A)** represented to highlight similarity and differences in RalGDS and RGL1 expression between these cancer cell lines. MiaPaCa2 and SW620 are chosen representative of the two groups for further experiments.

(A)

(B)

(C) Non-adherent MiaPaca2

(D) Non-adherent SW620

(E)

(F) Non-adherent MiaPaca2

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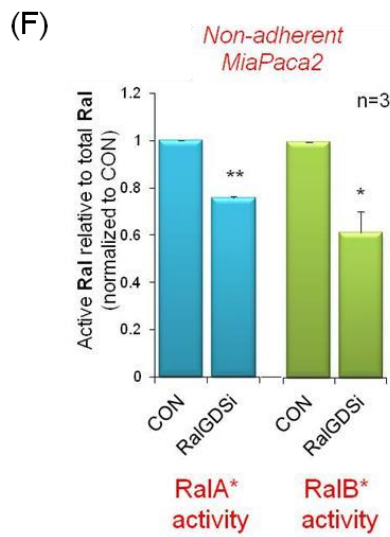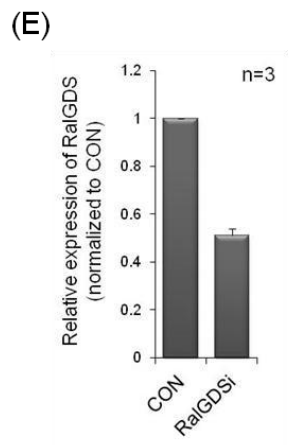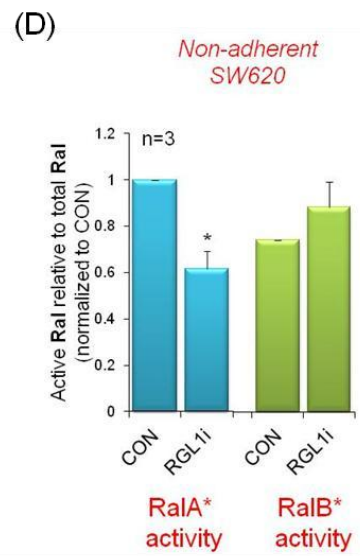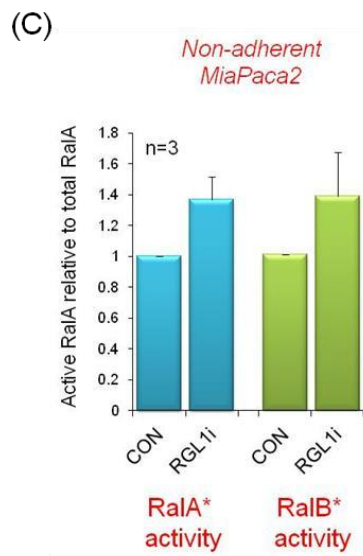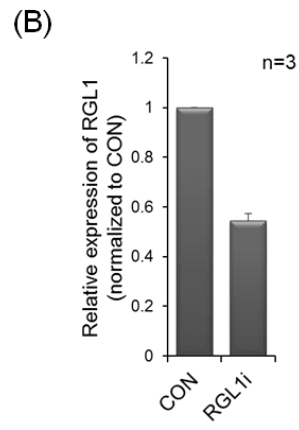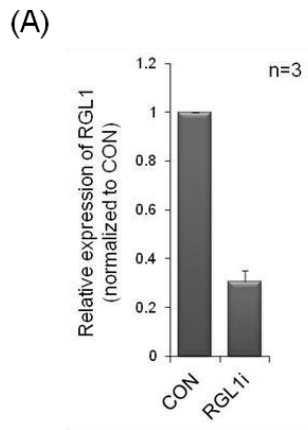
**Figure 5.14: RGL1 regulates anchorage independent RalA (not RalB) activation in SW620 colorectal cancer cell line (A)**, **(D)** Relative transcript levels of **(A)** RalGDS and RGL1 upon knockdown in MiaPaCa2 and **(D)** of RGL1 upon knockdown in SW620 cells determined by quantitative PCR. Actin was used as endogenous control. Error bars represents standard error data from three independent experiments. **(B,C)**, **(E,F)** Quantitation of active RalA/RalB pulled down by GST-Sec5-RBD (Sec5-RBD PD) and total RalA/RalB in the respective whole cell lysate (WCL) from **(B,C)** control (CON), RalGDS knockdown (RalGDSi) and RGL1 knockdown (RGL1i) in MiaPaCa2 cells and **(E,F)** CON and RGL1i in SW620 cells suspended for 120 minutes. Percentage active RalA/RalB levels were calculated as described in methods and normalized to respective CON. Graphs represent mean ± standard error data from three independent experiments. Statistical analysis was done using two tailed one sample T-test and significance represented (* p-value<0.05, ** p-value<0.01 or p-value represented above the bar).
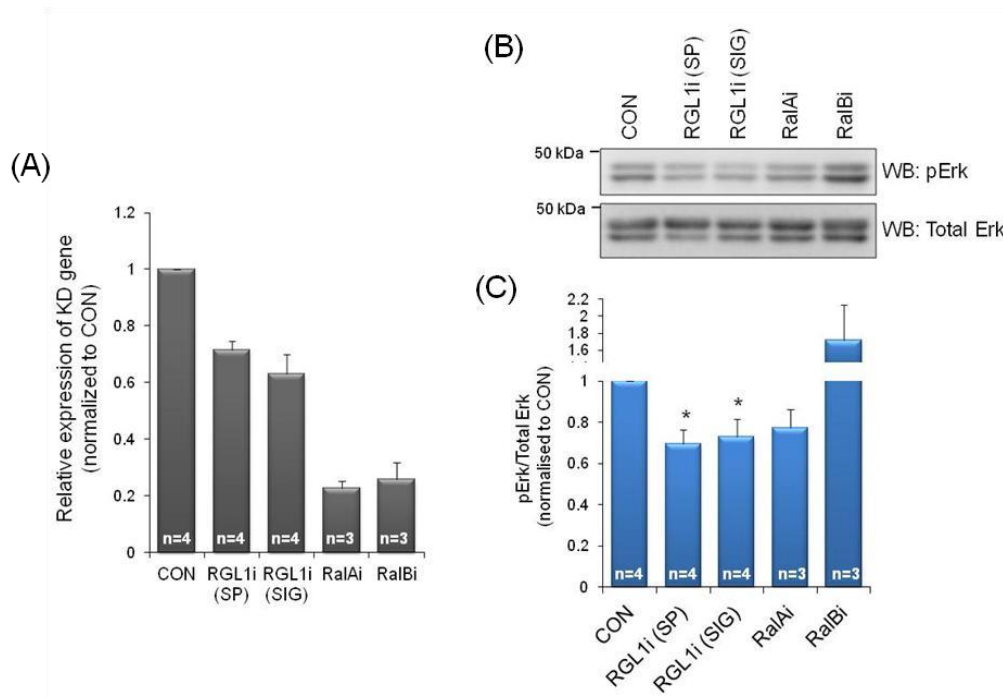
**Figure 5.15: RGL1 regulates anchorage independent Erk signalling in SW620 cells.**
**(A) (A)** Relative transcript levels of RGL1 upon knockdown with SMARTpool siRNA
(RGL1i-SP) or individual siRNA (RGL1i-SIG) and relative transcript levels of RalA and
RalB upon knockdown in SW620 cells determined by quantitative PCR. Actin was used as
endogenous control. Error bars represents standard error data from minimum of three and
maximum of four independent experiments. **(B)** Western blot (upper panel) and
quantitation (lower panel) of Erk1 phosphorylation at Thr202/Tyr204 residues (WB: pErk)
relative to total Erk (WB: total Erk) in whole cell lysates from control (CON), RGL1
knockdown by SMARTpool siRNA (RGL1i-SP), RGL1 knockdown by individual siRNA
(RGL1i-SIG), RalA knockdown (RalAi) and RalB knockdown (RalBi) in SW620 cells that
were held in suspension for 120 minutes with serum. Graph represents mean ± standard
error of the band intensity ratio of pErk/total Erk from minimum of three and maximum of
four independent experiments normalized to CON. Statistical analysis was done using two
tailed single sample T-test (* P-value <0.05).
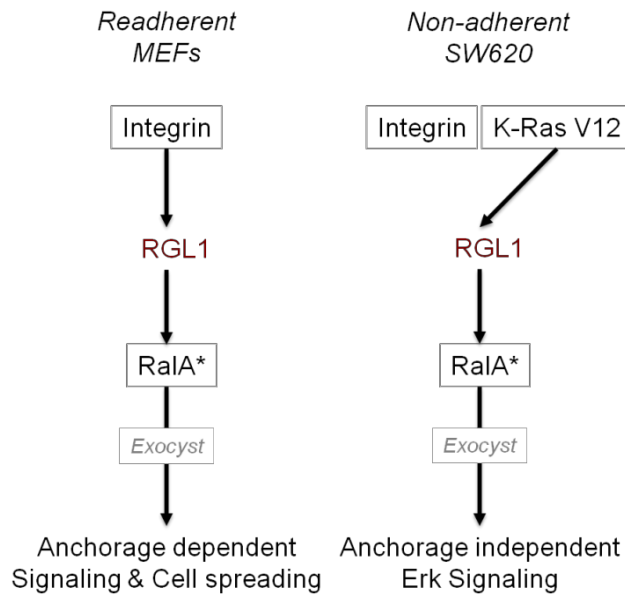
**Figure 5.16: RGL1 mediates RalA activation downstream of integrins and oncogenic Ras.** Schematic depicts role of RalGEF RGL1 as regulator of differential activation of Ral isoforms **(A)** downstream of integrins to mediate adhesion dependent cell spreading in MEFs and **(B)** downstream of oncogenic Ras in colorectal cancer cell line SW-620 which could contribute to its differential role in anchorage independent growth of these cells.

## 5.4 SUMMARY:

We have identified a Ras-dependent RalGEF- RGL1 to be the primary mediator of integrin dependent differential activation of RalA v/s RalB and adhesion dependent cell spreading in MEFs (Figure 5.14A). Spreading of Caveolin-1 null MEFs, known to be insensitive to RalA regulation, was unaffected by RGL1 knockdown further confirming its role. RalGEFs, RalGDS and RalGPS1, only marginally affected WTMEF spreading and their combined knockdown with RGL1 did not have an additive effect. This suggests that they are likely to act through a common downstream mediator, possibly RalA, in re-adherent cells. RGL1 depletion indeed affects adhesion dependent RalA (but not RalB) activation comparable to the joint knockdown of RGL1, RalGDS and RalGPS1. Constitutively active RalA (but not RalB) further reversed the defect in re-adherent cell spreading in RGL1 knockdown cells. RalB with a C-terminus tail of RalA was also able to rescue the cell spreading defect suggesting the differential localization of RalA and RalB to possibly mediate their differential regulation by RGL1. Supporting this speculation, in actively spreading cells we found RalA and RGL1 co-localized in membrane ruffles and focal adhesions. RGL1 was also found differentially regulates anchorage independent RalA (not RalB) activation downstream of Ras in SW620 colorectal cancer cells (Figure 5.14B) suggesting a role for RGL1 in cancers.

## 5.5 CONCLUSION:

**RalGEF RGL1 is an important mediator of the differential activation of RalA v/s RalB, playing a role along anchorage dependent pathways in normal cells and cancer cells.**

# CHAPTER 6:

# Discussion

## 6.1 Ral Arf6 crosstalk in MEFs and cancer cells

### 6.1.1 RalA activated downstream of multiple stimuli regulates Arf6 activation:

Integrin dependent adhesion of cells to extracellular matrix (ECM) regulates activation of RalA and Arf6 which in turn regulates the membrane raft microdomain recycling pathway (Balasubramanian et al., 2007, 2010). The present study explored the interdependence of these GTPases in the context of integrin dependent signalling in normal mouse embryonic fibroblasts (MEFs) and cancer cells. We found that upon re-adhesion of MEFs activation of Arf6 was dependent on RalA, but not vice versa, suggesting the presence of **integrin-RalA-Arf6 regulatory pathway** in MEFs. We find in these studies Arf6 activated downstream of Ral to be needed for active Ral mediated exocytic delivery of raft microdomains at the plasma membrane. However knowing that active Arf6 by itself is not sufficient for this delivery it suggests a role for active Ral with active Arf6 in this pathway (Figure 6.1-Adherent). This hence helps explain why active RalA was necessary and sufficient for regulating membrane raft exocytic trafficking; but active Arf6 was necessary but not sufficient (Balasubramanian et al., 2007, 2010; Pawar et al., 2016).The Ral-Arf6 crosstalk hence ensures co-ordination and spatial regulation of both these GTPases downstream of integrins that links them functionally as mediators of the membrane raft trafficking pathway that helps confer anchorage dependence in cells (Balasubramanian et al., 2007, 2010).

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In re-adherent MEFs, the Ral-Ar6 crosstalk works dependent on integrin engagement and clustering resulting in changes in membrane and cytoskeletal organization that likely provides a framework for RalA, Arf6 and their regulators to spatially come together at the actively ruffling plasma membrane (Honda et al., 1999; Ohta et al., 1999; Radhakrishna et al., 1999). It is possible that RalA activated by other stimuli, such as calcium efflux or Ras
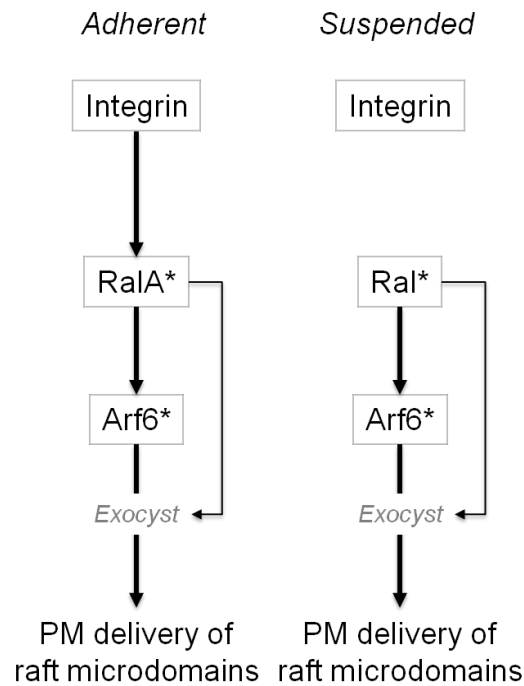
**Figure 6.1: Ral-Arf6 crosstalk and its function in MEFs.** Schematic depicts the linear Integrin-RalA-Arf6 crosstalk identified in this study. Since integrins activate RalA and not RalB, RalA (RalA*) specifically activates Arf6 in adherent cells. However, both active RalA and RalB (Ral*) are capable of inducing integrin independent Arf6 activation in suspended cells. RalA activated Arf6 along with RalA regulates exocyst function to mediate plasma membrane (PM) delivery of raft microdomains.

activation (Hofer et al., 1998; Wolthuis et al., 1998), could also activate Arf6 independent of integrins. To test this possibility we expressed constitutively GTP bound, active mutant of RalA in MEFs and found it to support Arf6 activation in non-adherent cells. This suggests that the presence of active Ral in cells could indeed activate Arf6 independent of adhesion (Figure 6.1- Suspended). This could be mediated by their spatial localization (Balasubramanian et al., 2007, 2010) and/or direct /indirect association in cells (Luo et al., 1998; Xu et al., 2003). This also means a role for Arf6 downstream of active Ral could exist along other pathways that regulate Ral.

As a first step in evaluating this we looked at oncogenic Ras mediated Ral activation in cancer cells and the role for this crosstalk and Arf6 in Ral function (Lim et al., 2005, 2006; Martin et al., 2011). When tested in bladder cancer, pancreatic cancer and fibrosarcoma cell lines we found anchorage independent activation of Arf6 in all these cell lines, though only in T-24 bladder cancer cells (expressing oncogenic H-Ras) was Arf6 activation seen to be Ral

dependent. In these cells RalA differentially regulates Arf6 in the serum starved and with serum conditions, further emphasizing this regulation to be stimulus dependent. Several different stimuli activate Ral in various cellular pathways such as calcium (in dense core granule exocytosis), glucose (in insulin secretion from pancreatic cells), insulin (in GLUT4 trafficking in adipocytes) and Fc-gamma receptor (in phagocytosis) (Chen et al., 2007; Corrotte et al., 2010; Hofer et al., 1998; Ljubicic et al., 2009; Vitale et al., 2005). These pathways are also reported to be regulated by Arf6 (Jayaram et al., 2011; Millar et al., 1999; Niedergang et al., 2003; Vitale et al., 2002). The role such a Ral-Arf6 crosstalk could have in mediating their function along these pathways would particularly be worth exploring and would help further establish the functional relevance of this crosstalk.

Another important regulatory aspect of this crosstalk is that Ral works upstream of Arf6 but not vice versa. It will be worth testing if the linear Ral-Arf6 nature of this crosstalk is conserved along other pathways that Ral and Arf6 regulate and determine how that could influence their functional roles in these pathways.

## 6.1.2 Both Ral isoforms, RalA and RalB can regulate Arf6, dependent on their activation status:

The observation that only RalA, and not RalB its 82% identical isoform, regulated Arf6 activation in re-adherent cells raised the possibility that this crosstalk could be isoform specific. However we know that (a) integrins differentially regulate RalA and RalB (Balasubramanian et al., 2010), (b) that the Ral-Arf6 crosstalk could exist independent of integrins (Pawar et al., 2016) and (c) that RalA and RalB share several common downstream effectors (van Dam and Robinson, 2006). We hence tested if active RalB could also regulate Arf6 activation and found constitutively active RalB could indeed support anchorage independent Arf6 activation. This suggests that both Ral isoforms (RalA and RalB) could possess the capability to regulate Arf6 independent of cell-ECM adhesion. Both Ral isoforms we further found to localize with and comparably bind Arf6 in immunoprecipitation studies. Downstream of oncogenic H-Ras in bladder cancer T-24 cells in low serum conditions, active RalA and RalB both comparably regulate anchorage independent Arf6 activation. These observations while establishing the crosstalk to be functional downstream of both Ral isoforms, does raise the possibility that their differential activation downstream of specific stimuli could differentially regulate Arf6 and downstream signalling. We have already seen

this to be the case downstream of integrins (that activates RalA not RalB) and tested this downstream of H-Ras in bladder cancer T-24 cells. In the presence of serum in T-24 cells RalB is seen to be significantly more active than RalA, which we then found prominently regulate Arf6 activation and resultant downstream Erk signalling. This hence suggests that as we envisage a role for Arf6 through this crosstalk in RalA driven processes, it could also play a role in RalB dependent cellular processes such as exocyst dependent cell migration and cancer cell survival (Chien et al., 2006; Rossé et al., 2006).

## 6.1.3 Role of Arf6 downstream of Ral in mediating its function:

While we identified the existence of a Ral-Arf6 crosstalk, a role for Arf6 in Ral function in cells will make the crosstalk functionally significant. In our studies such a role for Arf6 in Ral function is seen in, (1) Ral-exocyst mediated exocytosis of membrane raft microdomains and (2) Ral mediated anchorage independent growth signalling in cancer cells.

In non- adherent cells, while active RalA is sufficient for exocyst dependent delivery of raft microdomains to the plasma membrane, active Arf6 is not (Balasubramanian et al., 2007, 2010). Arf6 we have now found is needed for active RalA mediate delivery. This suggests the sufficiency of RalA observed in our earlier studies could in part result from its ability to activate Arf6 downstream. Knowing that active Arf6 by itself cannot complete this delivery suggests that the Ral-Arf6 crosstalk and their resulting joint activation to be needed for mediating the exocytic delivery of raft microdomains at the plasma membrane. There are several possible means by which RalA and Arf6 activated downstream can mediate this delivery. Active RalA binds two exocyst subunits- Sec5 and Exo84 and is speculated to facilitate delivery of vesicles by integrating    two exocyst sub-complexes, one plasma membrane anchored (harboring Sec5) and other vesicle anchored (harboring Exo84) (Moskalenko et al., 2003). Arf6 binding to exocyst complex subunits Sec10 (vesicle sub-complex) and Exo70 (plasma membrane sub-complex) could facilitate this process (Fielding et al., 2005; Prigent et al., 2003b). Additional interaction of Arf6 with SCAMP2- a t-SNARE protein at the plasma membrane (Liu et al., 2005) could also aid vesicle fusion at the plasma membrane. Active Arf6 also regulates PIP-5-kinase (Honda et al., 1999) and phospholipase D (Vitale et al., 2002) to increase local PI(4,5)P2 and phosphatidic acid levels that support Sec3

and Exo70 binding (Bendezú et al., 2012; He et al., 2007; Liu et al., 2007) and affect plasma membrane curvature and fluidity facilitating vesicle fusion (Aikawa and Martin, 2003; Bach et al., 2010; Caumont et al., 1998; Honda et al., 1999).

In T-24 bladder cancer cells the Ral-Arf6 regulatory crosstalk is seen to regulate anchorage independent Erk signalling. Loss of Arf6 had the most prominent effect on Erk signalling owing to its working downstream of Ral in the Ras-Ral-Arf6 pathway. Loss of RalA alone did not affect this pathway but its knockdown combined with Arf6 knockdown did substantially affect these pathways. Loss of RalB by significantly affecting Arf6 activation is seen to affect Erk in the presence of serum. This effectively is comparable to the joint RalB+Arf6 knockdown. This we speculate is the result of the fact that in the presence of serum growth factors RalB is more active than RalA and regulates Arf6 activation better than RalA. Taken together these results not only emphasize the role this crosstalk has in regulating this pathway but also highlight the role this could have in mediating Ral isoform specific function in cells.

Earlier studies have shown RalA mediated exocytosis and targeting of membrane rafts to the plasma membrane (which we now know requires downstream activation of Arf6) regulates anchorage independent growth signalling (Balasubramanian et al., 2010). This could indeed be a mechanism for Ral-Arf6 mediated regulation of Erk and Akt signalling in T-24 cells. Loss of Ral and Arf6 in T-24 cells however did not affect GM1 labelled membrane raft microdomain levels. While GM1 is a commonly used marker to label raft microdomains in fibroblasts (Norambuena and Schwartz, 2011; Del Pozo et al., 2004; del Pozo et al., 2005) we and others have seen its relative levels and trafficking in cancers to be variable and cell type dependent (Das et al., 2008; Dong et al., 2010; Furukawa and Fukuda, 2016; Zhang et al., 2006). Distinct heterogeneity in the surface levels of GM1 seen in T-24 cells also means their use as a marker to evaluate changes in surface raft levels could be compromised by the same. Testing membrane raft levels in T-24 cells and their changes in response to Ral-Arf6 depletion using other membrane rafts markers like GFP-GPI, Rac and Caveolin-1 would be needed to know confirm changes in membrane raft levels in T24 cells (Norambuena and Schwartz, 2011).

Studies have demonstrated a role for Arf6 in regulating the Erk pathway in epithelial cells, pre-adipocytes and cancer cells (Davies et al., 2014; Hu et al., 2012; Tague et al., 2004; Zhang et al., 2015). In three dimensional epithelial cell culture model, constitutively active Arf6 expression leads to PLD1 activation that generates high levels of phosphatidic acid (PA), known to increase Raf kinase plasma membrane localization and thus Erk activation (Tague et al., 2004). In suspended T-24 cells, the drastic decrease seen in Erk activation upon loss of Arf6 corroborates these findings. This change in Erk activation on loss of RalA, RalB and Arf6 indeed reflects in their anchorage independent growth in T-24 cells. The contribution Erk mediated regulation of proliferation has in mediating anchorage independent growth is something ongoing studies in the lab are testing.

Apart from T-24 cells (expressing oncogenic H-Ras), anchorage independent activation of Arf6 was detected in several cancer cell lines expressing oncogenic Ras and active Ral. These include pancreatic cancer cell line MiaPaCa2 (expressing K-Ras), bladder cancer cell line UM-UC-3 (expressing K-Ras) and fibrosarcoma cell line HT1080 (expressing N-Ras). Interestingly Arf6 activity in these cells was not dependent on Ral suggesting the presence of alternate mechanisms for sustaining its activation. One such known mechanism that can support Arf6 activation in cancers is the overexpression of ArfGEFs, for example BRAG2(GEP100) in breast cancer (Morishige et al., 2008), EFA6A in glioma (Li et al., 2006) and Cytohesin3 in hepatocellular carcinoma (Fu et al., 2014). Our studies looking at the role of these GEFs in T-24 cells suggests their non-involvement in these cells, but the role they could have in MiaPaCa-2, UM-UC-3 and HT1080 remains to be tested. The relative contribution Arf6 and Ral make to anchorage independent signalling and growth in these cells is also something we are aiming to explore. Interestingly, these cancer cell lines harbour either an active K-Ras or N-Ras mutation, unlike T-24 cells that carry H-Ras mutation. Studies by Xu et al have shown preferential binding of RalA to Arf6 in H-Ras-V12 transformed fibroblasts more than K-Ras-V12 transformed cells (Xu et al., 2003). Though the mechanism behind differential association of Ral and Arf6 in H-Ras v/s K-Ras background is not clear, the role this could have in mediating the crosstalk is again an open question that remains to be tested. The role Arf6 GAPs could also have in mediating the same is also open to speculation and of much interest (Onodera et al., 2005; Sabe et al., 2006).

## 6.2 Regulation of the Ral Arf6 crosstalk

Having confirmed presence of the Ral-Arf6 crosstalk and revealing the functional role such a crosstalk could have in normal and cancer cells, we were interested in understanding the mechanism of this crosstalk. Knowing the crosstalk is mediated by active Ral, the role direct association of Ral and Arf6 could have and the role Ral effectors could have in mediating the same was tested. The following section discusses our data evaluating both these possibilities in detail.

### 6.2.1 The role direct association has in mediating the Ral and Arf6 crosstalk:

RalA binds exocyst complex subunits Sec5 and Exo84 to mediate targeted delivery of vesicles carrying a variety of cargo molecules such as neuropeptides (Vitale et al., 2005), insulin (Ljubicic et al., 2009), GLUT4 receptor (Chen et al., 2007). Arf6 is also seen to bind components of the exocyst pathway (Sec10 and Exo70) suggesting RalA, Arf6 and exocyst could be part of a multimeric complex that binds membrane vesicles promoting their trafficking (Moskalenko et al., 2003; Prigent et al., 2003b). Supporting this hypothesis, earlier studies have observed active RalA and active Arf6 to localize with membrane raft component GM1 in intracellular vesicles and at plasma membrane ruffles (Balasubramanian et al., 2007, 2010). Accordingly in the present study we have also seen RalA and Arf6 to prominently co-localize in plasma membrane ruffles of actively spreading MEFs. We further tested this association and found active RalA and active Arf6 to co-precipitate with each other when co-expressed in HEK293T cells. Interestingly we also found the association of Arf6 with Ral to be comparable between WT-RalA and active V23–RalA suggesting this interaction to be independent of the activation status of RalA. Knowing that Ral effector proteins are known to preferentially bind active Ral (van Dam and Robinson, 2006; Moskalenko et al., 2002) this suggests Arf6 to not be a direct Ral effector. Since Arf6 activation is a function of Ral activation status, a Ral effector, that differentially binds WT v/s active Ral, could be speculated to be still involved in mediating the Ral-Arf6 association. It is hence that we have looked at the role Ral effectors have in mediating this crosstalk, discussed in the following section.

We also found that Arf6 precipitates and localizes comparably with wild type and active RalB as well as RalA in HEK293T cells. This agrees with our observations from earlier studies (as discussed in 6.1.2) that RalA and RalB can both regulate Arf6. RalA and RalB share common effectors (van Dam and Robinson, 2006) and that their differential association with such an effector could contribute to their differential regulation of Arf6 downstream of specific stimuli. We had also noted that the amount of Arf6 precipitated with RalA or RalB was very low (~ 0.01% of total Arf6) suggesting the crosstalk could be very transient at best and could possibly be spatially regulated in cells (like at the plasma membrane).

A common Ral and Arf6 effector that has a major role at the plasma membrane is PLD1. Increased RalA-Arf6 activities and their increased interaction with PLD1, observed in H-Ras transformed NIH3T3 cells supports its synergistic activation (Xu et al., 2003). Purified RalA and Arf1 proteins also exhibit this synergism in vitro by binding to purified PLD1 protein (Kim et al., 1998); but in cells this binding with RalA and joint function is shown to be specific to Arf6 and not Arf1 (Xu et al., 2003). This could be due to differential localization of Arf1 and Arf6 in cells, with Arf1 localizing predominantly at Golgi (Donaldson et al., 2005) while Arf6 localized at plasma membrane (D'Souza-Schorey et al., 1998) with RalA (Shipitsin and Feig, 2004). Interestingly, expression of active Arf6 or active RalA alone does not increase PLD1 activity, but their co-expression does (Xu et al., 2003). Xu et al proposed a model wherein Arf6 and RalA were independently activated by H-Ras leading to their translocation to a common site where they would bind PLD1 in a ternary complex and regulate its activity (Xu et al., 2003). Now knowing the existence of a RalA-Arf6 crosstalk, we can improve this model suggested by Xu *et. al.* by including a feed forward loop as follows: Active H-Ras induces RalA activation which activates Arf6 downstream. PLD1 independently binds active Arf6 in its switch region (Jovanovic et al., 2006) and RalA on its N-terminus (Luo et al., 1997) which in a ternary complex could allow for their spatial proximity enhancing the Ral-Arf6 crosstalk, further feeding into greater PLD1 activation through this activated complex. This model could also explain why RalA in an Arf6 dependent manner regulates PLD1 activation and downstream functions (Melendez et al., 2001; Vitale et al., 2005). Thus the Ral-Arf6 interaction through their regulatory crosstalk could actively bring together common and uncommon effectors mediating membrane trafficking and other cellular functions (Chen et al., 2007; Jayaram et al., 2011; Ljubicic et al., 2009; Millar et al., 1999; Vitale et al., 2002, 2005).

As much as effector proteins could regulate the spatial proximity of RalA and Arf6, upstream signals could also be contributing to the same. Looking at Ras as an upstream mediator of Ral activation, the differential role for H-Ras, K-Ras and N-Ras upstream of this crosstalk was evaluated. In H-Ras transformed NIH3T3 cells the RalA–Arf6 interaction is reported to be increased relative to K-Ras transformed cells (Xu et al., 2003). This interestingly agrees with the existence of a Ral-Arf6 regulatory crosstalk only in H-Ras expressing T-24 cells but not in K-Ras and N-Ras expressing MiaPaCa2, UM-UC-3 and HT1080 cells. These studies do need to be extended to other H and K –Ras transformed cancer cells to test if this is indeed conserved across cancers. RalA activated by H-Ras v/s K-Ras may bind different effectors and could be one of the reasons responsible for this differential Ral-Arf6 crosstalk. The differential spatial regulation and activation of Ral by H vs K-Ras could also contribute to the same. Our studies have shown oncogenic H-Ras to be able to support anchorage independent Arf6 activation in WTMEFs (Pawar et al., 2016) supporting the need to test this regulation downstream of other oncogenic Ras mutants.

In T-24 cells, better activation of RalB than RalA results in a prominent RalB-Arf6 crosstalk and regulation of downstream signalling. In serum starved T-24 cells, RalA and RalB comparably regulate Arf6. Whether Ral isoforms display differential interaction with Arf6 in low vs high serum conditions is not tested here due to our inability to immunoprecipitate endogenous Ral and Arf6 and study their associations in cells. This again could shed light on how upstream regulators mediate this crosstalk in cells. Collectively our data together with earlier studies indicate that Ral-Arf6 interaction and regulatory crosstalk could be dependent on the stimulus, cell type, Ral isoform expression and activation in normal and cancer cells.

### 6.2.2 Role of Ral effectors in Ral-Arf6 crosstalk:

Considering the **integrin-RalA-Arf6 pathway** is mediated by active Ral and knowing Arf6 is not a direct Ral effector, this crosstalk is likely to be mediated by a Ral effector. There are several known Ral effector proteins such as Sec5, Exo84, RalBP1, CyclinD1, Filamin-A, ZONAB that bind active Ral and mediate downstream signalling and function in cells (Cantor et al., 1995; Casanova, 2007; Fernández et al., 2011; Frankel et al., 2005; Moskalenko et al., 2002; Ohta et al., 1999). None of these however possess the Sec7 domain that is characteristic of ArfGEFs (Casanova, 2007) suggesting that they are unlikely to act as

direct GEFs for mediating Arf6 activation. Hence we speculate the Ral-Arf6 crosstalk to be mediated by at least two intermediate proteins – a Ral effector and an ArfGEF that is possibly recruited by direct/indirect association with the Ral effector. Knowing that Ral- Arf6 crosstalk controls anchorage independent signalling in cancer cells, these mediators could provide a target to decouple this GTPases and hence their identification was of much interest.

**(A) <u>Exocyst complex:</u>** In the integrin-RalA dependent membrane raft recycling pathway, an essential role for Ral effector Sec5 is demonstrated (Balasubramanian et al., 2010), making it a potential candidate in the Ral-Arf6 crosstalk as well. However, depletion of Sec5 did not disrupt adhesion dependent Arf6 activation suggesting it does not act as the mediator of the Ral-Arf6 crosstalk but likely works as part of exocyst complex downstream of RalA and Arf6 to mediate raft trafficking. We also attempted to test contribution of Exo84, the other exocyst component that RalA directly binds, but lack of consistent results from these experiments meant we could not clearly interpret the same. Though Sec5 and Exo84 are essential mediators of anchorage independence in Ras- transformed cells, their role in bladder cancer cells (exhibiting the Ral-Arf6 crosstalk) is not tested (Issaq et al., 2010; Martin et al., 2011) and would be of interest.

**(B) <u>RalBP1:</u>** Ral binding protein1 (RalBP1) or RLIP76 was tested as a strong candidate owing to its multidomain structure that could serve as a scaffold for recruiting ArfGEFs (Awasthi et al., 2003; Fenwick et al., 2010; Fillatre et al., 2012; Goldfinger et al., 2006; Han et al., 2009b). Indeed RalBP1 is known to bind the Arf GEF ARNO (Lee et al., 2014), seen to be essential for Arf6 activation and function in mediating membrane trafficking and migration (Caumont et al., 2000; Santy and Casanova, 2001). But unlike loss of RalA, RalBP1 depletion in MEFs did not disrupt Arf6 activation in re-adherent cells. Studies by Goldfinger *et al* have implicated RalBP1 to mediate adhesion dependent Arf6 activation downstream of R-Ras, but independent of Ral, regulating cell spreading (Goldfinger et al., 2006). These studies however looked Arf6 activation at early re-adhesion time points (5 minutes post re-adhesion), in contrast to our studies that have looked at RalA dependent Arf6 activation at 15 and 30 minutes after re-adhesion. Knowing the role R-Ras and Arf6 have in integrin recycling to plasma membrane (Conklin et al., 2010; Powelka et al., 2004), this R-Ras dependent Arf6 activation could facilitate binding of cells to ECM and their early spreading. This integrin activation mediated signalling could then induce the RalA-Arf6-

exocyst pathway that drives membrane raft trafficking to support further spreading. Hence it is possible that R-Ras-RalBP1-Arf6 pathway may precede the RalA-Arf6 pathway.

Even though RalBP1 does not regulate the RalA-Arf6 crosstalk in re-adherent cells we observed RalBP1 to be needed for maintaining active Arf6 levels in suspended cells.  This interestingly suggested a role for RalBP1 in regulating Arf6 activation independent of adhesion. Accordingly overexpression of RalBP1 in RalBP1 lacking cells was able to promote Arf6 activation effectively making it anchorage independent, similar to overexpression of constitutively active RalA or RalB. This raised the possibility that RalBP1 could work downstream of RalA in non-adherent cells to regulate Arf6, confirmed when loss of RalBP1 reduced active RalA mediated Arf6 activation and membrane raft delivery in non-adherent MEFs.  This further became of interest in context of the fact that RalBP1 is also over-expressed in bladder cancer cells (Smith et al., 2007) where we have detected the Ral-Arf6 crosstalk (T-24 cells). Indeed targeting of RalBP1 in T-24 cells affected anchorage independent Arf6 activation comparable to loss of Ral. The possibility that RalBP1 and Ral in these cells could still be working along distinct pathways was refuted by the fact that their combined knockdown had no  additive effect on Arf6  activation. Knowing  the role for RalB-Arf6 crosstalk in anchorage independent Erk signaling, the role RalBP1 could have in mediating the same was tested. RalBP1 depletion did affect Erk signaling in T-24 cells, which could contribute to its regulation of anchorage independent growth of T-24 cells.

The Arf6 GEF ARNO (Cytohesin2), that directly binds RalBP1, similarly affected active RalA supported Arf6 activation and membrane raft microdomain delivery in non-adherent MEFs. ARNO also was required for anchorage independent Arf6 activation in  T-24 cells. Loss of RalBP1 and ARNO also did not affect RalA and RalB activation in these cells, suggesting their regulation to be downstream of Ral. Collectively these results suggest the presence of a linear **Ral-RalBP1-ARNO-Arf6 pathway** in non-adherent MEFs and T24 cells (Figure 6.2). We simultaneously   tested for a possible role for other Arf6 GEFs in Ral-RalBP1-Arf6 pathway in T-24 cells. Tested using specific siRNA and inhibitors ArfGEFs – CYTH1, CYTH3, EFA6B, BRAG1, BRAG2 (implicated in other cancer) (Fu et al., 2014; Morishige et al., 2008; Weizhong et al., 2011; Zangari et al., 2014) or BIG1, BIG2 and GBF-1 did not affect Arf6 activation in T-24 cells. Our ongoing studies are actively testing the role other ArfGEF and ArfGAP proteins could have as mediators of the Ral-Arf6 crosstalk in these cells.
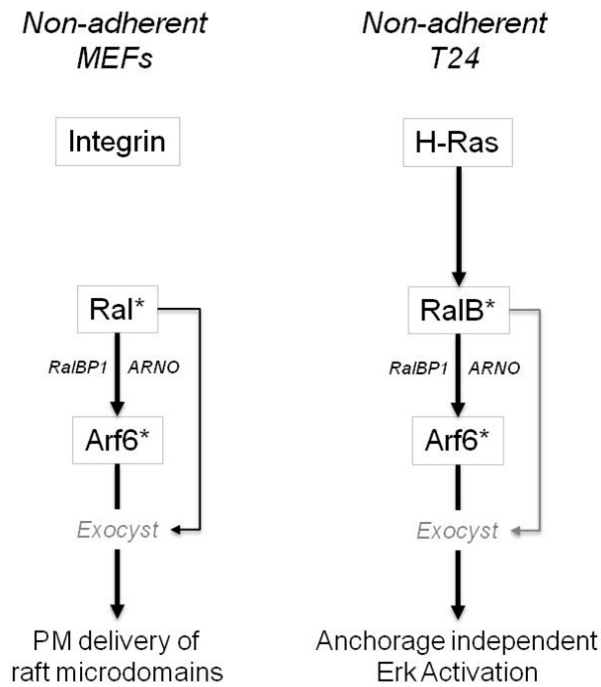
**Figure 6.2: Role of RalBP1 and Arf GEFs in regulating Ral-Arf6 crosstalk in MEFs and T-24 cells.** Schematic depicts role of by RalBP1 and its binding partner ArfGEF-ARNO in regulation of anchorage independent Ral-Arf6 crosstalk in mediating plasma membrane (PM) delivery of membrane raft microdomains and anchorage independent Erk signalling in non –adherent MEFs and T-24 cells respectively.

(C) **Phospholipase D1 (PLD1):** PLD1 as discussed earlier is a protein that binds Ral and Arf6. PLD1 is effector of Arf6 and mediates several of its functions downstream (Hiroyama and Exton, 2005a; Knizhnik et al., 2012; Muralidharan-Chari et al., 2009; Vitale et al., 2002). Ral binds to PLD1 independent of its activation status at its N-terminus (Jiang et al., 1995) but regulates PLD1 activity in Arf6 dependent manner (Luo et al., 1997; Vitale et al., 2005). Hence even though PLD1 is not a classical Ral effector and functions downstream of Arf6, we tested its contribution to integrin-Ral-Arf6 crosstalk in MEF due to the evidence supporting participation of PLD1 in functional complex with RalA and Arf6 (Luo et al., 1998; Xu et al., 2003). In our studies though loss of PLD1 did not regulate Arf6 activation in re-adherent cells, it however did promote RalA and Arf6 activation in suspended cells. It is however possible that the effect PLD1 directly has is limited to RalA, which through the Ral-Arf6 crosstalk regulates Arf6. It is however of much interest since loss of PLD

effectively makes Ral and Arf6 anchorage independent. The role PLD hence has in anchorage independent cancers is of much interest.

PLD1 in cells could work as a protein scaffold and/or through the regulation of its activity. siRNA mediated knockdown of PLD1 could affect both activation and function. We hence tested and found PLD activation in WTMEFs to decrease on loss of adhesion (relative to stable adherent and re-adherent cells). Knowing that RalA and Arf6 activity in these non-adherent WTMEFs also show a drop, this suggests that it is unlikely that PLD1 enzymatic activity regulates this pathway. Further studies are planned using PLD1 specific inhibitor to confirm the same (Lewis et al., 2009; Scott et al., 2009). If not through its activity, then PLD1 protein could be acting as a scaffold for RalA and Arf6 regulators. The phox domain of PLD1 is recently demonstrated to act as GAP domain for dynamin (Lee et al., 2006). Could such a GAP activity extend to Ral GTPase is an open question.

## 6.3 Possible role of Ral-Arf6 crosstalk in their cellular functions:

We have observed the Ral-Arf6 crosstalk downstream of integrins and active Ras and found it be important for Ral function in normal and cancer cells. This hence raises the question if this crosstalk could exist in other cellular pathways that activate RalA and how it could influence the same. Interestingly in many of these pathways a role for Arf6 is also demonstrated further supporting the speculation that the Ral-Arf6 crosstalk could be important here (Chen et al., 2006; Corrotte et al., 2010; Jayaram et al., 2011; Melendez et al., 2001; Vitale et al., 2002, 2005).

### 6.3.1 Ral-Arf6 crosstalk in cytokinesis?

Cytokinesis is a highly co-ordinated multistep process requiring active endosomal membrane recycling (Montagnac et al., 2008). Around late anaphase a cytokinetic furrow is initiated that progressively ingresses with formation of an intercellular bridge made of central spindle microtubules along the centrosomal axis. An electron dense structure of microtubule bundles called Flemming body or midbody develops in the centre of this bridge by the end of telophase. Abscission then occurs adjacent to the midbody via active membrane recycling

from endosomal compartments and fusion of these vesicles with plasma membrane (Chen et al., 2012). RalA is known to regulate early cytokinetic events where it localizes to the intercellular bridge and recruits the exocyst complex channeling active membrane delivery at this site. RalB is randomly distributed in early cytokinesis but localizes to midbody at later events and engages exocyst complex to drive abscission (Cascone et al., 2008; Chen et al., 2006). Differential roles for RalGEFs RGL1, RGL2, RalGDS and RalGPS1 are speculated in cytokinesis based on their localization with RalA/RalB and functional outcome of their depletion (Cascone et al., 2008). Interestingly Arf6 is localized at both the intercellular bridge and to the midbody and allows recruitment of Rab11 interacting proteins FIP3/FIP4 and exocyst component Exo70 at these sites to facilitate endosomal membrane delivery (Fielding et al., 2005; Schweitzer and D'Souza-Schorey, 2002, 2005; Takahashi et al., 2011). Arf6 is also shown to stabilize the midbody by competing with 14-3-3 proteins and directly binds mitotic kinesin like protein 1 -MKLP1, a midbody protein supporting cytokinesis (Joseph et al., 2012; Makyio et al., 2012). A surge in Arf6 activation is observed at around the onset of cytokinesis in HeLa cells and the specific localization of Arf6 to ingression furrow and midbody is dependent on its activation status (Schweitzer and D'Souza-Schorey, 2002; Ueda et al., 2013). How Arf6 is recruited at these sites is however not known. Knowing Ral-Arf6 crosstalk and essential role of Arf6 in mediating Ral function of membrane exocytosis, understanding whether Arf6 recruitment and activation are dependent on Ral GTPases would be of interest. Since our studies from T-24 cells have shown role Arf6 in mediating differential functions of RalA v/s RalB, it is possible that Arf6 also mediates differential roles of RalA and RalB during cytokinesis via its sequential engagement by them in early and late events. Exploring the Ral-Arf6 crosstalk in cytokinesis would help understand significance and penetrance of this crosstalk in other pathways of membrane trafficking.


## 6.3.2 Secretory functions of Ral and Arf6: Possible role of their crosstalk


Since Ral and Arf6 engage common effectors exocyst complex and PLD1, many of their functions involve delivery of cargo vesicles (assisted by exocyst) (Prigent et al., 2003b; Wang et al., 2004) and their fusion with the plasma membrane (assisted by PLD1) (Vitale et al., 2002, 2005). One of the earliest deciphered functions regulated by RalA and Arf6 is the calcium dependent secretion of dense core secretory granules containing hormones and peptides by neuroendocrine PC-12 and chromaffin cells (Vitale et al., 2002, 2005). Depolarization of these cells in high potassium buffer (stimulation) initiates calcium influx

that signals exocytosis of secretory granules. In stimulated cells, RalA and Arf6 were activated and localized with growth hormone (GH) enriched granules at plasma membrane. Expression of active RalA and Arf6 mutants in PC-12 cells enhanced granule release while their dominant negative mutants suppressed it (Vitale et al., 2002, 2005). ARNO co-expression with Arf6 enhanced GH release indicating ARNO might mediate Arf6 activation in this pathway (Caumont et al., 2000; Vitale et al., 2002). Downstream of active Arf6, its interaction and activation of PLD1 further help mediate this pathway (Caumont et al., 1998; Vitale et al., 2002). Interestingly RalA also binds and activates PLD1 in stimulated PC-12 cells. This Ral-PLD1 interaction is Arf6 dependent and critical for Ral dependent GH release (Vitale et al., 2005). Together this data further supports the possibility that the Ral-Arf6 crosstalk could help mediate the Ral dependent PLD1 activation and downstream function. Interestingly this pathway is independent of the RalA-RalBP1 interaction (Vitale et al., 2005) but dependent on ARNO suggesting that if the Ral-ARNO-Arf6 pathway is found to exist in these cells its regulation could be mediated by RalBP1 independent mechanisms.

Another secretory pathway that both RalA and Arf6 are implicated in is the secretion of insulin granules from pancreatic beta cells upon their stimulation by high glucose levels (Ljubicic et al., 2009; Millar et al., 1999). Similar to PC-12 cells, in these cells glucose induction leads to a high ATP/ADP ratio that triggers potassium channel dependent depolarization of plasma membrane followed by calcium influx. High calcium along with concurrent increase in cellular cAMP and membrane DAG and IP3 levels leads to release of pre-docked insulin granules (Seino, 2005). An exocyst complex dependent release of newly synthesized insulin granules in the second phase of secretion is also reported (Tsuboi et al., 2005). In 2003 for the first time, dependence of this pathway on Arf6 activation was reported followed by several studies that have described multiple aspects of this regulation (Lawrence and Birnbaum, 2003b). These include glucose mediated Arf6 activation by ARNO and Nm23-H1 (Jayaram et al., 2011; Veluthakal et al., 2013), and role for PLD1, PIP-5-Kinase and Erk activation downstream of Arf6 in this pathway (Jayaram and Kowluru, 2012; Millar et al., 1999). Further Lopez et al. implicated the exocyst complex and Tusboi et.al identified a role for RalA in regulating insulin secretion from pancreatic beta cells (Lopez et al., 2008; Tsuboi et al., 2005). Studies further revealed a role for the RalGEF RalGDS (Ljubicic et al., 2009) and calcium dependence of RalA activation in response to glucose stimulation (Xie et al., 2013). Active RalA activates PLD1 to control insulin secretion in these cells (Ljubicic et al., 2009). Understanding whether this pathway also uses the Ral-Arf6 crosstalk and the

possible role this could have in the co-ordinating the enhanced activation of Ral-Arf6 common effector PLD1 by the earlier proposed feed forward model, will be of much interest.

Other secretory and trafficking pathways that similarly use RalA and Arf6 include release of **exosomes or micro-vesicles from cells** (Hyenne et al., 2015; Muralidharan-Chari et al., 2009) and **recycling of GLUT4 receptor** in insulin stimulated adipocytes (Chen et al., 2011b, 2011c, 2007; Li et al., 2012; Millar et al., 1999). Cellular processes that require active membrane delivery at particular subcellular sites such as phagocytosis (Corrotte et al., 2010; Melendez et al., 2001), *Salmonella* invasion (Davidson et al., 2015; Humphreys et al., 2012, 2013; Nichols and Casanova, 2010), autophagy (Farré and Subramani, 2011; Martin et al., 2014; Moreau et al., 2012; Tracy et al., 2016), invadopodia formation (Hashimoto et al., 2004; Neel et al., 2012a; Onodera et al., 2005) and neurite branching (Cheung et al., 2014; Kobayashi and Fukuda, 2012; Lalli and Hall, 2005) are also shown to be regulated by both GTPases. Testing the existence and functional relevance of a Ral-Arf6 crosstalk across these processes could provide new insights into the role of the crosstalk in cellular function.

## 6.4 Integrin dependent regulation of RalA v/s RalB:

### 6.4.1 Role of RalGEFs in determining differential activation and function of Ral isoforms:

Ral isoforms RalA and RalB despite sharing 82% sequence identity are seen to differentially regulate cellular functions such as RalA (not RalB) regulating basolateral membrane delivery in epithelial cells (Shipitsin and Feig, 2004), Ras induced tumorigenesis (Lim et al., 2005), anchorage independence in colorectal and pancreatic cancers (Lim et al., 2006; Martin et al., 2011). On the other hand  RalB (not RalA) mediates cancer cell survival (Chien and White, 2003; Chien et al., 2006), development of tight junctions (Hazelett et al., 2011), migration of cancer cells (Oxford et al., 2005). Their differential roles along these cellular processes could be stimulus and cell type specific. Since RalA and RalB bind to same set of effectors downstream (van Dam and Robinson, 2006) their differential functions could be mediated by their differential activation and/or localization (Cascone et al., 2008; Neyraud et al., 2012; Shipitsin and Feig, 2004).

Few studies have supported the concept of differential activation of RalA v/s RalB determining their differential function. For example in colorectal cancers RalA promotes while RalB antagonizes anchorage independence (Martin et al., 2011). This is mediated by the increased activation of RalA in RalB depleted cells (Martin et al., 2011). Thus higher activation of RalA than RalB makes it the determinant of anchorage independence in these cells. A reverse proof is given by studies with K-Ras driven NSCLC mouse model with conditional knockout of RalA and RalB that have shown these isoforms to be intrinsically redundant in mediating their so far visualized isoform specific functions of cell proliferation and cell survival (Peschard et al., 2012). Hence differential functions of RalA and RalB could be the result of their differential activation status leading to preferential engagement of downstream effectors by the more active isoform. Our studies have revealed such a differential activation of Ral isoforms in mediating downstream Arf6 activation. Downstream of integrins activation of RalA (but not RalB) drives Arf6 activation; while in H-Ras expressing T-24 cells, RalB being more active than RalA, regulates anchorage independent Arf6 activation and Erk signaling.

Activation of Ral GTPases is regulated by RalGEF and RalGAP proteins that bind RalA/RalB in their Switch I and Switch II regions, incidentally carrying the highest sequence identity (95%) between them. This suggests the structure of individual Ral isoforms may not mediate their differential interaction with specific GEFs/ GAPs (Gentry et al., 2014). Both Ral isoforms could hence be comparably regulated by different Ral GEFs/GAPs, which makes their differential interaction with these regulators to likely be dependent on their relative spatio-temporal localization in cells. Studies by Cascone *et al* that evaluated the differential roles of RalA/RalB in cytokinesis supported this possibility. These studies showed RalGDS and RalGPS1 to localize with RalA to the nascent midbody and ingressed cytokinetic furrow to regulate early cytokinesis, while RGL1 and RalGPS2 localize with RalB at the mature midbody to regulate late cytokinesis (Cascone et al., 2008). This does imply the differential activation of RalA and RalB by distinct Ral GEFs to be differentially mediated during early v/s late cytokinesis, though this remains to be proven. Our studies identify for the first time a direct role for RGL1 in mediating differential activation of RalA v/s RalB downstream of integrins that directly implicates a RalGEF in mediating the same. Thus indeed downstream of specific stimuli individual GEFs could have a distinct role in mediating RalA vs RalB activation and hence function. The fact that this role for RGL1 is conserved in a colorectal cancer cell line SW620 cells implies this regulation could extend to

other cell types and act downstream of other stimuli like Ras. This hence makes this regulation particularly interesting.

## 6.4.2 Differential subcellular localization of Ral regulates RGL1 dependent activation and function:

Knowing integrin dependent RalA activation (and not RalB) was mediated by RGL1, we found constitutively active RalA rescued the defect in cell spreading observed in re-adherent RGL1 knockdown cells. Since we and others have shown that differential activation of Ral isoforms by upstream stimuli is the driver of their differential function, we asked if constitutively active RalB could also rescue the defect in spreading of RGL1 KD cells. Surprisingly we found that it could not, suggesting an intrinsic Ral isoform feature distinguished active RalA and RalB, which contributed to their regulation by RGL1. This feature could be their differential localization in cells through their C-terminal hypervariable region (amino acids 176-206) (Shipitsin and Feig, 2004). In adherent MDCK cells, active RalA localizes to the plasma membrane and recycling endosomes whereas active RalB localizes in dispersed cytoplasmic vesicles (Shipitsin and Feig, 2004). The active tail switch mutants (RalA72L/B- RalA with the tail of RalB and RalB72L/A-RalB with tail of RalA) are known to switch their localization (Shipitsin and Feig, 2004) and function(s), such as basolateral membrane delivery and anchorage independent growth (Lim et al., 2005; Shipitsin and Feig, 2004). In our studies these mutants also switched the ability of active RalA and RalB to rescue cell spreading in RGL1 knockdown cells leading us to speculate that the isoform specific localization in re-adherent cells is what allows RGL1 to specifically activate RalA downstream of integrins. Indeed RGL1 co-localized with RalA at ruffling plasma membrane edges and with focal adhesion markers– both of which are active sites of integrin signaling. Differential localization of RalA and RalB is observed in cytokinesis where RGL1 localizes with RalB at the midbody in late cytokinesis. Interestingly loss of RGL1 leads to similar defect in cytokinesis as seen on loss of RalB, indicating RGL1 could be regulating RalB activation at midbody during late cytokinetic events (Cascone et al., 2008). These results further emphasize that differential localization, and not structural differences between RalA and RalB would determine their differential activation by RGL1. It also raises the question if the redundancy in GEF mediated Ral activation means that their differential localization could allow for one or more GEF(s) to activate RalA and/or RalB depending on the stimulus.

While this reveals a role for RGL1 in mediating the recovery of RalA activation on re-adhesion, if regulation of RGL1 function has a role in mediating the basal Ral activation in stable adherent cells or the drop in Ral activity on loss of adhesion remains an open question. The organization of the plasma membrane and focal adhesions (possible sites for Ral-RGL1 crosstalk) are both altered in non-adherent cells (Gaus et al., 2006; Norambuena and Schwartz, 2011). RalA is known to be internalized along with membrane rafts in suspended cells (Norambuena and Schwartz, 2011) though the localization of RGL1 does remain to be tested in non-adherent cells. The role RalGAPs could have in mediating the same is again of relevance.

Collectively, our studies indicate that subcellular localization of RalA v/s RalB with RGL1 allows for their differential activation downstream of integrins. Knowing RalA, not RalB supports anchorage independence in pancreatic and colorectal cancer cells expressing oncogenic K-Ras (Lim et al., 2006; Martin et al., 2011) and that RGL1 is a Ras dependent GEF, its role in these cancers was worth exploring.

### 6.4.3 Role of RalGEFs and specifically RGL1 in cancer:

The last decade has unraveled the oncogenic nature of Ral GTPases in multiple cancer types and accordingly their regulation in cancer cells is of keen interest (Bodemann and White, 2008). Though constitutive Ral activation by oncogenic Ras mutation in cancers is the primary cue, Ral overexpression also has been detected in several cancer types (Guin et al., 2013; Smith et al., 2007; Wang et al., 2013). Recent studies have identified deregulated expression of RalGEFs and RalGAPs that promote tumorigenesis via aberrant Ral activation (Saito et al., 2013; Vigil et al., 2010a). RalGDS was the first Ras effector to be identified as RalGEF followed by RGL1, RGL2 and RGL3 (Neel et al., 2011). Though overexpression of RalGDS is shown in hepatocellular carcinoma and ovarian cancer and RGL2 in T-cell malignancies, expression of RGL1 is not evaluated so far (Ezzeldin et al., 2014; Vigil et al., 2010b; Wang et al., 2013). We hence compared expression of RalGDS and RGL1 in multiple cancer cell lines and found their relative expression to be differential in cancers. While the expression of RalGDS remained comparable across these cancers RGL1 expression was significantly lower in one group and was comparable to RalGDS in the other. The cause of this differential expression across these cancers from diverse origins remains unclear. As a

first step to understanding this differential expression, we tested if the role of these GEFs could indeed be influenced by their expression. We tested pancreatic cancer MiaPaCa2 (RalGDS > RGL1) and colorectal cancer SW620 (RalGDS = RGL1) cells for the relative contribution these GEFs make to RalA and RalB activation. Tumorigenic potential of these cell lines, measured by their ability to form colonies in soft agar, is known to be dependent on RalA and not RalB (Lim et al., 2006; Martin et al., 2011). Loss of RalGDS in MiaPaCa2 equally inhibited anchorage independent RalA and RalB activation, which was unaffected by the loss of RGL1. Interestingly in SW620 cells, that show the best RGL1 expression which is comparable to RalGDS, loss of RGL1 differentially regulates RalA and not RalB. This more than anything raises a set of very interesting questions on the relative roles RGL1 and RalGDS could have in cancers. Multiple attempts to knockdown RalGDS in SW620 cells with varying siRNA sequences failed to consistently give good knockdown. This meant we could not look at the role RalGDS has in SW620 cells. We are exploring alternate ways to target RalGDS and evaluate its relative role to RGL1. Studies are also planned to extend this analysis to other cancer cell lines from the screen to test if this differential regulation is indeed conserved. RGL1 is also known to be mutated (Y209S/V734M) in breast cancers with a frequency greater than the background frequency of random somatic mutations (Sjöblom et al., 2006). This RGL1 mutant expressed in HeLa cells was found to increase RalB activation more than RalA activation. Whether genomic RGL1 mutation can selectively activate one Ral isoform over the other was speculated to be cell context specific. (Tzuling Cheng, 2008).

Along with RalGEFs, a role for RalGAP proteins in deregulation of Ral in cancers is emerging. A common β subunit binds either of two catalytic subunits- α1 and α2 to make two heterodimeric complexes RalGAP1 and RalGAP2 (Gentry et al., 2014). These complexes deactivate Ral GTPases and hence could serve as tumor suppressors in Ral driven cancer. Accordingly reduced expression of α2 subunit is detected in bladder cancer cell lines and patient tissue samples correlating with poor prognosis in patients (Saito et al., 2013). Isoform specific regulation of RalA and RalB by RalGAP1 and RalGAP2 complexes is yet unknown. The possibility that RalGAPs may play a role in differential deactivation of RalA v/s RalB isoforms cannot be overruled.

In conclusion, these studies have helped address two major regulatory pathways downstream of integrins that drive anchorage dependent signalling and are hence exploited by oncogenic Ras. Arf6 is identified as a key downstream mediator of Ral isoforms required in adhesion

dependent pathways in normal and cancer cells. Our studies have also revealed a new understanding of how the Ral isoforms- RalA and RalB are differentially regulated and in turn regulate downstream signalling.

# Appendix

## APPENDIX TABLE 1: Primers for quantitative PCR (CHAPTER 3)

| Sr. number | Gene | Sequence (5' - 3') |
|---|---|---|
| 1 | mPLD1_F | TGACCCAAGTGAGGACCTTC |
| 2 | mPLD1_R | AAGGCACCGGAAGACCTTAT |
| 3 | mPLD2_F | ACCTCATCACCAAGGACTGG |
| 4 | mPLD2_R | TCCGTGTACAACCACTCCAA |
| 5 | mCYTH2_F | CCTTTGCCCAGAGATACTGC |
| 6 | mCYTH2_R | GGATTGTGAAGGCTGGTGTT |
| 7 | mSec10-F | TGCCAGGAGGGTGCTTACT |
| 8 | mSec10-R | CTGGATTACTAAAGATGTCTCCAACC |
| 9 | mEXO84-F | CCACCTGGCTGCCACAAC |
| 10 | mEXO84-R | TTCATGGGCGGATTGTCTTT |
| 11 | hCYTH1_F | GTTTGCCCAGCGATATTGTCAG |
| 12 | hCYTH1_R | GTGCAGACTGGTGTTCAACATG |
| 13 | mCYTH2_F | TGGTGGAGAATGAACTGCTG |
| 14 | hCYTH2_R | TCATGCAGATCCACAAAAGC |
| 15 | hCYTH3_F | TGCTACAGAGTTCCCCAGAA |
| 16 | hCYTH3_R | TTCATCCCTTTCACCCAGGTAG |
| 17 | hCYTH4_F | GTTCGCCAACCTCAACCTC |
| 18 | hCYTH4_R | GCAGTATCGAGTGGCAAAGG |
| 19 | hEFA6A_F | GCCAGTGACTACAGCAAGAGG |
| 20 | hEFA6A_R | GAGTGATCCAGGACTGCATCTG |
| 21 | hEFA6B_F | CTGGCTCGGAAAATGCATCAAG |
| 22 | hEFA6B_R | GAGAACCATCCCTCGCAGTAAG |
| 23 | hEFA6C_F | CGAGAAAAGCCGTTATGAGACC |
| 24 | hEFA6C_R | GCTTGAATGTGTCTTCCGGAGA |
| 25 | hEFA6D_F | GGAGGAGCAACTGAAGTCACAT |
| 26 | hEFA6D_R | GTCCTTGGCTTTGACCTTCTTG |
| 27 | hBRAG1_F | TGCAGGAGATGGAGAAATACCG |
| 28 | hBRAG1_R | GTCCCATTCACTGAGTCCTTGG |
| 29 | hBRAG2_F | GGTGGAGATGCTAGAACGAAAG |
| 30 | hBRAG2_R | CTTGTTCATCTGGTACTGGCG |
| 31 | hBRAG3_F | GCTGGTGGTAGGCATCTATGAG |
| 32 | hBRAG3_R | CACTGTCTTCATGCCCACAATG |
| 33 | hBIG1_F | TGCACGCATGGAAAACCAAG |
| 34 | hBIG1_R | CTCGTGATGGCTTACTGGAGAC |
| 35 | hBIG2_F | GGCAACTACCTTGGGAATTCC |
| 36 | hBIG2_R | TTCAGGCTCCCTTCTCTTTCAC |
| 37 | hGBF1_F | CGCATTGACTGTTTTCTCCCTC |
| 38 | hGBF1_R | TGTCCTAGTGTCGCCAGCAG |
| 39 | mActin_F | GCTACAGCTTCACCACCACA |
| 40 | mActin_R | TCTCCAGGGAGGAAGAGGAT |
| 41 | hActin_F | GATTCCTATGTGGGCGAC |
| 42 | hActin_R | GGTAGTCAGTCAGGTCCCG |

## APPENDIX TABLE 2: Primers for site directed mutagenesis (CHAPTER 1 and 3)

| Sr. # | Gene | Sequence (5' - 3') |
|---|---|---|
| 1 | RalA79L_SDM_A | GGCAGGAGGACTATGCTGCAATTCTAGACAACTACTTCCGA |
| 2 | RalA79L_SDM_B | TCGGAAGTAGTTGTCTAGAATTGCAGCATAGTCCTCCTGCC |
| 3 | hRalA*_SDM_A | CTGTTTTTTGCCTCTTCTACACTAACCTGTCTTTTATCTTCTAAATCTGATTTGT |
| 4 | hRalA*_SDM_B | ACAAATCAGATTTAGAAGATAAAAGACAGGTTAGTGTAGAAGAGGCAAAAAACAG |
| 1 | RalBP1#_5mut_A | TTGGAATTCCTTTGGCTGATGCAGTCGAACGAACTATGATGTATGATGGCATTCGGCTGC |
| 2 | RalBP1#_5mut_B | GCAGCCGAATGCCATCATACATCATAGTTCGTTCGACTGCATCAGCCAAAGGAATTCCAA |

## APPENDIX TABLE 3: Primers for quantitative PCR (CHAPTER 4)

| Sr. number | Gene | Sequence (5' - 3') |
|---|---|---|
| 1 | mRalGPS1_F | CTCTGAGACGGAAAACCCTG |
| 2 | mRalGPS1_R | CTTGGCTCCATAGTAAAGGAG |
| 3 | mRalGPS2_F | TGAACTAAGCGAAGAGACCTCA |
| 4 | mRalGPS2_R | CCGGAGCACACCTTGAATAG |
| 5 | mRalGDS_F | CATGAAGGACTATCTCTATGGG |
| 6 | mRalGDS-R | CTGACTGTAGCAACTTGATCTG |
| 7 | mRGL1_F | CAGCAGAATTCACGAACTTC |
| 8 | mRGL1_R | TATCCCGCTGAGACCAAATA |
| 9 | mRGL2_F | CCAAGTGTTATTAGTCGTGTCC |
| 10 | mRGL2_R | GAAGACGTTAGCTGAGTGTG |
| 11 | mRGL3_F | AAGCTGTCCCGAGAGAAGAA |
| 12 | mRGL3_R | AGGAGGCTCTCGGTTTCTAG |
| 13 | mActin_F | CTCCTAGCACCATGAAGATC |
| 14 | mActin_R | GACTCATCGTACTCCTGCTT |
| 15 | hRalGDS_F | GTCTCAGGGCTCTGCAACTC |
| 16 | hRalGDS-R | TCTTCAGCTTCCGGTCATCT |
| 17 | hRGL1_F | AACCACTCAGAGGCTGAGGA |
| 18 | hRGL1_R | AGACAGAGCGCTTGTGGATT |
| 19 | hActin_F | CTCCTGAGCGCAAGTACTCC |
| 20 | hActin_F | CCGGACTCGTCATACTCCTG |

# APPENDIX TABLE 4: siRNA procured from Sigma

| Sr. number | Gene | siRNA antisense Sequence (5'-3') | Reference |
|---|---|---|---|
| 1 | mRalA | AAGGCAGGUUUCUGUAGAA | (Balasubramanian et al., 2010; Vitale et al., 2005) |
| 2 | mRalB | GGUGGUUCUCGACGGAGAA | (Balasubramanian et al., 2010; Li et al., 2007) |
| 3 | sh-hmArf6 | AGCTGCACCGCATTATCAA | (Balasubramanian et al., 2007; Choi et al., 2006) |
| 4 | hmArf6#1 | UCCUCAUCUUCGCCAACAA | (Houndolo et al., 2005) |
| 5 | hmArf6#2 | GCACCGCAUUAUCAAUGACCG | (Béglé et al., 2009) |
| 6 | mRalBP1 | ACAUCAUGGUCCUCUCUAC | (Goldfinger et al., 2006) |
| 7 | hRalBP1 | GUAGAGAGGACCAUGAUG | (Lim et al., 2010) |
| 8 | hRalA | CAGAGCUGAGCAGUGGAAU | (Oxford et al., 2005) |
| 9 | hRalB | GGUGAUCAUGGUUGGCAGC | (Oxford et al., 2005) |
| 10 | hCyth3#1 | GUCGCCCAGUUCCUUUAUA | (Fu et al., 2014) |
| 11 | hCyth3#2 | CAGCAGAGAUCCCUUCUAU | (Fu et al., 2014) |
| 12 | hBRAG2#1 | AAGUGAAAUCACUGGCCGAGU | (Morishige et al., 2008) |
| 13 | hBRAG2#2 | GAUGCUAGAACGAAAGUAU | (Hu et al., 2013) |
| 14 | hBRAG2#3 | GCGAGAGCUAAAGACCAAU | (Hu et al., 2013) |
| 15 | hEFA6B | AATGAAGACTCAGGGGAAGAC | (Morishige et al., 2008) |
| 16 | mRalGPS1 | CGAUCCAACCAGAUGAACA | Designed by Ambion (ID 153738) |
| 17 | mRGL1 | GCAUCAGUGUAGAAGACAA | Designed by Ambion (ID 72932) |
| 18 | mRalGDS#1 | AGAAUGGACUAACCGAGAA | Custom designed |
| 19 | mRalGDS#2 | GGAAGGAGUUCGAAGUCAU | Designed by Ambion (ID 72927) |
| 20 | hRGL1#1 | CACAGTATCATTGTTAAGTGA | (Cascone et al., 2008) |
| 21 | hRGL1#2 | TCCCATAATACAGCTCCTAAA | (Cascone et al., 2008) |
| 22 | hRalGDS#1 | CTCGGGAACCGAAGCACGAAA | (Cascone et al., 2008) |
| 23 | hRalGDS#2 | CCCATCCGAGTCAGCCAGCAA | (Cascone et al., 2008) |
| 24 | hRalGDS#3 | CCAUCUUCCUGUGUACCUA | (Godin and Ferguson, 2010) |

**APPENDIX TABLE 5: siRNA procured from Dharmacon (On-Target Plus SMARTpool)**

| Sr. number | Gene | siRNA antisense Sequence (5'-3') | Reference |
|---|---|---|---|
| 1 | mARNO - SMARTpool | UAAGUGAAGCUAUGAGCGA | CAT# L-059077-01-0005 |
| | | CAGAAAAUUGAUCGAAUGA | |
| | | AAUUAAGAUUCCAGAACGU | |
| | | GCAAGAAAGAAGCGAAUUU | |
| 2 | hARNO - SMARTpool | UGGCAGUGCUCCAUGCUUU | CAT# L-011925-01-0005 |
| | | AAACCGAACUGCUUUGAAC | |
| | | GUAAGACCUUGCAACGGAA | |
| | | GAACACACCCGAGGAGAUC | |
| 3 | mSec5 - SMARTpool | AGAAGUAUUAGGUCGGAAA | CAT # L-042601-01-0005 |
| | | UCAACGUACUUCAGCGAUU | |
| | | CAGCAGAGAUUACACGUCA | |
| | | GUGAGUGGCUUGCGCAGUA | |
| 3 | mExo84 - SMARTpool | UGGAAUACGAUGCGGACCA | CAT # L-041268-01-0005 |
| | | AUGCCAAGAUCAAGCGCGA | |
| | | UGGACGAACGAGUGCGACA | |
| | | CAACAAAAUGGCUUCGUAA | |
| 3 | mSec10 - SMARTpool | GUAUAGUGUAAACGGUGUA | CAT # L-047583-01-0005 |
| | | GAGAACAACUUGCCAAUCU | |
| | | GAUGGAACUUGGAGUACGU | |
| | | GGCAUUGAUAGGACAUUAA | |
| 4 | mPLD1 - SMARTpool | UGUUAAUUGCUGACGACAA | CAT# L-040014-01-0005 |
| | | GGCCAGAGAUAUCGGGUGU | |
| | | CAGCACACGAGUUGAGAUA | |
| | | GCUCGAAACGCUACCAUAU | |
| 5 | mPLD2 - SMARTpool | GGGACUGCGUGGCGAGAUU | CAT# L-040015-01-0005 |
| | | GAAAGAUACACCAGCGGAU | |
| | | UUGAGGUCCAGGUCGGAAA | |
| | | CCAAGAAGAAGUUCCGACA | |
| 6 | mRalGDS- SMARTpool | GUUCAAAAGGUACGGUAGA | CAT# L-062150-01 |
| | | AGAAUGGACUAACCGAGAA | |
| | | GGCUAGGGUGUGAGAACGA | |
| | | GCAAGGUGCGCACGGUGAA | |
| 7 | mRalGPS1- SMARTpool | CGACAUUCCCAUCGGAGAA | CAT# L-059160-01 |
| | | GGACCUAACUGGCGGGUUC | |
| | | CAAGACUAGUUUCGUCCAA | |
| | | GGGCCAGAGCUGCGACUAU | |

## APPENDIX TABLE 5: siRNA procured from Dharmacon (On-Target Plus SMARTpool)

## (Continued from previous page)

| Sr. number | Gene | siRNA antisense Sequence (5'-3') | Reference |
|---|---|---|---|
| 8 | mRalGPS2-SMARTpool | UGACAAGAGUGCCGCGAAA | CAT# L-061034-01 |
| | | CAGAAGAAUACGCGGGUCA | |
| | | CGGAAGAUUUGGCGGUGCA | |
| | | CCGCAUAGGAGCACGGUGA | |
| 9 | mRGL1-SMARTpool | GAGCCAGAGUCAUCGAGAA | CAT# L-059274-01 |
| | | GAUCAACAUUGCUCACGAA | |
| | | CCUGGACAGCAGCGUGAAA | |
| | | ACGCAUAUCGUGUGUGUAU | |
| 10 | mRGL2-SMARTpool | GCUCAGAGUCACCGUGUAU | CAT# L-064793-01 |
| | | GUGCUGACCUCAUCCGAAA | |
| | | GCAAGGACACCUCGGCCAA | |
| | | GAGGAAGAUUGCACGGGCA | |
| 11 | mRGL3-SMARTpool | GUGCAGGGAACUACGAAAU | CAT# L-064000-01 |
| | | GAUACAGCCUUACCGGAUA | |
| | | CGCGUGAGGUCCUGCGAAU | |
| | | CCAACGUCUUCUACGCUAU | |
| 12 | hRGL1-SMARTpool | GGAAGAUCAUGUUCGAAUA | CAT# L-008387-00 |
| | | CAAGAGGUGUCUAAAUUCA | |
| | | GGACAAUGACUUUACCUAU | |
| | | UGUAAGAUCAGGACCAUAA | |
| 13 | hRalGDS | GAACUUCUCGUCACUGUAU | CAT# L-005193-00 |
| | | GGAUCCAGCUCCCUCACAA | |
| | | GCUACAACCUGUCGUGCGA | |
| | | GAAGACGUUUCCAGGGACA | |

# **REFERENCES**

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., et al. (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87–98.

Aikawa, Y., and Martin, T.F.J. (2003). ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis. J. Cell Biol. *162*, 647–659.

Anders, N., and Jürgens, G. (2008). Large ARF guanine nucleotide exchange factors in membrane trafficking. Cell. Mol. Life Sci. *65*, 3433–3445.

Awasthi, S., Singhal, S.S., Sharma, R., Zimniak, P., and Awasthi, Y.C. (2003). Transport of glutathione conjugates and chemotherapeutic drugs by RLIP76 (RALBP1): A novel link between G-protein and tyrosine kinase signaling and drug resistance. Int. J. Cancer *106*, 635–646.

Bach, A.-S., Enjalbert, S., Comunale, F., Bodin, S., Vitale, N., Charrasse, S., and Gauthier-Rouvière, C. (2010). ADP-ribosylation factor 6 regulates mammalian myoblast fusion through phospholipase D1 and phosphatidylinositol 4,5-bisphosphate signaling pathways. Mol. Biol. Cell *21*, 2412–2424.

Balasubramanian, N., Scott, D.W., Castle, J.D., Casanova, J.E., and Schwartz, M.A. (2007). Arf6 and microtubules in adhesion-dependent trafficking of lipid rafts. Nat. Cell Biol. *9*, 1381–1391.

Balasubramanian, N., Meier, J.A., Scott, D.W., Norambuena, A., White, M.A., and Schwartz, M.A. (2010). RalA-Exocyst Complex Regulates Integrin-Dependent Membrane Raft Exocytosis and Growth Signaling. Curr. Biol. *20*, 75–79.

Barale, S., McCusker, D., and Arkowitz, R.A. (2006). Cdc42p GDP/GTP cycling is necessary for efficient cell fusion during yeast mating. Mol. Biol. Cell *17*, 2824–2838.

Béglé, A., Tryoen-Tóth, P., de Barry, J., Bader, M.-F., and Vitale, N. (2009). ARF6 regulates the synthesis of fusogenic lipids for calcium-regulated exocytosis in neuroendocrine cells. J. Biol. Chem. *284*, 4836–4845.

Bendezú, F.O., Vincenzetti, V., and Martin, S.G. (2012). Fission yeast Sec3 and Exo70 are transported on actin cables and localize the exocyst complex to cell poles. PLoS One *7*, e40248.

Bernards, A., and Settleman, J. (2004). GAP control: regulating the regulators of small GTPases. Trends Cell Biol. *14*, 377–385.

Bodemann, B.O., and White, M.A. (2008). Ral GTPases and cancer: linchpin support of the tumorigenic platform. Nat. Rev. Cancer *8*, 133–140.

Bodemann, B.O., Orvedahl, A., Cheng, T., Ram, R.R., Ou, Y.H., Formstecher, E., Maiti, M., Hazelett, C.C., Wauson, E.M., Balakireva, M., et al. (2011). RalB and the exocyst mediate the cellular starvation response by direct activation of autophagosome assembly. Cell *144*, 253–267.

Bodempudi, V., Yamoutpoor, F., Pan, W., Dudek, A.Z., Esfandyari, T., Piedra, M., Babovick-Vuksanovic, D., Woo, R.A., Mautner, V.F., Kluwe, L., et al. (2009). Ral overactivation in malignant peripheral nerve sheath tumors. Mol Cell Biol *29*, 3964–3974.

Bos, J.L., Rehmann, H., and Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. Cell *129*, 865–877.

Brymora, A., Duggin, I.G., Berven, L.A., van Dam, E.M., Roufogalis, B.D., and Robinson, P.J. (2012). Identification and characterisation of the RalA-ERp57 interaction: evidence for GDI activity of ERp57. PLoS One *7*, e50879.

Camonis, J.H., and White, M. a. (2005). Ral GTPases: Corrupting the exocyst in cancer cells. Trends Cell Biol. *15*, 327–332.

Cantor, S.B., Urano, T., and Feig, L. a (1995). Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. Mol. Cell. Biol. *15*, 4578–4584.

Casanova, J.E. (2007). Regulation of Arf activation: the Sec7 family of guanine nucleotide exchange factors. Traffic *8*, 1476–1485.

Cascone, I., Selimoglu, R., Ozdemir, C., Del Nery, E., Yeaman, C., White, M., and Camonis, J. (2008). Distinct roles of RalA and RalB in the progression of cytokinesis are supported by distinct RalGEFs. EMBO J. *27*, 2375–2387.

Caumont,  a S., Vitale, N., Gensse, M., Galas, M.C., Casanova, J.E., and Bader, M.F. (2000). Identification of a plasma membrane-associated guanine nucleotide exchange factor for ARF6 in chromaffin cells. Possible role in the regulated exocytotic pathway. J. Biol. Chem. *275*, 15637–15644.

Caumont, A.S., Galas, M.C., Vitale, N., Aunis, D., and Bader, M.F. (1998). Regulated exocytosis in chromaffin cells. Translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. J. Biol. Chem. *273*, 1373–1379.

Cavenagh, M.M., Whitney, J.A., Carroll, K., Zhang, C. j, Boman, A.L., Rosenwald, A.G., Mellman, I., and Kahn, R.A. (1996). Intracellular distribution of Arf proteins in mammalian cells. Arf6 is uniquely localized to the plasma membrane. J. Biol. Chem. *271*, 21767–21774.

Chen, C.-T., Hehnly, H., and Doxsey, S.J. (2012). Orchestrating vesicle transport, ESCRTs and kinase surveillance during abscission. Nat. Rev. Mol. Cell Biol. *13*, 483–488.

Chen, C.X.J., Soto, I., Guo, Y.L., and Liu, Y. (2011a). Control of secondary granule release in neutrophils by Ral GTPase. J. Biol. Chem. *286*, 11724–11733.

Chen, E.H., Pryce, B.A., Tzeng, J.A., Gonzalez, G.A., and Olson, E.N. (2003). Control of myoblast fusion by a guanine nucleotide exchange factor, loner, and its effector ARF6. Cell *114*, 751–762.

Chen, Q., Quan, C., Xie, B., Chen, L., Zhou, S., Toth, R., Campbell, D.G., Lu, S., Shirakawa, R., Horiuchi, H., et al. (2014). GARNL1, a major RalGAP α subunit in skeletal muscle, regulates insulin-stimulated RalA activation and GLUT4 trafficking via interaction with 14-3-3 proteins. Cell. Signal. *26*, 1636–1648.

Chen, X., Leto, D., Xiong, T., Yu, G., Cheng, A., Decker, S., and Saltiel, A.R. (2011b). A Ral GAP complex links PI 3-kinase/Akt signaling to RalA activation in insulin action. Mol. Biol. Cell *22*, 141–152.

Chen, X., Leto, D., Xiao, J., Goss, J., Wang, Q., Shavit, J.A., Xiong, T., Yu, G., Ginsburg, D., Toomre, D., et al. (2011c). Exocyst function is regulated by effector phosphorylation. Nat. Cell Biol. *13*, 580–588.

Chen, X.W., Inoue, M., Hsu, S.C., and Saltiel, A.R. (2006). RalA-exocyst-dependent recycling endosome trafficking is required for the completion of cytokinesis. J. Biol. Chem. *281*, 38609–38616.

Chen, X.W., Leto, D., Chiang, S.H., Wang, Q., and Saltiel, A.R. (2007). Activation of RalA Is Required for Insulin-Stimulated Glut4 Trafficking to the Plasma Membrane via the Exocyst and the Motor Protein Myo1c. Dev. Cell *13*, 391–404.

Cherfils, J., and Zeghouf, M. (2013). Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiol. Rev. *93*, 269–309.

Chesneau, L., Dambournet, D., MacHicoane, M., Kouranti, I., Fukuda, M., Goud, B., and Echard, A. (2012). An ARF6/Rab35 GTPase cascade for endocytic recycling and successful cytokinesis. Curr. Biol. *22*, 147–153.

Cheung, H.N.M., Dunbar, C., Mórotz, G.M., Cheng, W.H., Chan, H.Y.E., Miller, C.C.J., and Lau, K.-F. (2014). FE65 interacts with ADP-ribosylation factor 6 to promote neurite outgrowth. FASEB J. *28*, 337–349.

Chien, Y., and White, M.A. (2003). RAL GTPases are linchpin modulators of human tumour-cell proliferation and survival. EMBO Rep. *4*, 800–806.

Chien, Y., Kim, S., Bumeister, R., Loo, Y.-M., Kwon, S.W., Johnson, C.L., Balakireva, M.G., Romeo, Y., Kopelovich, L., Gale, M., et al. (2006). RalB GTPase-mediated activation of the IkappaB

family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell *127*, 157–170.

Choi, S., Ko, J., Lee, J.-R., Lee, H.W., Kim, K., Chung, H.S., Kim, H., and Kim, E. (2006). ARF6 and EFA6A regulate the development and maintenance of dendritic spines. J. Neurosci. *26*, 4811–4819.

Choi, W.S., Kim, Y.M., Combs, C., Frohman, M. a, and Beaven, M.A. (2002). Phospholipases D1 and D2 regulate different phases of exocytosis in mast cells. J. Immunol. *168*, 5682–5689.

Cockcroft, S. (2001). Signalling roles of mammalian phospholipase D1 and D2. Cell. Mol. Life Sci. *58*, 1674–1687.

Cohen, L.A., Honda, A., Varnai, P., Brown, F.D., Balla, T., and Donaldson, J.G. (2007). Active Arf6 recruits ARNO/cytohesin GEFs to the PM by binding their PH domains. Mol. Biol. Cell *18*, 2244–2253.

Conklin, M.W., Ada-Nguema, A., Parsons, M., Riching, K.M., and Keely, P.J. (2010). R-Ras regulates beta1-integrin trafficking via effects on membrane ruffling and endocytosis. BMC Cell Biol. *11*, 14.

Corrotte, M., Nyguyen, A.P.T., Harlay, M.L., Vitale, N., Bader, M.-F., and Grant, N.J. (2010). Ral isoforms are implicated in Fc gamma R-mediated phagocytosis: activation of phospholipase D by RalA. J. Immunol. *185*, 2942–2950.

D'Souza-Schorey, C., van Donselaar, E., Hsu, V.W., Yang, C., Stahl, P.D., and Peters, P.J. (1998). ARF6 targets recycling vesicles to the plasma membrane: insights from an ultrastructural investigation. J. Cell Biol. *140*, 603–616.

Dai, Y., Walker, S.A., de Vet, E., Cook, S., Welch, H.C.E., and Lockyer, P.J. (2011). Ca2+-dependent monomer and dimer formation switches CAPRI Protein between Ras GTPase-activating protein (GAP) and RapGAP activities. J. Biol. Chem. *286*, 19905–19916.

van Dam, E.M., and Robinson, P.J. (2006). Ral: Mediator of membrane trafficking. Int. J. Biochem. Cell Biol. *38*, 1841–1847.

Das, T., Sa, G., Hilston, C., Kudo, D., Rayman, P., Biswas, K., Molto, L., Bukowski, R., Rini, B., Finke, J.H., et al. (2008). GM1 and tumor necrosis factor-alpha, overexpressed in renal cell carcinoma, synergize to induce T-cell apoptosis. Cancer Res. *68*, 2014–2023.

Davidson, A.C., Humphreys, D., Brooks, A.B.E., Hume, P.J., and Koronakis, V. (2015). The Arf GTPase-activating protein family is exploited by Salmonella enterica serovar Typhimurium to invade nonphagocytic host cells. MBio *6*.

Davies, J.C.B., Tamaddon-Jahromi, S., Jannoo, R., and Kanamarlapudi, V. (2014). Cytohesin 2/ARF6 regulates preadipocyte migration through the activation of ERK1/2. Biochem. Pharmacol. *92*, 651–660.

Donaldson, J.G., and Jackson, C.L. (2000). Regulators and effectors of the ARF GTPases. Curr. Opin. Cell Biol. *12*, 475–482.

Donaldson, J.G., and Jackson, C.L. (2011). ARF family G proteins and their regulators: roles in membrane transport, development and disease. Nat. Rev. Mol. Cell Biol. *12*, 362–375.

Donaldson, J.G., Honda, A., and Weigert, R. (2005). Multiple activities for Arf1 at the Golgi complex. Biochim. Biophys. Acta *1744*, 364–373.

Dong, Y., Ikeda, K., Hamamura, K., Zhang, Q., Kondo, Y., Matsumoto, Y., Ohmi, Y., Yamauchi, Y., Furukawa, K., Taguchi, R., et al. (2010). GM1/GD1b/GA1 synthase expression results in the reduced cancer phenotypes with modulation of composition and raft-localization of gangliosides in a melanoma cell line. Cancer Sci. *101*, 2039–2047.

Dorseuil, O. (1995). Bridging Ral GTPase to Rho Pathways. J. Biol. Chem. *270*, 22473–22477.

Dudek, S.M., Chiang, E.T., Camp, S.M., Guo, Y., Zhao, J., Brown, M.E., Singleton, P. a, Wang, L., Desai, A., Arce, F.T., et al. (2010). Abl tyrosine kinase phosphorylates nonmuscle Myosin light chain kinase to regulate endothelial barrier function. Mol. Biol. Cell *21*, 4042–4056.

Eades, G., Wolfson, B., Zhang, Y., Li, Q., Yao, Y., and Zhou, Q. (2015). lincRNA-RoR and miR-145 regulate invasion in triple-negative breast cancer via targeting ARF6. Mol. Cancer Res. *13*, 330–338.

Ebi, H., Corcoran, R.B., Singh, A., Chen, Z., Song, Y., Lifshits, E., Ryan, D.P., Meyerhardt, J.A., Benes, C., Settleman, J., et al. (2011). Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. J. Clin. Invest. *121*, 4311–4321.

Ezzeldin, M., Borrego-Diaz, E., Taha, M., Esfandyari, T., Wise, A.L., Peng, W., Rouyanian, A., Asvadi Kermani, A., Soleimani, M., Patrad, E., et al. (2014). RalA signaling pathway as a therapeutic target in hepatocellular carcinoma (HCC). Mol. Oncol. *8*, 1043–1053.

Falsetti, S.C., Wang, D., Peng, H., Carrico, D., Cox, A.D., Der, C.J., Hamilton, A.D., and Sebti, S.M. (2007). Geranylgeranyltransferase I inhibitors target RalB to inhibit anchorage-dependent growth and induce apoptosis and RalA to inhibit anchorage-independent growth. Mol. Cell. Biol. *27*, 8003–8014.

Farré, J.C., and Subramani, S. (2011). Rallying the exocyst as an autophagy scaffold. Cell *144*, 172–174.

Fenwick, R.B., Campbell, L.J., Rajasekar, K., Prasannan, S., Nietlispach, D., Camonis, J., Owen, D., and Mott, H.R. (2010). The RalB-RLIP76 complex reveals a novel mode of ral-effector interaction. Structure *18*, 985–995.

Fernández, R.M.H., Ruiz-Miró, M., Dolcet, X., Aldea, M., and Garí, E. (2011). Cyclin D1 interacts and collaborates with Ral GTPases enhancing cell detachment and motility. Oncogene *30*, 1936–1946.

Ferro, E., and Trabalzini, L. (2010). RalGDS family members couple Ras to Ral signalling and that's not all. Cell. Signal. *22*, 1804–1810.

Fielding, A.B., Schonteich, E., Matheson, J., Wilson, G., Yu, X., Hickson, G.R.X., Srivastava, S., Baldwin, S.A., Prekeris, R., and Gould, G.W. (2005). Rab11-FIP3 and FIP4 interact with Arf6 and the Exocyst to control membrane traffic in cytokinesis. EMBO J. *24*, 3389–3399.

Fillatre, J., Delacour, D., Van Hove, L., Bagarre, T., Houssin, N., Soulika, M., Veitia, R.A., and Moreau, J. (2012). Dynamics of the subcellular localization of RalBP1/RLIP through the cell cycle: the role of targeting signals and of protein-protein interactions. FASEB J. *26*, 2164–2174.

Fiucci, G., Ravid, D., Reich, R., and Liscovitch, M. (2002). Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene *21*, 2365–2375.

Frankel, P., Aronheim, A., Kavanagh, E., Balda, M.S., Matter, K., Bunney, T.D., and Marshall, C.J. (2005). RalA interacts with ZONAB in a cell density-dependent manner and regulates its transcriptional activity. EMBO J. *24*, 54–62.

Fu, Y., Li, J., Feng, M.-X., Yang, X.-M., Wang, Y.-H., Zhang, Y.-L., Qin, W., Xia, Q., and Zhang, Z.-G. (2014). Cytohesin-3 is upregulated in hepatocellular carcinoma and contributes to tumor growth and vascular invasion. Int. J. Clin. Exp. Pathol. *7*, 2123–2132.

Furukawa, K., and Fukuda, M. (2016). Glycosignals in Cancer: Mechanisms of Malignant Phenotypes (Springer).

Gamblin, S.J., and Smerdon, S.J. (1998). GTPase-activating proteins and their complexes. Curr. Opin. Struct. Biol. *8*, 195–201.

Garcia-Mata, R., Boulter, E., and Burridge, K. (2011). The "invisible hand": regulation of RHO GTPases by RHOGDIs. Nat. Rev. Mol. Cell Biol. *12*, 493–504.

Gaus, K., Le Lay, S., Balasubramanian, N., and Schwartz, M.A. (2006). Integrin-mediated adhesion regulates membrane order. J. Cell Biol. *174*, 725–734.

Gaynor, E.C., Chen, C.Y., Emr, S.D., and Graham, T.R. (1998). ARF is required for maintenance of yeast Golgi and endosome structure and function. Mol. Biol. Cell *9*, 653–670.

Gentry, L.R., Martin, T.D., Reiner, D.J., and Der, C.J. (2014). Ral small GTPase signaling and oncogenesis: More than just 15minutes of fame. Biochim. Biophys. Acta *1843*, 2976–2988.

Gentry, L.R., Nishimura, A., Cox, A.D., Martin, T.D., Tsygankov, D., Nishida, M., Elston, T.C., and Der, C.J. (2015). Divergent roles of CAAX motif-signaled posttranslational modifications in the regulation and subcellular localization of Ral GTPases. J. Biol. Chem. *290*, 22851–22861.

Gillingham, A.K., and Munro, S. (2007). The small G proteins of the Arf family and their regulators. Annu. Rev. Cell Dev. Biol. *23*, 579–611.

Godin, C.M., and Ferguson, S.S.G. (2010). The angiotensin II type 1 receptor induces membrane blebbing by coupling to Rho A, Rho kinase, and myosin light chain kinase. Mol. Pharmacol. *77*, 903–911.

Goldberg, J. (1998). Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. Cell *95*, 237–248.

Goldfinger, L.E., Ptak, C., Jeffery, E.D., Shabanowitz, J., Hunt, D.F., and Ginsberg, M.H. (2006). RLIP76 (RalBP1) is an R-Ras effector that mediates adhesion-dependent Rac activation and cell migration. J. Cell Biol. *174*, 877–888.

Golub, T., and Pico, C. (2005). Spatial control of actin-based motility through plasmalemmal PtdIns(4,5)P2-rich raft assemblies. Biochem. Soc. Symp. 119–127.

Guin, S., Ru, Y., Wynes, M.W., Mishra, R., Lu, X., Owens, C., Barn, A.E., Vasu, V.T., Hirsch, F.R., Kern, J.A., et al. (2013). Contributions of KRAS and RAL in non-small-cell lung cancer growth and progression. J. Thorac. Oncol. *8*, 1492–1501.

Hall, B., McLean, M. a, Davis, K., Casanova, J.E., Sligar, S.G., and Schwartz, M. a (2008). A fluorescence resonance energy transfer activation sensor for Arf6. Anal. Biochem. *374*, 243–249.

Hamada, M., Miki, T., Iwai, S., Shimizu, H., and Yura, Y. (2011). Involvement of RhoA and RalB in geranylgeranyltransferase I inhibitor-mediated inhibition of proliferation and migration of human oral squamous cell carcinoma cells. Cancer Chemother. Pharmacol. *68*, 559–569.

Han, F., Gu, D., Chen, Q., and Zhu, H. (2009a). Caveolin-1 acts as a tumor suppressor by down-regulating epidermal growth factor receptor-mitogen-activated protein kinase signaling pathway in pancreatic carcinoma cell lines. Pancreas *38*, 766–774.

Han, K., Kim, M.-H., Seeburg, D., Seo, J., Verpelli, C., Han, S., Chung, H.S., Ko, J., Lee, H.W., Kim, K., et al. (2009b). Regulated RalBP1 binding to RalA and PSD-95 controls AMPA receptor endocytosis and LTD. PLoS Biol. *7*, e1000187.

Hashimoto, S., Onodera, Y., Hashimoto, A., Tanaka, M., Hamaguchi, M., Yamada, A., and Sabe, H. (2004). Requirement for Arf6 in breast cancer invasive activities. Proc. Natl. Acad. Sci. U. S. A. *101*, 6647–6652.

Hatzoglou, A., Parrini, M., White, M. a, Chavrier, P., and Camonis, J. (2006). The Ral GTPases are essential components of the Ras reg- ulatory network and make a major contribution to Ras-in- machinery supporting tumor initiation and progression . Although it is known that Ral proteins mediate Carine Rosse RalB Mobilizes the Exocys. Mol Cell Biol *26*, 727–734.

Hazelett, C.C., Sheff, D., and Yeaman, C. (2011). RalA and RalB differentially regulate development of epithelial tight junctions. Mol. Biol. Cell *22*, 4787–4800.

He, B., Xi, F., Zhang, X., Zhang, J., and Guo, W. (2007). Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. EMBO J. *26*, 4053–4065.

Heider, M.R., and Munson, M. (2012). Exorcising the Exocyst Complex. Traffic *13*, 898–907.

Heikkilä, O., Susi, P., Tevaluoto, T., Härmä, H., Marjomäki, V., Hyypiä, T., and Kiljunen, S. (2010). Internalization of coxsackievirus A9 is mediated by {beta}2-microglobulin, dynamin, and Arf6 but not by caveolin-1 or clathrin. J. Virol. *84*, 3666–3681.

Hiroyama, M., and Exton, J.H. (2005a). Studies of the roles of ADP-ribosylation factors and phospholipase D in phorbol ester-induced membrane ruffling. J. Cell. Physiol. *202*, 608–622.

Hiroyama, M., and Exton, J.H. (2005b). Localization and regulation of phospholipase D2 by ARF6. J. Cell. Biochem. *95*, 149–164.

Hofer, F., Berdeaux, R., and Martin, G.S. (1998). Ras-independent activation of Ral by a Ca(2+)-dependent pathway. Curr. Biol. *8*, 839–842.

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A.J., Frohman, M.A., et al. (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell *99*, 521–532.

Hornia, A., Lu, Z., Sukezane, T., Zhong, M., Joseph, T., Frankel, P., and Foster, D.A. (1999). Antagonistic effects of protein kinase C alpha and delta on both transformation and phospholipase D activity mediated by the epidermal growth factor receptor. Mol. Cell. Biol. *19*, 7672–7680.

Houndolo, T., Boulay, P.-L., and Claing, A. (2005). G protein-coupled receptor endocytosis in ADP-ribosylation factor 6-depleted cells. J. Biol. Chem. *280*, 5598–5604.

Hu, Z., Du, J., Yang, L., Zhu, Y., Yang, Y., Zheng, D., Someya, A., Gu, L., and Lu, X. (2012). GEP100/Arf6 is required for epidermal growth factor-induced ERK/Rac1 signaling and cell migration in human hepatoma HepG2 cells. PLoS One *7*, e38777.

Hu, Z., Xu, R., Liu, J., Zhang, Y., Du, J., Li, W., Zhang, W., Li, Y., Zhu, Y., and Gu, L. (2013). GEP100 regulates epidermal growth factor-induced MDA-MB-231 breast cancer cell invasion through the activation of Arf6/ERK/uPAR signaling pathway. Exp. Cell Res. *319*, 1932–1941.

Humphreys, D., Davidson, A., Hume, P.J., and Koronakis, V. (2012). Salmonella virulence effector SopE and Host GEF ARNO cooperate to recruit and activate WAVE to trigger bacterial invasion. Cell Host Microbe *11*, 129–139.

Humphreys, D., Davidson, A.C., Hume, P.J., Makin, L.E., and Koronakis, V. (2013). Arf6 coordinates actin assembly through the WAVE complex, a mechanism usurped by Salmonella to invade host cells. Proc. Natl. Acad. Sci. U. S. A. *110*, 16880–16885.

Hutchins, J.R.A., Moore, W.J., and Clarke, P.R. (2009). Dynamic localisation of Ran GTPase during the cell cycle. BMC Cell Biol. *10*, 66.

Hyenne, V., Apaydin, A., Rodriguez, D., Spiegelhalter, C., Hoff-Yoessle, S., Diem, M., Tak, S., Lefebvre, O., Schwab, Y., Goetz, J.G., et al. (2015). RAL-1 controls multivesicular body biogenesis and exosome secretion. J. Cell Biol. *211*, 27–37.

Isomura, M., Okui, K., Fujiwara, T., Shin, S., and Nakamura, Y. (1996). Isolation and mapping of RAB2L, a human cDNA that encodes a protein homologous to RalGDS. Cytogenet. Cell Genet. *74*, 263–265.

Issaq, S.H., Lim, K., and Counter, C.M. (2010). Sec5 and Exo84 foster oncogenic ras-mediated tumorigenesis. Mol. Cancer Res. *8*, 223–231.

Jang, J.-H., Lee, C.S., Hwang, D., and Ryu, S.H. (2012). Understanding of the roles of phospholipase D and phosphatidic acid through their binding partners. Prog. Lipid Res. *51*, 71–81.

Jayaram, B., and Kowluru, A. (2012). Phagocytic NADPH oxidase links ARNO-Arf6 signaling pathway in glucose-stimulated insulin secretion from the pancreatic β-cell. Cell. Physiol. Biochem. *30*, 1351–1362.

Jayaram, B., Syed, I., Kyathanahalli, C.N., Rhodes, C.J., and Kowluru, A. (2011). Arf nucleotide binding site opener [ARNO] promotes sequential activation of Arf6, Cdc42 and Rac1 and insulin

secretion in INS 832/13 β-cells and rat islets. Biochem. Pharmacol. *81*, 1016–1027.

Jiang, H., Luo, J.Q., Urano, T., Frankel, P., Lu, Z., Foster, D.A., and Feig, L.A. (1995). Involvement of Ral GTPase in v-Src-induced phospholipase D activation. Nature *378*, 409–412.

Jin, R., Junutula, J.R., Matern, H.T., Ervin, K.E., Scheller, R.H., and Brunger, A.T. (2005). Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase. EMBO J. *24*, 2064–2074.

Joseph, N., Hutterer, A., Poser, I., and Mishima, M. (2012). ARF6 GTPase protects the post-mitotic midbody from 14-3-3-mediated disintegration. EMBO J. *31*, 2604–2614.

Jovanovic, O.A., Brown, F.D., and Donaldson, J.G. (2006). An effector domain mutant of Arf6 implicates phospholipase D in endosomal membrane recycling. Mol. Biol. Cell *17*, 327–335.

Jullien-Flores, V., Dorseuil, O., Romero, F., Letourneur, F., Saragosti, S., Berger, R., Tavitian, A., Gacon, G., and Camonis, J.H. (1995). Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. J. Biol. Chem. *270*, 22473–22477.

Kahn, R.A., and Gilman, A.G. (1984). Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. J. Biol. Chem. *259*, 6228–6234.

Karnik, R., Ludlow, M.J., Abuarab, N., Smith, A.J., Hardy, M.E.L., Elliott, D.J.S., and Sivaprasadarao, A. (2013). Endocytosis of HERG is clathrin-independent and involves arf6. PLoS One *8*, e85630.

Karnoub, A.E., and Weinberg, R.A. (2008). Ras oncogenes: split personalities. Nat. Rev. Mol. Cell Biol. *9*, 517–531.

Kashatus, D.F., Lim, K., Brady, D.C., Pershing, N.L.K., Cox, A.D., and Counter, C.M. (2011). RALA and RALBP1 regulate mitochondrial fission at mitosis. Nat. Cell Biol. *13*, 1108–1115.

Kim, J.H., Lee, S.D., Han, J.M., Lee, T.G., Kim, Y., Park, J.B., Lambeth, J.D., Suh, P.G., and Ryu, S.H. (1998). Activation of phospholipase D1 by direct interaction with ADP-ribosylation factor 1 and RalA. FEBS Lett. *430*, 231–235.

Kiyokawa, E., Aoki, K., Nakamura, T., and Matsuda, M. (2011). Spatiotemporal regulation of small GTPases as revealed by probes based on the principle of Förster Resonance Energy Transfer (FRET): Implications for signaling and pharmacology. Annu. Rev. Pharmacol. Toxicol. *51*, 337–358.

ten Klooster, J.P., Jaffer, Z.M., Chernoff, J., and Hordijk, P.L. (2006). Targeting and activation of Rac1 are mediated by the exchange factor beta-Pix. J. Cell Biol. *172*, 759–769.

Knizhnik, a. V., Kovaleva, O. V., Laktionov, K.K., Mochalnikova, V. V., Komelkov, a. V., Tchevkina, E.M., and Zborovskaya, I.B. (2011). Arf6, RalA, and BIRC5 protein expression in nonsmall cell lung cancer. Mol. Biol. *45*, 275–282.

Knizhnik, A. V, Kovaleva, O. V, Komelkov, A. V, Trukhanova, L.S., Rybko, V. a, Zborovskaya, I.B., and Tchevkina, E.M. (2012). Arf6 promotes cell proliferation via the PLD-mTORC1 and p38MAPK pathways. J. Cell. Biochem. *113*, 360–371.

Kobayashi, H., and Fukuda, M. (2012). Rab35 regulates Arf6 activity through centaurin- 2 (ACAP2) during neurite outgrowth. J. Cell Sci. *125*, 2235–2243.

Koo, T.H., Eipper, B.A., and Donaldson, J.G. (2007). Arf6 recruits the Rac GEF Kalirin to the plasma membrane facilitating Rac activation. BMC Cell Biol. *8*, 29.

Krugmann, S., Anderson, K.E., Ridley, S.H., Risso, N., McGregor, A., Coadwell, J., Davidson, K., Eguinoa, A., Ellson, C.D., Lipp, P., et al. (2002). Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices. Mol. Cell *9*, 95–108.

Kuo, J.-C., Han, X., Hsiao, C.-T., Yates III, J.R., and Waterman, C.M. (2011). Analysis of the myosin-II-responsive focal adhesion proteome reveals a role for β-Pix in negative regulation of focal adhesion maturation. Nat. Cell Biol. *13*, 383–393.

Lalli, G., and Hall, A. (2005). Ral GTPases regulate neurite branching through GAP-43 and the exocyst complex. J. Cell Biol. *171*, 857–869.

Lawrence, J.T.R., and Birnbaum, M.J. (2003a). ADP-ribosylation factor 6 regulates insulin secretion through plasma membrane phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. U. S. A. *100*, 13320–13325.

Lawrence, J.T.R., and Birnbaum, M.J. (2003b). ADP-ribosylation factor 6 regulates insulin secretion through plasma membrane phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. U. S. A. *100*, 13320–13325.

Lee, C.S., Kim, I.S., Park, J.B., Lee, M.N., Lee, H.Y., Suh, P.-G., and Ryu, S.H. (2006). The phox homology domain of phospholipase D activates dynamin GTPase activity and accelerates EGFR endocytosis. Nat. Cell Biol. *8*, 477–484.

Lee, S., Wurtzel, J.G.T., and Goldfinger, L.E. (2014). The RLIP76 N-terminus binds ARNO to regulate PI 3-kinase, Arf6 and Rac signaling, cell spreading and migration. Biochem. Biophys. Res. Commun. *454*, 560–565.

Leslie M. Shaw, A.S. and V.W.H.A.M.P.J.S.J.L.M.G. (2003). Co. Traffic *5*, 1–18.

Leto, D., Uhm, M., Williams, A., Chen, X., and Saltiel, A.R. (2013). Negative regulation of the RalGAP complex by 14-3-3. J. Biol. Chem. *288*, 9272–9283.

Lewis, J.A., Scott, S.A., Lavieri, R., Buck, J.R., Selvy, P.E., Stoops, S.L., Armstrong, M.D., Brown, H.A., and Lindsley, C.W. (2009). Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part I: Impact of alternative halogenated privileged structures for PLD1 specificity. Bioorg. Med. Chem. Lett. *19*, 1916–1920.

Li, G., Han, L., Chou, T.-C., Fujita, Y., Arunachalam, L., Xu, A., Wong, A., Chiew, S.-K., Wan, Q., Wang, L., et al. (2007). RalA and RalB function as the critical GTP sensors for GTP-dependent exocytosis. J. Neurosci. *27*, 190–202.

Li, J., Malaby, A.W., Famulok, M., Sabe, H., Lambright, D.G., and Hsu, V.W. (2012). Grp1 Plays a Key Role in Linking Insulin Signaling to Glut4 Recycling. Dev. Cell *22*, 1286–1298.

Li, M., Ng, S.S., Wang, J., Lai, L., Leung, S.Y., Franco, M., Peng, Y., He, M., Kung, H., and Lin, M.C. (2006). EFA6A enhances glioma cell invasion through ADP ribosylation factor 6/extracellular signal-regulated kinase signaling. Cancer Res. *66*, 1583–1590.

Li, T.T., Alemayehu, M., Aziziyeh, A.I., Pape, C., Pampillo, M., Postovit, L., Mills, G.B., Babwah, A. V, and Bhattacharya, M. (2009). Beta-arrestin/Ral signaling regulates lysophosphatidic acid-mediated migration and invasion of human breast tumor cells. Mol. Cancer Res. *7*, 1064–1077.

Lim, K., Brady, D.C., Kashatus, D.F., Ancrile, B.B., Der, C.J., Cox, A.D., and Counter, C.M. (2010). Aurora-A phosphorylates, activates, and relocalizes the small GTPase RalA. Mol. Cell. Biol. *30*, 508–523.

Lim, K.H., Baines, A.T., Fiordalisi, J.J., Shipitsin, M., Feig, L. a., Cox, A.D., Der, C.J., and Counter, C.M. (2005). Activation of RalA is critical for Ras-induced tumorigenesis of human cells. Cancer Cell *7*, 533–545.

Lim, K.H., O'Hayer, K., Adam, S.J., Kendall, S.D., Campbell, P.M., Der, C.J., and Counter, C.M. (2006). Divergent Roles for RalA and RalB in Malignant Growth of Human Pancreatic Carcinoma Cells. Curr. Biol. *16*, 2385–2394.

Liu, J., Zuo, X., Yue, P., and Guo, W. (2007). Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. Mol. Biol. Cell *18*, 4483–4492.

Liu, L., Liao, H., Castle, A., Zhang, J., Casanova, J., Szabo, G., and Castle, D. (2005). SCAMP2

interacts with Arf6 and phospholipase D1 and links their function to exocytotic fusion pore formation in PC12 cells. Mol. Biol. Cell *16*, 4463–4472.

Ljubicic, S., Bezzi, P., Vitale, N., and Regazzi, R. (2009). The GTPase RalA regulates different steps of the secretory process in pancreatic beta-cells. PLoS One *4*, e7770.

Lopez, J.A., Kwan, E.P., Xie, L., He, Y., James, D.E., and Gaisano, H.Y. (2008). The RalA GTPase is a central regulator of insulin exocytosis from pancreatic islet beta cells. J. Biol. Chem. *283*, 17939–17945.

Lu, Z., Hornia, A., Joseph, T., Sukezane, T., Frankel, P., Zhong, M., Bychenok, S., Feig, L.A., Foster, D.A., and Xu, L. (2000). Phospholipase D and RalA Cooperate with the Epidermal Growth Factor Receptor To Transform 3Y1 Rat Fibroblasts Phospholipase D and RalA Cooperate with the Epidermal Growth Factor Receptor To Transform 3Y1 Rat Fibroblasts. Mol. Cell. Biol. *20*, 462–467.

Luo, J.Q., Liu, X., Hammond, S.M., Colley, W.C., Feig, L.A., Frohman, M.A., Morris, A.J., and Foster, D.A. (1997). RalA interacts directly with the Arf-responsive, PIP2-dependent phospholipase D1. Biochem. Biophys. Res. Commun. *235*, 854–859.

Luo, J.Q., Liu, X., Frankel, P., Rotunda, T., Ramos, M., Flom, J., Jiang, H., Feig, L. a, Morris, a J., Kahn, R. a, et al. (1998). Functional association between Arf and RalA in active phospholipase D complex. Proc. Natl. Acad. Sci. U. S. A. *95*, 3632–3637.

Makyio, H., Ohgi, M., Takei, T., Takahashi, S., Takatsu, H., Katoh, Y., Hanai, A., Ueda, T., Kanaho, Y., Xie, Y., et al. (2012). Structural basis for Arf6-MKLP1 complex formation on the Flemming body responsible for cytokinesis. EMBO J. *31*, 2590–2603.

Male, H., Patel, V., Jacob, M. a, Borrego-Diaz, E., Wang, K., Young, D. a, Wise, A.L., Huang, C., Van Veldhuizen, P., O'Brien-Ladner, A., et al. (2012). Inhibition of RalA signaling pathway in treatment of non-small cell lung cancer. Lung Cancer *77*, 252–259.

Manser, E. (2002a). Small GTPases take the stage. Dev. Cell *3*, 323–328.

Manser, E. (2002b). Small GTPases take the stage. Dev. Cell *3*, 323–328.

Martin, T.D., and Der, C.J. (2012). Differential involvement of RalA and RalB in colorectal cancer. Small GTPases *3*, 126–130.

Martin, T.D., Samuel, J.C., Routh, E.D., Der, C.J., and Yeh, J.J. (2011). Activation and involvement of Ral GTPases in colorectal cancer. Cancer Res. *71*, 206–215.

Martin, T.D., Mitin, N., Cox, A.D., Yeh, J.J., and Der, C.J. (2012). Phosphorylation by protein kinase

Cα regulates RalB small GTPase protein activation, subcellular localization, and effector utilization. J. Biol. Chem. *287*, 14827–14836.

Martin, T.D., Chen, X.-W., Kaplan, R.E.W., Saltiel, A.R., Walker, C.L., Reiner, D.J., and Der, C.J. (2014). Ral and Rheb GTPase activating proteins integrate mTOR and GTPase signaling in aging, autophagy, and tumor cell invasion. Mol. Cell *53*, 209–220.

Matsubara, K., Kishida, S., Matsuura, Y., Kitayama, H., Noda, M., and Kikuchi, A. (1999). Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation. Oncogene *18*, 1303–1312.

Melendez, A.J., Harnett, M.M., and Allen, J.M. (2001). Crosstalk between ARF6 and protein kinase Calpha in Fc(gamma)RI-mediated activation of phospholipase D1. Curr. Biol. *11*, 869–874.

Millar, C.A., Powell, K.A., Hickson, G.R., Bader, M.F., and Gould, G.W. (1999). Evidence for a role for ADP-ribosylation factor 6 in insulin-stimulated glucose transporter-4 (GLUT4) trafficking in 3T3-L1 adipocytes. J. Biol. Chem. *274*, 17619–17625.

Miller, A.L., and Bement, W.M. (2009). Regulation of cytokinesis by Rho GTPase flux. Nat. Cell Biol. *11*, 71–77.

Mitin, N., Rossman, K.L., and Der, C.J. (2005). Signaling interplay in Ras superfamily function. Curr. Biol. *15*, R563–R574.

Miura, K., Jacques, K.M., Stauffer, S., Kubosaki, A., Zhu, K., Hirsch, D.S., Resau, J., Zheng, Y., and Randazzo, P.A. (2002). ARAP1: a point of convergence for Arf and Rho signaling. Mol. Cell *9*, 109–119.

Mollberg, N.M., Steinert, G., Aigner, M., Hamm, A., Lin, F., Elbers, H., Reissfelder, C., Weitz, J., Buchler, M.W., and Koch, M. (2012). Overexpression of RalBP1 in colorectal cancer is an independent predictor of poor survival and early tumor relapse. Cancer Biol. Ther. *13*, 694–700.

Montagnac, G., Echard, A., and Chavrier, P. (2008). Endocytic traffic in animal cell cytokinesis. Curr. Opin. Cell Biol. *20*, 454–461.

Moreau, K., Ravikumar, B., Puri, C., and Rubinsztein, D.C. (2012). Arf6 promotes autophagosome formation via effects on phosphatidylinositol 4,5-bisphosphate and phospholipase D. J. Cell Biol. *196*, 483–496.

Morishige, M., Hashimoto, S., Ogawa, E., Toda, Y., Kotani, H., Hirose, M., Wei, S., Hashimoto, A., Yamada, A., Yano, H., et al. (2008). GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion. Nat. Cell Biol. *10*, 85–92.

Moskalenko, S., Henry, D.O., Rosse, C., Mirey, G., Camonis, J.H., and White, M. a (2002). The exocyst is a Ral effector complex. Nat. Cell Biol. *4*, 66–72.

Moskalenko, S., Tong, C., Rosse, C., Mirey, G., Formstecher, E., Daviet, L., Camonis, J., and White, M.A. (2003). Ral GTPases Regulate Exocyst Assembly through Dual Subunit Interactions. J. Biol. Chem. *278*, 51743–51748.

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., and D'Souza-Schorey, C. (2009). ARF6-Regulated Shedding of Tumor Cell-Derived Plasma Membrane Microvesicles. Curr. Biol. *19*, 1875–1885.

Neel, N.F., Martin, T.D., Stratford, J.K., Zand, T.P., Reiner, D.J., and Der, C.J. (2011). The RalGEF-Ral Effector Signaling Network: The Road Less Traveled for Anti-Ras Drug Discovery. Genes Cancer *2*, 275–287.

Neel, N.F., Rossman, K.L., Martin, T.D., Hayes, T.K., Yeh, J.J., and Der, C.J. (2012a). The RalB Small GTPase Mediates Formation of Invadopodia through a GTPase-Activating Protein-Independent Function of the RalBP1/RLIP76 Effector. Mol. Cell. Biol. *32*, 1374–1386.

Neel, N.F., Rossman, K.L., Martin, T.D., Hayes, T.K., Yeh, J.J., and Der, C.J. (2012b). The RalB Small GTPase Mediates Formation of Invadopodia through a GTPase-Activating Protein-Independent Function of the RalBP1/RLIP76 Effector. Mol. Cell. Biol. *32*, 1374–1386.

Neely, A., and Hidalgo, P. (2014). Structure-function of proteins interacting with the α1 pore-forming subunit of high-voltage-activated calcium channels. Front. Physiol. *5*, 209.

Neyraud, V., Aushev, V.N., Hatzoglou, A., Meunier, B., Cascone, I., and Camonis, J. (2012). RalA and RalB proteins are ubiquitinated GTPases, and ubiquitinated RalA increases lipid raft exposure at the plasma membrane. J. Biol. Chem. *287*, 29397–29405.

Nichols, C.D., and Casanova, J.E. (2010). Salmonella-directed recruitment of new membrane to invasion foci via the host exocyst complex. Curr. Biol. *20*, 1316–1320.

Niedergang, F., Colucci-Guyon, E., Dubois, T., Raposo, G., and Chavrier, P. (2003). ADP ribosylation factor 6 is activated and controls membrane delivery during phagocytosis in macrophages. J. Cell Biol. *161*, 1143–1150.

Nielsen, E., Cheung, A.Y., and Ueda, T. (2008). The regulatory RAB and ARF GTPases for vesicular trafficking. Plant Physiol. *147*, 1516–1526.

Norambuena, A., and Schwartz, M.A. (2011). Effects of integrin-mediated cell adhesion on plasma membrane lipid raft components and signaling. Mol. Biol. Cell *22*, 3456–3464.

Ohta, Y., Suzuki, N., Nakamura, S., Hartwig, J.H., and Stossel, T.P. (1999). The small GTPase RalA targets filamin to induce filopodia. Proc. Natl. Acad. Sci. U. S. A. *96*, 2122–2128.

Onodera, Y., Hashimoto, S., Hashimoto, A., Morishige, M., Mazaki, Y., Yamada, A., Ogawa, E., Adachi, M., Sakurai, T., Manabe, T., et al. (2005). Expression of AMAP1, an ArfGAP, provides novel targets to inhibit breast cancer invasive activities. EMBO J. *24*, 963–973.

Osei-Sarfo, K., Martello, L., Ibrahim, S., and Pellicer, A. (2011). The human Rgr oncogene is overexpressed in T-cell malignancies and induces transformation by acting as a GEF for Ras and Ral. Oncogene *30*, 3661–3671.

Oude Weernink, P.A., López de Jesús, M., and Schmidt, M. (2007a). Phospholipase D signaling: orchestration by PIP2 and small GTPases. Naunyn. Schmiedebergs. Arch. Pharmacol. *374*, 399–411.

Oude Weernink, P.A., López de Jesús, M., and Schmidt, M. (2007b). Phospholipase D signaling: orchestration by PIP2 and small GTPases. Naunyn. Schmiedebergs. Arch. Pharmacol. *374*, 399–411.

Oxford, G., Owens, C.R., Titus, B.J., Foreman, T.L., Herlevsen, M.C., Smith, S.C., and Theodorescu, D. (2005). RalA and RalB: Antagonistic relatives in cancer cell migration. Cancer Res. *65*, 7111–7120.

Padovani, D., Folly-Klan, M., Labarde, A., Boulakirba, S., Campanacci, V., Franco, M., Zeghouf, M., and Cherfils, J. (2014). EFA6 controls Arf1 and Arf6 activation through a negative feedback loop. Proc. Natl. Acad. Sci. U. S. A. *111*, 12378–12383.

Palacios, F., Price, L., Schweitzer, J., Collard, J.G., and D'Souza-Schorey, C. (2001). An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. EMBO J. *20*, 4973–4986.

Palacios, F., Schweitzer, J.K., Boshans, R.L., and D'Souza-Schorey, C. (2002). ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. Nat.Cell Biol. *4*, 929–936.

Palamidessi, A., Frittoli, E., Garré, M., Faretta, M., Mione, M., Testa, I., Diaspro, A., Lanzetti, L., Scita, G., and Di Fiore, P.P. (2008). Endocytic trafficking of Rac is required for the spatial restriction of signaling in cell migration. Cell *134*, 135–147.

Palazzo, A.F., Eng, C.H., Schlaepfer, D.D., Marcantonio, E.E., and Gundersen, G.G. (2004). Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. Science (80-. ). *303*, 836–839.

Parrini, M.C., Sadou-Dubourgnoux, A., Aoki, K., Kunida, K., Biondini, M., Hatzoglou, A., Poullet,

P., Formstecher, E., Yeaman, C., Matsuda, M., et al. (2011). SH3BP1, an exocyst-associated RhoGAP, inactivates Rac1 at the front to drive cell motility. Mol. Cell *42*, 650–661.

Pasqualato, S., Ménétrey, J., Franco, M., and Cherfils, J. (2001). The structural GDP/GTP cycle of human Arf6. EMBO Rep. *2*, 234–238.

Pasqualato, S., Renault, L., and Cherfils, J. (2002). Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for "front-back" communication. EMBO Rep. *3*, 1035–1041.

Pawar, A., Meier, J.A., Dasgupta, A., Diwanji, N., Deshpande, N., Saxena, K., Buwa, N., Inchanalkar, S., Schwartz, M.A., and Balasubramanian, N. (2016). Ral-Arf6 crosstalk regulates Ral dependent exocyst trafficking and anchorage independent growth signalling. Cell. Signal. *28*, 1225–1236.

Peschard, P., McCarthy, A., Leblanc-Dominguez, V., Yeo, M., Guichard, S., Stamp, G., and Marshall, C.J. (2012). Genetic deletion of RALA and RALB small GTPases reveals redundant functions in development and tumorigenesis. Curr. Biol. *22*, 2063–2068.

Powelka, A.M., Sun, J., Li, J., Gao, M., Shaw, L.M., Sonnenberg, A., and Hsu, V.W. (2004). Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic *5*, 20–36.

Del Pozo, M.A., Alderson, N.B., Kiosses, W.B., Chiang, H.-H., Anderson, R.G.W., and Schwartz, M.A. (2004). Integrins Regulate Rac Targeting by Internalization of Membrane Domains. Sci. (New York, NY) *303*, 839–842.

del Pozo, M.A., Balasubramanian, N., Alderson, N.B., Kiosses, W.B., Grande-García, A., Anderson, R.G.W., and Schwartz, M.A. (2005). Phospho-caveolin-1 mediates integrin-regulated membrane domain internalization. Nat. Cell Biol. *7*, 901–908.

Prigent, M., Dubois, T., Raposo, G., Derrien, V., Tenza, D., Rossé, C., Camonis, J., and Chavrier, P. (2003a). ARF6 controls post-endocytic recycling through its downstream exocyst complex effector. J. Biol. Chem. *163*, 1111–1121.

Prigent, M., Dubois, T., Raposo, G., Derrien, V., Tenza, D., Rossé, C., Camonis, J., and Chavrier, P. (2003b). ARF6 controls post-endocytic recycling through its downstream exocyst complex effector. J. Cell Biol. *163*, 1111–1121.

Prior, I.A., Lewis, P.D., and Mattos, C. (2012). A comprehensive survey of Ras mutations in cancer. Cancer Res. *72*, 2457–2467.

Radhakrishna, H., Al-Awar, O., Khachikian, Z., and Donaldson, J.G. (1999). ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. J. Cell Sci. *112* ( Pt 6*, 855–866.

Randazzo, P.A., and Hirsch, D.S. (2004). Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodelling. Cell. Signal. *16*, 401–413.

Randazzo, P.A., Inoue, H., and Bharti, S. (2007). Arf GAPs as regulators of the actin cytoskeleton. Biol. Cell *99*, 583–600.

Rauen, K.A. (2013). The RASopathies. Annu. Rev. Genomics Hum. Genet. *14*, 355–369.

Rebhun, J.F., Chen, H., and Quilliam, L.A. (2000a). Identification and characterization of a new family of guanine nucleotide exchange factors for the ras-related GTPase Ral. J. Biol. Chem. *275*, 13406–13410.

Rebhun, J.F., Chen, H., and Quilliam, L.A. (2000b). Identification and characterization of a new family of guanine nucleotide exchange factors for the ras-related GTPase Ral. J. Biol. Chem. *275*, 13406–13410.

Ren, X.D., and Schwartz, M.A. (2000). Determination of GTP loading on Rho. Methods Enzymol. *325*, 264–272.

Ren, N., Oxford, G., Smith, S., Owens, C., and Theodorescu, D. (2007). Aurora-A and Ral GTPases as targets in the treatment of human bladder cancer. Cancer Res. *67*, 4360 – .

Rossé, C., Hatzoglou, A., Parrini, M.-C., White, M.A., Chavrier, P., and Camonis, J. (2006). RalB mobilizes the exocyst to drive cell migration. Mol. Cell. Biol. *26*, 727–734.

Sabe, H. (2003). Requirement for Arf6 in Cell Adhesion, Migration, and Cancer Cell Invasion. J. Biochem. *134*, 485–489.

Sabe, H., Onodera, Y., Mazaki, Y., and Hashimoto, S. (2006). ArfGAP family proteins in cell adhesion, migration and tumor invasion. Curr. Opin. Cell Biol. *18*, 558–564.

Sáenz, J.B., Sun, W.J., Chang, J.W., Li, J., Bursulaya, B., Gray, N.S., and Haslam, D.B. (2009a). Golgicide A reveals essential roles for GBF1 in Golgi assembly and function. Nat. Chem. Biol. *5*, 157–165.

Sáenz, J.B., Sun, W.J., Chang, J.W., Li, J., Bursulaya, B., Gray, N.S., and Haslam, D.B. (2009b). Golgicide A reveals essential roles for GBF1 in Golgi assembly and function. Nat. Chem. Biol. *5*, 157–165.

Saito, R., Shirakawa, R., Nishiyama, H., Kobayashi, T., Kawato, M., Kanno, T., Nishizawa, K., Matsui, Y., Ohbayashi, T., Horiguchi, M., et al. (2013). Downregulation of Ral GTPase-activating protein promotes tumor invasion and metastasis of bladder cancer. Oncogene *32*, 894–902.

Santy, L.C., and Casanova, J.E. (2001). Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. J. Cell Biol. *154*, 599–610.

Sanz-Moreno, V., Gadea, G., Ahn, J., Paterson, H., Marra, P., Pinner, S., Sahai, E., and Marshall, C.J. (2008). Rac activation and inactivation control plasticity of tumor cell movement. Cell *135*, 510–523.

Schmidt, A., and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. *16*, 1587–1609.

Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. (1995). INTEGRINS : Emerging Paradigms of Signal Transduction.

Schweitzer, J.K., and D'Souza-Schorey, C. (2002). Localization and activation of the ARF6 GTPase during cleavage furrow ingression and cytokinesis. J. Biol. Chem. *277*, 27210–27216.

Schweitzer, J.K., and D'Souza-Schorey, C. (2005). A requirement for ARF6 during the completion of cytokinesis. Exp. Cell Res. *311*, 74–83.

Schweitzer, J.K., Sedgwick, A.E., and D'Souza-Schorey, C. (2011). ARF6-mediated endocytic recycling impacts cell movement, cell division and lipid homeostasis. Semin. Cell Dev. Biol. *22*, 39–47.

Scott, S.A., Selvy, P.E., Buck, J.R., Cho, H.P., Criswell, T.L., Thomas, A.L., Armstrong, M.D., Arteaga, C.L., Lindsley, C.W., and Brown, H.A. (2009). Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. Nat. Chem. Biol. *5*, 108–117.

Seino, S. (2005). PKA-Dependent and PKA-Independent Pathways for cAMP-Regulated Exocytosis. Physiol. Rev. *85*, 1303–1342.

Selvy, P.E., Lavieri, R.R., Lindsley, C.W., and Brown, H.A. (2011). Phospholipase D: enzymology, functionality, and chemical modulation. Chem. Rev. *111*, 6064–6119.

Shipitsin, M., and Feig, L.A. (2004). RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. Mol. Cell. Biol. *24*, 5746–5756.

Shirai, Y., Morioka, S., Sakuma, M., Yoshino, K.-I., Otsuji, C., Sakai, N., Kashiwagi, K., Chida, K., Shirakawa, R., Horiuchi, H., et al. (2011). Direct binding of RalA to PKCη and its crucial role in morphological change during keratinocyte differentiation. Mol. Biol. Cell *22*, 1340–1352.

Shirakawa, R., Fukai, S., Kawato, M., Higashi, T., Kondo, H., Ikeda, T., Nakayama, E., Okawa, K., Nureki, O., Kimura, T., et al. (2009). Tuberous sclerosis tumor suppressor complex-like complexes

act as GTPase-activating proteins for Ral GTPases. J. Biol. Chem. *284*, 21580–21588.

Simons, K., and Gerl, M.J. (2010). Revitalizing membrane rafts: new tools and insights. Nat. Rev. Mol. Cell Biol. *11*, 688–699.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. *1*, 31–39.

Singhal, S.S., Awasthi, Y.C., and Awasthi, S. (2006). Regression of Melanoma in a Murine Model by RLIP76 Depletion. Cancer Res. *66*, 2354–2360.

Sjöblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N., et al. (2006). The consensus coding sequences of human breast and colorectal cancers. Science *314*, 268–274.

Smith, S.C., Oxford, G., Baras, A.S., Owens, C., Havaleshko, D., Brautigan, D.L., Safo, M.K., and Theodorescu, D. (2007). Expression of ral GTPases, their effectors, and activators in human bladder cancer. Clin. Cancer Res. *13*, 3803–3813.

Smith, S.C., Baras, A.S., Owens, C.R., Dancik, G., and Theodorescu, D. (2012). Transcriptional signatures of Ral GTPase are associated with aggressive clinicopathologic characteristics in human cancer. Cancer Res. *72*, 3480–3491.

Song, J., Khachikian, Z., Radhakrishna, H., and Donaldson, J.G. (1998). Localization of endogenous ARF6 to sites of cortical actin rearrangement and involvement of ARF6 in cell spreading. J. Cell Sci. *111 ( Pt 1*, 2257–2267.

Sot, B., Behrmann, E., Raunser, S., and Wittinghofer, A. (2013). Ras GTPase activating (RasGAP) activity of the dual specificity GAP protein Rasal requires colocalization and C2 domain binding to lipid membranes. Proc. Natl. Acad. Sci. U. S. A. *110*, 111–116.

Spaargaren, M., and Bischoff, J.R. (1994). Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap. Proc. Natl. Acad. Sci. U. S. A. *91*, 12609–12613.

Stalder, D., Barelli, H., Gautier, R., Macia, E., Jackson, C.L., and Antonny, B. (2011). Kinetic studies of the arf activator arno on model membranes in the presence of arf effectors suggest control by a positive feedback loop. J. Biol. Chem. *286*, 3873–3883.

Tague, S.E., Muralidharan, V., and D'Souza-Schorey, C. (2004). ADP-ribosylation factor 6 regulates tumor cell invasion through the activation of the MEK/ERK signaling pathway. Proc. Natl. Acad. Sci. U. S. A. *101*, 9671–9676.

Takahashi, S., Takei, T., Koga, H., Takatsu, H., Shin, H.-W., and Nakayama, K. (2011). Distinct roles of Rab11 and Arf6 in the regulation of Rab11-FIP3/arfophilin-1 localization in mitotic cells. Genes to Cells *16*, 938–950.

Tanabe, K., Torii, T., Natsume, W., Braesch-Andersen, S., Watanabe, T., and Satake, M. (2005). A novel GTPase-activating protein for ARF6 directly interacts with clathrin and regulates clathrin-dependent endocytosis. Mol. Biol. Cell *16*, 1617–1628.

Tatsumoto, T., Xie, X., Blumenthal, R., Okamoto, I., and Miki, T. (1999). Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. J. Cell Biol. *147*, 921–928.

Tchevkina, E., Agapova, L., Dyakova, N., Martinjuk, A., Komelkov, A., and Tatosyan, A. (2005). The small G-protein RalA stimulates metastasis of transformed cells. Oncogene *24*, 329–335.

Tracy, K., Velentzas, P.D., and Baehrecke, E.H. (2016). Ral GTPase and the exocyst regulate autophagy in a tissue-specific manner. EMBO Rep. *17*, 110–121.

Tsuboi, T., Ravier, M.A., Xie, H., Ewart, M.-A., Gould, G.W., Baldwin, S.A., and Rutter, G.A. (2005). Mammalian exocyst complex is required for the docking step of insulin vesicle exocytosis. J. Biol. Chem. *280*, 25565–25570.

Tsuchiya, M., Price, S.R., Tsai, S.C., Moss, J., and Vaughan, M. (1991). Molecular identification of ADP-ribosylation factor mRNAs and their expression in mammalian cells. J. Biol. Chem. *266*, 2772–2777.

Tzuling Cheng (2008). Evaluation of Chronic Ralgtpase Activation As a Core. Thesis.

Ueda, T., Hanai, A., Takei, T., Kubo, K., Ohgi, M., Sakagami, H., Takahashi, S., Shin, H.-W., and Nakayama, K. (2013). EFA6 activates Arf6 and participates in its targeting to the Flemming body during cytokinesis. FEBS Lett. *587*, 1617–1623.

Urano, T., Emkey, R., and Feig, L.A. (1996). Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. EMBO J. *15*, 810–816.

Veluthakal, R., Kaetzel, D., and Kowluru, A. (2013). Nm23-H1 regulates glucose-stimulated insulin secretion in pancreatic β-cells via Arf6-Rac1 signaling axis. Cell. Physiol. Biochem. *32*, 533–541.

Vetter, I.R., and Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. Science *294*, 1299–1304.

Vigil, D., Martin, T.D., Williams, F., Yeh, J.J., Campbell, S.L., and Der, C.J. (2010a). Aberrant

overexpression of the Rgl2 Ral small GTPase-specific guanine nucleotide exchange factor promotes pancreatic cancer growth through Ral-dependent and Ral-independent mechanisms. J. Biol. Chem. *285*, 34729–34740.

Vigil, D., Martin, T.D., Williams, F., Yeh, J.J., Campbell, S.L., and Der, C.J. (2010b). Aberrant Overexpression of the Rgl2 Ral Small GTPase-specific Guanine Nucleotide Exchange Factor Promotes Pancreatic Cancer Growth through Ral-dependent and Ral-independent Mechanisms. J. Biol. Chem. *285*, 34729–34740.

Vigil, D., Cherfils, J., Rossman, K.L., and Der, C.J. (2010c). Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? Nat. Rev. Cancer *10*, 842–857.

Vitale, N., Chasserot-Golaz, S., Bailly, Y., Morinaga, N., Frohman, M. a., and Bader, M.F. (2002). Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. J. Cell Biol. *159*, 79–89.

Vitale, N., Mawet, J., Camonis, J., Regazzi, R., Bader, M., and Chasserot-Golaz, S. (2005). The Small GTPase RalA controls exocytosis of large dense core secretory granules by interacting with ARF6-dependent phospholipase D1. J. Biol. Chem. *280*, 29921–29928.

Vojtek, A.B., and Der, C.J. (1998). Increasing complexity of the Ras signaling pathway. J. Biol. Chem. *273*, 19925–19928.

Volpicelli-Daley, L.A., Li, Y., Zhang, C.-J., and Kahn, R.A. (2005). Isoform-selective effects of the depletion of ADP-ribosylation factors 1-5 on membrane traffic. Mol. Biol. Cell *16*, 4495–4508.

Wang, H., Owens, C., and Chandra, N. (2010). Phosphorylation of RalB Is Important for Bladder Cancer Cell Growth and Metastasis Phosphorylation of RalB Is Important for Bladder Cancer Cell Growth and Metastasis. Cancer Res. *70*, 8760–8769.

Wang, K., Terai, K., Peng, W., Rouyanian, A., Liu, J., Roby, K.F., Wise, A.L., Ezzeldin, M., Larson, J., Woo, R.A., et al. (2013). The role of RalA in biology and therapy of ovarian cancer. Oncotarget.

Wang, L., Li, G., and Sugita, S. (2004). RalA-Exocyst Interaction Mediates GTP-dependent Exocytosis. J. Biol. Chem. *279*, 19875–19881.

Weizhong, Z., Shuohui, G., Hanjiao, Q., Yuhong, M., Xiaohua, Y., Jian, C., and Lisen, L. (2011). Inhibition of cytohesin-1 by siRNA leads to reduced IGFR signaling in prostate cancer. Brazilian J. Med. Biol. Res. *44*, 642–646.

Welch, H.C.E., Coadwell, W.J., Stephens, L.R., and Hawkins, P.T. (2003). Phosphoinositide 3-

kinase-dependent activation of Rac. FEBS Lett. *546*, 93–97.

Wennerberg, K., Rossman, K.L., and Der, C.J. (2005). The Ras superfamily at a glance. J. Cell Sci. *118*, 843–846.

White, M. a, Vale, T., Jacques, H., Schaefer, E., Michael, H., Camonis, J.H., and Wigler, M.H. (1996a). Communications : A Role for the Ral Guanine Nucleotide Dissociation Stimulator in Mediating A Role for the Ral Guanine Nucleotide Dissociation Stimulator in Mediating. 16439–16443.

White, M.A., Vale, T., Camonis, J.H., Schaefer, E., and Wigler, M.H. (1996b). A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. J. Biol. Chem. *271*, 16439–16442.

Wolthuis, R.M.F., Zwartkruis, F., Moen, T.C., and Bos, J.L. (1998). Ras-dependent activation of the small GTPase Ral. Curr. Biol. *8*, 471–474.

Wright, J., Kahn, R.A., and Sztul, E. (2014). Regulating the large Sec7 ARF guanine nucleotide exchange factors: the when, where and how of activation. Cell. Mol. Life Sci. *71*, 3419–3438.

Wu, Z., Owens, C., Chandra, N., Popovic, K., Conaway, M., and Theodorescu, D. (2010). RalBP1 is necessary for metastasis of human cancer cell lines. Neoplasia *12*, 1003–1012.

Xie, L., Kang, Y., Liang, T., Dolai, S., Xie, H., Parsaud, L., Lopez, J.A., He, Y., Chidambaram, S., Lam, P.P., et al. (2013). RalA GTPase tethers insulin granules to L- and R-type calcium channels through binding α2 δ-1 subunit. Traffic *14*, 428–439.

Xu, L., Frankel, P., Jackson, D., Rotunda, T., Boshans, R.L., Souza-schorey, C.D., Foster, D.A., and Iol, M.O.L.C.E.L.L.B. (2003). Elevated Phospholipase D Activity in H-Ras- but Not K-Ras-Transformed Cells by the Synergistic Action of RalA and ARF6. Mol. Cell. Biol. *23*, 645–654.

Yan, C., Liu, D., Li, L., Wempe, M.F., Guin, S., Khanna, M., Meier, J., Hoffman, B., Owens, C., Wysoczynski, C.L., et al. (2014). Discovery and characterization of small molecules that target the GTPase Ral. Nature *515*, 443–447.

Yang, C.Z., Heimberg, H., D'Souza-Schorey, C., Mueckler, M.M., and Stahl, P.D. (1998). Subcellular distribution and differential expression of endogenous ADP-ribosylation factor 6 in mammalian cells. J. Biol. Chem. *273*, 4006–4011.

Yano, H., Kobayashi, I., Onodera, Y., Luton, F., Franco, M., Mazaki, Y., Hashimoto, S., Iwai, K., Ronai, Z., and Sabe, H. (2008). Fbx8 makes Arf6 refractory to function via ubiquitination. Mol. Biol. Cell *19*, 822–832.

Yin, J., Pollock, C., Tracy, K., Chock, M., Martin, P., Oberst, M., and Kelly, K. (2007). Activation of the RalGEF/Ral Pathway Promotes Prostate Cancer Metastasis to Bone. Mol. Cell. Biol. *27*, 7538–7550.

Zangari, J., Partisani, M., Bertucci, F., Milanini, J., Bidaut, G., Berruyer-Pouyet, C., Finetti, P., Long, E., Brau, F., Cabaud, O., et al. (2014). EFA6B antagonizes breast cancer. Cancer Res. *74*, 5493–5506.

Zhang, Q., Furukawa, K., Chen, H.-H., Sakakibara, T., Urano, T., and Furukawa, K. (2006). Metastatic potential of mouse Lewis lung cancer cells is regulated via ganglioside GM1 by modulating the matrix metalloprotease-9 localization in lipid rafts. J. Biol. Chem. *281*, 18145–18155.

Zhang, Y., Du, J., Zheng, J., Liu, J., Xu, R., Shen, T., Zhu, Y., Chang, J., Wang, H., Zhang, Z., et al. (2015). EGF-reduced Wnt5a transcription induces epithelial-mesenchymal transition via Arf6-ERK signaling in gastric cancer cells. Oncotarget *6*, 7244–7261.

Zheng, Y., and Quilliam, L.A. (2003). Activation of the Ras superfamily of small GTPases. Workshop on exchange factors. EMBO Rep. *4*, 463–468.

Zipfel, P.A., Brady, D.C., Kashatus, D.F., Ancrile, B.D., Tyler, D.S., and Counter, C.M. (2010). Ral activation promotes melanomagenesis. Oncogene *29*, 4859–4864.

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