Adhesion dependent regulation of Aurora Kinase A and its role in RalA mediated anchorage-independence

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DECLARATION

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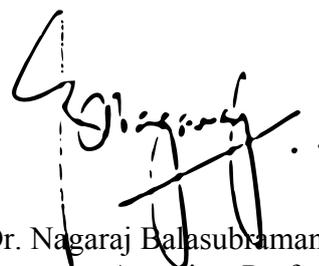Dr. Nagaraj Balasubramanian

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I certify that the thesis entitled '**Adhesion dependent regulation of Aurora Kinase A and its role in RalA mediated anchorage independence**' presented by **Ms. Siddhi Maruti Inchanalkar** represents her original work which was carried out by her at IISER, Pune under my guidance and supervision during the period from 24-07-2014 to 24-07-2020. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institutions. I further certify that the above statements made by her in regard to her thesis are correct to the best of my knowledge.

Dr. Nagaraj Balasubramanian,
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Commonly used Abbreviations

μM	micromolar
μg	microgram
μL	microliter
ECM	Extra cellular matrix
FN	Fibronectin
SA	Stable adherent
SUS	Suspension
GTPase	Guanosine triphosphatases
Ral GTPase	Ras-like GTPase
AURKA	Aurora Kinase A
AURKB	Aurora Kinase B
AURKC	Aurora Kinase C
Arf6	ADP-ribosylation factor-6
Ras	Rat Sarcoma
H-Ras	Harvey Sarcoma
K-Ras	Kristen Sarcoma
GEF	Guanine nucleotide exchange factor
GAP	GTPase activating protein
NOC	Nocodazole
CON	Control
V_{MLN}	Dextran polysaccharide encapsulated MLN8237
$V_{MLN+RhB}$	Encapsulated MLN8237 and Rhodamine-B
RhB	Rhodamine-B
ERK	Extracellular Receptor Kinase
MAPK	Mitogen activated protein kinase
DEX	Empty dextran polysaccharide scaffold
RBD	Ral-Binding domain

Inhibitors used in the study

MLN8237	Aurora Kinase inhibitor
AZD1152	Aurora Kinase B inhibitor
BQU57	Ral GTPase inhibitor

Abstract

Integrin-mediated adhesion regulates membrane trafficking to control anchorage-dependent signaling and growth that is deregulated in cancers. Downstream of integrins and oncogenic Ras, the small GTPase Ral, is a vital mediator of adhesion-dependent trafficking and signaling. While the role of RalA in these pathways is well established, what regulates the small GTPase downstream of integrin and oncogenic Ras is still elusive. In this study, we find a cell cycle protein Aurora Kinase A (AURKA) to be an important player that regulates RalA activity downstream of Integrin-mediated adhesion. We have developed and used a self-assembling dextran polymer nanovesicle (V_{MLN}) to deliver the AURKA inhibitor Alisertib (MLN8237) and specifically target AURKA in cells in 2D as well as 3D microenvironments. Using this tool to specifically inhibit AURKA, we find it regulates integrin-dependent RalA activity and cell spreading in early adhesion, through the RalGEF, RGL1. This could be mediated by the effect AURKA has on RalA and RGL1 localization. In anchorage-independent cancers, the AURKA-RalA crosstalk is conserved in Ras-independent cancers and remains to be tested in Ras-dependent cancers. Using V_{MLN} as a tool we have identified two Ras-independent cancer cell lines where targeting AURKA specifically inhibits RalA activity and RalA dependent anchorage-independent growth. Together these studies help identify AURKA as a key regulator of RalA, along adhesion dependent pathways in normal and Ras-independent cancers.

Synopsis

Introduction

Aurora Kinase A, B, and C belong to a family of Serine/Threonine Kinases that are all involved in various mitotic processes ranging from centrosome maturation to cytokinesis (Terada et al. 2003; Vader & Lens 2008). Dysregulation of these Kinases has hence been shown to cause severe defects in mitosis (Carmena et al. 2009; Khan et al. 2011). Owing to their role in mitosis, it was presumed that their role in carcinogenesis would largely be mediated by their deregulation of mitotic events. But recent studies have revealed that Aurora Kinases by themselves are weak oncogenes and manifest their oncogenic activity by enhancing p53 and Ras-mediated cellular transformation (Umstead et al. 2017; E. O. Dos Santos et al. 2016; Tseng et al. 2009; Tatsuka et al. 2004).

During mitosis, Aurora Kinase A phosphorylates and regulates a small GTPase RalA (but not RalB) which is indispensable for mitochondrial fission (Kashatus et al. 2011). Interestingly RalA is a major downstream regulator of Ras-mediated oncogenesis and anchorage-independence (Gentry et al. 2014). Studies have shown that RalA can be activated by integrins and oncogenic Ras. Active RalA, with the exocyst complex, mediates adhesion-dependent trafficking and targeting of membrane raft micro-domains to the plasma membrane to support anchorage-dependent signaling (N. Balasubramanian et al. 2010; Pawar et al. 2016). In cancer cells, constitutively activated RalA drives this trafficking pathway to support anchorage-independent growth signaling. Aurora Kinase A specifically has been found to be over-expressed in Ras (HRasV12 and KRasV12) driven carcinogenesis (Vader & Lens 2008).

Our studies aim to ask if Aurora Kinase A and Aurora Kinase B are regulated by integrin-mediated adhesion and if this contributes to the differential activation of RalA (vs RalB) in anchorage-dependent normal cells? Knowing the overlap that exists between integrin-dependent and Ras-dependent signaling pathways we also aim to test the contribution Aurora Kinase A makes downstream of oncogenic Ras and independent of Ras to support RalA activation in anchorage-independent cancer cells? We further aim to study the role of adhesion-regulated Aurora Kinase B activation in normal cells. We have divided the thesis into the following sub-aims to address these questions.

I. Study the role of Aurora Kinase A in regulating adhesion-dependent RalA activation

II. Evaluate a self-assembling Dextran nano-vesicle to efficiently deliver Aurora Kinase A inhibitor MLN8237 and specifically target AURKA-RalA crosstalk

III. Evaluate the significance of AURKA-RalA crosstalk in anchorage-independent growth of Ras-independent vs Ras-dependent cancers.

IV. Study the role of adhesion-growth factor crosstalk in regulation of Aurora Kinase activation and signaling

I. Study the role of Aurora Kinase A in regulating adhesion-dependent RalA activation

To evaluate whether Aurora Kinase A (AURKA) has any role in regulating RalA activity downstream of integrin-mediated adhesion, we first tested if integrins can regulate AURKA activity (autophosphorylation on Theronine 288) in wild-type Mouse Embryonic fibroblasts (WT-MEFs). To ensure growth factors do not affect these studies, WT-MEFs were serum starved for 12 hours (0.2% FBS). It is known that under these conditions re-adhesion to matrix fibronectin activates integrin-dependent signaling and function in WT-MEFs. Stable adherent (SA) WT-MEFs on loss of adhesion (suspended for 90mins) show a significant decrease in RalA activity (~40% drop) which is restored on re-adhesion to fibronectin (for 15mins) (N. Balasubramanian et al. 2010). Under these conditions, AURKA activity interestingly increases (~60%) on loss of adhesion and is restored on re-adhesion to fibronectin. This suggests that AURKA mediated activation of RalA could be decoupled on loss of adhesion and maybe re-adhesion as well.

Loss of adhesion has been shown cause cytokinesis failure (leading to G2-M arrest) which is reversed upon re-adhesion (De Santis Puzzonia et al. 2016). The change in AURKA activation on loss of adhesion could indeed be mediated by such an arrest. We hence tested the cell cycle profile of serum-deprived propidium iodide labeled WT-MEFs on loss of adhesion (Susp 90') and re-adhesion (FN 15'). Levels of cyclin D1 and A2 in these cells were further used to confirm the same (Carstens et al. 1996; Schwartz & Assoian 2001) (Pines & Hunter 1991; Baldin et al. 1993). Owing to the synchronization of cells at G1-S phase upon serum starvation (Langan & Chou 2011; Griffin 1976; Campisi et al. 1984), 65 ± 3 % of SA WT-MEFs were seen to be in G1 phase and 27.8 ± 4 % in S phase, reflected in their cyclin D1 levels being higher than cyclin A2. This distribution of cells and their cyclin levels did not change

significantly on loss of adhesion or re-adhesion. This suggests that changes in AURKA activation my not necessarily reflect a distinct change in cell cycle profile on loss of adhesion. What might cause the increase in AURKA activation on loss of adhesion in serum-deprived conditions hence remains unclear. Our studies show the presence of serum (10% FBS) to prevent this increase on loss of adhesion, reducing AURKA activation even below SA levels. The cell cycle profile of these serum-stimulated SA cells is distinctly different from serum-deprived cells. 44.86 ± 5 % of SA WT-MEFs were seen to be in the G1 phase and 44.17 ± 5 % in S phase, reflected in their cyclin D1 levels being similar to cyclin A2. This profile, like in serum-deprived cells, does not change significantly on loss of adhesion or re-adhesion. Taken together, these studies imply the cell cycle profile of WT-MEFs when they are detached and held in suspension could influence the differential activation of AURKA, though the mechanism mediating this remains unknown.

The primary focus of this thesis was evaluating the AURKA-RalA crosstalk and its regulation by cell-matrix adhesion. With earlier studies from the lab having established the regulation of RalA (vs RalB) in serum-deprived WT-MEFs suspended for 90min and re-plated on FN for 15min, we looked at the AURKA-RalA crosstalk under similar conditions. We hence asked how AURKA-dependent phosphorylation of RalA at Ser194 residue changes upon loss of adhesion and re-adhesion in WT-MEFs? Since the antibody that detects the S194RalA is specific to human-RalA, we expressed human-RalA in WT-MEFs and saw pS194-RalA levels to increase upon loss of adhesion where AURKA activity increases, but RalA activity decreases. On re-adhesion, pS194-RalA levels are restored to stable adherent levels, as is AURKA activity. RalA activity, however, does increase in re-adherent cells relative to suspended cells. Taken together these studies suggest that AURKA mediated regulation of S194-RalA phosphorylation might be indeed decoupled from its adhesion-dependent activation.

To further confirm this decoupling, we have used a self-assembling dextran nano-vesicle to encapsulate AURKA inhibitor MLN8237 (V_{MLN}) and inhibit AURKA activation in WT-MEFs. The development, characterization of V_{MLN} and its ability to specifically inhibit AURKA (without affecting AURKB) has been discussed in detail in AIM II. This allows us to treat WT-MEFs suspended for 60 mins for an additional 30min with V_{MLN} ($0.2\mu M$) and achieve up to 95% inhibition of AURKA (without affecting AURKB). This specific inhibition of AURKA

was retained in cells re-adherent on FN for 15min. This causes a significant ~40% decrease in RalA activity in suspended cells and a ~100% drop in re-adherent cells. S194 phosphorylation of RalA which is prominent in 90' suspended cell drops by ~40% on V_{MLN} treatment and by ~27% on re-adherent cells. RalB activity which is not known to be regulated by AURKA showed no difference upon the V_{MLN} treatment. These results imply that while the net AURKA activation and pS194-RalA profiles are different from RalA activity (GST-Sec5 pulldown) on loss of adhesion vs re-adhesion, the AURKA-RalA crosstalk does have a role in mediating adhesion-dependent RalA activation.

V_{MLN} treated cells upon re-adhesion show dramatically reduced cell spreading, known to be regulated by RalA activation (N. Balasubramanian et al. 2010). This regulation of RalA activity is likely dependent on known Ral GEFs and GAPs. RGL1 is one such RalGEF that we have implicated in adhesion-dependent RalA activation and spreading (Deshpande et. al., manuscript under preparation). We hence asked if RGL1 could have a role in AURKA mediated RalA activation downstream of matrix adhesion. RGL1 knockdown and AURKA inhibition (V_{MLN}) we found comparably affects RalA activation in re-adherent WT-MEFs. Their joint inhibition (RGL1 KD + V_{MLN} treatment) did not show any additive effect, suggesting that they likely work along the same pathway to regulate cell-matrix adhesion-dependent RalA activation and cell spreading. In re-adherent WT-MEFs, RGL1 and RalA co-localize to membrane ruffles and protrusions, sites of active integrin signaling. This suggests that localization plays an important role in RGL1 mediated RalA regulation. AURKA inhibition by V_{MLN} affects the localization of RGL1 and RalA at membrane protrusions and ruffles. This would, in turn, affect adhesion-dependent AURKA mediated RalA activation. Taken together, this suggests that AURKA does regulate integrin-dependent RalA activity, however, the possible role AURKA could have in RGL1 localization and its contribution to RalA activation remains to be tested.

II. Evaluate a self-assembling Dextran nano-vesicle to efficiently deliver Aurora Kinase A inhibitor MLN8237 and specifically target Aurora Kinase A in normal and cancer cells

The small GTPase RalA is a known mediator of anchorage-independent growth in cancers (Bodemann & White 2008; Camonis & White 2005) and is regulated by AURKA in anchorage-dependent normal cells. While there is a known inhibitor for Ral (BQU57), it is seen to be non-specific and inhibits both RalA and RalB(C. Yan et al. 2014), making it difficult to use it to

test the role of individual isoforms. Targeting AURKA, seen to specifically regulate RalA (not RalB), could provide a tool to inhibit RalA (without affecting RalB) in cells. MLN8237 (alisertib) is a known specific inhibitor of AURKA; its specificity compromised by its poor solubility and transport across the cell membrane (Carol et al. 2011; de Groot et al. 2015; Sells et al. 2015). In collaboration with the lab of Dr. Jayakannan (IISER, Chemistry) we helped develop and test a self-assembling dextran nano-vesicle to deliver and specifically inhibit AURKA in cancer cells. These nano-vesicles (<200 nm in size) carry MLN8237 (V_{MLN}) in their intermembrane space with up to 85% of it released by the action of esterase enzyme(s) in cells. Rhodamine B fluorophore trapped in the hydrophilic core of V_{MLN} ($V_{MLN+RhB}$) allows us to visualize its uptake and localization in cells in a 2D and 3D microenvironment. To test the efficiency of V_{MLN} mediated inhibition of AURKA we chose breast cancer cell line MCF-7, seen to express AURKA and RalA which is prominently phosphorylated on Serine 194, and shown to take up these nano-vesicles efficiently (Pramod et al. 2012). We tested AURKA inhibition across a range of concentrations (0.01μM and 0.1μM) of MLN8237 (Free drug) and V_{MLN} (dextran encapsulated MLN8237). Drug concentrations were chosen such that cells can be treated for 48 hours and keep their specificity for AURKA, without affecting AURKB. Treatment given at 0.02μM V_{MLN} for 48hour was seen to 8-fold better inhibit AURKA than the free drug, without affecting AURKB activity. The DEX nanovesicle scaffold by itself did not affect either. V_{MLN} further specifically targets RalA (but not RalB) downstream of AURKA, making it a useful tool and drug candidate for targeting cancer cells. V_{MLN} has also been used as a tool to study the role of AURKA in regulating RalA downstream of integrin-mediated adhesion in normal mouse fibroblasts in AIMI. In the next AIM, we have used V_{MLN} to evaluate the role of AURKA-RalA crosstalk in anchorage-independent growth of Ras-independent vs Ras-dependent cancers.

III. Evaluate the significance of AURKA-RalA crosstalk in anchorage-independent growth of Ras-independent vs Ras-dependent cancers

As discussed above, RalA has been shown to be regulated by two major signaling pathways: integrin-mediated adhesion and Ras – RalGEF mediated pathway. Cascone et. al., in 2008 reported that a Ras activated GEF RalGDS activates RalA and RGL1 activates RalB during cytokinesis (Cascone et al. 2008). However, it is known that all the Ras activated GEFs have the potential to activate both RalA and RalB, their differential regulation possibly mediated by

their localization. As discussed in Aim I, we have shown that AURKA and RGL1 are involved in the integrin-dependent regulation of RalA activation (Deshpande et.al. manuscript under prep). Thus, AURKA-RGL1 could act as a point of convergence for integrin and Ras-mediated regulation of RalA. To test this, we screened cancer cell lines for; (1) their levels of AURKA (2) AURKA activation (pThr288 AURKA) (3) RalA levels and (4) AURKA mediated Ser194 phosphorylation of RalA (pSer194 RalA) (5) effectiveness of V_{MLN} mediated AURKA inhibition (6) effect V_{MLN} has on pSer194 RalA levels and (7) Sec5-RBD pulldown of active RalA. This identified bladder cancer cell lines (T24 – HRAS G12V, UMUC3 – KRAS G12V), pancreatic cancer cell line (MIAPaCa 2 – KRAS G12V), fibrosarcoma cell line (HT1080 – NRAS Q61K), breast cancer cell lines (MCF-7 – No mutant Ras, MDAMB231 – KRAS G13D), glioblastoma cell line (U87MG – KRAS G12V) and ovarian cancer cell line (SKOV3 – No mutant RAS). All of the above cell lines showed V_{MLN} to inhibit AURKA significantly better as compared to free MLN8237. Interestingly, V_{MLN} treatment inhibited Ser194 phosphorylation of RalA only in the Ras-independent MCF-7 and SKOV3 cells. This further causes a significant decrease in GTP bound active RalA, in GST-Sec5RBD pulldown assays and a significant decrease in anchorage-independent growth of these cells. V_{MLN} treatment arrests MCF-7 and SKOV3 cells in the G2-M phase of the cell cycle, that inhibition of RalA has also been seen to cause (Kashatus et al. 2011). Taken together, the effect V_{MLN} has on AIG of Ras-independent cell lines can be mediated by (1) AURKA dependent inhibition of RalA-mediated vesicular trafficking and exocytosis reversing anchorage-independent growth signaling and (2) AURKA-RalA inhibition mediated G2-M arrest of cells thereby preventing cell proliferation and AIG of cancer cells.

We next wanted to evaluate the significance of this crosstalk in *in vivo* tumorigenesis by using V_{MLN} to target MCF-7 and SKVO3 induced tumours in mice. As a first step towards evaluating this, we have initiated studies to test the stability and detection limit of the V_{MLN} in mice by standardising the detection of MLN8237 by LC-MS/MS and DLS in mice serum. These studies reveal MLN8237 to be detectable at as low concentration as 10ppb by LC-MS/MS. We have also standardized the number of SKOV3 cells needed to make tumours of optimal desirable size in NOD SCID mice. These studies are now being further extended to MCF-7 cells and will be used to evaluate the impact of the AURKA-RalA crosstalk in tumorigenesis of these cancers.

IV. Study the role of adhesion-growth factor crosstalk in regulation of Aurora Kinase activation and signaling

As discussed in the previous studies, WT-MEFs show a significant decrease in RalA activity (~40% drop) on loss of adhesion (suspended for 90mins), which is restored on re-adhesion to fibronectin (for 15mins) (N. Balasubramanian et al. 2010). WT-MEFs also show a significant decrease in RalA activity (~40 % drop) on loss of adhesion when suspended for 30mins, restored upon re-adhesion to fibronectin (for 15mins). Under these conditions, AURKA activity decreases (~60%) on loss of adhesion for 30mins but is NOT restored back on re-adhesion to fibronectin. The role AURKA plays in regulating RalA under these conditions remains to be tested. Interestingly, under similar conditions, we observe another mitotic kinase; Aurora Kinase B (AURKB) activity (autophosphorylation on Threonine 232 residue) to drop in suspension (~40 %) and recover back to SA levels on re-adhesion to fibronectin, suggesting that AURKB activity is regulated by integrin-dependent signaling. Taken together these observations suggest regulation of Aurora kinases to be rather sensitive to the kinetics of loss and recovery of cell-matrix adhesion.

Crosstalk between integrins and growth factor receptors has been shown to be important in supporting cell proliferation, migration, and invasion *in vivo* (Eliceiri 2001; Schwartz 1997a). We hence tested the role integrin-growth factor crosstalk has on regulating Aurora kinases. The activation of both AURKA and AURKB known to drop in serum-deprived conditions continues to show this drop in the presence of serum growth factors (Susp 30'). The recovery of AURKB activity on re-adhesion to fibronectin for 15mins in serum-deprived conditions is interestingly lost in the presence of serum growth factors. AURKA activity stays low upon re-adhesion in the absence or presence of serum growth factors.

To further understand this differential regulation of AURKB we evaluated the cell cycle profile of WT-MEFs in the absence and presence of serum. As discussed in our previous studies, cells get synchronized at G1-S phase upon serum starvation (Langan & Chou 2011; Griffin 1976; Campisi et al. 1984), 76 ± 2.3 % of stable adherent cells were seen to be in G1 phase and 20 ± 1.6 % in S phase. This profile did not change significantly when these cells are detached and held in suspension for 30 mins, followed by re-plating on fibronectin for 15mins. In the presence of serum, the cell cycle profile was expectedly different, with 50 ± 0.7 % of stable adherent cells in G1 phase and 43 ± 1.8 % in S phase. This profile also did not change

significantly as cells were held in suspension for 30 mins, followed by re-plating on fibronectin for 15mins. Knowing that activation and levels of Aurora Kinases are different in different phases of the cell cycle (Carmena & Earnshaw 2003a), this distinct change in cell cycle profile that presence of serum causes could influence the differential effect re-adhesion has on AURKB activation. To evaluate this possibility, we tested if the rapid stimulation of serum-deprived cells with serum can regulate AURKB.

To further understand the impact of serum growth factors, serum-deprived WT-MEFs suspended for 30mins were transiently exposed to serum growth factors (10% FBS) for 15min and re-plated with serum. This prevented the recovery of AURKB activity upon re-adhesion as seen in WT-MEFs with serum, suggesting that transient growth factor stimulation can inhibit integrin-dependent recovery of AURKB activity. Such a 15min shot of serum is unlikely to change the cell cycle profile of the WT-MEF population significant enough to regulate AURKB activation, suggesting a direct regulation by a serum component. We hence tested if heat inactivation of serum (56°C, 30mins) can affect AURKB recovery and found it to not affect the same. This suggests the possible heat resistant components of serum that could mediate this regulation could be growth factors or any small molecules like amino acids, sugars, lipids or hormones (Honn et al. 1975). Identification of serum component(s) by dialysis or charcoal treatment (Stoikos et al. 2008) that might be involved in regulating AURKB activation could be of much significance in better understanding the effect integrin-growth factor crosstalk has on cellular function.

To establish the functional relevance of integrin-regulated AURKB activity, we have looked at whether integrin-growth factor-dependent AURKB activity regulates integrin-dependent signaling. We tested the effect absence/presence of Serum has on integrin-mediated activation of AKT, ERK and FAK (Eliceiri 2001) in WT-MEFs. Interestingly, while AKT and FAK activation recovered upon re-adhesion in the presence and absence of serum, ERK activation was differentially affected by the presence of serum (like AURKB is). ERK activity stayed low when AURKB activity recovered on re-adhesion in absence of serum and increased when AURKB activity was kept low by the presence of serum. To test if this reflects regulatory crosstalk, we inhibited AURKB activity using a specific small-molecule inhibitor AZD1152 in re-adherent cells in absence of serum (where AURKB is activated) and tested the effect it has on the reduced ERK activation status. Interestingly, inhibition of AURKB activity causes a

significant recovery in ERK activity upon re-adhesion. Immunofluorescence studies show this active phosphoERK to localize to membrane ruffles on AURKB inhibition. Together they suggest serum dependent AURKB activation can regulate ERK activation and localization downstream of integrin-mediated adhesion. ERK is also known to regulate AURKB activation and this could help create a regulatory feedback loop that ongoing studies will explore. Taken together our studies show that integrin-growth factor crosstalk regulates AURKA and AURKB differentially and this could contribute to their differential role(s) in normal and cancer cells.

Summary of Thesis project

In this study, we have found a cell cycle protein Aurora Kinase A (AURKA) to be an important regulator of RalA activity downstream of integrin-mediated cell-matrix adhesion. Targeting RalA and AURKA specifically have been long-standing challenges in the field owing to the lack of specific inhibitors against them. In our studies, we have helped develop and test a self-assembling dextran polymer nano-vesicle to deliver the AURKA inhibitor Alisertib (MLN8237) to specifically target AURKA (without affecting AURKB) and downstream RalA (without affecting RalB). We find that this MLN8237 encapsulated nano-vesicle (V_{MLN}) is taken up efficiently by cells in both 2D as well as 3D microenvironments making it a suitable tool to study the role of AURKA in regulating RalA in anchorage-dependent normal cells as well as anchorage-independent cancer cells. With the help of V_{MLN}, we were able to specifically target AURKA in anchorage-dependent WT-MEFs, and reveal that AURKA regulates integrin-dependent RalA activity and early adhesion-dependent cell spreading. We find that this AURKA mediated regulation of RalA is through the RalGEF, RGL1. Our studies also suggest that this regulation is mediated by the effect AURKA has on RalA and RGL1 localization. A comprehensive study to evaluate the mechanism of RGL1 regulation by AURKA is currently ongoing and will provide a complete understanding of the AURKA-RGL1-RalA pathway.

We had further hypothesized that considering the overlap between integrin-dependent and Ras-dependent pathways, the AURKA-RalA crosstalk could be conserved in anchorage-independent cancers and we have tested this using V_{MLN} as a tool to target AURKA in cancer cells. Using oncogenic Ras as a differentiating factor we have carried out a screen and selected a group of Ras-dependent and Ras-independent cell lines. We further compared their AURKA and RalA activity, the efficiency of V_{MLN} uptake and inhibition of AURKA using V_{MLN} over

free MLN. This screen identifies two Ras-independent cancer cell lines where AURKA regulates RalA phosphorylation and activity. This AURKA-RalA crosstalk we find also plays an important role in regulating the anchorage-independent growth of these cells. Ras-dependent cancers that were tested in this screen failed to show this regulatory crosstalk. Understanding the differential AURKA-RalA crosstalk and its relevance downstream of oncogenic Ras will be of much interest. AURKA regulated RalA is also known to control important processes like mitochondrial fission (Kashatus et al. 2011). It would be of interest to test if and how adhesion-dependent regulation of the AURKA-RalA pathway affects mitochondrial function in normal cells and the possible role this could have in oncogenic transformation of cancer cells. The role AURKB could have downstream of adhesion and possibly oncogenic Ras, in regulating ERK activation could further help understand the contribution Aurora kinases make in the regulation of cellular signaling.

Our studies have also revealed how the self-assembling dextran nano-vesicle delivery system for MLN8237 can be used as a tool to evaluate the role of AURKA and possibly RalA downstream in other physiological conditions. In combination with a general Ral inhibitor, this could further help discern the role of RalA *vs* RalB in anchorage-independent cancers. It would also be of interest to test the efficiency of V_{MLN} mediated drug delivery and its impact on tumour growth and regression *in vivo* in Ras-independent vs Ras-dependent cancers. Taken together, these studies help us better understand the intricate regulation and role AURKA-RalA and AURKB-ERK crosstalk have downstream of integrins and serum growth factors to support anchorage-dependence in cells. They further raise the need to evaluate these regulatory crosstalks in Ras-dependent and independent cancers and evaluate the implications targeting them could have on the anchorage-independent nature of cancers.

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Chapter 1

Introduction and

Review of literature

1.1 Integrin: Role and regulation.

1.1.1 Introduction

Integrins are the family of heterodimeric transmembrane cell adhesion receptors that are evolutionarily old and play crucial role in various developmental and pathological processes. The term 'Integrin' was first coined by Hynes and colleagues in 1987, when they first isolated a complex of cell surface glycoproteins from chicken fibroblasts which had the capability of binding to extracellular matrix glycoproteins causing cell-matrix adhesions (Hynes 1987; DeSimone et al. 1987; Tamkun et al. 1986).

Since the recognition of the integrin receptor family around three decades ago, an enormous amount of diverse research has been done that has made integrins the best-understood cell adhesion receptors (Hynes 2002; Barczyk et al. 2010; Schwartz 1997a; Anthis & Campbell 2011; Campbell & Humphries 2011). Integrins and their ligands have been demonstrated to play key roles in diverse cellular processes like cell adhesion, migration, growth, cell polarity, cytoskeleton re-arrangement and membrane trafficking (C. Singh et al. 2018; Caswell & Norman 2006; Berrier & Yamada 2007; Thuveson et al. 2019; Bridgewater et al. 2012; Caswell et al. 2009). These cellular functions at physiological level regulate development, immune responses, leukocyte traffic, hemostasis and cancer (Barczyk et al. 2010; Byzova et al. 2000; Schwartz 1997a; Yan Zhang et al. 2020; Vannini et al. 2019).

1.1.2 The Integrin Family: Structure

Till date, 24 canonical integrin heterodimers composed of non-covalently associated 18-α-subunits and 8-β-subunits have been reported (Hynes 2002; Takada et al. 2007). These heterodimers have also been shown to be expressed in tissue-specific manner and bind to different ligands carrying out distinct physiological functions (Barczyk et al. 2010). The overall shape and dimensions of integrins have been determined by electron microscopy, where each integrin heterodimer has a general structure with a 'head region' (amino terminus of α and β-subunits called the β-propeller and βA or hybrid domains, respectively) which provides a ligand binding site and 'leg region' (calf1, calf2 domains in α-subunit and EGF domains in β-subunit) that ends up in a transmembrane and cytoplasmic domain.
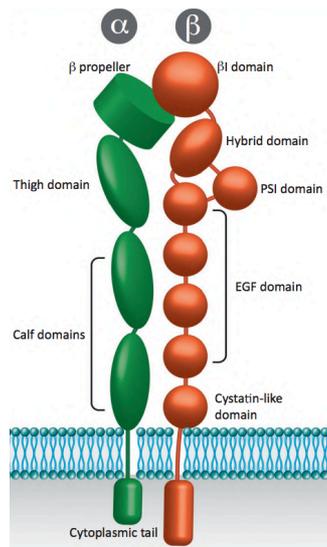
Figure 1.1. Schematic of integrin structure. Integrins are membrane receptors consisting of an α and β-subunit. The α-subunit (green) and β-subunit (brown) both have an extracellular domain which binds ligands, a single transmembrane helix, and a short cytoplasmic portion. Both α and β-subunits consist of different structural domains. The α-chain is composed of four or five head domains, a folded seven-bladed β-propeller domain, a thigh, and two calf domains. The β-subunit consists of a βI-like domain, a PSI (plexin/semaphoring/integrin) domain, a hybrid domain, four epidermal growth factor (EGF) repeats, and a membrane proximal-β tail (β TD). (Reproduced from (Marsico et al. 2018)).

1.1.3 Regulation of integrins

1.1.3.1 Ligand Binding

In mammals, integrin-ligand binding has been classified into four major groups, based on the type of molecular interaction (Figure 1.2). 18α and 8β-integrin isoforms combine to from 24 αβreceptors. These can be grouped based on the different extracellular domains of these α and β-subunit combinations and their preferred ligands or they can be grouped into ECM-binding receptors and leukocyte-binding receptors. Based on both of these integrins can be classified as (1) RGD tripeptide motif recognizing integrins (α5β1, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, and αIIbβ3), (2) collagen binding integrins (α1β1, α2β1, α3β1, α10β1, and α11β1), (3) laminins binding integrins ((α1β1, α2β1, α3β1, α6β1, α7β1, and α6β4) (4) leukocytes integrins ((αLβ2, αMβ2, αXβ2, and αDβ2) (Hynes 1987; Plow et al. 2000; Harris et al. 2000).

In addition to these canonical ligands, integrin ligands generated by proteolysis during various process have also been focus of recent studies. Few best-known examples of such ligands are endostatin, endorepellin and tumstatin. In addition, few integrin combinations can bind to

viruses and bacteria. Most of these interactions display distinct ligand-binding sites and characteristics as compared to canonical ligands (Barczyk et al. 2010).
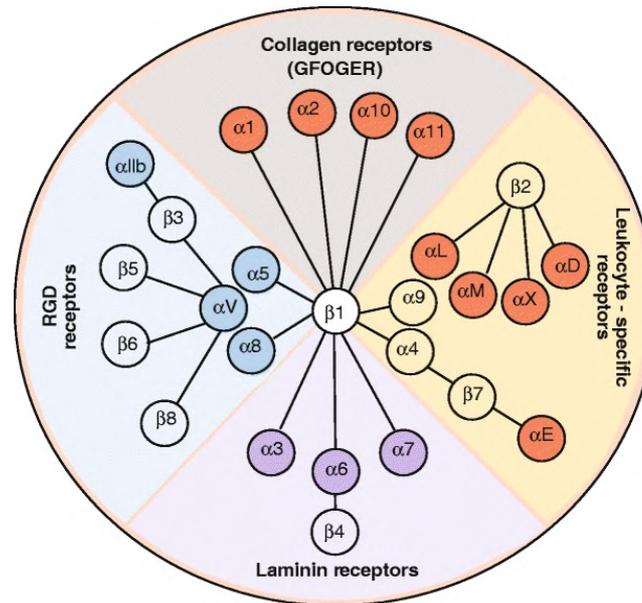
Figure 1.2. Classification of integrin-based on ligand binding. Integrins are broadly classified into 4 groups based on the type of ligand it binds. (Reproduced from (Barczyk et al. 2010)).

1.1.3.2 Activation and Bi-directional signaling

Integrins are not constitutively active in cells. They are often, but not always, expressed on cell surfaces in an inactive or "OFF" state, in which they do not bind ligands and do not signal (Gahmberg et al. 2009). The ligand-binding activity of integrins is regulated by their conformational changes; where integrins can adopt three major conformational states. These are a low affinity 'inactive' state, a 'primed' state or a high affinity 'active' state which is often but not always ligand occupied (Gahmberg et al. 2009; Campbell & Humphries 2011).

Unlike many other signaling receptors, integrins are bi-directional, involved in outside-in and inside-out signaling (Figure 1.3). Upon extracellular ligand binding, integrins undergo conformational changes that leads to the interaction of cytoplasmic domain of integrin with proteins like FAK, SRC, talin that ultimately activates signaling events that are complex and cell type specific, depending on the signaling cascades present and activated in the cell.
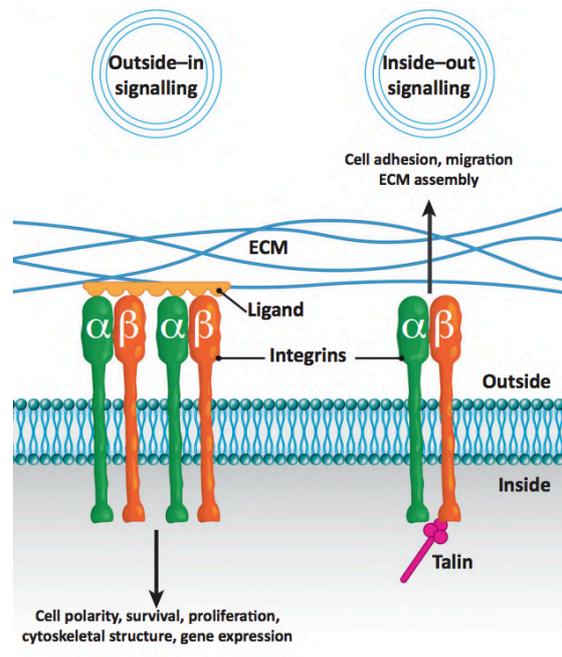
Figure 1.3. Model of Integrin bi-directional signaling. (Reproduced from (Marsico et al. 2018)).

The inside-out signaling mainly acts in cells like blood cells or immune cells, where integrin ligands and cells are in close proximity, but the interaction takes place only when integrins get activated in response to some external cues, like inflammatory response. Talins, filamins, FAK, kindlins and ILK have been reported to be involved in regulation of integrin activity. Although these two signaling events are viewed as two separate events, often these bi-directional signaling cascades are linked and set feedback loop to modulate cellular processes (Marsico et al. 2018).

1.1.4 Cellular functions regulated by integrins

Apart from their roles in adhesion to ECM ligands or complementary receptors on adjacent cells, integrins also serve as transmembrane mechanical links from ECM components to cytoskeleton inside cells. Integrin-mediated assembly of cytoskeletal linkages, in part (both in cause and effect) also triggers recruitment of variety of scaffolding proteins like talin, kindlins, vinculin (Calderwood et al. 2013; Anthis & Campbell 2011) and signal transduction kinases like SRC, FAK, JNK, ERK (M. Oktay et al. 1999; Fincham et al. 2000; Schwartz & Ginsberg 2002) (Figure 1.4). These scaffolding and signaling proteins ultimately regulate multiple cellular processes like cell adhesion (Calderwood et al. 2013; Schwartz 1997b), cell cycle and

proliferation (Schwartz & Assoian 2001; M. C. Jones et al. 2019), cell survival (Takada et al. 2007; Eliceiri 2001), cell polarity (Thuveson et al. 2019; Dix et al. 2018), cell migration (Caswell & Norman 2006) and membrane trafficking (endocytosis and exocytosis) (Caswell et al. 2009) establishing the fact that integrins are full-fledged signal transduction receptors, and as important to cells as more traditional growth factor receptors.
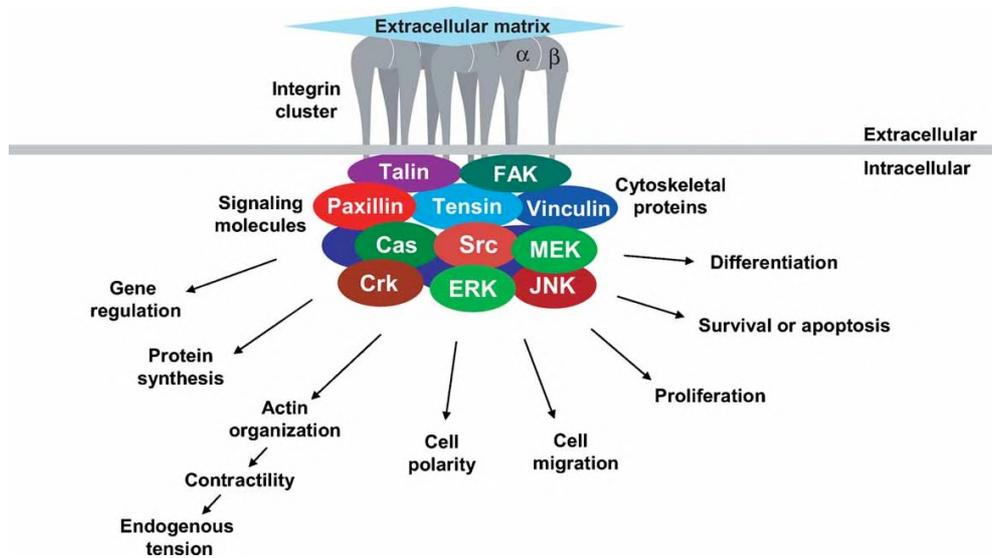
Figure 1.4. Integrin-dependent cell-matrix adhesion and downstream regulation of cellular processes. Activated integrin clusters at the cell-ECM adhesions recruit various scaffolding proteins and signal transduction kinases, which in co-operation with other extracellular receptor cues controls diverse cellular process and functions. (Reproduced from (Berrier & Yamada 2007)).

Integrin-mediated cell adhesion

Members of the integrin family of cell adhesion molecules play a crucial role in the interaction between cells and the extracellular matrix that regulates organisation, maintenance, and repair of several tissues. At least four different types of adhesion structures have been identified in fibroblasts, namely focal complexes, focal adhesions, fibrillary adhesion and 3D-matrix adhesions (Berrier & Yamada 2007). Recently, a new class of adhesion complex, called 'reticular adhesions' have been shown to be formed during interphase, and preserved at cell-ECM attachment sites throughout mitosis (Lock et al. 2018).

Most of the integrin receptors have the ability to localize to adhesion structures, however, certain integrin receptors are known to preferentially localize or concentrate at different cell-

matrix adhesion structures. Localisation of $\alpha_v\beta_3$ at focal complexes and focal adhesions (Berrier & Yamada 2007; Petit & Thiery 2000) and $\alpha_5\beta_1$ concentration in fibrillary adhesions (Suehiro et al. 2000; Mao & Schwarzbauer 2005) in fibroblasts adhered to 2D fibronectin matrix are examples of preferential localization of integrin receptors to distinct adhesions. Reticular adhesions are composed of $\alpha_v\beta_5$ integrin receptors (Lock et al. 2018). Thus, different integrin receptors recruit different cytoplasmic factors depending on extracellular stimuli and cell type and differentially control cellular signaling and functions.
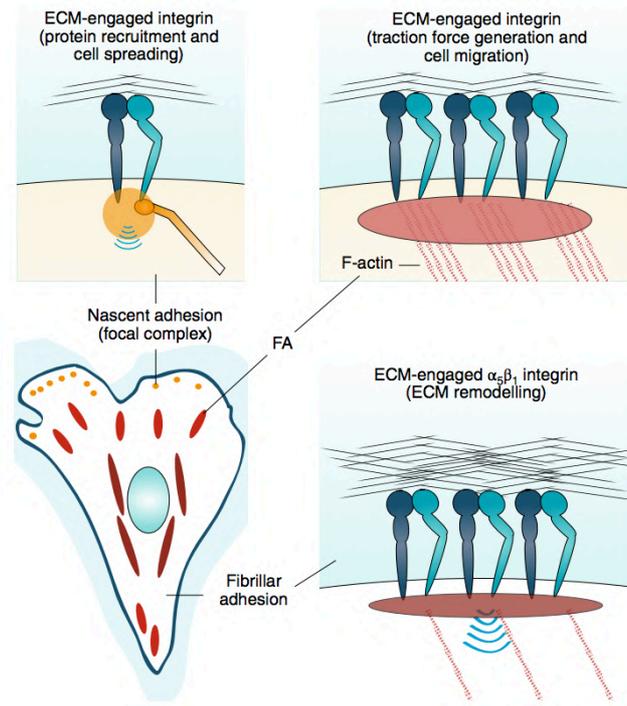
Figure 1.5. Integrin–ECM adhesions, are defined based on localization, components and maturation stage. Nascent adhesions also called focal complexes represent initial integrin receptor clustering in response to ECM engagement and the recruitment of adaptor and signalling proteins to the integrin tails. These small protein assemblies mature into focal adhesions (FAs), which serve to anchor actin stress fibres and are vital for the generation of contractile force. Fibrillar adhesions are mature α5β1 integrin adhesions and sites of fibronectin fibrillogenesis. (Reproduced from (Moreno-Layseca et al. 2019)).

Cell Migration

Migration of a cell is characterised by the dynamic interaction between a cell and the extracellular substratum to which it is attached and over which it migrates. Different modes of migration depending on various cell movements have been studied, ranging from individual cell migration classified as mesenchymal or amoeboid to collective cell migration where intercellular interactions are retained and groups of cells move in a coordinated manner upon

receiving external cues. Mesenchymal cell migration is a multiple process of protrusion, adhesion formation, stabilization at leading edge followed by cell body translocation and release of adhesions and detachment of cell's rear (Veevers-Lowe et al. 2011; Clark et al. 2003; Bear & Haugh 2014). Motile fibroblasts and some cancers show such mesenchymal migration (N et al. 2015; Klymkowsky & Savagner 2009; D. Yamazaki et al. 2005). On the other hand, amoeboid migration is characterized by gliding and rapid migration and it mostly observed in cells like neutrophils, lymphocytes and other immune cells (Friedl et al. 2001; Wolf et al. 2003).

Integrin receptors regulate two major aspects of cell migration: (1) they generate a traction force between the cells and its substratum by directly mechanically linking ECM to actin cytoskeleton and (2) they act as a tether for various scaffolding proteins and signaling kinases that play an important role in initiating and regulating cell migration (Huttenlocher & Horwitz 2011; Hood & Cheresh 2002). The genetic pertubation of individual integrin subunits and the resulting pathologies has established crucial and diverse roles for each type of integrin receptor in cell migration. For example, β1 integrin containing, α1β1, α2β1, α10β1 and α11β1 integrins are receptors for collagen and have been reported to support migration of leukocytes and chondrocytes through collagen rich ECM upon stimulation with specific stimuli like growth factors and chemokines (Huttenlocher & Horwitz 2011; Hood & Cheresh 2002). Thus, integrin receptors play indispensable role in cell migration, not just by their virtue of adhering to ECM, but also by regulating various intracellular signaling pathways that ultimately control cytoskeletal organisation, force generation to aid appropriate cell migration.

Cell cycle and mitosis

Although growth factors (GFs) are normally regarded as being the main extracellular regulators of cell cycle progression and mitosis, the extracellular matrix and integrin mediated adhesions provide equally important cues for the same in anchorage-dependent cells. Infact, the integration and interpretation of the intracellular signaling cascade that results from each of these cues regulates all the different cell fate decisions that they make including proliferate or exit cell cycle and survive or undergo apoptosis.

During the S phase of cell cycle also known as commitment phase, sustained integrin-dependent adhesion signaling is required to initiate DNA synthesis and supress apoptosis.

Integrin-mediated adhesion to ECM is required for the induction of cyclin-D1, inhibition of cyclin-dependent kinase (CDK) inhibitors and activation of MEK1 which are essential for progression of cells through the G1-S phase (Walker & Assoian 2005; M. Oktay et al. 1999; Schwartz & Assoian 2001). Further, extracellular force has also been reported to feed into cell cycle progression through integrin regulated FAK/Rac signaling axis and Hippo pathway (M. C. Jones et al. 2019; Hotchin & Hall 1995). Further, cells get arrested during cytokinesis, resulting in binucleated cells, when held in suspension for longer periods of time (loss of integrin-mediated signaling) (De Santis Puzzonia et al. 2016; Pugacheva et al. 2006; Pellinen et al. 2008; Ben-Ze'ev & Raz 1981; Kanada et al. 2005). There are also reports suggesting threonine phosphorylation of β1 integrin at 788-789 residues results in decrease in cell adhesion during mitosis and the inability of the integrins to interact with the actin cytoskeleton causing centrosome maturation and spindle assembly defects (Suzuki & Takahashi 2003). Taken together, these studies establish that integrin-mediated adhesion is required and necessary for cell cycle progression and mitosis.

1.1.5 Regulation of membrane trafficking by integrins

Membrane trafficking pathways are involved in sorting and transportation of cargos to various intracellular compartments and reciprocally to the plasma membrane (Rogers & Gelfand 2000). These pathways play an important role in integrating spatiotemporal regulation of signaling cascades that in turn regulates various cellular processes such as cell proliferation, division, migration and polarity (Wilson et al. 2018; Prekeris & Gould 2008). Various studies have identified integrin-cell matrix adhesion as regulators of exocytosis, endocytosis and recycling machinery (Caswell et al. 2009; del Pozo et al. 2004; Nolte et al. 2020).

Integrin regulates plasma membrane order

The lipid bilayer of the cells is highly asymmetric and this asymmetry is crucial for normal cell function and physiology (Holthuis et al. 2009). Plasma membranes are known to be sub-compartmentalized into lipid rafts, which are highly ordered, dynamic and enriched in cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins that form platforms or hubs for membrane trafficking and signaling (Edidin 1997; Parton 2004; van Meer 2002). Some of the regulatory interactions and components of these lipid rafts are provided by integrin-dependent cell adhesion machinery. Clustering of integrin receptors has been shown

to promote the assembly of lipids into an ordered state. The ordered state of the lipid bilayer is crucial for various cellular functions as these lipid raft microdomains act as platforms for downstream trafficking and signaling (Wickström & Fässler 2011).

Integrin-mediated membrane Exocytosis

Integrins have also been shown to regulate and fine tune the secretion of soluble as well as membrane associated proteins such as growth factors (transforming growth factor-β, VEGF and insulin) (Ortega-Velazquez et al. 2004; X. Wang et al. 2011; Kragl & Lammert 2010) and proteases (matrix metalloproteinase-2) (Agrez et al. 1999; Gu et al. 2002; Iyer et al. n.d.). This control of exocytosis represents an additional mechanism by integrins for regulating cellular activities. Few mechanisms by which integrins can regulate this process include cytoskeleton regulation, vesicle transport and protein complexes at plasma membrane to fine tune docking and fusion of vesicles (Wickström & Fässler 2011). This regulation has been reported to occur on at least three levels (Figure 1.6). (a) Integrin-ECM engagement recruits the scaffold protein integrin-linked kinase (ILK) that further regulates the recruitment of microtubule-stabilizing proteins to the cell cortex. This results in local capture and stabilization of MTs, releasing caveolin-1-containing vesicles from MT-based motility, which allows the transport of caveolin-1 to the plasma membrane, resulting in formation of caveolae (Wickström et al. 2010; N. Balasubramanian et al. 2007). (b) Integrins through FAK and Src can cause actin remodelling by the ARP2/3 complex, which allows exocytosis of secretory vesicles in neurons (Gupton & Gertler 2010). (c) Newly formed integrin-ECM adhesion sites can recruit the GTPase RalA, which through its interaction with the adhesion protein paxillin promotes exocyst assembly (N. Balasubramanian et al. 2010; Spiczka & Yeaman 2008).
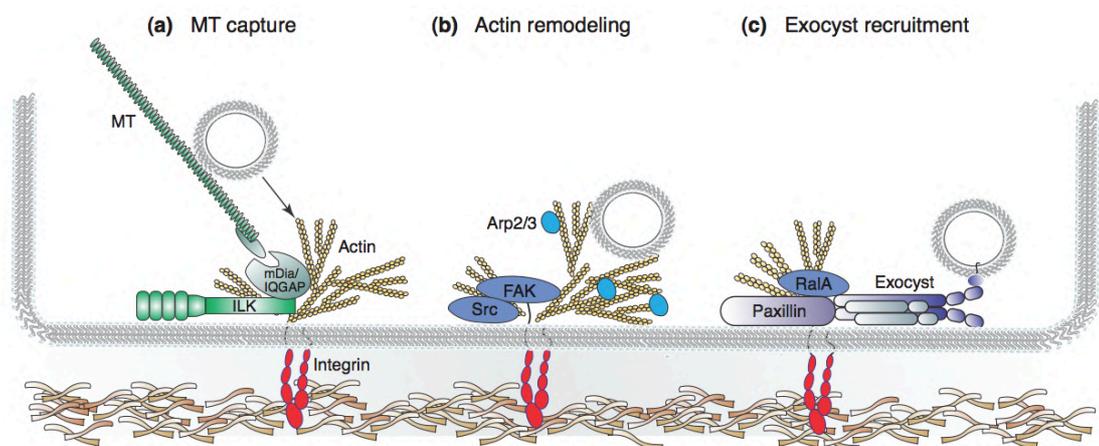
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Figure 1.6. Schematic of integrin-mediated regulation of exocytosis. Integrin-ECM engagement regulates exocytosis. (a) through the scaffold protein Integrin-linked kinase, ILK and microtubules (b) through the integrin-associated kinases FAK-Src and actin. (c) through RalA GTPase and paxillin during exocyst assembly and activation. (Reproduced from (Wickström & Fässler 2011)).

Integrin-mediated membrane Endocytosis

Integrin-mediated cell adhesion triggers caveolar and lipid raft endocytosis via focal adhesion kinase and Src kinase signals (Pelkmans et al. 2005). Loss of cell-matrix adhesion, triggers rapid internalization of caveolae and removal of active Rac tethered to the plasma membrane (del Pozo et al. 2004), subsequently inhibiting growth signaling pathways such as AKT and MEK/ERK (Grande-García et al. 2007; Wickström & Fässler 2011) . Recent studies have also suggested that besides regulating endocytosis of caveolae and rafts, integrins might also act as global regulators of endocytosis such as β1/2 integrin receptors being crucial for endocytosis of bacterial products (Morova et al. 2008; Hauck et al. 2012) and internalization of EGF and transferrin in HeLa cells (Collinet et al. 2010).

1.1.6 Integrin-dependent regulation of growth factor receptor signaling and trafficking

Integrins and growth factors share many common proteins in their signaling cascades, and hence there are multiple nodes where integrin signals can modulate growth factor signals and vice versa. In fact, integrin-ECM engagement has been shown to enable, if not always enhance, growth factor signaling. Mitogen-activated protein kinase (MAPK) pathway is the best characterized example for this (Schwartz 1997b). Here, activation of Ras that leads to downstream activation of Raf, MEK and ERK1/2, is dependent on integrin-ECM attachment, in absence of which there is only weak or transient activation of MAPK kinases ERK1/2 (Hotchin & Hall 1995). Multiple mechanism regulating different components of this signaling cascade have been shown to be under direct regulation of integrin-signaling, for example integrin-dependent PAK activity regulates Raf activation and FAK regulates MEK activity (Eliceiri 2001; Schwartz & Ginsberg 2002). These observations suggest that integrin-signaling feeds into growth factor pathways depending on cell type and external stimuli, however the final phenotype is strongly affected by the crosstalk between soluble stimuli and cell-matrix adhesion. Constitutive activation of the Ras effector pathways in cancer cells, hence overcomes the need for integrin-dependent regulation and promotes anchorage-independent signaling.

Recent studies have revealed that integrins can regulate growth factor signaling by growth factor receptor internalisation (Wickström & Fässler 2011). This regulation has been studied extensively in endothelial cells where αv integrin-dependent recycling of vascular endothelial growth factor receptors (VEGFR) has been shown to play an important role in tumor angiogenesis. In tumour cells, inhibition of αvβ3 integrin receptor has been shown to increase recycling of both EGFR and αvβ1 integrin (Caswell et al. 2008). Taken together, it seems that the major function of αvβ3 integrin is to control recycling of growth factor receptors and integrins back to the plasma membrane. Knowing the fact that, growth factor receptors can also regulate recycling of integrins, emphasizes the complex regulation that integrin-growth factor crosstalk seem to control in cells.
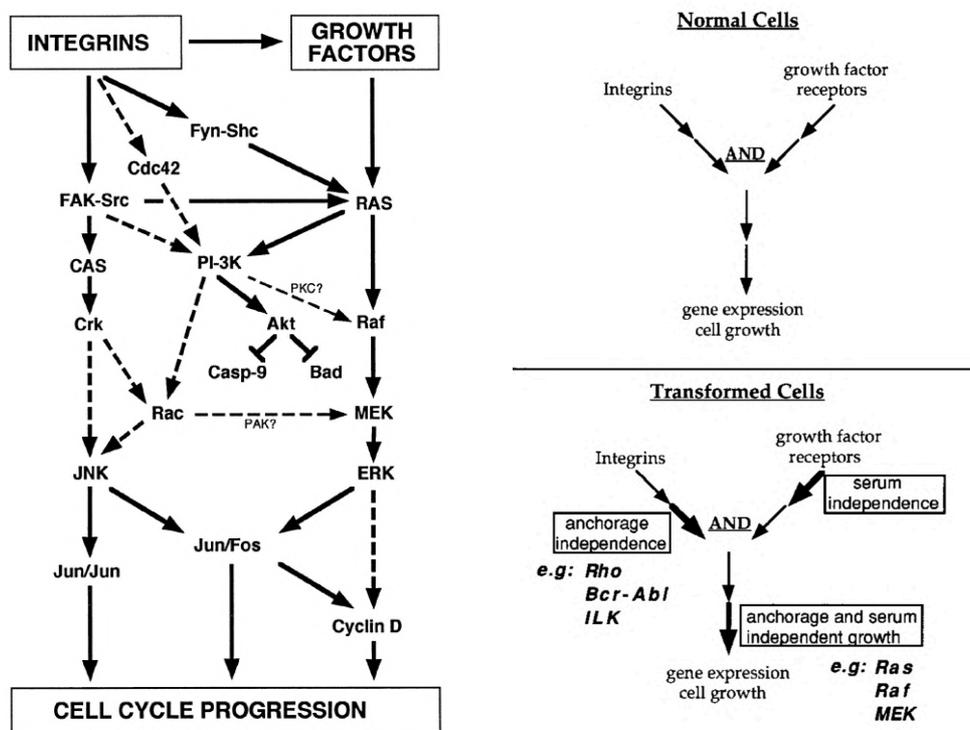
Figure 1.7. General scheme for convergence of integrin and growth factor–dependent pathways in which both are required for activation of gene expression and cell growth (left panel) in normal cells. In the right panel, constitutive activation is indicated by the boldface arrow. (Adapted from (Giancotti & Ruoslahti 1999; Schwartz 1997b))

1.2 Protein Kinases

The eukaryotic Protein kinases are one of the largest superfamily of enzymes catalysing protein phosphorylation. The human genome has been reported to contain 518 protein kinase genes, 478 of which belong to classical kinase family and 40 are atypical protein kinases. These family members although diverse significantly in their structures, regulation modes and substrate specificities, there are also many conserved structural motifs that provide clear indications as to how these enzymes manage to phosphorylate their residues. Phosphorylation is one of the crucial and dynamic mechanism for regulating a protein function and signaling cascade, that ultimately drives different cellular functions, such as cell proliferation, division, motility, differentiation, apoptosis, among others. Deregulation of a protein kinase activity has been shown to result in dramatic changes in these process, often if not always, leading to oncogenesis.

1.2.1. Classification of Protein Kinases

In 2002, Manning et al., classified the protein kinase complement of human genome primarily by sequence comparison of their catalytic domains, assisted by the information about their sequence and structure similarity, known biological functions and classification systems of yeast, worm and fly kinomes (Manning et al. 2002; Hanks & Quinn 1991; Hanks & Hunter 1995; Hanks et al. 1988).
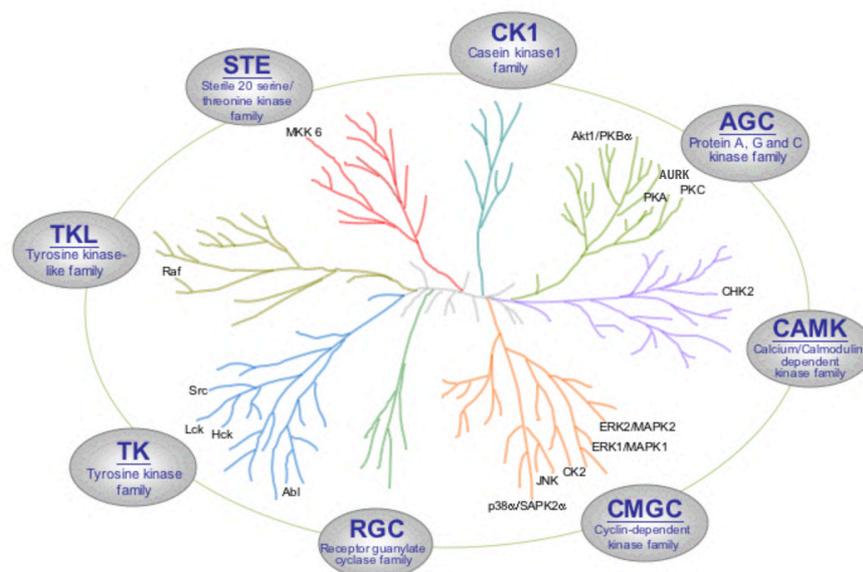
Figure 1.8. Classification of human protein kinases. (Adapted from (Manning et al. 2002))

The eukaryotic protein kinases are classified into nine groups, AGC group (consists of 63 members), CAMK group (74 members), CK1 group (12 members), CMGC group (61 members), STE group (47 members), TK (tyrosine kinase) group (90 members of which 58 are receptor kinases 32 non-receptor tyrosine kinases), TKL group (tyrosine-like kinases) (43 members), RCG (receptor guanylyl cyclase) (5 members) and a final group called others that contain 83 members (Hanks & Hunter 1995; Manning et al. 2002). Each of these protein kinases belong to complex network of signaling cascades and are stimulated by and respond to various extracellular and intracellular cues that together regulate physiologically important cellular functions.

1.2.2. Regulation of Protein Kinases: Structure-function relationship

Proteins kinases have been shown to be regulated by phosphorylation (sometimes by itself, called as cis-phosphorylation or auto-phosphorylation), by conformational change induced by binding to activator proteins or inhibitor proteins or small molecule messengers and by spatial regulation by controlling the intra-cellular localisation in the cell relative to their substrates (Hunter 1987; Nolen et al. 2004; Pellicena & Kuriyan 2006). This regulation of protein kinases has been sometimes referred to as switching 'on' or 'off' the kinase. Despite the conservation of overall fold of all protein kinases, there are differences in the sequence of activation loop and flanking regions of each protein that allows fine tuning of each kinase to respond to unique set of instructions to ultimately cause switching their activity to on or off state (Johnson et al. 1996). One of the common route of regulation of kinases is through conformation changes triggered by phosphorylation of activation segments or loops, a region of the protein kinase family that has gained a lot of attention in terms of understanding the structure-function relationship of kinases (Nolen et al. 2004; Adams 2002; Hanks et al. 1988). Phosphorylation of one or more residues in this activation segment allows the loop to refold itself, allowing it to position protein for substrate binding. In the protein kinases that are regulated by phosphorylation in this activation loop, the electrostatic interaction between a particular residue in the loop (primary phopsho-residue or phosphate) and a basic pocket (conserved among kinases; also called RD pocket) is a critical driving factor (Nolen et al. 2004). Further, each sub family has specific mechanisms of regulation, depending on the function and stimulus that they respond to. Tyrosine kinases that are receptors (RTKs) are activated by dimerization or clustering of the receptors to activate auto-phosphorylation and kinase activity, is one such example (Weiss & Schlessinger 1998).

1.2.3. General function of Protein kinases

Protein kinases catalyse the transfer of a phosphate molecule (phosphoryl group [(PO3)2-] to the target protein, that results in the activation or de-activation of the target protein, which is a key mechanism for regulating cellular and enzymatic functions in cell (Figure 1.9) (Z. Wang & Cole 2014). The phosphorylation by protein kinases is a highly dynamic process and can occur in few seconds or have kinetics spanning hours, both of which are highly coordinated to regulate complex cellular processes (Manning et al. 2002). Protein kinases also act as a means of amplification of a signal (where activation of a single molecule results in phosphorylation of multiple downstream targets) (Lemmon & Schlessinger 2010; Kettenbach et al. 2011; Hantschel 2012). Taken together, these functions of protein kinases provide an ideal means of appropriately regulating cellular functions by responding to multiple extra and intra cellular cues.
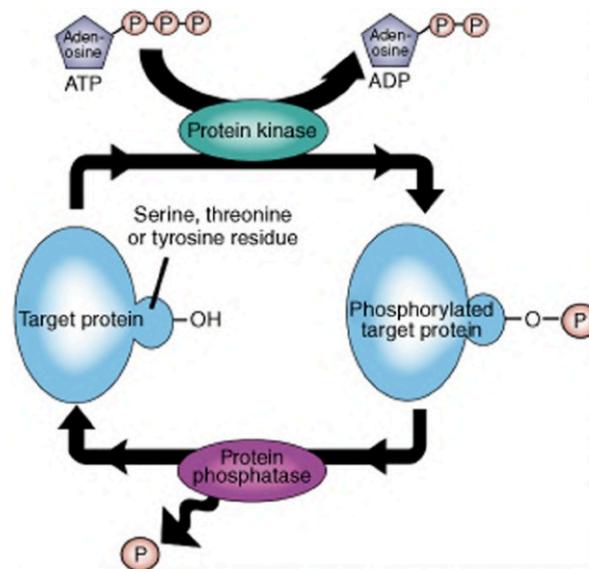
Figure 1.9. Schematic of the catalytic cycle for protein phosphorylation by a protein kinase. Protein kinases catalyse the transfer of a phosphate (P) from ATP to the hydroxyl group (OH) of a serine, threonine or tyrosine residue of the target protein. This phosphorylation acts as a 'molecular switch', which directly activates, or inactivates, the functions of proteins. However, protein phosphatases can oppose the kinase activities by catalysing the removal of the γ-phosphate from the targeted protein and reverses the effects of phosphorylation. (reproduced from (Patterson et al. 2014))

1.2.4. Protein kinases in cancer

Deregulated protein kinases are often found to be oncogenic and play a key role in the survival and spread of cancer cells. There are number of ways by which a kinase can be involved in cancers: de-regulated expression or amplification, mutation, aberrant phosphorylation,

chromosomal translocation and epigenetic regulation (Cicenas et al. 2018). Multiple databases have been developed to collate the information pertaining to human kinases. These databases contain the annotations describing the phosphorylation sites, substrates, de-regulation of kinase, like expression changes at mRNA and/or protein level or due to epigenetic regulation and genetic variants or splice variants of kinases associated with diseases and the potential use of kinases as biomarkers (diagnostic, prognostic or predictive) (Gaudet et al. 2015; Gosal et al. 2011).

Recently, amplifications and overexpression of kinases are being considered as predictive, diagnostic and prognostic biomarkers in numerous types of cancers. One of the best examples of kinase gene amplification is amplification of EGFR in NSCLC (non-small cell lung cancer) (Nukaga et al. 2017), bladder cancer (N. Chang et al. 2016), pancreatic cancer (C. Zhou et al. 2017.) and breast cancer (Cho et al. 2008). Similarly, AKT (Cheng et al. 1992), ERBB2 (Rasmussen et al. 2008), Aurora kinases (J. Zhu et al. 2005; Bonet et al. 2012) are examples of kinases that are often found to be over expressed in cancers and have a detrimental effect on progression of the same. Aberrant phosphorylation of kinases induced by or independent of mutations, such as ERK (Milde-Langosch et al. 2005), p38 (Fan et al. 2014), AKT (Cheng et al. 1992), EGFR and AURKA (Kitajima et al. 2007) have also been reported to be associated with poor prognosis in cancers and could serve as better biomarkers than amplification. Chromosomal translocations (for ex. Philadelphia chromosome) resulting in activation of kinases are also reported to be drivers of cancer (Advani & Pendergast 2002; Rabbitts 1994).

Owing to the central function of protein kinases in physiological cell process and their role in various cancers, an extensive search for kinase inhibitors has been carried out for several decades for both research as well as therapeutic purposes (Maurer et al. 2011; Fabian et al. 2005; Hidaka & Kobayashi 1992). Imatinib (Gleevec), was the first inhibitor against ABL1 and BCR-ABL1 fusion protein used for cancer therapy (Druker 2002). Several families of kinases, such as cyclin-dependent kinases, tyrosine kinases, aurora kinases, MAPKs have FDA approved inhibitors at different phases of clinical trials (Noble et al. 2004). Monoclonal antibodies is another approach that has been undertaken and studied extensively to inhibit various kinases in cancers (Sawyers 2002; Ivanov et al. 2008; Takai et al. 2005).

1.2.5 Aurora Kinases

Aurora Kinases belong to a family of serine/threonine protein kinases that were first identified in a screen for *Drosophila melanogaster* mutants that were defective in centrosome separation and spindle formation (Glover et al. 1995). The *aur* mutants result in failure of centrosome separation leading to monopolar spindle, hence they were given the name 'Aurora', reminiscent of the North pole. Since then many Aurora homologs have been identified in different species. Mammalian Aurora family comprises three members: Aurora Kinase A (AURKA), Aurora Kinase B (AURKB) and Aurora Kinase C (AURKC), located on chromosomes 20q13.2, 17p13.1 and 19q13.43, respectively (Carmena & Earnshaw 2003b). Aurora Kinases are involved in various mitotic processes ranging from centrosome maturation to cytokinesis. Owing to the fact that these are indispensable for mitosis, any dysregulation of these kinases leads to multiple defects in mitosis leading to aneuploidy or polyploidy. Aurora Kinases have also been reported to be over-expressed in many cancers, where they have been shown to support and enhance oncogenic potential of cancers. Considering the involvement of Aurora Kinases in cancer, many inhibitors are being developed for research purposes as well as therapeutic targeting.

1.2.5.1 Structural features and cellular geography of Aurora Kinases

The three members of human Aurora Kinase family, Aurora kinase A, B and C have been mapped on chromosome 20q13.2, 17p13.1 and 19q13.43, respectively. These kinases have amino acid sequence length ranging from 309 to 403 amino acids (Giet & Prigent 1999). All three kinases show a similar domain organization: N-terminal domain (39-129 amino acid), a protein kinase domain and a short C-terminal domain of 15-20 residues (Carmena & Earnshaw 2003b). As shown in Figure 1.10, the C-terminal catalytic domain of Aurora kinases shows high percentage of conservation. The Amino terminal domains share low sequence conservation. This differential amino terminal has been reported to play important role in localization and differential substrate interactions of different Aurora Kinases (S. Li et al. 2015). AURKA also has a silent C-Terminal D-Box (Destruction box) and an N-terminal A Box (also called D-box activating Domain(DAD)) (Crane 2004). The alignment of Crystal structure of AURKA to that of predicted structure of AURKB has shown that, the C-Terminal D box is present in Aurora Kinase B but N-Terminal A box is absent. This suggests a differential degradation pathway might be involved in degradation of AURKA and AURKB (Crane et al. 2004).
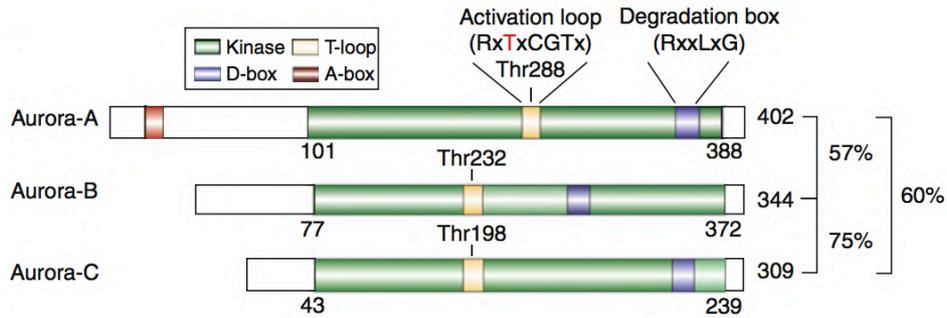
Figure 1.10: The organization of human Aurora kinase A, B and C. The position of the A box/D-box activating Domain (DAD) and the D-box is shown, as is the position of the activation loop. These features have been characterized most thoroughly in Aurora A and the Boxes shown for B and C are approximations (Reproduced from He L., Cheng J.Q, 2011).

Even though the members of Aurora Kinase family have high degree of sequence similarity, they have very different cellular localization. Aurora Kinase A localizes within the centrosomes from time of duplication of centrosomes until the mitotic exit. Indirect immunofluorescence studies have revealed that AURKA localizes to centrosomes, spindle poles, spindle from prophase to metaphase but predominantly to the spindle in telophase (Rannou et al. 2008). Aurora Kinase B localizes to the kinetochores from prophase to metaphase, in the midzone during anaphase and eventually in midbody during cytokinesis (Rannou et al. 2008). Very little is known about the third member of the family, Aurora Kinase C. Some of the studies have shown that Aurora Kinase C is specifically expressed at high levels in testis and has centrosomal localization from anaphase to telophase (Carmena & Earnshaw 2003b).
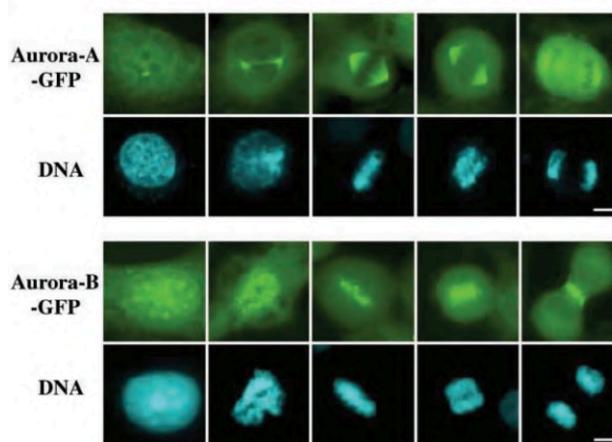
Figure 1.11. Cellular distribution of Aurora kinases during different phases of cell cycle. (Reproduced from (Fu, Bian, Q. Jiang & Zhang, 2007))

1.2.5.2 Regulation of Aurora Kinases

Transcriptional Regulation

Transcription of Aurora kinases is regulated in a cell cycle dependent manner. AURK promoters contain regulatory sequences CDE (cell cycle-dependent element) and CHR (cell cycle gene homology region). These sequences are involved in the transcriptional control of many G2/M regulators including Cyclin-A, CDC25C, CDK1, PLK and AURKA (Tanaka et al. 2002; Vader & Lens 2008; Crosio et al. 2002). For AURKB, E2F-1, E2F-4, DP-2 and FoxM1 transcription factor binding to CDE/CHR sequences within AURKB promoter has been shown to regulate its transcription during prophase (Kimura et al. 1999). AURKC genes till now have been shown to express mainly in meiotically dividing cells and a study shows that its transcription is controlled by a testis specific transcription factor called Testis zinc finger protein (Tzfp) (C. J. C. Tang 2001).

Regulation by Post translational modifications

All three members of aurora kinase family have been shown to be phosphorylated at specific residues upon co-factor binding during mitosis. As discussed previously, aurora kinases acquire an active conformation by regulation of the activation loop (Beenstock et al. 2016; Adams 2002). This includes auto-phosphorylation at specific residue in activation loop through an intermolecular (trans) binding within the two-lobed aurora kinase domain. In this method, the two catalytic lobes or domains form an asymmetric dimer, where one monomer acts as active enzyme and the other monomer acts as substrate (Zorba et al. 2014). Activity of Aurora Kinases is induced by auto-phophorylation at conserved Threonine residues (Threonine 288, Threonine 232 and Threonine 195 for AURKA, AURKB and AURKC, respectively) and co-factor binding followed by recruitment to specific mitotic structures (Carmena & Earnshaw 2003b; Willems et al. 2018).

Activation of AURKA during different stages of mitosis is mediated by auto-phosphorylation induced by several co-factors binding at each step. The best studied co-factors of AURKA are Ajuba, TPX2, Bora and TACC3. Ajuba has been shown to interact with N-Terminal region of AURKA and it plays an important role in the initial centrosomal activation of AURKA during late G2 phase (Hirota et al. 2003). It has been shown that the PreLIM domain of Ajuba induces autophosphorylation of the C-Terminal domain of AURKA by competitive binding to its N-terminal region (Bai et al. 2014). AURKA activation through the interaction with TPX2 is thought to be at-least partly due to PP1 antagonism (Kufer et al. 2002; Zorba et al. 2014).

TPX2- Aurora A binding occurs as an outcome of the Ran-GTP signaling pathway. TPX2 is usually in a complex with importins α or β. As cells enter mitosis, a gradient of Ran-GTP surrounding the chromosomes, promotes release of TPX2 from importins. This TPX2 then binds to AURKA that is kept in inactive form by protein phosphatase 1 γ (PP1). TPX2 interferes with the activity of PP1, allowing AURKA to auto-phosphorylate and activate itself and other substrates, including TPX2 (Kufer et al. 2002; Carmena & Earnshaw 2003b). The initial AURKA phosphorylation has been reported to cause a positive feedback phosphorylation loop which is responsible for (1) activation peak observed in AURKA activity from late G2 to pro-metaphase and (2) maintenance of active AURKA until anaphase.
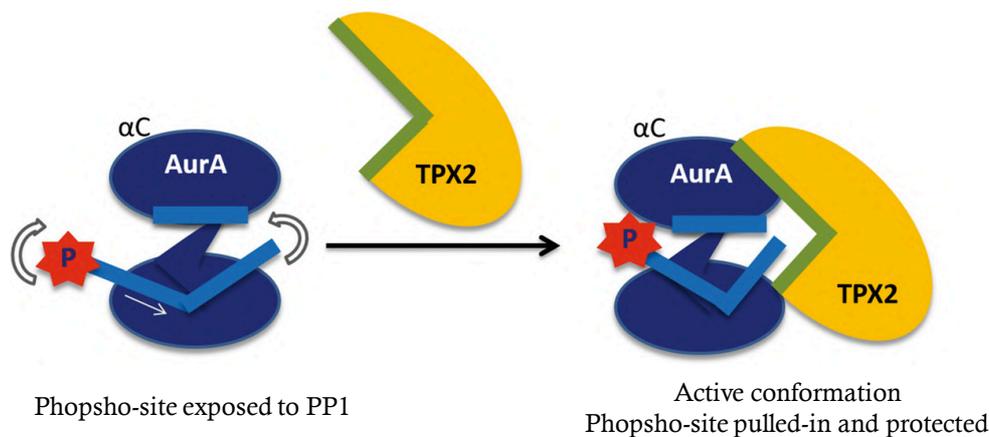
Figure 1.12. Diagrammatic representation of AURKA activation by TPX2. (Adapted from (Panicker et al. 2019))

AURKA can also be regulated by phosphorylation by upstream kinases at Threonine 288 residue (ex. mTOR (Platani et al. 2015), PKA and PAK-1 (Zhao et al. 2005)) and on other residues (ex. Thr287, Ser283/284) to activate and/or Ser342 to inhibit its kinase activity (Willems et al. 2018; Dodson & Bayliss 2012). AURKA can also be regulated by Ca2+/Calmodulin (CaM), which induces auto-phosphorylation at Ser51, Ser53/54, Ser66/67 and Ser98 residues (Plotnikova et al. 2010; Plotnikova et al. 2012). The different phosphorylation has been reported to be involved in protecting AURKA activity against degradation until the end of mitosis. Further, acetylation of AURKA at residues K75/K125 by ARD1 (Arrest Defective Protein 1) acetyl-transferase has been shown to help maintain its activity (Vo et al. 2017). Nevertheless, Theronine-288 auto-phosphorylation seems to be pre-requisite for activation of AURKA over any other mode of regulation (Zorba et al. 2014).

Auto-phosphorylation of AURKB occurs on Thr-232 in the activation segment in humans (Yasui et al. 2004). At the onset on prophase, AURKB binds to INCEP (Inner centromere protein) at a C-terminal motif, IN-box (Bolton et al. 2002; Sessa et al. 2005). This binding triggers the AURKB auto-phosphorylation and induces its kinases activity. This active AURKB further binds Survivin and Borealin to form CPC (Kollareddy et al. 2012; Carmena & Earnshaw 2003a). This integration of AURKB in CPC is essential for AURKB activity and correct localisation throughout mitosis. AURKC has been reported to phosphorylate same substrates as AURKB in meiotically-active cells and is subjected to similar regulation as AURKB (Vader & Lens 2008).
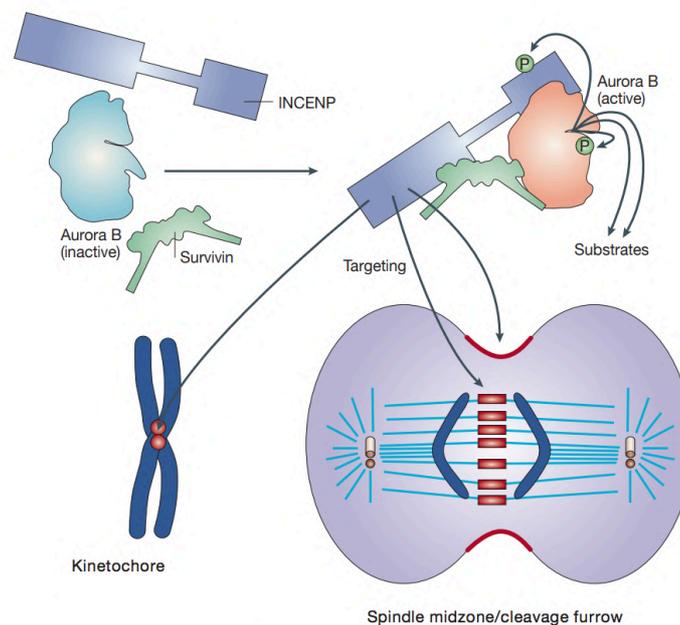
Figure 1.13. Diagrammatic representation of AURKB activation and targeting by CPC. (Adapted from (Carmena & Earnshaw 2003a))

Recently, several other kinases, such as Mps1, Chk1 and Tousled like kinase-1 were shown to be involved in proper activation of AURKB. The Msp1 is a checkpoint kinase that phosphorylates the CPC subunit of Borealin, which leads to activation of AURKB (Nhung Hoang et al. 2008.; Storchová et al. 2011). Chk1 is involved in direct binding and activating AURKB (Youwei Zhang & Hunter 2014; Mackay & Ullman 2015). Tousled like kinase1 present in *C. elegans,* was found to activate AURKB in a Kinase independent but INCENP dependent manner (Han et al. 2008). Recently, a protein TD 60 which shows chromosomal passenger like mitotic localization was reported to be involved in localization and activation of AURKB (Rosasco-Nitcher et al. 2008).

Regulation by Degradation

Degradation of Aurora Kinases is regulated by APC/C and is activated by the Cdh1 protein, which recognizes the destruction box signals (degrons) present in the proteins (Littlepage and Ruderman 2002). Upon activation, APC/C ubiquitinates and initiates the proteasome-mediated degradation of these kinases. AURKA degradation is initiated during mitotic phase and is completed during G1 phase, while AURKB and AURKC are degraded after cytokinesis (Floyd et al. 2008; Afonso et al. 2019). Ubiquitination of AURKs is reported to be induced by PP1 or PP2A phosphatase mediated de-phopshorylation at Ser51 residue (Kitajima et al. 2007). Further, APC/C mediated degradation of AURKA has been reported to be more efficient than AURKB. AURKB binds to a Microtubule-binding protein EB1, which protects it against degradation (Willems et al. 2018).

1.2.5.3 Effectors and Function of Aurora Kinases

The cell cycle is an ordered set of events that involves cell growth and culminates in division of a cell into two daughter cells. The process of cell division occurs at high fidelity to maintain the correct chromosome content in the daughter cells. Accurate chromosome segregation requires precise spatiotemporal co-ordination of many highly complex mitotic events (Carmena & Earnshaw 2003b). Aurora Kinase family members are one of the most important groups of proteins that control some of the most crucial processes in cell division.

Centrosome maturation

Aurora Kinase A in involved in the process of centrosome maturation that involves microtubule (MT) nucleation and peri-centriolar material (PCM) recruitment to the MTOC (microtubule-organizing center). The recruitment of AURKA and PLK1 (polo-like kinase 1) at centrosomes is favored by the increase in Cdk11 in prophase. The targeting of AURKA at centrosomes has also been reported to be mediated by Src kinase, that is activated at the site of Golgi-disorganization in G2 phase (Barretta et al. 2016). AURKA is majorly involved in centrosome growth rather than in centrosome duplication. Upon complete activation, AURKA recruits centrosomin (Terada et al. 2003) and γ-TuRC (γ-tubulin ring complex) (Sen et al. 2008), both of which are essential for nucleation and elongation of microtubules. AURKA also phosphorylates and recruits NDEL1, TACC, LATS2 and BRCA1 to the MTOC (Giet et al. 2002; Ouchi et al. 2004). AURKA regulated TACC binds to the microtubule binding protein CKAP4, that stabilizes the minus end of MT at centrosomes and initiates organization of actin

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cytoskeleton (Barros et al. 2005; LeRoy et al. 2007). LATS2 and BRCA1 are involved in recruitment of γ-tubulin that facilitates the MT nucleation during centrosome maturation (Barr & Gergely 2007a).

Additionally, AURKA has been shown to activate and target the cyclin B1-Cdk1 complex at centrosomes (Barr & Gergely 2007b), either by direct phosphorylation or by phosphorylation of cyclin-B1recruiting CDC25B phosphatase. Simultaneously, AURKA phosphorylates PLK1, suppressing the Wee1 inhibitor and activating CDC25B responsible for Cdk1 activation (Cazales et al. 2005; Dutertre et al. 2004). This cyclin B1-Cdk1 activation has been reported to be a first wave of its activation and is essential for the transition from the G2 phase to M phase of cell cycle (Gavet & Pines 2010; Jackman et al. 2003).

Centrosome separation and establishment of bipolar spindle assembly

Once the centrosomes have matured, they start migrating away from each other to define two poles of the bipolar mitotic assembly. Depletion or inhibition of AURKA causes monopolar spindle in several model systems (Glover et al. 1995). AURKA contributes to centrosome segregation by phosphorylating kinesin Eg5, that is involved in regulating the anti-parallel forces of the spindle MT (Asteriti et al. 2011; van Heesbeen et al. 2013). Additionally, AURKA regulates the cycles of microtubule assembly and disassembly to control mitotic spindle dynamics. Notably, AURKA (i) inhibits the Kinesin Family Member 2A (Kif2a) microtubule depolymerase (C.-Y. Jang et al. 2009), (ii) recruits TACC3, inducing the microtubule growth through CKAP5-a (Cytoskeleton-associated protein 5-A) (LeRoy et al. 2007) and (iii) stabilizes microtubules around the centrosomes by antagonizing Kinesin Family Member 2C (Kif2C) (Xin Zhang et al. 2008). AURKA has also been shown to affect the assembly and function of astral microtubules in a D-TACC dependent manner (Giet et al. 2002). These microtubules play important role in spindle assembly.

A second wave of AURKA-dependent phosphorylation of CyclinB1-Cdk1 has been shown to occur in late prophase. Here, activated cyclinB1-Cdk1 activates Ran GTPase dependent NEBD pathway and stimulates the release of a spindle assembly factor named TPX2 (Kufer et al. 2002). This activated TPX2 now binds to AURKA catalytic domain and confers it an active conformation, which allows Thr288 auto-phosphorylation, localization on astral microtubules and prevents inactivation of AURKA by PP1 and other phosphatases (Zorba et al. 2014).

Chromosome condensation, alignment and kinetochore anchoring

In the early stages of mitosis, chromatin starts condensing and folding into proper chromosomes. A complex of Condensin proteins (Condensin I and II) are responsible for this condensation process. Aurora Kinase B aids in association of condensin I complex with chromosomes and thereby brings about proper condensation of the chromosomes (Hochegger et al. 2013). Additionally, AURKB also phosphorylates the histone H3 at Ser10 residue, prompting the release of heterochromatin protein 1 (HP-1) from heterochromatin and to an epigenetic switch favoring active chromatin conformation (F. Wang et al. 2010). The phosphorylated H3 induces chromosome condensation and recruitment of AURKB to centromeres (A. E. Kelly et al. 2010). AURKC can also phosphorylate H3 in mitotic and meiotic cells, revealing an overlap in AURKB and AURKC functions (Willems et al. 2018).

AURKB has been shown to concentrate in the kinetochores during prophase. AURKB dependent phosphorylation of histone H2AX in kinetochore promotes auto-phosphorylation of AURKB (Shimada et al. 2016). Capture of the kinetochores by mitotic spindle is a stochastic process that gives rise to intermediate states of attachments like, Syntelic or Merotelic (Fu et al. 2007). These states occur during mitosis every time and they are eventually corrected to produce accurately attached chromosomes. Various studies have shown that these attachments are actively destabilized through AURKB activity (Buvelot et al. 2003; Petsalaki & Zachos 2013) (Buvelot et al. 2003; Tanaka et al. 2002). Two important kinetochore microtubule capture factors (the Ncd80/Hec1–and Dam1-complexes) (Tien et al. 2010) are subjected to phosphor-regulation by AURKB (Joukov & De Nicolo 2018). Additionally, AURKB also regulates MCAK (Kif2C) to depolymerize the incorrectly attached kinetochores (Lan et al. 2004; Gorbsky 2004) and these two things together contribute to correction of defective attachments. In conditions where unattached chromosomes or kinetochores are present the Spindle assembly checkpoint (SAC) is activated (Rieder & Maiato 2004). AURKB is thought to indirectly contribute to SAC activation by destabilizing wrongly attached kinetochores and creating un-attached kinetochores.

Chromosome separation

During pro-metaphase, AURKB along with members of CPC are recruited to centromeres and to the midzone to facilitate chromosome separation (Carmena et al. 2009). CULIN3-containing

ubiquitin ligase triggers the centromere targeting of CPC (Sumara et al. 2007). This CULIN3-containing ubiquitin ligase is known to be negatively regulated by CyclinB1-Cdk1 complex. During metaphase, cyclinB1 levels start decreasing, leading to a rapid drop of Cdk1 activity, facilitating the role of CULIN3-ubiquitin ligase (Sumara et al. 2007). During pro-metaphase, AURKB along with other components of CPC are recruited to the midzone, partially by the Mitotic Kinesin-like Protein (MKLP2 kinesin) (Gruneberg et al. 2004), where they are required for proper chromosome separation. Calmodulin (CaM) has also been reported to protect AURKB at midbody that is essential for regulating mitotic abscission, ensuring faithful mitotic progression (Mallampalli et al. 2013).

AURKA has been shown to be important for central spindle assembly during anaphase and lower levels of AURKA causes delocalization of MKLP1 and increase in MAP DCTN1 (dynactin subunit 1) levels at spindle poles, impairing bidirectional transport along microtubules and central spindle assembly (Reboutier et al. 2013; Barr & Gergely 2007a).

During telophase, AURKB is shown to cause dissociation of HP-1 and Rad21 (cleavable components of Cohesin complex) that allows for telomere dispersion. AURKB enhances chromosome condensation and telomere disjunction by phosphorylating Condensin complex subunit-2 (Cnd2) (Reyes et al. 2015). Additionally, AURKB recruits Shugoshin1 (SGO1) to the centromeres where it facilitates removal of cohesion to trigger sister chromatid separation during anaphase (Storchová et al. 2011).

Telophase and cytokinesis

AURKB has been reported to prevent nuclear envelope assembly, facilitating the inclusion of late-segregating acentric chromosomes that are prone to form damage-susceptible micronuclei (Warecki & Sullivan 2018). This function of AURKB is mediated by its regulation of HP-1 during telophase (Warecki & Sullivan 2018).

During cytokinesis, midbody localized AURKB activates the RhoA GTPase post RacGAP1 phosphorylation, thereby inducing actin polymerisation and myosin activation, both of which are indispensable for the contractile ring formation (Ma & Poon 2011; Carmena et al. 2009; Carmena & Earnshaw 2003a). Additionally, AURKB phosphorylates substrates like vimentin, desmin and GFAP to organize the cleavage furrow (Sessa et al. 2005; Carmena et al. 2009).

Thus, AURKB plays significant and diverse roles in maintaining genome integrity during telophase and in segregation of cytoplasmic factors during cytokinesis.

Regulating mitotic checkpoints

In untransformed normal cells, DNA damage by UV, radiation or chemicals, causes pausing of mitosis at predefined cell cycle checkpoints (G1/S/G2). The fate of a cell with altered DNA is determined at these checkpoints by various checkpoint associated proteins. For ex. Repair of damaged DNA (during cell cycle pauses at checkpoints), entry into G0 phase of cells whose DNA cannot be repaired or apoptosis are few of the outcomes of cells with DNA damage. The replication of damaged DNA is prevented in G1 checkpoint, S checkpoint prevents the progression of cells in mitosis before DNA synthesis and G2 checkpoint stops the mitotic entry of cells with double stranded DNA breaks (DSB) in newly replicated DNA. In response to DNA damage detected by sensor proteins (Rad50, Mre11, Nbs1), ATM (ataxia telangiectasia mutated) and ATR (ATM-and Rad3 related) kinases get activated (X. Tang et al. 2008), that further activate Chk1/Chk2 (checkpoint protein 1/2) known to supress AURKA activity (Zachos et al. 2007; Youwei Zhang & Hunter 2014). If DNA damage is repairable, then the checkpoint is overridden to proceed into mitosis, by inhibiting Chk1 thereby re-activating AURKA. Other mechanism of regulation of checkpoint involves induction of p-53-dependent transcription of p21 and Gadd45 (Growth arrest and DNA damage-inducible protein GADD45 alpha), that inhibits CDK1 induced mitotic entry (Taylor & Stark 2001). Centrosomal p53 proteins are also known to inhibit AURKA by its transcriptional (regulation of E2F3 by p21) and post-translational (ubiquitination and Ser215 phosphorylation) regulation thereby adding extra layer of regulation in mitotic checkpoint (Willems et al. 2018). In cancer, overexpresed AURKA can thereby overcome this checkpoint and mediate chemo- and radio-resistance by efficient homologous region dependent DSB repair (Nikonova et al. 2012).

Additionally, in response to DNA damage, AURKB is down-regulated by protein phosphatase 1 (PP1), which in turn is induced by ATM (X. Tang et al. 2008). This AURKB inhibition is further associated with Chk1 activation with delayed H3 phosphorylation, resulting in delayed chromosome replication and condensation (B. H. Chang et al. 2007). AURKB has also been reported to be involved in NHEJ repair pathway of DNA damage. Here, the Ku heterodimer (Ku70/Ku80), a component of NHEJ complex, inhibits AURKB activity upon irradiation (Fell et al. 2016). Further, in response to DNA damage, PARP1, a chromatin-associated DNA repair

enzyme, also represses AURKB to block mitosis and histone H3 phosphorylation (Monaco et al. 2005).
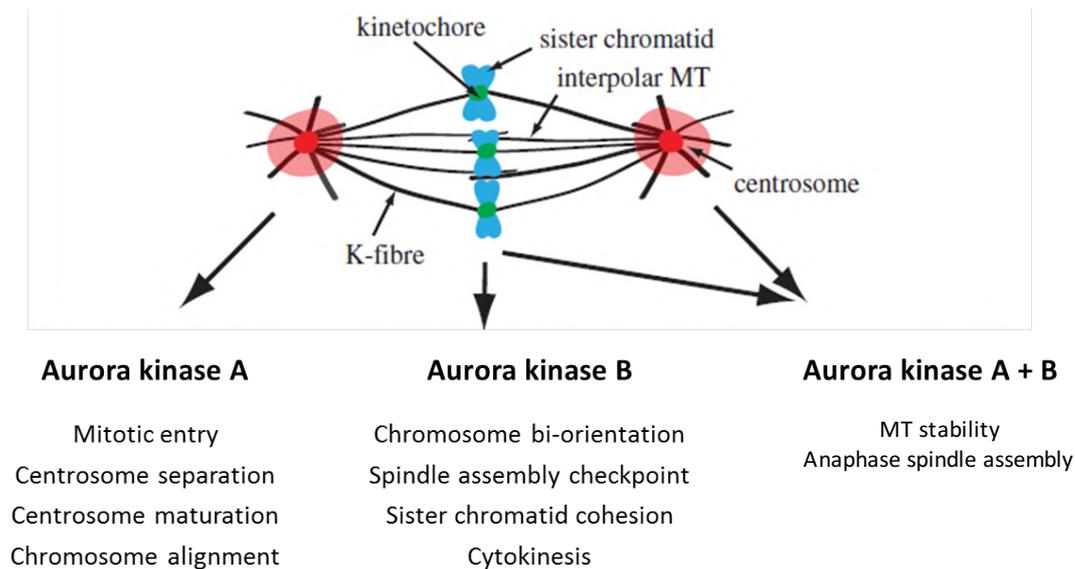
Figure 1.14: Overview of specific and combined functions of Aurora kinase A and Aurora kinase B. (Adapted from (Hochegger et al. 2013))

Non-mitotic roles of Aurora Kinases

The first piece of evidence for a role of Aurora kinases outside of mitosis came from the observation that activated AURKA (on Thr288) was found in close proximity of cilia in G0/G1 cells (Bertolin & Tramier 2020). The primary cilium acts as a docking platform for multiple signaling pathways, including the Notch, WNT, and the Hedgehog pathway. Additionally, cilium is necessary for sensing extracellular cues for cell cycle coordination. When the cells leave quiescent state and re-enter cell cycle, the cilia are dismantled. The activation of AURKA in close corporation with HEF1 (NEDD9) favours disassembly of cilia in G1 phase (Pugacheva et al. 2007). Further, calmodulin activation of AURKA has also been shown to promote ciliary disassembly (Plotnikova et al. 2012). Another role played by AURKA in G0/G1 phase was observed in post-mitotic neurons, that are large cells with extremely limited capacity to proliferate post differentiation. During neurite extension, AURKA was shown to be active in cells not actively cycling, where it phosphorylates NDEL1 in a PKC dependent manner to establish a functional MTOC during neuronal growth (D. Mori et al. 2009). Mahankali et al. reported that, AURKA is a central player in a non-mitotic cascade involving SRC, FAK and PLD2. They reported that, in interphase cells, AUKA activated by SRC and PLD2 can

positively regulate tubulin integration into microtubules in vitro in FAK dependent manner and that this PLD2-AURKA-SRC-FAK pathway helps COS7 cells sustain cell migration (Mahankali et al. 2015). Additionally, AURKA is also involved in formation of pre-replication complex, that ensures the initiation of DNA replication. AURKA phosphorylates and stabilizes a key component of this complex called Geminin (GMNN), which contributes to proper formation of pre-replication complexes when G1 begins (Tsunematsu et al. 2013).

At the cross-road between non-mitotic and mitotic roles of AURKA, is a role of kinase in maintenance of mitochondrial morphology and dynamics. AURKA contains an N-terminal mitochondrial targeting sequence (MTS) that drives its localisation and import in the mitochondrial matrix, irrespective of cell cycle phase (Bertolin et al. 2018). During cell division, AURKA localizes a small GTPase RalA and its effector RALBP1 at mitochondria, where they facilitate phosphorylation of Drp1 by CyclinB/Cdk1 complex (Lim et al. 2009; Kashatus et al. 2011). However, this function of AURKA is not limited to mitosis as interphase cells also show mitochondrial fragmentation by AURKA in Drp1 dependent but RalA-independent manner (Bertolin et al. 2018). A novel function for AURKB has been reported in neurons, where it regulates neuronal development and axonal growth in developing zebrafish (Gwee et al. 2018).
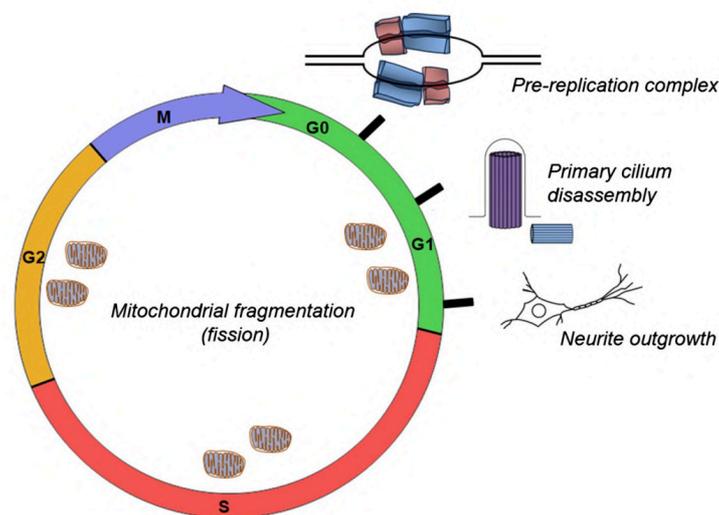

Figure 1.15. Schematic of the physiological non-mitotic roles of AURKA. Following roles are confined to G0/G1: disassembly of primary cilia, neurite outgrowth and the formation of the DNA pre-replication complex. The regulation of mitochondrial dynamics appears to take place throughout the interphase. (Reproduced from (Bertolin & Tramier 2020)).

1.2.5.4 Role of Aurora Kinases in cancers

The role of AURKA in tumorigenesis is supported by a large body of evidence, in contrast to role of AURKB/AURKC, whose oncogenic activities are not yet fully understood. Amplification, overexpression or hyper-activation of AURKA has been reported in several tumours including breast (Hole, Pedersen, Lykkesfeldt & Yde 2015b), colorectal (Bischoff et al. 1998), gastric (X. Liu et al. 2016), bladder (N. Zhou et al. 2013) and prostate cancers (Das et al. 2010). Further, expression of AURKA has been reported to predict patient prognosis in various cancers including, colorectal, breast, nasopharyngeal, bladder and gastric tumours (J. Xu et al. 2014; Nadler et al. 2008). AURKB is also found to be overexpressed in many human tumours and is a poor prognosis factor in NSCLC, oral squamous cell carcinoma and hepatocellular cancers (Nhung Hoang et al. 2008.). Aberrant expression of AURKC has been reported in some cancer cell lines including thyroid, cervical and colorectal cancers (Willems et al. 2018). Overexpression of AURKC has been shown to transform somatic cells and induce tumour progression, however, the mechanism of AURKC-mediated tumorigenesis is not yet clear.

Centrosome amplification and aneuploidy

It has long been implicated that alterations or aberrations of centrosome numbers can contribute to aneuploidy and tumorigenesis. Overexpression of AURKA in NIH3T3 cells confers transformed phenotype in cells that is characterized by centrosome amplification and aneuploidy (Carmena & Earnshaw 2003b). Overexpressed AURKA leads to centrosome overgrowth, multipolar spindle formation and unequal chromosome segregation thereby leading to aneuploidy in mitotically active cells, which can thereby corrupt into pre-cancerous cells (Meraldi et al. 2004). The role of AURKA in causing aneuploidy is majorly mediated by p53, BRCA1/2 and Ras association domain-containing protein 1 (RASSF1A). Under normal physiological conditions, when aneuploid or multinucleate cells are generated as a consequence of AURKA overexpression, they are subjected to p53-Rb checkpoint arrest in the G1 phase and then eliminated (Meraldi et al. 2002). However, in absence of a functional p53 checkpoint, such cells progress through extra rounds of DNA replication and give rise to polyploidy. This leads to chromosomal instability and aneuploidy which may ultimately lead to cellular transformation (Meraldi et al. 2002). Additionally, aberrant AURKA activity also causes monopolar spindles, defects in chromosome separation and chromatin bridges all ultimately leading to aneuploidy (Glover et al. 1995).

Aurora kinase B overexpression induced centrosome amplification also occurs in a manner similar to AURKA expression. But many other mechanisms seem to have a more profound effect on AURKB induced carcinogenesis. AURKB has been reported to induce chromosome lagging in metaphase, chromosome segregation error, SAC activation and errors in cytokinesis (Ota et al. 2002). AURKB overexpressing cells are also shown to remain in mitosis for longer periods, mostly through inhibition of a cyclin-dependent kinase inhibitor, p21Cip1 (González-Loyola et al. 2015).

Aurora Kinase C overexpression has also been shown to induce formation of multinucleate cells with more than two centrosomes (Khan et al. 2011). This also might be due to defect in cytokinesis, as multinucleate cells are observed. It has been reported that AURKC can disrupt the interactions of AURKB with INCENP leading to delocalization of AURKB. Thus overexpression of AURKC behaves as a dominant negative kinase for AURKB leading to cytokinesis defects and aneuploidy that can culminate into cancer (Sasai et al. 2004).

Spindle assembly checkpoint dysregulation and cytokinesis failure

It has been proposed that spindle checkpoint dysfunction is an important cause of aneuploidy in Human epithelial malignancies. Bub1, BubR1 and Mad2 are checkpoint proteins that are visualized at the kinetochores only when the spindle assembly checkpoint is active. They become almost non-existent when cells enter anaphase (Anand & Penrhyn 2003). Overexpression of AURKA causes cells to enter anaphase despite defective spindle formation. These cells show the presence of Mad2 at kinetochores even in anaphase suggesting that the spindle checkpoint stays active even after entering anaphase (Anand & Penrhyn 2003). Therefore, it might be possible that overexpression of AURKA disrupts the interaction between Mad2 and Cdc20 or it prematurely activates APC/C proteolytic functions. In one of the studies it was found that AURKA overexpression overrides the spindle checkpoint that had been triggered by nocodazole (Y. Jiang et al. 2003). Loss of activity of AURKB either by depletion of levels or expression of inactive forms in cell also compromises the spindle assembly checkpoint. This phenomenon occurs because AURKB is involved in the recruitment of many checkpoint proteins (Fu et al. 2007).

Tumor Suppressor gene dysfunction and Cell Viability

AURKA has been shown to interact with many tumor suppressor gene products like p53(Meraldi et al. 2002), BRCA1(Ouchi et al. 2004), NM23-H1(Du & Hannon 2002) and Chfr(Yu & Minter 2005). The interaction between AURKA and p53 has been well explored. AURKA phosphorylates p53 and inhibits its activity by promoting degradation of the protein (Katayama et al. 2004). On the other hand, p53 suppresses AURKA induced centrosome amplification and cellular transformation (Meraldi et al. 2002). Loss of p53 activity subsequently inhibits activity of downstream targets of p53, such as p21 and pTEN (Katayama et al. 2004). AURKA can also directly bind and phosphorylate BRCA1 at Ser308 residue. Loss of this phosphorylation has been shown to decrease the number of cells in M phase of cell cycle in Mouse embryonic fibroblasts (Ouchi et al. 2004). Thus, these interactions reveal that AURKA and tumor suppressor genes act in antagonistic way to control viability of a cell and maintenance of correct balance and interaction between these two is necessary to maintain normal growth of cells.

Cooperation in other oncogenic signaling pathways

In many of the human cancers, the Ras/Raf/ERK/MAP kinase pathway is known to be enhanced. This increases the responsiveness of a cell to growth signals and allows them to grow and form cancerous tissues. Recently it has been reported that AURKA and AURKB both act as downstream targets of MAPK/ER2 in pancreatic cancer and in melanoma cells, respectively (Furukawa et al. 2006). The overexpression of Aurora kinases owing to increased sensitivity of MAP kinase pathway may thereby allow the cells to divide faster and may lead to tumorigenesis.

Ectopic expression of AURKA also induces telomerase activity in Human ovarian and breast epithelial cell lines. AURKA stimulates the mRNA and promoter activities of human telomerase reverse transcriptase (hTERT). It has been previously shown that c-Myc binding sites of hTERT promoter are necessary for its activity. The overexpression of AURKA targets this c-Myc and upregulates its activity thereby stimulating hTERT promoter activity (H. Yang et al. 2004). This provides an additional mechanism for the role of AURKA in malignant transformation in addition to its role in cell cycle control.

Aurora activity might also modulate the oncogenic Ras signaling. The precise mechanism by which this can occur still needs to be understood. Some studies have been carried out and have reported that AURKA might target small G protein associated GAP molecules such as RasGAP, which mediated responsiveness to the oncogenic signals generated by the oncogenes such as G12VRAS during interphase (Tocque et al. 1997). Another protein Ajuba which is a cytoplasmic LIM domain protein has also been identified as a AURKA activation protein (Bai et al. 2014). Ajuba interacts with Grb2 and effects Ras signaling (Kimura et al. 1999) and presumably AuroraA/Ajuba complex also might modulate Ras signaling. Another signaling arm of oncogenic Ras pathway involves RalGEF-Ral effector signaling.
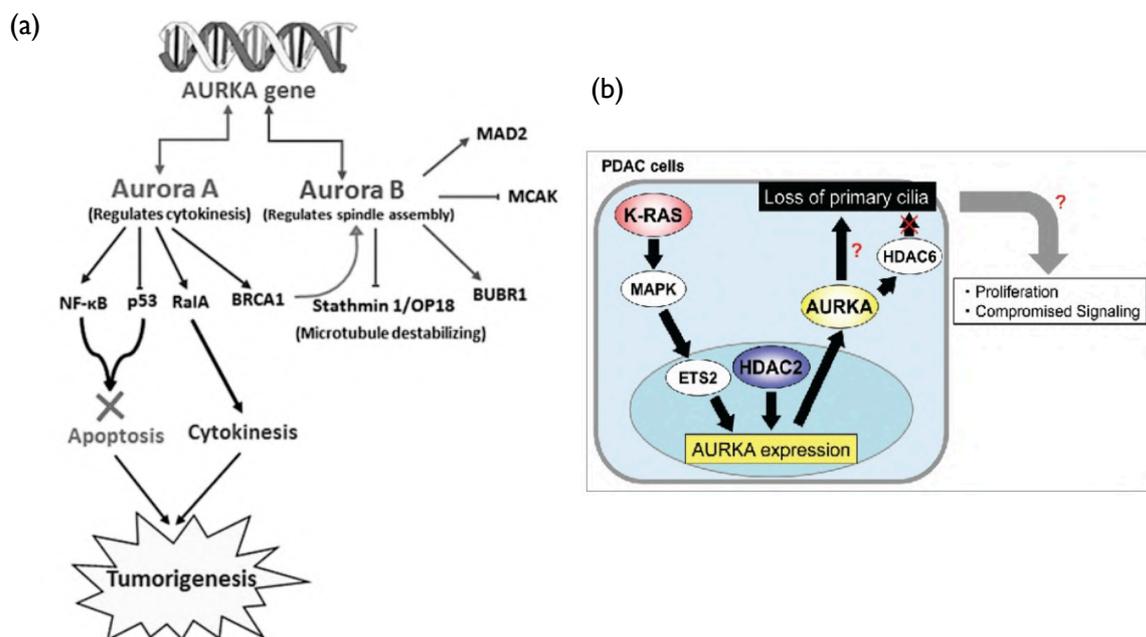
Figure 1.16. (a) Schematic of signaling pathways activated downstream of overexpressed or active AURKA and AURKB in cancers. (Adapted from (Suman & Mishra 2018)) **(b) Over-expression of AURKA by KRas,** inducing loss of primary cilia probably promoting proliferation in PDAC cells.(Reproduced from (Tetsuo Kobayashi 2017).

AURKA has been shown to phosphorylate RalA at Ser194 and enhance its activity. Studies have also shown that AURKA may act in concert with RalA to promote Collagen-1 induced cell motility and anchorage independent growth in MDCK epithelial cells (Wu et al. 2005). Thus, AURKA may converge upon oncogenic Ras signaling through RalA. *(This section has been reiterated in section 1.3.4 for ease of understanding).*

Stemness, epithelial-to-mesenchymal transition and migration

Higher nuclear AURKA expression has been shown to favour stemness and is associated with poor prognosis in breast cancer patients (Nadler et al. 2008). Additionally, AURKA is delocalized from the nucleus to cytoplasm in migrating cells. Taken together, these data suggest that disrupted AURKA localisation may mediate its oncogenic functions(Carmena et al. 2009; Das et al. 2010; Libertini et al. 2010).

1.2.5.5 Aurora kinase inhibitors as drug targets

Owing to the role of Aurora kinases that has been recognized in several cancers, these kinases have become attractive and potent targets of cancer therapy. In this regard, a series of Aurora kinase inhibitors (AKIs) have been designed and tested in the past decades and have been used into clinical trials (Kollareddy et al. 2012). The development of these inhibitors has typically involved structure-based in silico drug design, high throughput biochemical assays using purified proteins, cellular biomarker assays (primarily AURKAThr288 phosphorylation and AURKB-mediated phosphorylation of its canonical substrate, histone H3), cellular proliferation/cytotoxicity assays, and xenograft models in mice. Inhibition of expression or activity of Aurora Kinases by AKIs has been shown to indeed suppress proliferation, invasion and migration in cancer cells (Libertini et al. 2010; Bavetsias & Linardopoulos 2015; de Groot, 2015).
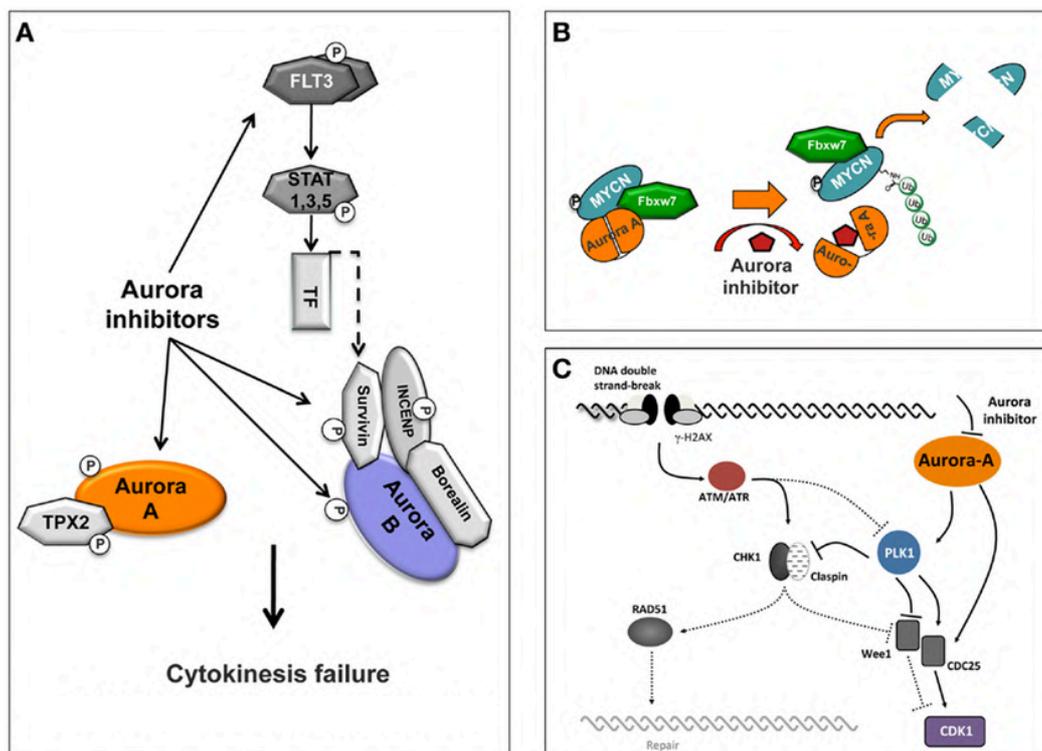
Figure 1.17. Schematic representation of Aurora inhibitor-targeted pathways in cancer. (A) Selective inhibition of AURKA or AUKRB or dual specificity Aurora/FLT3 compounds induces cytokinesis failure and inhibition of cancer cell proliferation. (B) Conformational changes of AURKA by an inhibitor causes disassembly of Aurora-A-FBXW7-MYCN complex leading to proteolytic degradation of MYCN. (C) Inhibition of AURKA known to be involved in DNA repair pathways bestows increased cellular sensitivity to cell death. (Adapted from (Bavetsias & Linardopoulos 2015))

Based on the current literature, MLN8237 (Alisertib) (Sells et al. 2015), one of the AURKA inhibitor and AZD1152 (Foote & Mortlock 2009), one of the AURKB inhibitor, are in phase III clinical trials and have attracted a lot of research attention (de Groot et al. 2015). Taken together, AKI have become promising therapeutic candidates in cancers, however the biggest challenge with these is the failure to distinguish the normal cells from cancer cells, thereby resulting in high toxicity, suggesting that targeting Aurora kinases is likely a double-edge sword. However, a lot of studies have reported the involvement of Aurora kinases in chemo- and radio-resistance in conventional therapy, and that, inhibition of Aurora kinases using AKIs somehow rescues this resistance. This makes cancer targeting through combination therapy an attractive possibility. However, a better understanding of the role of Aurora kinases in tumorigenesis may broaden our scope to invent/modify existing compounds and therapeutic strategies.

1.3 Small GTPase

Small GTPases are a large family of low molecular weight (20-40 kDa) G proteins that are defined by their basic biochemical activity of binding and hydrolysing GTP to GDP, a process called as guanosine triphosphate(GTP)/guanosine diphosphate (GDP) cycle (Figure 1.17) (Bourne et al. 1991). These proteins have been reported to respond to a wide variety of intracellular and extracellular cues to ultimately regulate cytoskeletal reorganisation, transcription, cell polarity, cell cycle progression, cytokinesis, membrane and protein cargo transport and many other significant events in cells, such as interaction with foreign particles (Manser 2002; X.-W. Chen et al. 2006). Even though the Small GTPases are very similar to heterotrimeric G protein α subunits in biochemistry and function, they are reported to function as monomeric G proteins (Wennerberg et al. 2005).

Small GTPases are often termed as molecular switches that are capable of turning ON and OFF several cellular processes depending on their GTP/GDP binding status. The regulation of these nucleotide binding states is tightly regulated by three elements: Guanine nucleotide Dissociation Inhibitors (GDIs), Guanine nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs) (Cherfils & Zeghouf 2013). Additionally, Small GTPases can also be regulated by post-translational modifications, like phosphorylation or ubiquitination, shown to be essential for protein stability and subcellular localization (Neyraud et al. 2012; Lim et al. 2009b; Martin et al. 2012).

The 'Active' form of Small GTPase is the GTP bound form and the GDP-bound form is the 'Inactive' form, both of which exhibit two distinct structural conformations, that have been shown to preferentially bind different regulators and effectors (Zheng & Quilliam 2003). GEFs bind to GDP bound state of Small GTPase and catalyses exchange of GDP for GTP and GAPs promote GTP hydrolysis by binding to GTP bound state. GDIs interact with small GTPases and prevent the exchange of GDP to GTP, additionally preventing the localization of Small GTPases at the membrane (Qu et al. 2019). The peculiar structural conformation of active GTP bound form enables interaction with multiple effectors such as kinases, phosphatases, transcription factors, scaffolding proteins that ultimately relay signals downstream into various signaling cascades (van Dam & Robinson 2006; Smith et al. 2007). The subcellular localization of the GTPase has been shown to play an important role in the relaying specific signals via specific effector interactions. For example, active Ras GTPase recruits RalGDS to plasma

membrane allowing its interaction with its substrate RalA known to localize at plasma membrane (Colicelli 2004). GAPs function to enhance the intrinsically low GTP hydrolysing activity of GTPase and thereby act as terminators of signals or initiators of new activation/inactivation cycle. Every cycle induces engagement of new effector molecules thereby amplifying the upstream signal. Both GEF and GAP proteins are in turn regulated by upstream stimuli and help determine what fraction of the total GTPase in cells is active at any given point (Manser 2002).
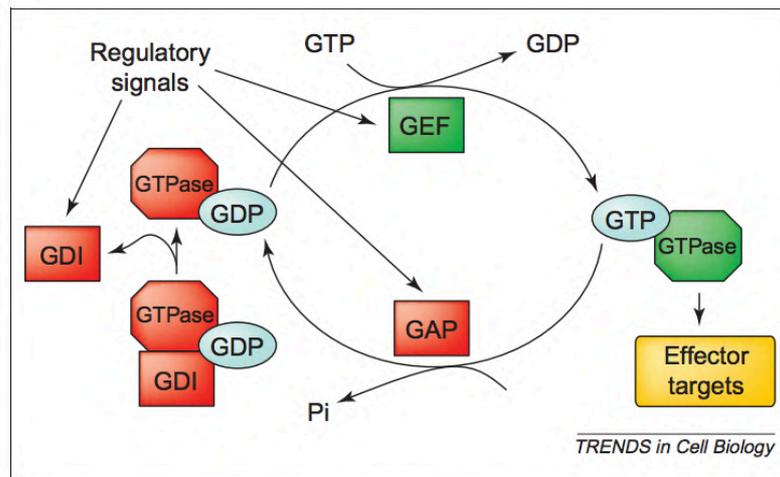
Figure 1.18 The GTPase cycle by GEFs/GAPs/GDIs. Most small GTPases cycle between inactive GDP bound or active GTP bound form. GEFs respond to upstream stimuli and induce exchange of GDP to GTP, activating the GTPase and allowing them to interact with so-called effector targets that ultimately produce a biological consequence. Like GEFs, GAPs can also respond to upstream stimuli, deactivating GTPase by assisting in GTP hydrolysis to terminate the signaling. (Adapted from (Bernards & Settleman 2004))

1.3.1. Classification of Small GTPases

Till date, 167 small GTPases have been identified in humans (W. N. Liu et al. 2017; Rojas et al. 2012). Depending on the sequence and functional similarity, the small GTPases have been divided into five main families, namely, Ras, Rho, Rab, ADP-ribosylation factor (Arf) and Ran (Wennerberg et al. 2005). Each of these families further have several subfamilies that share a subset of structural and functional features (Table 1.1). These proteins are found to be conserved across eukaryotes with orthologs 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

Table 1.1 Classification of Small GTPase. Families with subfamilies and the broad cellular functions they carry out– Ras, Rho, Arf, Rab and Ran. (Reproduced from (Kiyokawa et al. 2011))

1.3.2 Structural features of Small GTPases

All the proteins of small GTPase family have a 20kDa GTP-binding domain, called as the G-domain (Bourne et al. 1991). This domain is composed of five α helices (A1-A5), six β-strands (B1-B6) and five polypeptide loops (G1-G5). 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, Threonine35 and Glycine60 of Ras, make co-ordinate bonds with the γ-phosphate group of GTP and a magnesium ion to form the active site (Figure 1.19). 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).
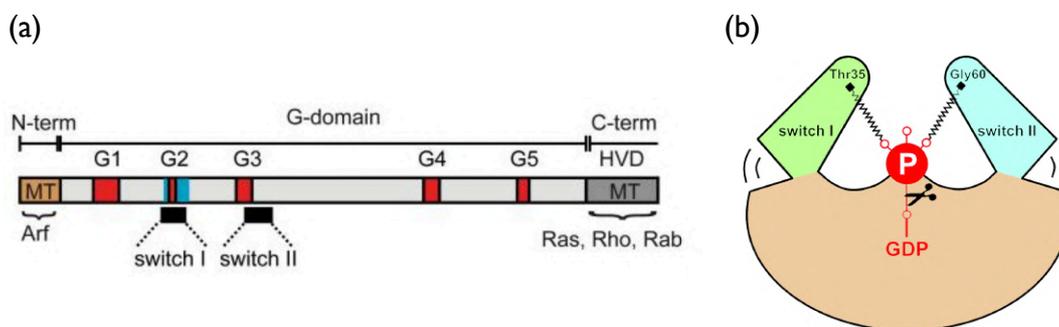

Figure 1.19 Structural features of the small GTPase. (a) Schematic depicting G box motifs G1-G5 in G domain. Membrane targeting (MT) domain located at C-terminus in the

hypervariable domain (HVD) for Ras, Rho and Rab family members and at N-terminus for Arf family members (Adapted from (Neely & Hidalgo 2014)) (b) Schematic for GTP-bound/GTP-loaded active spring conformation of Ras. The γ-phosphate of GTP (in red circle) interacts with residues from Switch I (Threonine35) and Switch II (Glycine60) and a Mg^{2+} in the active site (Adapted from (Vetter & Wittinghofer 2001)).

1.3.3 The Ras family of small GTPases

Ras proteins (H-Ras, K-Ras4A and 4B, and N-Ras) serve as crucial nodes in many signaling networks, that connect the diverse extracellular upstream stimuli to an even wider set of catalytically distinct downstream effectors, which regulate great assortment of cellular outcomes including regulating gene expression, cell cycle progression, migration, cytoskeletal changes, growth, apoptosis and senescence. Like all the small GTPases, Ras also undergoes a ON (Ras-GTP) and OFF (Ras-GDP) cycle, which is regulated by a definite set of Ras-GEFs and Ras-GAPs. At least three different protein families exhibiting GEF activity toward Ras, including, Sos, Ras-GRF and Ras-GRP (formerly denominated Cal-DAG GEF), have been identified in mammalian cells (Rojas et al. 2012). p120GAP, NF1 (neurofibromin) and GAP1 are the main, distinct, GAPs currently identified for Ras family proteins (Bernards & Settleman 2004).
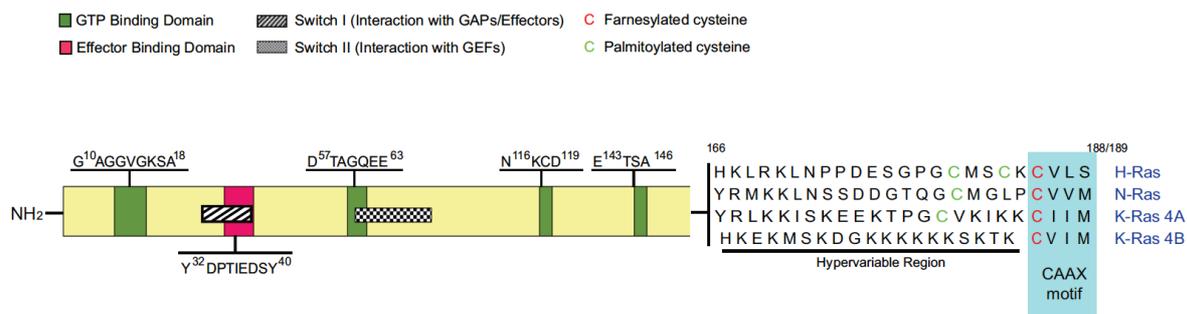
Figure 1.20. Structural features of Ras protiens. The structure of Ras proteins includes highly conserved domains that mediate binding and hydrolysis of guanine nucleotides, facilitate functional interaction with activators and effectors, and are responsible for attachment to membranes. The areas represented by the striped box (switch I) and the squared box (switch II) undergo conformational changes depending on Ras binding to GDP or GTP to facilitate effector binding. The unique residues of the CAAX motif in each Ras protein are subjected to diverse post-translational modifications that regulate their individual functions. (Reproduced from (Castellano & E. Santos 2011))

1.3.3.1 Ras effectors and signaling pathways

Ras functions in a cell are mediated through collaborative actions of multiple effectors, in that Ras has been known to act as a signaling node linking various extracellular stimuli to carry out diverse cellular functions via numerous effector signaling pathways.

Figure 1.21. Ras GTPase acting as a signaling node feeding various extracellular signals to intracellular effector pathways. (Adapted from Der, C. et al., 2006)

A protein is categorized as a Ras effector if it exhibits strong preferential binding to GTP-bound Ras and its binding is impaired by mutation in the core effector domain of Ras (Marshall 1996). Additionally, the function of the protein itself should be altered 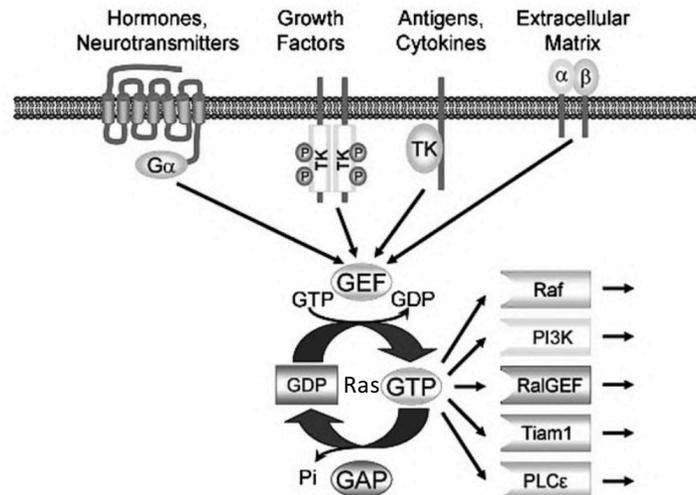by interaction with Ras, for example, change in intracellular localisation (recruitment), inherent catalytic activity (allosteric regulation), or binding and interaction with other signaling components (complex formation) (Castellano & E. Santos 2011). All the Ras effectors share common structural features including Ras-binding domains (RBDs) (characteristics of Raf and Tiam1 (T-lymphoma invasion and metastasis)), class IA p110 catalytic subunit of PI3Ks (PI3K-RBD), and the Ras-association domains (RA), all of which facilitate binding to Ras-GTPases (Nakhaeizadeh et al. 2016). Although these domains exhibit very little sequence identity, they share common tertiary structural motifs like ubiquitin fold-like protein conformations. However, not all proteins that contain RA domains act as Ras effectors, instead binding to Ras-related proteins (Malumbres & Barbacid 2003). In addition, one of the Ras effector called IMP (Impedes Mitogenic signal) does not exhibit any sequence homology with known Ras-interaction sequences (Matheny et al. 2004). Hence, although plethora of information exists

with respect to Ras effectors, as new signaling roles of Ras are getting uncovered, there is a continuous upgradation of proteins that can act as Ras effectors in various cell physiological and pathological conditions.

The best studies Ras-effector signaling pathway involves activation of Ras by epidermal growth factor tyrosine kinase receptor through the RasGEF SOS. GTP-bound Ras binds to and promotes translocation of a serine/threonine kinase Raf to the plasma membrane, where additional binding to scaffold proteins and phosphorylation events promote full activation of Raf kinase (Hancock 2003). This active Raf further catalysed the phosphorylation and activation of MEK1/2 dual specificity protein kinase, that further induces phosphorylation and activation of ERK1/2 mitogen-activated protein (MAP) kinase (Maurer et al. 2011). Activated ERK further translocates into nucleus, where it acts as transcription factor (TF) regulator by phosphorylating Ets-TF family members thereby activating Ets-responsive promoters (Sun et al. 2015).

The members of Ras superfamily are interconnected at various signaling nodes to regulate complex cellular functions. G-protein-coupled receptors (GPCR) and receptor tyrosine kinase (RTK) both can regulate Ras, Rap and Ral family members, leading to their activation in co-ordinated manner (Moore et al. 2020). Ras and Rap1 both are activated by increase in diacylglyceril (DAG), further leading to activation of related GEFs specific to Ras (Ras GRP) or Rap1 (CAlDAG-GEF1) (Colicelli 2004). Ras activation as previously discussed, leads to signaling through Raf/MEK/ERK pathway as well as through PI3-kinase and AKT pathway to activate anti-apoptotic protein Bad. GTP-bound Ras (active) has been shown to activate RalGDS and related GEFs to cause activation of Ral GTPases (Gentry et al. 2014). However, Ral GTPases have also been shown to be activated by calcium-dependent pathways (Clough et al. 2002), suggesting that Ras-independent pathways could also contribute and feed into the regulation of these GTPases. The RalGDS/Ral pathway further regulates Ral effector RalBP1, which is a GTPase activating protein for Rac1 and CDC42 small GTPases (Matsubara et al. 1997; Cantor et al. 1995).
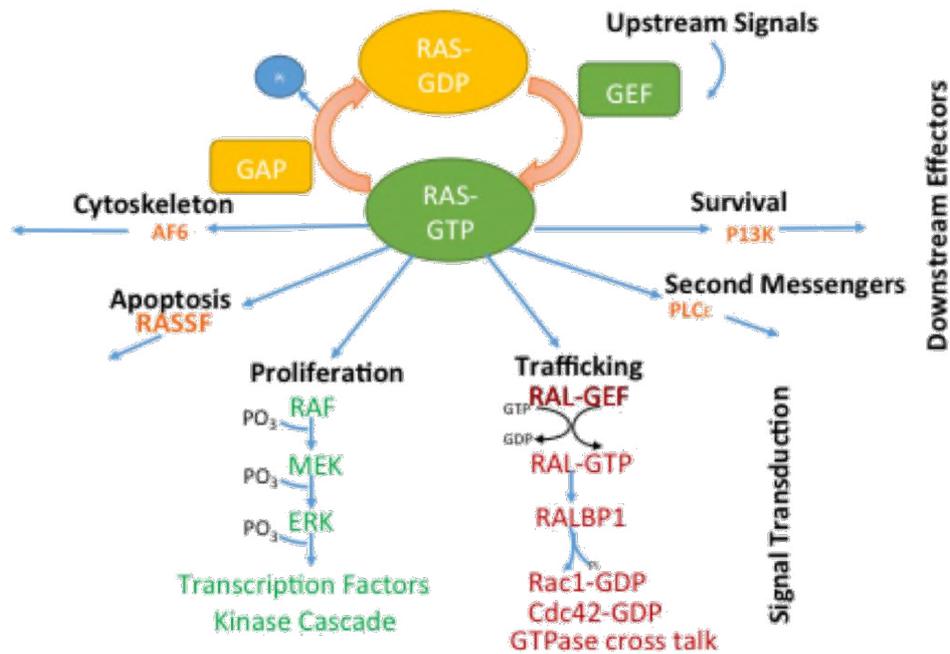
Figure 1.22. Downstream signaling pathways involving Ras effector interactions with the Ras-activated state (green circle) in processes such as cell proliferation (green extension) and trafficking (red extension), survival and cytoskeletal organisation. (Adapted from Chandrashekar and Adams, 2015)

1.3.3.2 Ral GTPases: downstream components of Ras signaling

The Ras-like subfamily of small GTPases, named Ral GTPases, was first identified through domain structure homology shared with Ras superfamily (van Dam & Robinson 2006; Chardin & Tavitian 1986). The two mammalian Ral isoforms, RalA and RalB are 80% identical in their protein sequence, but are differentially activated in response to variety of upstream stimuli and regulate distinct downstream pathways and cellular processes (Gentry et al. 2014). Both these isoforms however are regulated by a common set of GEFs and GAPs and bind to common effectors once activated. Some of the known Ral effectors include TF ZONAB, Filamin-A (actin modulator), phospholipaseD and phospholipase Cδ1 (phosphoinositide signaling component), cell secretory machinery exocyst complex and a multidomain protein RalBP1/RLIP76 (Bodemann & White 2008). Ral proteins have been reported to be key regulators in transcriptional regulation (C. Yan & Theodorescu 2018), cytokinesis (Cascone et al. 2008), exocytosis (X.-W. Chen et al. 2006), phagocytosis, apoptosis, autophagy (M. K. Singh et al. 2019) and mitochondrial fission dynamics (Kashatus et al. 2011a). The capability of Ral GTPases to regulate the exocyst complex and the multifunctional protein RalBP1 are central to their function.

Structural features and regulation of Ral GTPases

The basis of the functional distinction of Ral isoforms is proposed to be defined by their differential subcellular localization. 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). C-terminal tail switching between Ral isoforms, 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 Aurora kinase A (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).
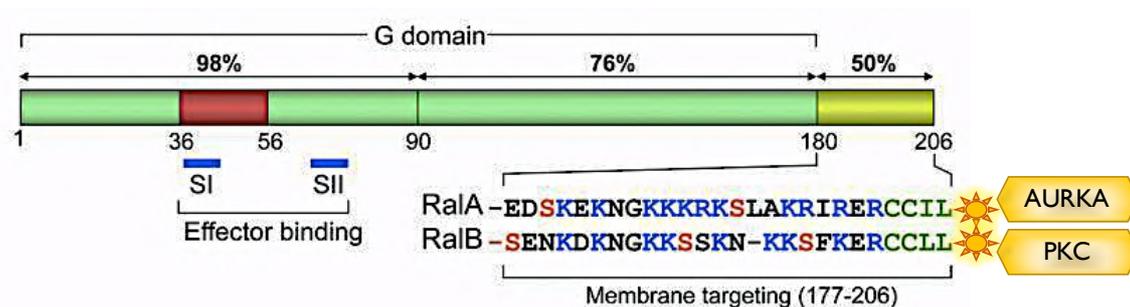
Figure 1.23. Schematic showing structural features if Ral GTPases. Amino acid sequence similarity between different regions of RalA and RalB with the C-terminal hypervariable region sequence elaborated to highlight differences (blue letters indicate basic residues and red letters are phosphorylation sites). AURKA and PKC phosphorylate RalA and RalB at Ser194 and Ser198 residues, respectively (Adapted from (Martin & Der 2012)).

Being small GTPases the Ral family members are also subjected to and regulated by a GTP/GDP cycle via a defined set of Ral-specific GEFs and GAPS. RalGEFs are divided in two groups depending on their ability to interact and be controlled by Ras GTPases as Ras-dependent GEFs and Ras-independent GEFs. 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. Ral GAPs have been shown to be 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 α1 or α2 respectively and a common βsubunit (Shirakawa et al., 2009).
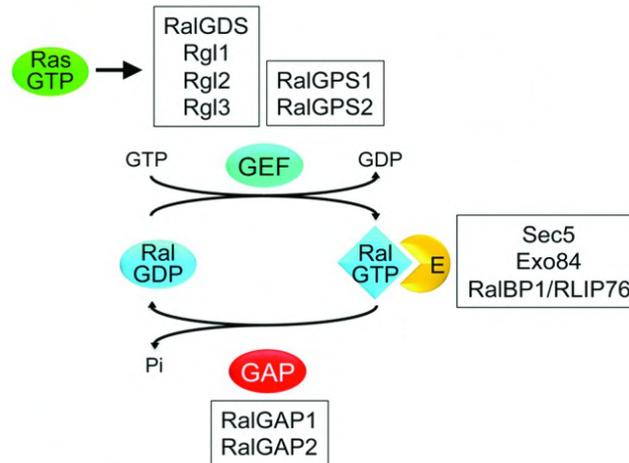
Figure 1.24. Ral GTP/GDP cycle and its regulators- Ras-dependent GEFs (RalGDS, RGL1, RGL2, RGL3), Ras independent GEFs (RalGPS1/2), RalGAP complexes (RalGAP1/2) and effectors Exo84, Sec5 and RalBP1. (Adapted from (Martin & Der 2012)).

Ral Effectors and signaling pathways

RalBP1 (RLIP6): RalBP1 is a multidomain, multifunctional protein that acts as an effector for RalA and RalB and as a GAP for Rho family GTPases. RalBP1 is an important signaling intermediate across multiple signaling pathways. RalA mediated mitochondrial fission via RalBP1 whereas RalB regulates invadopodia via RalBP1 interaction (Bodemann and White 2008). Additionally, active Ral GTPases have been shown to contribute to anchorage independent growth and invasion in colon and pancreatic cancers, a role dependent on their interaction with RalBP1.

SEC5/EXOcyst Complex 84: Exocyst complex is a conserved octameric complex that regulates polarized delivery of endosomal vesicles to specific plasma membrane sites. Previous studies have reported existence of two sub-complexes, one vesicle bound (Sec15, Sec10, Exo84) and other plasma membrane bound (Sec3, Sec5, Sec6, Sec8, Exo70). Ral GTPases have been shown to bind two important proteins of each of these sub-complexes, namely Sec5 (plasma membrane bound) and Exo84 (vesicle bound), facilitating fusion of these sub-complexes and delivery of vesicles (Camonis et al. 2005). Thus, Ral GTPases play an important

role in regulation of exocyst complex, thereby streamlining plethora of diverse cellular functions. The Ral GTPase regulated exocyst components has also been shown to play a significant role in various oncogenic pathways.

Role of RalA in integrin dependent membrane raft trafficking

As discussed in section 1.1.4, integrin-cell matrix adhesion plays an important role in regulating various membrane trafficking pathways involved in sorting and transportation of cargos to various intracellular compartments and *vice a versa* (Caswell et al. 2009). One such component that is subjected to integrin-cell matrix adhesion regulated trafficking are membrane raft micro-domains. Membrane rafts are dynamic cholesterol and sphingolipid enriched plasma membrane micro-domains that function as anchoring sites for proteins involved in growth factor signaling in plasma membrane (Caswell et al. 2009). Lipid raft micro-domains hence act as mediators of integrin-mediated adhesion and growth factor signaling crosstalk. Upon loss of cell-matrix adhesion, these rafts are internalized through caveolar endocytosis and held in recycling endosomal pool in non-adherent cells (Pelkmans et al. 2005). Upon re-adhesion, these rafts return back to plasma membrane, via RalA-Arf6-exocyst complex (Balasubramanian et al. 2010, Pawar et al. 2016). In absence of cell adhesion, internalisation of these signaling platforms from plasma membrane, switches off the anchorage-dependent growth signaling, thereby conferring anchorage-dependence in untransformed 'normal cells'. De-regulation of this pathway, either by downregulation of caveolin1 preventing endocytosis or activation of RalA-Arf6 promoting exocytosis, can hence drive anchorage-independent growth of cancer cells (Pawar et al. 2016).

RalA activity has been reported by integrin-mediated cell adhesion, where loss of adhesion shows a decrease in GTP-bound RalA which recovers back on re-adhesion. Further, this integrin-dependent RalA activation has been shown to regulate exocytosis of membrane rafts and confer anchorage-dependence to normal cells (Balasubramanian et al. 2010). Additionally, a fast cycling RalA mutant (RalA79L) induces AKT and ERK signaling in absence of cell-matrix adhesion, via regulating adhesion-independent raft micro-domain exocytosis (Balasubramanian et al. 2010). Taken together, this suggests that RalA plays an important role in maintaining anchorage-dependence of normal cells.
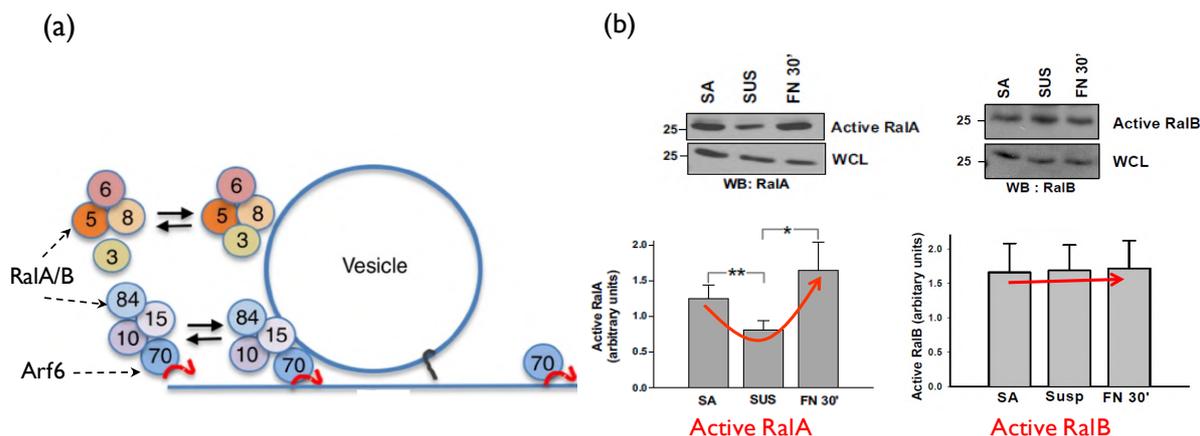
Figure 1.25. (a) Ral GTPases promote exocyst assembly through dual subunit interaction. RalA/B and Arf6 bind directly bind to their effectors Exo84 and Sec5 and this dual subunit interaction regulates exocyst function. Active Arf6 binds its effector Sec10 directly and Exo70 indirectly (Adapted from (Ahmed et al. 2018)) **(b) Regulation of Ral GTPases by integrin-mediated cell adhesion.** RalA but not RalB is regulated by integrin dependent adhesion to ECM. SA-Stable adherent cells, SUS-cells suspended/non-adherent, FN30' – Cells re-plated on fibronectin coated surface for 30mins. Graphs represent ratio of GTP-Ral to total Ral in whole cell lysate. (Adapted from (N. Balasubramanian et al. 2010))

1.3.4 Ras and Ral GTPases in cancers and as drug targets

The most prominent members and founders of Ras superfamily of small GTPases (H-Ras; Harvey-Ras, K-Ras; Kristen-Ras and N-Ras; Neuroblastoma-Ras) are defined proto-oncogenes and are among the most common drivers of cancer (Malumbres & Barbacid 2003). Ras mutations account for 20-30% of human tumours and have reported to show poor survival rates (Ryan & Corcoran 2018). Somatic mutations in one of the three Ras genes are most common events in tumorigenesis. Multiple studies over the years on different human tumours have identified 3 hotspots for Ras oncogenic mutation, located at Gly12, Gly13 and Gln61 in highly conserved coding sequences. These mutations are predominantly reported to impede the intrinsic and GAP-catalysed hydrolysis of GTP, resulting in accumulation of GTP-bound Ras, that in turn promotes pro-survival and proliferation signaling downstream (Fernández-Medarde & E. Santos 2011).

As discussed previously in section 1.3.3.1, mammalian cells exhibit atleast three major effector pathways that mediate Ras functions: Raf/MEK/ERK pathway, PI3K/AKT pathway and RalGEF-RalGTPase pathway (Marshall 1996; Hancock 2003). The discovery of Ras effector domain mutations, which can selectively engage distinct members of the effector family, has

facilitated the exploration of the relative contributions each of these effector pathways make to Ras functions in normal and cancer cells. These studies have emphasized the ability of Ral proteins to contribute to Ras-mediated transformation, however, the role of RalGEF-Ral signaling in Ras-driven tumours was initially overlooked as early studies in mouse fibroblasts found Raf but not PI3K or RalGEF-Ral pathways to be sufficient to mediate Ras-driven tumour formation (Urano et al. 1996). In 2002, the Counter group reported that, the RalGEF-Ral pathway, but not Raf or PI3K pathways, was important for Ras transformation of immortalized human cells (Hamad 2002). Further, it has been reported that RalA but not RalB is the effector protein that is important downstream of Ras, where it is required for anchorage-independent growth of cancer cells (Lim et al. 2005), whereas RalB was shown to be required for survival of human tumour cells (Chien & White 2003). Additionally, it has also been reported that Ral-Arf6 crosstalk plays an important role in regulating anchorage-independent signaling in H-Ras driven bladder cancers (Pawar et al. 2016). Subsequent studies have now established that, Ral GTPases play critical roles in both tumorigenesis and metastasis of diverse human cancers.

1.3.4.1 Role of Aurora Kinase A in Ras and Ral GTPase dependent cancers

Reiterating the contribution of Aurora kinase activity in cancers through regulating/feeding into oncogenic Ras signaling pathway that has been discussed in Section 1.2.5.2. AURKA has been reported to modulate the oncogenic Ras-RalGEF-Ral signaling cascade, however the precise mechanism by which this can occur still needs to be understood. In addition to the mechanisms discussed in section 1.2.5.2., recent reports have shown that, co-expression of AURKA along with H-Ras has been shown to increase the anchorage-independent potential of cells (Tatsuka et al. 2004a; Pérez de Castro et al. 2011). Further, the kinase domain of AURKA interacts with N-terminal domain of H-Ras and exists in a complex with Raf-1, which enhances the MAPK signaling. This functional link between AURKA and H-Ras/Raf1 protein complex provides a direct mechanism for AURKA's oncogenic activity through Ras/MAPK pathway (Umstead et al. 2017). AURKA expression has also been shown to be positively regulated in K-Ras positive H358 and A549 cell lines, and treatment with dual AURKA/AURKB inhibitor, reduced growth, viability, transformation, proliferation and induced apoptosis. Additionally, dual inhibition of AURKA and AURKB decreased growth, viability, transformation, and induced apoptosis in vitro in an oncogenic K-Ras-dependent manner, indicating that Aurora kinase inhibition therapy can specifically target K-Ras-transformed cells. AURKA mediated phosphorylation of RalA at Ser194 has also been reported to be essential in anchorage-independent growth and transwell migration in MDCK cells, abrogation of ser194 residue to a

non-phosphorylated form reverses the tumorigenic potential of these cells (Wu et al. 2005b). However, Neel et al., reported that the response to AURKA inhibition in PDAC cells is independent of RalA phosphorylation status (Neel et al. 2014), suggesting that further investigation needs to be carried out to understand the role of AURKA in Ras-Ral dependent cancers.
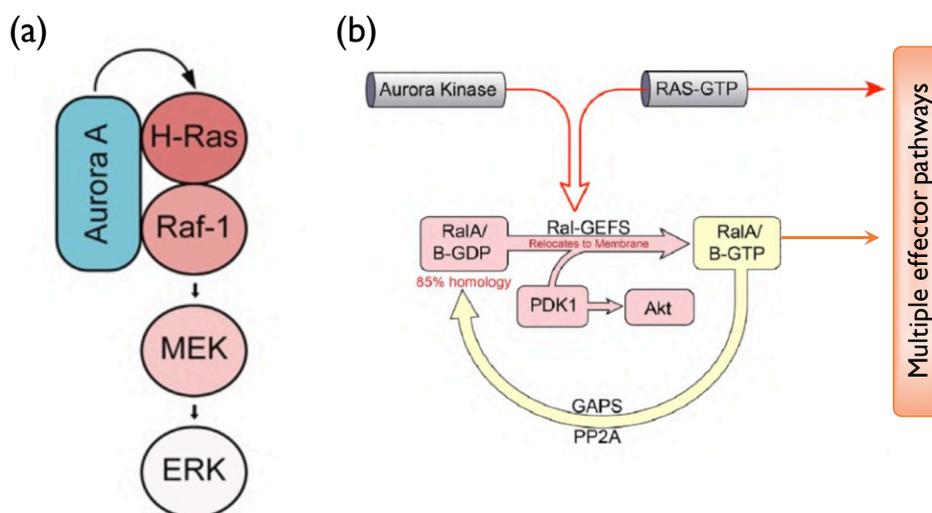

Figure 1.26. Role of AURKA in RAS-RAL regulation. (a) Aurora A forms a complex with H-Ras and Raf-1, acting through H-Ras to enhance ERK activation (Adapted from (Umstead et al. 2017)). (b) AURKA phosphorylates RalA and RalGEF RalGDS to translocate and activate it (Adapted from (Moghadam et al. 2017)).

1.3.4.2 Strategies for targeting Ras and Ral GTPases

The direct targeting of small GTPAase like Ras and Ral, represents a very challenging problem involving complex feedback mechanisms and signaling cascades which are not fully understood. Additionally, developing a compound that has the capability of discrimination between the various isoforms and close family members of active small GTPases is inherently difficult. Recently a comprehensive review describing strategies to directly targeting small GTPases has highlighted few examples of stabilizing the GTPase in GDP bound state (Figure 1.27) (Cromm et al. 2015a). Further, a small molecule inhibitor BQU57 has been developed against Ral GTPases but it lacks the distinction between RalA vs RalB (C. Yan et al. 2014). Most of the approaches to target small GTPases come with their own set of cons and hence various studies have now focused their attention towards co-targeting various members of a signaling cascade to achieve desirable effect. AURKA, due to its immense potential as anti-cancer target has attracted the synthesis and development of multiple inhibitors which are now

in different phases of clinical trials (described in detail in section 1.2.5.3). This makes co-targeting AURKA with Ras-Ral much more attractive approach to pursue.

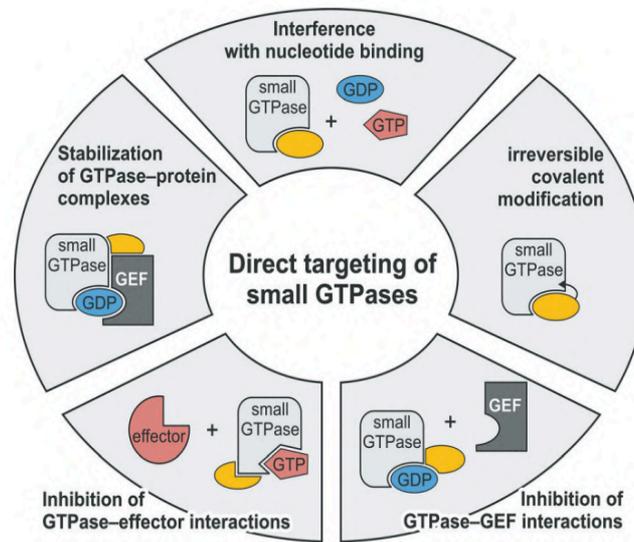**Figure 1.27. Strategies for targeting small GTPases** There are five different strategies for directly targeting small GTPases. (1) Interfere with binding of nucleotide; (2) inactivation of GTPase by irreversible covalent modification; (3) inhibition of GTPase–GEF interactions; (4) inhibition of GTPase–effector interactions; and (5) stabilization of GTPase–protein complexes. (Reproduced from (Cromm et al. 2015)).

1.4 Hypothesis and objectives of the thesis

Hypothesis: Can cell-matrix adhesion regulate activity of Aurora Kinases? How does this contribute to the differential activation of RalA (but not RalB) downstream of cell-matrix adhesion to regulate anchorage-dependence? If and how this pathway contributes to oncogenic-Ras mediated anchorage-independence?

This thesis tests the role cell-matrix adhesion has in regulating Aurora kinase A and Aurora kinase B activity and function in mouse embryonic fibroblasts. It further evaluates the significance Aurora Kinase A-RalA crosstalk has in regulating anchorage-dependence in normal fibroblasts and its deregulation in anchorage-independent cancers. It also aims to test the role this pathway has downstream of oncogenic Ras in cancers. **Our hypothesis is built on three major observations from literature. First**, integrin-dependent cell-matrix adhesion has been shown to regulate cell cycle progression by controlling mitotic rounding at the G2-M phase of cell cycle. Aurora Kinases are important cell cycle regulators that have been shown to respond to multiple cues at the onset of G2-M phase of cell cycle. The role cell-matrix adhesion could have on the regulation of these Aurora kinases is however largely unexplored. **Second**, AURKA is known to phosphorylate and regulate RalA during mitosis. RalA activity in turn is known to be regulated by cell-matrix adhesion. This makes it particularly relevant to ask if AURKA regulates RalA downstream of integrin-mediated cell adhesion. **Third**, knowing the existing overlap between adhesion-dependent and Ras-dependent pathways, the reported synergy between AURKA and Ras in enhancing tumorigenic potential is noteworthy. Whether the AURKA-RalA crosstalk exists in Ras-dependent and Ras-independent cancers and what relative role it might have in regulating anchorage-independence in these cancers is worth evaluating. The following objectives were hence designed to…

I. Study the role of Aurora Kinase A in regulating adhesion-dependent RalA activation

II. Evaluate a self-assembling Dextran nano-vesicle to efficiently deliver Aurora Kinase A inhibitor MLN8237 and specifically target AURKA-RalA crosstalk

III. Evaluate the significance of AURKA-RalA crosstalk in anchorage-independent growth of Ras-independent vs Ras-dependent cancers.

IV. Study the role of adhesion-growth factor crosstalk in regulation of Aurora Kinase activation and signaling

Chapter 2
Materials and Methodology

2.1 MATERIALS

2.1.1 Reagents

Human plasma fibronectin (Cat#F2006), nocodazole (Cat # M1404), DMSO (Cat # D2438), DAPI (Cat# D9542), Propidium iodide (Cat# P4170), Cesium chloride (Cat# C3032), sodium orthovanadate (Cat# S6508), sparfloxacin (Cat# 56968) and thiazolyl blue tetrazolium bromide (MTT, Cat# M2128) were purchased from Sigma, and Phalloidin-Alexa-488 (Cat# A12379), Phalloidin-Alexa-633 (Cat # A22284) was from Molecular Probes (Invitrogen). Fluoromount-G used to mount cells for imaging was obtained from Southern Biotech (Cat# 0100-01). Glutathione sepharose beads used for the Ral activity assay were from GE (Cat# 17075601). Crystal violet used for staining colonies in the AIG assay was from Amresco (Cat# 0528). Collagen for doing 3D uptake studies was purchased from Corning (Cat# 354236). Dextran (6000 M_W), dicyclohexylcarbodiimide (DCC), dimethylamino pyridine (DMAP), ethylchloroacetate, polyethylene glycol (PEG) (4600 M_W), were purchased from Sigma-Aldrich. Horse liver esterase was purchased from Sigma (Cat# 46069) and used as such. Alisertib (MLN8237) was purchased from Selleckchem (Cat# S1133). BQU57 (Ral inhibitor Cat# SML-1268), AZD1152 (AURKB inhibitor Cat# SML0268) was purchased from Sigma. N, N dimethyl formamide (DMF) and all of the necessary solvents were purchased from Spectrochem Laboratories. Carboxylic acid-substituted 3-pentadecylphenol (PDP-acid) was synthesized following our earlier report(Pramod et al. 2012). BCA protein estimation kit (Cat# 23227) was purchased from Thermo scientific. Nonidet P40 Substitute (Cat# 68387-90-6) and RNAase-A (Cat# 9001-99-4) was purchased from USB corporation. Trizol (Cat# 15596018) was purchased from Ambion. Immobilon western blot substrate (Cat# WBKLS0500) was purchased from Millipore.

2.1.2 Antibodies

Antibodies used for western blotting include anti-phospho-aurora A (Thr288)/aurora B (Thr232)/ aurora C (Thr198) (Cat #2914), anti-AURKB (Cat #3094), anti-phospho AKT-Ser473 (Cat# 9271) (1:2000 dilution), anti-phospho-FAK-Tyr397 (Cat# 3283), anti-phospho-p44/p42 ERK1/2 (Thr202/Tyr204) (Cat# 4370) (1:2000 dilution), anti-p44/p42 ERK1/2 (Cat# 4695) (1:2000 dilution), anti-FAK (Cat#3285), anti-AKT (Cat# 4691) antibodies were purchased from Cell Signaling Technologies and used at 1:1000 dilution unless mentioned

otherwise. Anti-AURKA (Cat #610939) and anti-RalA (Cat# 610221, clone 8) were purchased from BD Transduction Laboratories used at 1:1000 dilution. Anti-Phospho-RalA (Ser194) (Cat# 07-2119) was purchased from Millipore and Anti-RalB was from R&D Laboratories (Cat# AF3204) both used at 1:1000 dilution. Anti-beta actin (Cat# Ab3280) antibody was purchased from Abcam used at 1:2000 dilution. Secondary antibodies conjugated with HRP were purchased from Jackson Immunoresearch and were used at a dilution of 1:10000.

Antibodies used for immunofluorescence include Anti-myc (Cat# sc-789) obtained from Dr. Sanjeev Galande's Lab (IISER, Pune) used at dilution of 1:200 and anti-phospho-p44/p42 ERK1/2 (Thr202/Tyr204) (Cat# 4370) from Cell signaling technologies used at 1:200 dilution. Secondary antibodies with Alexa conjugate (488 or 594) were purchased from Invitrogen Molecular Probes (Cat. No. # A12379 and A12381) and were used at a dilution of 1:1000.

2.1.3 Plasmids and Primers

pRK5-Myc-WT-RGL1 was kind gift from Dr. Michael White's lab. WT human RalA construct was a gift from the Theodorescu Lab. Myc-RGL1* (siRNA insensitive mutant) was made by site-directed mutagenesis of Myc-RGL1. Primers were designed using QuikChange tool (Agilent Technologies) with sequences as follows:
Primer1
5'AATGAAGACACCTGCATAATCCG**G**AT**A**AG**C**GTGGA**G**GA**T**AATAACGGCAACATGTACAAGAG3'
Primer 2
5'CTCTTGTACATGTTGCCGTTATTATCCTCCACGCTTATCCGGATTATGCAGGTGTCTTCATT 3'

All constructs were sequenced to confirm their identity before being used in our studies. RNA interference sequences used for RGL1 knockdown studies were procured from Sigma or as On-Target Plus smartpools from Dharmacon and are as listed below.

Dharmacon smart pool mRgl1 (Cat No # L-059274-01),
1) GAGCCAGAGUCAUCGAGAA,
2) GAUCAACAUUGCUCACGAA,
3) CCUGGACAGCAGCGUGAAA,
4) ACGCAUAUCGUGUGUGUAU,

Individual oligo from sigma

mRGL1: 5'- GCAUCAGUGUAGAAGACAA-3' (designed by Ambion) (ID s72932)

Primers used for RT-PCR to detect RGL1 KD efficiency (forward and reverse) were designed using Primer 3 software. Primers were selected for use based on the size of the transcript, their GC content and Tm. Listing below shows the forward and reverse primer sequences (5' to 3').

mActin - CTCCTAGCACCATGAAGATC, GACTCATCGTACTCCTGCTT

mRGL1- CAGCAGAATTCACGAACTTC, TATCCCGCTGAGACCAAATA

Two primer sets used for RT-PCR to detect myc-RGL1* expression efficiency designed using IDT primer quest spanning the *myc-BamH1–RGL1* start site unique to the siRNA resistant myc-hRGL1*. Listing below shows the forward and reverse primer sequences (5' to 3').

Primer Set A -GGACCTGGGATCCAGCTCGATT, CAGCCTGTTGAATCTGGACTCTTT

Primer Set B - CCTGGGATCCAGCTCGATT, CCTAGCCATCTTGCTCCTTTATTG

2.2 Methods commonly used throughout the study

2.2.1 Cell culture and transfections

Wild-type Mouse embryonic fibroblasts (WT-MEFs) obtained from Dr. Richard Anderson (University of Texas Health Sciences Centre, Dallas TX), MCF-7 cells obtained from Dr. Amit Dutt (ACTREC, Mumbai), T24, UMUC3, HT1080, A549, Calu1, SKOV3, CFPAC-1, MDA-MB-231 and U87MG obtained from ATCC or ECACC were cultured in DMEM (Cat# 11995-065) supplemented with 5% (v/v) fetal bovine serum (FBS) (Cat# 26140-079) and 1% (v/v) penicillin-streptomycin (Cat# 15140-122) (all from Invitrogen) at 37°C under 5% CO_2 humidified atmosphere. MIA-PaCa-2 and DLD1 were cultured in RPMI1640 and SW620 in L15 media supplemented with 5% FBS and 1% PS at 37°C. Cells were treated regularly with an antimycoplasma agent sparfloxacin from Sigma (Cat# 56968) and, when needed, with mycozap from Lonza (Cat# VZA-2011) to keep them mycoplasma-free. Cells were detached using 0.05% trypsin (Cat# 25300-062) and seeded in 60 mm dishes (Eppendorf) for all transfection and inhibition assays and in 6-well plates (Eppendorf) for uptake studies unless mentioned otherwise.

For siRNA mediated knockdowns, WT-MEFs seeded in 60 mm dishes were transfected using the RNAiMax transfection reagent (Invitrogen) with 25pmoles siRNA smart pool oligo (Dharmacon) or 100pmols individual siRNA oligo (Sigma). These transfections were repeated after 24 hours and cells were used 48 hours after the second transfection. For expression of human RalA, 2×10^5 WT-MEFs seeded in 60 mm dishes were transfected with 4μg plasmid using PEI 1mg/ml (Sigma) and used 48hours post transfection.

2.2.2 Suspension assay

Cells were serum starved (0.2% FBS) for at least 12 hours, detached with 1X trypsin-EDTA, which was diluted with low serum medium and cells were held in suspension for 30 or 90 minutes with 1% methylcellulose in low serum DMEM. Post incubation for respective time points cells were carefully washed twice with 0.2% FBS DMEM at 4°C to avoid clumping and collected.
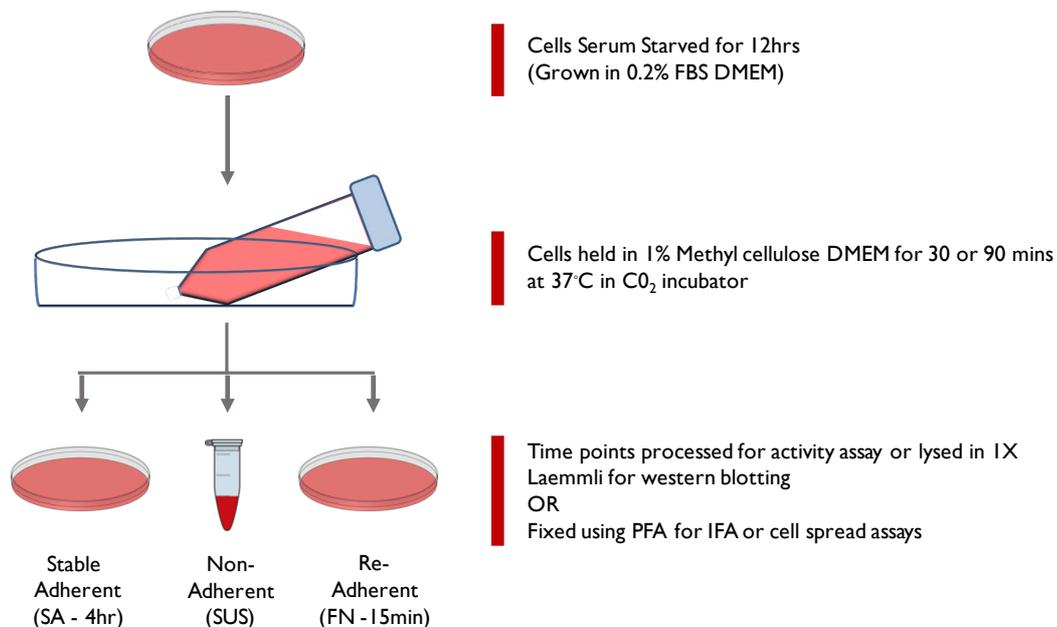

Cells Serum Starved for 12hrs
(Grown in 0.2% FBS DMEM)

Cells held in 1% Methyl cellulose DMEM for 30 or 90 mins at 37°C in CO_2 incubator

Time points processed for activity assay or lysed in 1X Laemmli for western blotting
OR
Fixed using PFA for IFA or cell spread assays

Stable Adherent (SA - 4hr)   Non-Adherent (SUS)   Re-Adherent (FN -15min)

Figure 2.1 Schematic of the suspension assay. WT-MEFS cultured in 5% FBS DMEM in 100 mm dishes to ~ 65 % confluency were incubated with medium containing 0.2% FBS (serum starved) for 12 hours detached using Trypsin-EDTA, washed with 0.2% FBS containing DMEM. These are then held in suspension with 1% methylcellulose containing low serum DMEM (0.2% FBS) for the required time. serum DMEM. Post incubation for respective time points cells were washed twice with 0.2% FBS DMEM and collected. These were re-plated on dishes or coverslips coated overnight with fibronectin at 4°C for 15minutes or 4 hours. When needed for western blotting these cells were lysed in required volume of 1X laemmli, heated at 95°C for 5mins and stored at -80°C. For confocal microscopy cells were fixed with 3.5% paraformaldehyde (PFA) for 15 mins at room temperature (RT), washed with PBS thrice, stained and mounted. *Schematic credit: Archana Pawar.*

These washed cells were re-plated on dishes or coverslips coated overnight with fibronectin at 4°C (2μg/ml or 10μg/ml as indicated in figure legends) for 15minutes or 4 hours. When needed for western blotting these cells were lysed in required volume of 1X laemmli, heated at 95°C for 5mins and stored at -80°C. For confocal microscopy cells were fixed with 3.5% paraformaldehyde (PFA) for 15 mins at room temperature (RT), washed with PBS thrice, stained and mounted.

2.2.3 Cell cycle analysis by flow cytometry

For evaluating adhesion-dependent cell cycle profile, WT-MEFs grown in either 0.2% FBS DMEM or 10% FBS DMEM were detached using trypsin and held in suspension for 30 or 90 minutes. Post suspension cells were carefully washed, collected and divided into three equal proportions: one processed immediately as suspension time point (SUS), one re-plated on 10μg/ml fibronectin coated dishes for 15minutes (FN 15') and last re-plated on 10μg/ml fibronectin coated dishes for 4 hours (SA). These cells were washed twice with PBS and fixed using chilled 70% ethanol and stored at 4^oC till further use (not more than 18hours). For evaluating effect of AURKA inhibition using V_{MLN} (dextran nano-vesicle encapsulated MLN8237) on cell cycle profile, 3×10^5 MCF-7 and SKOV3 cells were seeded in 100mm dish, followed by 48 hours incubation with 0.02μM MLN8237 or V_{MLN} along with volume equivalent DMSO and empty dextran scaffold as respective solvent controls. Post 48 hours cells were washed twice with PBS and fixed using chilled 70% ethanol and stored at 4^oC till further use (not more than 18hours). On the day of flow cytometer measurement cells were treated with 100μg/ml RNAse A and labelled with 10μg/ml of propidium iodide. DNA content was analysed for cell cycle status in a FACScan flow cytometer BD Calibur for adhesion-dependent experiments and in BD LSRFortessa SORP cell analyzer for AURKA inhibition experiments. 10000 events were recorded for each treatment and time point to obtain percentage of cells in different phases of cell cycle. The cell cycle profiles were calculated by using ModFit software that gives percentages of cells in G0- G1, S and G2-M phase and were compared across different treatments and time points. This method was used to evaluate the cell cycle profile of WT-MEFs that are stable adherent, held in suspension for 30 or 90 minutes and re-adherent on fibronectin for 15 minutes in presence and absence of serum growth factors. It was also used to evaluate the effect AURKA inhibition using free drug vs encapsulated drug has on cell cycle profile.

2.2.4 Ral activity assay

For adhesion-dependent Ral activity, serum starved WT-MEFs were detached, counted using hemocytometer and 0.6 million cells per time point (1.8 million for three time points) were held in suspension as described in 2.2.1. Post suspension, cells at their respective time points were frozen at -80°C (not more than 24 hours). To determine Ral activity post MLN8237/BQU57 treatment, cells were treated with 0.02 μM of free inhibitor (MLN) or nanovesicle-encapsulated inhibitor (V_{MLN}) or BQU57 (5 μM) for 48 h. Following the above treatments, cells were frozen at at -80°C (not more than 24 hours). The frozen cells were revived together on ice and were then lysed with Ral activity assay buffer (50mM Tris pH 7.4, 150mM NaCl, 10mM MgCl$_2$, 0.1% NP-40, 1mM NaF, 0.1mM Na-orthovanadate and 1X PIC). Cell scrapper was used to scrap out and lyse the re-plated time point cells from the dishes in 1ml Ral activity assay buffer. 180 μl of lysate was added to 45 μl of 5X laemmli buffer to make whole cell lysate (WCL). 400 μl of lysate were incubated with 60 μg GST-Sec5-RBD bound to Glutathione Sepharose beads for 35 minutes at 4°C on a rotary mixer. Post incubation beads were washed thrice with activity assay buffer at 4°C and eluted with 20 μl of 2X laemmli buffer to make pulled down lysate (Sec5-PD).
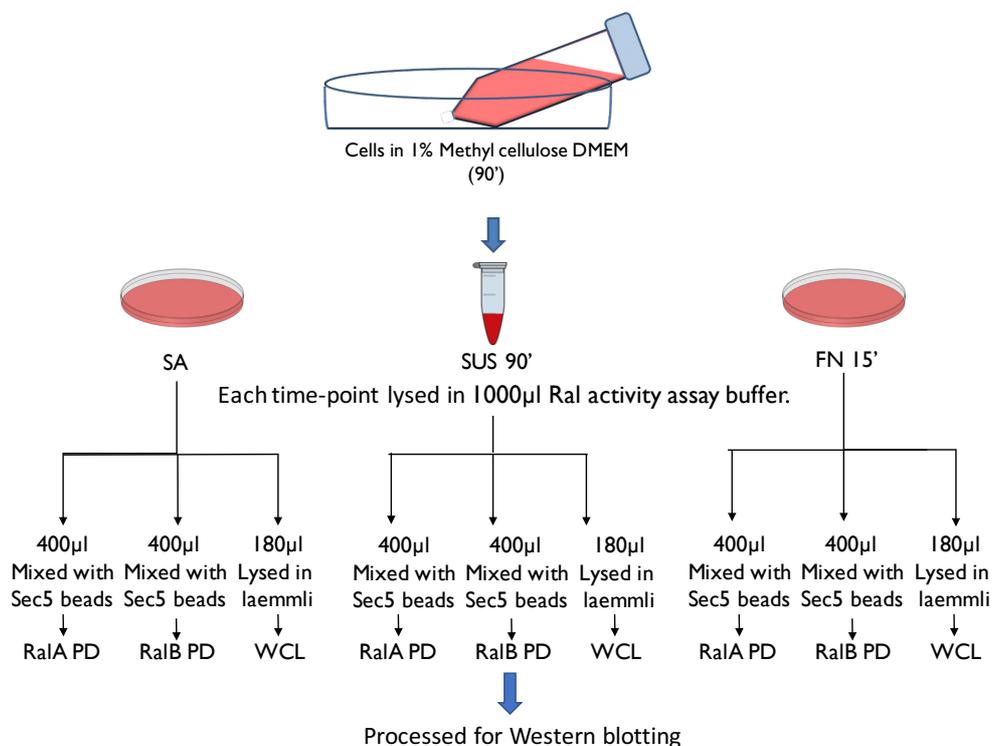

Figure 2.2. Schematic of Ral activity assay. WT-MEFs suspended in Methylcellulose for

90mins or re-plated on fibronectin for 15mins or 4 hours post suspension were collected and lysed in 1000μl of Ral activity assay buffer. 800μl of this lysate was divided into two equal parts, one used for RalA and remaining for RalB pull down. 400μl of lysates was incubated with 60μg GST-Sec5-RBD bound to Glutathione Sepharose beads for 35 minutes at 4°C on a rotary mixer. Post incubation beads were washed thrice with activity assay buffer at 4°C and eluted with 20 μl of 2X laemmli buffer to make pulled down lysate (Sec5-PD). Remaining 180μl was lysed with laemmli buffer for whole cell lysate. These lysates where then subjected to western blotting.

30 μl of WCL and all of the Sec5-PD lysate were resolved by 12.5% SDS-PAGE and transferred to PVDF membrane (Millipore). Blots were blocked with 5% milk in 0.1% Tween-20 containing Tris-buffered saline (TBST) for 1 hour at room temperature and incubated with anti-RalA or anti-RalB antibody diluted in 5% BSA or 2.5% milk respectively at 4°C overnight. Blots were then washed thrice with TBST and incubated with anti-mouse HRP and anti-goat HRP for RalA and RalB respectively for an hour, followed by detection of RalA and RalB using chemiluminescent substrates from Pierce and Millipore. LAS4000 (Fujufilm-GE) was used to image the blots and densitrometric band analysis was done using Image-J software (NIH). To determine the Percentage active RalA and RalB following calculation was used:

$$\text{Percentage activity} = \frac{\text{Pulldown Band Intensity} \times 100}{\text{Corresponding WCL Band Intensity} \times \text{Dilution factor}}$$

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 resolved by SDS PAGE in the whole cell lysate (WCL) lane (24 μl WCL + 6 μL 5X Lamelli buffer). The dilution factor was hence $400 \div 24 = 16.66$. This ratio was kept constant in all experiments. Active RalA and Active RalB levels under different treatment conditions were normalized to Stable adherent (SA) or control (CON). The calculation has also been used in previous studies from lab {Pawar:2016ju}.

2.2.5 Cell spread assay

WT-MEFs were serum starved with 0.2% FBS containing DMEM (low serum DMEM) for 12 hours, detached, held in suspension for 90 minutes and replated on fibronectin (2 μg/ml) as described in 2.2.1. For AURKA inhibition experiments, serum starved WTMEFs were detached, held in suspension for 60 mins followed by 30mins incubation with Empty DEX (CON) or V_{MLN} (0.2 μM) and re-plated on fibronectin (2 μg/ml) with or without inhibitor. Re-

adherent cells were fixed with 3.5% paraformaldehyde after 15 minutes of re-plating, stained with Phalloidin-Alexa-488/Phalloidin-Alexa-594, mounted and imaged using a Zeiss LSM 710 laser confocal-Anisotropy or LSM780 multiphoton microscope with a 40x objective. Images were analyzed using the Image J software (NIH), thresholded to define the cell edge to create a mask and the cell spread area inside the mask measured. Values obtained from at least 100 cells per treatment in an experiment were analysed, collated and compared between treatments.

2.2.6 Quantitative RT PCR to determine knockdown efficiency

Total RNA was isolated from cells using Trizol reagent followed by cDNA preparation using Reverse Transcriptase and Oligo-dT primers (BioRad iScript Kit). Quantitative Real Time PCR reactions were set up using SYBR FAST qPCR master mix reagent from Kapa Biosystems (Catalog No KK4601) using the BioRad CFX96 Real-Time System. The list of specific primers was used for measuring transcript levels in qPCR are mentioned above in section 2.1.3. Their optimal Tm was determined using a gradient PCR. For determining percentage knockdown of the target gene in siRNA-treated cells (Target KD) relative to treatment control (CON) cells, actin was used as reference gene. $\Delta\Delta$Ct was calculated as follows:

$$\Delta Ct\ (CON) = Ct_{\text{Target}}\ of\ CON - Ct_{\text{Actin}}\ of\ CON$$
$$\Delta Ct\ (TARGET\ KD) = Ct_{\text{Target}}\ of\ Target\ KD - Ct_{\text{Actin}}\ of\ Target\ KD$$
$$\Delta\Delta Ct = \Delta Ct\ (TARGET\ KD) - \Delta Ct\ (CON)$$

Fold change was determined by calculating $2^{(-\Delta\Delta Ct)}$. Fold change was then converted to percentage knockdown as follows, Percentage knockdown = 100 - (100 × fold change).

2.2.7 Immunofluorescence assay

WT-MEFs were transfected with 4µg of myc-RGL1 using Lipofectamine 2000 reagent. Post 48 hours of transfection cells were serum starved with 0.2% FBS containing DMEM (low serum DMEM) for 12 hours, detached, held in suspension for 90mins and re-plated on fibronectin (2µg/ml) as described earlier. Re-adherent cells were fixed with 3.5% paraformaldehyde after 15minutes of re-plating. 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 1:200 rabbit anti-myc (Santa Cruz Biotechnology), 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-568 and anti-rabbit-Alexa-488) or 1:500 diluted phalloidin-Alexa 633 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 or LSM780 multiphoton microscope with a 63x objective.

2.2.8 Wound healing migration assay

siRNA mediated knockdowns (smartpool or individual) in WT-MEFs were done as described earlier in the presence of 5%FBS. 24 hours post second siRNA shot, cells were detached with 1X trypsin and 0.2 million cells were plated in duplicate wells of a 24-well plate for each treatment in the presence of serum. 15 hours post plating; cells were incubated with 10 ug/ml Mitomycin C for 2 hours at 37°C in 5% CO2. V_{MLN} (0.2μM) or volume equivalent Empty DEX(CON) were added to media in last 1hour of Mitomycin C treatment before a linear wound scratch was made in the centre of the well using a 10ul tip. 0.06μM of V_{MLN} was maintained throughout the assay in the treated wells. At least 3 regions of interest (ROI) were identified for each scratch per well and they were imaged every 2 hours from 0 to 24 hours using the "time lapse" feature of the EVOS FL auto cell imaging system from Life Technologies. Images captured were opened using TScratch software and wound area values calculated for each ROI at each time-point. Values for open wound area obtained from TScratch analysis for each of the four ROIs were used to calculate average wound area for each time-point. This was then compared to average wound area at 0 hours and percentage closed wound area per time-point was determined as shown below.

$$\text{Percentage closed wound area at "X" hours time-point} = 100 - \left[\frac{\text{Average wound area at "X" hours}}{\text{Average wound area at 0 hours}} \times 100 \right]$$

2.2.9 Preparation of MLN8237-Loaded Polysaccharide Vesicles

Amphiphilic dextran (DEX-PDP) was synthesized as reported earlier (Pramod et al. 2012) by chemically conjugating the hydrophobic unit of carboxylic acid-substituted 3-pentadecylphenol (PDP) on the hydrophilic dextran backbone (Mw= 6,000) by an enzyme-responsive aliphatic ester chemical linkage. The degree of substitution of PDP in dextran was kept at 5%. To encapsulate MLN8237 in the polysaccharide (dextran) vesicles, 20 mg of DEX-PDP and 0.2 mg of MLN8237 were dissolved in 2 mL of DMSO, and then 2 mL of Milli-Q water was added. The resulting solution was stirred for 12hours in the dark and dialyzed (MWCO = 3500) against Milli-Q water for 48hours to remove any unencapsulated drug. The resulting solution was filtered, lyophilized, and stored at 4°C. This powder was reconstituted for use as needed. The drug loading content (DLC) and drug loading efficiencies (DLE) were determined by dissolving a known amount of a lyophilized drug loaded sample in methanol and estimating its drug content by absorption spectroscopy. For this purpose, the molar extinction coefficient of MLN8237 was determined as 76500 L mol−1 cm−1 in methanol. The DLC and DLE were determined as 0.40% and 56%, respectively.

In Vitro Drug Release Studies for MLN8237.

MLN8237-loaded vesicles (V_{MLN}, 3.0 mg) or MLN8237 and Rh-B dual- loaded vesicles ($V_{MLN+RhB}$) were dissolved in 1 mL of phosphate buffer saline (PBS) (pH 7.4) and placed in the dialysis tube. The tube was then immersed in 10 mL of PBS in a beaker incubated at 37°C. At fixed time intervals, 2 mL of media was removed and replaced with fresh PBS. The absorbance of each aliquot taken out was measured and the amount of MLN8237 or Rh-B present was determined using Beer's law. For esterase assisted release studies, the same protocol was used with the addition of 10U of esterase.

Stability of the Dextran Nanovesicles in Serum Using DLS Technique.

To study the intracellular stability of the dextran vesicles, 0.1 mg/mL concentration of the nano-vesicles was made in a PBS, Dulbecco's modified eagles medium (DMEM) with 5% fetal bovine serum (FBS) and 5% FBS alone, incubated at 37°C for 24 h. The size of the nano-vesicles in these solutions was measured by DLS every hour for the initial 6hours and then at 9, 12, and 24hours, respectively. The DLS data thus obtained was plotted using Origin 8.0 software and compared.

2.2.10 Cellular Uptake of Encapsulated MLN8237 (V_{MLN}) by Confocal Microscopy.

Cells were seeded at a density of 1×10^5 cells on coverslips coated with 2 μg/mL of fibronectin in 6-well plates containing DMEM or RPMI 1640 (depending on cell line) with 5% FBS and incubated at 37°C for 18hours. Cells were then incubated with the required concentration of the DEX loaded with MLN8237 and Rh-B ($V_{MLN+RhB}$) for 48hours, with or without nocodazole at a concentration of 10 ng/mL (to activate Aurora kinases). After 48hours, the drug-containing medium was removed, cells were washed twice with PBS (1 mL per wash) and fixed with 3.5% paraformaldehyde solution in PBS for 15 min at room temperature. They were then washed with PBS and stained with phalloidin conjugated to Alexa 488 (Invitrogen) diluted 1:400 in 5% BSA solution in PBS for 45 min in dark. Excess dye was washed from the cells, coverslips were incubated with DAPI (0.05 mg/mL) for 2 min to stain the nucleus, and finally they were mounted on slides using Fluoromount-G mounting medium (Southern Biotech). Slides were then dried overnight at room temperature in the dark and imaged using an LSM780 multiphoton microscope with the λ 405 nm (blue channel), λ 568 (red channel), and λ 488 (green channel) lasers.

Cellular Uptake of Encapsulated MLN8237 ($V_{MLN+RhB}$) in 3D Gels.

MCF-7 cells growing in DMEM with 5% FBS were trypsinized, and 2.5×10^5 cells were mixed with 1.5 mg/mL of collagen diluted with PBS and polymerized using NaOH in glass bottom Lab-Tek chambers. The gel with cells was allowed to polymerize for 30 min at 37 °C in a CO_2 incubator and 400 μL of DMEM containing 5% FBS with 5 μM Rh-B encapsulated in the DEX vesicle (V_{RhB}) or with MLN8237 ($V_{MLN+RhB}$). Cells were incubated for 3hours at 37°C and fixed with 3.5% paraformaldehyde solution in sucrose for 15 min at room temperature. Cells were then imaged using a LSM-710 anisotropy microscope with a λ 568 (red channel) laser.

Deconvolution of Z-Stacks Using Huygen's Professional Image Analysis Software.

V_{RhB} or $V_{MLN+RhB}$-labeled MCF-7 cells in 3D collagen gels were imaged using a confocal microscope and cross-section images collected as a Z-stack 0.2 μm apart. All of the images were processed and analyzed using the Huygens Professional software (version 16.10 825) (Scientific Volume Imaging, The Netherlands, http://svi.nl). Deconvolution of these z-stacks was done using their deconvolution add-on using the following settings: iterations, 30; threshold, 0.0001; signal to noise ratio (SNR), 20; and background estimation radius, 1.

Deconvoluted Z-stacks were processed using the maximum intensity projection (MIP) tool to render images with a 15% threshold.

2.2.11 Treatment of cells using MLN8237 and dextran encapsulated MLN8237 (V_{MLN})

Cells (3×10^5 cells) were seeded in 60 mm dishes and allowed to attach for 24hoursfollowed by a treatment with 0.02 μM MLN8237 as free drug or in the nanovesicle (V_{MLN}) for 48hours. For standardisation experiments with MCF-7 cells nocodazole (10 ng/mL, 33 nM) was simultaneously added to the cells with the drug for this period. Four untreated controls, (a) cells without nocodazole and MLN8237, (b) cells without nocodazole and with an empty DEX scaffold (equivalent to the highest amount DEX added with the encapsulated drug), (c) cells with nocodazole and without MLN8237, and (d) cells with nocodazole and empty DEX, were used in each experiment. In all other inhibition experiments, DMSO (CON) and empty dextran scaffold (DEX) were used as solvent controls for MLN8237 (MLN) and encapsulated MLN8237 (V_{MLN}), respectively.

2.2.12 Treatment of cells using BQU57 (Ral inhibitor) and AZD1152 (AURKB inhibitor)

BQU57 treatment

MCF-7 cells seeded at low density in 100mm dishes were treated with 5 μM BQU57 inhibitor for 48hours and processed for Ral activity assay as discussed in section 2.2.3. The percentage active RalA and RalB values for BQU57 treated cells were normalized to DMSO treated control cells (equated to 1) and represented.

AZD1152 treatment

WT-MEFs were serum starved at ~70% density, detached using trypsin and held in suspension for 30minutes in presence of 2 μM AZD1152 or volume equivalent DMSO. Post suspension cells were carefully washed with DMEM containing 2 μM AZD1152 or volume equivalent DMSO and re-plated in coverslips coated with 2 μg/ml fibronectin or dishes coated with 10 μg/ml coated fibronectin for 15 minutes in presence of 2 μM AZD1152 or volume equivalent DMSO. The cells on dishes were lysed in 1X laemmli buffer and used for western blotting to detect AURKB and ERK activity. And the cells on coverslips were fixed with 3.5% PFA and

used for IFA to detect phopsho-ERK localization and cell spread area. These cells were also used to determine the distribution profile of cells with ruffled edges vs protruding edges.

2.2.13 Cell viability Assay (MTT Assay)

To observe the effect of free MLN8237 and DEX encapsulated MLN8237, a cell viability assay was performed in MCF-7 cells using the tetrazolium salt, 3-4,5 dimethylthiazol-2,5- diphenyl tetrazolium bromide (MTT). Cells were seeded in a fibronectin (2ug/ml) coated 96-well plate (Corning) at a density of 2×10^3 cells per well in DMEM with 5% FBS and allowed to adhere for 24 hours. Prior to drug treatment, existing medium was aspirated and fresh medium with 0.05μM of MLN and DEX encapsulated MLN (V_{MLN}) was added to each well. A control with untreated cells, a DEX scaffold control (having Empty DEX scaffold) and a blank control only DMEM in absence of cells, were used in each experiment. All the control and treated experiment wells were in triplicates. Cells were incubated for 72 hours without change in medium. After 72 hours, drug containing medium was aspirated and freshly prepared stock of MTT in Sterile PBS(5mg/ml) was diluted to 50μg/ml in 100μl DMEM and added to cells. Cells were incubated with MTT at 37°C for 4 hours. At the end of 4 hours existing medium was aspirated and the purple formazan crystals formed as a result of reduction of MTT by mitochondrial dehydrogenase enzymes from cells were dissolved in 100μl of 100% DMSO per well. The absorbance from formazan crystals was measured using microplate reader at 570nm (Varioscan Flash) and is representative of number of viable cells per well.

2.2.14 Anchorage-Independent Growth (AIG) Assay

Cells treated with 0.02 μM of free inhibitor (MLN) or dextran-encapsulated inhibitor (V_{MLN}) were trypsinized after 24hours, and 5000 cells were mixed with 0.3% agar containing DMEM and layered on top of 0.5% agar base per well in 6-well plates. Each of the control and inhibitor-treated cells were plated in duplicates. The agar was allowed to solidify, and 1.5 mL of DMEM containing 5% FBS and 0.02 μM MLN8237 (as free drug or nanovesicle-encapsulated inhibitor V_{MLN}) was added. These dishes were maintained for 15 days with their medium changed every 3 days. Freshly reconstituted free drug or V_{MLN} at the same used concentration were added to the medium during this change. A similar protocol was followed to study the AIG of MCF-7

cells treated with 5 µM BQU57 (Ral inhibitor). The colonies formed in agar at the end of the 15-day incubation were stained with 0.05% crystal violet dissolved in 20% ethanol for 1hour at room temperature and de-stained by repeated washing with distilled water until stained colonies were clearly visible. The colonies were then imaged on an Olympus MVXC10 microscope at 0.63X zoom in the HDR mode and counted using the particle analysis tool of Image J software.

2.2.15 Statistical analysis

All analysis was done using Prism Graphpad analysis software. Statistical analysis of data was done using the two-tailed unpaired Student's T-test, two-tailed paired T test and when normalized to respective controls using the two-tailed single sample T-test. Distribution profile data was analysed using Chi-square test.

Chapter 3

Study the role of Aurora Kinase A in regulating adhesion-dependent RalA activation

3.1 Rationale

During mitosis, two distinct pools of Aurora Kinase A namely microtubule-associated and centrosome associated are activated by different regulators (Carmena & Earnshaw 2003a). Microtubule-associated Aurora Kinase A is activated by auto-phosphorylation at Threonine 288 residue through the interaction with TPX2 (Kufer et al. 2002), which is released from an inactive complex as a result of the Ran-GTP activation when cells enter mitosis (Kufer et al. 2002). Apart from TPX2, Astrin has been shown to activate microtubule AURKA in different stages of mitosis (Jian Du et al. 2008). Centrosomal Aurora Kinase A is in turn activated by Ajuba, Bora, and Nucleophosmin/B23 (Hirota et al. 2003). Two of the regulators of centrosomal AURKA also include PAK1 kinase and HEF1/NEDD9 scaffolding protein that are present at both focal adhesions and centrosomes (Pugacheva & Golemis 2005; Parrini et al. 2005). Both PAK1 and HEF1/NEDD9 activate AURKA by directly phosphorylating Thr-288 residue and stabilising it (Zhao et al. 2005; Pugacheva et al. 2007). HEF1/NEDD9, in turn, is phosphorylated and regulated by FAK and SRC kinases (Tikhmyanova et al. 2010). The focal adhesion localization of these proteins suggests that they define a pool of AURKA that belongs to a signaling pathway linking loss of cell-matrix adhesion or mitotic cell rounding (typical of cell division process) with mitotic centrosomal events and mitotic entry. However, if and how Integrin-mediated cell-matrix adhesion regulates the activity of Aurora Kinase A is still unexplored.

Mitotically activated AURKA phosphorylates a small GTPase RalA at Serine 194, a residue which is absent in RalB (Lim, Brady, Kashatus, Ancrile, Der, Cox & Counter 2009b) and translocates it to mitochondria where it activates and binds to its effector RalBP1 aiding mitochondrial fission (Kashatus, Lim, Brady, Pershing, Cox & Counter 2011b). This small GTPase RalA is a part of the vesicular trafficking pathway that regulates the anchorage-dependent growth of cells. RalA is activated downstream of Integrin-mediated cell-matrix adhesion where along with the exocyst complex it regulates exocytosis of membrane raft microdomains (N. Balasubramanian et al. 2010) to the plasma membrane to regulate anchorage-dependent growth signalling. Loss of adhesion decreases RalA activity ensuring minimal exocytosis of raft microdomains and diminished growth signalling. Conversely, re-adhesion of cells activates RalA triggering membrane raft exocytosis and plasma membrane delivery to restore growth signaling(N. Balasubramanian et al. 2010). Since both AURKA and RalA independently help in adhesion-dependent regulation of cell proliferation, and AURKA regulates RalA during mitosis, it will be interesting to explore if and how AURKA regulates

RalA-dependent vesicular trafficking pathway downstream of Integrin-mediated adhesion in anchorage-dependent cells.

In this chapter, we hence evaluated what happens to Aurora Kinase A activity in anchorage-dependent Wild Type-Mouse Embryonic Fibroblasts (WT-MEFs) on their 'loss of adhesion' and 're-adhesion' to fibronectin matrix and whether this activity is important for RalA activation and function. The role Ral GEFs have in adhesion and AURKA-dependent regulation of RalA was also explored. The possible contribution cell cycle profile of WT-MEFs could make to this regulation was also tested.

3.2 Results

3.2.1 Cell-matrix adhesion regulates Aurora kinase A activity

To evaluate whether Aurora Kinase A (AURKA) has any role in regulating RalA downstream of integrin-mediated cell-matrix adhesion, we first tested if adhesion can regulate AURKA activity (autophosphorylation on Threonine 288) in a time frame where RalA is regulated. To ensure crosstalk from growth factors does not affect these studies and ascertain cell-matrix adhesion is the primary regulator of signaling leading to AURKA and RalA activity, Wild type Mouse embryonic fibroblasts (WT-MEFs) were serum-starved for 12 hours (grown in medium with 0.2% serum). Under these conditions, re-adhesion to matrix fibronectin activates integrin-dependent signaling and function in WT-MEFs.

To study the adhesion-dependent regulation of AURKA serum-starved stable adherent WT-MEFs were detached and held in suspension for 90mins in 1% methylcellulose (SUS 90'), and re-plated on fibronectin for 15mins (FN 15') or 4hours (stable adherent - SA). We first evaluated the effect on downstream AKT activation (detected using Serine473 phosphorylation) in these time frames. Immunoblots revealed that in suspension (SUS 90') AKT activation was significantly reduced and rapidly restored in 15 mins of re-adhesion to fibronectin (Figure 3.1b). Under these conditions, AURKA activity interestingly increases (~60%) on the loss of adhesion and is restored to stable adherent levels on re-adhesion to fibronectin (Figure 3.1a). This suggests that integrin-mediated adhesion does regulate AURKA activity. However, knowing the fact that AURKA is a cell cycle kinase that is under tight regulation of cell cycle phase (Carmena & Earnshaw 2003a; Tanaka et al. 2002; Goldenson & Crispino 2014a), we evaluated the possible contribution adhesion-dependent cell cycle regulation could have in AURKA activation.
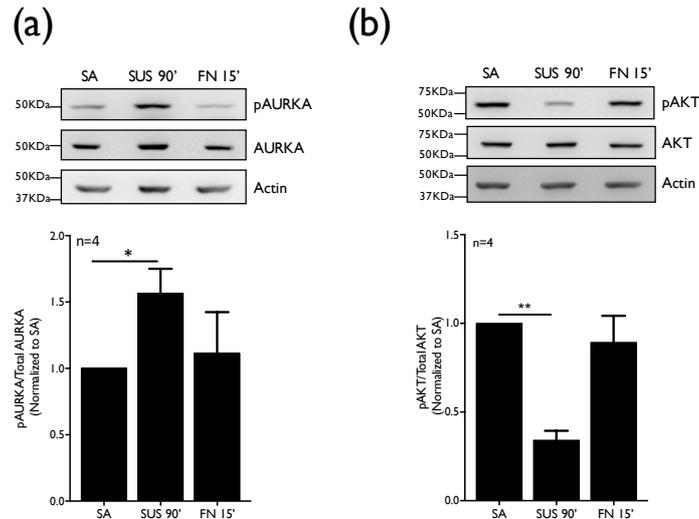
Figure 3.1: Adhesion dependent regulation of AURKA activation in WT-MEFs. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on Threonine 288 residues of AURKA (pAURKA), total AURKA and actin and, (b) phosphorylation on the serine 473 residues of AKT (pAKT), total AKT and actin in the lysates from serum-starved WT-MEFs stable adherent (SA), suspended for 90 mins (SUS 90') and re-adherent on fibronectin for 15mins (FN 15'). The ratios of pAKT/AKT, pAURKA/AURKA were normalized to respective SA (equated to 1), and these values are represented in the graph as mean ± SE from four independent experiments. Statistical analysis of all the above data was done using the single sample *t*-test and significance if any was represented in graph (* p-value <0.05, ** p-value <0.01).

3.2.2 Cell-matrix adhesion regulates cell cycle profile of WT-MEFs

Loss of adhesion has been shown to cause cytokinesis failure (leading to G2-M arrest) which is reversed upon re-adhesion (Avri Ben-Zeev and Avraham Raz,1981) (De Santis Puzzonia et al. 2016). Knowing the fact that AURKA activity increases during G2-M phase, the changes in AURKA activation on the loss of adhesion could indeed be mediated by an arrest in cell cycle. Conversely, such a change in AURKA activity could in-turn affect cell cycle profile. Understanding this cause-effect relationship will hence be useful in exploring if and how integrin-mediated adhesion (and their crosstalk with growth factors) regulate Aurora Kinases.

As a first step to addressing this question, we asked if adhesion affects the cell cycle profile of serum-deprived WT-MEFs, by propidium iodide labelling, on the loss of adhesion (90' SUS) and re-adhesion (FN 15'). Levels of cyclin D1 and A2 in these cells were further used to confirm the same (Carstens et al. 1996; Schwartz & Assoian 2001) (Pines & Hunter 1991; Baldin et al. 1993). The synchronization of cells at G1-S phase upon serum starvation (Langan

& Chou 2011; Griffin 1976; Campisi et al. 1984) is reflected in 65 ± 3 % of SA WT-MEFs being in the G1 phase and 27.8 ± 4 % in the S phase (Figure 3.2a and 3.2b). This is reflected in their cyclin D1 levels being higher than cyclin A2 (Figure 3.2c). Although, the cyclin levels did not change significantly on loss of adhesion, the percentage of cells in G1 phase decrease significantly accompanied by corresponding increase in S phase cells (Figure 3.2b). However, the percentage of cells in G2-M phase did not change significantly upon loss of adhesion or re-adhesion to fibronectin. As majority of known regulators of AURKA activity function during G2-M phase (Pugacheva & Golemis 2005; Tanaka et al. 2002; Goldenson & Crispino 2014a; Carmena & Earnshaw 2003a), it is unlikely that the increase in AURKA activity upon loss of adhesion is a causation of change in cell cycle profile. However, to exclude out this possibility we have tested the activity of AURKA upon loss of adhesion for a shorter time of 30 mins, and the same will be discussed in chapter 6. Knowing the overlap that exists between integrin-growth factor pathways that regulate cell cycle, we further evaluated the role serum growth factors could have in adhesion-dependent regulation of cell cycle and AURKA activity.
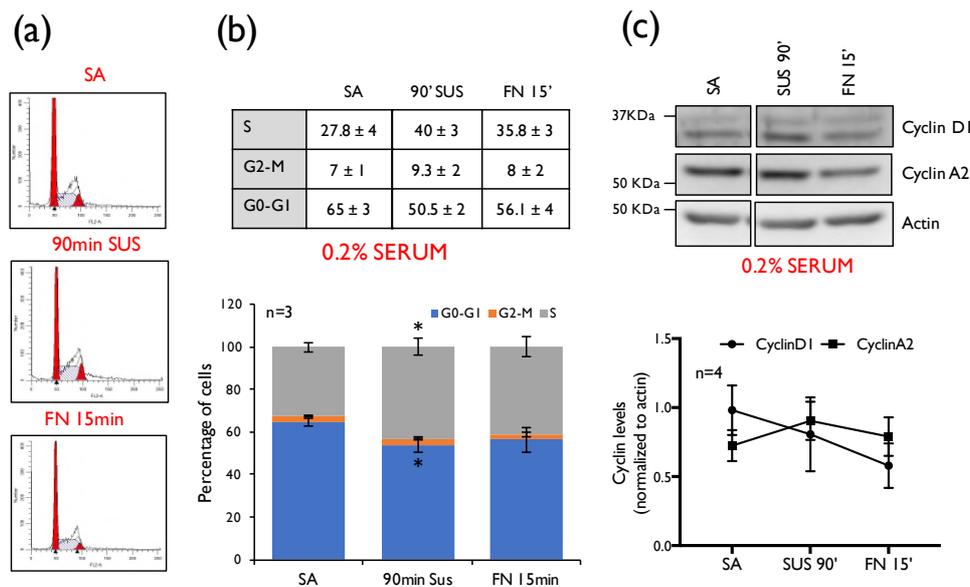

Figure 3.2: Adhesion-dependent regulation of Cell cycle profile. (a) Representative histogram of 3 independent experiments (b) Percentage of cells present in G2-M, S and G0-G1 phase are shown in table (upper panel) and graph (lower panel). (b) Western blot detection (upper panel) and quantitation (lower panel) of CyclinD1 and CyclinA2 in lysates from serum-starved WT-MEFs stable adherent (SA), suspended for 90 mins (SUS 90') and re-adherent on fibronectin for 15mins (FN 15') are represented. Samples compared and represented in blots were all run and developed together. Blots are cropped to remove blank lanes in between only for representation. The graph represents the mean ± SE from at least 3 independent experiments. Statistical analysis was done using Students T-Test and significance represented (* p-value <0.05).

3.2.3. Adhesion-growth factor crosstalk regulates AURKA activity

There are multiple mechanisms by which crosstalk between integrin-mediated adhesion and growth factors exists and is very well documented in the literature (Schwartz & Assoian 2001) (Danen and Yamada, 2001). The objective of performing above experiments in serum-deprived conditions stems from this same reason. By depriving cells of serum growth factors, we could isolate the contribution integrin-dependent adhesion has in regulating AURKA activity. However, it is of interest to ask if growth factors can influence, through known crosstalk with integrin signaling pathways, adhesion-dependent AURKA activity.

Our studies show the presence of serum growth factors (10% FBS) causes a decrease in AURKA activity upon loss of adhesion that does not recover back on re-plating to fibronectin for 15mins (Figure 3.3a). Under these conditions, AKT activity was significantly reduced and it was rapidly triggered in 15 mins of re-adhesion to fibronectin (Figure 3.3b). The cell cycle profile of these serum-cultured SA cells is also distinctly different from serum-deprived cells. 44.86 ± 5 % of SA WT-MEFs were seen to be in G1 phase and 44.17 ± 5 % in S phase (Figure 3.4b), reflected in their cyclin D1 levels being similar to cyclin A2 (Figure 3.4c). Upon loss of adhesion the cells in G1 phase enter S-phase as seen by the significant decrease in percentage of cells in G1 phase accompanied by corresponding increase in S phase cells (Figure 3.4b). The percentage of cells in G2-M phase decrease significantly upon loss of adhesion and does not change significantly upon re-adhesion suggesting cell cycle might be contributing to the drop in the AURKA activity upon loss of adhesion in presence of serum growth factors. Taken together, these studies imply that the differential cell cycle profile of WT-MEFs in presence and absence of serum could influence the differential activation of AURKA, though the mechanism mediating this remains unknown. A comprehensive study to determine the regulators and contribution of cell cycle on AURKA activity upon loss of adhesion remains to be initiated. However, the regulation and role of AURKA activity on re-adhesion is explored further in studies reported here.
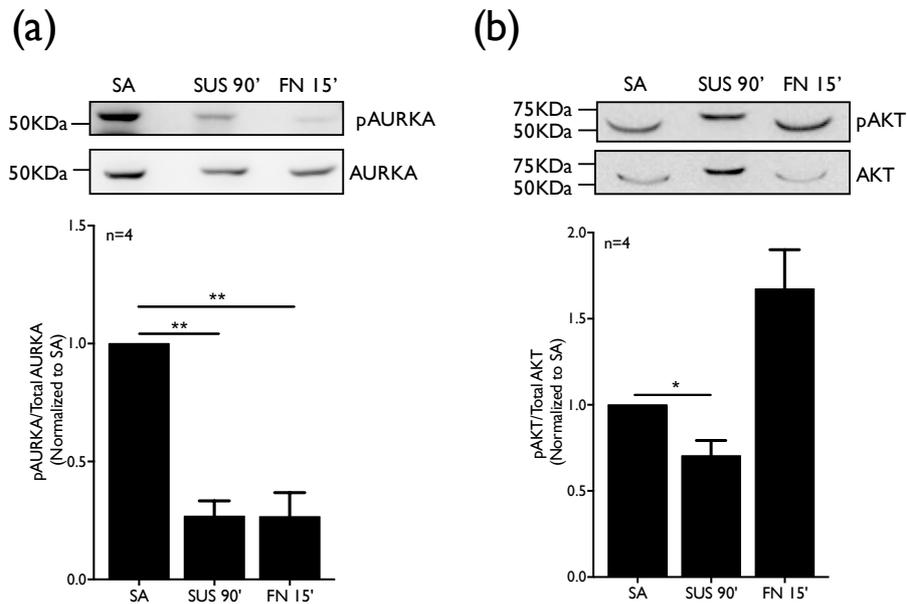
Figure 3.3: Adhesion-Growth factor crosstalk dependent regulation of AURKA activity in WT-MEFs. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on Threonine 288 residues of AURKA (pAURKA), total AURKA, (b) phosphorylation on Serine 473 residues of AKT (pAKT), total AKT from lysates obtained from 10% FBS grown WT-MEFs stable adherent (SA), suspended for 90 mins (SUS 90') and re-adherent on fibronectin for 15mins (FN 15'). The ratios of pAKT/AKT, pAURKA/AURKA were normalized to their respective SA (equated to 1), and these values are represented in the graph as mean ± SE from four independent experiments. Statistical analysis of all the above data was done using the single sample *t*-test and significance represented (* p-value <0.05, ** p-value <0.01).
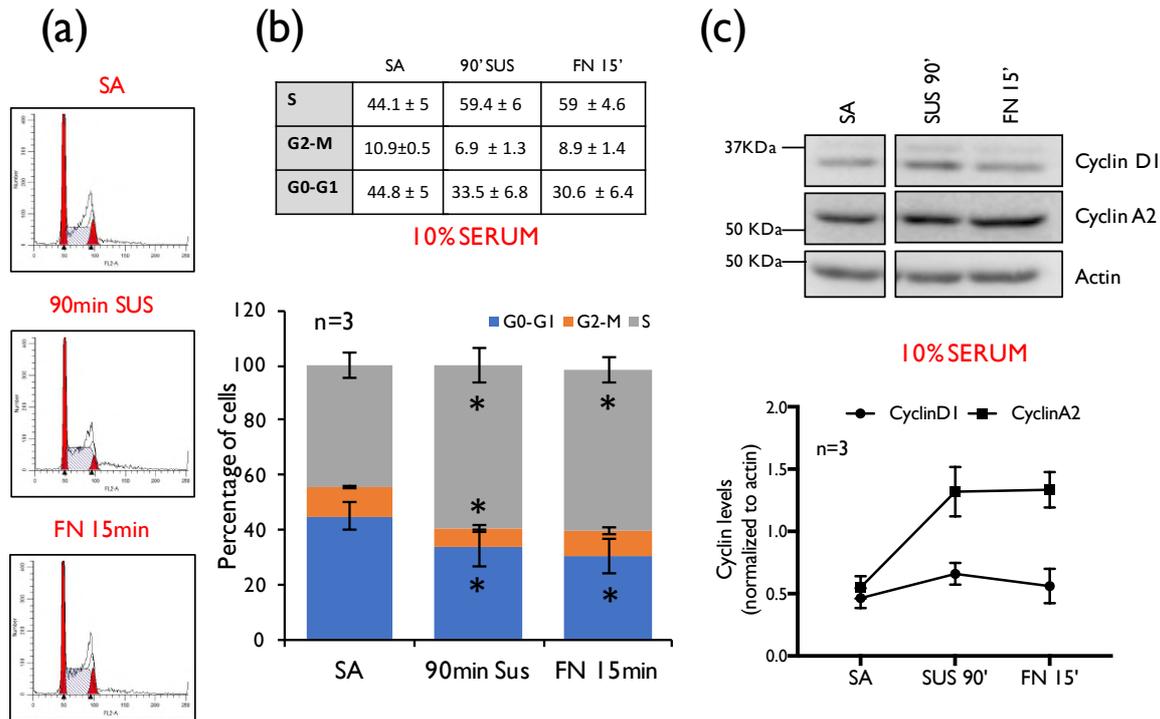
Figure 3.4: Adhesion-Growth factor crosstalk dependent regulation of cell cycle in WT-MEFs (a) Representative histogram of 3 independent experiments (b) Percentage of cells present in G2-M, S and G0-G1 phase are shown in table (upper panel) and graph (lower panel). (b) Western blot detection (upper panel) and quantitation (lower panel) of CyclinD1 and CyclinA2 in lysates from 10% FBS grown WT-MEFs stable adherent (SA), suspended for 90 mins (SUS 90') and re-adherent on fibronectin for 15mins (FN 15') are represented. Samples compared and represented in blots were all run and developed together. Blots are cropped to remove blank lanes in between only for representation. The graph represents the mean ± SE from three independent experiments. Statistical analysis was done using Students T-Test and significance represented (* p-value <0.05).

3.2.4. Aurora Kinase A regulates adhesion-dependent RalA activation and cell spreading

The primary focus of this thesis was evaluating the AURKA-RalA crosstalk and its regulation by cell-matrix adhesion. With earlier studies from the lab having established the regulation of RalA (vs RalB) (Figure 3.5a and 3.5b) in serum-deprived WT-MEFs suspended for 90min and re-plated on FN for 15min, we looked at the AURKA-RalA crosstalk under similar conditions. We hence asked how AURKA-dependent phosphorylation of RalA at Ser194 residue changes upon loss of adhesion and re-adhesion in WT-MEFs? Since the antibody that detects the S194RalA is specific to human-RalA, we expressed human-RalA in WT-MEFs and saw pS194-RalA levels to increase upon loss of adhesion (Figure 3.6) where AURKA activity increases (Figure 3.1a), but RalA activity decreases (Figure 3.5a). On re-adhesion, pS194-

RalA levels are restored to stable adherent levels, as is AURKA activity. RalA activity, however, does increase in re-adherent cells relative to suspended cells. Taken together these studies suggest that AURKA mediated regulation of S194-RalA phosphorylation might be decoupled from its adhesion-dependent activation. In cancer cells the phosphorylation of RalA at Serine 194 by AURKA is known to be important for its oncogenic activity including anchorage independent growth and tumorigenesis (Wu et al. 2005), however our experiments suggest that in adhesion-dependent WT-MEFs the phosphorylation of RalA by AURKA might not play a significant role in regulating GTP-GDP cycling downstream of integrin signaling. Further as discussed earlier, AURKA regulates RalA phosphorylation during G2-M phase of cell cycle to help mitochondrial fission (Kashatus et al. 2011), whereas in our suspension experiments with WT-MEFs, majority of cells are in G1-S phase and this could be the reason why phosphorylation of RalA and its activity are decoupled downstream of integrin-dependent signaling
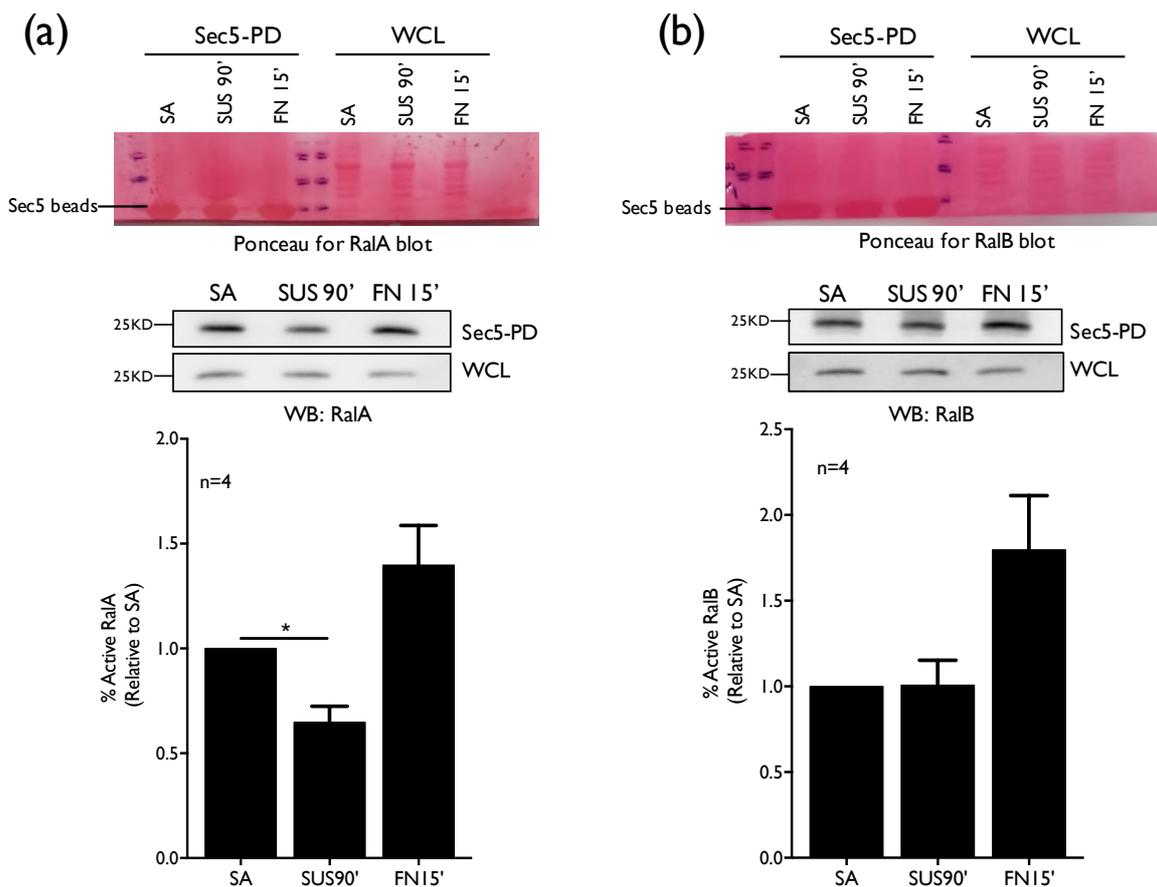

Figure 3.5: Adhesion-dependent activation of RalA and RalB. (a) Active RalA, (b) Active RalB pulled down by GST-Sec5, and the total RalA/RalB in WCL was done from WT-MEFs stable adherent (SA), suspended for 90 mins (SUS 90') and re-adherent on fibronectin for 15mins (FN 15'). Representative Ponceau-S stained blots for GST-Sec5 pulldowns show the GST fusion protein levels being comparable across treatments. RalA and RalB activities were

calculated as discussed in Section 2.2.4 of Methods chapter. The activites were normalized to their respective SA (equated to 1), and these values are represented in the graph as mean ± SE data from four independent experiments. Statistical analysis was done using the single-sample t-test and p values, if significant, are represented in the graph (* $p < 0.05$).
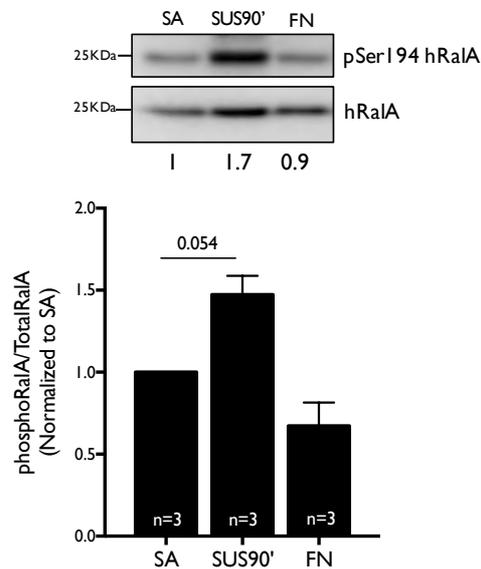

Figure 3.6: Adhesion-dependent regulation of Phosphorylation of RalA at Serine 194. Western blot detection (upper panel) and quantitation (lower panel) of phosphorylation on Serine 194 residues of human RalA (pSer194hRalA) and total human RalA in lysates from serum-starved WT-MEFs stable adherent (SA), suspended for 90 mins (SUS 90') and re-adherent on fibronectin for 15mins (FN 15'). The ratio of pRalA/TotalRalA was normalized to SA (equated to 1), and these values are represented in the graph as mean ± SE from three independent experiments. Statistical analysis of all the above data was done using the single sample *t*-test and the p-value represented in the graph.

To evaluate whether AURKA has any role in regulating adhesion-dependent RalA, we have used a self-assembling dextran nano-vesicle to encapsulate AURKA inhibitor MLN8237 (V_{MLN}) (made in house and described in detail in chapter 4 and (Inchanalkar et al. 2018)) and inhibited AURKA activation in WT-MEFs. The development, characterization of V_{MLN} and its ability to specifically inhibit AURKA (without affecting AURKB) has been discussed in detail in Chapter 4. For this experiment, WT-MEFs serum-starved for 12hours (grown in 0.2% FBS) were detached and held in suspension for 60mins in 1% methylcellulose followed by additional 30mins in suspension along with 0.2μM of V_{MLN} (V_{MLN} SUS 90') or volume equivalent empty dextran nano-vesicle scaffold (CON SUS 90'). These cells further were re-plated on fibronectin for 15mins with 0.2μM V_{MLN} or empty dextran scaffold, respectively. This V_{MLN} treatment allows us to achieve up to 95% inhibition of AURKA in suspended cells that is maintained at these levels in cells re-adherent on fibronectin for 15min (Figure 3.7a). This inhibition of

AURKA causes a significant ~40% decrease in RalA activity in suspended cells and a ~100% inhibition of its recovery in re-adherent cells (Figure 3.7b). Serine 194 phosphorylation of RalA which is prominent in 90min suspended cells drops by ~40% on V_{MLN} treatment (Figure 3.7c). In re-adherent cells this S194RalA phosphorylation is reduced by ~50% relative to suspended cells, and drops by an additional ~27% on V_{MLN} treatment (Figure 3.7c). Thus, V_{MLN} mediated inhibition of AURKA in suspended and re-adherent cells does disrupt adhesion-dependent RalA activation in re-adherent cells. RalB activity which is known to not be regulated by AURKA is unaffected by this inhibition (Figure 3.7d). These results suggest that even though AURKA activation and Serine 194-RalA phosphorylation profiles are distinctly different from RalA activity detected by GST-Sec5 pulldown on the loss of adhesion vs re-adhesion, AURKA is still required for recovery of adhesion-dependent RalA activation, confirming the existence of adhesion-AURKA-RalA signaling pathway in WT-MEFs.

In these experiments, WT-MEFs suspended with V_{MLN} show complete inhibition of AURKA activity without completely inhibiting RalA S194 phosphorylation, suggesting that, other known regulators of RalA S194 phosphorylation, namely protein phosphatase 2Aβ (Sablina et al. 2007) and/or Protein kinase A (Gentry et al. 2014) might also be involved in regulating RalA in WT-MEFs. Investigating the role of these proteins in adhesion-dependent regulation of RalA hence is an interesting open question.

Integrin-mediated adhesion specifically regulates RalA in WT-MEFs to support adhesion-dependent membrane raft trafficking and cell spreading (Balasubramanian et al., 2010). Knowing the role AURKA has in mediating RalA activation and phosphorylation we tested the effect this has on cell spreading. Serum starved WT-MEFs held in suspension for 60mins followed by 30mins 0.2μM V_{MLN} treatment were re-plated on fibronectin for 15mins in presence of 0.2μM V_{MLN}. These cells post-fixation were stained with phalloidin-488 to calculate cell spread area. V_{MLN} treated cells upon re-adhesion show dramatically reduced cell spreading (Figure 3.8), supporting the presence of an adhesion-AURKA-RalA-cell spreading pathway.
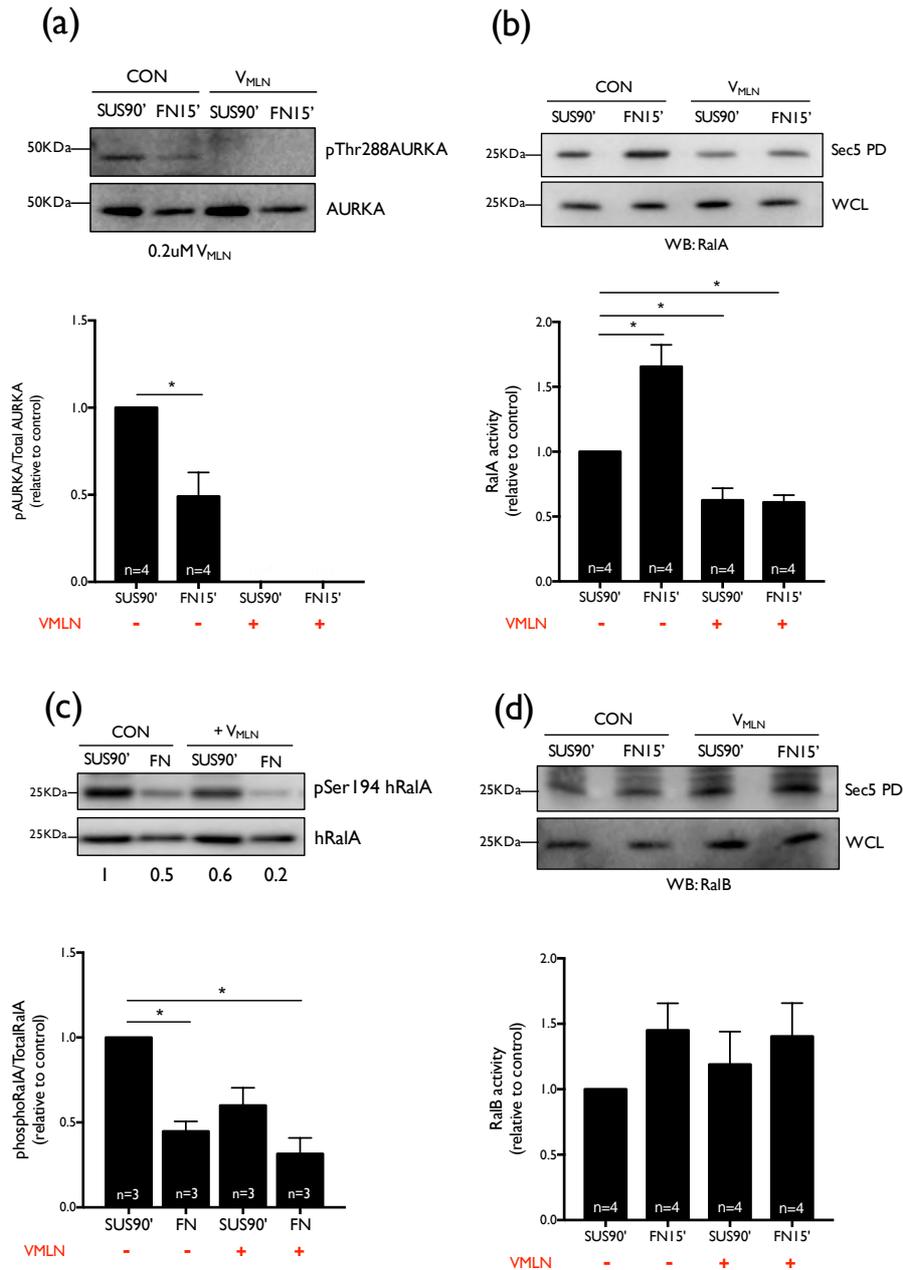
Figure 3.7: Inhibition of AURKA using V$_{MLN}$ and its effect on adhesion-dependent RalA activity in WT-MEFs. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on Threonine 288 residues of AURKA (pThr288AURKA) and total AURKA (b) Active RalA pulled down by GST-Sec5, and the total RalA in WCL (c) phosphorylation on Serine 194 residues of human RalA (pSer194hRalA) and total human RalA, and (d) Active RalB pulled down by GST-Sec5, and the total RalB in lysates from serum-starved WT-MEFs stable adherent (SA), suspended for 60 mins + 30mins with V$_{MLN}$/Empty DEX (SUS 90') and re-adherent on fibronectin for 15mins with V$_{MLN}$/Empty DEX (FN 15'). The ratios of pAURKA/TotalAURKA, pRalA/TotalRalA and active RalA and RalB were normalized to respective 90' suspension without V$_{MLN}$ (equated to 1). The graph represents the mean ± SE data from at least three independent experiments. Statistical analysis was done using the single-sample t-test and p values, if significant, are represented in the graph (* $p < 0.05$) .
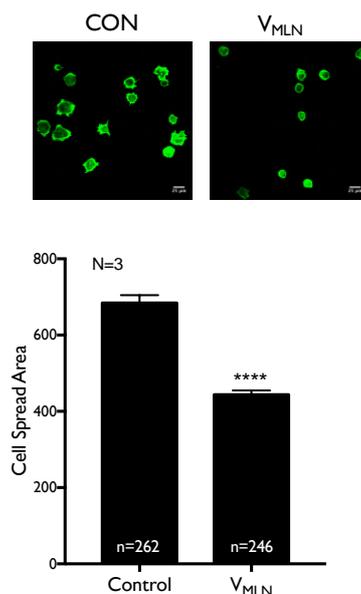
Figure 3.8: Effect of AURKA inhibition on RalA-dependent cell spreading in WT-MEFs. Inhibition of AURKA using dextran nano-vesicle encapsulated MLN8237 (V_{MLN}) significantly decreases cell spreading in WT-MEFs re-adherent on fibronectin for 15mins, relative to empty dextran nano-vesicle control (Control). The graph represents the mean ± SE of cell spread area from three independent experiments. Representative cell spread images are shown in the upper panel. Statistical analysis of all data was done using the two-tailed non-parametric T-Test and p-values are as indicated (**** $p < 0.0001$).

3.2.5 Ral GEF, RGL1 regulates RalA (but not RalB) activation in spreading WT-MEFs

Adhesion-dependent regulation of RalA activity is likely dependent on Ral GEFs and GAPs. Ral GEFs are classified as Ras dependent / Ras independent. RGL1 is one such Ras-dependent RalGEF that we have implicated in adhesion-dependent RalA activation and spreading (Deshpande et. al., manuscript in review). Work from the lab by Neha Deshpande using both individual siRNA and smart pool mediated RGL1 knockdown (Figure 3.9e, 3.9f, 3.10e and 3.10f) significantly inhibits adhesion-dependent recovery of RalA activation (Figure 3.9a and 3.9c), and cell spreading (Figure 3.11) upon re-adhesion to fibronectin for 10mins. This regulation is not seen at later (20mins) re-adhesion time points (Figure 3.10a and 3.10c). This knockdown of RGL1 does not affect RalB activity in re-adherent cells at either early (10min) or late (20min) time points (Figure 3.9b, 3.9d, 3.10b and 3.10d). My studies further confirmed this by reconstitution of individual siRNA mediated RGL1 knockdown cells with a siRNA resistant mutant RGL1 (Figure 3.12c and d) that restores RalA activation (Figure 3.12a and b) in re-adherent cells (10mins on fibronectin), further establishing the specificity of this regulation. To further test the nature of this RGL1-RalA regulation, we examined the

localization of endogenous RalA and Myc-tagged RGL1 during cell spreading. Both proteins were seen to localize extensively in membrane ruffles and occasionally in membrane protrusions (Figure 3.13) both sites of active integrin signaling. While a complete overlap in RalA and RGL1 was detected at membrane ruffles and protrusions in most of the re-adherent cells, few cells also show a partial overlap of these proteins. This suggests localization at sites of active integrin signaling could help mediate RGL1-dependent differential activation of RalA. Together these observations confirm the presence of an integrin-RGL1-RalA pathway that regulates adhesion-dependent RalA activation and cell spreading.
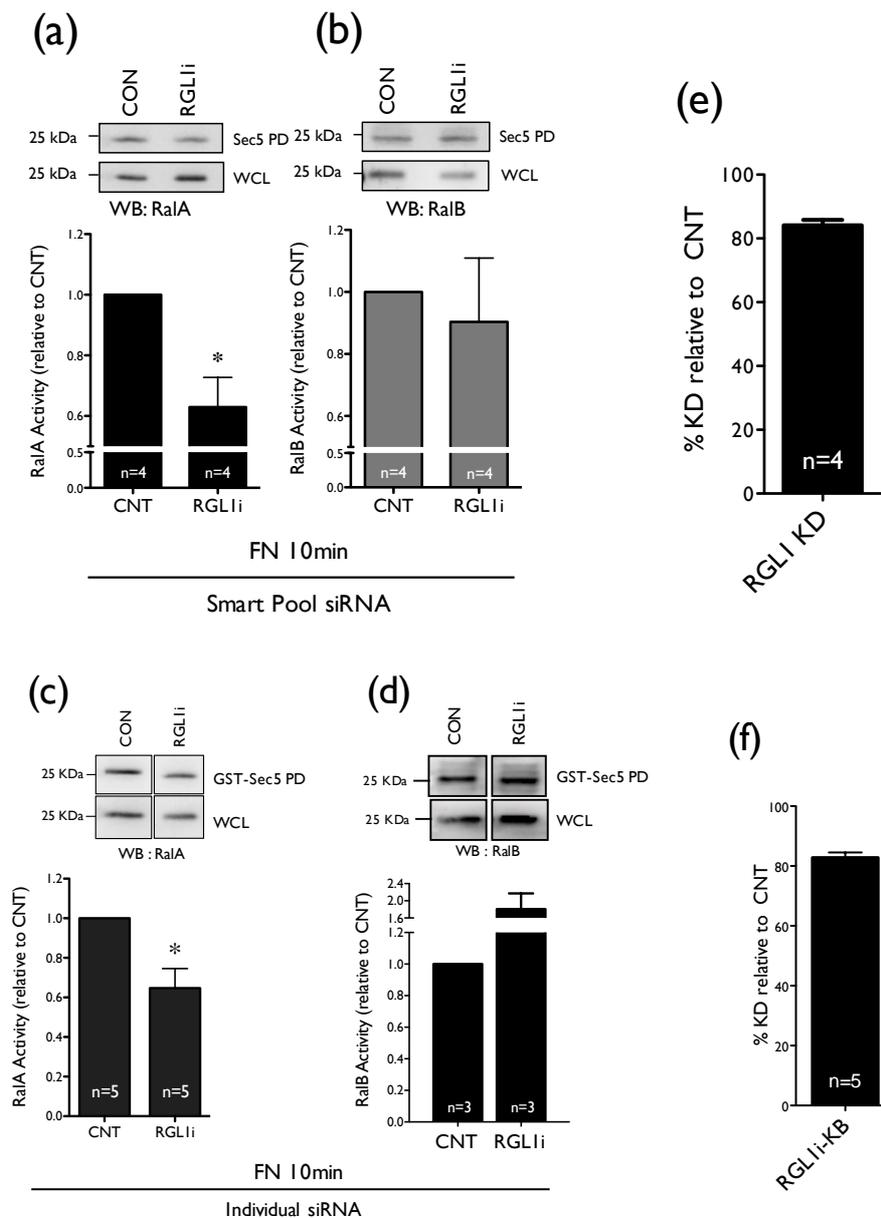
Figure 3.9: Effect of RGL1 knockdown with smart pool and individual siRNA on adhesion-dependent RalA activation post-10mins re-plating. Western blot detection (upper panel) and quantitation (lower panel) of (a and c) active RalA and (b and d) active RalB pulled

down by GST-Sec5, and the total RalA/total RalB in whole-cell lysates from serum-starved Control (CNT/CON) and RGL1 knockdown (RGL1i) WT-MEFs re-adherent on fibronectin for 10min (FN 10min). Samples compared and represented in blots were all run and developed together. Blots are cropped to remove blank lanes in between only for representation. (e) and (f) Graph representing RGL1 KD efficiency determined using qRT-PCR w.r.t. control. The graph represents the mean ± SE data from at least three independent experiments. Statistical analysis was done using the single-sample t-test and p-values, if significant, are represented in the graph (* p < 0.05). *Data credit: Neha Deshpande*
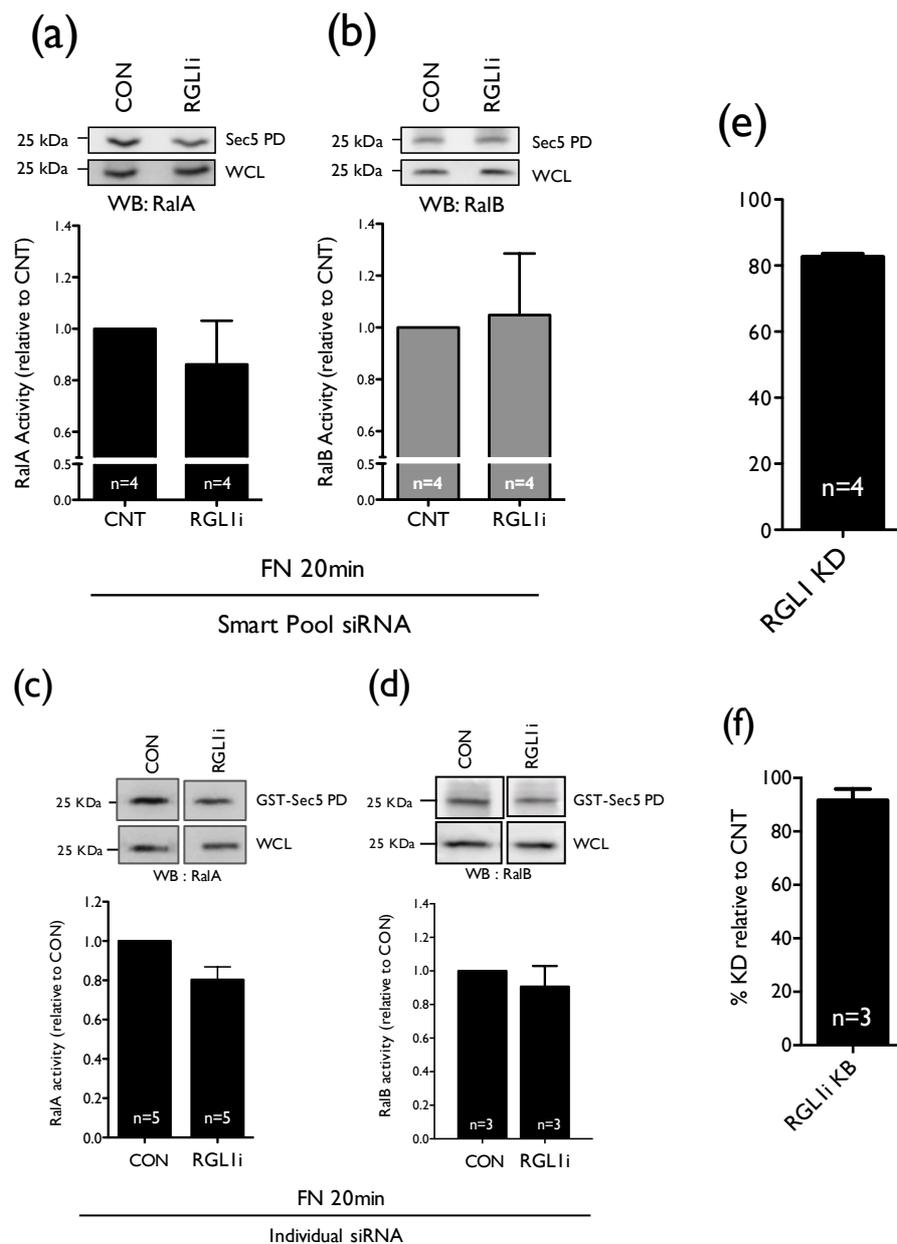

Figure 3.10: Effect of RGL1 knockdown with smart pool and individual siRNA on adhesion-dependent RalA activation post-20mins re-plating. Western blot detection (upper panel) and quantitation (lower panel) of (a and c) active RalA and (b and d) active RalB pulled down by GST-Sec5, and the total RalA/total RalB in whole-cell lysates from serum-starved

Control (CNT/CON) and RGL1 knockdown (RGL1i) WT-MEFs re-adherent on fibronectin for 20min (FN 20min). Samples compared and represented in blots were all run and developed together. Blots are cropped to remove blank lanes in between only for representation. (e) and (f) Graph representing RGL1 KD efficiency determined using qRT-PCR w.r.t. control. The graph represents the mean ± SE data from at least three independent experiments. Statistical analysis was done using the single-sample t-test and p-values, if significant, are represented in the graph (* p < 0.05, ** p < 0.01). *Data credit: Neha Deshpande*
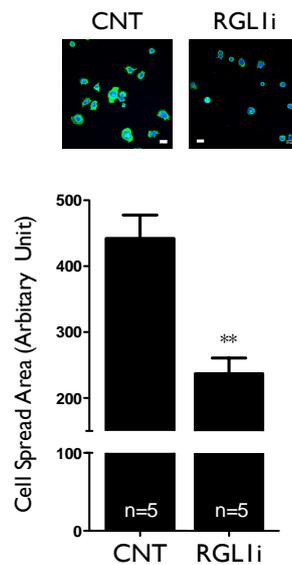

Figure 3.11: Effect of RGL1 knockdown on RalA-dependent cell spreading. Knockdown of RGL1 using individual siRNA (RGL1i) significantly decreases cell spreading in WT-MEFs re-adherent on fibronectin for 10mins, relative to control (CNT). The graph represents the mean ± SE of cell spread area from five independent experiments. Representative cell spread images are shown in the upper panel. Statistical analysis of all data was done using the two-tailed non-parametric T-Test and p values are as indicated (** p < 0.01). *Data credit: Neha Deshpande*
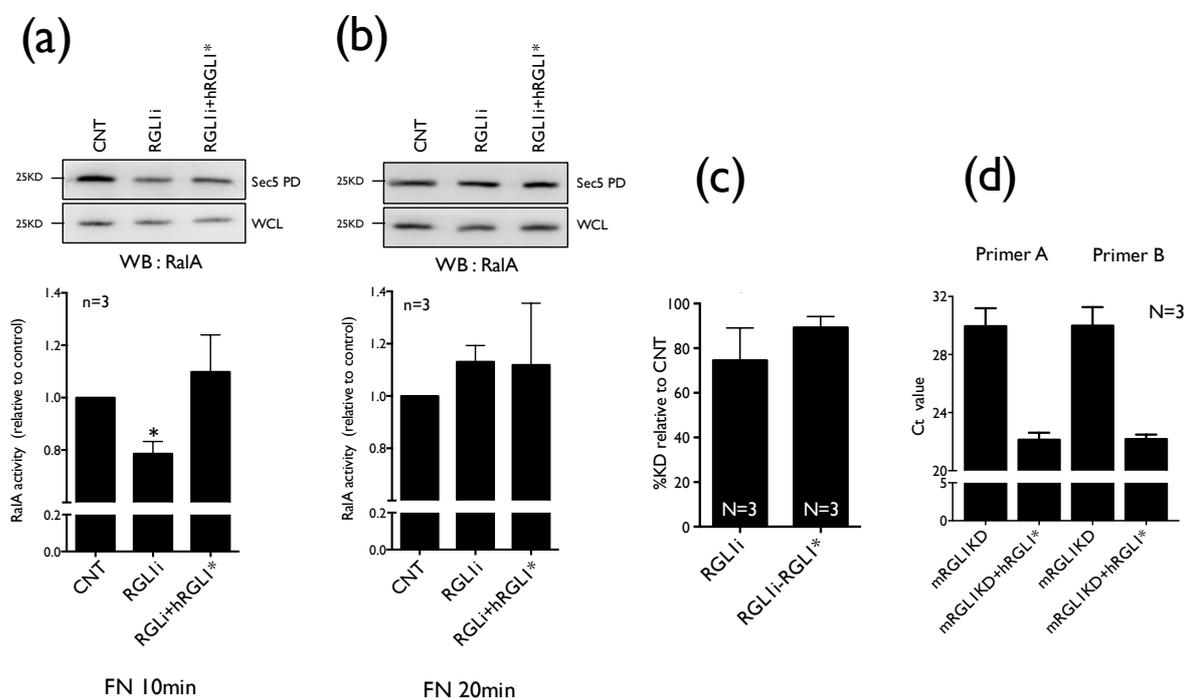
Figure 3.12: Effect of siRNA resistant RGL1 on adhesion-dependent RalA activity in WT-MEFs. (a, b) Western blot detection and quantitation of active RalA pulled down by GST-Sec5 (GST-Sec5-PD) and total RalA in the whole-cell lysate was done from serum-starved control (CNT), RGL1 knockdown MEFs (RGL1i) and RGL1 knockdown reconstituted with siRNA resistant RGL1(RGL1i+hRGL1*), re-adherent on fibronectin for 10min (FN 10min) (a) and 20min (FN20min) (b) respectively. Calculated percentage of active RalA levels were normalized to respective control (CNT) (equated to 1). The graph represents mean ± standard error three independent experiments. Statistical analysis of all data was done using the single sample T-Test and p-values are as indicated (* $p<0.05$, **$p<0.01$). (c) RGL1 KD efficiency and (d) expression efficiency of siRNA resistant RGL1 was determined in RGL1 (RGL1i) knockdown WT-MEFs (RGL1i) and RGL1 knockdown WT-MEFs expressing siRNA resistant human RGL1 (RGL1i+hRGL1*) using RT-PCR.
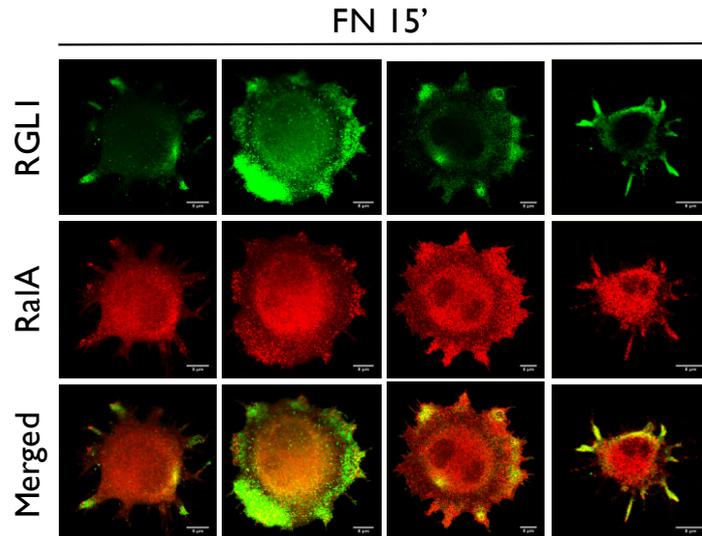
Figure 3.13: Localization of endogenous RalA and Myc-tagged RGL1 during cell spreading. Re-adherent WT-MEFs expressing Myc-tagged RGL1 detected with an anti-myc antibody (Myc-RGL1) and endogenous RalA with the anti-RalA antibody (RalA) shows their co-localization in membrane ruffles and membrane protrusions. Data is representative of 30 cells from three independent experiments.

3.2.6. Aurora Kinase A regulates adhesion-dependent RalA activation through RGL1.

Knowing the role AURKA and RGL1 independently have in regulating adhesion-dependent RalA activity and cell spreading, we asked if RGL1 and AURKA work synergistically to mediate this regulation. For this we compared re-adhesion mediated activation of RalA in RGL1 knockdown (RGL1i), AURKA inhibited (V_{MLN}) and RGL1 knockdown + AURKA inhibited WT-MEFs held in suspension for 90mins and re-plated on fibronectin for 15mins with respective treatments. We find that RGL1 knockdown and AURKA inhibition comparably affect RalA activation in re-adherent WT-MEFs (Figure 3.14a). Their joint inhibition (RGL1 KD + V_{MLN} treatment) did not show any additive effect (Figure 3.14a), suggesting that they likely work along the same pathway to regulate cell-matrix adhesion-dependent RalA activation and cell spreading. Comparable inhibition of AURKA (Figure 3.14c) and knockdown of RGL1 (Figure 3.14d) was observed among the treatments. Neither AURKA inhibition nor RGL1 KD had any effect on RalB activity in these cells (Figure 3.14b). As discussed earlier, in re-adherent WT-MEFs, RGL1 and RalA co-localize to membrane ruffles and protrusions, sites of active integrin signaling (Figure 3.13). This suggests that localization plays an important role in RGL1-mediated RalA regulation. AURKA inhibition by V_{MLN} affects re-adherent cell spreading (Figure 3.7) and localization of RGL1 and RalA at membrane protrusions and ruffles (Figure 3.15). This could, in turn, affect adhesion-dependent

AURKA-mediated RalA activation. Taken together, this suggests that AURKA does regulate adhesion-dependent RalA activity through RGL1. The possible role AURKA could have in mediating RGL1 localization as it regulates RalA, remains to be tested.
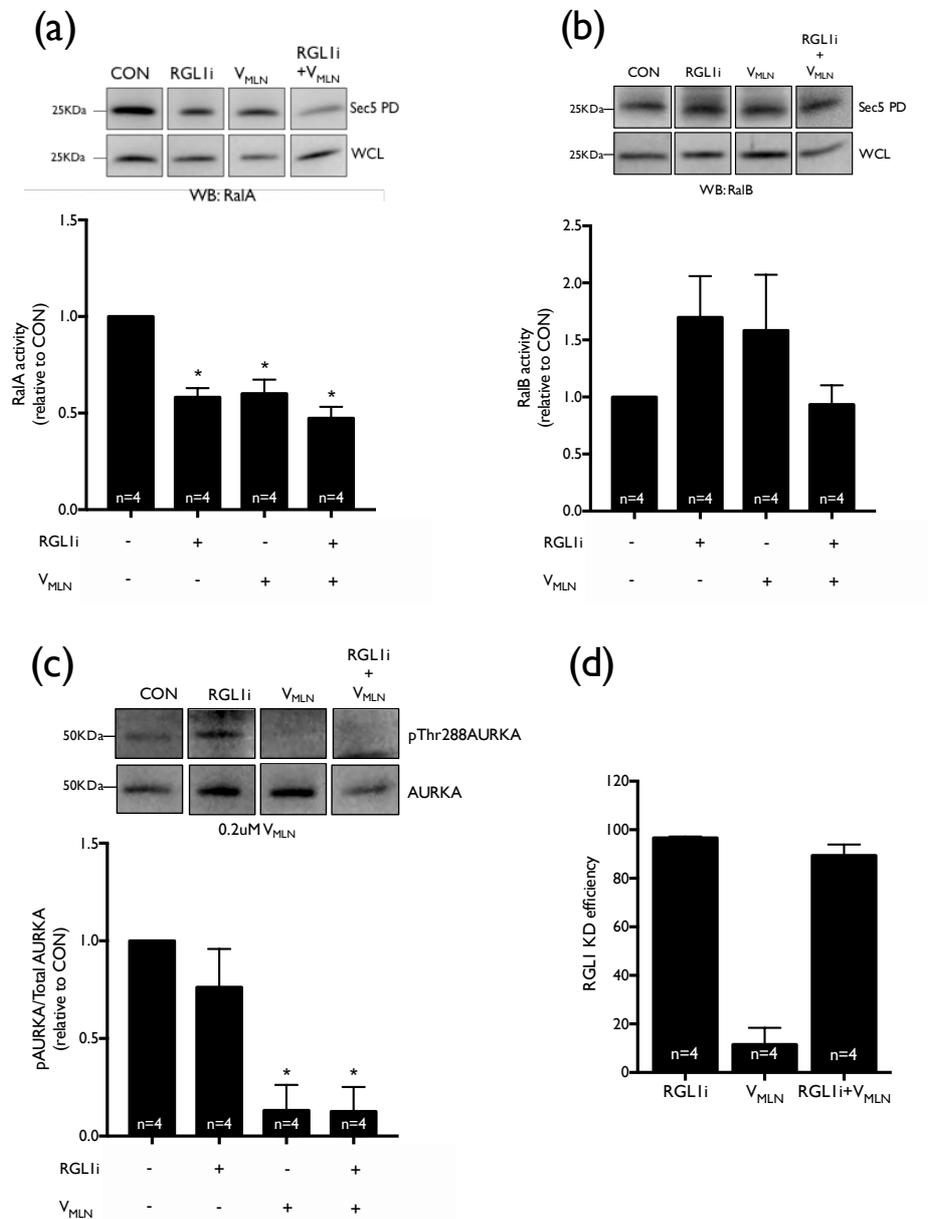

Figure 3.14: Effect of combined RGL1 knockdown and AURKA inhibition on adhesion-dependent RalA activity. Western blot detection (upper panel) and quantitation (lower panel) of (a) Active RalA and (b) Active RalB pulled down by GST-Sec5, and the total RalA/RalB in WCL, (c) Phosphorylation on Threonine 288 residues of AURKA (pThr288AURKA) and total AURKA (d) RT-PCR quantitation for knockdown efficiencies in lysates from serum-starved, control or RGL1 knockdown WT-MEFs, suspended for 60 mins + 30mins with V_{MLN}/Empty DEX and re-adherent on fibronectin for 15mins with V_{MLN}/Empty DEX. The ratio of pAURKA/TotalAURKA, active RalA and Active RalB were normalized to the respective SA (equated to 1). The graph represents the mean ± SE data from four independent experiments. Samples compared and represented in blots were all run and developed together.

Blots are cropped to remove blank lanes in between only for representation. Statistical analysis was done using the single-sample t-test and p values, if significant, are represented in the graph (* $p < 0.05$).
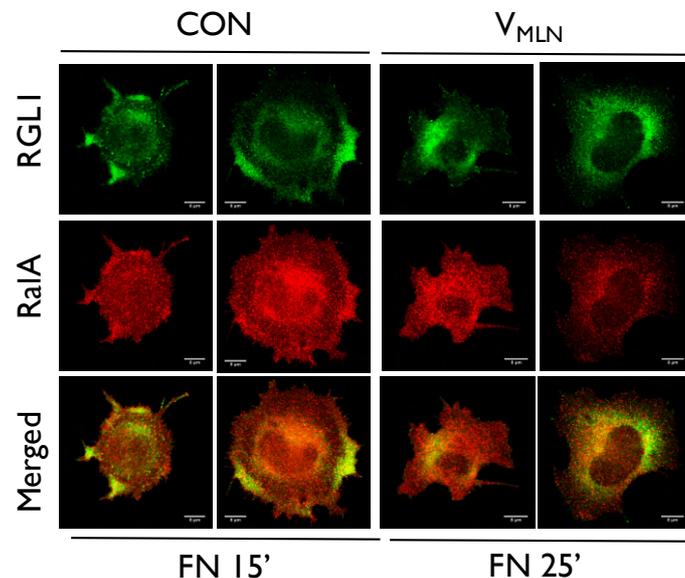

Figure 3.15: Effect of AURKA inhibition on the localization of endogenous RalA and Myc-tagged RGL1 in re-adherent cells. Re-adherent WT-MEFs expressing Myc-tagged RGL1 detected with an anti-myc antibody (Myc-RGL1) and endogenous RalA with the anti-RalA antibody (RalA) shows their co-localization in membrane ruffles and membrane protrusions in control cells which are altered in V_{MLN} treated WT-MEFs. Data is representative of 30 cells from three independent experiments. Scale bars in images are set at 8 μm.

3.2.7 Ral GEF, RGL1 regulates RalB (but not RalA) activation in migrating WT-MEFs

Role of RalA vs RalB in migration of untransformed non-cancer cells has been previously studied in normal rat kidney cells (epithelial cells) where RalB regulated delivery of secretory vesicles at plasma membrane necessary for directional movement (Rossé et al. 2006). Two other studies have reported the role of RalB in cancer cell migration and invasion, in that one study stated that RalA and RalB perform non-overlapping and antagonistic functions in bladder (UMUC3) and prostate (DU145) cancer cell migration, where inhibiting RalB expression causes significant decrease in migration but inhibiting both RalA and RalB simultaneously had no effect on migration (Oxford et al. 2005).

In another study with 9 pancreatic cancer cell lines, it was shown that both RalA and RalB are required for tumor metastasis, but RalB played a more important role than RalA in this process. In this study, suppression of RalA but not RalB caused a significant increase in migration of Panc-1 cells and suppression of RalA or RalB had no effect in Capan-2 and AsPc-1 cells (Lim

et al. 2006). Together these studies suggest that the relative contribution of RalA and RalB in cell migration might be cell type specific. We hence test the role RGL1 could have in the regulation of RalA vs RalB in migrating WT-MEFs in the presence of serum growth factors. Knowing the importance of localization of Ral isoforms and GEFs in mediating their activation and function, we also compared their relative localization in re-adherent early spreading cells vs actively migrating cells.

RGL1 and RalB knockdown cells migrate significantly faster as compared to control WT-MEFs reflecting in an early wound closure (Figure 3.16 a and b). RalA knockdown cells, on the other hand, migrated comparable to control cells (Figure 3.16a and b). The knockdown efficiencies of all three proteins were comparable as seen by RT-PCR analysis (Figure 3.16c). This raises the possibility that RGL1 mediated regulation of Ral, might be altered in serum growth factor stimulated migrating cells, possibly regulating RalB but not RalA in actively migrating WT-MEFs in the presence of serum growth factors. To confirm the same, we tested the activation of RalA vs RalB in RGL1 knockdown migrating WT-MEFs in the presence of 5% FBS. RGL1 knockdown by individual siRNA as well as smart pool both caused a significant drop in RalB activity (Figure 3.17b) in migrating cells as opposed to RalA activity in the same cells (Figure 3.17a), confirming RGL1 specificity to indeed be altered to regulate RalB (but not RalA) in migrating cells. We further find the localization of RGL1 to be significantly different in migrating cells (almost entirely intracellular and not at the cell membrane) as compared to re-adherent early spreading cell (cell membrane protrusions) (Figure 3.18). Unlike RGL1 which is intracellular, we found RalA to be evenly distributed in the cell (Figure 3.18). We could not compare the localization of endogenous RalB with RGL1 as multiple attempts of immunostaining endogenous RalB were unsuccessful. Together these results suggest that in migrating WT-MEFs, localization of RGL1 is altered which as per reported literature (Cascone et al. 2008) might be one of the contributing factors of differential regulation of RalB (but not RalA) in migrating cells.
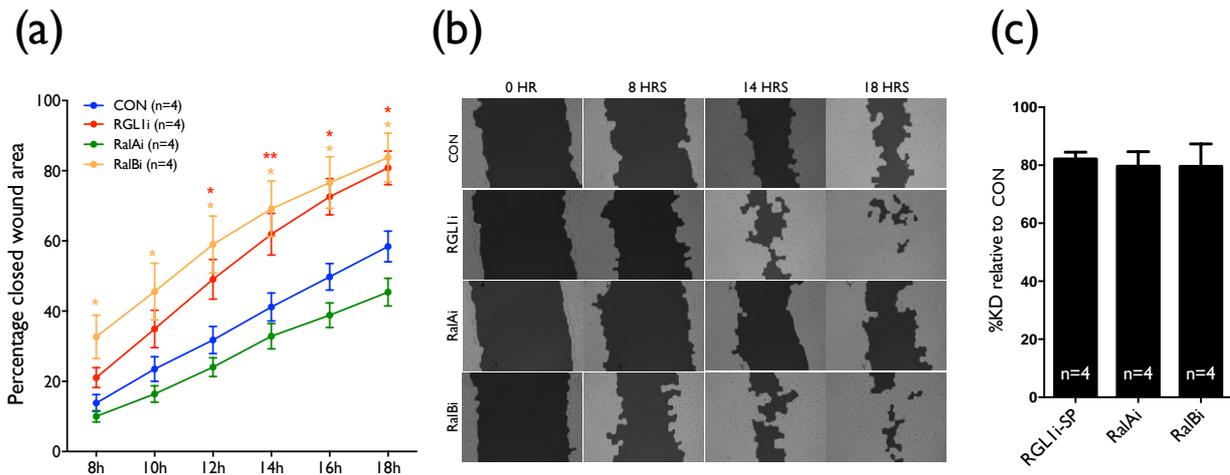
Figure 3.16: Effect of RGL1, RalA and RalB knockdown on the migration of WT-MEFs in wound healing assay. Calculated percentage wound area healed (b) and representative ROI (a) are shown from smart pool siRNA treated Control (CON), RGL1 (RGL1i), RalA (RalAi) and RalB (RalBi) knockdown WT-MEFs post 8, 14 and 18hours of scratch formation. Aleast three beacons per group were analyzed for each experiment. Data was obtained from results of four independent experiments. Student t test was used to calculate significance (* p < 0.05, ** p < 0.01) (c) shows the respective KDs to be >70% for each protein. *Data credit: Neha Deshpande*
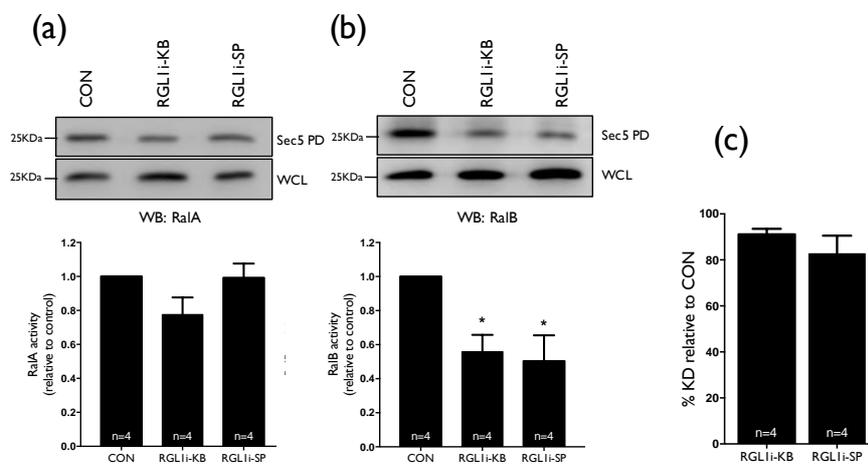

Figure 3.17: Effect of RGL1 knockdown on Ral activation in actively migrating WT-MEFs. Western blot detection and quantitation of (a) active RalA and (b) active RalB pulled down by GST-Sec5 (Sec5 PD) and total RalA/RalB in the whole-cell lysate (WCL) was done from control (CON), individual siRNA (RGL1i-KB) and smart pool siRNA (RGL1i-SP) targeting RGL1 treated WT-MEFs. Calculated percentage of active RalA/RalB levels were normalized to respective control (CON) (equated to 1). The graph represents the mean ± standard error from four independent experiments. Statistical analysis of all data was done using the single sample T-Test and P values are as indicated (* p<0.05). (c) RGL1 KD efficiency using individual siRNA and the smart pool was determined using RT-PCR.
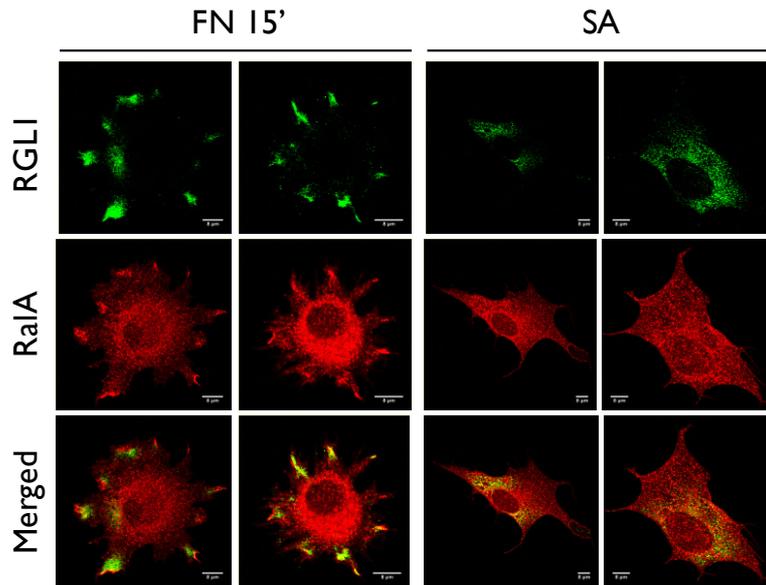
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Figure 3.18: Localization of Myc-tagged RGL1 and endogenous RalA during cell spreading in re-adherent cells and migrating stable adherent WT-MEFs. Re-adherent WT-MEFs expressing Myc-tagged RGL1 detected with an anti-myc antibody (RGL1) and endogenous RalA with the anti-RalA antibody (RalA) shows their co-localization in membrane ruffles and membrane protrusions in serum deprived re-adherent WT-MEFs (Left panel – FN 15'). Stable adherent WT-MEFs actively migrating in the presence of serum growth factors show RGL1 to be intracellular with RalA also showing some localization to membrane edges (Right panel - SA). Data is representative of 30 cells from three independent experiments. Scale bars in images are set at 8 μm.

3.2.8 AURKA does not affect RGL1 dependent RalB activation in migrating WT-MEFs.

Knowing the ability of AURKA to phosphorylate RalA and its role in regulating RGL1-RalA crosstalk downstream of integrin-mediated adhesion, we next asked if it could have any role in regulating RGL1-RalB crosstalk during cell migration. Inhibition of AURKA with V_{MLN} (0.2μM for 1 hour) was followed by wound scratch and incubation with 0.06μM V_{MLN} for the duration of the wound healing experiment. AURKA inhibition did not affect the rate of wound closure as compared to control cells (Figure 3.19a), neither did it affect RalA (Figure 3.20b) or RalB (Figure 3.20b) activity. This while confirming that AURKA is not involved in regulating RalB activation also suggests that the regulation of RalA in adherent migrating WT-MEFs to indeed be independent of AURKA. We tested the localization of RGL1 in V_{MLN} treated stable adherent WT-MEFs to evaluate whether the localization is effected like in early spreading event (Figure 3.15). The localization of RGL1 and RalA are both not effected by V_{MLN} treatment (Figure 3.21), confirming that AURKA mediated regulation RGL1 and RalA to
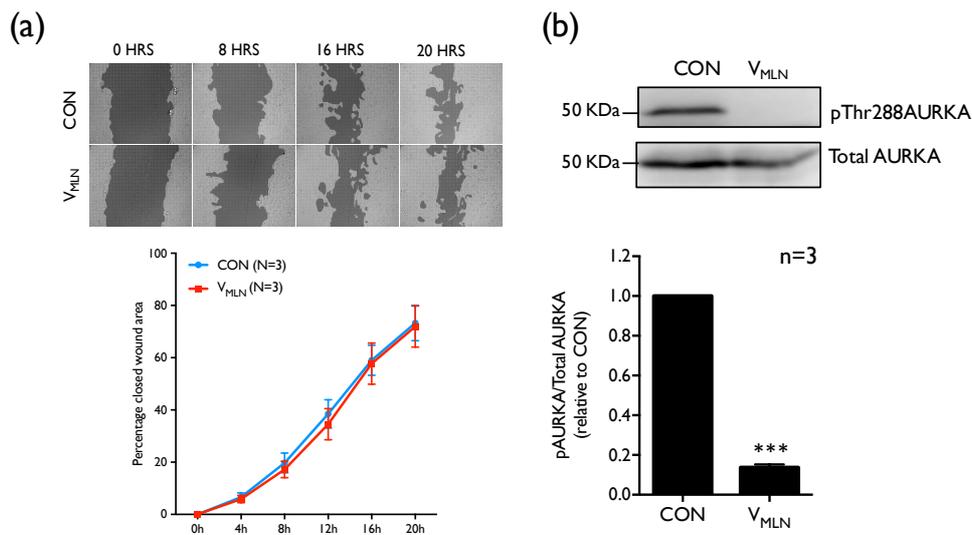
indeed be different in migrating WT-MEFs.

Figure 3.19: Effect of AURKA inhibition using V_{MLN} on the migration of WT-MEFs in wound healing assay. (a) Calculated percentage wound area healed and representative ROI are shown from Control and V_{MLN} treated WT-MEFs post 8, 16 and 20hours of scratch formation. (b) Western blot detection (upper panel) and quantitation (lower panel) of phosphorylation on Threonine 288 residues of AURKA (pThr288AURKA) and total AURKA were done from lysates of control (CON) and 0.06μM V_{MLN} treated (V_{MLN}) WT-MEFs in presence of serum at end of migration assay. The calculated ratio of pAURKA/TotalAURKA was normalized to respective control (CON). The graph represents the mean ± standard error from three independent experiments. Statistical analysis of all data was done using the single sample T-Test and P values are as indicated (***p<0.001). *Data credit: Mayuresh Konde*
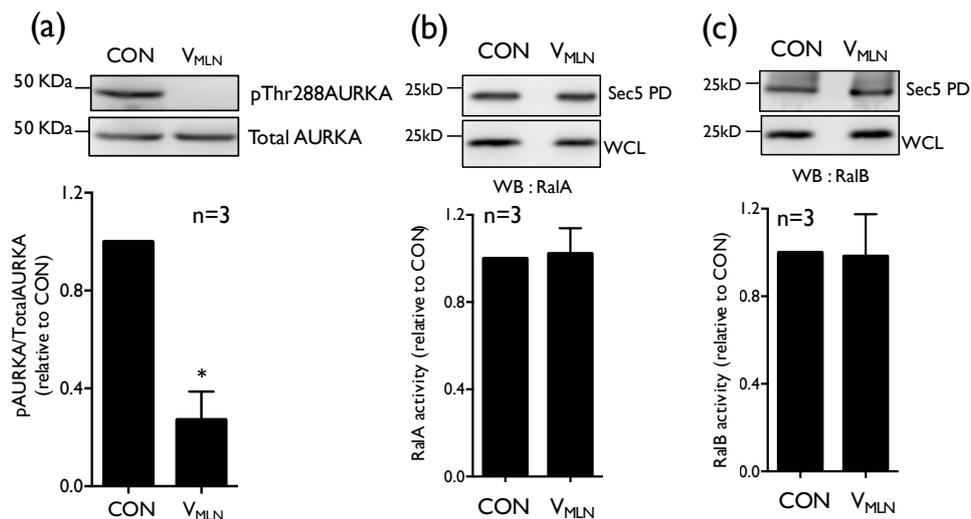

Figure 3.20: Effect of AURKA inhibition on Ral activation in actively migrating WT-MEFs. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on Threonine 288 residues of AURKA (pThr288AURKA) and total AURKA (b) Active RalA and (c) Active RalB pulled down by GST-Sec5, and the total RalA/RalB in

the whole-cell lysate (WCL) was done from control (CON) and V_{MLN} (0.06μM) treated (V_{MLN}) WT-MEFs in presence of serum. Calculated percentage of active RalA/RalB levels and the ratio of pAURKA/TotalAURKA were normalized to respective control (CON) (equated to 1). The graph represents the mean ± standard error from three independent experiments. Statistical analysis of all data was done using the single sample T-Test and p-values if significant are indicated in the graph (* $p<0.05$, **$p<0.01$). *Data credit: Mayuresh Konde*
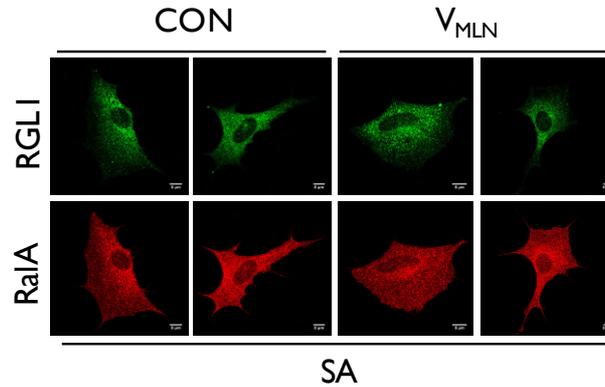

Figure 3.21: Effect of AURKA inhibition on the localization of endogenous RalA and Myc-tagged RGL1 in migrating WT-MEFs. WT-MEFs expressing Myc-tagged RGL1 detected with an anti-myc antibody (Myc-RGL1) and endogenous RalA with the anti-RalA antibody (RalA) shows their internal localisation which remains the same in V_{MLN} treated WT-MEFs. Data is representative of 30 cells from three independent experiments. Scale bars in images are set at 8 μm.

3.3 Summary and Open questions

In this chapter, we report that cell-matrix adhesion regulates the activity of Aurora Kinase A (AURKA). This regulation we find is dependent on serum growth factors and could partly be mediated by the adhesion-dependent control of the cell cycle. Dextran encapsulated AURKA inhibitor MLN8237 (V_{MLN}) developed in the lab was used to inhibit AURKA and revealed it to regulate cell-matrix adhesion-dependent RalA phosphorylation, activation and spreading. Inhibition of AURKA significantly reduces (but not completely abolishes) RalA phosphorylation (Serine 194) and activity that does not recover on re-adhesion to matrix fibronectin. This suggests that AURKA regulates RalA phosphorylation and activity downstream of integrin-mediated adhesion in WT-MEFs. The residual phosphorylation of RalA observed in suspended cells under the complete inhibition of AURKA suggests that other regulators of RalA S194 phosphorylation, namely PP2A β or Protein Kinase A may have a role alongside AURKA to regulate adhesion-dependent RalA activation. Having multiple stimuli regulating the activation is a hallmark of small GTPases and hence exploring regulatory crosstalks involving AURKA/PKA becomes an interesting open question.

Ral activation in cells is known to be regulated by RalGEFs and RalGAPs. Knockdown of the individual GEFs revealed a Ras-dependent GEF, RGL1 to mediate adhesion-dependent RalA activation and early cell spreading. Rescue experiment using a siRNA resistant RGL1 construct restores adhesion-dependent RalA activation confirming the role of RGL1 in the same. The joint targeting of AURKA and RGL1 further reveals that they likely work along the same pathway to regulate cell-matrix adhesion-dependent RalA activation. In re-adherent WT-MEFs, RGL1 and RalA co-localize to membrane ruffles and protrusions, sites of active integrin signaling, that we observe is dependent on AURKA activity. This membrane localization of RGL1 and RalA is altered by AURKA inhibition using V_{MLN}, suggesting that AURKA regulates the localization of not just RalA as has been previously reported (Lim, Brady, Kashatus, Ancrile, Der, Cox & Counter 2009b)but also RGL1, which could in turn contribute to adhesion-dependent regulation of RalA. AURKA has been previously reported to phosphorylate and activate a RalGEF RalGDS, and hence might be involved in phosphorylating and regulating RGL1. However, the exact mechanism of AURKA mediated regulation of RGL1 localization and activation in early spreading cells remains to be tested. A comprehensive in-silico analysis of probable AURKA phosphorylation sites on RGL1 has been

initiated in the lab. Further, in-vitro evaluation of this regulation will help us understand the adhesion-AURKA-RGL1-RalA pathway better and also open new avenues to study other physiological functions where this regulatory crosstalk might be playing significant roles (cell cycle and mitochondrial dynamics are few such cellular processes that could be explored).

It has been previously reported that RalGEFs can regulate Ral isoforms differentially in response to specific stimuli. In our studies, we find RGL1 seen to regulate RalA (not RalB) in early re-adherent cell spreading, regulates RalB (not RalA) in migrating WT-MEFs in the presence of serum growth factors. This RGL1 dependent regulation of RalB we find is independent of AURKA activity. Further, the inhibition of AURKA also does not alter the localization of RGL1 and RalA in actively migrating WT-MEFs in the presence of serum growth factors. As discussed earlier, we could not compare the localization of endogenous RalB with RGL1 as multiple attempts of immunostaining endogenous RalB were unsuccessful. Together these results suggest that in migrating WT-MEFs, localization of RGL1 is altered which might be one of the contributing factors of differential regulation of RalB (but not RalA) in these cells. The possible role serum growth factors could have in RGL1-RalB-migration however remains to be evaluated.

Together our studies provide a better understanding of how Ral isoforms are regulated differentially by Aurora Kinase A and RGL1 downstream of distinct stimuli to perform significantly diverse physiological functions. These studies make it evident that Ral isoforms integrate multiple upstream signals to manoeuvre divergent physiological roles downstream of adhesion. Moreover, these studies provide another compelling example of how sub-cellular localization determines the regulation of Ral isoforms by same RalGEF. Further, exploring the functional relevance of such crosstalk in other cellular process where each of the players are individually involved will be of great interest.
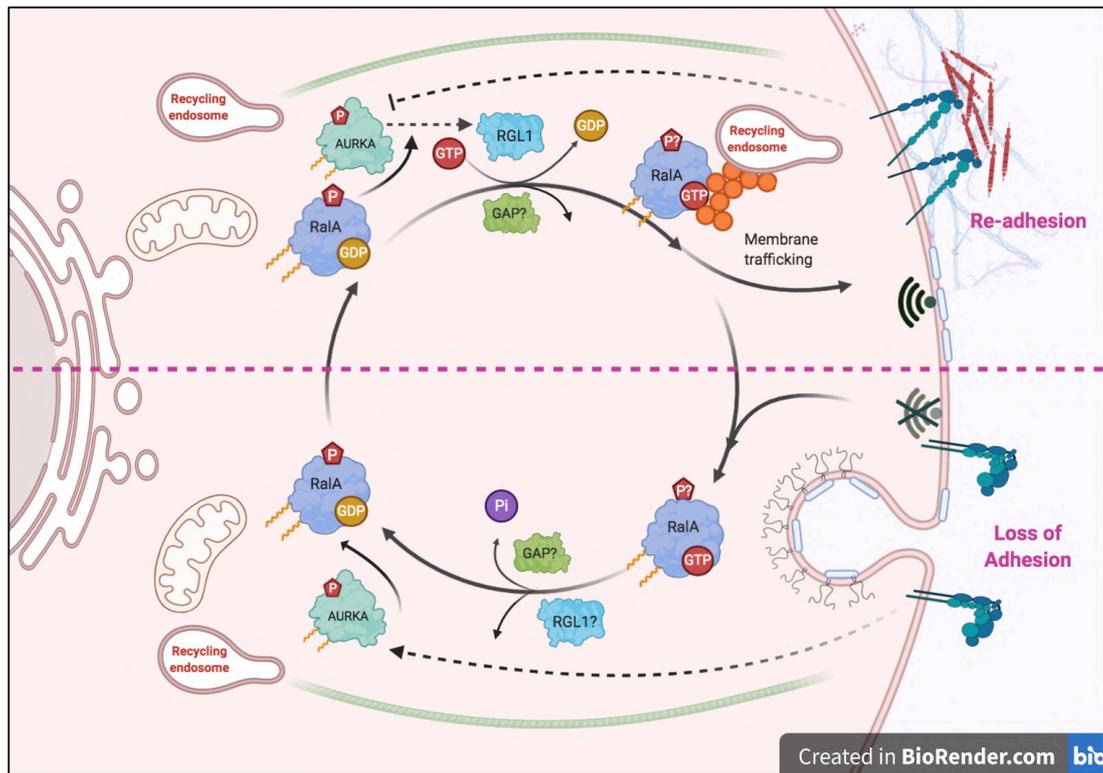
Figure 3.22: Schematic of the proposed model of adhesion-dependent regulation of RalA in WT-MEFs. Loss of adhesion triggers caveolae-mediated endocytosis of membrane raft micro-domains and inactivation of RalA (RalA-GDP). Loss of adhesion also causes an increase in AURKA activity (auto-phosphorylation) which in-turn phosphorylates RalA at Serine194 altering its localisation. Upon re-adhesion, AURKA activity is re-stored to stable adherent levels causing a decrease in RalA phosphorylation simultaneously increasing RGL1-mediated GTP loading. The GTP-bound RalA along with the exocyst complex restores the membrane rafts and facilitates cell spreading. AURKA also regulates RGL1 localisation to membrane edges in re-adherent cells, however, the exact mechanism of this regulation remains to be evaluated. AURKA is necessary for RGL1-mediated GTP loading, as inhibition of AURKA fails to restore RalA-GTP activity and cell spreading upon re-adhesion. (Note: Components of the figure are for representational purpose and not true to scale) (Created using Biorender).

3.4. Conclusion

Cell-matrix adhesion regulates Aurora Kinase A which in turn controls adhesion-dependent RalA (but not RalB) activation through the Ral GEF, RGL1, to regulate cell spreading. Further, RGL1 regulates RalB (but not RalA) activation, independent of AURKA, in migrating cells.

Chapter 4

Evaluate a self-assembling Dextran nano-vesicle to efficiently deliver AURKA inhibitor MLN8237 and specifically target AURKA-RalA

4.1 Rationale

RalA and RalB belong to the Ras superfamily of small GTPases and were first identified based on their sequence similarity to the H-Ras, K-Ras, and N-Ras proteins. RalA and RalB, although being 82% identical (Gentry et al. 2014), are reported to play significantly divergent roles in normal and cancer phenotypes (Martin & Der 2012). These isoforms differ significantly in their C-terminal domain (50% identical), facilitating their differential post-translational modifications. This further contributes to their distinct subcellular localization and function (Gentry et al. 2015). RalA is necessary for the anchorage-independent growth of cancer cells and RalB is critical for the survival of tumour cells (Bodemann & White 2008; Camonis & White 2005; Martin et al. 2011; Y. Yamazaki et al. 2001). Anchorage-independent signaling and the growth are vital contributors to the tumour phenotype of cancer cells, that aid in tumour cell invasion and metastasis (S. Mori et al. 2009). Inhibiting RalA hence is an attractive method to target the oncogenic potential of cancer cells. Creating effective drug candidates against small GTPases is inherently difficult and Ral isoforms with 82% sequence identity are unlikely to be distinguished by an inhibitor. The only know specific inhibitor for Ral, BQU57 (C. Yan et al. 2014), is seen to act on both Ral isoforms (RalA and RalB) (C. Yan et al. 2014). This lack of specificity could compromise its effectiveness. Hence targeting an upstream regulator of Ral GTPases that can differentially regulate their activation and function could not only allow for specific inhibition of RalA vs RalB but it could also become a tool to evaluate their differential involvement in cellular functions.

Aurora kinase A (AURKA), as discussed in earlier chapters is one such regulator that can differentially phosphorylate and regulate RalA activity in cells. AURKA phosphorylates RalA at Serine194 (a site missing in RalB) during mitosis and causes it to localize to the mitochondrial membrane where RalA, with the help of RalBP1, regulates mitochondrial fission (Kashatus et al. 2011). AURKA, in turn, belongs to a family of serine/threonine kinases that play crucial roles in mitotic entry and exit during cell division. AURKA and Aurora Kinase B (AURKB) are structurally similar, with 72% identity in their kinase domains and have similar protein substrate preferences in vitro (Vader & Lens 2008; Ohashi et al. 2006; Ferrari et al. 2005). In vivo, their unique substrate specificities and differential functions arise from their distinct cellular localization leading to discrete interactions with binding partners (Carmena & Earnshaw 2003a; S. Li et al. 2015). AURKA and AURKB activation are regulated by their phosphorylation on the Threonine288 (Thr288) and Threonine232 (Thr232) residue,

respectively, in the activation loop (Carmena & Earnshaw 2003a) (Bodemann & White 2008; Feig 2003). As discussed in chapter 3, AURKA regulates adhesion-dependent activity of RalA in WT-MEFs, supporting its role in anchorage-dependent signaling (N. Balasubramanian et al. 2010; Pawar et al. 2016). The presence and contribution of such an AURKA−RalA crosstalk in cancers and the possible role targeting this could have can be tested by specifically inhibiting AURKA. In the last two decades, several aurora kinase inhibitors have been identified and entered clinical trials (A. Yan et al. 2011; Gautschi et al. 2006; Libertini et al. 2010). Known AURKA inhibitor MLN8237 (Alisertib), seen in Phase II clinical trial was limited by its lack of solubility in water which greatly impairs its uptake by cells. (de Groot et al. 2015). This poor solubility makes the drug delivery across the cell membrane inefficient, needing higher amounts to be administered to achieve necessary intracellular concentrations compromising the specificity of these drugs (de Groot et al. 2015; Bavetsias & Linardopoulos 2015; Melichar et al. 2015). We have hence in collaboration with the lab of Dr Jayakannan (IISER, Chemistry) developed a new drug delivery protocol that would allow for a more efficient delivery of poorly water-soluble AURKA inhibitor which could significantly help better target AURKA and hence AURKA-RalA crosstalk in cancer cells.

Earlier studies from the lab have established the use of a unique class of dextran (polysaccharide) nano-vesicles to transport drugs, such as cisplatin, doxorubicin, and camptothecin, in breast and colon cancers (Pramod et al. 2012; Pramod, Shah, et al. 2015; Pramod et al. 2014). These studies have also identified caveolin-1 lacking cancer cells (like breast cancer MCF-7 cells) to better uptake these nano-vesicles (Pramod et al. 2014). In this chapter, we have evaluated the efficiency of a self-assembling dextran nano-vesicle encapsulated MLN8237 (V_{MLN}) to preferentially and significantly inhibit AURKA over free MLN8237 in breast cancer MCF-7 cells. The uptake of the V_{MLN} in 2D, as well as 3D micro-environments, has also been tested. The role AURKA has in regulating anchorage-independent RalA activity in cancer cells has also been evaluated.

4.2 Results

4.2.1. Developing Dextran based nano-vesicle as a delivery system for Aurora Kinase A inhibitor MLN8237

The hydrophilic dextran was made amphiphilic by substituting its backbone with a renewable hydrophobic unit carboxylic acid-substituted 3-pentadecylphenol (PDP) via aliphatic ester linkages, which can be cleaved in the enzyme-rich intracellular lysosomal compartment (Pramod et al. 2012). The percentage of PDP relative to the dextran backbone was kept constant at 5% to balance the amphiphilicity in the newly designed dextran derivative (Figure 4.1b). The substituted dextran polymer and AURKA inhibitor MLN8237 (Figure 4.1a) were dissolved in DMSO + water and subjected to nanoprecipitation, and then dialyzed against Milli-Q water for 48hours using a semipermeable membrane. The vial in Figure 4.1c shows vesicles loaded with the MLN8237 to be a clear solution, suggesting enhanced aqueous solubility of the drug in the vesicular nano-scaffold. The DLS histogram of the drug-loaded vesicles (Figure 4.1c) confirmed its monomodal size distribution of 200 ± 20 nm. The formation of spherical nanostructures in an aqueous medium was confirmed by AFM analysis of the MLN8237-loaded nano-objects, which shows a typical two hump height profile corresponding to their vesicular geometry (Figure 4.1c). HR-TEM microscopic analysis of these vesicles showed the existence of a distinct hydrophobic thin-layer separating their inner and outer walls in the nano-scaffold (Figure 4.1c). Together, these studies show the water-insoluble MLN8237 to be effectively loaded in the hydrophobic layer of the dextran vesicles with a Drug loading efficiency (DLE) of 56% and Drug loading content (DLC) of 4.0 μg/mg/mL. This MLN8237-loaded dextran vesicle is hereafter referred to as V_{MLN}.

(a) **(b)** **(c)**
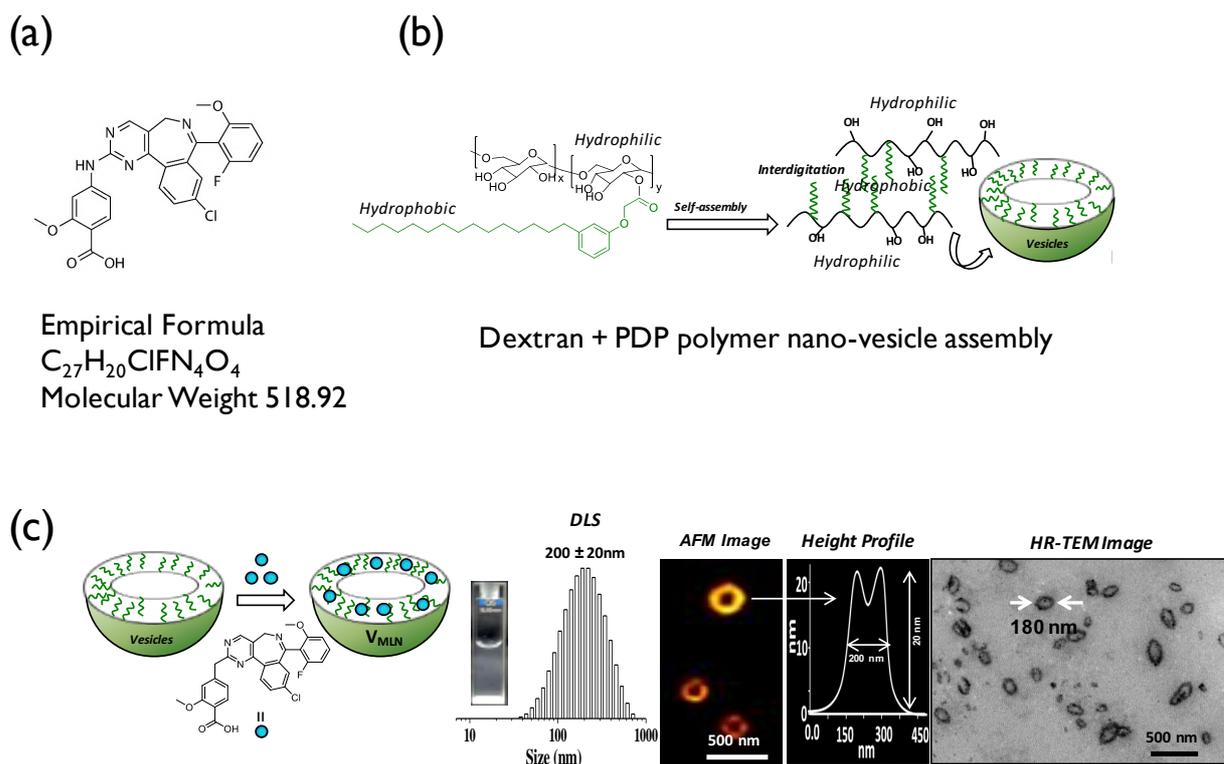
Empirical Formula
$C_{27}H_{20}ClFN_4O_4$
Molecular Weight 518.92

Hydrophilic

Hydrophobic

Self-assembly

Hydrophilic

DLS
200 ± 20nm

Vesicles V_MLN

Size (nm)

AFM-Image

180 nm

Pico Image Elements 7.1.7106

Figure 4.1: Characterisation of MLN8[...]
(V_MLN). (a) Structure of AURKA inhibit[...]
assembly of the newly designed dextran [...]
(V_MLN), DLS histogram of V_MLN, AFM [...]
V_MLN (arrows mark the size and distinct [...]
Deshpande

The stability and release of MLN8237 fr[...]

7.4 at 37 °C (Figure 4.2a), V_MLN was fou[...]

over 25hours. As the dextran nano-vesicle was made through the conjugation of a hydrophobic

PDP subunit via a biodegradable aliphatic ester linkage, these vesicles show more than ~85%

of MLN8237 to be released in the presence of 10U of esterase enzyme at pH 7.4 and 37 °C

(Figure 4.2a).

Earlier computational docking studies from our lab have revealed the aliphatic chemical

linkage connecting the hydrophilic dextran backbone and hydrophobic PDP tail to perfectly

lock in the enzymatic pocket of the esterase enzyme (Pramod, N. U. Deshpande, et al. 2015).

Thus, cleavage of this aliphatic ester linkage will disturb the amphiphilicity in the scaffold,

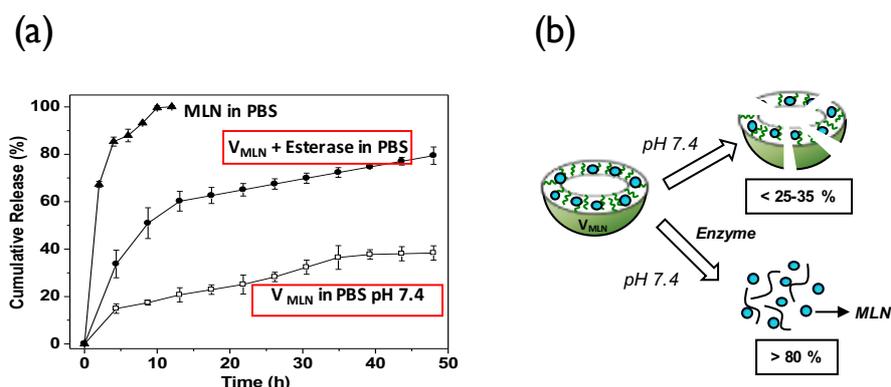causing it to disassemble and hence release the drug (MLN8237).

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Figure 4.2: Release profile for MLN8237 from V$_{MLN}$. (a) The cumulative release profile of MLN8237 from V$_{MLN}$ in PBS in the presence or absence of esterase at pH 7.4, 37 °C. (b) Schematic representation of enzyme responsiveness of V$_{MLN}$ releasing MLN8237. *Data credit - Nilesh Deshpande*

4.2.2. Encapsulated MLN8237 (V$_{MLN}$) is taken up by MCF-7 cells in 2D as well as 3D micro-environments

We further tested the uptake of V$_{MLN}$ in breast cancer MCF-7 cells in 2D as well as 3D micro-environments. MCF-7 cells in our earlier studies were seen to better take up the dextran nano-vesicle, relative to normal fibroblasts (Pramod et al. 2014). As MLN8237 is not inherently fluorescent, visualization of its cellular uptake as V$_{MLN}$ or tracking its release in cells is not possible. A water-soluble fluorescent dye Rhodamine B was hence loaded in the hydrophilic core of the dextran vesicles along with MLN8237. These Rhodamine B and MLN8237 containing vesicles are called V$_{MLN+RhB}$ (Figure 4.3a).
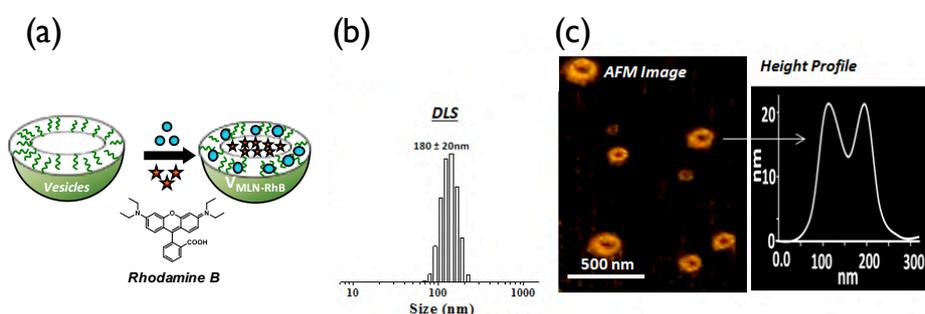

Figure 4.3: Characterization of MLN8237 + Rhodamine-B dual loaded Dextran nano-vesicles (a) Schematic of MLN8237 and Rhodamine B dual loading in dextran vesicles (b) DLS histogram of the dialysed V$_{MLN+RhB}$ recorded in PBS at pH 7.4 and 37°C (c) AFM image of V$_{MLN+RhB}$ on mica surface. *Data credit - Nilesh Deshpande*

The DLS histogram (Figure 4.3b) of these dual-loaded vesicles also showed a monomodal distribution corresponding to the size of 180 ± 20 nm. Further, AFM images of $V_{MLN+RhB}$ (Figure 4.3b) confirmed their preservation of the vesicular geometry reported earlier in these dextran nano-vesicles. The optical properties of the Rhodamine B loaded vesicles were tested by recording their absorbance and emission spectra (Figure 4.4b and c). The absorbance spectrum confirmed absorbance peaks at maxima λ 310 and 550 nm, corresponding to MLN8237 and Rhodamine B chromophores (Figure 4.4b).
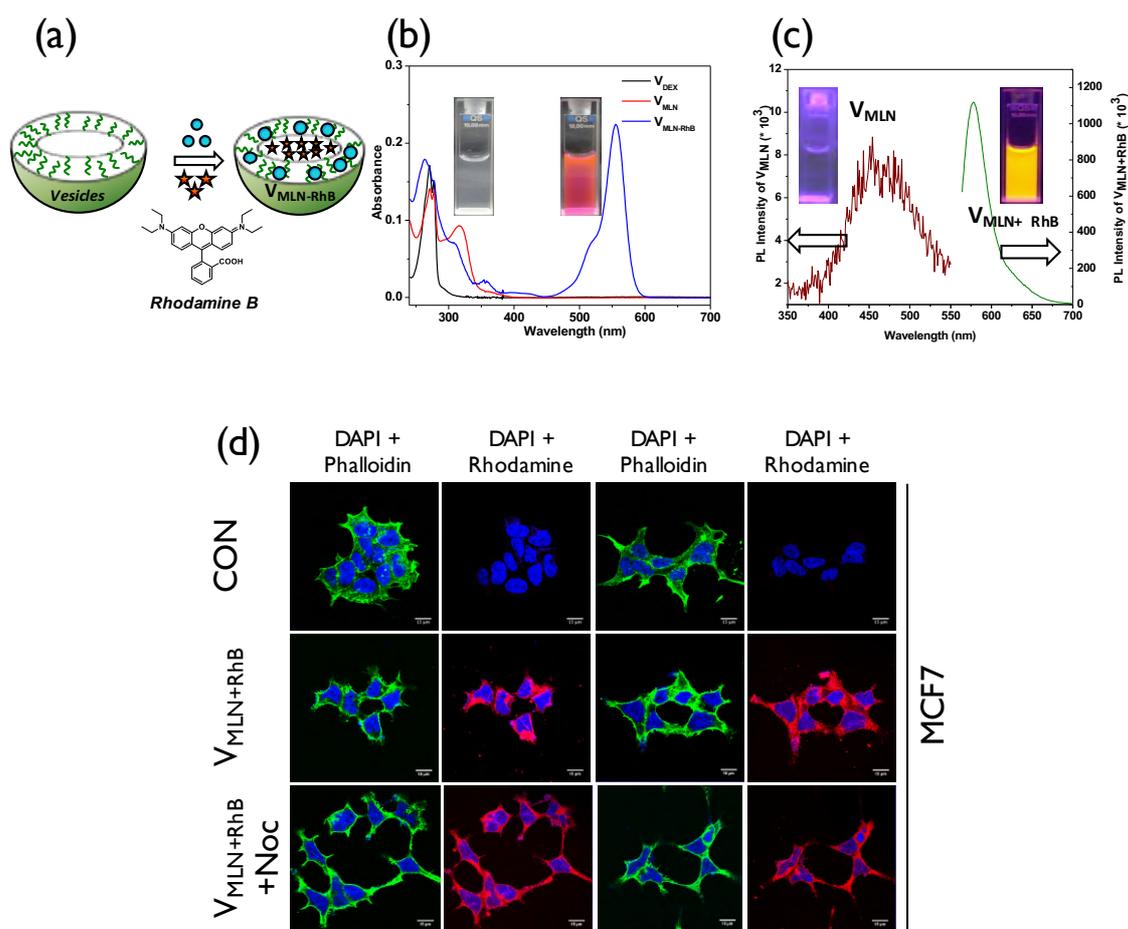

Figure 4.4: Characterisation and uptake of $V_{MLN+RhB}$ in MCF-7 cells. (a) Schematic of MLN8237 and Rhodamine B dual loading in dextran vesicles (b) Absorbance spectra of V_{MLN}, $V_{MLN+RhB}$, and V_{DEX}. (c) Emission spectra of V_{MLN} and $V_{MLN+RhB}$. (d) Uptake of dual loaded DEX nano-vesicle with RhB (1.37 μM) and MLN8237 (0.5 μM) ($V_{MLN+RhB}$) in the absence or presence of 10 ng/mL nocodazole ($V_{MLN+RhB}$ + Noc) was visualized by confocal microscopy in MCF-7 cells treated for 48hours relative to untreated CON. The nucleus was counterstained with DAPI, and the actin cytoskeletal network stained with phalloidin conjugated to Alexa-488 (DAPI + phalloidin). The images shown are representative of three independent experiments that gave similar results.

The emission spectra of $V_{MLN+RhB}$ exhibited a strong red-luminescence with maxima at 580 nm (Figure 4.4c). This allows for $V_{MLN+RhB}$ to be used to visualize the cellular uptake of the drug-containing nano-vesicle in breast cancer MCF-7 cells. Stable adherent MCF-7 cells treated with $V_{MLN+RhB}$ for 48hours show robust fluorescence which is uniformly distributed throughout the cell (Figure 4.4d). MCF-7 cells embedded in 3D collagen gels incubated with $V_{MLN+RhB}$ for 3 hours also show fluorescence evenly distributed throughout the cell body (Figure 4.5), suggesting that self-assembling dextran nano-vesicle is a suitable tool to efficiently deliver MLN8237 in cells in both 2D as well as 3D microenvironments.
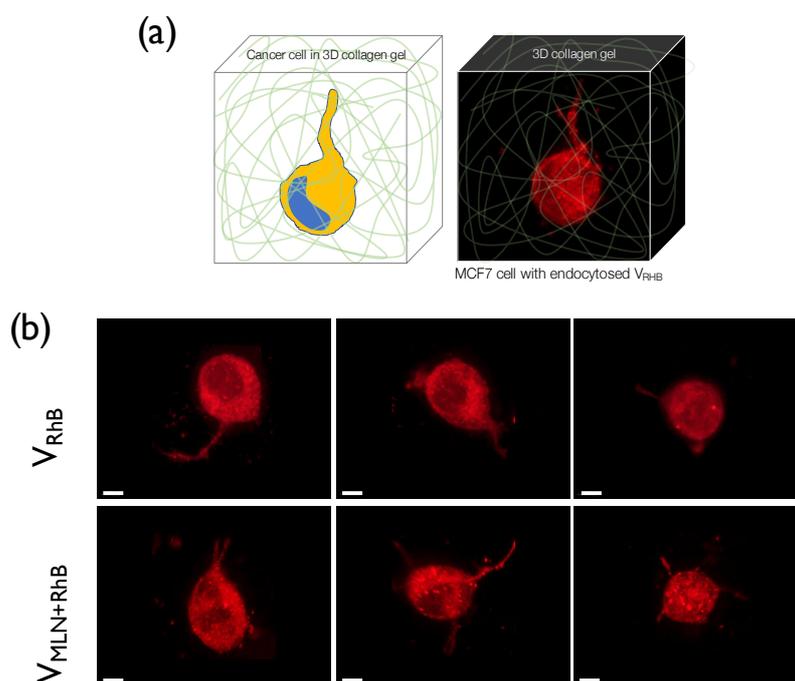

Figure 4.5: Uptake of DEX-encapsulated MLN8237 ($V_{MLN+RhB}$) by MCF-7 in the 3D microenvironment. (a) Schematic of embedding of cells in 3D collagen gels. (b) Uptake of DEX nano-vesicle with Rh-B (5 μM) alone or nano-vesicle with Rh-B (5 μM) and MLN823 (1.3 μM) was visualized by confocal microscopy in MCF-7 cells treated for 3h. The images shown are representative of two independent experiments that gave similar results (scale bar 5 μm). *Data credit: Vishakha Kasherwal*

4.2.3. Encapsulated MLN8237 (V_{MLN}) inhibits Aurora Kinase A better than free MLN8237

Being able to specifically inhibit AURKA over AURKB has been the basis of designing inhibitors like MLN8237. However, its poor solubility in water and reduced bioavailability does suggest concentrations needed for significantly inhibiting AURKA could likely affect AURKB. Hence, we tested if the efficient uptake of V_{MLN} could result in the specific and

significantly better inhibition of AURKA in MCF-7 cells. MCF-7 cells show the high activity of AURKA and RalA which is prominently phosphorylated on Serine 194 (Figure 4.6).
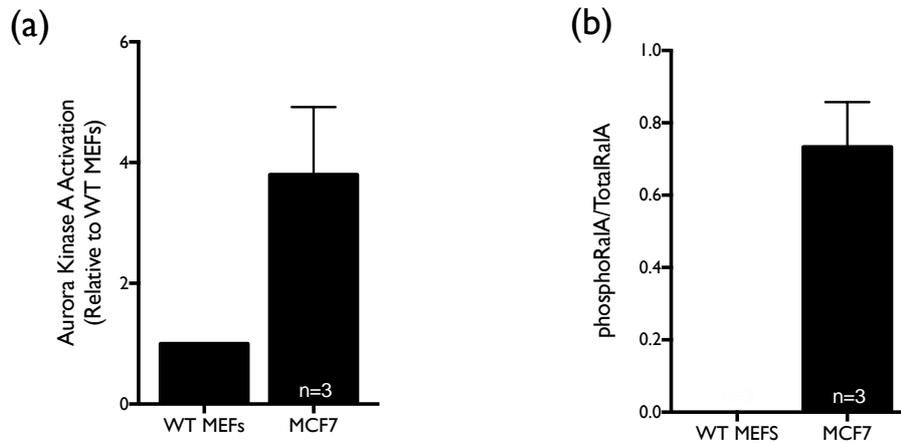

Figure 4.6: Activity of AURKA and phosphorylation of RalA in MCF7 cells. Phosphorylation of (a) AURKA at Threonine 288 residue with respect to the same in WT MEFs and (b) RalA at Serine 194 residue in MCF-7 cells. The graph represents the mean ± SE of pAURKA/TotalAURKA, pRalA/TotalRalA from three independent experiments.

To evaluate the efficiency of inhibition with V_{MLN} over free MLN, we first arrested cells at the G2-M phase by treating cells with nocodazole to boost up the phosphorylated levels of AURKA and AURKB. This causes up to a 2-fold increase in basal AURKA (Figure 4.7a) and AURKB activation (Figure 4.7b). Nocodazole-treated MCF-7 cells were incubated with a range of concentrations ranging from 0.01μM to 0.1μM of V_{MLN} and Free MLN for 24hours (Figure 4.8). Treatments with concentrations of 0.02μM and 0.04μM showed inhibition of AURKA activity by V_{MLN} to be significantly better (~4- and ~8- fold, respectively) than the free MLN8237 (Figure 4.8b). This suggests V_{MLN} could work as well as the free drug at less than half of its concentration. In these studies, the DEX nano-vesicle scaffold by itself only very marginally affected AURKA activity (Figure 4.8a and 4.8b). Next, we tested the efficiency of V_{MLN} to inhibit basal AURKA activity in asynchronous cells and although needing higher Western blot exposures, we found a 24hour treatment of V_{MLN} to significantly better inhibit AURKA at lower concentrations (0.02 μM) than free MLN8237 at same concentrations (Figure 4.9).
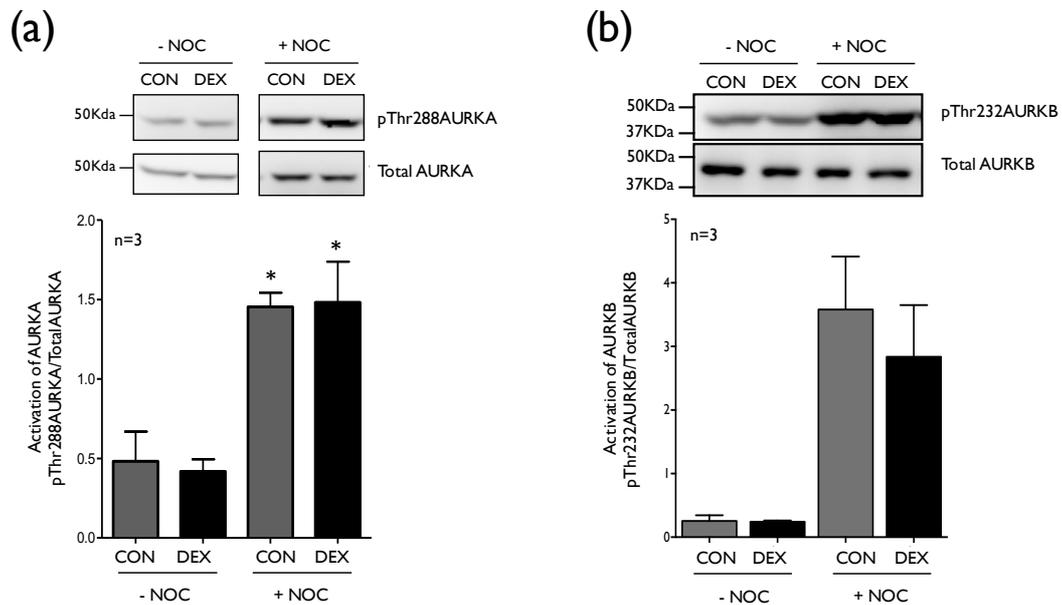
Figure 4.7: Nocodazole treatment of MCF-7 cells. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation of Threonine 288 residue (pThr288 AURKA) and total AURKA and (b) phosphorylation of Threonine 232 residue (pThr232 AURKB) and total AURKB, from lysates of MCF-7 cells treated with 10ng/ml of nocodazole for 24hours in presence of DMSO (CON) or empty nano-vesicle scaffold (DEX). Samples compared and represented in blots were all run and developed together. Blots are cropped to remove blank lanes in between only for representation. The ratio of pAURKA/AURKA and pAURKB/AURKB are represented in the graph as mean $\pm$ SE from three independent experiments. Statistical analysis was done using a paired Students t-test, and p values, if significant, are represented in the graph (* $p < 0.05$).

Figure 4.8: Inhibition of AURKA using Free MLN8237 and V$_{MLN}$. Western blot detection (a) and quantitation (b and c) of phosphorylation of Threonine 288 residue of AURKA, total AURKA and actin, from lysates of MCF-7 cells treated with 10ng/ml nocodazole and 0.01µM to 0.1µM of either Free MLN8237 (MLN) or nano-vesicle encapsulated MLN8237 (V$_{MLN}$). DMSO was used as the control for MLN and empty nano-vesicle scaffold (DEX) was used as the control for V$_{MLN}$. The ratio of pAURKA/AURKA treated with MLN or V$_{MLN}$ were normalized to their respective nocodazole treated controls (NOC 10) (equated to 1), and these values are represented in the graph as mean ± SE from three independent experiments. Statistical analysis was done using a paired Students t-test, and p values, if significant, are represented in the graph (* $p < 0.05$).
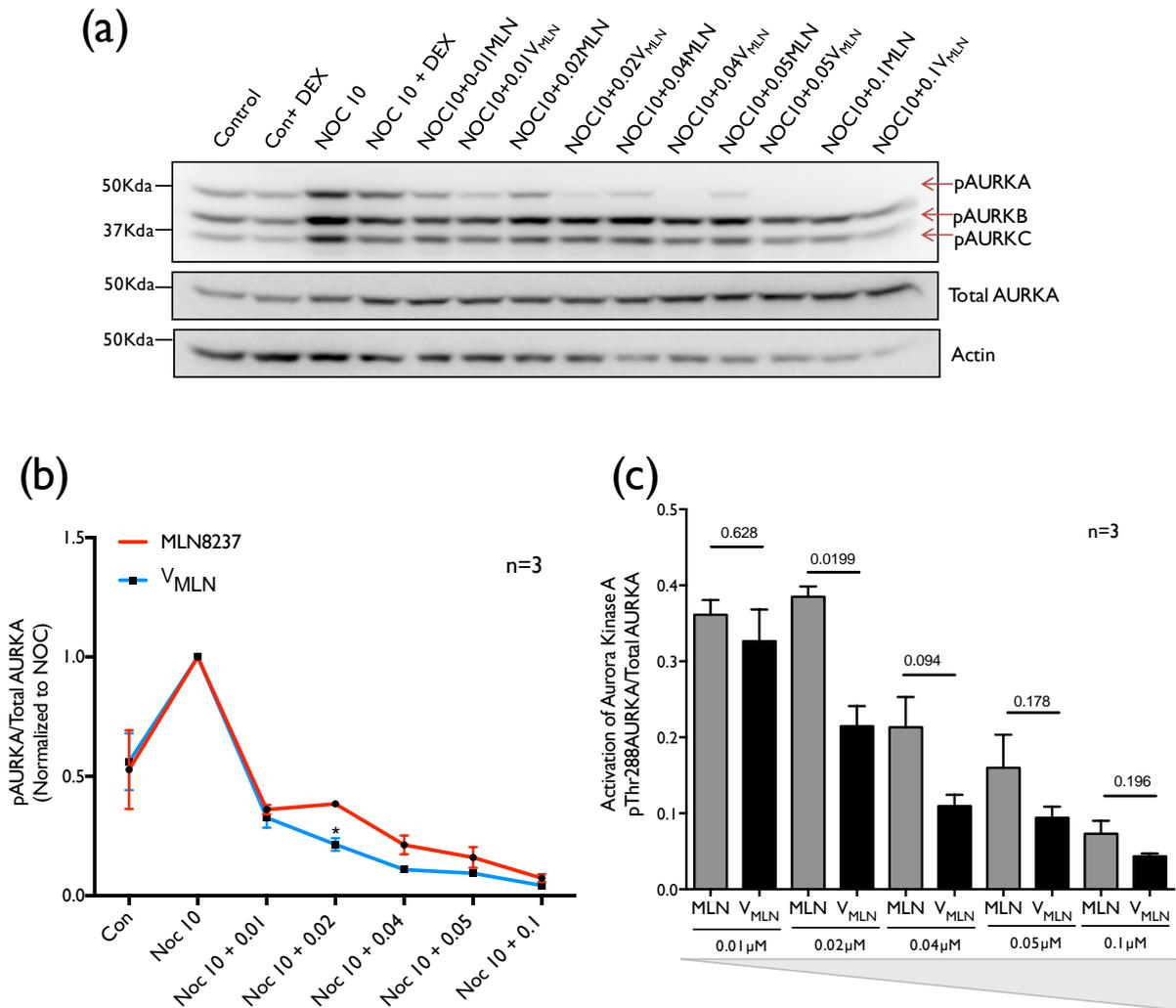
Figure 4.9: Inhibition of endogenous AURKA using Free MLN8237 and V$_{MLN}$. Western blot quantitation of phosphorylation of Threonine 288 residue of AURKA and total AURKA, from lysates of MCF-7 cells, treated 0.02μM to 0.08μM of either Free MLN8237 (MLN) or nano-vesicle encapsulated MLN8237 (V$_{MLN}$). The ratio of pAURKA/AURKA is represented in the graph as mean ± SE from five independent experiments. Statistical analysis was done using a paired Students t-test, and p values, if significant, are represented in the graph (* $p <$ 0.05).
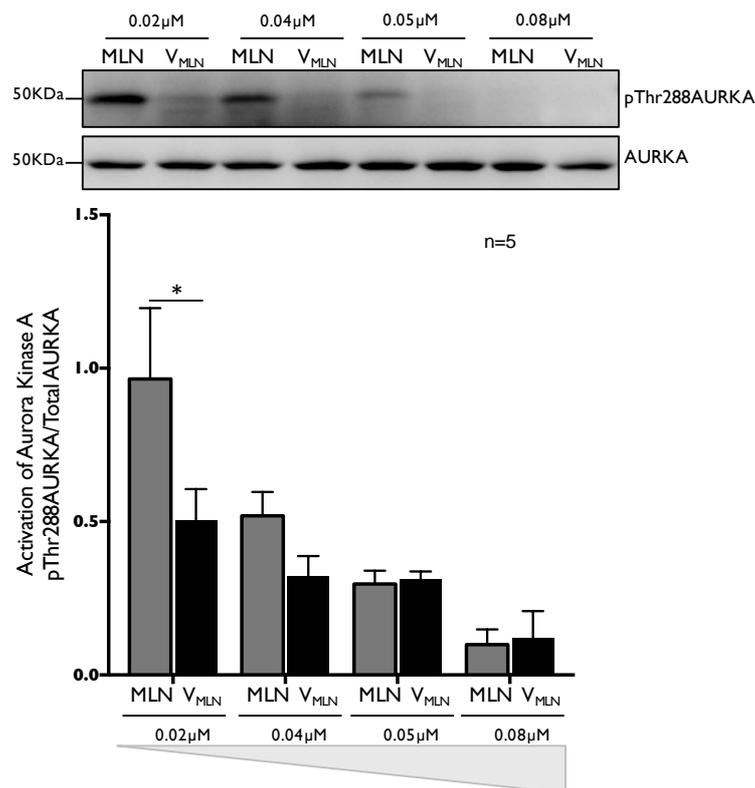
4.2.4 V$_{MLN}$ preferentially inhibits Aurora Kinase A over Aurora Kinase B

We further found V$_{MLN}$ (at 0.02 and 0.04 μM) to significantly better inhibit AURKA over AURKB, unlike the free drug that comparably affected both (Figure 4.10a and b). This confirms the nano-vesicle-mediated delivery of MLN8237 at low concentrations to indeed be more effective against AURKA in MCF-7 cells. This inhibition was done in nocodazole treated cells to ease the detection of phosphorylation of AURKA and AURKB in cells. We hence further evaluated the effect V$_{MLN}$ has on inhibition of basal AURKA and AURKB activation (without nocodazole) in these cells.

While needing higher Western blot exposures for their detection, basal aurora kinase activation in MCF-7 cells incubated with V$_{MLN}$ (0.02 μM) for 48hours showed ~94% inhibition of AURKA activity, relative to control and DEX- treated cells (Figure 4.11 a). This is

significantly better than the effect free MLN8237 has at the same concentration (~56% inhibition). V_{MLN} also did not affect AURKB activation, unlike the free drug (~28% inhibition) (Figure 4.11b). This makes V_{MLN} at the concentration and time used here (0.02µM and 48hours) a significantly more potent version of MLN8237.
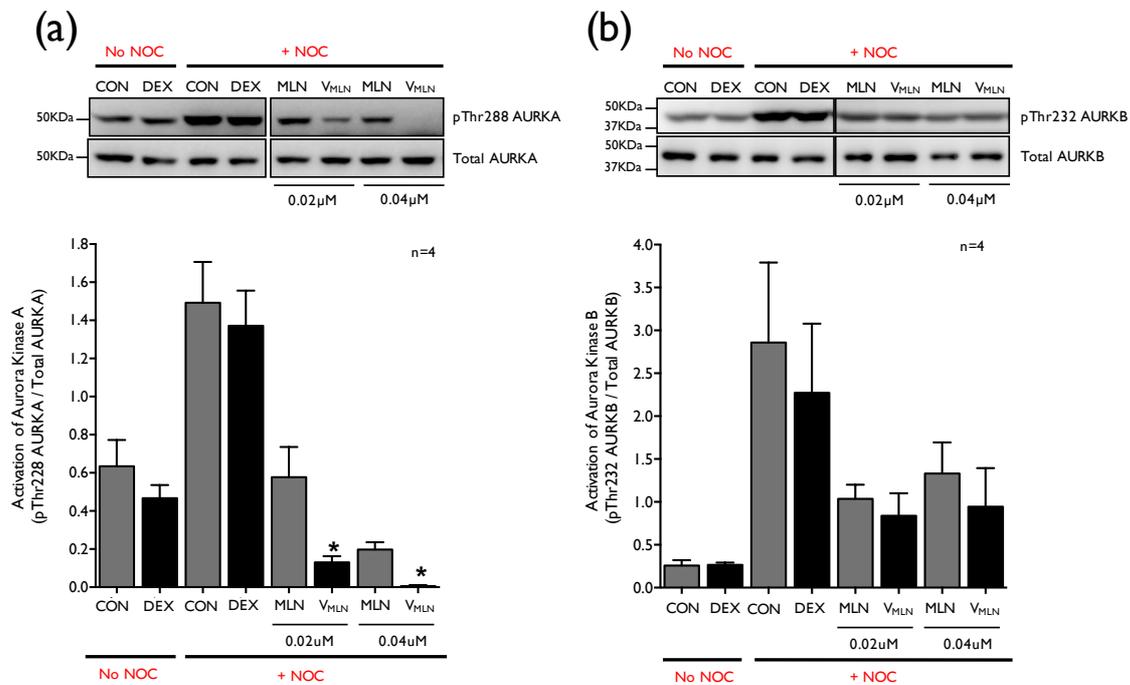

Figure 4.10: Inhibition of AURKA vs AURKB activity with Free MLN vs V_{MLN}. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation of Threonine 288 residue (pThr288 AURKA) and total AURKA and (b) phosphorylation of Threonine 232 residue (pThr232 AURKB) and total AURKB, from lysates of MCF-7 cells treated with 10ng/ml of nocodazole for 24hours in presence of DMSO (CON) or empty nano-vesicle scaffold (DEX), 0.02µM Free MLN or V_{MLN} and 0.04µM Free MLN or V_{MLN}. Samples compared and represented in blots were all run and developed together. Blots are cropped to remove blank lanes in between only for representation. The ratio of pAURKA/AURKA and pAURKB/AURKB are represented in the graph as mean ± SE from four independent experiments. Statistical analysis was done using paired Students t-test, and p values, if significant, are represented in the graph (* $p < 0.05$).
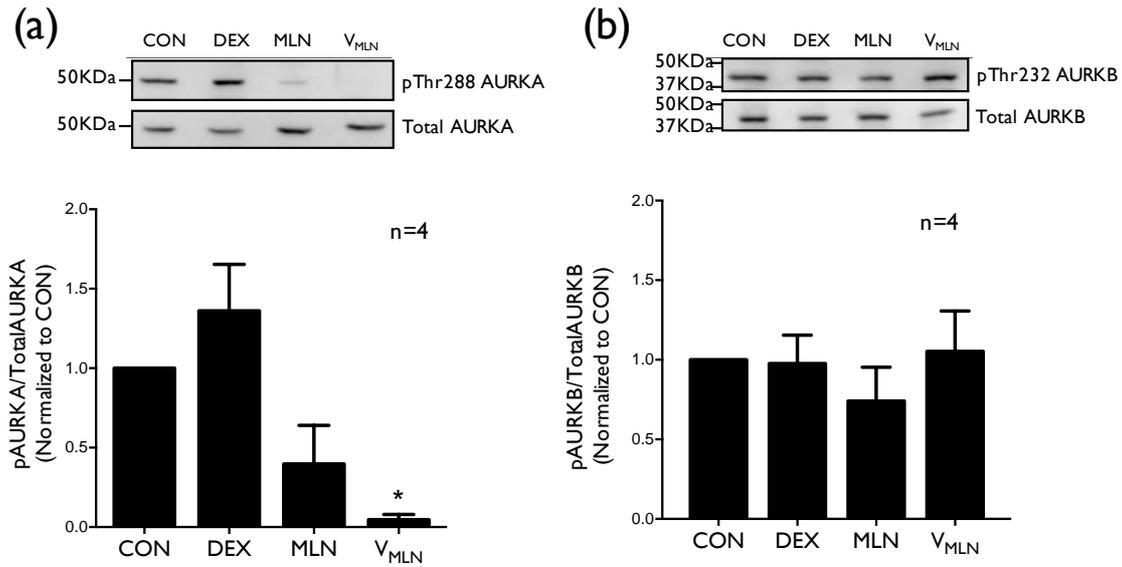
Figure 4.11: Inhibition of endogenous AURKA vs AURKB activity with Free MLN vs V_{MLN}. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation of Threonine 288 residue (pThr288 AURKA) and total AURKA and (b) phosphorylation of Threonine 232 residue (pThr232 AURKB) and total AURKB, from lysates of MCF-7 cells treated with 0.02µM Free MLN (MLN) or encapsulated MLN (V_{MLN}). DMSO and empty nano-vesicle scaffold were used as controls for MLN and V_{MLN}, respectively. The ratio of pAURKA/AURKA and pAURKB/AURKB were normalized to their respective controls (CON) (equated to 1) and were represented in the graph as mean ± SE from four independent experiments. Statistical analysis was done using a single-sample t-test, and p values, if significant, are represented in the graph (* $p < 0.05$).

4.2.5 V_{MLN} mediated inhibition of Aurora Kinase A specifically inhibits RalA (not RalB) in MCF-7 cells

Knowing that we could now specifically target AURKA using V_{MLN}, we next tested if and how V_{MLN}-mediated specific inhibition of AURKA affects downstream activation of RalA (vs RalB) in MCF-7 cells. To address this query, we used V_{MLN} at 0.02µM shown to inhibit AURKA (Figure 4.11a) in stably adherent MCF-7 cells in the presence of serum growth factors. This, we find, specifically affects RalA phosphorylation at Serine 194 residue (Figure 4.12) known to be regulated by AURKA, suggesting that AURKA might be involved in regulating RalA in anchorage-independent MCF-7 cells. To further evaluate if this phosphorylation regulates the activity of RalA in these cells we did GST-Sec5 pull-down assay from V_{MLN} and Free MLN treated MCF-7 cells comparing them to untreated (CON) and only DEX treated (DEX) MCF7 cells. The V_{MLN} treated cells show a significant (~25%) decrease in RalA activity (Figure 4.13a), without effecting RalB activity (Figure 4.13b) suggesting

inhibition of AURKA does effect RalA phos

an AURKA-RalA pathway in stably adhere

while showing a 40% drop in RalA phosph

RalA, indicating that, the phosphorylation

regulated in these cells, paving way for an

pathway along with AURKA-mediated one

these cancer cells.

Figure 4.12: Inhibition of RalA phospho
detection (upper panel) and quantitation (low
(pSer194RalA) and total RalA, from lysates
nano-vesicle scaffold (DEX), 0.02µM Free
normalized to CON (equated to 1) and is r
independent experiments. Percentage value
relative to control. Statistical analysis was
significant, are represented in the graph (* p

Knowing the significant role RalA plays i

Meier, Dasgupta, Diwanji, N. Deshpan

Balasubramanian 2016b), we further evalu

anchorage-independent MCF-7 cells suspen

of MCF-7 cells with V_{MLN} (0.02µM) caus

activity (Figure 4.14), which is seen to be

vesicle scaffold treated (DEX) cells. Taken

Pico Image Elements 7.1.7106

regulates stable adherent as well

significance of this AURKA-Ra

formation in these cells will be d

(a)

25kDa — 25kD —

25kDa — 25kD —

WB

RalA activity (relative to control)

1.25

1.00

0.75

0.50

0.25

0.00

CON   DE

0.0
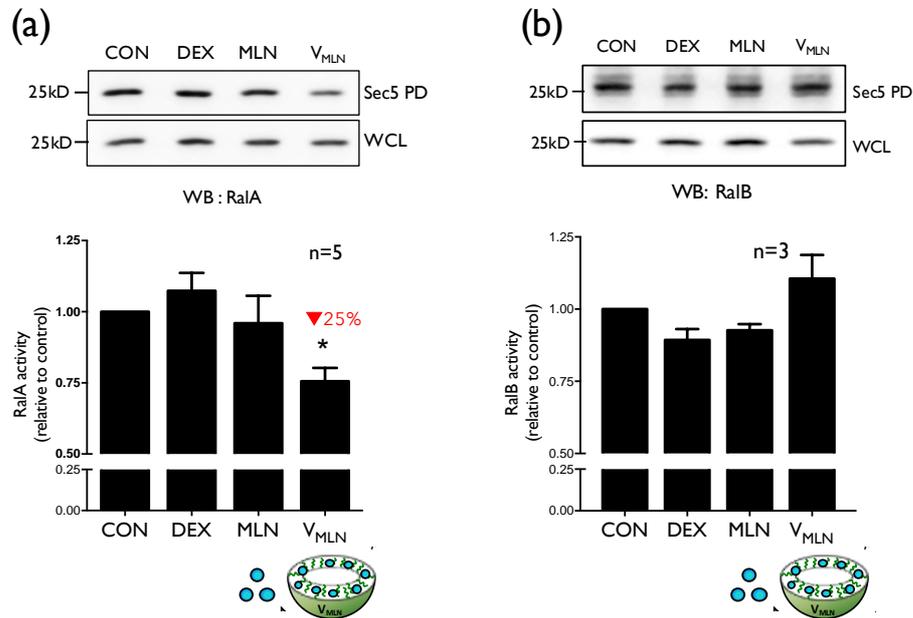
Figure 4.13: Inhibition of RalA detection (upper panel) and quan and total RalA and RalB, respecti empty nano-vesicle scaffold (DE normalized to their respective co ± SE from at least three indep inhibition for the treatment relati t-test, and p values, if significant

Size (nm)

1200

1000

800

600

400

200

0   5   V$_{MLN}$

CON   DEX   MLN   V$_{MLN}$

25KDa —

25KDa —

WB: RalA

RalA Activity (relative to CON)

1.5

n=3

1.0

0.5

0.0

*

CON   DEX   MLN   V$_{MLN}$

Pico Image Elements 7.

Pico Image Elements 7

from three independent experiments. Statistical analysis was done using single sample t-test, and p values, if significant, are represented in the graph (* $p < 0.05$)

4.3. Summary and open questions

One of the aims of the Thesis was to develop a drug delivery system for Aurora Kinase inhibitor MLN8237 to increase its specificity to inhibit AURKA. Once developed this drug delivery system was used as a tool to target AURKA and study its role in anchorage-dependence of non-cancerous WT-MEFs and anchorage-independence of cancerous cells. However, the objective of using a cancer cell line to develop and evaluate the efficacy of the drug delivery system was two-fold, one being that the ultimate long-term goal of the study is to be able to target AURKA-RalA driven tumours using V_{MLN} (dextran nano-vesicle encapsulated MLN8237) in mice models and evolve it as a targeted therapy against 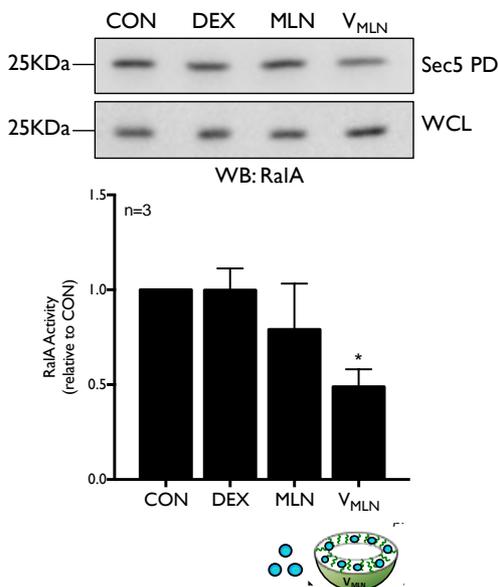AURKA-RalA. We have hence selected a breast cancer cell line, MCF-7 where AURKA has reported to be over-expressed and RalA has shown to be active in anchorage-independent condition in absence of oncogenic Ras. Secondly, in the previous study from the lab where the dextran nano-vesicle was tested for delivery of other chemotherapy drugs (Doxorubicin and camptothecin), it was observed that MCF-7 cells show a better uptake of the vesicle as compared to WT-MEFs (Pramod et al. 2014). It was hence decided that for developing and testing the efficacy of V_{MLN}, MCF-7 would be a better cell line system.

In this chapter, we have developed a polymer nano-vesicle delivery system to encapsulate AURKA inhibitor MLN8237 to preferentially target AURKA and RalA downstream. To test the efficiency of V_{MLN} mediated inhibition of AURKA we chose breast cancer cell line MCF-7, seen to express AURKA and RalA which is prominently phosphorylated on Serine 194, and shown to take up these nano-vesicles efficiently in our previous studies (Pramod et al. 2012). These nano-vesicles (<200 nm in size) carry MLN8237 (V_{MLN}) in their intermembrane space with up to 85% of it released by the action of esterase enzyme(s) in cells. Further, a Rhodamine B fluorophore trapped in the hydrophilic core of V_{MLN} ($V_{MLN+RhB}$) allowed us to visualize its uptake and localization in MCF-7 cells in a 2D and 3D microenvironment. We have tested AURKA inhibition across a range of concentrations (0.01μM and 0.1μM) of MLN8237 (Free drug) and V_{MLN} (dextran nano-vesicle encapsulated MLN8237). Treatment given at 0.02μM V_{MLN} for 48hour was seen to inhibit AURKA 8-fold better than the free drug, without affecting AURKB activity. The dextran nano-vesicle scaffold by itself did not affect either AURKA or AURKB activity. Hence, for further experiments, we chose 0.02μM V_{MLN} for use. V_{MLN} treated cells further show specific inhibition of RalA (but not RalB) downstream of AURKA, making it a useful tool and drug candidate for targeting cancer cells. V_{MLN} can be used as a

tool, as seen in studies evaluating the role of AURKA in regulating RalA downstream of integrin-mediated adhesion in normal mouse fibroblasts (Chapter 3). V_{MLN} can also been used to target and evaluate the role AURKA-RalA crosstalk has in anchorage-independent growth of Ras-independent vs Ras-dependent cancers, as detailed in Chapter 5.
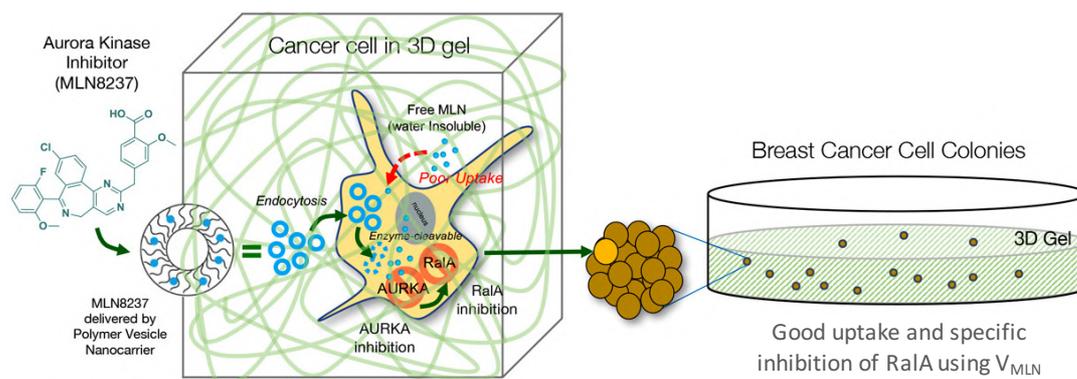
Figure 4.15: Schematic of nano-vesicle mediated delivery of MLN8237 in MCF-7 cells

The fact that V_{MLN} can cause this dramatically improved inhibition of AURKA (over AURKB) in cells makes it a particularly significant tool in understanding the differential role AURKA vs AURKB have in cellular processes, like mitosis, migration, ciliary disassembly, centrosome amplification, aneuploidy, and cell viability. The effect V_{MLN} mediated AURKA inhibition has on RalA (but not RalB) activation, makes is particularly better than the free drug that affects neither. This allows for us to detect the AURKA−RalA crosstalk and test its effect on anchorage independent growth of cancer cells. AURKA-mediated differential regulation of RalA is known to be through its phosphorylation on the Serine194 residue (missing in RalB), which affects its localization in cells. The differential roles RalA and RalB have downstream of integrin-mediated adhesion, is known to be dependent on their localization (our unpublished data). V_{MLN} has provide a tool for us to evaluate the role AURKA has in regulating Ral localization and activation in normal and cancer cells.

V_{MLN} although shows preferential inhibition of AURKA over AURKB, the exact mechanism by which this specificity is mediated remains to be fully explored. MLN8237 is inherently specific to AURKA and does not affect AURKB at low concentrations. The fact that we can now deliver the drug using V_{MLN} at these low concentrations ensures this specificity is visible

in how the drug acts in cells. This inherent specificity of the drug is mediated by the ability of the drug to bind with significantly higher affinity to AURKA. Hence, while the localisation of AURKA vs AURKB could contribute to their regulation and function, this may not be the cause of their differential inhibition by V_{MLN}. Improved bio-availability, improved delivery, better uptake and relative activity of AURKA vs AURKB in different cancer cell types could all contribute to the same. The relative ability of V_{MLN} to inhibit AURKA could vary between cancers and a comprehensive study to determine its effectiveness across different types of cancers has been initiated in the lab.

4.4. Conclusion

Self assembling Dextran polymer nano-vesicle allows for the efficient delivery of MLN8237 (V_{MLN}) at low concentrations in normal and cancer cells to preferentially inhibit Aurora Kinase A (over Aurora Kinase B). This further targets the AURKA mediated activation of RalA (but not RalB), unlike the free drug. This confirms the existence of an AURKA-RalA crosstalk in breast cancer MCF-7 cells.

Chapter 5

Evaluate the significance of AURKA-RalA crosstalk in anchorage-independent growth of Ras-independent vs Ras-dependent cancers

5.1. Rationale

Aurora kinases are known to be overexpressed and activated in a wide range of human cancers (Giet et al. 2005; Mehra et al. 2013). AURKA overexpression has been reported in primary breast tumours, colorectal cancers, and cancer cell lines, including colon, prostate, breast, ovarian, neuroblastoma, and cervical cancer cell lines (Jeng et al. 2004; Furukawa et al. 2006; Katsha et al. 2015). AURKA overexpression has also been suggested to be associated with a higher tumour grade and poor prognosis in hepatocellular carcinoma (Jeng et al. 2004). Preclinical studies have also demonstrated the oncogenic potential of AURKA activation, as it promotes in vitro and in vivo transformation of rodent fibroblast cells, supporting the formation of multipolar mitotic spindles inducing genome instability (Y. Jiang et al. 2003). Overexpression of AURKA in cancer cells has also been reported to enhances oncogenic Ras-mediated transformation (Umstead et al. 2017; E. O. Dos Santos et al. 2016; Tseng et al. 2009; Tatsuka et al. 2004a). Ras-RalGEF-RalGTPases is a major downstream effector pathway of oncogenic Ras and is vital to Ras-mediated transformation in breast, pancreatic, colon, and other cancers (Neel et al. 2011). Downstream of Ras, RalA and RalB can differentially regulate anchorage-independent growth and cell survival (Bodemann & White 2008; Feig 2003). As discussed in earlier chapter, AURKA along with RGL1 regulates integrin-mediated adhesion-dependent activity of RalA, supporting its role in anchorage-dependent signaling (N. Balasubramanian et al. 2010; Pawar et al. 2016a). Knowing the overlap that exists between integrin and Ras-mediated signaling, AURKA-RGL1 could hence act as a point of convergence for integrin and Ras-mediated regulation of RalA. Moreover, AURKA could also contribute to Ras-independent Ral signaling in cancers. The presence and contribution of such an AURKA−RalA crosstalk in cancers and the possible role inhibiting this using V_{MLN} could have in targeting their oncogenic potential is detailed in Chapter 4.

In this chapter, we have used V_{MLN} to inhibit AURKA and evaluate its role in regulating RalA activity and anchorage independent growth (AIG) in Ras-dependent and Ras-independent cancers. The efficiency of uptake of V_{MLN} in different cancer cell lines and its ability to inhibit AURKA and RalA was tested. We have also evaluated the effect AURKA inhibition has on the cell cycle profile of Ras-independent cancers.

5.2. Results

5.2.1 Screen for Aurora kinase A and RalA expression and activation in Ras-dependent and Ras-independent cancer cells

To select the cancer cell lines where AURKA-RalA crosstalk could be evaluated we first screened cancer cell lines for; (1) their levels of AURKA (2) AURKA activation (pThr288 AURKA) (3) RalA levels and (4) AURKA mediated Ser194 phosphorylation of RalA (pSer194 RalA). This identified following cell lines that showed good expression levels of AURKA and RalA and increased activity of AURKA as compared to WT-MEFs (Figure 5.1a and 5.1b): bladder cancer cell lines (T24 – HRAS G12V, UMUC3 – KRAS G12V, J82-HRAS WT over-expressed), pancreatic cancer cell line (MIAPaCa 2 – KRAS G12V, CFPAC-1 – KRAS G12V, AsPC1 – KRAS G12D), Lung cancer cell lines (CALU-1 – KRAS, A549 – KRAS) fibrosarcoma cell line (HT1080 – NRAS Q61K) and ovarian cancer cell line (SKOV3 – No mutant RAS).
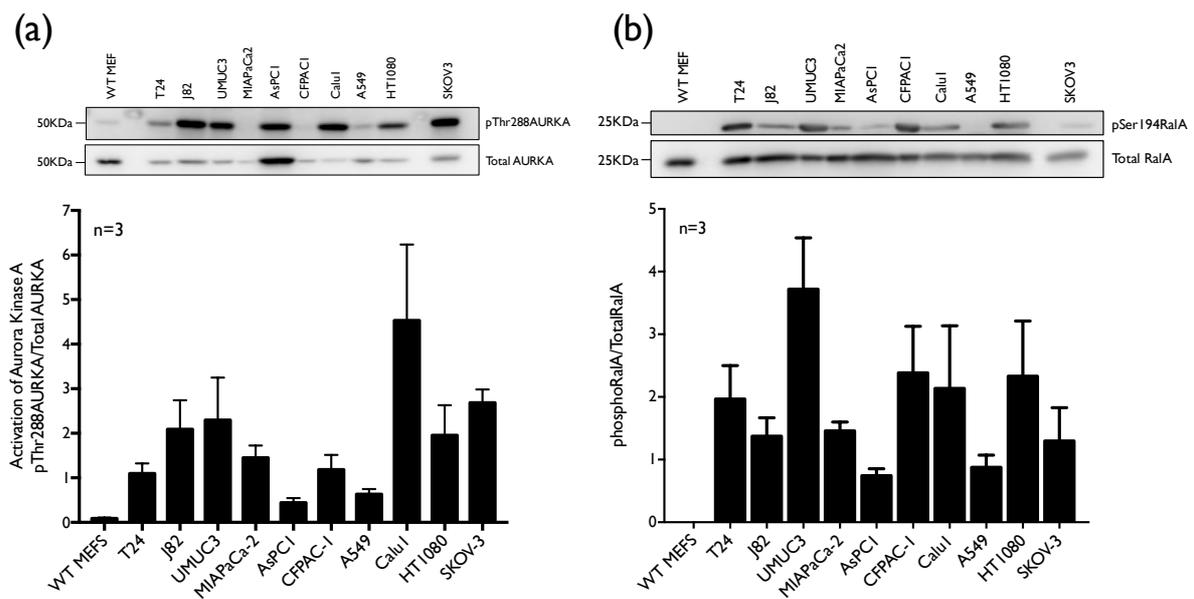
Figure 5.1: Screen for selection of cancer cell lines to evaluate AURKA-RalA crosstalk. Activity of (a)AURKA and (b) RalA in lysates from Ras-dependent and Ras-independent cancer cell lines. Blots on the top represent AURKA and RalA phosphorylation on the Threonine288 (pThr288 AURKA), and Serine194 (pSer194RalA) residue, respectively, and the total AURKA/RalA levels in cells. The graph represents the mean ± SE of the pThr288 AURKA/total AURKA, and pSer194RalA/RalA ratio from three independent experiments.

As the antibody that detects phosphorylation of Ser194 on RalA does not work for mouse protein we could not compare the increase in RalA phosphorylation in cancer cells as compared

to WT-MEFs. In comparison to AURKA activity, the activation of AURKB was not significantly increased with respect to WT-MEFs in most of the cancer cell lines screened (Figure 5.2).

Figure 5.2: AURKB activity in cancer cell lines. Activity of AURKB in Ras-dependent and Ras-independent cancer cell lines. Blots on the top represent AURKB phosphorylation on the Threonine232 (pThr232 AURKB), and the total AURKB levels in lysates from cancer cells. The graph represents the mean ± SE of the pThr232 AURKB/total AURKB, ratio from three independent experiments.

5.2.2 Inhibition of Aurora Kinase A activity using Free MLN8237 vs Encapsulated MLN8237 (V_{MLN}) in cancer cells

As most of the cell lines showed good expression and phosphorylation of AURKA and RalA, we did a secondary screen to further narrow down the selected cell lines. In this secondary screen, we evaluated cancer cell lines for; (1) effectiveness of V_{MLN} mediated AURKA inhibition relative to free MLN and (2) Uptake of V_{MLN} in the cancer cells. All of the cell lines showed V_{MLN} to inhibit AURKA significantly better as compared to free MLN8237 (Figure 5.3). However, V_{MLN} mediated inhibition of AURKA activity was >50% as compared to Free MLN in T24, UMUC3, SKOV3, DLD1, MDA MB 231, U87MG, MIAPaCa-2 and SW620 cells (Figure 5.3). <50% inhibition of AURKA activity with V_{MLN} was seen in HT1080 and

Calu1 cells. In order to evaluate if the significantly better inhibition was due to better uptake of V_{MLN}, we looked at the uptake of V_{MLN} in these cell lines.
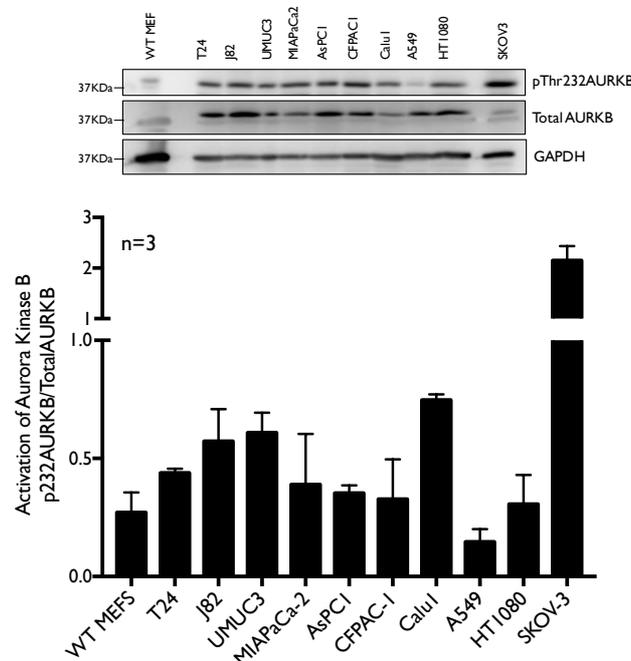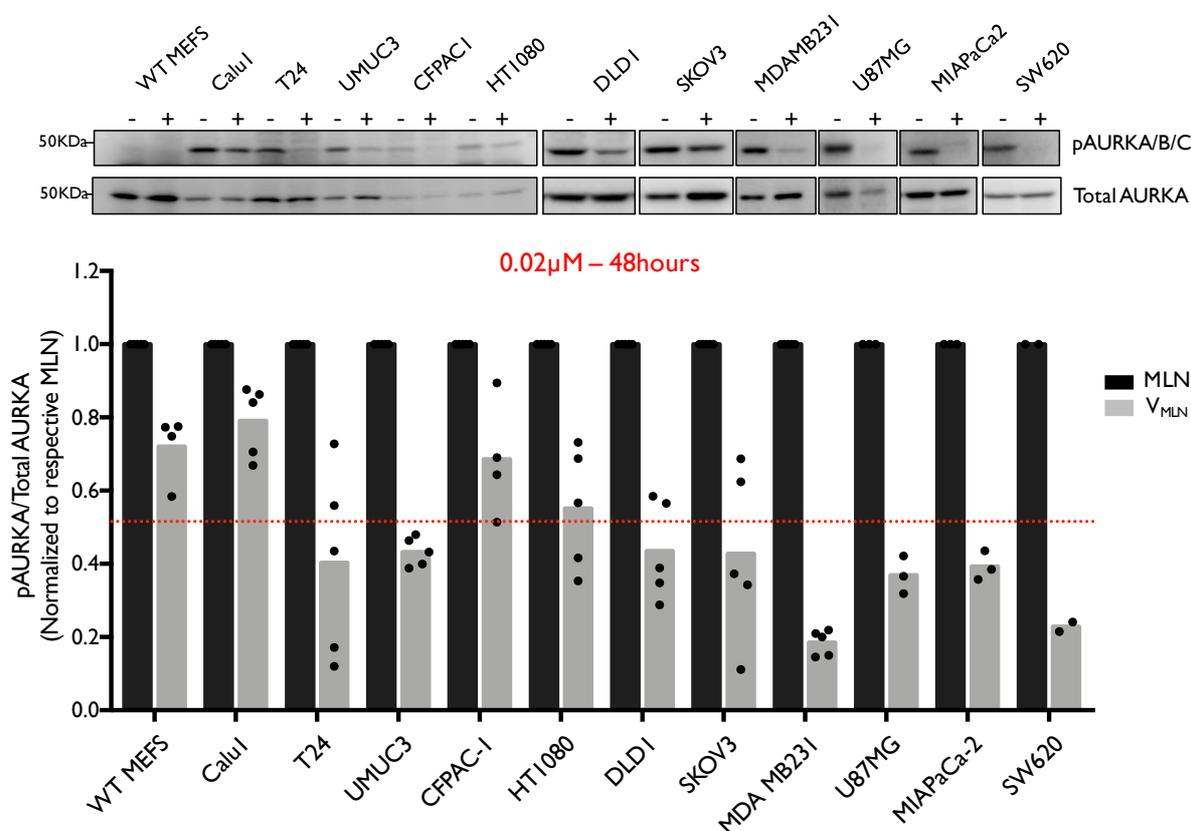

Figure 5.3: V_{MLN} mediated inhibition of AURKA in cancer cell lines. Activity of AURKA in Ras-dependent and Ras-independent cancer cell lines. Blots on the top represent AURKA phosphorylation on the Threonine288 (pThr288 AURKA), and the total AURKA levels in lysates from cancer cells treated either with 0.02μM free MLN8237 (MLN-black bars) or dextran encapsulated MLN8237 (V_{MLN} – grey bars) for 48hours. The graph represents the mean ± SE of the pThr288 AURKA/total AURKA, ratio from atleast three independent experiments except in SW620 which is from two experiments.

5.2.3 Uptake of $V_{MLN+RhB}$ in cancer cells

As described in Chapter 4, dual loaded dextran nano-vesicles with hydrophilic dye Rhodamine B and hydrophobic MLN8237 ($V_{MLN+RhB}$) (Figure 4.4) was used to study the uptake of nano-vesicles in cancer cells. The dual loaded nano-vesicle ($V_{MLN+RhB}$) is taken up efficiently in all the cancer cell lines (Figure 5.4) suggesting that the bioavailability of the drug might not be the reason for variable inhibition of AURKA in different cancer cell lines. The inherent activity of AURKA, upstream regulators, site of drug release, kinetics of drug release (governed by the

concentration of esterases present) could be some of the factors that can contribute to the differential inhibition of AURKA by V_{MLN} in different cancer cell lines.
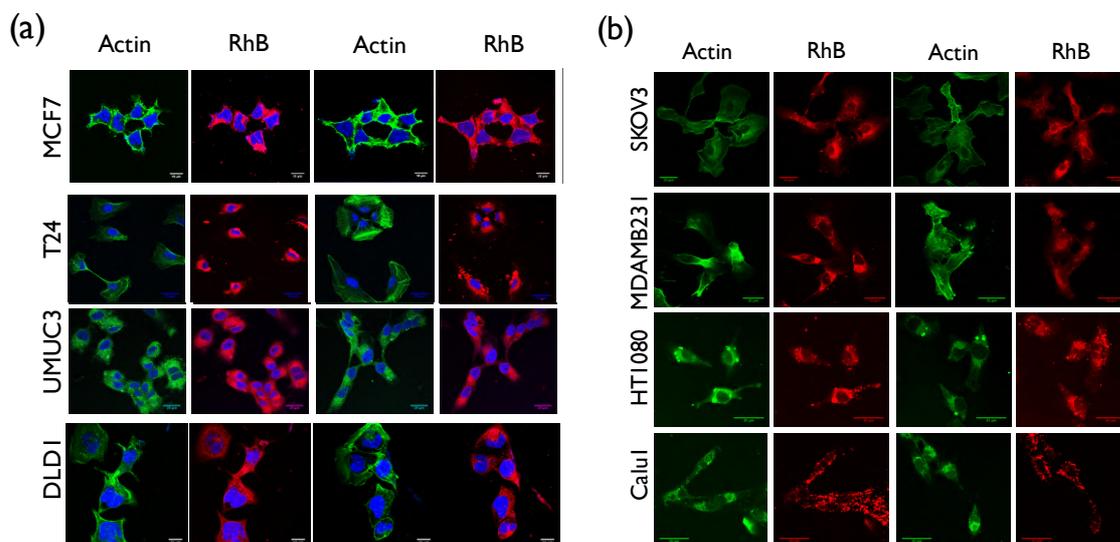
Figure 5.4: Uptake of $V_{MLN+RhB}$ in cancer cells. Uptake of DEX nano-vesicle with RhB (1.37 µM) and MLN8237 (0.5 µM) ($V_{MLN+RhB}$) was visualized by confocal microscopy in cells treated for 48hours. The actin cytoskeletal network stained with phalloidin was conjugated to Alexa-488. Nucleus of (a) panel cells was counterstained with DAPI. The images shown are representative of three independent experiments that gave similar results

5.2.4. Inhibition of RalA phosphorylation and activity using Free MLN8237 *vs* V_{MLN} in Ras-independent *vs* Ras-dependent cancer cells

Knowing that V_{MLN} is taken up efficiently and inhibits AURKA significantly better than free MLN in all the selected cancer cell lines, we next evaluated whether this AURKA inhibition effects RalA phosphorylation and activity in these cells. We first tested it in **two Ras-independent cell lines**- MCF-7 (Evaluated in Chapter 4) and SKOV3. As described in detail in Chapter 4, in MCF-7 cells, V_{MLN} inhibits AURKA significantly better than Free MLN with empty dextran scaffold (DEX) showing no inhibition of AURKA activity (Data in Figure 5.5a reproduced from Figure 4.11). We observe similar trend in Ras-independent SKOV3 cell line too, in that, V_{MLN} inhibits AURKA significantly better than free MLN (Figure 5.5d). Empty dextran scaffold showing no effect on AURKA activity (Figure 5.5d). In both these cell lines, V_{MLN} mediated inhibition of AURKA causes significant decrease in phosphorylation of RalA at serine 194 (Figure 5.5b (reproduced from Figure 4.12) and 5.5e). This is accompanied by a significant decrease in RalA activity as determined by GST-Sec5 pulldown assay (Figure 5.5c (reproduced from Figure 4,13a) and 5.5f). The phosphorylation of RalA at Ser194 has been

previously shown to be important for activation (GTP-binding) of RalA in in vitro system with over-expressed proteins (Lim et al. 2009; Wu et al. 2005). Additionally, phosphorylation of RalA at S194 has been reported to be essential for maintaining anchorage-independent growth and tumorigenesis of a range of pancreatic cancer cells lines (Lim, Brady, Kashatus, Ancrile, Der, Cox & Counter 2009c). Taken together, our studies re-establish the observation that phosphorylation by AURKA might play an important role in activation (GTP-binding) of Ral in Ras-independent MCF-7 and SKOV3 cells. On the other hand, inhibition of AURKA using Free MLN8237 while causing a distinct decrease in AURKA activation (be it significantly less than V_{MLN}) does not significantly affect phosphorylation or activation of RalA in either MCF-7 or SKOV3 cell line (Figure 5.5), suggesting that a threshold level of AURKA activation might be enough to maintain the RalA phosphorylation in these cells and inhibition of AURKA beyond that (which might be the case with V_{MLN} inhibition) might translate downstream into RalA inhibtion.

Knowing that AURKA-RalA crosstalk exists in Ras-independent cell lines, we next tested whether AURKA regulates RalA in **Ras-dependent cancer cells**. The cell lines selected for this were: Bladder cancer cell lines - T24 (H-Ras) and UMUC3 (K-Ras), Breast cancer cell line – MDA MB 231 (K-Ras), Glioblastoma cell line – U87MG (K-Ras), Pancreatic cancer cell line (K-Ras) and Colorectal cancer cell line – SW620 (K-Ras).
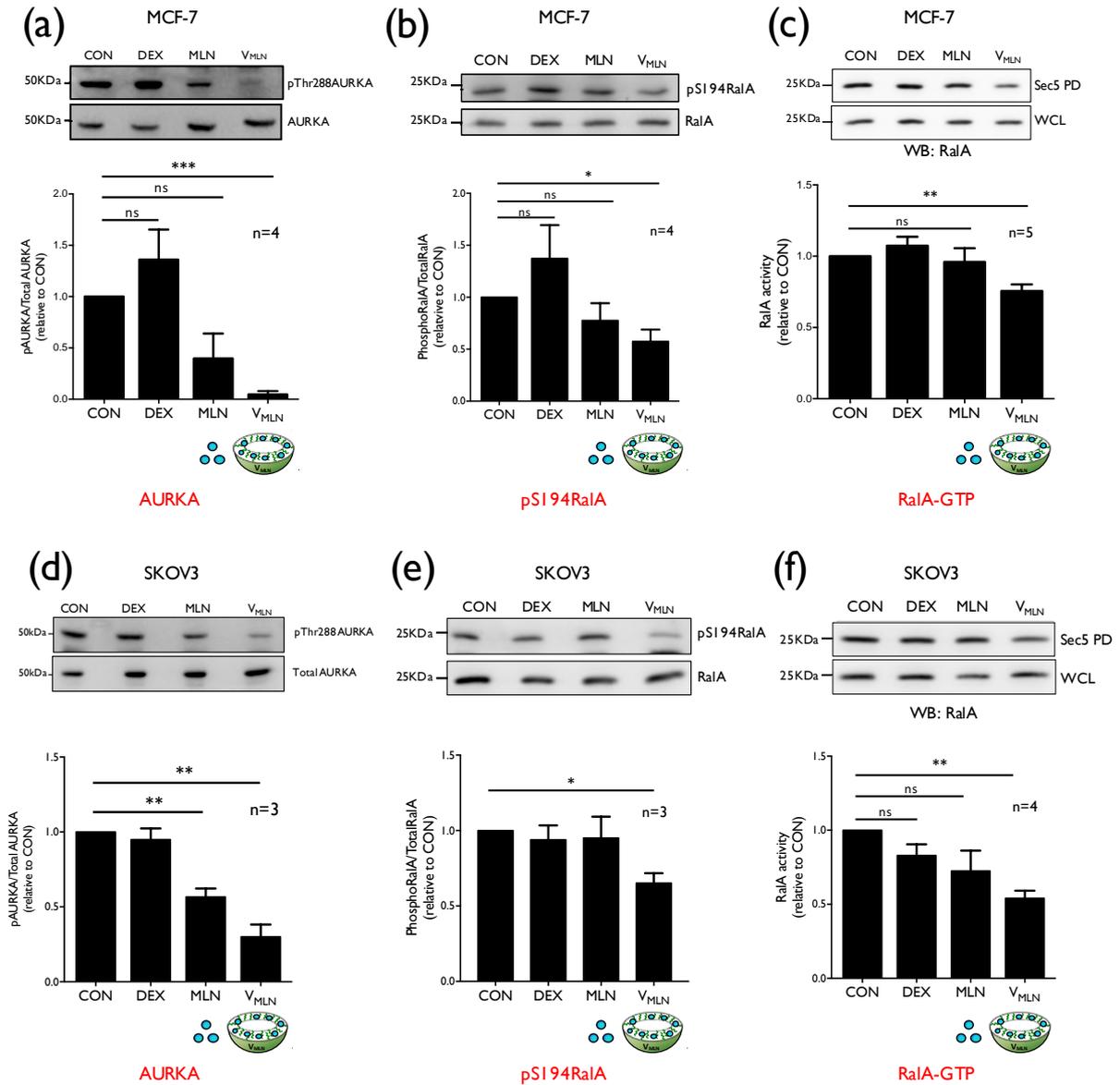
Figure 5.5: Inhibition of RalA phosphorylation and activity in V_{MLN} treated MCF-7 and SKOV3 cells. Western blot detection (upper panel) and quantitation (lower panel) of (a nad d) phosphorylation of Threonine 288 residue (pThr288 AURKA) and total AURKA, (b and e) phosphorylation of Serine 194 residue (pS104RalA) and total RalA, and (c and f) active RalA pulldown using GST-Sec5 beads (Sec5) and total RalA (WCL), from lysates of MCF-7 (a, b, c) and SKOV3 (d, e, f) cells treated with 0.02μM Free MLN (MLN) or 0.02μM encapsulated MLN (V_{MLN}). DMSO (CON) and empty nano-vesicle scaffold (DEX) were used as controls for MLN and V_{MLN}, respectively. The ratio of pAURKA/AURKA, pRalA/RalA and active RalA were normalized to their respective controls (CON) (equated to 1) and were represented in the graph as mean ± SE from at least three independent experiments. Statistical analysis was done using single-sample t-test, and p values, if significant, are represented in the graph (* $p < 0.05$, ** $p<0.01$, *** $p<0.001$). To allow for comparison and better understanding of the MCF-7 and SKOV3 data, Figure 4.11a, 4.12 and 4.13a are adapted as Figure 5.5a, 5.5b and 5.5c, respectively.

(a) T24

(d) U87MG

(c) CON DEX MLN V_MLN

n=3

n=3

*
***
**
ns

Figure 5.6: I...
blot detection ...
288 residue (p...
MDA MB 231 ...
MLN (MLN) ...
scaffold (DEX...
pAURKA/AU...
(equated to 1) ...
experiments e...
and p values, i...

Pico Image Elements 7.1.7   Pico Image Elements 7.1.71   Pico Image Elements 7.1.7106

V_{MLN} inhibits ...

(Figure 5.3 and 5.6) with empty dextran nano-scaffold showing no visible effect on AURKA activity (Figure 5.6). Of particular interest is MDA MB 231 cell line shows a dramatic inhibition of AURKA activity with V_{MLN} where Free MLN had no effect at all (Figure 5.6c).

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The bladder cancer cell lines T24 and UMUC3 both show significant inhibition of AURKA by free MLN8237, though the inhibition by V_{MLN} is found to be significantly better in both cell lines (Figure 5.6a and 5.6b). U87MG and MiaPaCa2 cell lines show Free MLN to only modestly inhibit AURKA activity, while V_{MLN} significantly better inhibits AURKA (Figure 5.6d and 5.6e). Preliminary studies using SW620 cell line also show V_{MLN} to better inhibit AURKA than Free MLN (Figure 5.6f), however further studies with this cell line are currently ongoing in lab. AURKB activity when probed for in same lysates did not show any significant change upon V_{MLN} treatment in any of the tested cell lines (Data not shown). Taken together, these results suggest that, V_{MLN} is not only taken up efficiently inside cells, but it also increases the bioavailability (entry of drug and release in cells) of the drug into the cells as compared to free drug that solely depends on diffusion for entering the cells, thereby allowing lower concentrations of the drug to inhibit AURKA significantly better than the equivalent concentration given as free drug.

Now that we had established that V_{MLN} at 0.02 μM concentration significantly inhibits AURKA in all the selected Ras-dependent cancer cell lines we next tested if this inhibition can affect RalA phosphorylation at Serine 194 residue. Contrary to expectation, V_{MLN} mediated inhibition of AURKA did not show any significant change in phosphorylation of RalA at Serine 194 in all the tested Ras-dependent cell lines (Figure 5.7a, b, c, d, e) except in SW620 cells (Figure 5.7f) (preliminary data). This raises a few possibilities, 1) AURKA does not regulate RalA phosphorylation in these cancers, and does so only in some cancers like SW620, 2) in these Ras-dependent cell lines other known regulators of S194 RalA phosphorylation, like PP2A (Sablina et al. 2007) or Protein Kinase A (Gentry et al. 2014) might have a more prominent role that than AURKA, 3) inhibition of AURKA could affect the regulation of PP2A and/or PKA is altered to sustain RalA Serine 194 phosphorylation, 4) RalA is de-localised in these cancers owing to upstream regulators other than AURKA (probably Ras-dependent GEFs), thereby overcoming regulation by AURKA and 5) depending on our results with V_{MLN} and free MLN, it is evident that a threshold inhibition of AURKA is necessary for having an impact on RalA phosphorylation (Figure 5.5b and 5.5e). Taking this into account, it is highly probable that to begin with, these cell lines have over-expression and hyper-activation of AURKA and the residual AURKA activity remaining post V_{MLN} treatment is still enough to sustain RalA phosphorylation. Together, this data suggests that testing the role of Serine phopsho-mimetics

and -deficients

worth exploring

(a) T24

CON DEX MLN V_{MLN}

25kDa — pS194Ral

25KDa — Total RalA

(d) U87MG

CON DEX MLN V_{MLN}

25KDa — pS194R

25KDa — RalA

Figure 5.7: Inh
Western blot d
Serine 194 resid
MB 231, (d) U
(MLN) or 0.02µ
(DEX) were used
in each cell line
represented in t
SW620 cell lin
differences were

Pico Image Elements 7.1.7   Pico Image Elements 7.1.71   Pico Image Elements 7.1.71

We further tested if and how S19...

effector binding in GST-Sec5 pu...

T24 and UMUC3. We find that...

free MLN or V_{MLN} treatment) in...

(a)
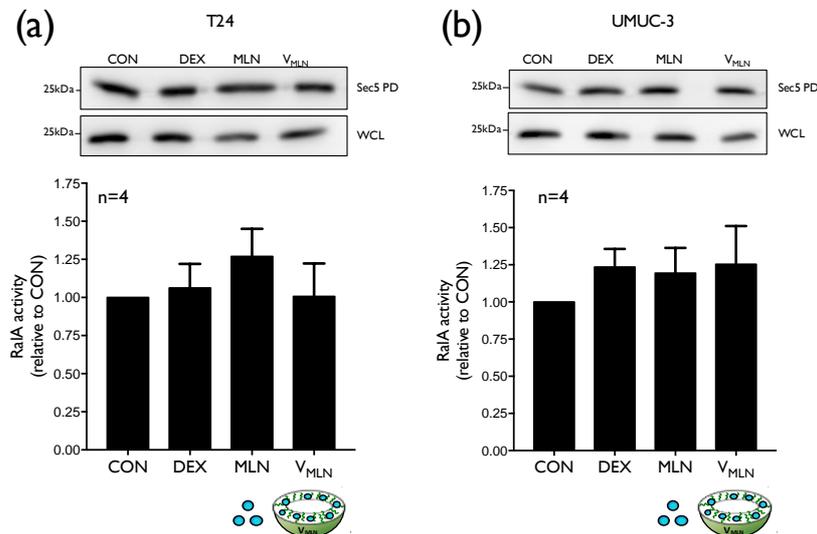
Figure 5.8: Inhibition of RalA...
and UMUC3 cells. Western blo...
Active RalA and total RalA, from...
(CON), empty nano-vesicle sca...
were normalized to their respecti...
mean ± SE from four independ...
sample t-test, and none of the dif...

Taken together these results sug...

RalA activity in these Ras-dep...

phosphorylation and activity in...

cell lines (Figure 5.5 e and 5.5...

AURKA-RalA crosstalk could...

MCF-7 and SKVO3 cell lines. The possible differential regulation of AURKA-RalA crosstalk in Ras-dependent and Ras-independent cancer could be of much interest going forward.

5.2.5. Effect of V_{MLN} mediated inhibition of AURKA-RalA crosstalk on anchorage-independent growth of MCF-7 and SKOV3 cells

RalA is a known regulator of anchorage-independent growth of cancer cells (Bodemann & White 2008; Camonis & White 2005; Martin et al. 2011; Y. Yamazaki et al. 2001). Anchorage-

independent signaling and the

cells, that aid in tumour cell in

AURKA at the Ser194 of ac

phenotype and transwell migra

MDCK cells (Wu et al. 2005).

negatively regulate anchorage-

cancer cells lines (Lim et al. 2

AURKA regulated RalA mig

pancreatic cancers (Neel et al.

we see in Ras-independent cell

growth of these cells. V_{MLN} tre

in soft agar and colonies obse

treated Control (DEX) (Figur

AIG. This suggests that AURF

of Ras-independent MCF-7 an

(a)
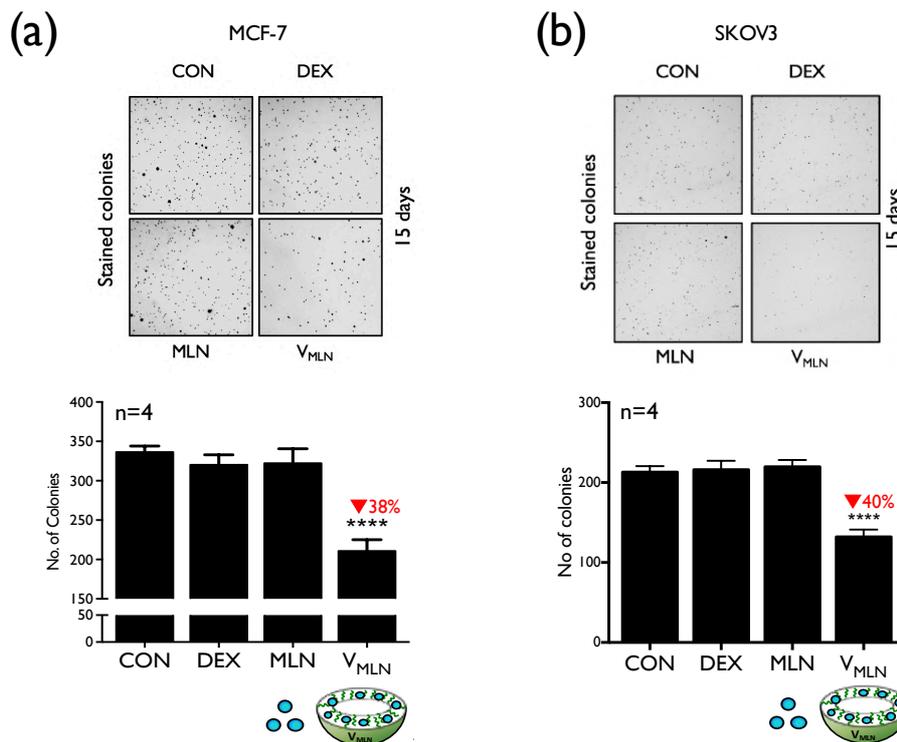
MCF-7

Figure 5.9: Inhibition of Al
DMSO-treated CON, empty
(MLN), and V_{MLN}-treated (a)
and incubated for 15 days wit
stained and counted as descri

from four independent experiments. Statistical analysis was done using the paired Students t test, and p values, if significant, are represented in the graph (**** $p < 0.0001$).

To further confirm this role of RalA, we have tested the effect of a Ral inhibitor (BQU57) (C. Yan et al. 2014b) on anchorage-independent growth of MCF-7 cells. It is however worth noting that Ral inhibitor (BQU57) targets both RalA and RalB (C. Yan, Liu, Li, Wempe, Guin, Khanna, Meier, Hoffman, Owens, Wysoczynski, Nitz, Knabe, Ahmed, Brautigan, Paschal, Schwartz, D. N. M. Jones, Ross, Meroueh & Theodorescu 2014b) and lacks the isoform specificity that V_{MLN} offers. Treatment of MCF-7 cells with BQU57 we find inhibits RalA (~50%) and RalB (~60%) to similar extents (Figure 5.10a and 5.10b) and distinctly better that AURKA mediated inhibition of RalA (~25%). A significant inhibition in the number of colonies in soft agar assay is seen on BQU57 treatment (~31%) (Figure 5.10c). This decrease in the number of colonies upon Ral inhibition (Figure 5.10c) is comparable to that seen upon V_{MLN} (~38%) mediated inhibition of AURKA and RalA (Figure 5.9a) suggesting the AURKA-RalA crosstalk could indeed be a vital regulator of anchorage-independent growth in Ras-independent cancer cell lines.
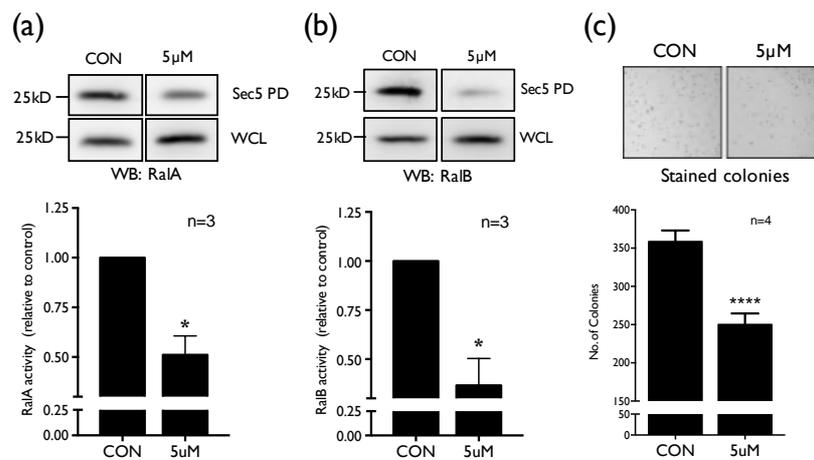
Figure 5.10: Western blot detection and quantitation of (a) active RalA and (b) active RalB pulled down by GST-Sec5 (Sec5 PD), and the total RalA/RalB in the whole cell lysate (WCL) was done from DMSO-treated CON and 5μM BQU57-treated MCF-7 cells. The graph represents mean ± SE data from three independent experiments. (c) Cells treated similarly were embedded in the agarose gel and incubated for 15 days with DMSO or 5μM BQU57, and the colonies formed were stained and counted. The activity assay data (a and b) are normalized to their respective DMSO-treated CON (equated to 1). The images in (c) are representative images of stained colonies. Statistical analysis was done using the single sample t test (a and b) and the paired Students t test (c), and p values, if significant, are represented in the graph (* $p < 0.05$, **** $p < 0.0001$).

Both AURKA and RalA have been reported to play important roles during mitosis, with AURKA being necessary for early mitotic events like centrosome maturation and spindle formation (Carmena & Earnshaw 2003a; Goldenson & Crispino 2014b). RalA is seen to be indispensable for completion of cytokinesis (X.-W. Chen et al. 2006; Cascone et al. 2008; Prekeris & Gould 2008). The AURKA-RalA crosstalk is further seen to be important for regulation of mitochondrial dynamics during mitosis (Kashatus, Lim, Brady, Pershing, Cox & Counter 2011a; Bertolin et al. 2018). Inhibiting one or both might hence have a distinct effect on cell cycle progression which in turn could result affect cell growth dependent colony formation in soft agar. We hence tested and found that V_{MLN} mediated inhibition of AURKA-RalA crosstalk causes a significant arrest of both MCF-7 and SKOV3 cells in G2-M phase of cell cycle (Figure 5.11a and 5.11b). This also results in reduced cell survival of MCF-7 cells as determined by MTT assay (Figure 5.12). Taken together, the effect V_{MLN} has on AIG of Ras-independent cell lines can be mediated by AURKA-RalA mediated (1) targeting of RalA dependent vesicular trafficking and exocytosis impacting anchorage-independent growth signaling and (2) AURKA-RalA inhibition mediated G2-M arrest of cells thereby preventing cell proliferation and AIG of cancer cells. The relative contribution both make to AIG in these cells remains to be tested and confirmed.
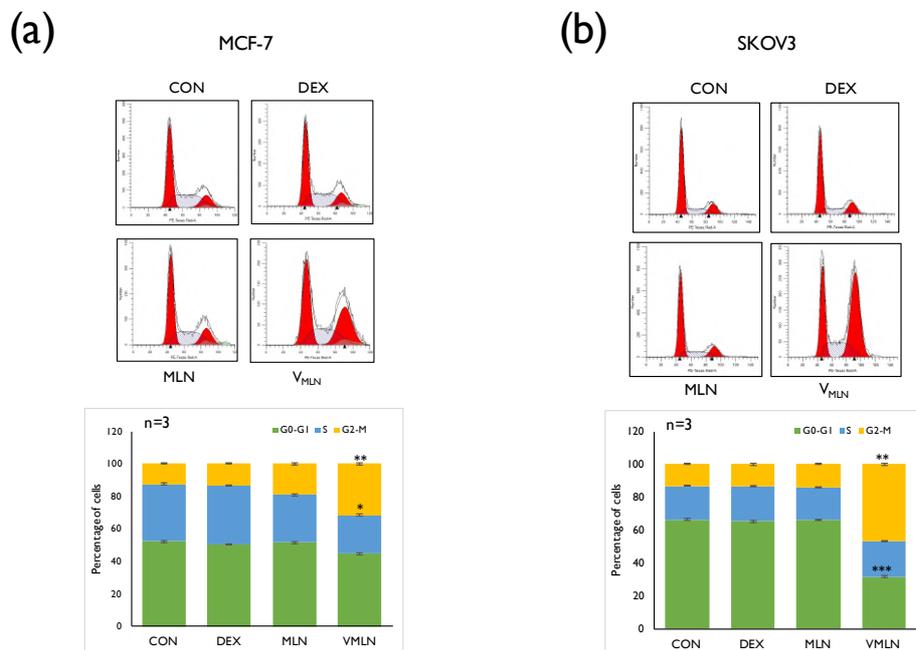
Figure 5.11: Cell cycle profile of V_{MLN} treated MCF-7 and SKOV3 cells. Representative histograms (Upper panel) and quantitation of percentage of cells in G0-G1, S and G2-M phase of cell cycle were determined from DMSO treated control (CON), empty dextran nano-vesicle treated (DEX), 0.02μM Free MLN8237 treated (MLN) and 0.02μM encapsulated MLN8237 treated (V_{MLN}) (a) MCF-7 and (b) SKOV3 cells by flow cytometry. The graph represents mean

± SE data from three independent experiments. Statistical analysis was done between treated and control cells using paired Students t test and p values, if significant, are represented in the graph (* p < 0.05, **p<0.01 *** p < 0.001).
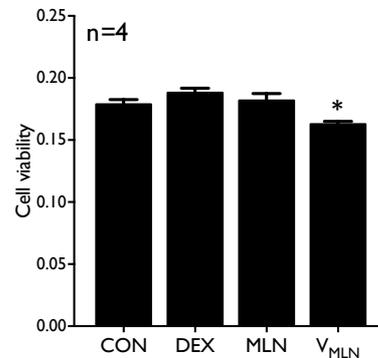
Figure 5.12: Viability of V_{MLN} treated MCF-7 cells. Absorbance values of MTT stained MCF7 cells measured from DMSO treated control (CON), empty dextran nano-vesicle treated (DEX), 0.02μM Free MLN8237 treated (MLN) and 0.02μM encapsulated MLN8237 treated (V_{MLN}) MCF-7 cells. The graph represents mean ± SE data from four independent experiments. Statistical analysis was done between treated and control cells using paired Students t test and p values, if significant, are represented in the graph (* p < 0.05).

	SKOV3 Ovarian	MCF7 Breast	T24 Bladder	UMUC3 Bladder	MDA-231 Breast	U87MG Glioblastoma	MIAPaca2 Pancreatic	SW620 Colon
Ras	No mutant Ras	No mutant Ras	H-Ras G12V	K-Ras G12V	K-Ras G13D	K-Ras G12V	K-Ras G12V	K-Ras G12V
AURKA inhibition (MLN vs V_{MLN}) (How much better is VMLN relative to MLN alone	~53% (N=3,n=6)	~75% (n=4)	~50% (n=5)	~30% (n=5)	~83% (n=3)	~64% (n=3)	~60% (n=3)	~78% (n=2)
pRalA inhibition (MLN vs V_{MLN})	~32% (N=3,n=6)	~26% n=4	No Effect n=5	No Effect n=5	No Effect n=3	No Effect n=3	No Effect n=3	~25% (n=2)
RalA activity assay (CON/DEX/MLN/ V_{MLN})	~10% (n=5)	~25% inhibition	No Effect n=5	No Effect n=5				
Cell Cycle analysis using FACS(Increase in G2 population of VMLN vs MLN)	144%	30%						
AIG inhibition	~40% (n=4)	~38% (n=4)						

Not selected for further studies

Further in-vitro studies on-going

Selected for in-vivo tumor studies

Figure 5.13: Summary of *in-vitro* studies to select and validate AURKA-RalA crosstalk in Ras-independent and Ras-dependent cell lines.

5.3. Summary and Open questions

RalA GTPase is known to be regulated by two major signaling pathways: integrin-mediated adhesion and Ras–RalGEF mediated pathway. Cascone et. al., in 2008 reported that a Ras activated GEF RalGDS activates RalA and RGL1 activates RalB during cytokinesis (Cascone et al. 2008). However, it is known that all Ras activated GEFs have the potential to activate both RalA and RalB, which combined with their spatial localization regulating their distinct downstream signalling.

As discussed in Aim I, we have shown that AURKA and RGL1 are both involved in the integrin- dependent regulation of RalA activation (Deshpande et.al. manuscript under prep). Thus, AURKA-RGL1 could act as a point of convergence for integrin and Ras-mediated regulation of RalA. To test for this possibility, we screened cancer cell lines for; (1) their levels of AURKA (2) AURKA activation (pThr288 AURKA) (3) RalA levels and (4) AURKA mediated Ser194 phosphorylation of RalA (pSer194 RalA) and (5) effectiveness of V_{MLN} mediated AURKA inhibition over Free MLN8237. This identified bladder cancer cell lines (T24 – H-Ras G12V, UMUC3 – K-Ras G12V), pancreatic cancer cell line (MIAPaCa2 – K-Ras G12V), fibrosarcoma cell line (HT1080 – N-Ras Q61K), breast cancer cell lines (MCF-7 – No mutant Ras, MDAMB231 – K-Ras G13D), glioblastoma cell line (U87MG – K-Ras G12V) and ovarian cancer cell line (SKOV3 – No mutant Ras). All of the above cell lines showed V_{MLN} to inhibit AURKA significantly better as compared to free MLN8237. Interestingly, V_{MLN} treatment inhibited Ser194 phosphorylation of RalA only in the Ras-independent MCF-7 and SKOV3 cells. This we find further caused a significant decrease in GTP bound active RalA, in GST- Sec5 pulldown assays and a significant decrease in anchorage-independent growth of these cells (Figure 5.18). V_{MLN} treatment arrests MCF-7 and SKOV3 cells in the G2-M phase of the cell cycle, that inhibition of RalA has also been seen to cause (Kashatus et al. 2011).

Taken together these studies suggest that the effect V_{MLN} has on anchorage-independent growth of Ras-independent cell lines could be mediated by (1) AURKA dependent inhibition of RalA-mediated vesicular trafficking and exocytosis reversing anchorage-independent growth signaling and (2) AURKA-RalA inhibition mediated G2-M arrest of cells thereby preventing cell proliferation and anchorage-independent growth of cancer cells. However, the relative

contribution of the effect of cell cycle and vesicular trafficking on anchorage-independent growth of these cells still remains to be explored.
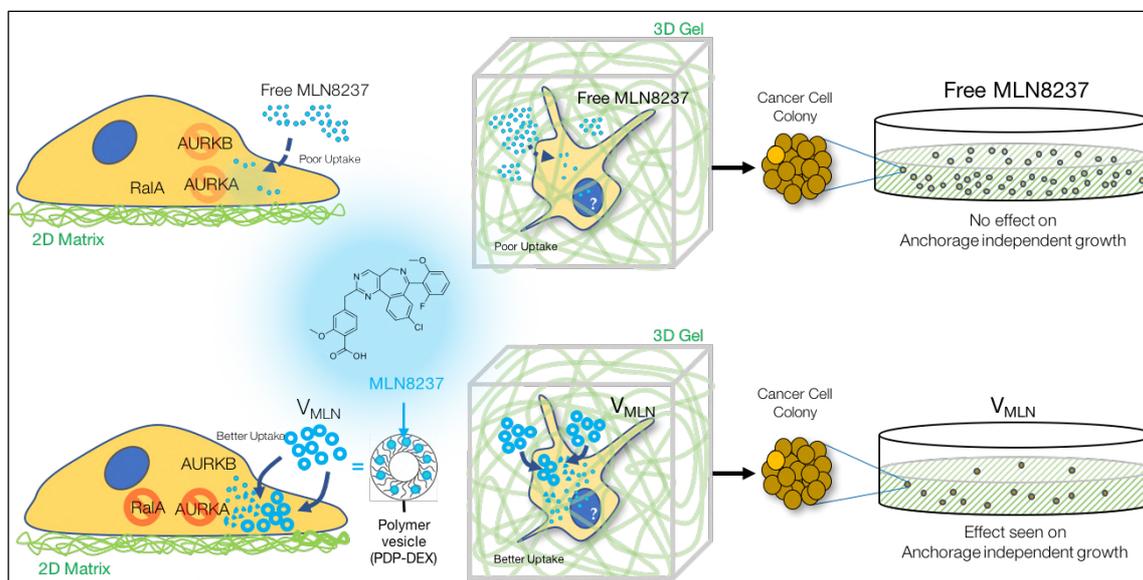
Figure 5.14: V$_{MLN}$ mediated inhibition of AURKA-RalA crosstalk significantly reduces anchorage-independent growth in Ras-independent MCF-7 and SKOV3 cells. (Upper panel) Poor solubility of MLN8237 results in poor uptake of the drug in cells in 2D and 3D microenvironments, causing modest inhibition of both AURKA and AURKB. (Lower panel) Drug delivered in a polymer nano-vesicle, on the other hand, is taken up efficiently in cells, resulting in low concentrations of the drug now significantly better inhibiting AURKA (without affecting AURKB) in both 2D and 3D microenvironments. This inhibition of AURKA targets downstream RalA activation suppressing the anchorage-independent growth of MCF-7 and SKOV3 cells. This identifies a role for the AURKA−RalA crosstalk in maintaining the anchorage-independence of these Ras-independent cancer cells (Reproduced from (Inchanalkar et al. 2018)).
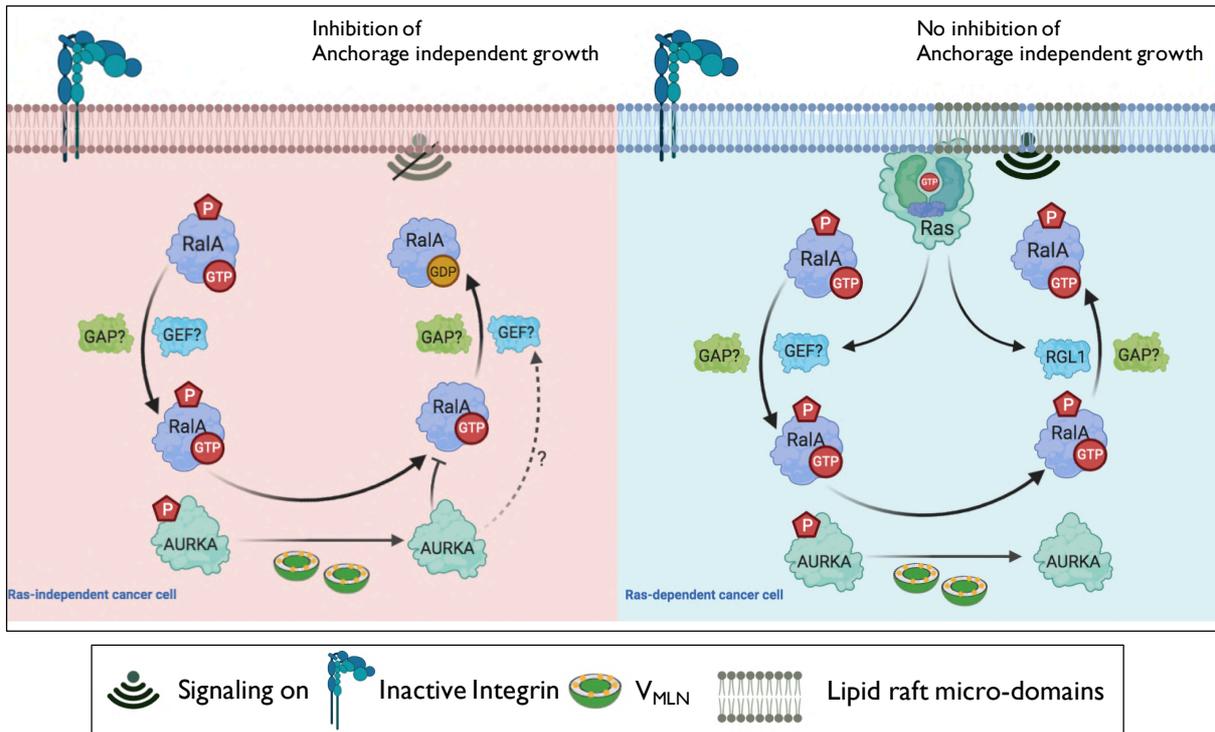
Figure 5.15: Proposed model for role of AURKA-RalA crosstalk in cancers. In Ras-independent cancer cells, inhibition of AURKA using V_{MLN} causes inhibition of phosphorylation and activity of RalA (RalA-GDP). This ultimately causes reduction in anchorage-independent growth of cancer cells. On the other hand, in Ras-dependent cancer cells, inhibition of AURKA using V_{MLN} does not cause significant inhibition of either RalA phosphorylation or activity. Together these results suggest that, AURKA-RalA crosstalk plays an important role in anchorage-independent growth of only Ras-independent cancers but not tested Ras-dependent cancers. (Note: Components of the figure are for representational purpose and not true to scale) (Created using Biorender)

5.4. Conclusion

V_{MLN} mediated inhibition of AURKA-RalA crosstalk significantly reduces anchorage-independent growth in Ras-independent MCF-7 and SKOV3 but not Ras-dependent cancer cells.

Chapter 6
Study the role of adhesion-growth factor crosstalk in regulation of Aurora Kinase activation and signaling

6.1 Rationale

The mitotic kinases Aurora Kinase A (AURKA) and Aurora Kinase B (AURKB) share about 71 percent identity in their catalytic domain (Carmena & Earnshaw 2003a). Despite this similarity, AURKA and AURKB are found to be differentially activated in response to variety of upstream stimuli and also regulate distinct downstream pathways during mitosis (Barr & Gergely 2007a; Nair et al. n.d.; Lan et al. 2004; Goldenson & Crispino 2014a). AURKA is essential in centrosome maturation, centrosome separation, formation of bipolar spindle assembly, G2-M transition (Terada et al. 2003). AURKB is involved in chromosome condensation, sister chromatid cohesion, mitotic spindle assembly, regulation of spindle assembly checkpoint, cytokinesis and daughter cell spreading (Crosio et al. 2002; Ferreira et al. 2013). All these functions are crucial for cell proliferation, with de-regulation of these kinases leading to incomplete and improper cell division leading to aneuploidy and effecting cell viability (Khan et al. 2011; Umstead et al. 2017).

During mitosis, human cells undergo rounding up, decreasing their adhesion to extracellular matrix substrates (Uroz et al. 2018; Y. Li & Burridge 2018). Spatiotemporal regulation of various mitotic kinase activity aids in the extensive cytoskeletal remodelling mechanisms that prevent detachment from epithelia, while aiding successful completion of cytokinesis (Champion et al. 2017; Petridou et al. 2019). AURKB is one such dynamic mitotic kinase that has been reported to have multiple roles during different phases of cell cycle. Apart from its extensively studied functions as a part of chromosome passenger complex (CPC), in early G1 phase cell cortex associated pool of AURKB has been found to be associated with cell spreading wherein it interacts and phosphorylates a formin, FHOD1, that is known to be essential to organize cytoskeleton after cell division (Floyd et al. 2013). In another study by Ferreira et al., in late cytokinesis, a gradient of AURKB activity has been shown to ensure that microtubules (MT) in the furrow region remain phosphorylated and the ones in vicinity of extracellular matrix stay de-phosphorylated, restricting the MT growth at the cell-matrix interphase and allowing for stabilization of focal adhesion, aiding co-ordinated daughter cells spreading (Ferreira et al. 2013). A membrane raft protein Flotillin-1 has been recently shown to regulate AURKB activity and CPC function providing a direct point of regulation between extracellular cues and cell cycle progression (Gómez et al. 2010). However, if and how integrin-mediated cell-matrix adhesion regulates the activity of Aurora Kinase B and the

relative contribution of serum growth factors and cell cycle stage has on the same remains unclear.

In this chapter, we have hence tested if integrin-dependent adhesion regulates AURKB activity in anchorage-dependent WT-MEFs and if this contributes to integrin-dependent signaling (ERK, FAK, AKT) and cell spreading. The role serum growth factors have regulating adhesion-dependent AURKB activation and the possible contribution cell cycle profile has on this regulation were also tested.

6.2 Results

6.2.1 Cell-matrix adhesion differentially regulates AURKB vs AURKA activation

As discussed in chapter 3, WT-MEFs show a significant drop in RalA activity on loss of adhesion (suspended for 90mins), which is restored on re-adhesion to fibronectin (for 15mins). Under similar conditions, AURKA activity increases on loss of adhesion (suspended for 90mins) and is restored on re-adhesion to fibronectin. Loss of adhesion for 90min is however seen to significantly affect the cell cycle profile of serum-deprived fibroblasts which could contribute to the regulation of AURKA seen in these cells. A shorter 30min incubation on loss of adhesion could allow us to evaluate the impact re-adhesion of fibroblasts have on AURKA (and AURKB) independent of changes in their cell cycle profile. WT-MEFs suspended in 1% methylcellulose for 30mins and re-plated on fibronectin show a significant decrease in RalA activity (~40 % drop) on loss of adhesion, restored upon re-adhesion to fibronectin (for 15mins) (Figure 6.1A). Under these conditions, AURKA activity decreases (~60%) on loss of adhesion for 30mins but is not restored on re-adhesion to fibronectin (Figure 6.1B). This suggests adhesion to not regulate AURKA activation in re-adherent cells. The basal AURKA activity in these cells could still contribute to adhesion-dependent RalA activation, which remains to be tested. Interestingly, under similar conditions, we find AURKB activity (autophosphorylation on Threonine 232 residue) to drop in suspension (~40 %) and recover back to stable adherent levels on re-adhesion to fibronectin (Figure 6.1C), suggesting that AURKB activity is indeed regulated by integrin mediated adhesion. AKT activation known to be regulated by adhesion, expectedly drops on loss of adhesion (SUS 30') and recovers on re-plating for 15mins (FN 15') (Figure 6.2B). Taken together these observations suggest integrin mediated adhesion to regulate RalA activation. It also highlights adhesion to differentially regulate AURKA and AURKB activation in mouse fibroblasts. It is this differential regulation we wanted to explore further.
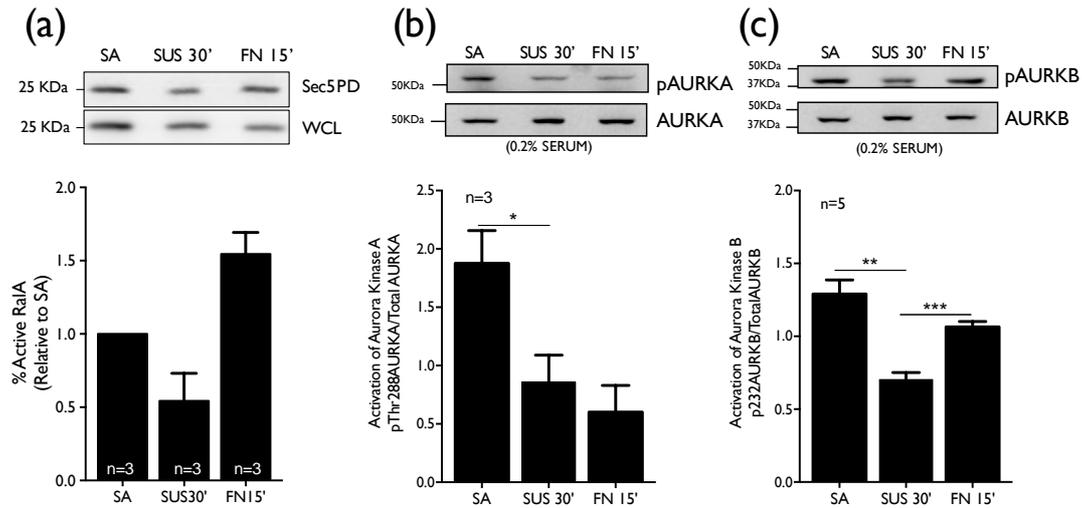
Figure 6.1: Adhesion-dependent regulation of RalA, AURKA and AURKB. Western blot detection (upper panel) and quantitation (lower panel) of (a) Active RalA pulled down by GST-Sec5 beads and total RalA in WCL, (b) phosphorylation on Threonine 288 residues of AURKA (pAURKA), total AURKA and, (c) phosphorylation on the Threonine 232 residues of AURKB (pAURKB), total AURKB in the lysates from serum-starved WT-MEFs stable adherent (SA), suspended for 30 mins (SUS 30') and re-adherent on fibronectin for 15mins (FN 15'). The ratios of pAURKA/AURKA and pAURKB/AURKB and percent Active RalA were normalized to respective SA (equated to 1), and these values are represented in the graph as mean ± SE from at least three independent experiments. Statistical analysis of all the above data was done using the single sample *t*-test for normalized and two-tailed unpaired Student's *t*-test for non-normalized data and significance if any was represented in graph (* p-value <0.05, ** p-value <0.01, *** p-value <0.0001).

6.2.2 Adhesion mediated regulation of Aurora Kinase B activity is independent of cell cycle regulation

Knowing that percentage of cells in G2-M cells does not change significantly in 90mins suspension, we highly suspected that the cell cycle profile will change in 30mins suspension. Fibroblasts when serum deprived as reported in chapter 3, synchronize cells at G1-S phase (Langan & Chou 2011; Griffin 1976; Campisi et al. 1984) with 76 ± 2.3 % of stable adherent cells seen to be in G1-G0 phase and 3 ± 0.8 % in G2-M phase (Figure 6.2A). This profile does not change significantly when the cells are detached and held in suspension for 30 mins, followed by re-plating on fibronectin for 15mins (Figure 6.2A). The absence of any significant change in the cell cycle profile of fibroblasts suggests the regulation of Aurora Kinase B by adhesion is indeed independent of the cell cycle.

Figure 6.2: Adhesion-dependent regulation of Cell cycle profile. (a) Representative histogram of 3 independent experiments (left panel) and Percentage of cells present in G2-M, S and G0-G1 phase (right panel) are shown in table (upper panel) and graph (lower panel), (b) Western blot detection (upper panel) and quantitation (lower panel) of phosphorylation at Serine 473 residue of AKT and total AKT in lysates from serum-starved WT-MEFs stable adherent (SA), suspended for 30 mins (SUS 30') and re-adherent on fibronectin for 15mins (FN 15') are represented. The graph represents the mean ± SE from at least 3 independent experiments. Statistical analysis was done using two-tailed unpaired Student's *t*-test and significance represented (* p-value <0.05, ** p-value <0.01).

6.2.3 Adhesion-growth factor crosstalk regulates Aurora Kinase B activity

Studies have reported extensive crosstalk between growth factor and adhesion dependent signalling pathways (Schwartz & Assoian 2001; Eliceiri 2001; Reverte et al. 2006). Aurora Kinases themselves are regulated by various growth receptor signaling pathways either directly or through indirect mechanisms as discussed in detail in section 1.2.5.2 in chapter 1. Importantly, MAPK pathway has been shown to play an important role in regulating both AURKA (D'Assoro et al. n.d.) and AURKB (Eves et al. 2006; K. Oktay et al. 2014) activities. Earlier studies in serum-deprived conditions help isolate the contribution integrin-dependent adhesion has in regulating AURKB and AURKA activity. These studies were now repeated in the presence of serum growth factors to test the impact growth factors could have in regulating adhesion dependent Aurora Kinase activation. On loss of adhesion (suspension 30min) activation of both AURKB and AURKA, known to drop in serum-deprived conditions, (Figure 6.1c and 6.1b) continue to show a drop in the presence of serum growth factors (Figure

6.3a and 6.3b). The recovery on re-adhesion to fibronectin for 15mins of AURKB activity in serum-deprived conditions (Figure 6.1c) is interestingly lost in the presence of serum growth factors (Figure 6.3a). AURKA activity that does not recover on re-adhesion, in the absence of serum, remains un-affected in the presence of serum growth factors as well (Figure 6.1b and 6.3b). AKT activity drops on loss of adhesion and recovers back on re-plating to fibronectin even in the absence and presence of serum (Figure 6.4b).
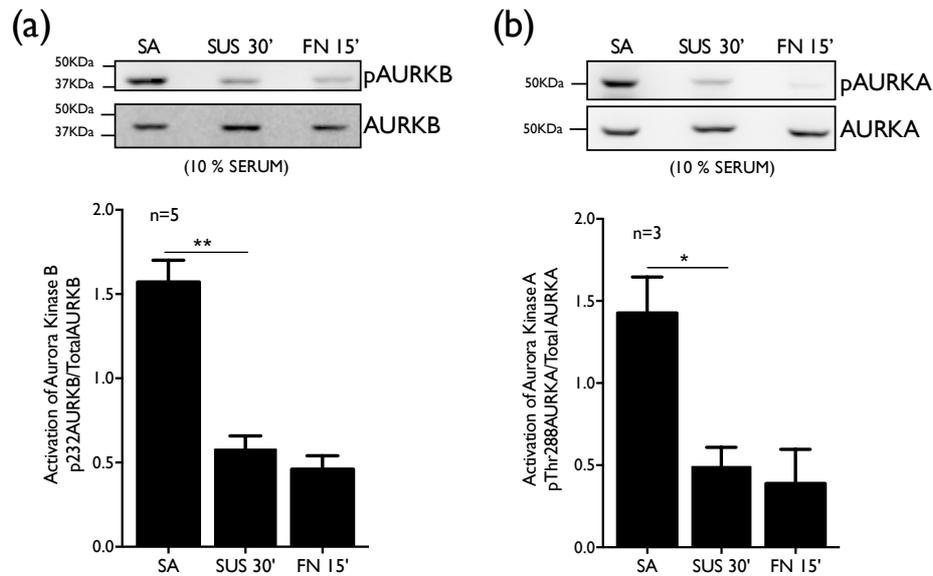
Figure 6.3: Adhesion-serum growth factor crosstalk mediated regulation of AURKB and AURKA. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on the Threonine 232 residues of AURKB (pAURKB), total AURKB and, (b) phosphorylation on Threonine 288 residues of AURKA (pAURKA) and total AURKA in the lysates from WT-MEFs stable adherent (SA), suspended for 30 mins (SUS 30') and re-adherent on fibronectin for 15mins (FN 15') in presence of serum growth factors. The ratios of pAURKB/AURKB and pAURKA/AURKA were normalized to respective SA (equated to 1), and these values are represented in the graph as mean ± SE from at least three independent experiments. Statistical analysis of all the above data was done using the two-tailed unpaired Student's t-test and significance if any was represented in graph (* p-value <0.05, ** p-value <0.01).

To further understand this differential regulation of AURKB we determined adhesion-dependent regulation of the cell cycle profile of WT-MEFs in the presence of serum. When grown with serum 50 ± 0.7 % of stable adherent WT-MEFs are seen to be in the G1-G0 phase and 43 ± 1.8 % in S phase (Figure 6.4a). This profile also did not change significantly when these cells were held in suspension for 30 mins, followed by re-plating on fibronectin for 15mins (Figure 6.4a). In the presence of serum (Figure 6.4a), the cell cycle profile was expectedly different as compared to profile of these cells in absence of serum (Figure 6.2a) and

we next evaluated if this could be a contributing factor to regulate Aurora Kinase activity.

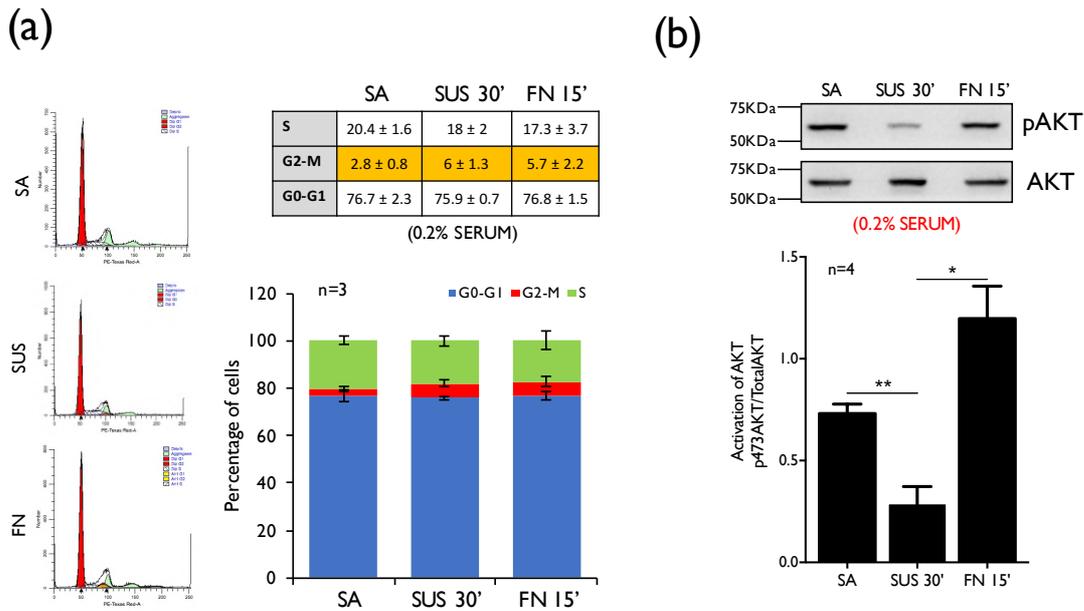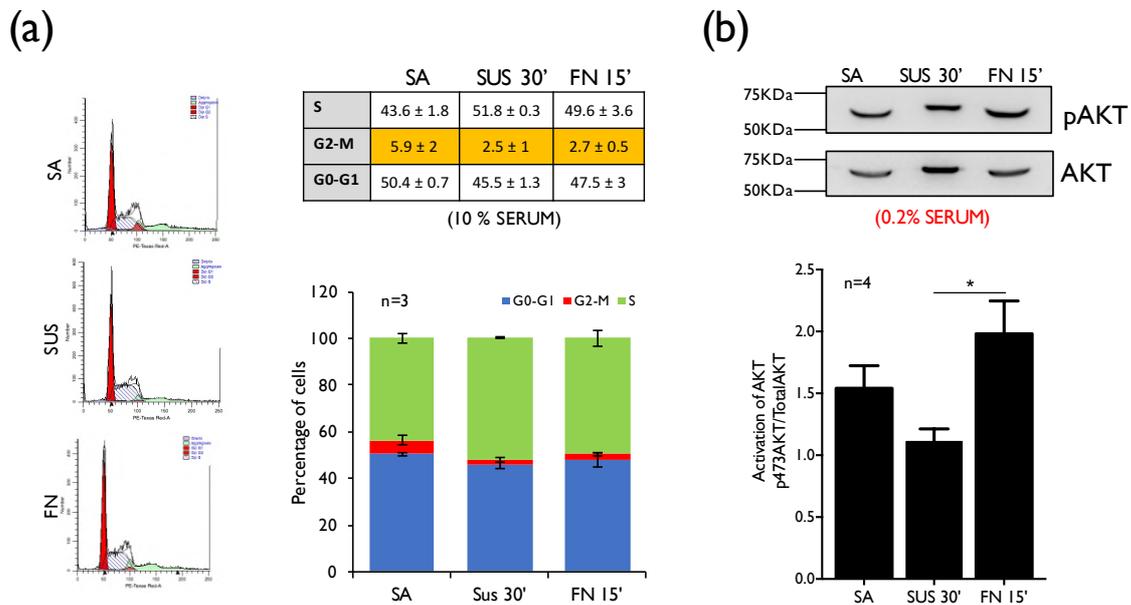**Figure 6.4: Adhesion-growth factor crosstalk dependent regulation of Cell cycle profile.** (a) Representative histogram of 3 independent experiments (left panel) and Percentage of cells present in G2-M, S and G0-G1 phase (right panel) are shown in table (upper panel) and graph (lower panel), (b) Western blot detection (upper panel) and quantitation (lower panel) of phosphorylation at Serine 473 residue of AKT and total AKT in lysates from WT-MEFs stable adherent (SA), suspended for 30 mins (SUS 30') and re-adherent on fibronectin for 15mins (FN 15') grown in presence of serum growth factors are represented. The graph represents the mean ± SE from at least 3 independent experiments. Statistical analysis was done using two-tailed unpaired Student's *t*-test and significance represented (* p-value <0.05).

The expression levels and activation of Aurora Kinases are seen to vary at different phases of the cell cycle (Carmena & Earnshaw 2003c; Goldenson & Crispino 2014b). A small but significant change in the cell cycle profile that presence of serum causes could in part contribute to the differential effect serum growth factors have on re-adhesion mediated AURKB activation. To evaluate this possibility, we tested if rapid (15min) stimulation of serum-deprived cells with serum growth factors affects the re-adhesion mediated regulation of AURKB. This rapid serum stimulation is unlikely to affect cell cycle profile of mouse fibroblasts. Serum-deprived WT-MEFs suspended for 30mins were treated for 15min with 10% FBS and re-plated with serum on fibronectin. This treatment was enough to prevent the recovery of AURKB activity upon re-adhesion (Figure 6.5a) as seen in WT-MEFs grown with 10% serum (Figure 6.5a and 6.3a). This suggests serum growth factors can indeed inhibit adhesion-dependent activation of AURKB.

This could have implications for the functional behaviour of fibroblasts grown in the presence of serum in tissue culture experiments. We hence tested if heat inactivation of serum (56°C, 30mins) can disrupts the adhesion-dependent AURKB activation and find it does not affect the same (Figure 6.5a).

Figure 6.5: Effect of serum stimulation on adhesion-growth factor crosstalk dependent regulation of AURKB activity. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on the Threonine 232 residues of AURKB (pAURKB), total AURKB and, (b) phosphorylation on Tyrosine 397 residues of FAK (pFAK) and total FAK in the lysates from: serum starved WT-MEFs (-Ser 12hrs) suspended for 45mins and re-plated on fibronectin in presence of 0.2% serum, serum starved WT-MEFs suspended for 30 mins followed by 15mins in heat inactivated 10% FBS DMEM (+ΔSer 30mins) and re-adherent on fibronectin for 15mins (FN 15') in presence of heat inactivated 10% serum growth factors, serum starved WT-MEFs suspended for 30 mins followed by 15mins in 10% FBS DMEM (+Ser 30mins) and re-adherent on fibronectin for 15mins (FN 15') in presence of 10% serum growth factors or WT-MEFS suspended and replated in presence of 10% FBS DMEM. The ratios of pAURKB/AURKB and pFAK/FAK were normalized to respective SUS (equated to 1), and these values are represented in the graph as mean ± SE from four independent experiments. Statistical analysis of all the above data was done using the single sample *t*-test and significance if any was represented in graph (* p-value <0.05).

Focal adhesion kinase (FAK) activity is known to be regulated by integrin mediated adhesion (M. Oktay et al. 1999; Eliceiri 2001) and was hence found to drop upon loss of adhesion and recover back upon re-plating on fibronectin irrespective of presence or absence of serum

growth factors or heat inactivated serum (Figure 6.5b). Taken together these results suggest that integrin-mediated adhesion differentially regulates AURKB (but not AURKA) and this regulation is further dependent on the integrin-growth factor crosstalk.

6.2.4 Adhesion-growth factor crosstalk regulates ERK activation

To evaluate the functional relevance adhesion and growth factor mediated control of AURKB activation could have in cellular signalling, we tested whether if and how it could affect integrin-dependent signalling or cell spreading. We tested the effect absence or presence of serum has on integrin-mediated activation of ERK and FAK (Eliceiri 2001) in WT-MEFs. FAK activation drops on loss of adhesion and recovers on re-adhesion in the presence and absence of serum (Figure 6.6c). Adhesion-dependent ERK activation however was seen to be differentially affected by the presence or absence of serum (Figure 6.6a), as was AURKB (Figure 6.6b, 6.3a and 6.1c). As expected, basal ERK activity was found to be high in all experiments carried out in the presence of 10% FBS DMEM (Figure 6.7b). In low serum conditions ERK activation that drops on loss of adhesion, further decreases on re-adhesion to fibronectin (Figure 6.6a) as AURKB activation increases (Figure 6.6b). In the presence of serum, ERK activation was high on loss of adhesion and stays elevated on re-adhesion (Figure 6.6a), as AURKB activation is seen to be low under both of these conditions (Figure 6.6b). Presence of serum did not have any effect on regulation of FAK activity by adhesion (Figure6.6c). Taken together this suggests an inverse correlation could exists between ERK and AURKB activation in re-adherent cells.

Further, as shown in Figure 6.5a, rapid stimulation of serum-deprived cells with complete serum as well as heat inactivated serum for 15minutes can both cause a drop in AURKB activity on re-plating (Figure 6.5a). These cells when probed show an increase in ERK activity (Figure 6.7a) further suggesting the adhesion-stimulated AURKB activation to inversely regulates ERK activity in re-adherent cells. This highlighted the presence of a AURKB-ERK crosstalk in re-adherent cells that we wanted to further confirm.
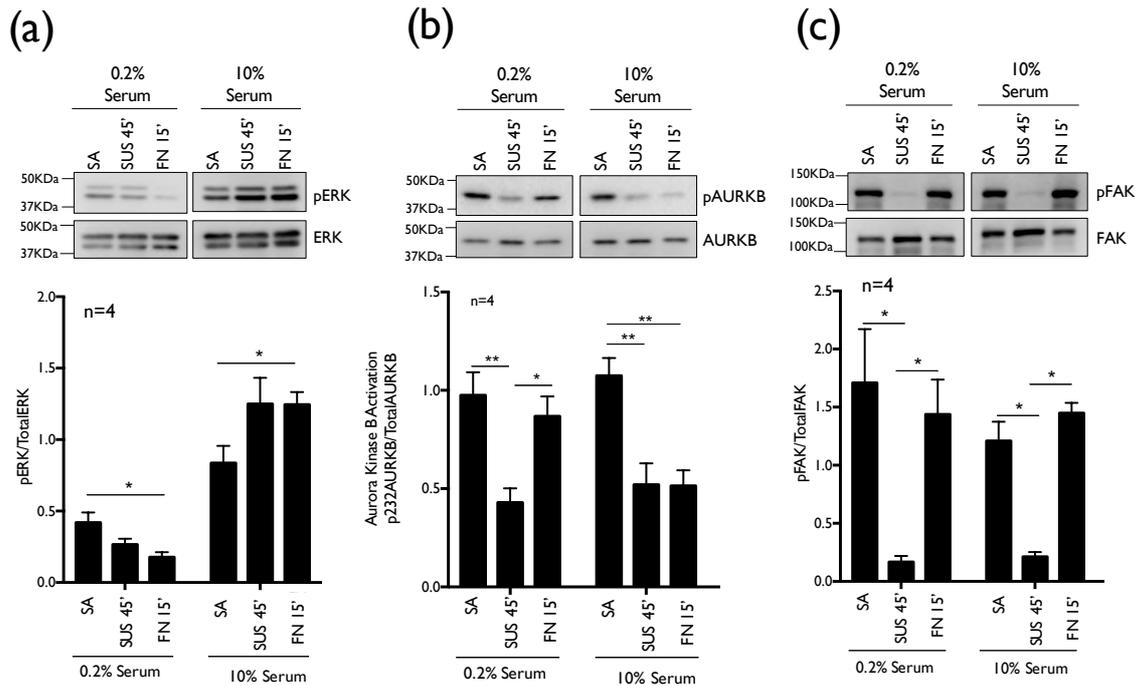
Figure 6.6: Effect of adhesion-growth factor crosstalk on ERK activity. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on Threonine202/Tyrosine204 residues of p44/p42 ERK1/2 (pERK) and Total p44/p42 ERK1/2 (TotalERK), (b) phosphorylation on the Threonine 232 residues of AURKB (pAURKB), total AURKB and, (c) phosphorylation on Tyrosine 397 residues of FAK (pFAK) and total FAK in the lysates from serum starved WT-MEFs suspended for 45mins and re-plated on fibronectin in presence of 0.2% serum and WT-MEFS suspended and replated in presence of 10% FBS DMEM. The ratios of pERK/TotalERK, pAURKB/AURKB and pFAK/FAK are represented in the graph as mean ± SE from four independent experiments. Statistical analysis of all the above data was done using the two-tailed unpaired Student's *t*-test and significance if any was represented in graph (* p-value <0.05, ** p-value<0.01).
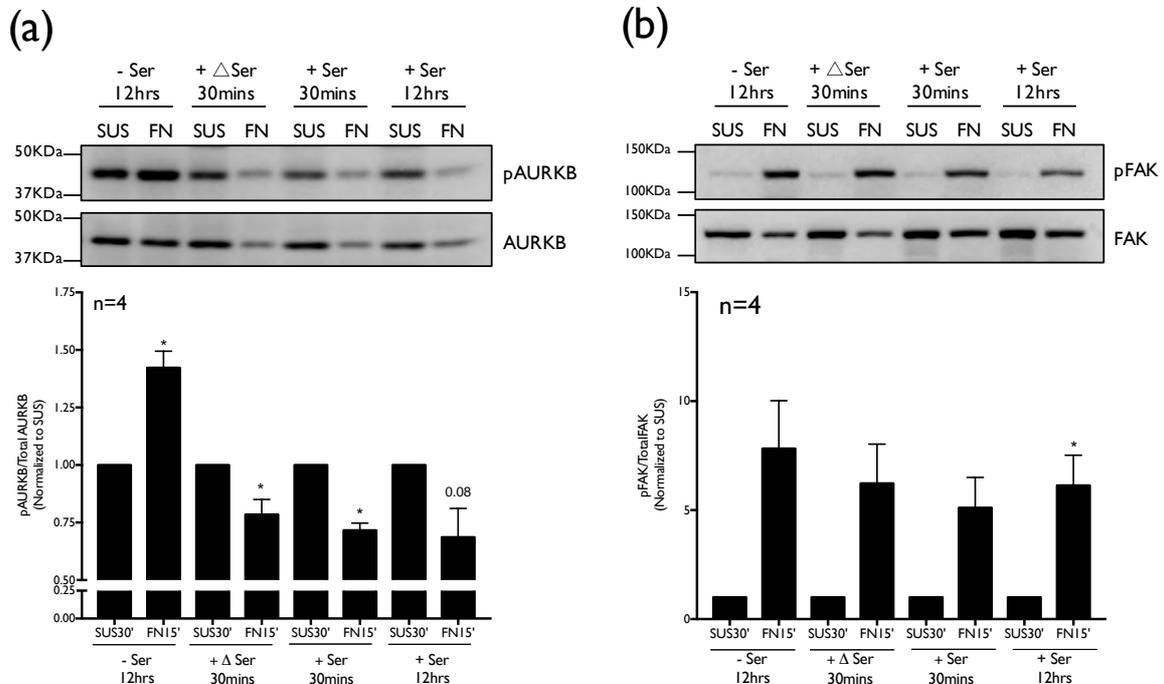
Figure 6.7: Effect of serum stimulation on adhesion-growth factor crosstalk dependent regulation of ERK activity. Western blot detection (upper panel) and quantitation (lower panel) of phosphorylation on Threonine202/Tyrosine204 residues of p44/p42 ERK1/2 (pERK) and Total p44/p42 ERK1/2 (TotalERK), in the lysates from, (a) serum starved WT-MEFs (-Ser 12hrs) suspended for 45mins and re-plated on fibronectin in presence of 0.2% serum, serum starved WT-MEFs suspended for 30 mins followed by 15mins in heat inactivated 10% FBS DMEM (+ΔSer 30mins) and re-adherent on fibronectin for 15mins (FN 15') in presence of heat inactivated 10% serum growth factors, serum starved WT-MEFs suspended for 30 mins followed by 15mins in 10% FBS DMEM (+Ser 30mins) and re-adherent on fibronectin for 15mins (FN 15') in presence of 10% serum growth factors or WT-MEFS suspended and re-plated in presence of 10% FBS DMEM and (b) 45 mins suspended cells under similar conditions. The ratios of pERK/TotalERK were normalized to respective SUS (equated to 1) for panel a, and these values are represented in the graph as mean ± SE from four independent experiments. Statistical analysis of all the above data was done using the single sample *t*-test for normalized data and two-tailed unpaired Student's *t*-test for non-normalized data and significance if any was represented in graph (* p-value <0.05).

6.2.5 Aurora Kinase B inhibition increases adhesion-dependent ERK activation and localization

To further establish the adhesion-dependent AURKB-ERK crosstalk, we tested the effect inhibition of AURKB activity has on ERK activation. We used a specific small-molecule inhibitor AZD1152 (Foote & Mortlock 2009; de Groot et al. 2015) to treat re-adherent cells in

absence of serum (where AURKB is activated) (Figure 6.1c and 6.6b) and tested the effect this has on ERK activation. Treatment of WT-MEFs with 2μM of AZD1152 for 30mins causes a significant inhibition of AURKB (Figure 6.8a and 6.9a). This inhibition of AURKB activity causes a significant increase in ERK activity upon re-adhesion (Figure 6.8b) but not in suspended cells (Figure 6.9b). This confirms the presence of a regulatory AURKB-ERK crosstalk in re-adherent cells. Inhibition of AURKB using AZD1152 had no effect on FAK activation in suspended as well as re-adherent WT-MEFs (Figure 6.10), suggesting the effect of its inhibition to be unique to ERK activation.
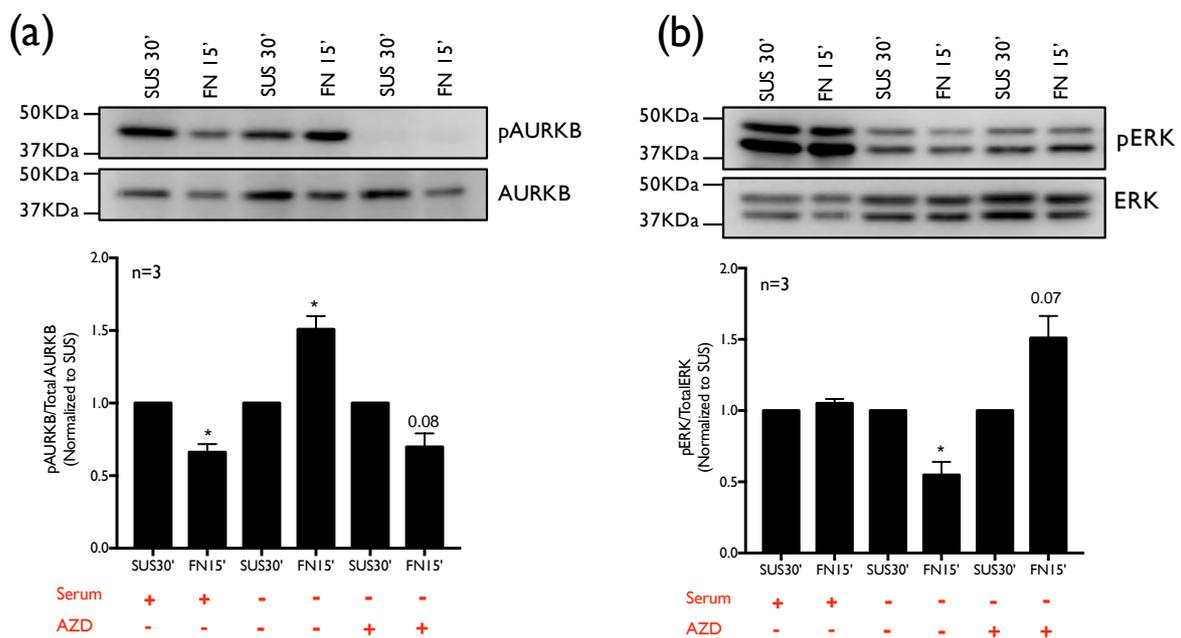
Figure 6.8: Effect of AURKB inhibition on re-adherent ERK activity. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on the Threonine 232 residues of AURKB (pAURKB), total AURKB and, (b) phosphorylation on Threonine202/Tyrosine204 residues of p44/p42 ERK1/2 (pERK) and Total p44/p42 ERK1/2 (TotalERK) in the lysates from serum starved WT-MEFs suspended for 30mins (with or without 2μM AZD1152) and re-plated on fibronectin in presence of 0.2% serum (with or without 2μM AZD1152) and WT-MEFS suspended and replated in presence of 10% FBS DMEM. The ratios of pERK/TotalERK and pAURKB/AURKB are represented in the graph as mean ± SE from three independent experiments. Statistical analysis of all the above data was done using the single sample *t*-test and significance if any was represented in graph (* p-value <0.05).

Figure 6.9: Effect of AURKB inhibition on ERK activity in suspended WT-MEFs. Western blot detection (upper panel) and quantitation (lower panel) of (a) phosphorylation on the Threonine 232 residues of AURKB (pAURKB), total AURKB and, (b) phosphorylation on Threonine202/Tyrosine204 residues of p44/p42 ERK1/2 (pERK) and Total p44/p42 ERK1/2 (TotalERK) in the lysates from serum starved WT-MEFs suspended for 30mins (with or without 2μM AZD1152) and WT-MEFS suspended in presence of 10% FBS DMEM. The ratios of pERK/TotalERK and pAURKB/AURKB are represented in the graph as mean ± SE from three independent experiments. Statistical analysis of all the above data was done using the two-tailed unpaired Student's *t*-test and significance if any was represented in graph (* p-value <0.05, ** p-value<0.01, *** p-value<0.001).

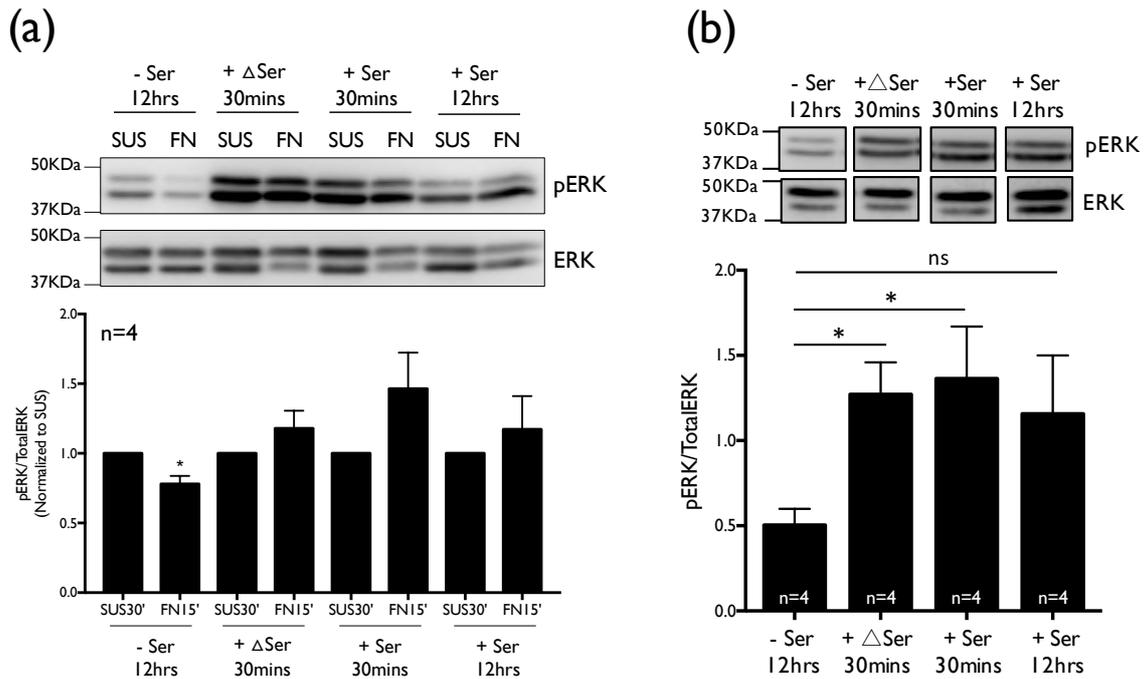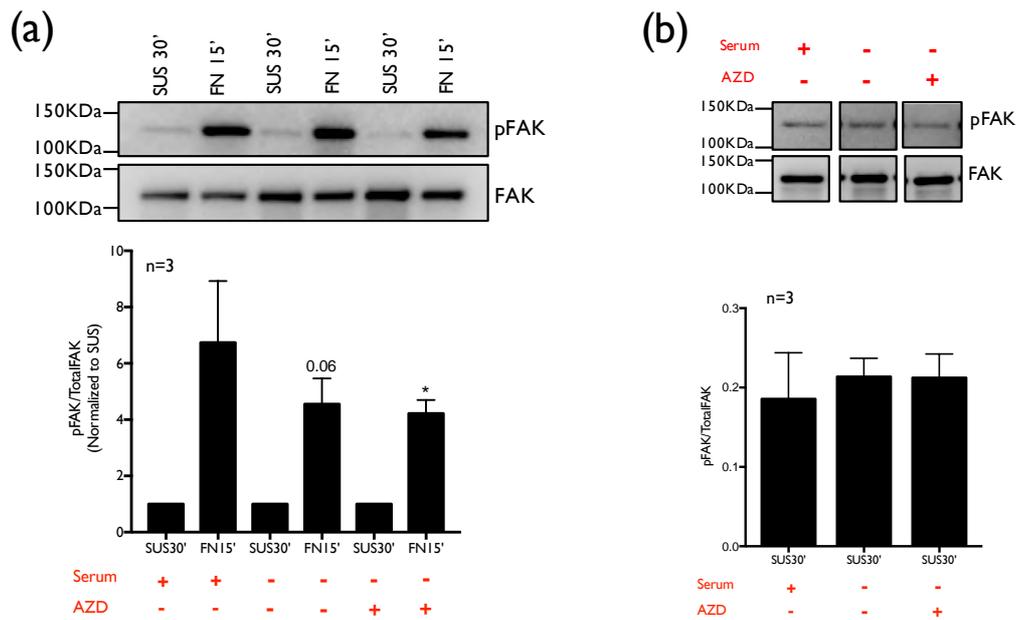**Figure 6.10: Effect of AURKB inhibition on FAK activity.** Western blot detection (upper panel) and quantitation (lower panel) of phosphorylation on Tyrosine 397 residues of FAK (pFAK) and total FAK in the lysates from (a) serum starved WT-MEFs suspended for 30mins (with or without 2μM AZD1152) and re-plated on fibronectin in presence of 0.2% serum (with or without 2μM AZD1152) and WT-MEFS suspended and re-plated in presence of 10% FBS DMEM, and, (b) 30 mins suspended cells under similar conditions. The ratios of pFAK/TotalFAK are represented in the graph as mean ± SE from three independent experiments and normalized to SUS (equated to 1) in case of panel a. Statistical analysis of all the above data was done using the the single sample *t*-test for normalized data and two-tailed unpaired Student's *t*-test for non-normalized data and significance if any was represented in graph (* p-value <0.05, ** p-value<0.01).

We further evaluated the localization of active ERK (phosphorylated ERK) in re-adherent cells and tested the impact AURKB inhibition mediated stimulation of ERK activation has on the same. In serum-deprived cells active phosphoERK is seen to localize to membrane ruffles on AURKB inhibition (Figure 6.11a) which is accompanied by an increase in the number of cells exhibiting a ruffling phenotype (Figure 6.11b). This is comparable to the phosphoERK localization and ruffling phenotype in cells treated with 10% FBS (FIGURE 6.11b).

Together these results confirm AURKB activation to regulate ERK activity and localization downstream of integrin-mediated adhesion. This regulation of AURKB and ERK is seen to be dependent on the adhesion-growth factor crosstalk.
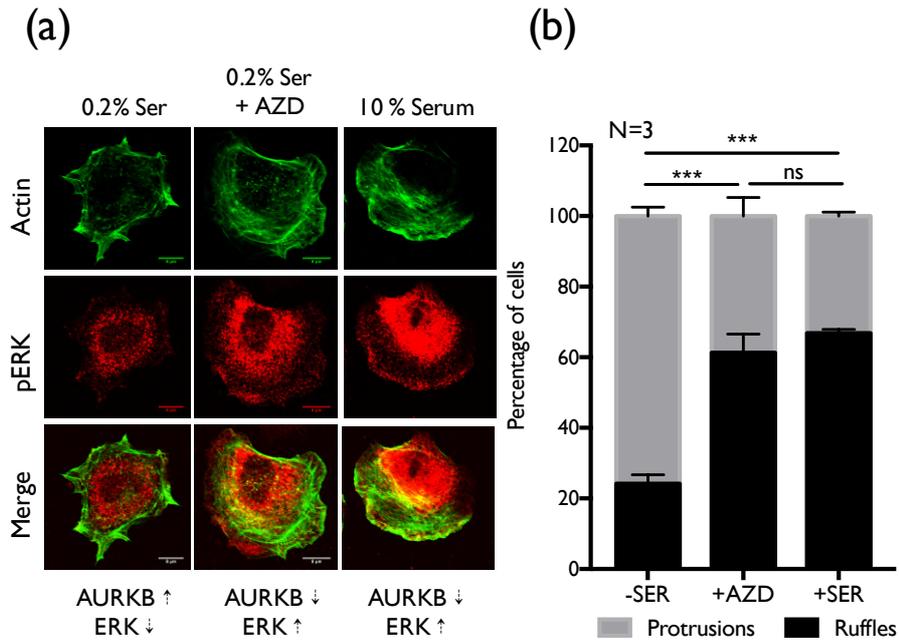
Figure 6.11: Effect of AURKB inhibition on ERK localisation and membrane ruffling in WT-MEFs. (a) pERK detected using specific antibody against phosphorylation on Threonine202/Tyrosine204 residues of p44/p42 ERK1/2 (pERK) at membrane ruffles in re-adherent spreading WT-MEFs in presence of 0.2% FBS, 0.2% FBS + 2 μM AZD1152 or 10% FBS DMEM. (b) Percentage distribution of cells with ruffled and protrusion phenotypes in re-adherent WT-MEFs in presence of 0.2% FBS, 0.2% FBS + 2 μM AZD1152 or 10% FBS DMEM, was determined by counting 100 cells per time point from three independent experiments. Statistical analysis was done using Chi-square test two-tailed for distribution profile (b) data and p-values are as indicated (**$p<0.01$, *** $p < 0.001$). Scale bar in (a) is set at 8 μm.

6.3 Summary and Open questions

In this chapter, we report that cell-matrix adhesion differentially regulates the activity of Aurora Kinase B) and Aurora Kinase A (AURKA), where AURKB activity drops on loss of adhesion and recovers back on re-adhesion to fibronectin matrix. This recovery of AURKB activity upon re-adhesion is inhibited in presence of serum growth factors suggesting the regulation of Aurora kinase B to be sensitive to the adhesion-growth factor crosstalk.

To further understand this differential regulation of AURKB we evaluated the cell cycle profile of WT-MEFs in the absence and presence of serum. The cell cycle profile was expectedly different between the two conditions, with ~76% cells being arrested at G1 phase in absence of serum. In both these conditions (with and without serum) the cell cycle profile did not change significantly upon loss of adhesion or re-adhesion suggesting that adhesion-growth factor crosstalk dependent regulation of AURKB might be independent of its cell cycle regulation. However, knowing that activation and levels of Aurora Kinase B are different in different phases of the cell cycle (Carmena & Earnshaw 2003), the distinct change in cell cycle profile that presence of serum causes could influence the differential effect re-adhesion has on AURKB activation. To evaluate this possibility, we tested if the rapid stimulation of serum-deprived cells with serum can regulate AURKB.

Serum-deprived WT-MEFs suspended for 30mins were transiently exposed to serum growth factors (10% serum DMEM) for 15min and re-plated with 10% serum DMEM. This prevented the recovery of AURKB activity upon re-adhesion as seen in WT-MEFs with serum, suggesting that transient growth factor stimulation can inhibit adhesion-dependent recovery of AURKB activity. Such a 15min shot of serum is unlikely to significantly change the cell cycle profile of WT-MEFs to affect AURKB activation. This suggests a direct regulation of AURKB by a serum component. Heat inactivation of serum (56°C, 30mins) did not affect its regulation of AURKB in re-adherent cells suggesting the serum regulatory component to not be heat-sensitive. Possible heat resistant components of serum that could mediate this regulation could be growth factors or small molecules like amino acids, sugars, lipids or hormones (Honn et al. 1975). Identification of serum component(s) by dialysis or charcoal treatment (Stoikos et al. 2008) that might be mediating AURKB activation could be of much significance in better understanding the adhesion-growth factor crosstalk and its impact on cellular function.

To establish the functional relevance of adhesion-regulated AURKB activity, we tested the effect absence/presence of serum has on integrin-mediated activation of AKT, ERK and FAK (Eliceiri 2001) in WT-MEFs. Interestingly, while AKT and FAK activation recovered upon re-adhesion in the presence and absence of serum, ERK activation was differentially affected by the presence of serum (like AURKB is). ERK activity stayed low when AURKB activity recovered on re-adhesion in absence of serum and increased when AURKB activity was kept low by the presence of serum. To test if this reflects regulatory crosstalk, we inhibited AURKB activity using a specific small-molecule inhibitor AZD1152 in re-adherent cells in absence of serum (where AURKB is activated) and tested the effect it has on the reduced ERK activation status. Inhibition of AURKB activity causes a significant recovery in ERK activity upon re-adhesion. Immunofluorescence studies show this active phosphoERK to localize to membrane ruffles on AURKB inhibition promoting membrane ruffling in re-adherent cells. Together these results suggest AURKB can regulate ERK activation and localization downstream of integrin-mediated adhesion. ERK is also known to regulate AURKB activation and this could create a regulatory feedback loop that future studies will explore. Taken together our studies show that adhesion-growth factor crosstalk regulates AURKA and AURKB differentially and this could contribute to their control of downstream signalling (like ERK activation) in cells.

6.4. Conclusion

Cell-matrix adhesion differentially regulates AURKB vs AURKA in the presence of serum. This regulation is independent of the cell cycle. Adhesion-dependent AURKB activation negatively regulates ERK activation and localization in re-adherent spreading WT-MEFs.

Chapter 7

Discussion

The work presented in this thesis discusses the role of cell-matrix adhesion in regulating the activity and function of two mitotic kinases, Aurora Kinase A and Aurora Kinase B in anchorage-dependent mouse embryonic fibroblasts. Studies further determine the intricate regulation and role AURKA-RalA and AURKB-ERK1/2 crosstalk have downstream of integrin and serum growth factors to support anchorage-dependence in cells. This further supports the need to evaluate these regulatory crosstalks in Ras-dependent and -independent cancers and evaluate how targeting them could disrupt anchorage-independence in cancers.

7.1 A novel and differential regulation of Aurora Kinase A and Aurora Kinase B by cell-matrix adhesion.

7.1.1 Integrin-growth factor crosstalk regulates Aurora Kinase activity

Progression of mammalian cells through different stages of cell cycle requires the attachment of cells to extracellular matrix (ECM), to other cells (cell-cell adhesion) and soluble factors (growth factor peptides, mitogens, inhibitors, hormones) (Y. Li & Burridge 2018; Champion et al. 2017; Pugacheva et al. 2006). The transition of cells from interphase to mitosis and back is also often marked by profound changes in cellular attachment (Uroz et al. 2018; Aguilar-Aragon et al. 2020; M. C. Jones et al. 2019). The most economical way to achieve this coordination is to engage and re-use same signaling cascade proteins to govern both cell attachment and cell cycle. Integrin-dependent signaling is one such regulatory pathway that has been reported to be intricately involved in programmed cell division (Reverte et al. 2006). Integrin engagement by ECM at focal adhesions activates many signaling proteins, including focal adhesion kinase (FAK), SRC family kinases and Cas family members (HEF-1/NEDD9 and p130Cas). These proteins engage with multiple effector pathways to transmit pro-proliferative signals downstream that ultimately activate various cell cycle phase specific cyclins and cyclin-dependent kinases (CDKs) (Walker & Assoian 2005; Schwartz & Assoian 2001). One of the focal adhesion localized Cas family member protein HEF1 has been reported to translocate to centrosome at the G2-M interface, where it activates a cell cycle kinase Aurora Kinase A (Pugacheva & Golemis 2005). This centrosome activated Aurora Kinase A then phosphorylates and helps activate Cdk1/cyclin B complex and TPX2 to drive formation of mitotic spindle (Kufer et al. 2002; Carmena & Earnshaw 2003a). This suggests cell attachment proteins might be involved in fine tuning the regulation and activation of Aurora Kinases in cells.

In this thesis, I have explored for the first time, the regulatory crosstalk between cell-matrix adhesion and activation of Aurora Kinases and its functional consequences in anchorage-dependent mouse embryonic fibroblasts.

I find that detachment of serum-starved anchorage-dependent mouse embryonic fibroblasts from ECM results in decrease in both AURKA and AURKB activity (Chapter 6). However, on re-adhesion to fibronectin matrix for 15mins, only AURKB activity recovers back but not AURKA. The rapid nature of this regulation on re-adhesion of suspended cells to fibronectin in absence of serum supports a role of integrin-mediated adhesion in differentially regulating AURKB vs AURKA. I further find cell cycle profile to not change significantly upon loss of adhesion and re-adhesion, suggesting adhesion-mediated regulation of AURKB might be independent of its cell cycle regulation. I further find serum growth factors to negatively regulate the adhesion-dependent AURKB activation but have no effect on AURKA activity. Taken together, though loss of adhesion and re-adhesion are extreme conditions that do not exactly mimic cell rounding during mitosis and re-adhesion post cytokinesis, it has helped me establish the regulatory control integrin-serum growth factor crosstalk has on Aurora Kinase activation.

Integrins form a large family of α/β heterodimeric receptors that have been classified into different groups, based on the components of extracellular matrix that they interact with (eg collagen, RGD, Laminin-binding integrins) (J. T. Yang & Hynes 1996; Hynes 2004; Tamkun et al. 1986; C. Singh et al. 2018). Different integrin combinations and integrin-dependent signaling cascades play crucial roles throughout different stages of cell cycle and are involved in formation of focal adhesions in interphase cells vs reticular adhesions in mitotic cells (Lock et al. 2018; LaFlamme et al. 2008; Y. Li & Burridge 2018). Fibronectin is the extracellular matrix component used in my studies to evaluate integrin-dependent regulation of Aurora kinases. Fibronectin is known to bind various combinations of integrins (α2β1, α3β1, α4β1, α4β7, α5β1, α8β1, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, αIIbβ3) to mediate downstream signaling in the cell (J. T. Yang & Hynes 1996; Tamkun et al. 1986; Bowditch et al. 1991). It is hence important to identify specific integrins that are involved in regulating adhesion-Aurora Kinase pathway. Further, investigating whether and how other ECM components like collagen, fibrinogen, laminin etc can regulate Aurora Kinase activity is also an interesting open question.

In my study, serum-deprived WT-MEFs suspended for 30mins when transiently stimulated with serum growth factors, prevented the recovery of AURKB activity upon re-adhesion,

suggesting that transient growth factor stimulation can inhibit adhesion-dependent recovery of AURKB activity. This in turn suggests a direct regulation of AURKB by a serum component. Heat inactivation of serum (56°C, 30mins) did not affect its regulation of AURKB in re-adherent cells suggesting the serum regulatory component to not be heat-sensitive. This has implications in the field as heat-inactivated serum is widely used for various experiments and making sure the regulation of AURKB activity is not variable between serum that is heat-inactivated or not ensures the pathway is conserved in all cells. Possible heat resistant components of serum that could mediate this regulation could be growth factors or small molecules like amino acids, sugars, lipids or hormones(Honn et al. 1975). A report on how thyroid hormone can modulate AURKB activity during transcriptional activation of pituitary genes (Tardáguila et al. 2011) solidifies the observation that serum component can be involved in AURKB regulation. Hence, identification of serum component(s) by dialysis or charcoal treatment (Stoikos et al. 2008) that might be mediating AURKB regulation could be of much significance in better understanding the adhesion-growth factor crosstalk and its impact on cellular function.

Aurora Kinase A and Aurora Kinase B share similar protein structure, but exhibit distinct localization, functions, substrates specificity and regulatory partners (Carmena & Earnshaw 2003a). The functional divergence of Aurora kinases is determined by their spatial compartmentalization, and their divergent N-termini domains that contributes to their spatial and functional differentiation (S. Li et al. 2015). Low levels of AURKA are diffusely distributed throughout the cytoplasm of interphase cells whereas AURKB is majorly localized to nucleus of interphase cells (Carmena & Earnshaw 2003a). During mitosis, AURKA levels increase and it accumulates at the centrosomes with the help of C-terminal domain and at proximal microtubules with the help of N-terminal domain. On the other hand, AURKB localization is governed majorly through its C-terminal domain. Hence, the mitotic roles of each Aurora member seem to mostly rely on their expression, temporal restriction and localization, rather than on their kinase activity. On the other hand, non-mitotic roles of Aurora kinases are not yet shown to depend on their localization in interphase cells. The differential regulation of AURKA and AURKB by integrin-mediated adhesion that we see in our studies with WT-MEFs might hence stem from the possibility that these two kinases localize differentially in suspended and re-adherent cells. Evaluating the spatial and temporal activation of AURKA and AURKB in suspended vs re-adherent cells in presence and absence of serum could hence provide necessary insights into adhesion-growth factors-Aurora Kinase regulatory

pathway. One of the ways this could be evaluated is using AURKA and AURKB specific FRET sensors (Bertolin et al. 2016; Fuller et al. 2008; Bertolin et al. 2019; Afonso et al. 2019) which will help establish whether localisation plays any role in adhesion-dependent differential Aurora Kinase activation and regulation.

7.1.2 Regulators of integrin-growth factor crosstalk-dependent Aurora Kinase activity

Loss of adhesion (for 30mins) in absence of serum causes a decrease in AURKA and AURKB activity (Chapter 6), however the activity of both these kinases is recovered when kept in suspension for longer (for 90mins) (Chapter 3), suggesting Aurora kinases to be rather sensitive to the kinetics of loss and recovery of cell-matrix adhesion. Identifying what signaling components downstream of integrins could be involved in this regulation hence is an important question. During mitosis, centrosomal AURKA is activated by PAK1 kinase and HEF1/NEDD9 scaffolding protein, both of which are translocated at centrosomes from focal adhesions as the cells enter mitotic phase or rather at the onset of mitotic cell rounding (Parrini et al. 2005; Pugacheva & Golemis 2005). Both PAK1 and HEF1/NEDD9 activate AURKA by directly phosphorylating Thr-288 residue and stabilising it (Pugacheva et al. 2007; Zhao et al. 2005). These proteins could hence be involved in recovery of AURKA activity when cells are held in suspension for longer. Mahankali et al, have also reported direct activation of AURKA by SRC kinases that aids in cell migration through regulation of tubulin polymerisation (Mahankali et al. 2015). This regulation could contribute to the drop in AURKA activity observed immediately on loss of adhesion. Testing if and how one or combination of these regulators could be involved in controlling AURKA activation downstream of adhesion in serum-starved WT-MEFs becomes an important hypothesis to test. Very little is known about the regulation of AURKB in non-mitotic cells. One of the regulatory pathways that has been reported is where a lipid raft resident protein, Flotillin-1 has been shown to regulate AURKB activity (Gómez et al. 2010). It might be hence worth testing the role of Flotillin-1 in regulating adhesion-dependent AURKB activity in WT-MEFs.

Integrins engagement with ECM is known to trigger an increase in cytosolic calcium levels in several cell lines (Shankar et al. 1993; Kwon et al. 2017; L. Balasubramanian et al. 2007). Integrin-induced Ca^{2+} signaling is mediated through focal adhesion proteins like FAK, Src, paxillin, talin, and cytoskeletal elements (actin and microtubules) (Giannone et al. 2004; M. U. Naik & U. P. Naik 2003; Kirchhofer et al. 1991). It has been reported that multiple stimuli

causing release of Ca^{2+} from ER, rapidly and transiently activate AURKA through calmodulin binding (Plotnikova et al. 2010). This activation of AURKA by Ca^{2+}/CaM is required for mitotic progression, cytokinesis during mitosis and in ciliary disassembly during interphase (Plotnikova et al. 2012). Calmodulin binding has also been reported to be important for stability and activity of AURKB at midbody during mitosis to prevent chromosomal segregation errors (Mallampalli et al. 2013). These reports coupled with the fact that Ca^{2+} signaling can dissipate rapidly through the cell raises the possibility that cell-adhesion mediated regulation of calcium signaling could contribute to adhesion-dependent Aurora Kinase activity.

7.2 Functional significance of cell matrix adhesion-regulated Aurora Kinase A activity in anchorage-dependent WT-MEFs and anchorage-independent cancers

7.2.1 Role of Aurora Kinase A in regulating RalA activity in normal and cancer cells

Integrin-mediated adhesion regulates RalA activity that promotes exocytosis of raft micro-domain which is necessary for anchorage-dependent growth signaling of normal cells (N. Balasubramanian et al. 2010; Pawar et al. 2016). This when deregulated promotes anchorage-independent growth of cancer cells (Lim et al. 2005; Martin & Der 2012; Pawar et al. 2016). During mitosis, AURKA phosphorylates and translocates RalA from plasma membrane to cytoplasmic pool at mitochondria where activated RalA along with its effector RalBP1 causes mitochondrial fission (Lim et al. 2009; Kashatus et al. 2011). This pathway hence provided an attractive system to test the role adhesion-regulated AURKA might have in regulating RalA activation and function in anchorage-dependent WT-MEFs and also study the consequence of disruption of the same in anchorage-independent cancer cells. In this study, targeting re-adherent AURKA activity using a dextran polysaccharide encapsulated small molecule inhibitor MLN8237 (V_{MLN}) disrupts re-adherent RalA phosphorylation, activity and early cell spreading (Chapter 3). This suggests the presence of an adhesion-AURKA-RalA pathway in re-adherent cells. However, RalA phosphorylation on Serine 194 is also known to be regulated by a serine threonine protein phosphatase PP2A Aβ (Sablina et al. 2007) and Protein kinase A (Gentry et al. 2014). A comprehensive study, testing the relative role of these proteins would provide a better understanding of adhesion-dependent regulation of RalA activity.

Ectopic expression of AURKA-WT or AURKA-T288 (constitutively active) in 293 cells has been shown to significantly increase phosphorylation of RalA at S194. However, expression of AURKA-WT but not AURKA-T288 (constitutively active) increases GTP-bound RalA

(active) levels{Lim:2009gla}. This suggests that the relationship between phosphorylation of RalA (S194) and its activation could be variable. Both AURKA-WT and -T288 do cause a change in RalA localisation to internal membranes and cytoplasm from the plasma membrane. This effect is significantly more prominent on the expression of AURKA-T288 mutant {Lim:2009gla}. This suggests active AURKA to more prominently re-localize RalA away from the plasma membrane and associated Ral GEFs. This is supported by the fact that the phospho-mimetic RalA-S194D is enriched on internal membranes and is less active (GTP bound) {Lim:2009gla}.

Studies correlating RalA phosphorylation and their GTP loading have been done in cancer cells, where the role of AURKA-RalA crosstalk in tumorigenic activities was evaluated. The correlation between RalA phosphorylation and GTP loading in these studies is however variable. In MDCK cells, constitutively phosphorylated RalA (WT) does not support anchorage independent growth. Constitutively active RalA (V23) needs to be phosphorylated at S194 residue to support anchorage independent growth. Together this suggests while phosphorylation does not drive activation, its effect on RalA localisation could support RalA function in cancers {Wu:2005cy}. In CFPac-1 cells, activation and function of RalA was not dependent on its phosphorylation at S194 residue, however, in Capan-1 and HPAC cells, S194 phosphorylation was required for RalA activation and function {Lim:2009glb}. In our studies with WT-MEFs, we find integrin-dependent RalA phosphorylation to be directly regulated by AURKA. This however does not correlate to RalA activation that is regulated by RalGEF RGL1.

We can speculate that phosphorylation of RalA by AURKA can possibly effect its activation by either (1) altering its localisation and hence proximity to GEF/GAP, (2) inducing a conformational change in structure effecting GTP loading, (3) altering binding affinities to GEFs/GAPs, (4) altering other post-translational modifications (geranylgeranylation, ubiquitination).

Both the Ral GTPAses, RalA and RalB contain an N-terminal Ca^{2+}-independent and a C-terminal Ca^{2+}- dependent CaM binding domain, which has been demonstrated to be involved in thrombin-induced activation of RalA and RalB in human platlets (Clough et al. 2002). In neuroendocrine cells like chromaffin cells, Ca^{2+}-dependent RalA activation has been shown to occur in response to membrane depolarization which triggers calcium influx through voltage-

gated Ca^{2+} channels (Vitale et al. 2005). This combined with the fact that AURKA activity is also regulated by Ca^{+2}/CaM signaling (Plotnikova et al. 2012; Plotnikova et al. 2010), makes it worth exploring whether Ca^{2+} signaling is involved in adhesion-dependent AURKA-RalA regulation in fibroblast cells.

The intrinsic GDP-GTP exchange and GTP hydrolysis of Ral GTPases are reported to be weak, with each process accelerated by Ral-specific guanine nucleotide exchange factors (RalGEFs) and GTPase activating proteins (RalGAPs), respectively (Gentry et al. 2014). In our studies, the RalGEF, RGL1 is involved in regulation of adhesion-dependent RalA activation and cell spreading. Further we find, individual vs joint targeting of AURKA and RGL1 comparably effect RalA activation in re-adherent cells, suggesting they work along a common pathway. Targeting AURKA we find also alters the localization of RalA and RGL1 in re-adherent cells, suggesting AURKA might regulate RGL1 to further regulate RalA activity downstream of adhesion. RalGEF, RalGDS was identified as a phosphorylation target of AURKA in a small pool expression screening (Wu et al. 2005) suggesting AURKA might control RalGEFs to ultimately regulate RalA activity. Few studies have reported the involvement of a priming kinase which translocates the protein of interest to desired intracellular location where the activating proteins acts upon it (Kettenbach et al. 2011). AURKA could act as a priming kinase that phosphorylates RalA and translocates it where RGL1 could catalyse GDP-GTP exchange. Our preliminary *insilico* analysis suggests the presence of probable AURKA phosphorylation sites in RGL1 (data not shown). Whether adhesion-stimulated AURKA can indeed phosphorylate RGL1, still remains to be tested. The role such adhesion-AURKA-RGL1 crosstalk could have in mitosis where AURKA (Goldenson & Crispino 2014a), Ral (X.-W. Chen et al. 2006) and RGL1 (Cascone et al. 2008) are all implicated will be worth exploring.

All RalGEFs are capable of catalysing GDP-GTP exchange of both RalA and RalB differentially in response to specific stimuli (Gentry et al. 2014). The differential functions that Ral isoforms perform in cells could be attributed to their spatial activation by distinct GEFs (Cascone et al. 2008). In my studies, RGL1 seen to regulate RalA (not RalB) in early re-adherent cell spreading, however, regulates RalB (not RalA) in migrating WT-MEFs in the presence of serum growth factors. Previous studies have reported RalB to be an important player in migration (Rossé et al. 2006). This study reveals RGL1 to also be involved in regulation of cell migration through its effect on RalB (not RalA) activation. Knockdown of both RalB and RGL1 stimulates migration of adherent WT-MEFs in the presence of serum. In

these cells, RGL1 localises differently from serum deprived re-adherent cells, which could contribute to its differential regulation of Ral isoforms. AURKA is not involved in RGL1-RalB dependent cell migration, suggesting the earlier speculated adhesion-dependent AURKA-RGL1 crosstalk could also be spatially regulated. Additionally, serum growth factors have been known to play an important role in fibroblast migration (W. Li et al. 2003) and could contribute to RGL1-RalB crosstalk. Late-adhesion dependent fibroblast migration when viewed in context of the early-adhesion mediated cell spreading reveals how Ral isoforms can be regulated differentially by AURKA and RGL1 downstream of distinct stimuli to support diverse physiological functions.

On evaluating whether AURKA-RalA crosstalk is exploited in cancer cells to promote anchorage-independence, I find that AURKA regulates RalA activity and anchorage-independent growth (AIG) of only Ras-independent cell lines but not Ras-dependent cancer cell lines that I tested in this study. Previous studies have reported inhibition of RalA signaling pathway by using a Aurora Kinase A inhibitor to cause reduction in *invivo* tumorigenesis of non-small cell lung cancer cells (Male et al. 2012) that are K-Ras driven. However, there are also contradictory reports suggesting that decrease in oncogenic properties of K-Ras driven pancreatic cancers using Aurora Kinase A inhibitor is not mediated by decrease in RalA activity (Neel et al. 2014). Taken together this suggests that, the role of Aurora Kinase A mediated regulation of RalA activity and function could indeed be cell type or cancer specific. However, it would be worth evaluating whether there is a underlying common driver that increases the significance of AURKA-RalA crosstalk in cancer cells. From our earlier studies in anchorage-dependent cells, we know AURKA regulates RalA activity with the help of RalGEF, RGL1, it would be hence worth exploring whether in anchorage-independent cells the same GEF, RGL1 is exploited by AURKA to regulate RalA.

In these studies, targeting AURKA-RalA crosstalk in Ras-independent cancers causes a significant inhibition of AIG of these cells. Both AURKA and RalA are known to play indispensable roles during mitosis (Goldenson & Crispino 2014a; X.-W. Chen et al. 2006; Cascone et al. 2008), suggesting that the effect V_{MLN} has on anchorage-independent growth of Ras-independent cell lines could be mediated by (1) AURKA dependent inhibition of RalA-mediated vesicular trafficking and exocytosis that reverses anchorage-independent growth signaling and/or (2) AURKA-RalA inhibition mediated G2-M arrest of cells thereby preventing cell proliferation and anchorage-independent growth of cancer cells. However, the

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relative contribution that regulation of cell cycle and vesicular trafficking have on anchorage-independent growth of these cells remains to be confirmed.

James Bischoff in 1998 for the first time reported that over-expression of Aurora Kinase A in NIH 3T3 and Rat 1 fibroblasts leads to transformation of these cells (Bischoff et. al. 1998). However, in 2005 Tatsuka et. al., reported that the magnitude of induction of Aurora Kinase A activity in transformed cells was only 1/50 – 1/100 times that observed following the transfection of various viral oncogenes. And repeat experiments in their lab with BALB/c 3T3A31 cells did not show transformation when Aurora Kinase A was over-expressed. It is hence speculated that Aurora Kinase A has only weak oncogenic activity on its own. Various studies have reported that Aurora Kinase A enhances oncogenic transformation by interacting with and controlling different binding partners and effectors. AURKA phosphorylates and inhibits the transcriptional activity of p53 and enhances MDM2-mediated p53 degradation (D Zhang et al. 2008; Katayama et al. 2004). AURKA overexpression has also been reported to inactivate BRCA-1 and together with BRCA-2 mutation enhance Ras-mediated tumorigenesis (Ouchi et al. 2004; Fu, Bian, Q. Jiang & Zhang 2007b). AURKA and H-Ras have been shown to synergistically enhance tumorigenic potential of cells by regulating Ras-MAPK pathway (Tatsuka et al. 2004; Umstead et al. 2017) . In this study, where I find AURKA-RalA crosstalk to not exist in Ras-dependent cancer cells, it would still be worth testing to see if, Ras through its downstream effectors can regulate AURKA and anchorage independent growth.
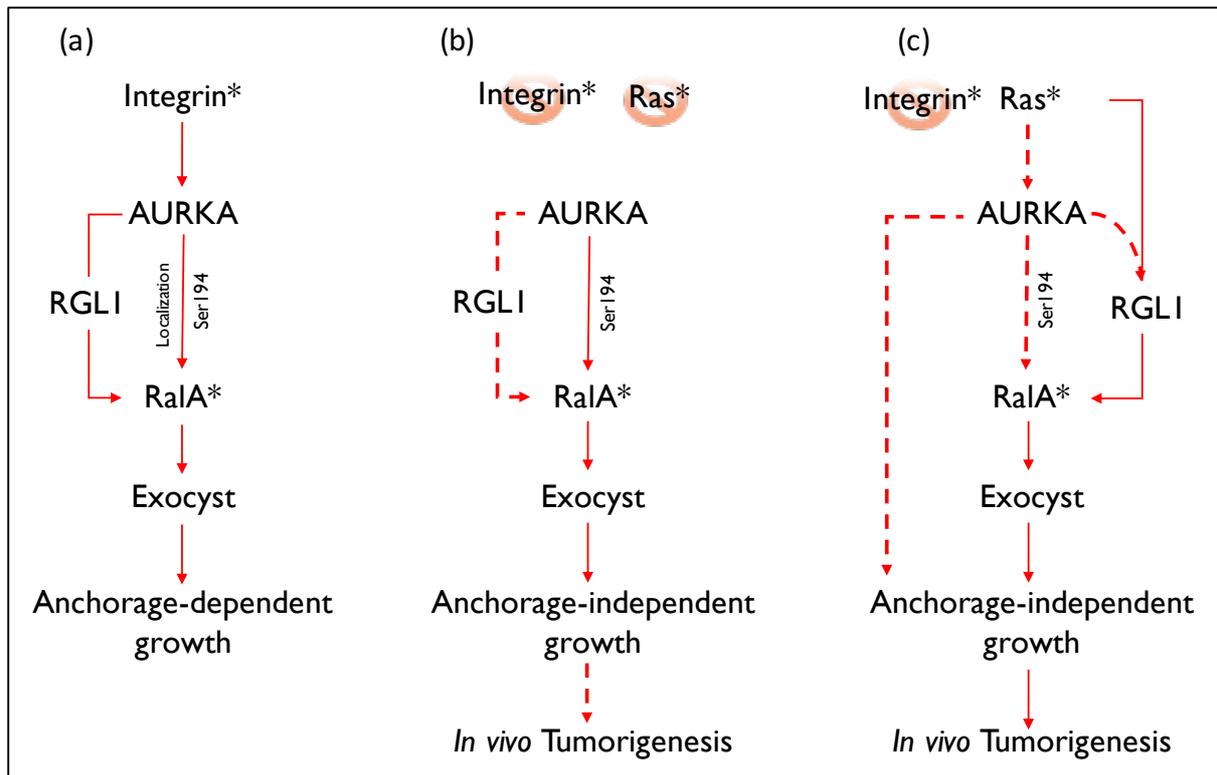
Figure 7.1. Schematic of proposed model of AURKA-RalA crosstalk. Schematic represents regulation of (a) Integrin-AURKA-RalA crosstalk in RGL1 dependent manner in anchorage-dependent WT-MEFs, (b) AURKA-RalA crosstalk and its role in Ras-independent and, (c) Ras-dependent cancers to regulate anchorage-independent growth and tumorigenesis. The solid lines represent experimentally proved regulations and dotted lines represents proposed regulations that need to be evaluated.

7.2.2 Role of Aurora Kinase A in regulating organelle structure and functions in normal and cancer cells

In addition to the well-characterised roles in mitosis, AURKA has been also reported to be recruited to the mitochondrial matrix during interphase by physically interacting with TOMM complex (Bertolin et al. 2018). In mitochondria, AURKA is capable of interacting with other multiple mitochondrial proteins. This import and function of AURKA to mitochondria during interphase is independent of RalA suggesting that, AURKA can impact mitochondria functions by two pathways: first being a RalA-independent pathway during interphase (Bertolin et al. 2018) and second RalA-dependent pathway during mitosis (Kashatus et al. 2011). It would be interesting to study whether adhesion regulated AURKA activity regulates mitochondria organisation and function and whether it does so in RalA-dependent or -independent manner in normal cells. Over-expression of AURKA leads to the higher levels of mitochondrial pool of AURKA that regulates the fusion of interconnected organelles and controls ATP production

(Bertolin et al. 2018). Mitochondria with high metabolic capacity are be able to sustain high metabolic needs of cancers and provide a selective advantage for cancer progression. Whether this function of AURKA is dependent on RalA is still an open question. However, taken together targeting mitochondrial hyperactivity together with AURKA inhibition could represent an innovative approach in the development of anti-cancer treatments.

A series of reports have shown that Golgi partitioning during G2-M phase induces centrosome recruitment and activation of AURKA, which is essential for G2-M transition (Cervigni et al. 2011). G2 block of cell cycle progression caused due to Golgi partitioning block is found to be overcome by over expression of AURKA, indicating that AURKA is a major effector of Golgi checkpoint (Cervigni et al. 2011). Further this recruitment of AURKA is promoted by activated SRC kinase at Golgi. Upon Golgi ribbon fragmentation SRC phosphorylates AURKA at Tyr-148 residue, required for its localization at centrosomes. This process is reported to be pivotal for centrosome maturation and a prerequisite for proper spindle formation and chromosome segregation (Barretta et al. 2016). Reciprocally, it has also been reported that AURKA activity is necessary for maintaining Golgi apparatus (Kimura et al. 2018). Knockdown or inhibition of AURKA causes Golgi dispersal during interphase which is rescued by over expression of AURKA (Kimura et al. 2018). Earlier studies from the lab have reported that cell-matrix adhesion regulates Golgi organisation and function in WT-MEFs, in that loss of adhesion causes rapid disorganisation of Golgi apparatus which is rescued by re-adhesion to fibronectin matrix (V. Singh et al. 2018). It would be hence interesting to test if adhesion-dependent AURKA activity has any role in regulating Golgi organisation in these cells. Knowing the fact that AURKA is over expressed and Golgi organisation and function is deregulated in many cancers, it would be interesting to evaluate whether AURKA-Golgi regulation exists and could be targeted in cancer cells.

7.3 Functional significance of adhesion-regulated AURKB activity in anchorage-dependent WT-MEFs and anchorage-independent cancers

Intracellular signal transduction pathways for cell cycle progression are dependent on proper integration and interpretation of multiple growth regulatory stimuli (including growth factor receptor pathways and integrin-cell-matrix adhesion pathways) (Schwartz & Assoian 2001; Uroz et al. 2018). Intricate mechanisms have evolved to ensure the fidelity of cell division in anchorage-dependent cells. One such player in regulation of cell growth and division is the

MAP kinase pathway (Sun et al. 2015). The extracellular signal-regulated kinases (ERKs) are a subfamily of MAPKs that have been reported to be activated via signaling cascades involving Ras, Raf kinase and MEK (Sun et al. 2015). Raf-1 is a key regulator of ERK activation (Minden et al. 1994; Galabova-Kovacs et al. 2006). During mitosis, Raf-1/ERK1,2 is known to be associated with kinetochores and spindle poles from prometaphase to anaphase and with midbody during later stages of mitosis, under the control of Raf kinase inhibitory protein (RKIP) (Eves et al. 2006). Further, this RKIP controlled Raf-1/MEK/ERK cascade has been shown to regulate Aurora Kinase B activity and spindle checkpoint, where depletion of RKIP causes an increase in Raf-1/ERK1,2 activity which downstream causes a decrease in AURKB activity, indicative of a negative regulation of AURKB by ERK1/2 (Eves et al. 2006).

In our studies with WT-MEFs, we find ERK1/2 activation on re-adhesion to fibronectin to be differentially affected by the presence or absence of serum (like AURKB is). ERK1/2 activity stayed low when AURKB activity recovered on re-adhesion in absence of serum and increased when AURKB activity was kept low by the presence of serum. Further, we find AURKB inhibition using a specific small-molecule inhibitor AZD1152 in re-adherent cells in absence of serum (where AURKB is normally, activated) causes a significant increase in ERK1/2 activity upon re-adhesion. Immunofluorescence studies show this active phosphoERK1/2 localizes to membrane ruffles on AURKB inhibition promoting membrane ruffling in re-adherent cells. Together these results suggest AURKB can regulate ERK1/2 activation and localization downstream of integrin-mediated adhesion in WT-MEFs. Although the exact mechanism of this regulation is not known, these results along with previous report where ERK1/2 negatively regulates AURKB activation in RKIP dependent manner, are suggestive of the existence of a regulatory crosstalk between AURKB-ERK1/2 in these cells. Hence, determining the functional relevance of AURKB-ERK1/2 crosstalk is an interesting open question that might help us better understand the contribution it makes in maintaining the fidelity of the cell cycle in anchorage-dependent cells. Further, knowing the fact that ERK1/2 and AURKB are both individually involved in cell spreading (Fincham et al. 2000; F. Xu et al. n.d.; Floyd et al. 2013; Ferreira et al. 2013) and migration (Matsubayashi et al. 2004; Sun et al. 2015; L. B. Zhu et al. 2014; Lifang Xie 2013), it is worth testing whether the adhesion-dependent AURKB-ERK1/2 crosstalk observed in our studies is important for these cellular processes.

Contrary to this, in anchorage-independent cancers, the AURKB and ERK1/2 have been

reported to synergistically enhance tumorigenic potential and aid in radio-resistance of cancer cells (Marampon et al. 2014; Niermann et al. 2011). In melanoma cells, BRAF/ERK axis has been shown to control AURKB expression at the transcriptional level, through the transcriptional factor FOXM1 (Bonet et al. 2012). In gynaecological cancers MEK/ERK cascade has been shown to regulate AURKB signalling to sustain colony forming potential, invasion and migration along with altering their radiation response (Marampon et al. 2014). These reports give us a compelling reason to explore the role AURKB-ERK1/2 crosstalk might have in cancers that have over expression of AURKB. This crosstalk could also be an attractive target for developing therapeutic drugs.

In all my studies, I have tested these hypotheses in two-dimensional (2D) cell cultures, however, in physiology the cells experience three-dimensional (3D) microenvironment. Integrin engagement and downstream signaling pathways have been reported to be different in cells that are embedded in a 3D matrix as compared to a 2D surface (Kapałczyńska et al. 2018; Davidenko et al. 2016; Martino et al. 2009; Duval et al. 2017). Additionally, levels of ERK1/2 activity in primary human fibroblasts grown in cell-derived 3D fibronectin matrix were found to be 2.5fold higher as compared to the same cells grown on fibronectin coated flat surfaces (Damianova et al. 2008). Hence, comparing AURKB-ERK1/2 crosstalk in 2D vs 3D microenvironments will provide a better insight on how this pathway might be regulated *invivo*.

7.4 V_{MLN} as a novel approach to modulate AURKA activity: a unique avenue for investigation in cancer therapy.

From the time when the role of Aurora Kinases became evident in cancers, the field has focused on discovering small molecule inhibitors that can target them and be used as anti-cancer drugs (Bavetsias & Linardopoulos 2015; Libertini et al. 2010). Several Aurora kinase inhibitors have been identified and augmented through multi-step organic synthesis. However, the majority of these inhibitors have heterocyclic polyaromatic structures, that makes them poorly soluble in an aqueous medium, affecting their availability and delivery across cell membrane. Due to this, the inhibitors have to be administered at a higher concentration severely compromising their specificity (de Groot et al. 2015). We have hence, developed with Dr. Jayakannan's lab in IISER, a novel and efficient drug delivery system for poorly-water soluble AURKA inhibitors in the form of a unique class of dextran polysaccharide nanovesicle.

Polymer vesicles (or polymersomes) are emerging as a new class of synthetic polymer nano-

scaffolds, that improve the delivery, bioavailability and efficiency of drugs supporting more effective treatment of diseases like cancers (Mizrahy & Peer 2012; Discher 1999; Rodríguez-Hernández & Lecommandoux 2005). They also provide a unique opportunity to repurpose drug candidates that despite their specificity are limited by issues such as solubility and bioavailibity. The effectiveness of dextran nanovesicles that we have developed is further supported by their ability to deliver both water-soluble (hydrophilic) and insoluble (hydrophobic) drugs in a single nano-vesicle (Pramod et al. 2014). Till date, liposomal formulations or nano-objects, have been one of the excellent candidates for drug delivery, with liposomal formulations such as DOXIL (Barenholz 2012) and lipoplatin (Canta et al. 2011), already evaluated in clinical trials. However, these are limited by their half-life time in blood which is found to be just 12hours. Further, these systems also require an additional protection layer, like a layer of long-chain PEG to protect them against the corona-effect caused by blood proteins (Palchetti et al. 2016). Synthetic polymer vesicles like the one we have designed provide an excellent alternative, in part due to their prolonged stability in blood plasma. An additional advantage of these polymer vesicles is that they can be structurally modified to conjugate drugs and be stimuli-responsive (Hoai et al. 2011; Feng & J. Yuan 2014; Jianzhong Du & O'Reilly 2009; W.-S. Jang et al. 2016). Polysaccharide vesicles like the one we have developed, also have the benefit of being made from a renewable resource (Pramod et al. 2012) and their surface lectins promote their binding and uptake in cells (Martin J Allen et al. 2001; N. U. Deshpande & Jayakannan 2018). These vesicles have also been used in our previous studies to deliver drugs, such as doxorubicin, paclitaxel, camptothecin and cisplatin (Pramod et al. 2012; Pramod et al. 2014; N. U. Deshpande & Jayakannan 2016).

The study presented in my thesis, is a first approach of exploring a polymer nano-vesicle platform for the delivery of Aurora Kinase A inhibitor MLN8237 in mammalian cells. We find that dextran nano-vesicle encapsulated MLN8237 (called as V_{MLN} for vesicular MLN) is taken up efficiently in cells in 2D (MCF7, SKOV3, T24, UMUC3, MIAPaCa2, Calu1, DLD1) as well as 3D (MCF7, T24, UMUC3) microenvironments. It significantly and specifically inhibits AURKA activity without effecting AURKB activity in these cells. Improved bio-availability, delivery, uptake and drug release kinetics when considered in context of the relative activity and localisation of AURKA (vs AURKB) in different cancer cell types could contribute to its better inhibition. The relative ability of V_{MLN} to inhibit AURKA could vary between cancers and a comprehensive evaluation of its effectiveness across different types of cancer would broaden the scope of V_{MLN} applications.

Further we find, V_{MLN} treated cells show specific inhibition of RalA (but not RalB) downstream of AURKA inhibition. In our studies in normal WT-MEFs, V_{MLN} mediated inhibition of AURKA revealed a role for it in regulating adhesion-dependent RalA activity in cells. Similarly, in anchorage-independent MCF7 and SKOV3 cells, V_{MLN} mediated inhibition of AURKA revealed a role for AURKA in regulation of RalA activity and anchorage-independent growth.
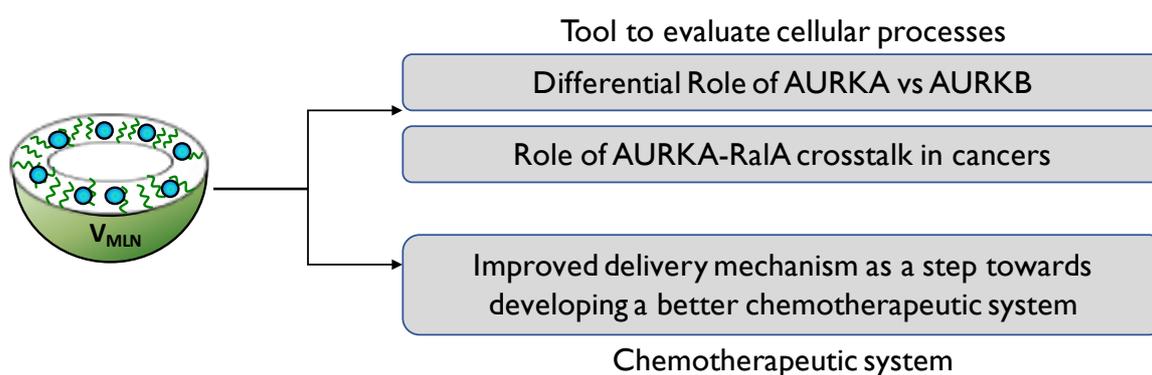
Figure 7.2. Applications of V_{MLN} in determining AURKA regulated signaling pathways in normal and cancer cells and as drug-delivery systems in cancers.

Taken together these studies identify V_{MLN} as a useful tool to evaluate AURKA-RalA signaling in cells as well as a potent drug-delivery candidate for targeting anchorage-independent cancers. Targeting members of Ras-RalGEF-Ral pathway has been a longstanding challenge in the field and being able to inhibit AURKA that phosphorylates and activates RalA (not RalB) could have vital implications in cancers.

Till date numerous Aurora Kinase inhibitors have been developed, however unfortunately, none of these have been approved for clinical use, because of cell toxicity issues (Boss et al. n.d.; Borisa & Bhatt 2017; Hyman et al. 2017; K. R. Kelly et al. 2014). A recent study demonstrated AURKB inhibitor, AZD2811 delivered by the nanoparticle 'accurin' increases its efficacy in mouse tumour xenograft models while reducing side effects (Bearss 2016). My study, with encapsulation of MLN8237 in V_{MLN}, shown to increase efficiency of AURKA inhibition and better uptake in cancer cells, thus also holds promise in tumour xenograft models. An additional advantage of V_{MLN} lies in the design of the nano-vesicle scaffold, as it

can be customized to conjugate inhibitors in its structure, and also be modified to repond to specific stimuli (ex. pH, esterase) (Pramod, Shah, et al. 2015). Thus, depending on the cancer and the proteins to be targeted multiple drug molecules can be brought together in the same nano-scaffold structure to be effectively delivered (eg. Hydrophilic Rhodamine B dye along with hydrophobic MLN8237 (Inchanalkar et al. 2018), covalently-stitched cisplatin along with encapsulated hydrophilic doxorubicin and hydrophobic camptothecin (N. U. Deshpande & Jayakannan 2016)) (L. Wang et al. 2017).

With Aurora Kinases shown to be involved in the development of resistance against various anti-cancer drugs, being able to simultaneously deliver an AURKA inhibitor with additional drugs could become particularly relevant in these cancers. Recent studies have identified AURKA overexpression to contribute to Cisplatin-based chemotherapy resistance in NSCLC (J. Xu et al. 2014). AURKA is also involved in platinum-resistance and administration of either MLN8237 or VX-680 re-sentisizes cells to platinum based treatment and diminishes the migration capacity of platinum-resistant NSCLC cells (J. Xu et al. 2014; Kuang et al. 2017). AURKA has been reported to activate SMAD5 oncogenic signaling leading to down-regulation of estrogen receptor α, causing estrogen resistance in ERα^+ breast cancers (Opyrchal et al. 2014). Treatment of these cells with tamoxifen and MLN8237 abrogated the estrogen resistance (Opyrchal et al. 2014). Similarly, AURKB overexpression has been shown to induce tamoxifen resistance and poor prognosis in breast cancer (Hole et al. 2015). Furthermore, AURKA inhibitor MLN8237 and AURKB inhibitor AZD1152 have been reported to exhibit enhanced tumour responsiveness to radiotherapy in p53$^{-/-}$ cancer cells (Venkataraman et al. 2012) and androgen-resistant prostate cancers (Niermann et al. 2011), respectively. A combination of pan-Aurora kinase inhibitors R763 and EGFR antibody cetuximab, activated G2-M cell cycle checkpoint and induced apoptosis in cetuximab-resistant squamous cell carcinoma of head and neck (SCCHN) (Hoellein et al. 2011). Taken together, Aurora kinases have become biomarkers in cancer prediction and prognosis and simultaneous inhibition of Aurora kinases could help overcome drug resistance thereby enhancing the anti-tumour effect of traditional drugs. Our polymer nano-vesicle architecture provides a unique opportunity in these scenarios making it possible to deliver multiple drugs simultaneously in cancer cells and hence hold great potential to be developed as a chemotherapeutic drug delivery system.

Conclusion

The work carried out in my thesis has helped unravel two significant regulatory crosstalks downstream of integrin-dependent cell-matrix adhesion in mouse embryonic fibroblasts. The first one shows Aurora Kinase A to regulate adhesion-dependent RalA activity, mediated by the Ras dependent RalGEF, RGL1. We further find RGL1 differentially regulates RalA and RalB activation in spreading vs migrating cells. Additionally, the AURKA-RalA crosstalk promotes anchorage-independent growth in Ras-independent cancer cell lines (MCF-7 and SKOV3). This crosstalk was not detected in five Ras-dependent cancer cell lines we tested. My study also reveals a second crosstalk, where Aurora Kinase B negatively regulates adhesion-dependent ERK1/2 activity and localisation in the presence of serum growth factors. Together these observations have added to our understanding of how Aurora Kinases – AURKA and AURKB are regulated by adhesion and in turn regulate downstream signaling. My work has also helped establish a novel dextran nanovesicle as a reliable means of delivering MLN8237 (V_{MLN}) to specifically target AURKA. This has not only helped evaluate the role of AURKA in normal cell function but also supported its prospective testing as a drug candidate in cancers.

Annexure 1

Preliminary studies for evaluating significance of AURKA-RalA crosstalk in *in-vivo* mice models

Knowing the fact that AURKA-RalA crosstalk plays a significant role in regulating anchorage-independent growth of Ras-independent cancers, we wanted to evaluate the significance of this crosstalk in *in vivo* tumour formation and tumour growth. Two strategies have been designed to study this, the first being to inject V_{MLN} treated MCF-7 and SKOV-3 cells in NOD SCID mice and continue V_{MLN} treatment to study if AURKA-RalA crosstalk is necessary for tumorigenesis or tumour formation and second is to start V_{MLN} treatment post tumour formation to study if it causes tumour regression and inhibits metastasis. As a first step towards evaluating this, we have collaborated with Dr. Siddhesh's lab in IISER, Biology and initiated studies to test the stability and detection limit of the V_{MLN} in mice serum by standardising the detection of MLN8237 by LC-MS/MS. Pilot studies to determine the number of MCF-7 and SKOV3 cells needed to form tumours of suitable size were also carried out in 9 NOD SCID mice (6 females + 3 males). 1, 2 and 3 million MCF-7 or SKOV3 cells were injected in flanks of each female and male mice to test tumour formation in both female and male mice.

Ax.1 Materials and Methodology

Ax.1.1 Detection of MLN8237 using LC-MS/MS

MLN8237 was dissolved in Acetonitrile with 1% Formic acid to get a final concentration of 0.1mg/ml. IDA method was used in Dr. Siddhesh's Lab (IISER, Pune) during preliminary standardisations. Three dilutions were used to determine the linearity and sensitivity of detection- 0.1 mg/ml, 0.01 mg/ml and 0.001mg/ml. Further experiments to determine stability and recovery of MLN8237 were done in CAMS facility in Venture Centre, Pune. Dilutions used for the same were from 10ppb to 100ppb. To determine the stability of MLN8237 and recovery, MLN8237 was dissolved in mice serum followed by acetoniterile extracted for LC-MS/MS analysis.

Ax.1.2 Collection of mice serum from NOD-SCID mice

1.5ml (for blood collection) and 0.6ml (For experiment) Eppendorf tube were coated with

0.05mg/ml Heparin and stored at 4°C. Blood was collected from 6-8-week-old NOD-SCID mcie by retro-orbital route into Heparin coated 1.5ml tubes and immediately kept on ice. To separate plasma the blood was centrifuged at 14,000 rpm for 5 mins at 4°C. The plasma was transferred to separate heparin coated tube and store at -20°C till further use. This protocol was followed from the following source; https://www.jax.org/research-and-faculty/research-centers/aging-center/blood-plasma-and-serum-collection.

Ax.1.3 Extraction of MLN8237 from mice serum

250µl acetonitrile was added to 50µl blood plasma with or without MLN8237 followed by vortex mixing at highest speed for 10minutes. Post vortex mixing the sample was centrifuged at 4000 rpm for 20mins at 4°C and supernatant was loaded directly onto LC-MS/MS system.

Ax.1.4 Pilot experiment for determining cell number to be injected for tumour formation

Pilot studies to determine the number of MCF-7 and SKOV3 cells needed to form tumours of suitable size were carried out in 9 NOD SCID mice (6 females + 3 males). 1, 2 and 3 million MCF-7 or SKOV3 cells were injected in flanks of each female and male mice to test tumour formation in both female and male mice (Table 2.1). Tumours were dissected out post 8 weeks of injection. MCF-7 cells however did not form tumours even 12 weeks post injection and pilot with modified protocol are currently ongoing.

Treatment groups	MCF7 group	SKOV3 group	MCF7 + SKOV3
1 million cells	1F	1F	1M
2 million cells	1F	1F	1M
3 million cells	1F	1F	1M
Total	3	3	3

MCF7 injected on right flank & SKOV3 injected on left flank in MCF7 +SKOV3 group

Table Ax.1 Pilot study to determine the number of cells to be injected to form palpable tumours in NOD SCID mice.

Ax.2 Results and Discussion

Method standardisations reveal a single peak of 519.1 Da corresponding to MLN8237 is detected (Figure Ax1a and b) over a liner range of concentration ranging from 1ng/ml to 100ng/ml. These studies reveal MLN8237 to be detectable at as low concentration as 10ppb by

Figure Ax2: Detection of 10ppb MLN8237 in ACN. Data credit: CAMS facility at Venture centre

Sample Concentration	Replicate No	RT (min)	PEAK AREA	AVG PEAK AREA	RECOVERY (%)
100 ppb in ACN	1	7.017	1524365	1503275.5	
	2	6.915	1482186		33.12516568
100 ppb in Serum	1	7.01	501762	497962.5	
	2	7.015	494163		
50 ppb in ACN	1	6.972	739607	727898.5	
	2	6.981	716190		50.80667153
50 ppb in Serum	1	7.06	362482	369821	
	2	7.06	377160		
20 ppb in ACN	1	7.009	268694	270936.5	
	2	7.031	273179		27.0683352
20 ppb in Serum	1	6.959	66947	73338	
	2	7.08	79729		

* Recovery= (Avg peak area in serum/Avg peak area in ACN)*100

Table Ax2. Recovery of MLN8237 in mice serum. Data credit: CAMS facility at Venture centre
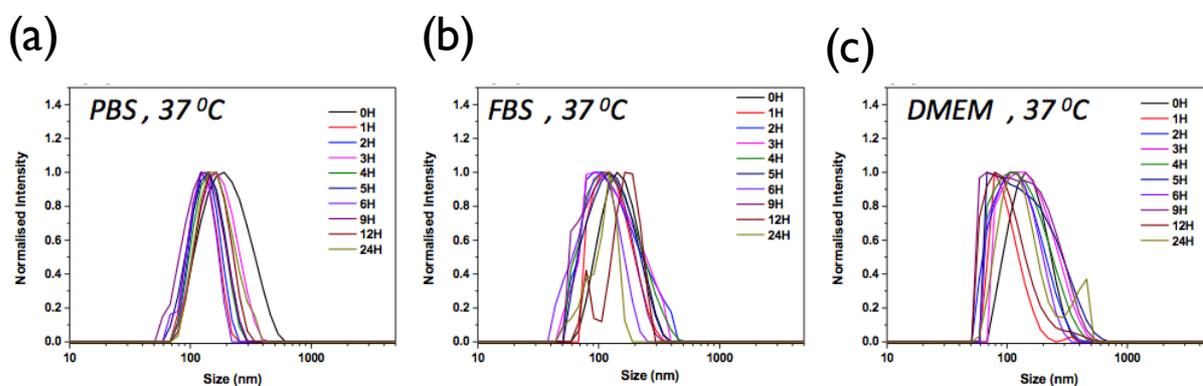
Figure Ax3: DLS histograms showing stability of V$_{MLN+RhB}$ in (a) PBS, (b)FBS and (c) 5% FBS DMEM. *Data credit: Nilesh Deshpande*

1 million

SKOV3 group

2 million

Tumors have been fixed and stored for further experiments.

3 million
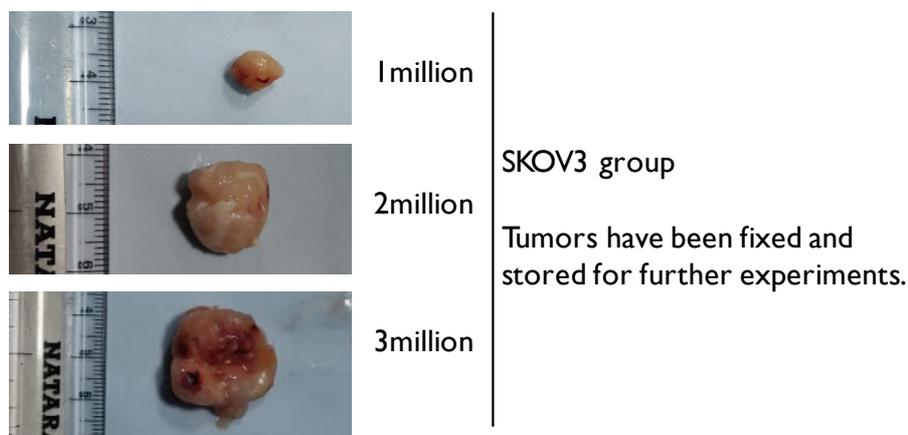
Figure Ax4: Tumours of SKOV3 cells dissected out from NOD SCID mice 8 weeks post injection. *Dissection credit: Keerthi Harikrishnan*

Noticeable tumours were observed in SKOV3 injected mice 2 weeks post injection and tumours were dissected out post 8 weeks of injection (Figure Ax4). MCF-7 cells however did not form tumours even 12 weeks post injection and pilot with modified protocol are currently ongoing. Taken together these pilot studies provide much information needed to carry out actual experiment to evaluate the impact of the AURKA-RalA crosstalk in tumorigenesis of these cancers.

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