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ROLE OF SMALL GTPases IN REGULATION OF BREAST CANCER CELL MIGRATION

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CONTENTS

ABBREVIATIONS

ABPs – Actin binding protein
AMBRA1 – Activating molecule in beclin 1 regulated autophagy protein 1
ANGPT1 – Angiopoietin 1
ARP 2/3 – Actin receptor protein 2/3
ATF2 – Activating transcription factor 2
BECLIN1 – Bcl-2 interacting protein
CDC42 – Cell division cycle 42
CDK4 – Cyclin-dependent kinase 4
CRL4 – Cullin-RING 4 ligase
CRL5 – Cullin-RING 5 ligase
DIC – Differential interference contrast
ECM – Extracellular matrix
EDTA – Ethylenediaminetetra acetic acid
EPAC – Exchange protein directly activated by cAMP
ERK – Extracellular signal-regulated kinase
EZH2 – Enhancer of zeste homolog 2
FA – Focal adhesions
FAKs – Focal adhesion kinases
FBS – Fetal bovine serum
FRET – Fluorescence resonance energy transfer
G12C – Glycine at position 12 mutated to Cysteine
GAP – GTPase-activating protein
GAPDH – Glyceraldehyde-3-phosphate dehydrogenase
GDP – Guanosine diphosphate
GEF – Guanine nucleotide exchange factor
GPPcP – Guanosine-5'-[(β, γ)-methyleno triphosphate]
GTP – Guanosine triphosphate
GST-RBD – Glutathione S transferase - ras binding domain
HEK293T – Human embryonic kidney 293T cells
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H-Ras – Harvey rat sarcoma viral oncogene homolog
ITG – Integrin subunit beta

ITGB7 – Integrin subunit beta 7
K-Ras – Kirsten rat sarcoma viral oncogene homolog
MAP4K4 – Mitogen-activated protein 4 kinase 4
MLCs – Myosin light chains
MMP2 – Matrix metallo proteins 2
MYC – C-myelocytomatosis oncogene
N-Ras – Neuroblastoma ras viral oncogene homolog
PBS – Phosphate buffer saline
PCW-Cas9 – Plasmid, constitutive, tetracycline-inducible CRISPR-9 enzyme
PCR – Polymerase chain reaction
P190RhoGAP – P190 rho guanosine triphosphate-activating protein
PI3K – Phosphatidylinositol-3 kinase
PLA – Proximity ligation assay
PMSF – Phenyl methane sulfonyl fluoride
PVDF – Poly vinylidene difluoride
QPCR – Quantitative polymerase chain reaction
Rab40 – Ras associated binding protein 40
Rab40a – Ras associated binding protein 40 A isoform
Rab40b – Ras associated binding protein 40 B isoform
Rab40c – Ras associated binding protein 40 C isoform
Rab40c-4A – Ras associated binding protein 40 C isoform-4A
Rab40-CRL5 – Ras associated binding protein 40/Cullin-RING Ligase 5
Rap1 – Ras-associated protein 1
Rap2 – Ras-associated protein 2
RhoA – Ras homologous family member A
RNF – Ring finger protein
ROI – Region of Interest
RT-PCR – Reverse transcriptase polymerase chain reaction
SiRNA – Small interfering ribonucleic acid
SNAI2 – Snail family transcriptional repressor 2
SOCS – Suppressor of cytokine signalling
TBST – Tris buffer saline with tween
TGFB2 – Transforming growth factor beta-2

TKO – Triple knock-out
TKS5 – Tyrosine kinase substrate with 5 SH3 domains
ULK 1 – Unc-51 like autophagy activating kinase 1
VPS34 – Vacuolar protein sorting 34
WASP – Wiscott-aldrich syndrome protein
WHO – World Health Organization
WDR77 – WD repeat domain 77
WD40 – WD repeat domain 40
MDA-MB-231 – MD Anderson Metastatic Breast-231 (Cell line)

PH.D. CANDIDATE'S CONTRIBUTIONS

Study I. Contributed to the development of research methodologies, experimental design, and conceptualization of the research. Performed single-cell migration assays at the Laboratory of Cell Culture, LSMU. Participated in the ubiquitination and protein validation assays.

Study II. Conducted single-cell random migration assays, immuno-fluorescence staining for cortactin, Myosin IIB, Phospho-Paxillin, as well as GFP-paxillin-transfected live imaging in control and Rap2 *Knockout* (KO) cells. Contributed to the overall planning and optimization of experiments, actively participated in the workflow at the University of Colorado (USA) and the LSMU Cell Culture Laboratory

Study III. Contributed to western blot validation of protein-protein interaction between Rab40-CRL5 and Rap1 using pull-down assays, followed by immunofluorescence staining. Participated in experimental design. All the experiments were conducted at the University of Colorado, USA.

1. INTRODUCTION

According to the American Cancer Society and Siegel's group from 2025, breast cancer remains the most frequently diagnosed cancer globally among women and is the leading cause of cancer-related deaths worldwide, recorded from World Health Organization (WHO) statistics [1]. Despite emerging advancements in primary detection, early diagnosis, and therapeutic interventions, the metastatic nature of colonizing breast tumor cells from one organ to another continues to present a set of major challenges in cancer-related mortalities [2,3]. Cell migration is a crucial hallmark of metastasis, dynamically coordinated, fundamental not only to wound healing, immune response, and embryonic development but also includes the ailments in cancer metastasis, all of which contribute to migration [4,5]. During the progression of cancer, the disrupted regulation of collective migration enables tumor cells to detach from the primary tumor and invade the surrounding tissues, enter the vasculature, and colonize distant sites [6]. Necessarily, it's crucial to uncover molecular mechanisms governing cell migration for the development of effective and metastatic therapies.

Although migration is one of the hallmarks of metastasis, efficient cell motility requires cells to move spatiotemporally, where lies, the remodelling of actin cytoskeleton dynamics. These dynamics promote the architecture and structural framework for the cells to establish a proper form and shape, aiding in changing and directing the motility in cells [7]. The arrangement of the cytoskeleton promotes the formation of dynamic protrusions such as lamellipodia and filopodia at the leading edge, referred to as the front of the cell. This process is associated with focal adhesion assembly and disassembly to form traction forces, which are essential for a cell to move forward, determining the direction. Any disruption in these tightly regulated dynamic mechanisms mostly promotes migration, invasiveness, and metastatic potential for the cancer cells to establish their niche [8,9].

Despite the involvement of numerous structural and regulatory proteins in orchestrating these processes, a central and foundational question remains: how are these cytoskeletal and adhesion-related events precisely regulated in space and time within the cell? This has led to growing interest in small GTPases, molecular switches belonging to the Ras superfamily, known to have emerged as major players in regulating signaling networks that navigate cytoskeletal dynamics and cell migration [10]. They act as molecular switches between the GTP-bound state, as active, and the GDP-bound state, as inactive, accounting for polarity, adhesion turnover, and mobility in cells. Within this superfamily, particular attention has been drawn to the

Rap subfamily, especially Rap1 and Rap2, due to their established roles in modulating integrin-mediated adhesion and directional cell migration [11,12]. It also enhances the integrin bond, mediates cell adhesion, and promotes focal adhesions by facilitating directional migration [13-15]. However, our group's findings show that Rap2 regulates lamellipodia formation and cell polarity, leading to processes crucial for migration [16].

Despite the advances and approximately 60 % functional similarity [17,18], the contradictory roles of Rap1 and Rap2 demonstrate that the dense connection between Rap1 and Rap2 and small GTPase signalling in cell migration remains obscure, requiring further investigation. Alternatively, Rab GTPases are a set of proteins primarily responsible for regulating membrane trafficking, such as the movement of transporting vesicles that carry the adhesion and signalling proteins inside the cell, promoting migration by modulating adhesion molecules like integrins [19].

Moreover, the investigation of novel regulatory proteins, such as the activating molecule in Beclin1-regulated autophagy protein 1 (AMBRA1), is emerging as one of the leading regulators for migration, along with various pathological processes such as tumorigenesis, cancer cell proliferation, [20-22]. Latest findings suggest that AMBRA1 not only participates in the cell cycle regulation and autophagy, but also influences transcription of key gene complexes, such as ANGPT1, TGF, ITG, involved in cell migration. [23,24].

The studies conducted over the past decade highlight the crucial need to discover how these dynamic signalling pathways are tightly coordinated to synchronize the spatio-temporal regulating mechanisms in breast tumor migration, along with potential therapeutic targets aiming to inhibit metastasis and invasion. This dissertation investigates the oncogenes in three different studies, regulating roles of Rap2, AMBRA1, Rap1, and RabGTPases in breast tumor cell migration, highlighting the significance of their molecular interplay with cytoskeletal dynamics and adhesion machinery to facilitate metastatic dissemination.

1.1. Aim of the study

The aim of this study is to elucidate how AMBRA1, Rap2, and Rap1 regulate breast cancer cell migration through coordinated control of cytoskeletal dynamics, focal adhesion remodeling, and GTPase signaling.

1.2. Objectives of the study

1. To investigate the role of AMBRA1 in regulating focal adhesion and migration of breast tumor cells and its interaction with the Rab40-CLR5 complex.
2. To determine how Rab40-CLR5-mediated mono-ubiquitination controls Rap2 membrane localization and lamellipodia positioning, and the influence of RhoA on Rap2 activity.
3. To determine the effect of mono-ubiquitination on Rap1 localization and activity, and Rap1's role in regulating focal adhesion and migration of breast tumor cells.

1.3. Novelty and relevance of the research

Although small GTPases and ubiquitin ligases are known regulators of cell migration, how mono-ubiquitylation spatially controls GTPase activity and adhesion dynamics remains unclear. This study explores how Rab40-CRL5-dependent ubiquitylation affects transcriptional regulation, focal adhesion turnover, and cytoskeletal remodeling during breast cancer cell migration. Novel discoveries of this work include the identification of AMBRA1 as a Rab40c substrate whose mono-ubiquitylation promotes a migratory transcriptional role and regulates focal adhesion organization beyond its established role in autophagy. Additionally, the Rab40-CRL5-mediated mono-ubiquitylation spatially regulates Rap2 activity at lamellipodia, where Rap2 controls RhoA-dependent contractility, by coordinating focal adhesion dynamics and maintaining balanced protrusion-retraction mechanisms. Finally, we further demonstrate that Rab40-CRL5-mediated mono-ubiquitylation regulates Rap1 activation and localization, thereby controlling focal adhesion dynamics in breast cancer cells during directional migration.

1.4. Research structure

The thesis consists of 3 studies, I, II, and III, the first two of which are published in a peer-reviewed journal. In study I, we investigated how AMBRA1 regulates cell migration by regulating focal adhesion turnover and transcriptional networks. We further explored its interaction with signaling components involved in cytoskeletal dynamics, discovering a novel role apart from autophagy. Secondly, in Study II, we examined how the Rab40-CRL5-mediated mono-ubiquitylation regulates Rap2 activation, membrane localization, and its role in counteracting RhoA activity during lamellipodial retraction. This uncovered a novel post-translational mechanism essential for dynamic cell migration. Finally, in Study III, we explored the

regulating role of Rap1 GTPase. Rap1 is ubiquitylated only at conserved lysines, and this modification influences its activation and subcellular localization, suggesting shared yet distinct regulatory mechanisms (Fig.1).

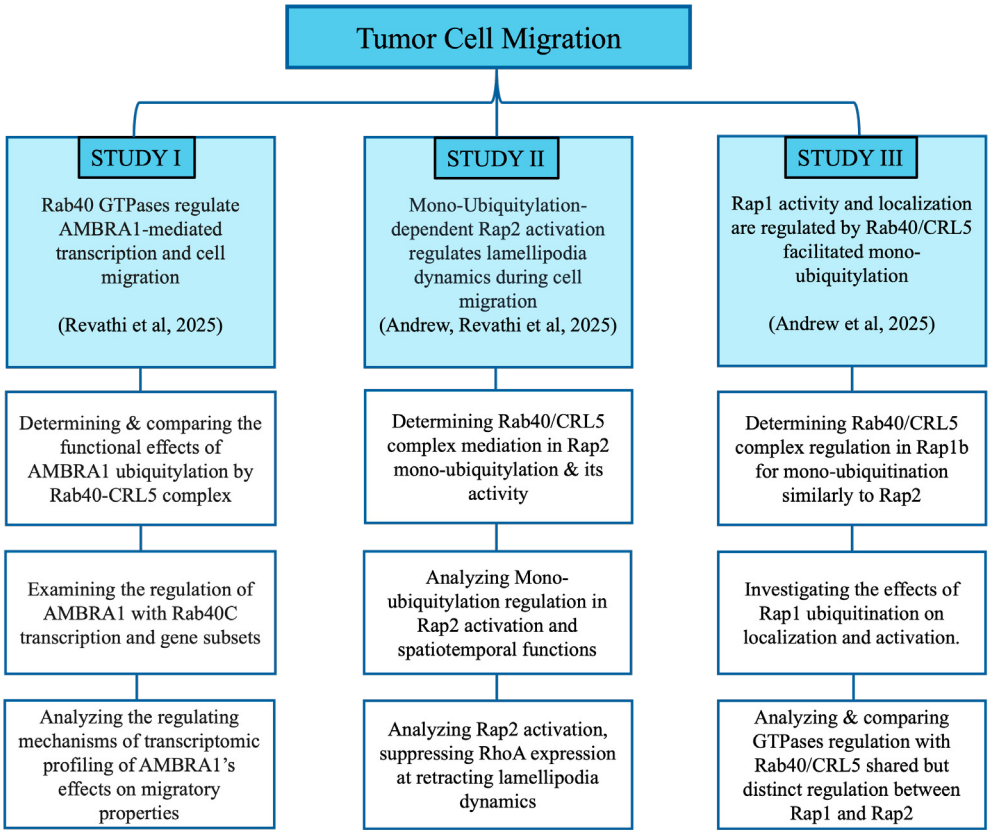


Fig. 1*. The outline of the thesis with 3 descriptive studies*

2. LITERATURE REVIEW

2.1. Cell migration and regulating mechanisms

Cell migration is an important biological process for allowing the cells to move from one point to another, which is crucial to cell development, wound healing, immune responses, and maintaining the proper organization of multicellular organisms [25].

This movement is achieved by the formation of leading-edge protrusions at the front of the cell, adhesions to the extracellular matrix (ECM), and rear-edge retraction at the back of the cell. These dynamics are regulated by the actin cytoskeleton [26], providing both a protrusive nature and necessary contractile forces for the movement [27-29]. Actin tends to polymerize at the leading edge, known as actin polymerization, which helps to push the cell forward, leading to the formation of structures known as lamellipodia and filopodia, defining the direction of the cells [30,31]. In order to establish a direction and location, Lamellipodia are thin protrusions generally nucleated by the Arp2/3 complex [32], made up of actin filaments, also supported by filopodia [33] (Fig. 2). They are finger-like projections consisting of bundled actin filaments [34].

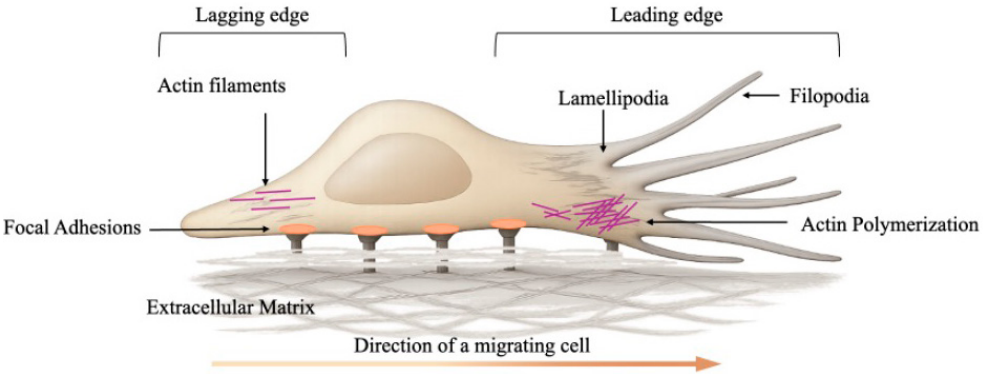


Fig. 2. *Schematic illustration of a polarized migrating cell on the ECM. Actin-driven lamellipodia and filopodia form at the leading edge, while focal adhesions provide traction, enabling directional movement (created with Illustrae.co)*

These actin-based protrusions of lamellipodia and filopodia are responsible for orchestrating the dynamic movement of cells by generating the driving force for forward migration [35]. Simultaneously, the term actomyosin is established at the cell rear with contractility, generated by myosin II [36],

pulling on actin stress fibres, which helps retract the trailing edge [37,38]. The dynamic coordination of these forces, which involves protrusions at the front and contraction at the back end, drives the cell forward.

Actin cytoskeleton dynamics are an important mechanism for effective cell migration, under which actin polymerization, another mechanism involved in this dyamics is not only responsible for the forces to move but also regulates focal adhesion dynamics at the leading edge of the cell. Two functions are composed in actin polymerization, at the leading edge, which consists of assembly, providing traction for the protrusion to stabilize [39]. Another one with the disassembly of actin filaments in older adhesions is crucial for it to detach from the surface [40].

These dynamic actin filaments turnover movements are tightly regulated by various actin-binding proteins (ABPs), such as assembly and disassembly are needed for movement. These turnovers are managed by the constant formation of linear actin, unbranched filaments present at the rear edge of the cells, and branched actin filaments, which accumulate at the front or leading edge of the cells, leading to polymerization of the actin filaments [41]. Also, there are various regulators for actin cytoskeleton remodelling, such as the actin-related protein 2/3, Arp2/3 complex, which is a known regulator for actin polymerization for establishing the lamellipodia, as mentioned above, by activating Wiskott-Aldrich syndrome protein, WASP, another active protein, and actin monomers [42]. Meanwhile, formin proteins are known to promote the linear actin filament structures and growth in filopodia [43]. All these mechanisms and regulators together ensure that the actin cytoskeleton networks promote signalling pathways, where upstream signals are controlled by the assembly of the actin networks. Overall, the process of cell migration relies on a tightly packed actin cytoskeleton, through which actin polymerization drives plasma membrane protrusion and new adhesion formation. For an efficient and directed cell migration, it’s crucial to have proper regulation of actin filaments.

2.2. Rac/Rho GTPases and their role in regulating migration

Cell migration is highly dependent, central to the actin cytoskeleton and small GTPases belonging to the Rho family, a large family of hydrolase enzymes binding to the nucleotide guanosine triphosphate (GTP), having a switch ON state in the active state, and when it hydrolyzes to guanosine diphosphate (GDP) as a switch OFF state (Fig. 3) [10] [44]. These small GTPases, from the Rho family, especially Rac1, RhoA, and Cdc42, are the master regulators of this process, acting as molecular switches between the

GTP-bound and GDP-bound state, following spatially channelled within migrating cells to guarantee polarity, adhesion, and protrusions dynamics.

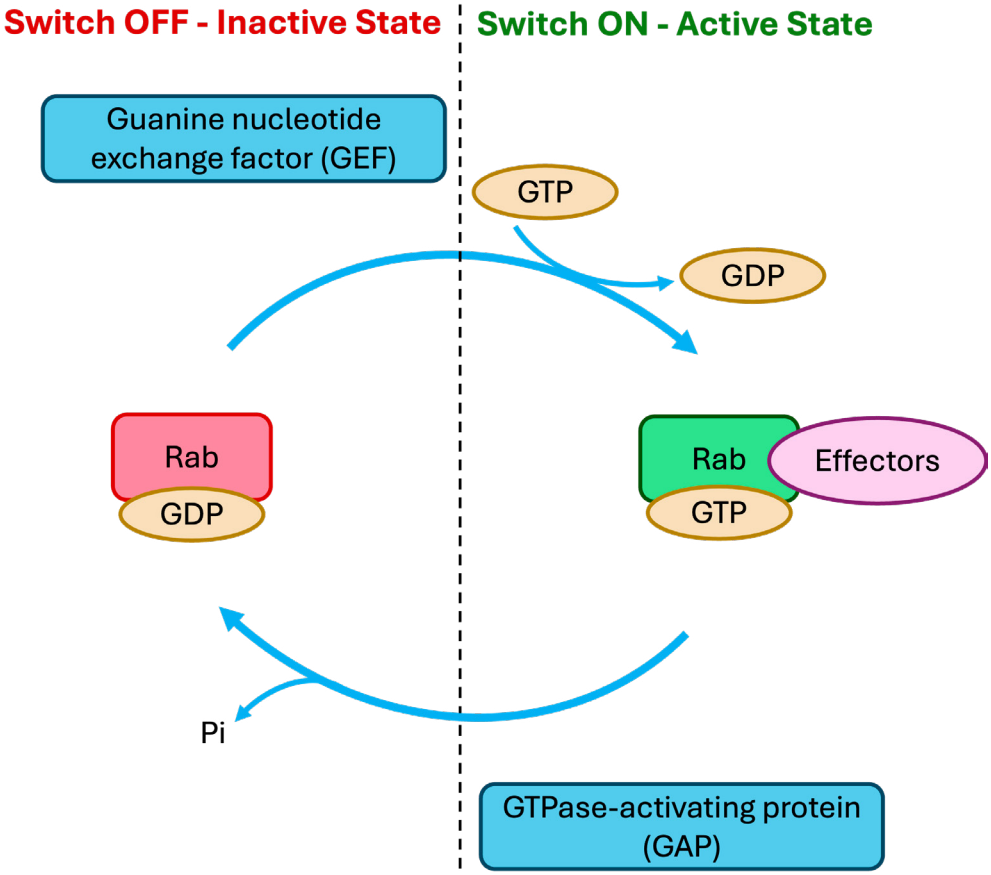


***Fig. 3**. Mechanism of small GTPases cycle between a GTP "Switch-On" and a GDP "Switch-Off", illustration adapted from Charest PG et al., 2007*

Among these GTPases, Rac1 is primarily known for forming lamellipodia at the leading edge of migrating cells. When it gets activated, Rac1 binds with effector proteins, stimulating the Arp2/3 complex, leading to branched actin networks essential for lamellipodia formation. For instance, in fibroblasts, Rac1 tends to produce ruffled membranes and protrusions, leading to persistent migration [45]. On the other hand, RhoA regulates and is responsible for actomyosin contractility and formation for stress fiber formation at the lagging edge of the cells. RhoA exhibits its effects through effectors like Rho-associated kinases, in turn promoting the myosin light chains (MLC), leading to enhanced actin-myosin contraction [46].

Consequently, the process of RhoA and Rac1 acting together comes with dual roles and is critical during the contraction of the rear end of retraction. Thus, while Rac1 is moving forward, RhoA is known to detach the cell's trailing edges, together promoting the process of retraction [47,48].

In contrast, an important feature of these GTPases is their antagonistic behaviour, with Rac1 and RhoA being spatially exclusive. Rac1 is active at the front of the cell, while RhoA is located at the back. This segregation helps to modulate front and rear polarity and prevents the potential signals that could disrupt these synchronized mechanisms. From recent literature studies, live-cell imaging analysis has shown that activation of Rac1 at the leading edge inhibits RhoA at the same location, promoting smoother lamellipodial extension [48].

Cdc42 is another crucial member of the Rho GTPases family, known as a regulator of actin cytoskeleton organization, leading to the polarization of the cells and filopodia formation [49]. Upon Cdc42 activation, it leads to the assembly of finger-like structures responsible for managing microtubules, which organize the Golgi apparatus towards the front of the cell, ensuring directional secretion and supporting persistent movement. When there is a loss in Cdc42, disorganisation of migration and loss of polarity are seen [50].

The Rac1, RhoA, and Cdc42 are regulated by upstream proteins and molecules such as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). They are known as multi-domain proteins having spatiotemporal control of small GTPase activation [51]. These GEFs facilitate GTP activation and loading, while GAPs promote GTP hydrolysis, inhibiting the GTPases. These processes allow cells to rapidly react to migration signals, like a set of growth factors. For example, one of the GEFs (Tiam1) is known to activate Rac1 from the integrin response with ECM proteins, whereas among GAPs, the p190RhoGAP downregulates RhoA activity to lessen the adhesion strength at the leading edge of migrating cells [52-54].

Disruption of any hindrances in Rac1 and RhoA signalling can lead to migration defects. Overproduction of RhoA can result in higher contractility and loss of its shape of protrusions, leading to rounding of cells, preventing forward movement. On the contrary, unregulated Rac1 activity can lead to unstable and excessive protrusions that fail to form proper adhesions. This balance nature in migrating cancer cells is essential, so usually deregulation in any of these signalling pathways often results in elevated metastasis and invasion [55]. Further studies and understanding how these small monomeric GTPases function in different cellular contexts are crucial to target diseases, where abnormal migratory behaviors contribute to metastasis.

2.3. Role of Rab GTPases and mechanisms during migration

Rabs, on the other hand, are known as onco-proteins and a tumor suppressor, depending on specific mechanisms, belonging to the family of small GTPases. Rabs also play a huge role in vesicle trafficking, membrane dynamics, and maintaining homeostasis [56,57]. Rabs are constantly known to appear in cancer progression, by influencing invasion, autophagy, and migration [58,59]. Among 50 [60] Rabs; Rab 11, Rab35, and Rab27a, Rab27b stood out to play a huge role in exosome secretion, depicting solid tumor progression or immune suppression [61-64]. Hence, targeting the Rab GTPases could give insights for a potential strategy to uncover cancer progression, as they are also involved in prognostic factors for cancer [65].

2.4. The role of focal adhesions during migration

The focal adhesions (FAs) are complex, dynamic multi-protein complexes that play a critical role in linking the ECM and actin cytoskeleton, serving a pivotal role in migration. Their functions are mainly acting as an anchoring point that can transfer chemical signals, ensuring that cells adhere, attach, and move in a direction.

These FAs are composed of complex proteins, transmembrane integrin receptors that directly bind to ECM proteins such as collagen and fibronectin. In detail, there is a set of signalling proteins, such as talin, vinculin, paxillin, and focal adhesion kinases (FAKs), and a network of adaptor proteins that connect through integrins, connecting to the actin cytoskeleton (Fig. 4) [174]. This assembly creates a structural and dynamic signalling process that regulates adhesion motility.

Recent data showed FAs are organized into three well-defined layers, such as integrin signalling, transduction, and the actin regulatory layer [66]. All three layers are arranged to promote efficient signal transduction and mechanical forces during cell movement [66].

It has been observed that during migration, cells manage to form new FAs at the leading edge while disassembling previous ones at the rear, known as FAs turnover. This dynamic remodelling is needed for moving forward. Mechanosensors manage to sense and respond to various signals, and mechanotransduction and FAs act as mechanosensors, transmitting mechanical forces from the ECM to chemical signals. The rigidness of ECM promotes stiffness in FA composition and stability, promoting cell motility. The rigid behaviour comes from a phenomenon called durotaxis, where migrating cells tend to move towards stiffer substrates, which is mediated by FA signalling pathways, including Rho GTPases and actomyosin contractility [67]. During

FA turnover, it has been found that the role of calcium signalling has been implicated in regulating FA turnover. These communications between the Endoplasmic Reticulum (ER) together with FAs promote activation of calpain, which cleaves FA components, leading to disassembly. This crosstalk between FA and ER is crucial during cell migration [68-70].

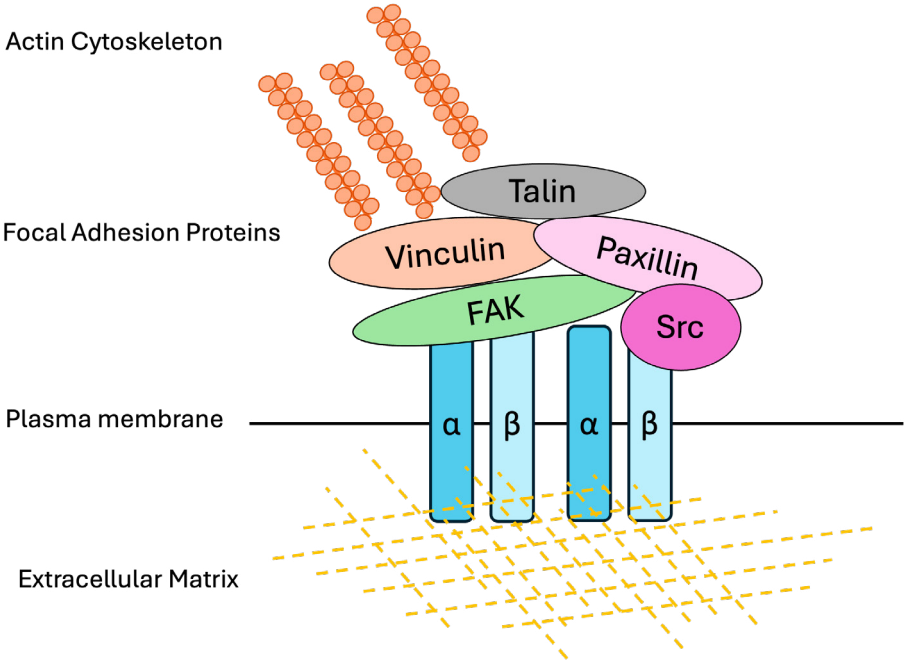


Fig. 4. *Schematic components of the FAKs. Integrin α/β heterodimers connect the extracellular matrix to intracellular proteins, including talin, vinculin, paxillin, FAK, & Src, linking to a complex to the actin cytoskeleton. Image acquired from Katoh K.et al., 2024*

Explicitly, FAs are central to dynamics, and remodelling contributes to cancer cell metastasis and invasion. Either overexpression or activation of FA or FAs components, like FAK, and paxillin, vinculin, and talin are being observed in various cancer mechanisms and functions. Corresponding with increased migratory and invasive phenotypes. Solely targeting the FA signalling pathways could be a path to a potential therapeutic approach to limit cancer progression [71]. On a comprehensive note, the FAs are an integral part of cell migration, having a signalling mechanism between the ECM and acto-skeleton network, making their dynamic assembly and disassembly primary to movement, for efficient migration. Understanding these molecular mechanisms that govern the functions of dynamics provides insights for more cancer-related interventions.

2.5. Current Implications of small GTPases: Ras, Rap1, and Rap2 in cancer progression

Small monomeric GTPases are well-known regulators for many biological, cellular, and various signalling processes. These GTPases are GTP-binding proteins, mostly found in eukaryotes [72]. Reported as well as for differentiation, proliferation, adhesions, migration, apoptosis, actin cytoskeletal reorganization, polarity of cells, and notably, cell progression [73-76]. There are almost 150 members of GTPases present at the moment [77]. Among these, Ras oncogenes are members belonging to the Ras superfamily [78], having played a significant role in cancer progression and invasion over the years [79,80].

2.5.1. Ras GTPases and oncogenesis

Ras oncogene proteins consist of H-Ras, K-Ras, and N-Ras; these are the most studied oncogenes in mammalian cancers. Any disruptions or genetic mutations in Ras genes result in activation of Ras signalling, exclusively to codons at 12, 13, and 61 [81]. The above-mentioned mutations not only impair the GTPases' activity but also the interconnection with GAPs, leading to prolonged downstream signalling pathways like MAPK/ERK and PI3K/ AKT pathways [82-84]. K-Ras is one of the common mutations associated with pancreatic, colorectal, and lung cancer, enhancing the aggressiveness of the cancer and often leading to resistance towards targeted chemotherapies. Focusing and targeting Ras directly has proven challenging in recent therapeutic works; nevertheless, certain progress has emerged as inhibitors against K-Ras G12C, shown to be promising in clinical trials [85-88].

2.5.2. Rap1 in cancer signalling

Rap, inclusive of isoforms Rap1A and Rap1B, is relatively related to Ras but has distinct functions when it comes to cellular signalling roles (Fig. 5) [96], specifically in adhesion mechanisms and migration. Rap1 is known to regulate and activate integrins, which control the cell-cell junctions, and processes involved critically in tumor invasion and metastasis. While Rap1 mutations are less frequent, their activity is dysregulated in cancers through altered expression of their regulatory proteins, like GEFs, such as C3G, or GAPs, such as Rap1GAP [89-92]. Meanwhile, there is a reduced expression of Rap1Gap seen in prostate cancer, leading to Rap1 activation, and tumor cell migration and metastasis [93]. Likewise, in melanoma cells and pancreatic tumor cells, the active Rap1 promotes the integrin-mediated adhesions to the ECM, enhancing migration. Rap1 stabilized the cell-to-cell

junctions, contradicting the fact that Rap1 has also been shown to have dual roles, surprisingly, to suppress the roles depending on the tissue type and tumor stage [94-96].

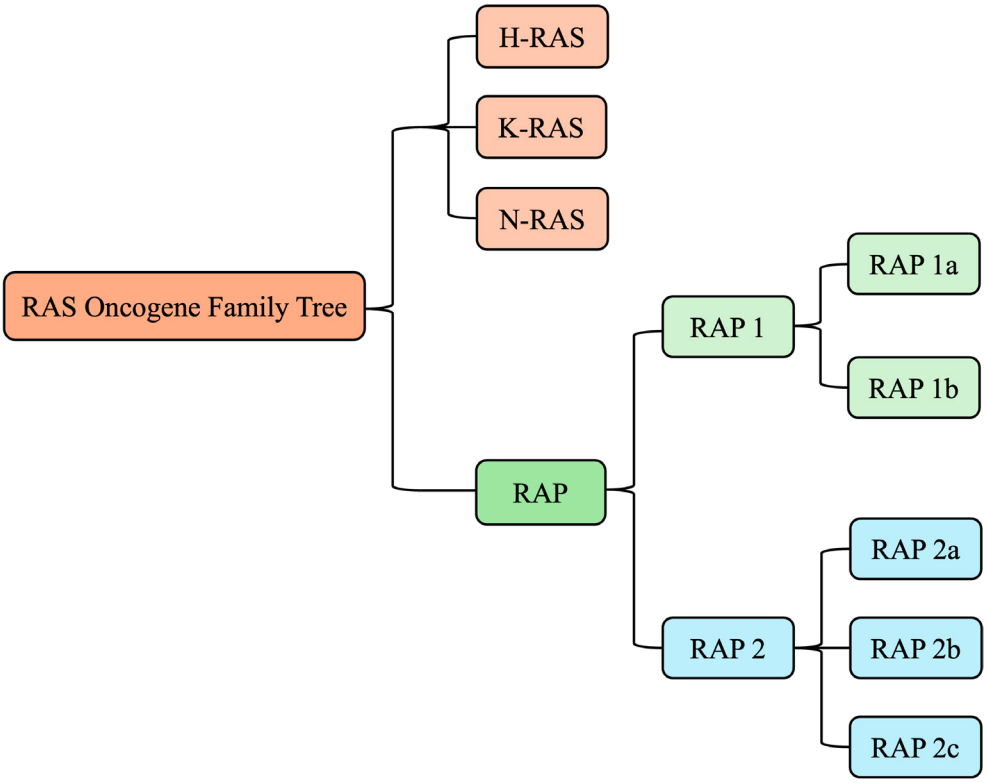


Fig. 5. *Schematic illustration of the Ras oncogene superfamily tree image acquired from Rio-Vilariño et al., 2021*

2.5.3. Rap2 and its emerging roles in cancer

We know that Rap2 GTPases are an emerging family of signalling pathways, consisting of three known isoforms, such as Rap2A, Rap2B, and Rap2C [97-100]. Although they are less understood than Rap1, they are increasingly mentioned in tumor implications. Rap2 activates integrins but not as Rap1; instead, it regulates cell shape, actin cytoskeleton dynamics, and polarity. A set of recent investigations has revealed that Rap2 can promote cancer cell migration and invasion, affecting actin cytoskeleton organization and cell contractility. For instance, in renal cell carcinoma (RCC), there was an upregulation of Rap2a and enhanced cell migration and invasion, promoting metastasis [101]. Correspondingly, in lung cancer and glioblastoma cells,

Rap2b contributes to invasive growth by activating the mitogen-activated protein kinase (MAPK) signalling, influencing actomyosin contraction, and activating increased levels of matrix metalloprotease enzyme 2 (MMP2), known to help in migration and metastasis, influencing actomyosin contraction, hence migration [102,103]. Another exciting and unique feature Rap2 holds is its ability to be regulated by post-translational modifications, such as ubiquitination [104,105]. Where these modifications are affecting Rap2 localization and activation, enhancing the contribution to invasive behaviours.

2.5.4. Therapeutic implications

Uncovering the mechanisms of Ras, Rap1, and Rap2 in cancer therapeutic implications. While Ras is the sole oncogenic protein and target for therapy, Rap1 and Rap2 are emerging as one of the cancer cell behaviors for influencing migration and invasion [106,107]. Targeting Rap1GAP, EPAC/ RapGEF Inhibitors, or the mainstream signalling pathways of Rap2 with ERK pathways can benefit the promising strategies to restrain metastasis. Further studies are needed to better understand how to modulate these new pathways effectively.

2.6. Rap1 and Rap2 GTPases regulating mechanisms in lamellipodia and focal adhesion dynamics

It's shown that Rap1 and Rap2 have similar functionalities and are closely related members of the Ras superfamily of oncogenes, and while they also share structural similarities, they perform distinct roles in regulating cell migration. Both get involved in the actin cytoskeleton by influencing adhesions, polarity, and protrusions. Even then, their functions diverge in significance when it comes to migration.

2.6.1. Rap1 and focal adhesion dynamics

Two isoforms of Rap1, Rap1a and Rap1b, significantly play a central role in regulating the cell-to-cell matrix adhesion by facilitating integrins and FAs assembly. When Rap1 gets activated, there is an affinity for integrin accumulations, a set of transmembrane receptors that bind to ECM. When integrin is activated, Rap1 recruits the adhesions between the cell and the surrounding cells to ensure stable protrusions.

Rap1 acts with the help of effector proteins, such as RIAM and Talin, which tend to bind with activated integrins to the actin cytoskeleton. This binding enables the intracellular and traction forces required for cell

movement [108]. In epithelial cells, Rap1 activity recruits integrin activation at the leading edge, where naive adhesions start to form. At the lagging edge, the activity of Rap1 is suppressed, where disassembly happens, and retraction can be observed [109]. Dysregulation in Rap1 signalling pathways can lead to excessive adhesions or reduced adhesions, in the case of inactive Rap1, altogether, which results in impairment of directional migration. A study showed that the downregulation of Rap1 using siRNA in endothelial cells leads to fewer focal adhesions and migration, highlighting Rap1's role in regulating these structures [110,111].

In support of that, Rap1 essentially influences polarity in cells; it does so by regulating while maintaining the structures of the microtubule and the Golgi apparatus, aligning the machinery for migration. Polarization is an essential part of remodelling the cytoskeleton by forming protrusions and is a crucial player for persistent directional migration [112-114].

2.6.2. Rap2 and lamellipodia formation

Rap2, having three isoforms such as Rap2a, Rap2b, and Rap2c, was primarily involved in cell shape and morphology in migrating cells, along with regulating functions like Rap1, by recruiting cytoskeletal dynamics for movement. It has been observed or can be viewed that, like Rap1, Rap2 isn't essentially activating integrins, but it mostly regulates the front and rear polarity and lamellipodia dynamics well. Rap2 activity is more inclined towards the leading edge, by supporting stretches of lamellipodia, wide actin-rich protrusions driven by Arp2/3 mediated complex. Backing up this statement, a recent study has demonstrated that loss of Rap2a leads to a decrease in lamellipodium extensions, actin branches, and diminished directional migration [16,115,116].

Rap2 seems to be tuning the actin dynamics by modulating signalling pathways that antagonize RhoA activity. RhoA activates actomyosin contractility, which in turn, reduces lamellipodia extensions when overactive at the front of the cell [117,118]. Rap2 is negatively regulating RhoA at the leading edge, allowing Rac1 to promote actin polymerization and extensions of lamellipodia, leading to the conclusion that Rap2 supports the cytoskeletal dynamics [119]. In addition to this, Rap2 is involved in defining the adhesion turnover's location, through its effect on the cytoskeleton and tension at the leading edge, where it can favor adhesions with many turnovers, essential for protrusion cycles, where it can re-establish cytoskeleton dynamics [120].

2.6.3. Interaction between Rap1 and Rap2

Despite Rap1 and Rap2 being activated by the same upstream signals, such as EPAC1 and GEFs, as mentioned before, they tend to localize in different places in cells and contribute non-redundant mechanisms. Rap1 regulates adhesion molecules and integrins, and Rap2 regulates actin remodelling and cell shape.

It's compelling that when there is a functional division between two GTPases for migration, the balance between these two mechanisms accounts for the ability to move and migrate. Excessive adhesion mechanisms can lead to strong anchoring, and excessive protrusions without traction forces can result in inefficient movement. For a synchronized cell movement, this remodelling is crucial. The recent (FRET) Forster Resonance Energy Transfer analysis elucidates the spatial separation and coordination during migration [121].

Altogether, these investigations highlight the fundamental gap in the recent understanding. While Rap1 and Rap2 roles are being established independently, the mechanisms that coordinate their spatiotemporal interplay remain poorly defined or yet to be explored. What signaling crosstalk ensures their non-overlapping activation? How are their activities integrated with upstream effectors like EPAC1, or with downstream targets such as RhoA and cytoskeletal proteins, to regulate the migration? And crucially, how is this balance dysregulated in metastatic cells? Understanding these unanswered questions is essential for several reasons, such as;

1. In terms of migratory properties, when there is a disruption of Rap1 and Rap2 balance, it may drive cancer cell invasion; targeting these GTPases could restore proper migratory behavior or arrest metastatic spread.
2. When it comes to signalling factor, if the same GEFs activate both proteins, then what molecular determinants (e.g., scaffolds, membrane localization, post-translational modifications) confer specificity?
3. Different cancer subtypes may differentially exploit Rap1 or Rap2 functions. A better mechanistic understanding can help stratify tumors for targeted intervention.
4. Most studies use overexpression or knockout models. There is a possibility to explore more on dynamic modulation and real-time imaging of both GTPases in complex environments.

Thus, exploring and emphasising the Rap proteins is not only specifically justified but crucial, as they act as signalling regulators, balancing the cell's ability to both move and adhere with a dual role, which defines and deter-

mines the migration of the cell. In the context of breast cancer metastasis, the role becomes a determinant of whether a cell maintains to localization or successfully invades the distant tissues.

2.7. The role of Rab40 in GTPase signaling with ubiquitination of Rap2 by the Rab40-CRL5Complex

We know that Rap2 is tightly regulated by the classic mechanism of GTP/GDP, but also by post-translational modifications affecting localization, stability, and regulating functions. To support, we know the key to supporting migration through the ECM is between actin polymerization, assembly, and disassembly of FAs, and secreting specific MMPs [122,123]. One of the main regulatory mechanisms is ubiquitination. In recent findings from our group, we presented that a key role for the small GTPases Rab40 and the CRL-5 ubiquitin complex (CRL5) in regulating the Rap2 activation and localization is through ubiquitination [16]. Here, we show the additional controlling mechanisms over Rap2's role during cell migration. Despite Rab40 being denoted with three isoforms, Rab40a, Rab40al, Rab40b, and Rab40c, containing an extended C-terminal containing SOCS box (Suppressor of Cytokine Signalling), with genetic duplication patterns [124-126]. Along with this, our group has also investigated the Tks5 (tyrosine kinase substrate at 5 SH3 domain, an adaptor & invadopodia regulatory protein for Rab40b [123,127]). Among these, Rab40b emerged as a crucial and master regulator for cell migration, potentially leading to a strategic target [128-130].

2.7.1. Rab40: dual functions of GTPases and E3 ligase adapter

Rab40 isoforms from Rab40b and Rab40c, particularly, are unique and specific among other Rab GTPases with SOCS Box at their C-terminal. And especially the Rab40 proteins tend to bind to the Cullin 5 complex to function as part of a Cullin-RING E3 ubiquitin ligase complex. This complex consists of a scaffold protein Cullin5, a SOCS box-containing protein, such as Rab40, the adaptor proteins Elongin B and Elongin C, and the RING protein Rbx1/2 (Fig. 6). This unique SOCS box validates the Rab40 GTPases to interact with CRL-5, forming an E3 ubiquitin ligase complex. It demonstrates that Rab40b binds to Rap2a, leading to ubiquitination through CRL5. Specifically, the modification is non-degradative, where it modifies with monounbiquitination molecules; this complex process is denoted as tri-monoubiquitination in Duncan and the group's work [126].

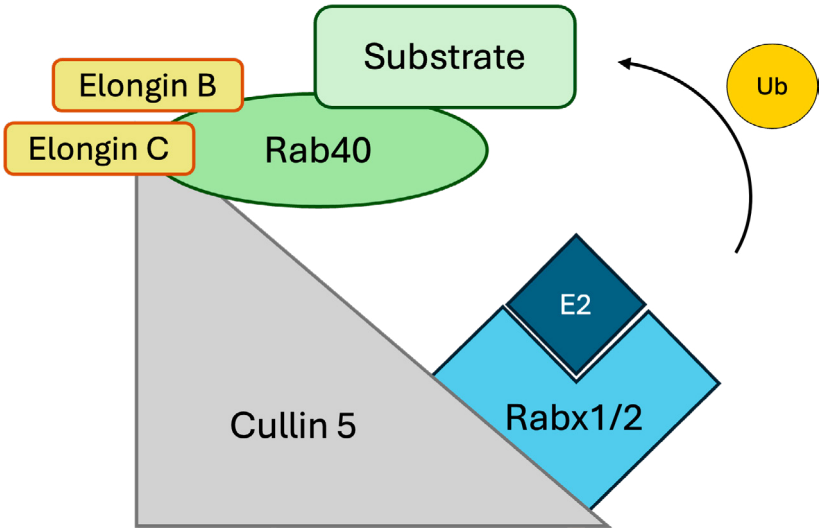


Fig. 6. *Graphical representation of Rab40 protein binding to Cullin5 Complex, adapted from Neumann et al., 2023*

2.7.2. Types of ubiquitination

As mentioned above, it's a post-translational modification, where ubiquitin binds to proteins and plays functional and essential roles in migration [131]. As demonstrated in Fig. 7, [131] when there is an addition of a single ubiquitin molecule, there is a modulation of proteins and their functions without degradation. For instance, Fascin is an actin-binding protein, and monoubiquitination of Fascin impairs its ability to form, basically interrupting the protrusions from forming during migration [132]. Polyubiquitination, on the other hand, attaches the whole chain of series, affecting protein mechanisms and activity, where K63 of EZH2 poly-ubiquitination promotes the popular EMT pathway [133]. And K48 targeting WDR77 to degrade RNF, activating migration-promoting transcription factor EGR1 [134,135]. For Triubiquitination, there are still fewer studies, but it consists of short chains, while known to interrupt another ubiquitination process, and could help similarly in protein regulation [136,137].

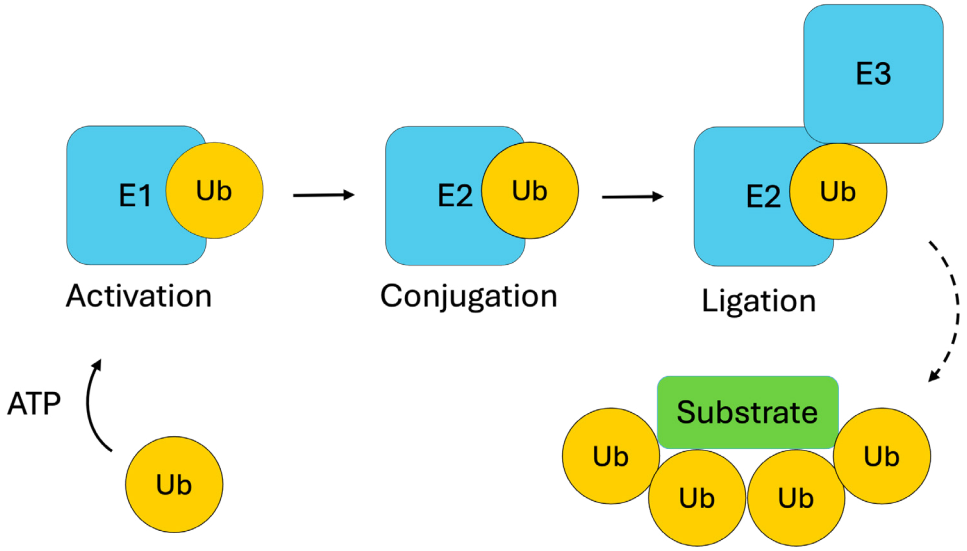


***Fig.** 7. Schematic diagram of ubiquitination, illustrating the sequence of the cascade from E1, E2, and E3 Enzymes resulting in Polyubiquitination. Image is adapted from Swatek KN et al., 2016, with modifications to present the study*

2.7.3. Effects of ubiquitination in Rap2

With subcellular localization, the ubiquitination signalling has two major effects on Rap2; one is that the effect of localization alters the trafficking route for tri-monoubiquitination. Usually, the Rap is localized to the membrane and endocytic pathway, present at the leading edge of a migrating cell. When ubiquitinates Rab40b-CRL5, Rap2 is internalized within the cell and assembled in the recycling pathway, rather than degraded in lysosomes, which recruits Rap2 to recycle back to the leading edge of the cell, resulting in the accumulation of Rap2 to promote actin protrusions. Another aspect is to navigate and understand the regulating activity, where ubiquitination affects Rap2's state, by hypothesising that monoubiquitination impairs the activity if GAPs are to inhibit Rap2. This leads to the GTP-bound state of Rap2, enhancing the signal. Coincidentally, resonates with the statement that Ras monoubiquitination inhibits the GAP-mediated inactivation [138]. Overall, stating that Rab40-CRL5-mediated ubiquitination is not only for degradation but is also responsible for signalling pathways where Rap2 activity is involved.

2.7.4. Functional impact on cell migration

The ubiquitination modification is considered crucial for migrating cells and establishing polarity [139,140]. When there is an absence of Rab40 and Rap2 cannot be ubiquitinated (mutation in Rap2), cells tend to display fewer lamellipodia protrusions and defective front-rear polarity, which can also be validated with live imaging results, revealing that these cells lose the Rap2 localization at the leading edge and form disorganized actin extensions [16,141]. Additionally, when Rap2 is not ubiquitinated, it loses its activity to establish stable protrusions during migration. It features the importance of recycling Rap2 and re-establishing it to localize protrusions and adhesions at the leading edge.

2.7.5. Ubiquitination: Significance in cell behaviour and cancer

The Rab40-CRL5signalling cascades are directly involved and can be relevant to the invasion and migration of cancer, since Rap2 is recognized as one of the master regulators of tumor cell migration [142,143]. Rap2's invasive behaviours can be modified with ubiquitination activity and its localization. Likewise, an elevated degree of Rab40 or imbalances in CRL5 signalling activity may result in Rap2 overexpression, hence migration and invasion. Interestingly, Rab40 has emerged as a potential target for therapeutic strategies in the context of cancer cell migration and invasion, as it is predominant in GTPases signalling, by regulating cytoskeletal dynamics and establishing polarity [129,144,145].

The investigation on Rab40-CRL5complex as a main regulator for Rap2 could give a lot of insights to understand the small monomeric GTPases signalling. From ubiquitination pathways, it's important to consider that Rabb40 is involved in both Rap2 localization and the role in activating Rap2, which enables the establishment of the polarized nature of the cells and lamellipodium extensions. The tightly regulated mechanism of Rap2 activity is found at the leading edge of the cells, and together with the Rab40 complex, it can be further represented as a novel mechanism to coordinate the efficient and directed cell migration. There is a clear need to investigate Rab40 and Rap2 mechanisms to uncover migration phenotypes.

2.8. AMBRA1: Beyond autophagy, role in cell division, gene transcription, focal adhesions remodeling, and cell migration

AMBRA1 (Activating Molecule in Beclin-regulated Autophagy) is a multifunctional protein primarily characterized by its crucial role in autophagy [146,147]. Nevertheless, as mentioned before, in recent studies, AMBRA1

has appeared as a major player in multifunctional cellular processes, not in autophagy, but also in cell division, transcription, actin cytoskeletal remodeling, and cell migration [20,22,24,148,149,150]. Multiple factors are responsible for modulating the role mediated through its interactions with signalling molecules and ubiquitin complexes [150].

2.8.1. AMBRA1: Central regulator of autophagy

While AMBRA1 accounts for mostly autophagy activity by initiating and activating the Beclin1/Vps34 complex. When AMBRA1 undergoes cellular stress, AMBRA1 is phosphorylated by ULK1 kinase; this interaction leads to the removal of the dynein motor complex, recruiting Beclin1 to the endoplasmic reticulum, leading to the formation of an autophagosome [151]. Despite the autophagy machinery, which regulates the autophagy-dependent removal of cellular components, it maintains mitochondrial homeostasis, resulting in specific autophagy pathways only [152]. The basic mechanisms set the foundation for other signalling pathways and influence wider opportunities for further studies.

2.8.2. AMBRA1: Role in cell cycle and protein stability

Advanced development is needed for AMBRA1's broader mechanisms to come up with the discovery, which provides the substrate receptor for CRL4, which is broadly known as a ubiquitin ligase complex, targeting Cyclin D for degradation [153]. Cyclin D is known to act as an important regulator for the G1/S phase cell cycle, through the ubiquitination of CRL4/AMBRA1, where AMBRA1 confirms that Cyclin D gets degraded at a specific time to avoid unnecessary overproliferation [154,155]. When there is an increased loss of AMBRA1, a stabilized amount of Cyclin D can be observed with abnormal cell cycle and resistance to drugs such as CDK4/6, used in lung and breast cancer [156,157]. Thus, proposing the idea for AMBRA1 as a potential tumor suppressor, having dual functions for proliferation control, and therapeutic implications.

2.8.3. AMBRA1: Focal adhesions and cytoskeletal regulation

From recent publications, studies have uncovered that AMBRA's localization is not only based on or limited to the nucleus or cytoplasm but also to the FAs sites, where cells tend to bind like an anchor to the ECM during movement and migration of a cell, supporting our idea. AMBRA1 is known to play a significant role in FAK scaffolding components of FAs signalling dynamics networks. Schoenherr et al and team demonstrated that AMBRA1

is recruited to FAs through communication with FAKs, modulating the dynamics of Src kinase activity by governing it onto autophagosomes, a term coined as autophagy-dependent Src clearance. This process avoids excessive accumulation of Src at FA sites, thereby reducing accumulation, leading to fewer hyperadhesions and inhibiting effective migration. Resulting in a statement that AMBRA1 promotes FAs turnover, which is a crucial mechanism for effective migration. Cells with Loss of AMBRA1 are observed to display higher levels of FA size and stability, facilitating slower movements and depletion in invasiveness. AMBRA1 promotes a direct association between adhesion dynamics and autophagy, in which signalling proteins generated by autophagy aid in resetting adhesion sites during cell migration [158].

2.8.4. AMBRA1: Role of transcriptional regulation

Recent studies have mentioned AMBRA1 to be found in mechanisms regulating the nuclear scaffolding [159]. This certainly binds with the ATF2 complexes, resulting in the allowance of AMBRA1 assembling to transcriptional hubs that control genes involved in cell-to-cell adhesion. cell migration and ECM remodelling [160]. For example, we already know AMBRA1 also regulates the expression cascade of TGFB2, ANGPT1, and integrins, recruiting the migratory phenotypes to initiate movement [161]. Simultaneously, it affects the C-Myc expression of the targeting genes, which tends to take part in oncogenic transcriptional activity [162]. Hence, it has been established that, in most of the cancers, loss of the AMBRA1 gene promotes dysregulated expression of genes, leading to invasion nd metastasis, additionally confirming its crucial relation to transcription activity [158].

2.9. AMBRA1: Crosstalk between autophagy and cell migration

The compromises between autophagy and migration are one of the developing trends in cell biology, while emphasizing AMBRA1 and how it can link both mechanisms. Autophagy, on one hand, is a cellular recycling pathway impacting migration and regulating the turnover of FAs, responsible for movement on the other hand. Whereas autophagy can specifically target and degrade the components of FAs and other active signalling complexes, enhancing the disruption of FAa assembly required for the motility of the cell. In other words, AMBRA1 is involved in the remodeling of adhesions, necessary for detachment and movement. Extending the migration and autophagy link to the nucleus and gene expression, it seems to influence the long-term migratory phenotypes and cell state. Our recent work on RNA sequencing established that AMBRA1 tends to control the expression of quite a lot of cell adhesion and migratory phenotypic genes. To be specific, integrins

such as ITGb7 and ITGb8, ECM recruiters such as ANGP1 and TGFB [163]. Overall, the interplay can influence therapy responses, autophagy, and cell migration, together adapting their mechanisms both in autophagic and non-autophagic roles, ensuring the cells can dynamically respond to the environment while activating migration. Discovering this regulation opens avenues for targeted therapy. Therefore, understanding how AMBRA1 and small GTPases coordinate these processes is essential for uncovering the molecular mechanisms underlying cancer cell migration and metastasis, and may provide new opportunities for therapeutic targeting of invasive tumor cells.

3. MATERIALS AND METHODS

3.1. Cell culture conditions

MDA-MB-231 (ATCC) triple-negative breast cancer cells and HEK293T (ATCC) human embryonic kidney cells were used throughout all three studies conducted in this research work. The MDA-MB-231 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Corning, Mediatech) supplemented with 4.5 g/L glucose and 5.84 g/L L-glutamine (Corning). Further, the culture medium was further supplemented with 1 % sodium pyruvate (Gibco), 1 % non-essential amino acids, 1 µg/ml insulin (Gibco), 1 % penicillin/streptomycin (Corning), and 10 % fetal bovine serum (FBS; Phoenix Scientific). Another cell line, HEK293T (ATCC) cells, was cultured under similar conditions in DMEM containing 4.5 g/L glucose and 5.84 g/L L-glutamine, supplemented with 1 % penicillin/streptomycin and 10 % FBS. Both the cell lines were routinely maintained in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. To ensure consistent growth and optimal cell health, cells were passaged when they reached approximately 70–80 % confluency, using trypsin-EDTA, thereby maintaining stable and reproducible cell culture conditions across all experiments.

3.2. Generation of CRISPR/Cas9 KO cell lines

CRISPR/Cas9 gene editing was used to generate knockout (KO) cell lines targeting AMBRA1, all three Rap2 family of isoforms (Rap2a, Rap2, and Rap2c; hereafter referred to as Rap2 KO), and the Rab40 family (Rab40a, Rab40b, and Rab40c; referred to as Rab40 TKO).

MDA-MB-231 cells stably expressing Tet-inducible Cas9 were grown to 70 % confluency and then treated with 1 µg/ml doxycycline for up to 24 hours to induce Cas9 expression in the cells. Later, transfected with crRNA (CRISPR RNA): tracrRNA (trans-activating CRISPR RNA) mix using DharmaFECT Duo Transfection reagent (Horizon discovery), as per the manufacturer's protocol, and incubated for 1 day, followed by trypsinization and processing for clones. Cells were transfected and selected with puromycin (5µg/mL) for ~ 48–72 h to enrich edited populations. Surviving clones were expanded, and knockout efficiency was verified by Western blotting to confirm loss of protein expression.

3.3. Plasmid transfection and generation of constructs

MDA-MB-231 cells were transfected with jetPRIME (Polyplus) reagent throughout the study, while HEK293T cells were transfected using either Lipofectamine RNAiMAX (Invitrogen) or the standard calcium phosphate precipitation method to achieve high expression efficiency, grown with 60–70 % confluency. Transfection efficiency was verified by fluorescence microscopy and immunoblotting. MDA-MB-231 cells were grown to 80–90 % confluence and transfected with 10 µg plasmid per 100-mm petri dish for single-gene expression or up to 30 µg for co-transfection of three plasmids using jetPRIME. siRNA transfections in HEK293T and MDA-MB-231 cells were performed using Lipofectamine RNAiMAX.

3.4. Co-Immunoprecipitation and western blot

Cells with 80–90 % confluence, from a 100-mm petri dish, were lysed on ice under native conditions with buffer containing protease inhibitors containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2mM EDTA, 1 % Triton X-100, and 10 % glycerol to preserve protein complexes. Later to lysates were centrifuged and incubated with the appropriate antibody or control IgG, such as mouse IgG (Sigma); rabbit IgG (Sigma) I5006, for approximately 4 hours, at 4 °C, then supplemented with protein G beads (Cytiva). Further, protein G beads were pelleted and washed three times with lysis buffer containing 0.5 M NaCl, and bound proteins were eluted in 1xSDS sample buffer. For denaturing immunoprecipitation, cells were lysed with 1 % SDS, heated at 95 °C, diluted to 0.1 % SDS, immunoprecipitated using 5 µg anti-FLAG antibody, and further analyzed by SDS-PAGE and immunoblotting using a ChemiDoc MP system (Bio-Rad).

For protein validation, cells were lysed on ice in HEPES-based buffer, clarified by centrifugation at 15,000 g in pre-cooled at 4 °C, and protein concentration was determined using a Bradford assay. Lysate samples were prepared in 5× SDS loading dye, boiled for 5 min at 95 °C, separated by SDS-PAGE, and gels were transferred to PVDF membranes and analyzed by immunoblotting with primary and fluorescent secondary antibodies, followed by imaging on a Li-Cor Odyssey CLx system (LI-COR Biosciences).

3.5. Ubiquitylation assays

HEK293T cells were cotransfected with protein tags FLAG, Myc (amino acid sequence EQKLISEEDL), and HA (amino acid sequence YPYDVPDYA) tagged vectors (e.g. FLAG-Rap2a, FLAG-Rap1b, FLAG-AMBRA1, Myc-Rab40b, HA-ubiquitin). After 24 h, cells were lysed under

denaturing conditions, immunoprecipitated with anti-FLAG antibody and protein G beads, and analyzed by SDS-PAGE and immunoblotting, with signals detected using a ChemiDoc MP system (Bio-Rad).

3.6. Immunofluorescence microscopy and image analysis

Immunofluorescence microscopy was performed to analyze protein localization, cytoskeletal organization, and lamellipodial dynamics in AMBRA1 KO, Rap2 KO, Rap1 mutant, and Rab40 TKO cells. Cells were seeded on glass coverslips, coated with collagen (rat tail collagen, isolated in- house at Prekeris Lab) when required for a specific experiment, fixed with 4 % paraformaldehyde, permeabilized with 1 % Triton X-100, and blocked with 1 % BSA. Primary antibodies included anti-GFP, anti-FLAG, anti-HA, and anti-paxillin, followed by fluorescent secondary antibodies. Phalloidin was used to visualize F-actin, and DAPI or Hoechst staining marked nuclei of the cells. Samples were imaged using confocal or spinning-disk microscopy under consistent acquisition settings across the conditions. Fluorescence intensity and localization analyses were performed using Fiji, an add-on of ImageJ software (Version 1.54f). Imaging was performed on a widefield inverted Zeiss Axiovert 200M microscope using a 63× oil objective, QE charge-coupled device camera (Sensicam), and Slidebook v. 6.0 software (Intelligent Imaging Innovations). Images were analyzed in a blinded manner to ensure a consistent threshold was applied.

For study I:

FA number analysis: FAs amount was analyzed in ImageJ from maximum-intensity projected images with selected z-stacks, where 15 to 20 non-dividing and isolated cells per replicate were chosen and captured, using background subtraction, manual thresholding, and the ‘Analyze Particle’ command in ImageJ to identify every single FA present in the cell. The ratio of peripheral FAs was calculated as the number of FAs within a 4-µm membrane region / total FAs in the whole cell.

For study II:

Cell morphology analysis: The actin cytoskeleton was outlined to define the cell perimeter (ROI, region of interest), and area and perimeter were measured. Circularity was calculated as , and the aspect ratio as the major/ minor axis of the fitted ellipse. *Cortactin-enrichment analysis:* segments were

measured as a fraction of the total perimeter (segment length/cell perimeter), and enrichment was calculated as , with each segment quantified separately.

RhoA Biosensor analysis: RhoA biosensor enrichment was calculated from line scans across ruffles as peak fluorescence intensity / first valley fluorescence intensity. Ruffle longevity was measured by counting enriched frames and multiplying by 5 seconds.

FA Dynamics: FA number and size were measured from thresholded phospho-paxillin images, and periphery localization was calculated as (whole cell puncta area − intracellular puncta area) / whole cell puncta area × 100.

MyoIIb analysis: MyoIIb intracellular ROI and a thresholded stress fiber presence were defined from the actin channel. MyoIIb enrichment was calculated as stress fiber presence, MyoIIb mean grey value / intracellular ROI MyoIIb mean grey value.

PLA analysis: Whole-cell and intracellular ROIs were defined from the GFP-Rap2a signal, and thresholded PLA puncta were counted within each ROI. Membrane-localized PLA puncta were calculated as (whole cell puncta − intracellular puncta) / whole cell puncta × 100. Whole-cell puncta density was calculated as the number of PLA puncta / whole-cell ROI area.

For study III:

The ratio of integrated density in MDA-MB-231 cells was calculated/ defined as intracellular – background/ whole background, with a value indicating 1 as intracellular signal.

3.7. Focal adhesions analysis

Focal adhesion analysis was performed to evaluate adhesion number, distribution, and turnover (assembly & disassembly) in AMBRA1 KO and Rap2 KO cells. These cells were plated on 1x collagen-coated coverslips, fixed, and stained with antibodies against focal adhesion markers such as paxillin or phospho-paxillin. For FA dynamic analysis, control and Rap2 KO cells were transfected with GFP-paxillin and imaged live under temperature- and CO_2-controlled conditions using a stage-top incubator maintained at 37 °C. Time-lapse videos were acquired at 30 to 60 second intervals for up to 30 minutes per independent replicate. FA dynamics were quantified using Fiji & TrackMate plugin add-ons from ImageJ to measure adhesion lifetime or

lifespan, assembly and disassembly rates, and velocity. For fixed-cell analysis, adhesion structures were segmented using thresholding algorithms, and parameters such as adhesion number, aspect ratio, fluorescence intensity, and peripheral versus central distribution were quantified. Identical acquisition settings were maintained across all conditions and replicates, and at least three independent experiments were performed.

3.8. SACED - Straboscopic Analysis of Cell Dynamics

SACED was performed to quantify membrane ruffling and lamellipodial retraction dynamics activity in control and Rap2 KO cells, and this method was acquired from previous work [172]. Cells were plated on collagen-coated glass-bottom petri dishes and imaged using the 64x magnification differential interference contrast (DIC) channel. Time-lapse images were acquired for 8 minutes at 1 frame per second with 100 ms exposure. Only cells maintaining focus at actively ruffling edges were selected and analyzed. Kymographs were generated in Fiji by drawing perpendicular lines across the ruffling membrane. Membrane extension, retraction distance, and ruffle periodicity were quantified from these space-time plots.

3.9. RNA seq analysis

RNA-seq was performed using total RNA for MDA-MB-231 AMBRA1 WT and KO isolated with TRIzol, and libraries were prepared using the KAPA mRNA HyperPrep kit (Roche) with polyA selection from 50 ng RNA. After fragmentation, cDNA synthesis, adapter ligation, and 13-cycle PCR amplification, libraries were sequenced on an Illumina platform to generate paired-end reads. Raw FASTQ files were quality trimmed, aligned to the human reference genome (hg38), and quantified. Differential gene expression between WT and KO cells was analyzed using standard statistical pipelines with FDR < 0.05 and fold-change thresholds, pooled, and s equenced on NovaSeq 6000 (Illumina). Gene ontology and pathway enrichment analyses were performed for each replicate and visualized using volcano plots and heatmaps.

3.10. Random migration assay

Random single-cell migration assays were performed to assess the individual cell movement in AMBRA1 KO, Rap2 KO, Rab40a KO, Rab40b KO, Rab40c KO, and Rab40 TKO (isoforms of Rab40a, b, and c were knocked out altogether, Rab40abc KO in MDA-MB-231 cells). 1.5×10^4 cells/cm^2 were calculated and plated uniformly for accurate single-cell tracking on

fibronectin (Sigma-Aldrich) diluted in 300 µls (1 µg/cm^2) of PBS, coated 35 mm glass-bottom petri dishes. After overnight adhesion, time-lapse imaging was conducted using an Olympus IX83 inverted confocal microscope at the facility in the laboratory of the Cell Culture, LSMU. Equipped with a temperature-controlled chamber, a bottom-top incubator, maintained at 37 °C, along with 5 % CO_2.

Images were set to acquire automatically at a defined interval, for every 20 minutes for 12 hours for long-term tracking, which includes 36 frames in total. Only isolated cells that remained in focus and did not divide until the last time frame were analyzed. The geographic centre or the nucleus of the cells was selected to focus the trajectory of the individual cell. Single-cell movement trajectories were tracked using Excellence Pro Software (Gradientech), from which the length, distance, and speed data were generated. Migration parameters, including length, total distance travelled, speed (velocity rate), and directional persistence (calculated by distance/length), were calculated. At least three independent biological replicates were performed per condition, with 30 to 50 cells analyzed per replicate.

3.11. Flow cytometry analysis

Flow cytometry was performed to assess cell-cycle distribution in MDA-MB-231 Cas9 control and AMBRA1 KO cells. Cells were harvested at 70–80 % confluency, fixed in cold 70 % ethanol, and stained with propidium iodide (PI) for DNA content analysis. Doublets were excluded using peak-versus-integral gating strategies to ensure accurate single-cell measurements. Cell cycle phase analysis was performed with a Beckman Coulter Gallios flow cytometer, and data were processed using ModFit LT software (Verity Software House).

3.12. Proximity ligation assay (PLA)

PLA was performed to detect in situ association between Rap2 and ubiquitin in MDA-MB-231 cells expressing GFP-Rap2a WT or K3R (K117R/K148R/K150R, hereinafter GFP-Rap2a-K3R). Cells were fixed, permeabilized, and processed using the Duolink PLA kit (Sigma Aldrich) with anti-GFP and anti-ubiquitin antibodies. Z-stack images were acquired and max-projected for analysis in the ImageJ add-on plugin with Fiji. ROIs were defined to distinguish whole-cell and intracellular compartments. PLA puncta were thresholded and quantified using particle analysis, and membrane-localized puncta were distinguished from intracellular signals. Three biological replicates were performed.

3.13. Glutathione S-transferase (GST) binding assay

GST pull-down assays were performed to assess protein-protein interactions between Rab40b binds to Rap1b. Lysates from cells expressing FLAG-Rab40b WT or 4A mutant were incubated with GST or GST-Rap1b under defined nucleotide-loading conditions, such as GDP or GppCp. Glutathione beads were used to isolate complexes. Bound proteins were analyzed by SDS-PAGE and Western blotting. Coomassie staining confirmed GST protein loading, and samples were imaged on Li-Cor Odyssey CLx.

3.14. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software (version 10.4.1 (532)) throughout the research work. All the mentioned data represent at least three independent biological replicates. For comparisons between two groups, unpaired two-tailed Student's t-tests were used. For three or more groups, one-way ANOVA was applied. Biological replicates were analyzed independently, and technical replicates were averaged within each biological replicate. Significance was defined as $p \leq 0.05$. Error bars represent standard deviation unless otherwise stated.

4. RESULTS AND DISCUSSION

4.1. Study I - Rab40 GTPases regulate AMBRA1-mediated transcription and cell migration

The detailed results of Study I are a collaborative project with Dr. Ke-Jun Han, from Prekeris lab, University of Colorado, USA, and published in The Company of Cell Biologists Journal, "Rab40 GTPases regulate AMBRA1 mediated transcription and cell migration." Published in April 2025, https://doi.org/10.1242/jcs.263707

AMBRA1 (Activating Molecule in Beclin-1-Regulated Autophagy 1) has become recognized as a versatile regulator of autophagy, cell cycle progression, and transcription [20,22]. Initially, it was known for its role in autophagy initiation through the Beclin1 and Vps34 complex, but it is now also linked to other cellular pathways, such as regulating the cell cycle via ubiquitin-mediated degradation of Cyclin D and controlling gene transcription through its interaction with chromatin-remodeling complexes [151]. Regarding migration, AMBRA1 has been involved in focal adhesion remodeling and the transcriptional regulation of genes related to the cytoskeleton and cell movement [23,27].

Recent studies from the literature suggest that AMBRA1 is itself regulated by Rab40-CRL5-mediated ubiquitination, which alters its functional output without targeting it for degradation. This non-canonical ubiquitination influences AMBRA1's nuclear activity and ability to control gene expression programs associated with adhesion and migration. Transcriptomic analyses of AMBRA1-deficient cells have revealed dysregulation of genes including SNAI2, ITGB7, and MAP4K4, highlighting its potential role in fine-tuning the migratory behavior of cancer cells [163]. Taken together, identifying AMBRA1-dependent transcriptional programs that influence migration. This work seeks to advance the current understanding of how migration is regulated at the interface of vesicular trafficking, cytoskeletal remodeling, and transcriptional control.

4.2. AMBRA1 is a substrate of Rab40c/CRL5 Complex

4.2.1. AMBRA1 interacts with GTPases

Previous studies have established that Rab40 family members regulate cell migration by forming CRL5 ubiquitin ligase complexes that ubiquitylate specific substrates [16, 145]. While Rab40a and Rab40b substrates have been characterized, the targets of Rab40c remain unclear in autophagy [126,141].

This approach interacts with a previously characterized mutation in the SOCS box of Rab40 proteins, in which the 211, LPLP,216 region motif was mutated to 211, AAAA,216 (Rab40-4A). This mutation reduces binding to Cullin-5 and consequently enhances the association of Rab40 proteins with their potential ubiquitylation substrates. Using this hypothesis, immunoprecipitation followed by mass spectrometry identified AMBRA1 as a candidate Rab40c-associated protein. Specifically, AMBRA1 peptides were detected in FLAG-Rab40c-4A immunoprecipitates but not in FLAG-Rab40c WT precipitates, suggesting that AMBRA1 could be able to interact with a substrate of the Rab40c-CRL5 complex (Fig. 8A). To validate this binding, the endogenous Rab40c was immunoprecipitated from MDA-MB-231 cell lysates using anti-Rab40c antibodies, where immunoblot analysis demonstrated that endogenous AMBRA1 presence of co-immunoprecipitated Rab40c, confirming that these proteins interact under physiological conditions (Fig. 8B). HEK293T cells were transfected with either FLAG-Rab40c wild-type or the FLAG-Rab40c-4A mutant construct. Following immunoprecipitation with anti-FLAG antibodies, immunoblotting showed that endogenous AMBRA1 was strongly enriched in FLAG–Rab40c-4A precipitates, while a weaker binding was detected with WT Rab40c (Fig. 8C). This result is consistent with the substrate-presence of the 4A mutant and supports the interaction between AMBRA1 and Rab40c.

To determine whether this interaction is specific to Rab40c, HEK293T cells were transfected with FLAG-tagged Rab40a, Rab40al, or Rab40b constructs, followed by FLAG immunoprecipitation. Immunoblot analysis revealed that endogenous AMBRA1 co-immunoprecipitated with all Rab40 subfamily members tested, indicating that AMBRA1 can interact with multiple Rab40 proteins (Fig. 8D). To identify the AMBRA1 region responsible for this interaction, FLAG-tagged AMBRA1 deletion mutants were co-expressed with HA-Rab40c in HEK293T cells. Immunoprecipitation and immunoblotting showed that Rab40c predominantly binds to the C-terminal region of AMBRA1, specifically within the 706–861 amino acid region overlapping with the WD40 domain (Fig. 8E).

Altogether, the ubiquitylation assays showed that Rab40c promotes the mono-ubiquitylation of AMBRA1 rather than targeting it for degradation, which shows that Rab40c-mediated modification of AMBRA1 involves regulatory mechanisms. Given that Rab40-CRL5 regulates focal adhesion dynamics during migration [126,127], and on the other hand, AMBRA1 has been implicated in migration-related signaling [162], our findings suggest a potential role for AMBRA1 in regulating migration-associated pathways that ultimately influence migratory phenotypes. Together, these results identify AMBRA1 as a Rab40c-binding protein and novel Rab40c-CRL5

substrate, establishing a direct biochemical link between Rab40c-mediated ubiquitylation and AMBRA1-dependent cellular functions.

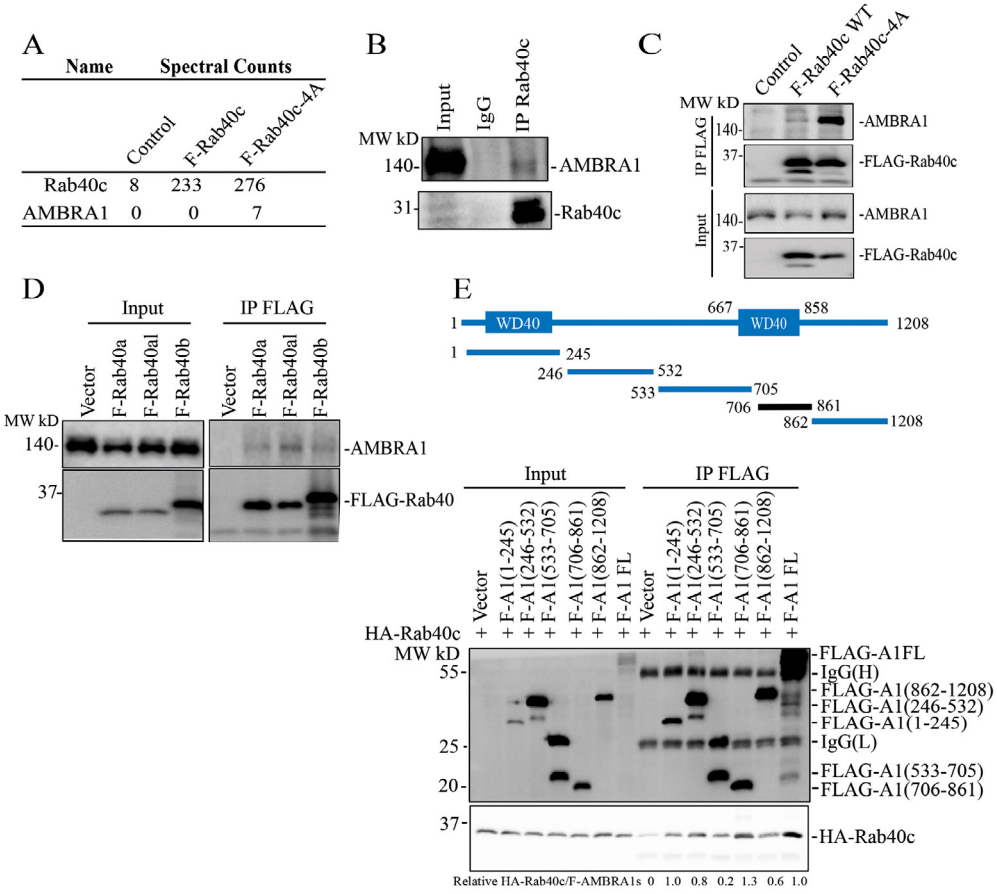
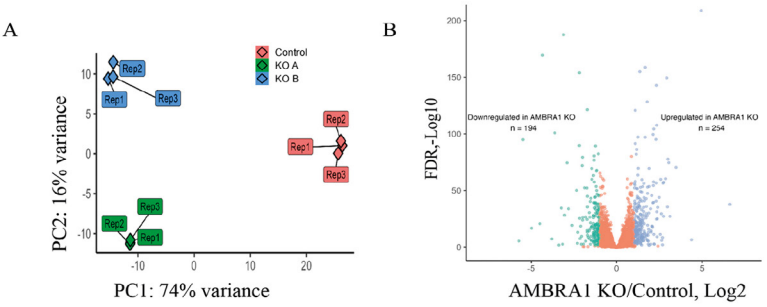


Fig. 8. *Ambra1 interacts with Rab40c. (A) Proteomics screening of AMBRA1 in FLAG-Rab40c-4A immunoprecipitates. (B) Co-immunoprecipitation binding of Rab40c and AMBRA1. (C) FLAG-Rab40c and FLAG-Rab40c-4A immunoprecipitation with AMBRA1 binding. (D) AMBRA1 interacts with Rab40 subfamily members. (E) Domain mapping shows Rab40c binding to the C-terminal region of AMBRA1 (706–861 aa) overlapping with the WD40 domain*

4.2.2. AMBRA1 involvement transcription and FA dynamics

Our previous data indicated that AMBRA1 regulates Rab40c expression at the transcriptional level, suggesting that AMBRA1 may have dual functions: participation in ubiquitylation and regulation of gene transcription [24,160]. To further define the transcriptional program controlled by AMBRA1, RNA sequencing was performed in control and two independent AMBRA1 knockout MDA-MB-231 cell lines. Principal component analysis demonstrated strong reproducibility among biological replicates and showed that the transcriptomes of AMBRA1-deficient cells clustered together but were clearly separated from control samples, indicating a distinct transcriptional profile upon AMBRA1 loss (Fig.9A).

Later, Differential expression analysis revealed widespread transcriptional changes in AMBRA1-KO cells. Compared with control cells, 254 genes were upregulated, and 194 genes were downregulated [KO/control, log2(fold change)>2, $P<0.05$] (Fig. 9B). Gene Ontology analysis further indicated that many of these genes were associated with cell migration–related processes, including growth factor binding, extracellular matrix organization, collagen binding, and cell–cell adhesion (Fig. 9C). Notably, RAB40C mRNA levels were significantly increased in AMBRA1-KO cells (Fig. 9D), consistent with earlier observations. To validate RNA-seq results, quantitative PCR analysis was performed on several migration-related genes identified in the dataset, including paxillin (PXN), MAP4K4, GRAMD1B, PXDN, and SNAI2. These analyses confirmed that AMBRA1 loss alters the expression of genes involved in adhesion and migration pathways (Fig. 9D,E).



C GO Molecular Functions

Term ID	Term Name	Padj
GO:001938	growth factor binding	$2.2x10^{-8}$
GO:0005201	extracellular matrix constituent	$6.6x10^{-6}$
GO:000518	collagen binding	$9.0x10^{-5}$
GO:0019899	enzyme binding	$2.5x10^{-4}$
GO:0018636	phenanthrene 9,10-monooxygenase activity	$5.1x10^{-3}$

GO Biological Process

Term ID	Term Name	Padj
GO:0048583	regulation of reponse to stimulus	$1.5x10^{-17}$
GO:0030198	extracellular matrix organization	$7.2x10^{-4}$
GO:0098742	cell-cell adhesion	$1.4x10^{-3}$
GO:004292	homeostatic process	$2.5x10^{-3}$
GO:200630	positive regulation of miRNA metabolism	$3.9x10^{-3}$

D

Gene	log2FoldChange	P value
Rab40c	0.37	0.001
Paxillin	0.5	2.9E-12
MAP4K4	1.81	4.95E-56
GRAMD1B	-4.49	4.77E-23
PXDN	-14.68	5.36E-69
SNAI2	-1.2	2.8E-34

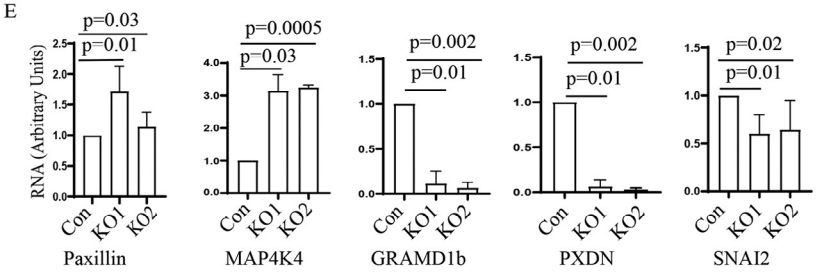


Fig. 9. *AMBRA1 regulates gene transcription. (A) PCA analysis of RNA-seq data showing separation between control and AMBRA1-KO cells. (B) Volcano plot of differentially expressed genes in AMBRA1-KO cells. (C) GO enrichment analysis of altered genes. (D–E) qPCR validation of selected genes (PXN, MAP4K4, GRAMD1B, PXDN, SNAI2)*

Further, to analyse if AMBRA1 regulates migration-associated genes and interacts with members of the Rab40 family, next investigated whether AMBRA1 influences focal adhesion organization. Western blot analysis confirmed altered expression of key migration-associated proteins, showing increased paxillin levels and reduced SNAI2 levels in AMBRA1-KO cells

compared with control cells (Fig. 10A). Because paxillin is a focal adhesion adaptor protein involved in adhesion formation and signaling, these changes suggested that AMBRA1 may affect focal adhesion dynamics.

To validate this possibility, focal adhesion distribution was quantified in control and AMBRA1-KO cells. In control MDA-MB-231 cells, paxillin-positive focal adhesions appeared as small puncta-like (or dot-like structure; usually represents the bright signals) predominantly localized at the cell periphery, particularly within leading-edge lamellipodia (Fig. 10B & C). In contrast, AMBRA1-KO cells displayed enlarged paxillin-positive adhesions that were distributed throughout the cell body rather than concentrated at the periphery (Fig. 10 B & C). Further, quantification confirmed a reduction in peripheral FA localization relative to total adhesions in AMBRA1-KO cells (Fig. 10B), indicating altered spatial organization of adhesion structures.

Because FA organization is closely linked to cell motility, we next wanted to examine whether AMBRA1 loss directly affects cell migration or not? For that, we performed random cell migration assay, where time-lapse tracking of individual cells revealed that AMBRA1-KO cells exhibited increased migration velocity compared with control cells (Fig. 10D). Interestingly, a similar increase in migration velocity was observed in Rab40 TKO (Rab40a, Rab40b, and Rab40c, triple knockout cells), suggesting that Rab40 GTPases regulate migration in part through their interaction with AMBRA1. In addition to increased speed, AMBRA1-deficient cells also exhibited enhanced migration directionality, as determined by the ratio of net displacement to total path length during time-lapse analysis (Fig. 10E).

Together, these results indicate that AMBRA1 regulates a transcriptional program linked to cell adhesion and migration and contributes to the spatial organization of focal adhesions. AMBRA1 KO alters the expression of migration-related genes, disrupts focal adhesion distribution, and enhances cell motility, supporting a role for the Rab40-AMBRA1 pathway in regulating focal adhesion function and cell migration.

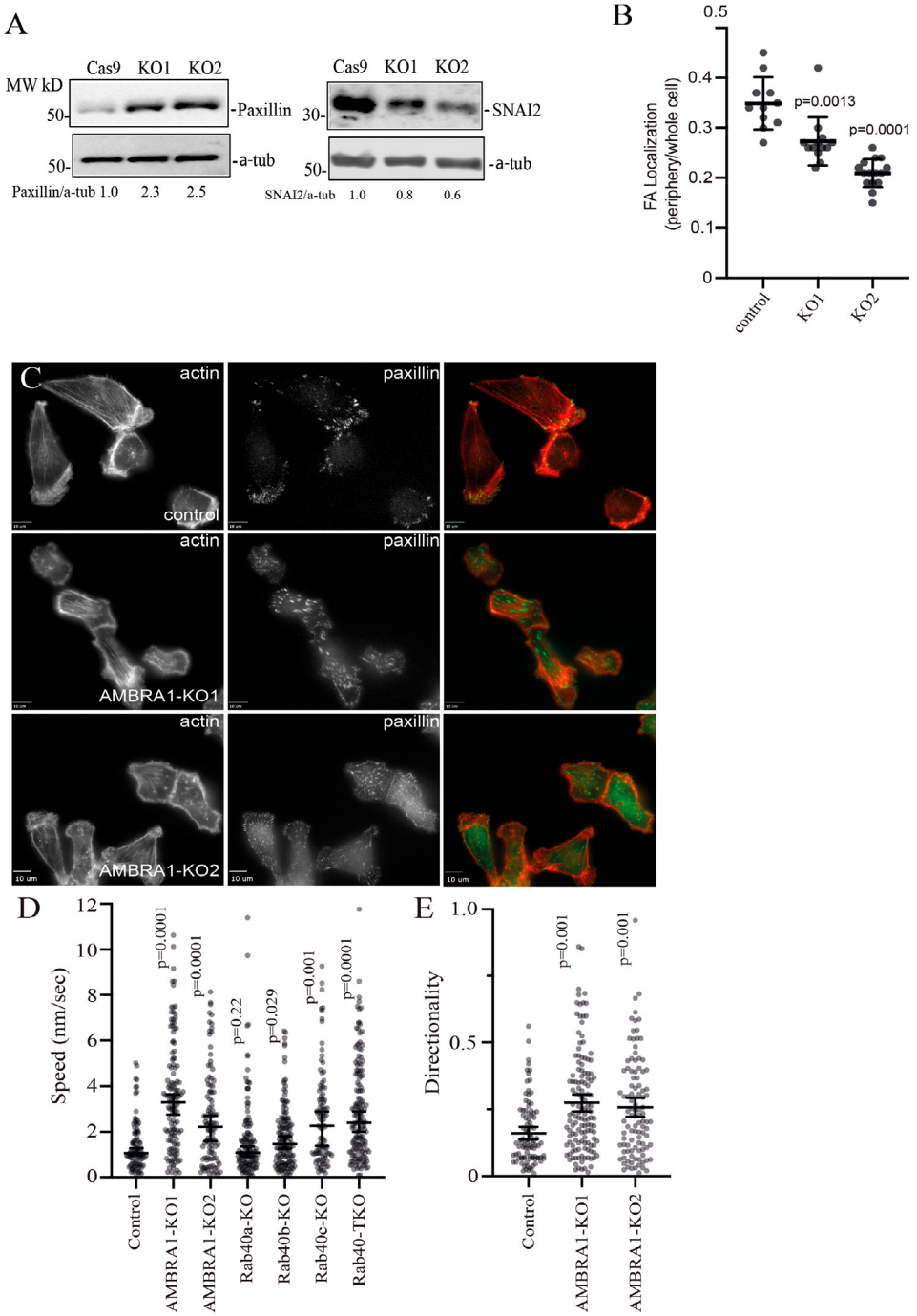


Fig. 10. *AMBRA1 regulates focal adhesion dynamics and cell migration. A) Western blot analysis of paxillin and SNAI2 in control and AMBRA1-KO*

cells. α-tubulin was used as a loading control. (B) Quantification of focal adhesion (FA) localization at the cell periphery relative to the whole cell. (C) Immunofluorescence images of control and AMBRA1-KO cells stained for actin (phalloidin) and paxillin. Scale bar: 10 μm. (D) Quantification of migration velocity in control, AMBRA1-KO, Rab40 single KO, and Rab40 TKO cells. (E) Quantification of migration directionality from time-lapse analysis

4.2.3. Discussion

Altogether, these findings support the hypothesis that AMBRA1 functions not only as part of an E3 ubiquitin ligase complex but also as a separate transcriptional regulator, and this transcriptional activity depends on its interaction with Rab40 GTPases rather than on CRL4 complex formation. Some studies uncover a previously unrecognized role of AMBRA1 in breast cancer cell migration by acting as a transcriptional regulator independent of its canonical function in autophagy. AMBRA1 has traditionally been characterized as a scaffold protein essential for the initiation of autophagy through its interaction with Beclin1 and ULK1 [146][163]. However, emerging evidence suggests that AMBRA1 exhibits versatile regulatory roles, extending into cell cycle control, transcription, and now, as demonstrated in this study, cell migration through modulation of gene expression [156]. In this work, AMBRA1 was shown to regulate the expression of multiple migration-related genes, including SNAI2, PAXILLIN, and MAP4K4 in MDA-MB-231 breast cancer cells, suggesting that its function in transcriptional control is broad and impactful.

Notably, loss of AMBRA1 led to downregulation of SNAI2, a master regulator of epithelial-to-mesenchymal transition (EMT), and upregulation of PAXILLIN, a core component of focal adhesions. These transcriptional alterations translated into functional consequences at the cellular level: AMBRA1 KO cells revealed increased cell migration speed and altered FA distribution, with larger and more dispersed adhesions that were no longer restricted to the leading edge. Importantly, this transcriptional regulatory function of AMBRA1 appears to be independent of its association with the CRL4 ubiquitin ligase complex. Despite being a substrate adaptor for CRL4, AMBRA1 mutants lacking the CRL4-binding domain were still capable of restoring SNAI2 expression in KO cells, indicating that its transcriptional role does not require CRL4-mediated ubiquitylation. Instead, the study highlights the central role of Rab40 GTPases and the CRL5 complex in modulating AMBRA1 function through a unique non-degradative ubiquitination

mechanism [163]. Together, these data support a model in which AMBRA1 transcriptionally coordinates the expression of adhesion-related genes to control breast cancer cell motility.

Although this study identifies the Rab40 relation with AMBRA1 in regulating migration and transcription, several questions remain. The exact mechanisms by which ubiquitinated AMBRA1 controls gene expression are still unclear, including whether it directly interacts with chromatin signalling or works through any other transcription factors, with the downstream signalling pathway.

In addition to that, the work relies predominantly on the in vitro breast cancer cell, limited to the MDA MB 231 cell line, and therefore, the physiological relevance of these mechanisms must be tested in in vivo studies, which remain to be established and validated. Overall, this work positions AMBRA1 as a dual-function protein that orchestrates cancer cell migration through both protein ubiquitylation and transcriptional regulation. By modulating the expression of genes that govern FA turnover, cytoskeletal tension, and cell polarity, AMBRA1 exerts a central influence over migratory behavior in aggressive breast cancer cells. The modulation of its activity by Rab40 GTPases further reveals a sophisticated layer of regulation through non-proteolytic ubiquitination, offering new insights into the plasticity of cancer cell behavior.

4.3. Study II - Investigating Rap2 GTPase role in breast cancer cell migration

The detailed results of Study II are a collaborative project with Andrew Neumann, from Prekeris lab, University of Colorado, USA, and published in the Journal of Cell Science, "Ubiquitylation-dependent Rap2 activation regulates lamellipodia dynamics during cell migration," Published in November 2025, doi.org/10.1242/jcs.264375

In recent years, increasing attention has been paid to the spatial and temporal regulation of signaling proteins that orchestrate cell migration. While the core components of the migratory machinery, such as actin polymerization, focal adhesion dynamics, and Rho-family GTPases (RhoA, Rac1, and Cdc42), are well established, much less is known about how their activity is finely tuned at specific subcellular sites through post-translational mechanisms. Small GTPases function as molecular switches that control cellular polarity and directional motility. Among these, the Ras-related GTPase Rap1 has been extensively studied for its role in promoting integrin activation and adhesion stability. In contrast, the related GTPase Rap2 has remained relatively understudied. Although Rap2 shares high sequence homology with Rap1,

its distinct functions in membrane trafficking and cytoskeletal remodeling have only recently begun to be elucidated. The Rap2 subfamily, comprising Rap2a, Rap2b, and Rap2c, has long been considered enigmatic, especially in the context of leading-edge protrusion and cell migration dynamics.

This study addresses a critical gap in our understanding of Rap2 regulation by demonstrating that Rap2 activity is modulated by mono-ubiquitylation, a non-degradative post-translational modification. We identify the Rab40-CRL5 E3 ubiquitin ligase complex as the upstream regulator responsible for mono-ubiquitylating Rap2 at lysine residues K117, K148, and K150. This modification is shown to be both necessary and sufficient for Rap2 activation, membrane localization, and functional engagement with its guanine nucleotide exchange factors (GEFs), particularly EPAC1. Mutation of these lysine residues (K3R mutant) prevents Rap2 GTP loading and membrane retention, even when Rap2 is forcibly tethered to the plasma membrane, indicating that ubiquitylation actively promotes its signaling state rather than merely serving as a membrane anchor.

Functionally, Rap2 is found to act as a spatial inhibitor of RhoA activity at lamellipodia. During lamellipodial retraction, Rap2 is recruited to dynamic ruffle membranes, where it locally suppresses RhoA-driven actomyosin contractility. In the absence of Rap2, cells exhibit excessive retraction, enlarged and stabilized focal adhesions, and enhanced stress fiber formation, all of which impair migratory capacity. These observations position Rap2 as a key regulator of actin dynamics by tempering RhoA activity in space and time.

Importantly, this work challenges the traditional view of mono-ubiquitylation as a degradation signal, revealing instead its role as a molecular switch for small GTPase activation. Furthermore, it expands the functional repertoire of the Rab40-CRL5 complex from trafficking regulation to direct control of cell migration signaling pathways. These findings have broader implications, particularly in cancer biology, where precise regulation of cell migration underpins invasion and metastasis. By identifying mono-ubiquitylation as a mechanistic checkpoint for Rap2 activation, this study highlights a potential therapeutic axis for modulating cell motility.

Altogether, this Study introduces a novel mechanism of Rap2 regulation via Rab40-CRL5-mediated mono-ubiquitylation, which governs Rap2 activation, localization, and downstream effects on actin-mediated motility. These results not only deepen our understanding of Rap2 biology but also provide a framework for exploring ubiquitin-dependent spatial regulation across other Ras-family GTPases.

4.3.1. Rap2: Essential for cell migration and lamellipodia formation

To understand the functional requirement of Rap2 during breast cancer cell migration, we generated a complete Rap2 KO MDA-MB-231 line by deleting three isoforms of Rap2a, Rap2b, and Rap2c simultaneously. This strategy ensured full depletion of total Rap2 activity, allowing us to evaluate its necessity for cellular motility. Using random migration assays, we observed that Rap2 KO cells exhibited markedly reduced migration velocity and net displacement compared with control cells, confirming that Rap2 is essential for productive movement (Fig. 11A–C)

To understand how Rap2 supports migration, the structural organisation of the actin cytoskeleton was examined. Control cells displayed the expected front-to-rear polarity, forming a broad lamellipodium at the leading edge and a compact trailing rear. In contrast, Rap2 KO cells failed to establish this polarity, instead presenting a rounded or flattened morphology without a pronounced leading edge (Fig. 11D & E). Quantification of the aspect ratio, which is a measure of cell elongation, supported this observation, demonstrating a significant loss of front-to-back structural organisation. This morphological collapse indicates that Rap2 is required for the establishment of directional polarity. Because lamellipodia are the dominant actin-based protrusions guiding cell movement, we next assessed the presence and quality of lamellipodia in Rap2 KO cells. Cortactin immunostaining revealed that while most control cells form large cortactin-rich lamellipodia, only a fraction of Rap2 KO cells were able to generate lamellipodia-like structures, as illustrated in (Fig. 11G–J). When present, these structures were smaller and less enriched in cortactin, indicating defective branched actin assembly. Collectively, these findings illustrate that Rap2 is indispensable for the formation and maintenance of lamellipodia and for the organisation of polarity structures required for efficient migration.

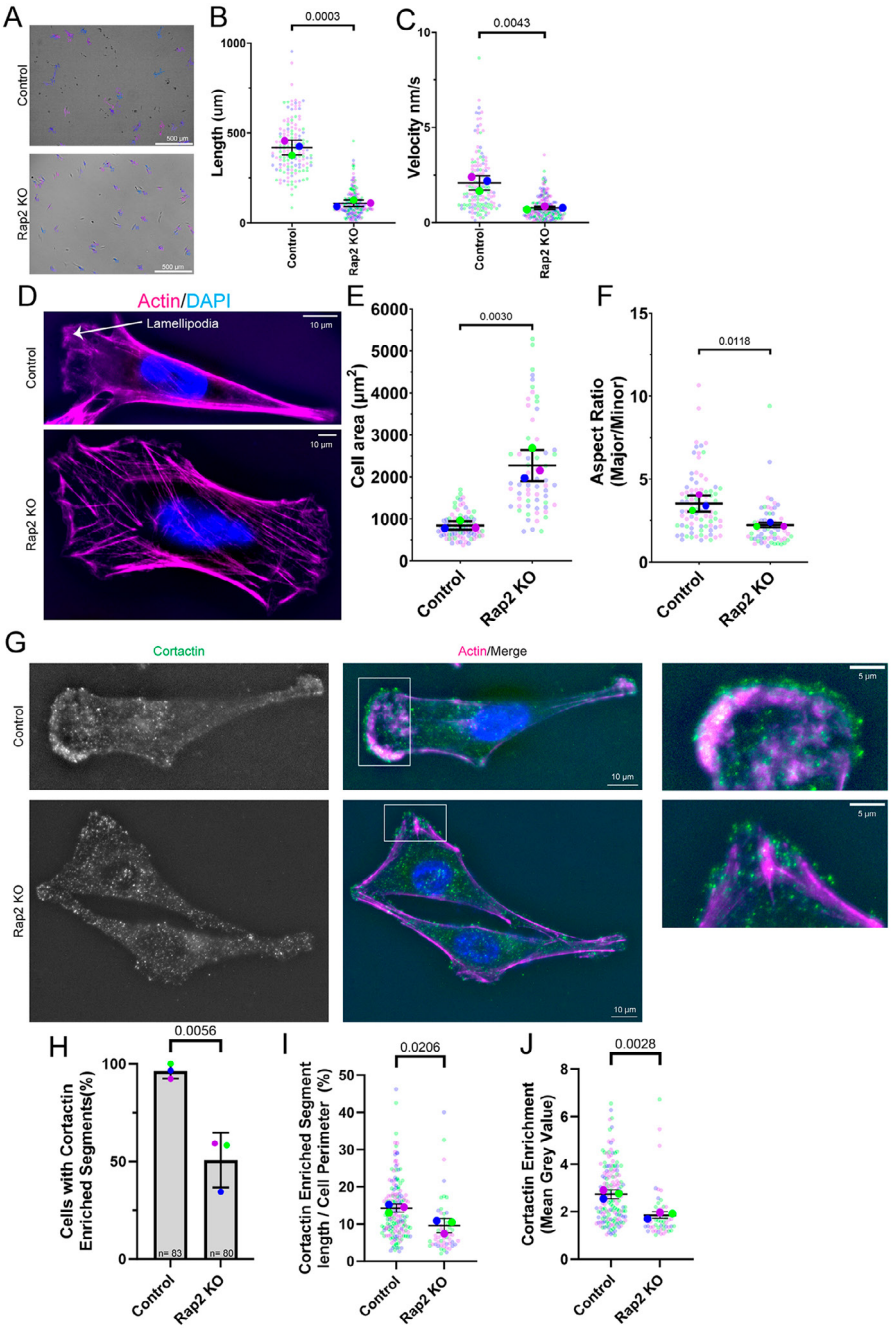


Fig. 11. *Rap2 is an absolute requirement for cell migration and lamellipodia formation. (A–C) Time-lapse migration assays show that Rap2-KO cells move more slowly and cover shorter distances than control cells, and in the section from D to F, the phalloidin staining reveals that*

Rap2-KO cells are larger and less elongated, indicating a loss of front–back polarity. (G) Phalloidin and cortactin staining were used to assess lamellipodia organisation. (H–J) Rap2-KO cells display fewer cortactin-positive lamellipodia, reduced lamellipodial coverage, and lower cortactin enrichment, demonstrating impaired lamellipodia dynamics.

4.3.2. Rap2 Regulates RhoA activity in retraction of lamellipodia ruffles

To investigate how Rap2 controls lamellipodia behaviour, we analysed the spatiotemporal dynamics of GFP Rap2 during migration. Time-lapse imaging revealed that Rap2 does not localise strongly during membrane extension but instead accumulates specifically at retracting lamellipodia ruffles, as shown in Fig.12. D–G. This pattern suggested that Rap2 may function as a regulator that restrains excessive retraction (Fig.12. A–C). Kymograph analysis shows that Rap2 KO cells display altered ruffle periodicity and increased membrane retraction compared with controls. Consistent with this hypothesis, Rap2 KO cells displayed dysregulated ruffle dynamics. Kymograph-based quantification showed that while extension rates were comparable to controls, Rap2 KO cells had greater retraction distances and reduced ruffling periodicity (Fig.12B & C). These characteristics represent a failure to terminate RhoA-driven retraction pulses, a process necessary to maintain balanced protrusion and retraction cycles. It was observed that Rap2 accumulates specifically at retracting lamellipodia, suggesting a different path from its previously described function in promoting actin polymerisation. Since lamellipodia retraction is driven by RhoA-dependent contractility, these localisation patterns raise a question that leads to a hypothesis whether Rap2 can influence RhoA activity during retraction or not? Examining these interactions between Rap2 and active RhoA at the leading edge is needed.

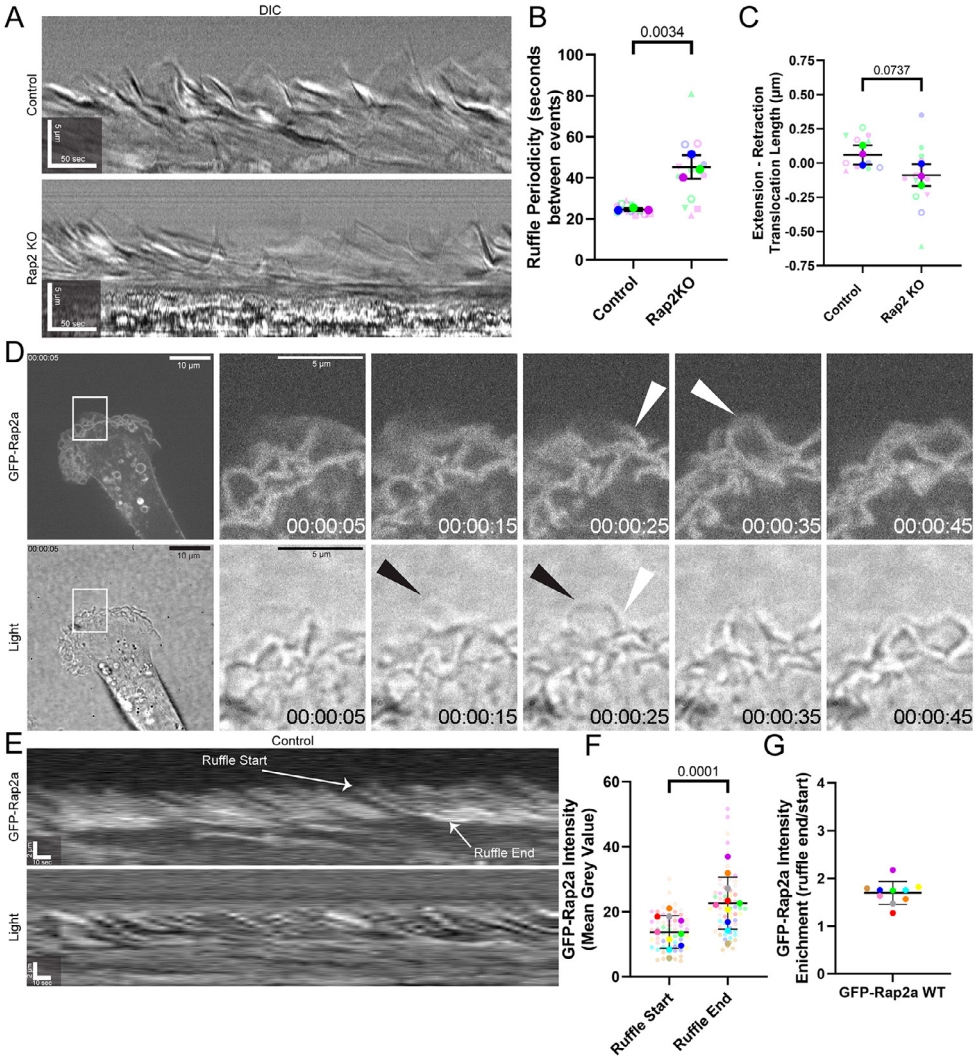


Fig. 12. *Rap2 localizes to retracting ruffles and regulates leading-edge dynamics. (A–C) From SACED, kymograph analysis shows that Rap2 KO cells display altered ruffle periodicity and increased membrane retraction compared with controls. (D) Time-lapse imaging of GFP Rap2a reveals that Rap2 is absent from extending ruffles but accumulates specifically in retracting ruffles. (E–G) Kymograph quantification confirms that GFP Rap2a signal increases from the start to the end of a ruffle, demonstrating progressive Rap2 enrichment during retraction.*

Using a fluorescent RhoA biosensor, there was a strong colocalization between active RhoA and Rap2 at the retracting ruffles (Fig.13A & B). In control cells, RhoA activity peaked sharply during the onset of retraction and declined rapidly as retraction ended. To determine whether this colocalisation reflected functional regulation, it was necessary to compare the RhoA activity patterns in control and Rap2 KO cells. If Rap2 acts as a negative regulator of RhoA during retraction, then Rap2's absence should lead to exaggerated or prolonged RhoA activity. Indeed, Rap2 KO cells displayed stronger, longer-lasting RhoA signals and uncoordinated ruffle movements, including lateral farther rather than inward retractions. This behaviour indicates a loss of spatial and temporal control over RhoA signalling. The prolonged persistence of RhoA activity in Rap2 KO cells further supports the conclusion that Rap2 normally functions to diminish or terminate RhoA activation once retraction is initiated. However, in Rap2 KO cells, RhoA activity was abnormally elevated and prolonged, and ruffles moved not only inward but also laterally across the lamellipodium (Fig.13C–E). This disorganisation reflects spatial and temporal misregulation of RhoA. Taken together, these findings show that Rap2 accumulation at retracting ruffles is not a passive event but rather reflects its functional role as a regulator that restrains RhoA activity, ensuring that retraction is properly moderated and lamellipodia dynamics remain well coordinated during migration.

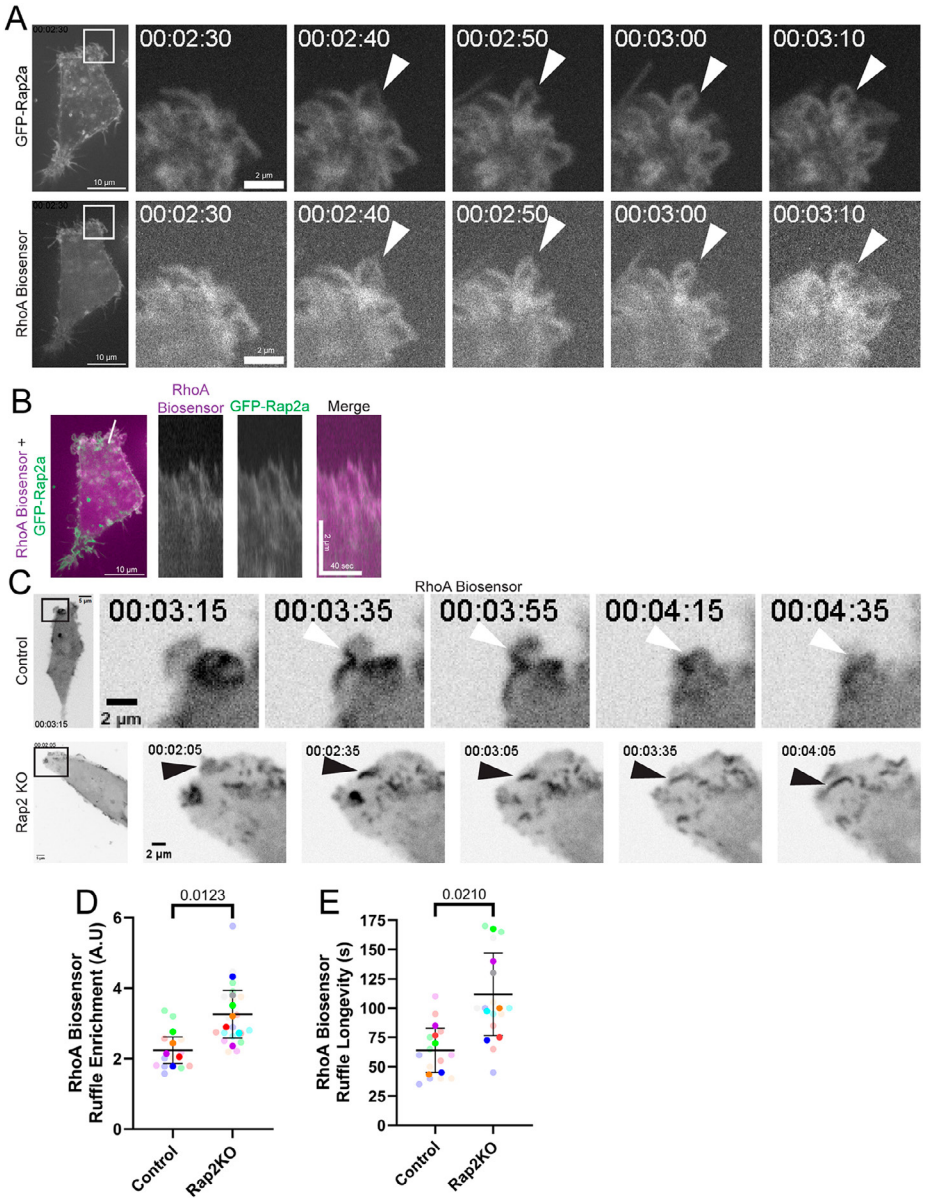


Fig.13. *Rap2 regulates the RhoA activity at the lamellipodia. (A–B) Selected part of time-lapse imaging of cells co-expressing GFP Rap2a and a RhoA biosensor shows that Rap2 and active RhoA colocalize specifically at retracting lamellipodial ruffles. (C–E) In control cells, RhoA-enriched ruffles retract in a directed manner, whereas Rap2 KO cells show stronger, longer-lasting, and more disorganized RhoA activity, which confirms the increased RhoA presence and prolonged signal duration in the depletion of Rap2.*

4.3.3. Rap2 regulates focal adhesion formation and dynamics

To know deeper why Rap2 cells developed widespread morphological defects, it was essential to consider how Rap2 influences the broader RhoA actin regulatory network. Our earlier findings showed that Rap2 restrains RhoA activity specifically during lamellipodial retraction, leading to maintaining balanced protrusion-to-retraction cycles at the leading edge.

However, Rap2 KO cells exhibited not only defects at the lamellipodium but also some noticeable changes in cell shape, including a more spread morphology and reduced polarity features commonly associated with elevated RhoA activity throughout the cell. Because RhoA is a key driver of actin contractility, focal adhesion maturation, and stress fiber assembly, we reasoned that, when there is a loss of Rap2, it might lead to excessive or misregulated RhoA signalling beyond the lamellipodium. This reasoning motivated us to examine whether focal adhesion formation and turnover processes are highly sensitive to RhoA activity, which was altered in Rap2-deficient cells. By analysing GFP-paxillin dynamics in live-cell imaging with dedicated time periods (Fig.14A), we could get a clear statement on whether Rap2 plays a localized role at the leading edge or a more global role in controlling adhesion turnover across the entire cell surface.

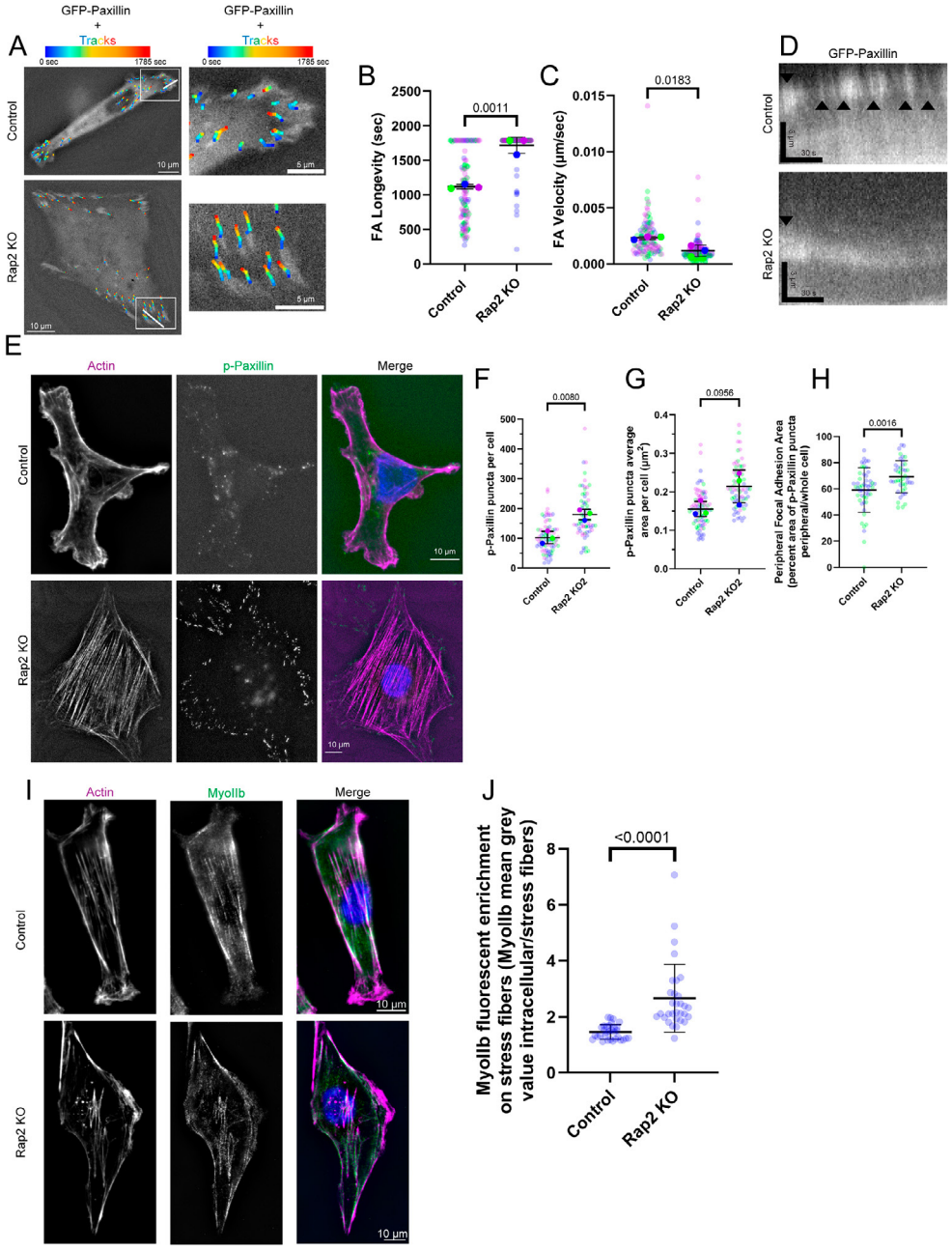


Fig. 14. *Rap2 functions regulating the focal adhesion formation and dynamics. From A–D, Time-lapse imaging of cells expressing GFP-paxillin, depicting the movement of focal adhesion puncta over time. Paxillin puncta lifetime and movement speed were quantified, and kymographs illustrate the appearance and turnover of new adhesions. (E–H) Control and Rap2*

KO were stained for phalloidin and phosphorylated-paxillin. The number, size, and distribution of paxillin-positive adhesions were quantified. (I–J) Control and Rap2-KO cells were stained for Myosin IIb to evaluate acto-myosin contractility. Quantification shows increased Myosin IIB expression accumulation on stress fibers in Rap2 KO.

Following this logic, we next experimented with fixed-cell analysis to evaluate endogenous focal adhesion organisation and acto-myosin contractility. If Rap2 loss increases the RhoA-driven adhesion maturation and contractile tension, then Rap2 KO cells should exhibit more numerous, larger, and more stable focal adhesions, accompanied by stronger recruitment of myosin II to stress fibers. We then performed Immunofluorescence staining with phospho-paxillin antibodies, which confirmed the predicted hypothesis that Rap2 KO cells displayed enlarged phospho-paxillin-positive adhesions or bright dot-like signals in Fig.14E, which were more peripheral and less dynamic than compared to those in control cell lines. These dynamic distributions suggested impaired turnover and reduced ability to translocate inward across the ventral surface, a hallmark of over-stabilized adhesions. Finally, increased enrichment of non-muscle myosin IIb along stress fibers indicated heightened acto-myosin contractility, consistent with elevated RhoA activity as illustrated in Fig.14 I & J. Together, these findings justify our investigation and support the conclusion that Rap2 is required not only for regulating leading-edge dynamics but also for maintaining appropriate focal adhesion turnover and contractile balance across the entire cell.

4.3.4. Rab40-CRL5 Ubiquitylation is essential for Rap2 activation, membrane targeting, and the migratory phenotypes

Given that Rab40-CRL5 mono-ubiquitylates Rap2, next it's important to determine how this modification regulates Rap2 function. To understand this, GFP Rap2a WT with a non-ubiquitylatable triple mutant (K117R/K148R/K150R; "K3R" mutation). Rescue experiments in Rap2 KO cells revealed that WT Rap2 restored the polarity back, lamellipodia formation, and focal adhesion organisation, whereas the K3R mutant failed to rescue any of these phenotypes (Fig.15A–F).

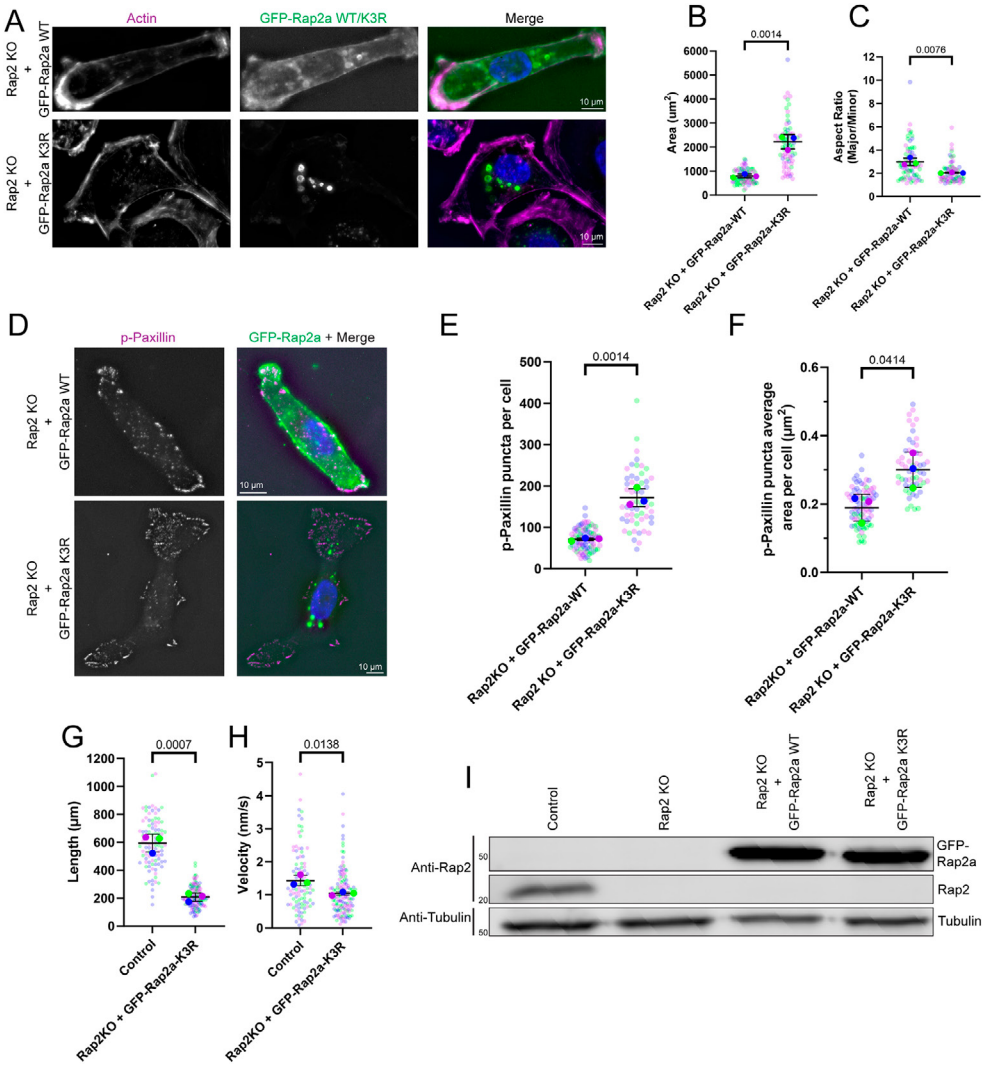


Fig.15*. Ubiquitylation is an absolute requirement for Rap2 functions during cell migration. (A–C) Rap2 KO cells were rescued with either GFP Rap2a WT or the non-ubiquitylatable GFP Rap2a K3R mutant and stained with phalloidin. Cell size and aspect ratio were measured to assess changes in cell shape. (D–F) Control and Rap2 KO cells were stained for phalloidin and phosphorylated paxillin to evaluate focal adhesion points. The number and size of paxillin-positive adhesions were quantified. (G–H) Shows random migration assays comparing Rap2 KO cells rescued with either Rap2a-WT or Rap2a-K3R. (I) Western blot confirming expression of GFP Rap2a constructs in rescue cell lines compared with endogenous Rap2 levels in control cells*

Understanding the importance of Rap2 ubiquitylation required us to first consider the unresolved question in our earlier findings: although Rap2 is clearly regulating lamellipodia dynamics, cell polarity, and focal adhesion turnover partly; we still did not know how Rap2 becomes properly activated or positioned at the plasma membrane to perform these functions. Our previous work confirms and suggests that Rab40-CRL5 ubiquitylation leads to Rap2 localization, but it was unclear whether this modification is truly required for the role of Rap2 in cell migration. This gap in knowledge created a logical need to find out whether ubiquitylation is a mechanistic requirement for Rap2's ability to restore polarity and cytoskeletal organisation.

To address this, we used Rap2 KO lines and reintroduced either WT Rap2, which can be ubiquitylated, or a K3R mutant that cannot undergo Rab40-CRL5-mediated ubiquitylation. By comparing these rescue conditions (Fig.15A–C), we could directly determine whether Rap2 ubiquitylation is necessary for establishing front-to-back polarity. Being consistent with this hypothesis, WT Rap2 lines restored polarity, but the K3R mutant did not, clearly indicating that ubiquitylation is a prerequisite for Rap2 to function correctly at the leading edge.

Building on this reasoning, we next asked whether Rap2 ubiquitylation is also required for its broader cytoskeletal interaction roles, specifically the regulation of focal adhesions and acto-myosin stress fibers. Since KO lines of Rap2 display remarkably stable focal adhesions and enhanced contractility, both indicative of elevated RhoA activity, rescue experiments allowed us to test whether WT Rap2, but not K3R, could reverse these phenotypes.

Indeed, Rap2 WT corrected the adhesion and stress-fiber abnormalities, whereas the K3R mutant failed to rescue any defects, clearly stating that ubiquitylation is essential for the function of Rap2 in adhesion turnover mechanisms. This led us to predict that Rap2 ubiquitylation would also be required for restoring migration behaviour. Random migration assays confirmed this: the K3R mutant behaved like a Rap2 KO line, whereas Rap2 WT restored normal migration (Fig.15 G & H). Although the K3R mutant was expressed at slightly lower levels, both constructs exceeded endogenous Rap2 expression, indicating that expression differences do not account for the failed rescue (Fig.15 G-I). All together, these experiments provide a stepwise demonstration that Rab40-CRL5-mediated ubiquitylation at K117, K148, and K150 is essential for Rap2 function, controlling its ability to establish polarity, regulate adhesions, control contractility, and support the forward migration. This necessity was crucial for a deeper investigation into how ubiquitylation mechanistically controls Rap2 activation and membrane targeting.

4.3.5. Discussion

This study reveals a novel and critical role for Rap2 GTPases in orchestrating breast cancer cell migration, with implications for understanding cytoskeletal regulation and spatial signaling during metastasis. Using CRISPR-mediated deletion of all three Rap2, a, b, and c, isoforms in MDA-MB-231 cells, we demonstrate that Rap2 is indispensable for lamellipodial persistence, front-to-rear polarity, and directional motility. Live-cell imaging revealed that Rap2 TKO cells are deficient in forming stable protrusions at the leading edge. Rather than maintaining broad and persistent lamellipodia as seen in control cells, Rap2-depleted cells exhibited fragmented and unstable extensions that retracted rapidly. This impairment in protrusion correlated with disrupted cellular polarity, evidenced by randomized Golgi orientation in migration assays. As polarity and sustained protrusions are key features of efficient mesenchymal motility, these findings highlight Rap2 as a central regulator of directional migration. The reduction in migration speed and directional persistence in Rap2-deficient cells further confirms that Rap2 coordinates essential structural features of cell motility.

A key mechanistic insight gained in this study is that mono-ubiquitylation of Rap2 is a prerequisite for its activation and function. This discovery challenges the conventional view of ubiquitylation solely as a signal for protein degradation or trafficking. By identifying lysines K117, K148, and K150 as modification sites, we establish that mono-ubiquitylation is required not only for membrane localization but also for GTP loading and interaction with GEFs such as EPAC1. The triple K→R mutant Rap2 K3R, which cannot undergo mono-ubiquitylation, failed to localize at the membrane and showed reduced activation even when membrane-tethered. These findings indicate that the mono-ubiquitylation event is not a passive localization signal but an active regulatory switch governing spatial Rap2 signaling.

The inability of Rap2 K3R to interact with EPAC1 further supports the notion that mono-ubiquitylation enhances GEF accessibility or binding affinity. This adds a novel layer of regulation to GTPase biology, suggesting that post-translational modifications can gate interactions with upstream activators. The data also imply that spatial activation of Rap2 at the leading edge relies not merely on local expression but on a post-translational licensing step, expanding our understanding of how small GTPases are differentially regulated in time and space. Downstream of Rap2, we identified suppression of RhoA as a key functional outcome. In the absence of Rap2, RhoA was hyperactivated, particularly at retracting edges of the lamellipodia. This hyperactivation was associated with excessive stress fiber formation and enlarged focal adhesions, both of which are hallmarks of increased contractility and reduced motility.

These changes phenocopy a migration-inhibited state where contractile forces dominate over protrusive activity, a condition unfavorable for forward movement. Restoration of Rap2 expression reversed RhoA hyperactivity and normalized focal adhesion turnover, whereas the non-ubiquitylatable K3R variant failed to do so. This demonstrates that Rap2 acts upstream of RhoA in maintaining the dynamic balance between protrusion and retraction required for efficient cell migration.

The functional consequences of these findings are significant in the context of front-to-rear polarity. Rap2 appears to act as a spatially restricted suppressor of RhoA signaling at the leading edge, thereby supporting persistent lamellipodia and directional migration. This complements previous work that has focused on Rap1's role in adhesion stabilization. Unlike Rap1, which enhances integrin activity and adhesion maturation, Rap2 fine-tunes actin protrusions and polarity. Together, these data support a model in which Rap1 and Rap2 execute parallel, but distinct tasks. Rap1 anchors the cell, and Rap2 steers its direction. This spatial division of labor adds complexity to Ras-family GTPase signaling during migration and suggests that therapeutic strategies may need to target these pathways with isoform-specific precision. Previously implicated in trafficking, Rab40-CRL5 is shown here to act as a direct upstream activator of GTPase signaling [62]. This places it at the intersection of trafficking and motility regulation, expanding its known biological functions. Rab40-CRL5-mediated ubiquitylation promotes Rap2's interaction with EPAC1, confirming its role not just in localization but in activation control.

Finally, the implications of this study extend into cancer therapeutics. The role of Rap2 in regulating migration and polarity links it to metastatic potential in breast cancer. By identifying mono-ubiquitylation as a regulatory checkpoint, we open the door to targeting this pathway pharmacologically. Inhibiting CRL5 or blocking Rap2 ubiquitylation may suppress cancer cell migration and invasion. Furthermore, since K117 ubiquitylation is conserved among Ras-family GTPases, this mechanism may represent a broader paradigm in spatial GTPase regulation. Thus, Rap2 is not just a molecular switch but a therapeutic target whose modulation can be disrupted in the metastatic cascade in invasive cancers.

4.4. Study III - Rap1-dependent regulation of cancer cell migration

The detailed results of Study III are a collaborative project with Andrew Neumann at Prekeris Lab, University of Colorado, USA, and published in a special journal in Micro publication Biology, "Rap1 activity and localization is regulated by Rab40-CRL5 facilitates mono-ubiquitylation," Published in

June 2025, doi.org/10.17912/micropub.biology.001629. This work reports a single core observation but doesn't extend into broader research.

Over the past decade, extensive work has revealed that cell migration is coordinated by tightly regulated signaling networks that control actin cytoskeleton remodeling, focal adhesion turnover, and front-to-rear polarity. While the core players such as RhoA, Rac1 [45], and Cdc42 have been extensively characterized, much less is known about how small GTPases of the Rap subfamily integrate spatial and temporal cues to fine-tune these processes through post-translational regulation [11-14].

Rap1 has been well established to promote integrin-mediated adhesion and stabilize focal EPAC1 EPAC1adhesions, contributing to the anchoring of the leading-edge during migration. In contrast, the closely related Rap2 isoforms Rap2a, Rap2b, and Rap2c share over about 90 % sequence similarity with Rap1 but have distinct cellular functions that remain poorly understood. While Rap1 enhances adhesion strength, Rap2 appears to coordinate dynamic membrane remodeling and actin cytoskeletal rearrangements, particularly at the lamellipodia and leading edge of migrating cells.

Here, we demonstrate that the E3 ubiquitin ligase complex Rab40-CRL5 mono-ubiquitylates Rap2 at three specific lysine residues (K117, K148, and K150). This modification is both necessary and sufficient for Rap2 activation and membrane targeting. Mechanistically, mono-ubiquitylation enhances Rap2 interaction with its guanine nucleotide exchange factor, GEF, EPAC1, facilitating conversion to the GTP-bound state and enabling its retention at the plasma membrane. In contrast, mutation of these residues, as the Rap2 K3R mutant, abolishes mono-ubiquitylation, leading to loss of membrane localization, reduced GTP loading, and impaired cell migration.

Functionally, Rap2 acts as a local suppressor of RhoA activity at the lamellipodia. By inhibiting RhoA-driven actomyosin contractility in specific subcellular regions, Rap2 prevents premature retraction of lamellipodia, thereby maintaining protrusive activity and promoting efficient forward movement. Loss of Rap2 results in excessive RhoA activity, stabilized focal adhesions, increased stress fiber formation, and severely impaired migration dynamics [121]. Together, these findings identify a mechanism that spatially regulates Rap2 activation and, consequently, breast cancer cell motility.

Importantly, this discovery also extends the known biological role of the Rab40-CRL5 complex beyond its previously defined function in vesicle trafficking. It demonstrates that Rab40-CRL5 directly influences cell migration signaling by controlling Rap2 activation through mono-ubiquitylation. This mechanistic insight represents a conceptual advancement in understanding how small GTPases achieve spatial precision in signaling during cell movement. From a translational perspective, these findings have significant

implications for breast cancer metastasis. Directional migration and invasion depend on coordinated control of cytoskeletal tension and adhesion dynamics. By defining mono-ubiquitylation as a molecular checkpoint for Rap2 activity, this work identifies a potential therapeutic target for modulating invasive behavior in aggressive breast cancer subtypes.

4.4.1. Rap1 is a substrate of the Rab40-CRL5 E3 ligase complex

To determine whether Rap1 GTPases are bona fide substrates of the Rab40-CRL5 E3 ligase complex, a series of in vitro binding assays and co-immunoprecipitation experiments was performed. (Fig.16A), sequence alignment across Rap1A, Rap1B, with 2 isoforms, and Rap2 isoforms revealed conserved lysine residues in their C-terminal hypervariable regions, highlighting potential ubiquitylation sites. (Fig.16B), GST pull-down assays using GST-Rap1B with FLAG-tagged Rab40B from HEK293T lysates confirmed that Rap1B physically interacts with Rab40B. Importantly, this binding was markedly diminished when Rap1B was mutated at conserved lysines (K117/159R), indicating that ubiquitylation is likely dependent on these residues (Fig.16D). The specificity of this interaction was further validated by quantification of FLAG-Rab40B binding to WT versus lysine mutant GST-Rap1B proteins, as visualized in (Fig.16C & E). The reduction in binding to the lysine mutants underscores the critical importance of these lysine residues for Rab40B interaction and potential ubiquitylation.

To validate these interactions in a cellular context, co-immunoprecipitation experiments were performed using tagged proteins, which indicated the distinct ubiquitylation banding patterns, such as bands #1 to #3 in Fig.16F, for Rap1B in the presence of Rab40B and Cullin5, further implicating the CRL5 complex in direct modification of Rap1. Interestingly, ubiquitylation was reduced in the lysine mutants and absent without Rab40B or Cullin5 complex, emphasizing the specificity and necessity of this E3 ligase complex for Rap1 modification.

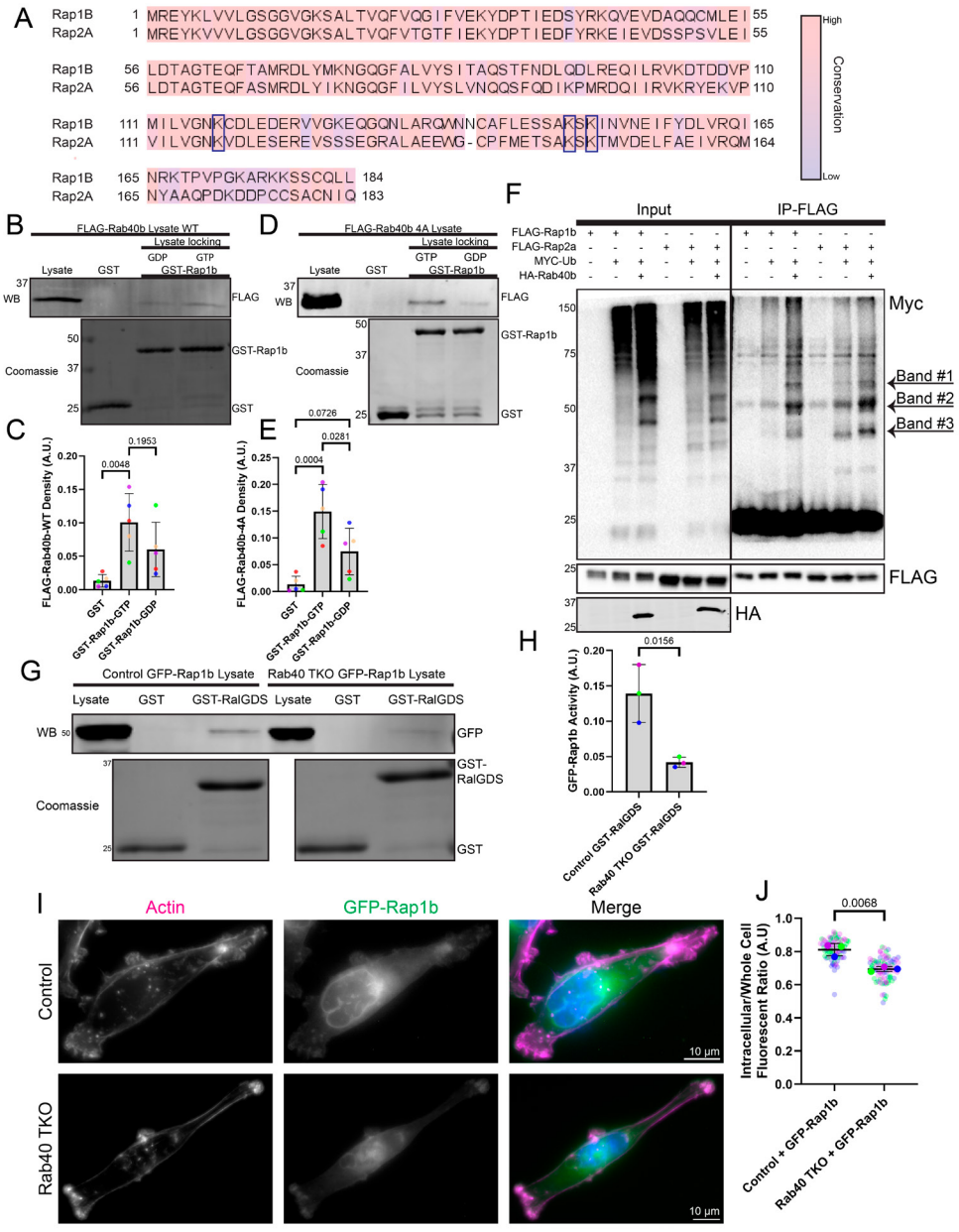


Fig. 16. *Rab40-CRL5targets Rap1 and its regulating mechanisms. (A) Alignment of Rap1b and Rap2a shows strong similarity in sequence conservation, including conserved lysine residues targeted by Rab40-CRL5. (B–E) Binding assays using GST Rap1b show that FLAG Rab40b WT binds Rap1b, whereas the Rab40b-4A mutant shows reduced binding. Representative blots and quantifications are shown.*

(F) Ubiquitylation assays in HEK293T cells show that Rab40b promotes ubiquitylation of both Rap1b and Rap2a. (G–H) GST-RalGDS pulldowns were used to assess Rap1b activity in control and Rab40 TKO cells. Loss of Rab40 reduces Rap1b activation. (I–J) Control and Rab40-TKO cells expressing GFP-Rap1b were stained with phalloidin to examine Rap1b localization. Quantification shows reduced membrane localization in Rab40-TKO cells.

4.4.2. Mono-Ubiquitylation regulates the Rap1 localization

Following the biochemical evidence for Rap1 ubiquitylation, functional assays were conducted to determine whether this modification influenced Rap1 localization. GST-RALGDS pull-down experiments were performed (Fig.16G), it reveals a significant reduction in GTP-bound Rap1B in Rab40 TKO cells compared to controls, confirming that the loss of Rab40 impairs Rap1B activation. Quantification in (Fig.16F) second verified this reduction, ultimately supporting the notion behind this, that ubiquitylation by Rab40 is necessary for proper Rap1 signaling and functioning.

Immunofluorescence analysis from fixed imaging provided further mechanistic insights into the functional consequences of this ubiquitylation. In control MDA-MB-231 cells expressing GFP-Rap1B displayed distinct focal adhesion-like puncta, co-localizing with actin-rich protrusions (Fig.16I). Conversely, Rab40 TKO cells exhibited diffused cytoplasmic GFP-Rap1B expression, with fewer discrete focal adhesion-associated signals, and distant or fewer signals. The merged images clearly showed a lack of membrane-associated Rap1B in the absence of Rab40, suggesting that mono-ubiquitylation is essential for spatial targeting of Rap1B to sites of dynamic adhesion. Quantitative analysis in (Fig.16J) supported this observation, showing a significant reduction in the number of intracellular adhesion contacts per cell in the Rab40 TKO background. Collectively, these data demonstrate that mono-ubiquitylation of Rap1B governs its membrane retention and localization to focal adhesion sites, which are crucial for downstream adhesion dynamics.

4.4.3. Ubiquitylation promotes Rap1 activation and focal adhesion dynamics

The observed shift in Rap1B localization was paralleled by a substantial reduction in its activation status, as shown in the biochemical assays above. This provides compelling evidence that ubiquitylation is not merely a structural modification but a prerequisite for GTP loading and activation.

The pull-down of active GTP-bound Rap1B using the RALGDS domain, as shown in Fig.16G–H, underscores this regulatory mechanism, confirming that Rab40-CRL5-dependent ubiquitylation directly facilitates activation.

Functionally, this loss of Rap1 activation impairs the ability of the cells to establish and maintain focal adhesions. Imaging of focal adhesion dynamics in Fig.16I section demonstrated that in the absence of Rab40, Rap1B fails to be present at leading-edge adhesion zones. As focal adhesion assembly and disassembly are central to directed migration, the spatial mislocalization of Rap1B likely leads to the observed reduction in intracellular adhesion points, thereby compromising coordinated migration. Thus, mono-ubiquitylation of Rap1B serves as a dual-function regulatory mechanism: it promotes its interaction with membranes and focal adhesion components and enables GTP-loading necessary for downstream signaling. These findings extend the role of ubiquitylation beyond protein turnover, positioning it as a signaling determinant critical for Rap1 function.

4.4.4. Rap1 Mono-Ubiquitylation is required for directional migration

Directional cell migration requires a pronounced front-to-back polarity, established by distinct leading and lagging edges formed through tightly regulated actin remodeling. Small GTPases orchestrate this polarization, and among them, the Rap subfamily (where Rap1 has Rap1a, Rap1b and Rap2a/b/c) has emerged as key modulators of migration. In particular, Rap1 is known to regulate integrin trafficking during migration, contributing to the formation and turnover of cell adhesions that drive persistent movement. We hypothesized that post-translational mono-ubiquitylation of Rap1 is crucial for these functions, ensuring that Rap1 is activated and localized in a manner that promotes sustained directional migration. Consistent with this idea, our results demonstrate that Rab40-CRL5-dependent mono-ubiquitylation of Rap1b is indispensable for full Rap1 activation and proper spatial distribution, which in turn underlies the cell's ability to migrate directionally.

Experiments using GST-RalGDS pull-down assays revealed that loss of Rap1 ubiquitylation severely impairs Rap1b activation. In Rab40 TKO cells, where it is lacking the Rab40a/b/c E3 ligase complex responsible for Rap1 ubiquitylation, the amount of GTP-bound Rap1b that bound to RalGDS was significantly reduced compared to control lines (Fig.16H). This indicates that mono-ubiquitylation is required for Rap1b to attain its active GTP-bound state, a precondition for engaging its downstream effectors. In the context of migration, an inability to activate Rap1 would disrupt its known role in promoting integrin recycling and adhesion turnover, thereby compromising

the cell's directional persistence. In addition to reduced activity, we observed marked changes in Rap1b localization when ubiquitylation was revoked.

Under normal conditions, GFP-Rap1b localizes strongly to the perinuclear region, notably the nuclear envelope, and is also detectable at the plasma membrane within leading-edge ruffles. Strikingly, Rab40 TKO cells showed an inversion of this distribution: Rap1b was depleted from the nuclear envelope and instead abnormally accumulated at dynamic ruffling edges, as quantified and shown in Fig.16I & J. This mislocalization was unexpected and suggests that, unlike Rap2, which requires ubiquitylation to remain at the plasma membrane lamellipodia, Rap1b without ubiquitylation becomes trapped at the cell periphery. The ectopic enrichment of Rap1 at the leading edge may disturb the balanced spatial signaling needed for polarity. Indeed, Rap1 and Rap2 are thought to have antagonistic roles in regulating protrusive structures of a cell, and their proper segregation depends on ubiquitylation-dependent targeting mechanisms. Thus, in cells lacking Rap1 mono-ubiquitylation, the normal partitioning of Rap1 and Rap2 signaling is likely to be lost, leading to disorganized lamellipodia dynamics and impaired directional persistence.

In summary, our findings establish that mono-ubiquitylation of Rap1 is a critical requirement for efficient directed migration. By promoting Rap1 activation and by restricting Rap1 to appropriate subcellular locations, while preventing its aberrant accumulation at the leading edge, Rab40-CRL5-mediated ubiquitylation enables Rap1 to execute its functions in contexts with Rap2 and other regulators. Cells in which Rap1 is not ubiquitylated fail to maintain a stable front-back polarity, presumably due to defective adhesive cycling and perturbed lamellipodial signaling, ultimately resulting in a loss of directional migratory phenotypic behaviours. These results reveal a novel mechanistic link between a specific ubiquitin modification and the persistence of cell movement, underscoring that mono-ubiquitylation of Rap1 is indispensable for maintaining the polarized cytoskeletal and adhesion dynamics that drive directional migration.

4.4.5. Discussion

The present study identifies Rap1b as a bona fide substrate of the Rab40-CRL5 E3 ubiquitin ligase complex and demonstrates that mono-ubiquitylation is required for Rap1 activation, membrane targeting, and directional migration in breast cancer cells. Together with findings from Study II on Rap2, these data establish mono-ubiquitylation as a shared regulatory mechanism controlling spatial activation of Rap GTPases. Unlike degradative ubiquitin signaling, this modification acts as a non-proteolytic signaling determinant that fine-tunes adhesion dynamics and polarity. Comparison with Previous Literature

Rap1 has long been recognized as a key regulator of integrin-mediated adhesion and cell migration [15,17]. Several studies demonstrated that Rap1 enhances integrin affinity and focal adhesion stability, thereby promoting cell attachment and persistent migration [11,108,109]. However, these studies primarily focused on GEF/GAP regulation and membrane recruitment via lipid modifications.

In contrast, ubiquitin-dependent regulation of Rap proteins has remained largely unexplored. While mono-ubiquitylation has been shown to regulate small GTPases such as Ras and Rho family members [95,104], its role in Rap1 spatial activation has not previously been established. Our findings overlap with the broader literature in demonstrating that Rap1 activity is tightly linked to its subcellular localization. However, we extend this concept by identifying mono-ubiquitylation as a prerequisite for proper localization and GTP loading. When we talk about similarities, there are several reports that suggest the ubiquitin modifications influence membrane trafficking or degradation of signaling. However, in contrast to canonical degradative ubiquitination, we observe a non-proteolytic function for mono-ubiquitylation.

Our data reveal that the absence of mono-ubiquitylation causes aberrant Rap1 redistribution to leading-edge regions, disturbing polarity. This contrasts with earlier models, where Rap1 loss generally reduced adhesion strength without altering spatial partitioning. Why might these findings differ? Because many previous studies used fibroblasts or immune cells, whereas our system employs aggressive breast cancer cells, MDA-MB-231, which exhibit high migratory plasticity. In terms of genetic context, the Rab40 TKO model introduces the regulatory aspects that were absent in earlier work. Spatiotemporal quantification, such as live-cell imaging, provides finer subcellular insights than the bulk adhesion molecular assays. Interestingly, while Rap2 mono-ubiquitylation promotes retention at lamellipodia (Study II), Rap1 mono-ubiquitylation appears to prevent aberrant leading-edge accumulation. This suggests isoform-specific effector engagement. Rap1 preferentially interacts with integrin and adhesion complexes, whereas Rap2 modulates RhoA activity and actin dynamics. Thus, mono-ubiquitylation does not simply “activate” Rap proteins; it imposes spatial constraints that preserve front-to-back polarity.

The importance and the novelty of the Rap1b protein, which provides an insight and first demonstration that it is directly mono-ubiquitylated by the Rab40-CRL5, and its mono-ubiquitylation regulates the Rap1 activation in a non-degradable manner. By integrating ubiquitin signaling with small GTPase spatial control, this work advances the conceptual framework of how directional migration is maintained in metastatic breast cancer. Despite its strengths, this study has several limitations: Cell line specificity; findings are

derived primarily from MDA-MB-231 cells. While widely used, this model represents triple-negative breast cancer and may not reflect luminal subtypes. The functional consequences of Rap1 mono-ubiquitylation were not validated in animal metastasis models. Although localization was assessed by imaging, real-time dynamics of ubiquitylation cycling were not measured, and these results will give the temporal resolution.

CONCLUSIONS

1. AMBRA1 regulates the focal adhesion dynamics through Rab40-CRL5 and E3 ligase complex, and its loss leads to disrupted front-to-rear polarity of breast cancer cells. In addition, AMBRA1 regulates genes involved in adhesion, cytoskeletal remodeling, and motility, demonstrating that AMBRA1 functions as a dual regulator in breast cancer cells.
2. Rap2 regulation via Rab40-CRL5-mediated mono-ubiquitylation is crucial for Rap2 membrane localization. Disruption of ubiquitination promotes inactive Rap2 formation, increases RhoA-dependent cell contraction, stabilizes focal adhesion, and reduces migration. Without Rap2, cells show excessive retraction with strong stress fibres, and are unable to maintain the lamellipodial persistence, suggesting that ubiquitinated Rap2 functions as a RhoA inhibitor, required for coordinated cytoskeletal reorganization.
3. Rab40-CRL5-mediated mono-ubiquitylation regulates Rap1 activity and localization. Loss of ubiquitylation inactivates Rap1 and prevents its accumulation at the nuclear envelope and plasma membrane, which is necessary for the regulation of cell migration.

SANTRAUKA

ĮVADAS

Remiantis Pasaulio sveikatos organizacijos duomenimis krūties navikai išlieka dažniausiai diagnozuojama moterų onkologinė liga pasaulyje bei viena pagrindinių su piktybiniais navikais susijusių moterų mirčių priežasčių [1]. Nepaisant ankstyvos diagnostikos ir pažangos gydymo srityse, metastazavusių krūties navikų ląstelių plitimas į kitus organus išlieka viena didžiausių onkologinių problemų, lemiančių pacientų mirtingumą [2,3]. Metastazavimo procesas yra glaudžiai susijęs su ląstelių migracija, kuri atlieka svarbų vaidmenį tiek homeostazėje, tiek onkologinių ligų progresavime [4,5]. Navikui vystantis sutrinka ląstelių migracijos valdymas, o tai leidžia naviko ląstelėms atsiskirti nuo pirminio naviko, patekti į aplinkinius audinius ir kraujagyslėmis pasiekti nutolusius organus [6]. Siekiant sukurti veiksmingas gydymo strategijas, kurios užkirstų kelią metastazėms, būtina išsiaiškinti ląstelių migraciją reguliuojančius molekulinius mechanizmus.

Ląstelėms judant kinta citoskeleto struktūra – formuojamas dinamiškas struktūrinis karkasas, leidžiantis ląstelėms keisti formą ir kryptingai judėti [7]. Citoskeleto persitvarkymo metu ląstelės judėjimo kryptimi formuojasi lamelipodijos bei filopodijos. Šis mechanizmas glaudžiai susijęs su židininių sukibimo vietų (angl. *focal adhesions,* FA) formavimu ir išardymu, o šių procesų metu susidarančios traukos jėgos lemia ląstelės judėjimą ir kryptingumą. Bet koks šių griežtai reguliuojamų dinaminių mechanizmų sutrikdymas gali paskatinti naviko metastazavimą [8,9].

Nors literatūroje gausu tyrimų, kuriuose nagrinėjami už ląstelių migraciją atsakingi baltymai, vis dar trūksta duomenų, paaiškinančių, kaip erdvėje ir laike koordinuojami viduląstelinio citoskeleto persitvarkymo ir adhezijos procesai. Mažųjų GTPazių superšeima, apimanti Ras baltymų šeimą, atlieka svarbų vaidmenį reguliuojant signalinius kelius, susijusius su citoskeleto dinamika ir ląstelių migracija [10]. Ras baltymai veikia kaip molekuliniai jungikliai. Aktyvioje būsenoje Ras, susijungęs su GTP, skatina citoskeleto persitvarkymą, būtiną ląstelės judėjimui. Tuo tarpu su GDP susijungęs Ras pereina į neaktyvią būseną ir jo sąveika su migraciją skatinančiais baltymais nutrūksta.

Ląstelių migracijos tyrimuose ypatingas dėmesys taip pat skiriamas Rap pošeimio baltymams Rap1 ir Rap2 [11,12]. Šie baltymai stiprina ryšį su integrinais, tokiu būdu skatindami FA formavimąsi ir kryptingą ląstelių judėjimą [13-15]. Mūsų grupės tyrimų rezultatai tai pat parodė, kad Rap2 reguliuoja lamelipodijų formavimąsi ir ląstelių poliškumą – procesus būtinus migracijai [16]. Nors Rap1 ir Rap2 turi maždaug 60 proc. funkcinių panašumų, jų ryšys

su kitais mažų GTPazių valdomais signaliniais keliais, dalyvaujančiais ląstelių migracijoje, lieka neaiškus bei reikalauja tolimesnių tyrimų [17,18]. Be to, svarbūs migracijos moduliatoriai yra ir Rab GTPazės, kurios atsakingos už membraninės pernašos reguliavimą. Rab GTPazės reguliuoja transportinių pūslelių judėjimą ląstelių viduje, taip skatindamos sukibimo su substratu molekulių, tokių kaip integrinai, pernašą [19].

Literatūros duomenimis AMBRA1 baltymas yra svarbus onkogenezės reguliatorius [20-22]. Naujausi duomenys rodo, kad AMBRA1 ne tik dalyvauja ląstelės ciklo reguliavime bei autofagijoje, bet veikia ir svarbių už migraciją atsakingų genų kompleksų, tokių kaip ANGPT1, TGF ir ITG, transkripciją [23,24].

Šioje disertacijoje buvo tiriamos Rap1, Rap2 ir AMBRA1 baltymų molekulinės sąveikos, jų įtaką krūties naviko ląstelių judėjimui bei šių baltymų ryšys su GTPazėmis.

Darbo tikslas

Šio darbo tikslas yra ištirti, kaip AMBRA1, Rap2 ir Rap1 baltymai reguliuoja krūties naviko ląstelių migraciją, koordinuodami citoskeleto dinamiką, židininių sukibimo vietų persitvarkymą ir GTPazės signalo perdavimą.

Darbo uždaviniai

1. Ištirti AMBRA1 baltymo vaidmenį, reguliuojant krūties naviko ląstelių židininio sukibimo vietas ir migraciją bei jo sąveiką su Rab40-CLR5 kompleksu.
2. Nustatyti, kaip Rab40-CRL5 komplekso tarpininkaujamas monoubikvitinimas kontroliuoja Rap2 lokalizaciją ir lamelipodijų padėtį bei įvertinti RhoA įtaką Rap2 aktyvumui.
3. Nustatyti monoubikvitinimo poveikį Rap1 lokalizacijai ir aktyvumui bei Rap1 vaidmenį, reguliuojant krūties naviko ląstelių židininį sukibimą ir migraciją.

Darbo mokslinis naujumas ir aktualumas

Nors mažos GTPazės ir ubikvitino ligazės yra gerai žinomi ląstelių migracijos reguliatoriai, tačiau vis dar nėra aišku, kaip monoubikvitinimas erdviškai kontroliuoja GTPazių aktyvumą ir FA dinamiką. Šiame darbe pirmą kartą ištyrėme kaip nuo Rab40-CRL5 priklausomas ubikvitinimas reguliuoja genų transkripciją, FA dinamiką ir citoskeleto persitvarkymą krūties naviko ląstelių migracijos metu. Taip pat nustatėme, kad AMBRA1, kuris buvo žinomas kaip svarbus autofagijos reguliatorius, yra ir Rab40c substratas. Nustatėme,

kad Rab40-CRL5 tarpininkaujamas monoubikvitinimas kontroliuoja Rap2 lokalizaciją lamelipodijose ir nuo RhoA priklausomą jų susitraukimą. Nuo Rab40-CRL5 priklausomas monoubikvitinimas reguliuoja Rap1 aktyvinimą ir lokalizaciją, taip kontroliuodamas FA dinamiką krūties naviko ląstelėse kryptingos migracijos metu.

Darbo struktūra

Šią disertaciją sudaro trys dalys – I, II ir III studijos (1.pav.).

I-ojoje studijoje nagrinėjome, kaip AMBRA1 reguliuoja ląstelių migraciją, veikdamas židininių sukibimo vietų apykaitą ir transkripcinius tinklus. Taip pat ištyrėme AMBRA1 sąveiką su signaliniais komponentais, veikiančiais citoskeleto dinamiką.

II-ojoje studijoje nustatėme, kaip Rab40-CRL5 sukeltas monoubikvitinimas reguliuoja Rap2 aktyvaciją ir lokalizaciją membranoje. Parodėme, kad lamelipodijų susitraukimo metu Rap2 sąveikauja su RhoA. Tai atskleidė naują, ląstelių migracijai būtiną, potransliacinį mechanizmą.

III-ojoje studijoje nagrinėjome Rap1 GTPazės vaidmenį krūties navikinių ląstelių migracijoje.

Doktorantės indėlis

I- studija. Prisidėjo prie tyrimo metodologijų kūrimo, eksperimentų planų kūrimo ir tyrimo koncepcijos. Atliko vienos ląstelės migracijos tyrimus LSMU Ląstelių kultūrų laboratorijoje. Dalyvavo ubikvitinimo ir baltymų validacijos eksperimentuose.

II-studija. Atliko vienos ląstelės atsitiktinės migracijos tyrimus, kortaktino, miozino IIB bei fosfopaksilino imunofluorescencinį dažymą, taip pat atliko GFP-paksilinu transfekuotų kontrolinių ląstelių ir ląstelių, kuriose išsveiklintas Rap2, vaizdinimą. Prisidėjo prie bendro eksperimentų planavimo ir optimizavimo, aktyviai dalyvavo darbo eigoje Kolorado universitete (JAV) bei LSMU Ląstelių kultūrų laboratorijoje

III-studija. Prisidėjo prie Rab40-CRL5 ir Rap1 baltymų sąveikos įrodymo Western blot metodu bei imunofluorescencinio dažymo. Dalyvavo eksperimentų planavime. Visi eksperimentai buvo atlikti Kolorado universitete (JAV).

MEDŽIAGOS IR METODAI

Ląstelių kultūros

MDA-MB-231 (ATCC) ląstelės buvo kultivuojamos DMEM (DMEM, pagal angl. *Dulbecco's Modified Eagle Medium*) terpėje (Corning, Mediatech), papildytoje 10 proc. fetaliniu veršelio serumu (angl. *fetal bovine serum*, toliau FBS), 1 proc. penicilino-streptomicino tirpalu (P/S) (Corning), 1 proc. neesminių aminorūgščių (Gibco), 1 μg/ml insulino (Gibco), ir 2 mM Lglutamino (Corning). Ląstelės buvo kultivuojamos 37 °C temperatūros inkubatoriuje su 5 proc. CO_2. Plazmidinių konstruktų dauginimui dėl didelio transfekcijos efektyvumo buvo naudojamos HEK293T (ATCC) ląstelės, kultivuotos DMEM su 4,5 g/l gliukozės, 5,84 g/l L-glutamino (Corning), 1 proc. P/S ir 10 proc, 1 proc. natrio piruvato (Gibco), FBS (FBS; Phoenix Scientific). Siekiant išlaikyti nuoseklias augimo sąlygas, ląstelės buvo persėjamos pasiekus 70–80 proc. konfluentiškumą, atlipimui buvo naudotas 0,05 proc. tripsino-EDTA tirpalas.

CRISPR/Cas9 ląstelių linijų su išveiklintais genais konstravimas

CRISPR/Cas9 genų redagavimas buvo naudojamas kuriant ląstelių linijas su išveiklintais genais (ang. *knock-out*, toliau KO): AMBRA1-KO, Rap2-KO (išveiklintos izoformos a/b/c), Rap40-TKO (išveiklintos izoformos a/b/c; angl. *triple knock-out*, TKO). MDA-MB-231 ląstelės, stabiliai ekspresuojančios tetraciklino indukuojamą Cas9, buvo auginamos iki ~70 proc. padengimo, tuomet ~ 24 val. apdorojamos 1 μg/ml doksiciklino tirpalu, siekiant indukuoti Cas9 raišką ląstelėse. Vėliau ląstelės buvo transfekuotos CRISPR RNR ir transaktyvuojančiu CRISPR RNR mišiniu, naudojant *DharmaFECT Duo Transfection* reagentą (Horizon discovery), pagal gamintojo protokolą. Ląstelių atrankai naudotas puromicinas (5μg/ml), o išveiklinimo efektyvumas buvo patikrintas baltymų imunoblotingu.

Transfekcija

MDA-MB-231 ląstelių transfekcijai atlikti buvo naudojamas jetPRIME (Polyplus) reagentas pagal gamintojo rekomendacijas, o HEK293T ląstelės buvo transfekuojamos naudojant Lipofectamine RNAiMAX arba standartinį kalcio fosfato nusodinimo metodą. Transfekcijos efektyvumas buvo patikrintas fluorescencine mikroskopija ir imunoblotingu. siRNR transfekcijos HEK293T ir MDA-MB-231 ląstelėse buvo atliktos naudojant Lipofectamine RNAiMAX (Invitrogen).

Imunoprecipitacija ir baltymų imunoblotingas

Ląstelės buvo auginamos 100 mm Petri lėkštelėse, lizuojamos ant ledo, naudojant buferį, papildytą proteazių inhibitoriais, 20 mM Tris-HCl, pH 7,4, 150 mM NaCl, 2 mM EDTA, 1 proc. Triton X-100 ir 10 proc. glicerolio. Lizatai buvo centrifuguojami ir inkubuojami su atitinkamu antikūnu arba kontroliniu imunoglobulinu G (toliau IgG), pvz., pelių IgG (Sigma), triušių IgG (Sigma) maždaug 4 valandas, 4 °C temperatūroje, tada buvo pridedama G-baltymo dalelių. Toliau, G-baltymo dalalės (Cytiva) buvo centrifuguotos ir tris kartus nuplautos lizės buferiu, kuriame buvo 0,5 M NaCl, o surišti baltymai buvo atskirti 1xSDS buferiu. Denatūruojančiai imunoprecipitacijai ląstelės buvo lizuojamos 1 proc. SDS, kaitinamos 95 °C temperatūroje, praskiedžiamos iki 0,1 proc. SDS, imunoprecipituojamos naudojant 5 µg antikūno prieš baltymų žymėjimo oktapeptidą (aminorūgščių seka DYKDDDDK) FLAG ir toliau analizuojamos panaudojant elektoforezę ir baltymų imunoblotingą, vizualizavimui buvo naudota ChemiDoc MP sistema (Bio-Rad).

Baltymų validavimui ląstelės buvo lizuojamos ant ledo HEPES buferyje, centrifuguotos 15 000 g greičiu 4 °C temperatūroje, o baltymų koncentracija buvo nustatyta naudojant Bradfordo testą. Lizatų mėginiai buvo paruošti naudojant 5× SDS dažus, kaitinti 5 minutes 95 °C temperatūroje, atskirti SDS-PAGE metodu, geliai buvo perkelti ant PVDF membranų, jos buvo inkubuotos pirminiais ir po to fluorescenciniais antriniais antikūnais, membranos vaizdinamos naudojant Li-Cor Odyssey CLx sistemą (LI-COR Biosciences).

Ubikvitinimo tyrimai

HEK293T ląstelės buvo kotransfekuotos FLAG, Myc (aminorūgščių seka EQKLISEEDL) ir HA (aminorūgščių seka YPYDVPDYA) vektoriais (pvz., FLAG-Rap2a, FLAG-Rap1b, FLAG-AMBRA1, Myc-Rab40b, HA-ubikvitinu). Po 24 valandų ląstelės buvo lizuojamos denatūruojančiomis sąlygomis, imunoprecipituojamos su anti-FLAG antikūnu ir G-baltymo dalelėmis, ir analizuojamos SDS-PAGE ir baltymų imunoblotingo metodu, signalai buvo matuojami naudojant ChemiDoc MP Imaging sistemą (Bio-Rad).

Imunofluorescencinė mikroskopija ir vaizdų analizė

Tyrimams ląstelės buvo sėjamos ant objektinių stikliukų, padengtų kolagenu. Ląstelės buvo fiksuojamos 4 proc. paraformaldehidu, permeabilizuojamos 1 proc. Triton X-100 ir blokuojamos 1 proc. galvijų serumo albuminu. Ląstelės buvo inkubuojamos su pirminiais antikūnai prieš žaliai fluorescuojantį baltymą (angl. *green fluorescent protein,* toliau GFP, antikūnai anti-GFP), FLAG, HA ir paksiliną, po to inkubuojamos su antriniais antikūnais

su fluorescentinėmis žymėmis. Faloidinas buvo naudojamas F-aktino vizualizavimui, o DAPI arba Hoechst dažai naudoti ląstelių branduolių žymėjimui. Visos fiksuotos ląstelės buvo vaizdinamos invertuotu Zeiss Axiovert 200M mikroskopu su QE krūvio sąsajos kamera (Sensicam), naudojant 63× alyvinį objektyvą. Mėginių analizė buvo atlikta naudojant Fiji/ImageJ programinę įrangą (Version 1,54f).

Židininių sukibimo vietų analizė

FA dinaminei analizei atlikti kontrolinių ir Rap2KO ląstelių transfekcija GFP-paksilinu buvo atlikta naudojant lipofektaminą pagal gamintojo instrukcijas. Gyvos ląstelės buvo vaizdinamos 37 °C temperatūroje, esant 5 proc. CO_2 koncentracijai. Ilgalaikio registravimo metu vaizdai buvo fiksuojami kas 30–60 sekundžių iki 30 minučių kiekvienam nepriklausomam pakartojimui. FA dinamika kiekybiškai įvertinta naudojant ImageJ programos papildininius Fiji ir TrackMate, siekiant išmatuoti FA gyvavimo trukmę, formavimosi ir degradacijos dažnį bei greitį. Fiksuotų ląstelių analizei ląstelės buvo auginamos ant kolagenu dengtų objektinių stikliukų, tada užfiksuotos ir dažytos antikūnais prieš paksiliną ar fosfopaksiliną. FA struktūrų skaičius, ilgis, kraštinių santykis, fluorescencijos intensyvumas ir periferinis bei centrinis pasiskirstymas buvo įvertinti kiekybiškai naudojant Fiji/ImageJ programą.

Ląstelių kraštų dinamikos straboskopinė analizė

Ląstelių kraštų dinamikos straboskopinė analizė buvo atlikta siekiant kiekybiškai įvertinti membranos raukšlių ir lamelipodijų susitraukimų dinamiką kontrolinėse ir Rap2 KO ląstelėse. Ląstelės buvo auginamos ant kolagenu dengtų plonadugnių lėkštelių ir vaizdinamos naudojant DIC su ×64 objektyvu. Vaizdų kimografai buvo sugeneruoti naudojant Fiji/ImageJ programą.

RNR sekoskaita

RNR sekoskaita buvo atlikta naudojant bendrą RNR, išskirtą iš MDA-MB-231 AMBRA1-WT ir AMBRA1-KO ląstelių naudojant TRIzol reagentą. Bibliotekos buvo paruoštos naudojant KAPA mRNA HyperPrep (Roche) rinkinį su polyA atranka, pradedant nuo 50 ng bendros RNR. Paruošimo metu RNR buvo fragmentuota, po to atlikta komplementarios DNR sintezė, adapterių prijungimas ir bibliotekų amplifikacija naudojant PGR (13 ciklų). Paruoštos bibliotekos buvo sekvenuojamos Illumina platformoje, generuojant porinių skaitymų sekas. Gauti FASTQ failai buvo apdoroti atliekant kokybės filtravimą ir trumpinimą, po to sekos suderintos su žmogaus etaloniniu genomu (hg38) ir kiekybiškai įvertintos. Genų raiškos skirtumai tarp WT ir

KO ląstelių buvo nustatyti naudojant FDR ribą < 0,05 ir nustatytus kartotinio pokyčio slenksčius. Bibliotekos buvo sujungtos ir sekvenuotos naudojant NovaSeq 6000 platformą (Illumina). Toliau buvo atlikta genų ontologijos ir signalinių kelių praturtinimo analizė kiekvienam biologiniam pakartojimui. Rezultatai buvo vizualizuoti naudojant sklaidos diagrama ir spalvų intensyvumo žemėlapius.

Ląstelių migracijos tyrimas

Siekiant įvertinti atskirų ląstelių judėjimą AMBRA1-KO, Rap2-KO, Rab40a-KO, Rab40b-KO, Rab40c-KO, Rab40-TKO ir Rab40abc-KO MDA-MB-231 ląstelėse buvo atlikti vienos ląstelės atsitiktinės migracijos tyrimai. Ląstelės buvo užsėtos $1,5 \times 10^4$ ląstelių/cm^2 ant fibronektinu (Sigma-Aldrich) dengtų plonadugnių lėkštelių. Ilgalaikio registravimo tyrimai buvo atlikti naudojant Olympus IX83 invertuotą mikroskopą su inkubatoriumi, palaikančiu 37 °C temperatūrą ir 5 proc. CO_2 koncentraciją. Vaizdai buvo registruojami automatiškai 12 valandų kas 20 minučių. Vienos ląstelės judėjimo trajektorija buvo analizuota naudojant Manual Tracking Excellence Pro programinę įrangą, apskaičiuojant ląstelės nueitą atstumą, greitį ir kryptingumą. Kiekvienomis sąlygomis buvo atlikti mažiausiai trys nepriklausomi biologiniai pakartojimai.

Tėkmės citometrija

Ląstelės buvo auginamos iki 70–80 proc. padengimo, tada surinktos centrifūgavimo būdu, fiksuotos šaltame 70 proc. etanolyje ir pažymėtos propidžio jodido dažu. Ląstelių ciklo fazių analizė buvo atlikta su (Beckman Coulter Gallios flow cytometry) citometru, o duomenys apdoroti naudojant ModFit LT programinę įrangą (Verity software house).

Baltymų-baltymų sąveikos analizė

Baltymų-baltymų sąveikos analizė buvo atlikta siekiant nustatyti Rap2 ir ubikvitino sąsają *in situ* MDA-MB-231 ląstelėse, ekspresuojančiose GFP-Rap2a-WT arba GFP žymėtą neubikvitinamą Rap2a mutantą (K117R/K148R/K150R, toliau tekste GFP-Rap2a-K3R). Ląstelės buvo fiksuotos, permeabilizuotos, apdorotos naudojant Duolink PLA (Sigma Aldrich) rinkinį pagal gamintojo instrukcijas ir tada nudažytos antikūnais prieš GFP ir ubikvitiną. Z-plokštumų vaizdai buvo apdoroti Fiji/ImageJ programa.

Glutationo S-transferazės surišimo analizė

Glutationo S-transferazės (toliau GST) surišimo tyrimai buvo atlikti siekiant įvertinti baltymų-baltymų sąveiką tarp Rab40b ir Rap1b. Ląstelių, ekspresuojančių FLAG-Rab40b-WT arba 4A, lizatai buvo inkubuojami su GST arba GST-Rap1b nukleotidų užkrovimo sąlygomis. Kompleksams izoliuoti buvo naudojamos glutationo dalalės. Surišti baltymai buvo analizuojami SDS-PAGE ir baltymų imunoblotingo metodu. GST baltymų analizė buvo atlikta dažant Coomassie, o pavyzdžiai vaizdinami Li-Cor Odyssey CLx.

Statistinė analizė

Statistinė duomenų analizė buvo atlikta naudojant GraphPad Prism, version 10.4.1 (532) programinę įrangą. Visi duomenys atspindi mažiausiai tris nepriklausomus biologinius pakartojimus. Statistinė analizė buvo atlikta naudojant dvipusį Stjudento t-testą. Trijų ar daugiau grupių atveju buvo taikoma ANOVA analizė. Duomenys buvo laikomi statistiškai reikšmingais, kai $p \leq 0,05$. Jei nenurodyta kitaip, grafikuose paklaidos pavaizduotos standartiniu nuokrypiu.

REZULTATAI IR JŲ APTARIMAS

AMBRA1 yra Rab40-CRL5 komplekso substratas

Ankstesni tyrimai parodė, kad Rab40 šeimos baltymai reguliuoja ląstelių migraciją, formuodami CRL5 ubikvitino ligazės kompleksus, kurie ubikvitina specifinius substratus [16,145]. Ir nors Rab40a ir Rab40b substratai yra aprašyti ankstesniuose tyrimuose, tačiau Rab40c taikiniai autofagijoje kol kas nėra žinomi. Su Rab40c susijusių baltymų identifikavimui buvo naudotas Rab40c-4A mutantas, turintis SOCS dėžutę, kuris didina substratų sulaikymą ir palengvina Rab40c sąveikų identifikavimą. Proteominės analizės rezultatai parodė, kad AMBRA1 yra Rab40c-CRL5 substratas (8A pav).

Koimunoprecipitacijos eksperimentai patvirtino, kad endogeninis AMBRA1 sąveikauja su Rab40c, o sąveika su mutuotu Rab40c-4A baltymu buvo stipresnė nei su laukinio tipo baltymu (8B pav). Taip pat nustatėme, kad AMBRA1 sąveikauja ir su kitais Rab40 pošeimio baltymais. Siekiant detaliau įvertinti šią sąveiką, buvo atlikta domenų funkcinių sričių analizė (8C pav.), kuri parodė, kad Rab40c specifiškai jungiasi prie AMBRA1 706–861 aminorūgščių ir persidengia su C-galiniu WD40 domenu (8D pav.). Vėlesni ubikvitinimo tyrimai parodė, kad Rab40c skatina AMBRA1 monoubikvitinimą, bet neveikia jo degradacijos (8E pav.). Apibendrinant, gauti rezultatai pademonstravo, kad AMBRA1 jungiasi su Rab40c ir yra Rab40c-CRL5

komplekso substratas. Tai parodo, kad Rab40c vykdomas ubikvitinimas yra tiesiogiai susijęs su AMBRA1 atliekamomis ląstelės funkcijomis.

AMBRA1 vaidmuo transkripcijos ir židininių sukibimo vietų dinamikos reguliacijoje

Mūsų grupės tyrimai rodo, kad AMBRA1 reguliuoja Rab40c ekspresiją transkripciniu lygmeniu [163], leidžiant manyti, jog AMBRA1 gali atlikti dvi funkcijas: dalyvauti nuo CRL4 priklausomame ubikvitinime ir veikti kaip genų transkripcijos reguliatorius [163]. Todėl tolimesnis tyrimų tikslas buvo nustatyti, ar CRL5 taip pat galėtų dalyvauti panašiame reguliaciniame kelyje.

Siekiant apibrėžti AMBRA1 vaidmenį reguliuojant genų ekspresiją, buvo atlikta RNR sekoskaita ir atlikta palyginamoji analizė tarp kontrolinių ląstelių ir ląstelių, kuriose buvo išveiklintas AMBRA1 (AMBRA1-KO). Atlikus pagrindinių komponentų analizę, nustatėme (9A pav.), kad AMBRA1 išveiklinimas lėmė padidėjusią 254 genų ir sumažėjusią 194 genų ekspresiją, iš kurių daugelis yra susiję su ląstelių migracijos ir adhezijos signaliniais keliais. Ypač svarbu pabrėžti, kad AMBRA1-KO ląstelėse RAB40C informacinės RNR kiekis buvo reikšmingai padidėjęs (9D pav.), o tai atitinka kiekybinės PCR analizės duomenis (9E pav.), kuriuose nustatytas su ląstelių migracija susijusių genų, pavyzdžiui paksiliną, ekspresijos padidėjimas.

Atsižvelgiant į tai, kad AMBRA1 reguliuoja su migracija susijusių genų ekspresiją ir sąveikauja su Rab40 šeimos baltymais, toliau siekėme nustatyti, ar AMBRA1 veikia vieną iš pagrindinių migracijos mechanizmų – FA dinamiką. Rezultatai parodė, kad AMBRA1 išveiklinimas reikšmingai pakeitė FA formavimąsi ir padidino MDA-MB-231 ląstelių judrumą. AMBRA1-KO ląstelėse padaugėjo FA, sutriko jų formavimosi mechanizmas ir pakito lokalizacija. Kontrolinėse ląstelėse FA buvo mažesnės, labiau dinamiškos ir sutelktos ląstelės vedančiajame gale, tuo tarpu AMBRA1-KO ląstelėse FA buvo didesnės bei pasiskirstė po visą ląstelę, o tai rodo, kad buvo sutrikdyta erdvinė FA reguliacija (10B,C pav.). Toks AMBRA1-KO ląstelėse stebėtas FA fenotipas koreliuoja su padidėjusia paksilino ekspresija (10A pav.), o tai gali būti susiję su FA stabilizavimu ir jų sutrikusiu išsiardymu. Poliarizuoto adhezijų pasiskirstymo praradimas taip pat rodo susilpnėjusią į vedantįjį galą nukreiptą traukos jėgą.

Ankstesni tyrimai parodė, kad AMBRA1 lokalizuojasi FA ir per autofaginius mechanizmus reguliuoja Src signalinį kelią [24,160]. Kadangi Src-FA kinazės (angl. *focal adhesion kinases,* FAK) aktyvumas didina FA stabilumą, o AMBRA1 praradimas lemia sutrikdytą Src aktyvumą, todėl susidaro hiperstabilios FA. Pažymėtina, kad nors AMBRA1-KO ląstelėse formavosi daugiau FA, šios ląstelės judėjo greičiau. Šį paradoksą galima paaiškinti pakitusiu su

migracija susijusių genų transkripcijos aktyvumu, įskaitant padidėjusią PXN ir sumažėjusią SNAI2 ekspresiją. Šio tyrimo trūkumas yra tai, kad Src-FAK aktyvumas nebuvo tiesiogiai įvertintas. Apibendrinant, duomenys rodo, kad AMBRA1 reguliuoja genų ekspresiją ir FA dinamiką, o jo išveiklinimas skatina migraciją ir su metastazėmis siejamą ląstelių elgseną.

Rap2 reguliuoja lamelipodijų dinamiką ir RhoA signalo perdavimą

Toliau buvo tiriami Rap2 veikimo mechanizmai ir jų vaidmuo reguliuojant navikinių ląstelių migracijos savybes. Literatūros duomenys rodo, kad Rap2 yra siejamas su citoskeleto reguliaciniais mechanizmais, moduliuojant aktino dinamiką ir Rho GTPazių signalo perdavimą [75,118]. Siekiant nustatyti Rap2 reikšmę krūties navikinių ląstelių judrumui, MDA-MB-231 ląstelėse buvo išveiklintos visos trys Rap2 izoformos – Rap2a, Rap2b ir Rap2c. Ilgalaikio registravimo mikroskopijos tyrimų rezultatai parodė, kad Rap2 yra būtinas efektyviai ląstelių migracijai. Nustatėme, kad ląstelėse, kuriose išveiklintas Rap2 (Rap2-KO), reikšmingai sumažėjo judėjimo greitis (11A-C pav.). Imunofluorescencinė F-aktino analizė (11D–F pav.) parodė, kad pakito Rap2-KO ląstelių morfologija - jos buvo apvalesnės lyginant su kontrolinėmis ląstelėmis. Rap2-KO ląstelės formavo mažiau lamelipodijų ir jos buvo mažesnio dydžio (11G-J pav.). Taip pat, ląstelėse, kuriose buvo išveiklintas Rap2 sumažėjo kortaktino, kuris jungiasi prie aktino filamentų Arp2/3 komplekso [32], kaupimasis lamelipodijose. Tai rodo, kad lamelipodijose yra sutrikęs šakotų aktino struktūrų formavimasis.

Gyvų GFP-Rap2a ekspresuojančių ląstelių vaizdinimo tyrimo rezultatai (12D-G pav.) parodė, kad Rap2 nėra randamas besiplečiančiuose membranos kraštuose, bet kaupiasi lamelipodijų raukšlėse. Šie rezultatai leidžia daryti išvadą, kad Rap2 svarbus lamelipodijų susitraukimo mechanizmo reguliavimui ląstelių migracijos metu. Lamelipodijų raukšlių kimografinė analizė (12A-C pav.) parodė, kad Rap2-KO ląstelės išlaiko normalų raukšlių formavimosi greitį, tačiau pasižymėjo padidėjusiais atstumais ir sutrikusiu raukšlių formavimosi periodiškumu. Literatūros duomenimis, RhoA yra pagrindinis aktomiozino kontraktilumo ir FA formavimosi reguliatorius [36].

Siekiant išsiaiškinti, kaip Rap2 sąveikauja su RhoA bei kokią įtaką ši sąveika turi lamelipodijų formavimuisi, tolimesniuose eksperimentuose įvertinome RhoA kaupimąsi lamelipodijų raukšlėse (13A-B pav.). Rezultatai parodė, kad aktyvus RhoA susitraukiančiose lamelipodijų raukšlėse lokalizuojasi kartu su Rap2. Kontrolinėse ląstelėse susitraukimo metu RhoA aktyvumas trumpam padidėdavo, tačiau vėl greitai sumažėdavo. Tuo tarpu Rap2-KO ląstelėse buvo stebėtas užsitęsęs ir erdviškai neorganizuotas RhoA aktyvumas lamelipodijų raukšlėse (13C-E pav.).

Apibendrinant, šie duomenys rodo, kad Rap2 yra svarbus lamelipodijų formavimuisi, kryptingam ląstelių poliškumui ir subalansuotai lamelipodijų susitraukimo dinamikai. Parodėme, kad Rap2 veikia kaip erdvinis RhoA aktyvumo reguliatorius susitraukimų metu, užtikrinantis koordinuotą lamelipodijų dinamiką. Rap2 išveiklinimas šią kontrolę sutrikdo, todėl prarandamas poliškumas bei sutrinka efektyvi ląstelių migracija.

Rap2 reguliuoja židininių sukibimo vietų apykaitą ir nuo RhoA priklausomą susitraukimą

Literatūros duomenimis Rap2 buvo siejamas su integrinų pernaša ir citoskeleto reguliacija [108], o mūsų gauti rezultatai parodė, kad Rap2 kontroliuoja RhoA aktyvumą susitraukiančiose lamelipodijose. Siekiant nustatyti, ar Rap2 taip pat reguliuoja FA dinamiką, kontrolinės ir Rap2-KO MDA-MB-231 ląstelės buvo transfekuotos GFP-paksilino vektoriumi ir atlikta GFP-paksilino dinamikos analizė (14A–D pav.). Gyvų ląstelių vaizdinimas atskleidė, kad kontrolinėse ląstelėse formuojasi trumpalaikės, dinamiškos FA, o Rap2-KO ląstelėse buvo stebimos ilgai išliekančios, lėtai judančios FA. Kiekybinė analizė Rap2-KO ląstelėse patvirtino padidėjusį FA gyvavimo laiką ir sumažėjusią apykaitą (14 C,D).

Imunofluorescencinė fosforilinto paksilino analizė (14E–H pav.) parodė, kad Rap2-KO ląstelėse FA buvo daugiau ir jos buvo didesnės, dažniausiai lokalizuotos ląstelės periferijoje. Kadangi FA formavimasis yra susijęs su susitraukimo metu susidariusia įtampa [69,70], buvo ištirtas miozino IIb pasiskirstymas kontrolinėse ir Rap2-KO MDA-MB-231 ląstelėse (14I–J pav.). Rap2-KO ląstelėse nustatytas reikšmingai padidėjęs miozino IIb kaupimasis mikrofilamentuose, o tai rodo padidėjusį aktomiozino kontraktilumą. Apibendrinant, mūsų duomenys rodo, kad Rap2 sutrikdo RhoA aktyvinamą FA formavimąsi. Rap2 išveiklinimas lemia itin stabilias FA, padidėjusį mikrofilamentų formavimąsi ir sutrikusią FA dinamiką.

Rap2 tarpininkaujamai ląstelių migracijai svarbus Rab40-CRL5 ubikvitinimas

Ankstesniais tyrimais taip pat buvo parodyta, jog nuo Rab40-CRL5 priklausomas ubikvitinimas reguliuoja Rap2 lokalizaciją [16] tačiau nebuvo nustatyta, ar ši modifikacija yra būtina Rap2 funkcijai ląstelių migracijos metu. Tyrėme, ar MDA-MB-231 Rap2-KO ląstelėse, Rap2 ekspresija bei funkcija gali būti atkurta, panaudojus laukinio tipo (angl. *wild-type, WT*) GFP-Rap2a (GFP-Rap2a-WT) vektorių arba GFP-Rap2a-K3R vektorių, kuris koduoja neubikvitinamą Rap2a formą. Imunofluorescencinė aktino analizė (15A pav.) parodė, kad ląstelių ekspresuojančių GFP-Rap2a-WT morfologija iš dalies

buvo atstatyta, tuo tarpu GFP-Rap2a-K3R ekspresuojančios ląstelės nesiskyrė nuo Rap2-KO. Kiekybinė analizė parodė, kad ląstelės ekspresuojančios GFP-Rap2a-K3R pasižymėjo mažesniu ilgio ir pločio santykiu, bei didesniu plotu (15B, C pav.). Kadangi Rap2 išveiklinimas stabilizuoja FA, jų dinamikai įvertinti buvo atlikta fosforilinto paksilino imunofluorescencinė analizė (15D pav.). Ląstelėse, ekspresuojančiose GFP-Rap2a-WT, sumažėjo tiek FA skaičius, tiek jų dydis, tuo tarpu GFPRap2aK3R ekspresuojančios ląstelės pasižymėjo fenotipu, panašiu į Rap2-KO (15E,F pav., 15 F,G). Ląstelių migracijos tyrimai parodė, kad GFP-Rap2a-K3R ekspresuojančios ląstelės judėjo panašiai kaip Rap2-KO (15G,H pav., Fig.11 B,C.).

Nuo Rab40-CRL5 priklausomas ubikvitinimas reguliuoja Rap2 lokalizaciją, o ubikvitinimo slopinimas nukreipia Rap2 į lizosomas [16,141] Siekiant nustatyti Rap2 ubikvitinimo vietą ląstelėse, tyrėme galimas baltymų sąveikas pasitelkdami PLA. Ląstelėse, ekspresuojančiose GFP-Rap2a-WT buvo stebima daugiau ubikvitinto Rap2 signalinių taškų, lyginant su ląstelėmis, kurios buvo transfekuotos GFP-Rap2a-K3R. Be to, ląstelėse, ekspresuojančiose GFP-Rap2a-WT ubikvitinto Rap2 signaliniai taškai daugiausia lokalizavosi lamelipodijų raukšlėse.

Šie duomenys rodo, Rap2 yra būtinas koordinuotai ląstelių migracijai, kadangi jis dalyvauja reguliuojant lamelipodijų ir FA dinamiką. Rap2 išveiklinimas lemia padidėjusį ir užsitęsusį RhoA aktyvumą, sustiprintą kontraktilumą, padidėjusias FA ir sutrikusį ląstelių poliškumą. Rap2 kaupiasi lamelipodijų raukšlėse, kuriose riboja RhoA signalo perdavimą ir užtikrina lamelipodijų kontraktilumą. Nuo Rab40-CRL5 priklausomas ubikvitinimas kontroliuoja Rap2 membraninę lokalizaciją ir funkciją migracijos metu.

Nuo Rab40-CRL5 priklausomas monoubikvitinimas valdo Rap1 aktyvumą ir lokalizaciją

Rap1 gerai žinomas kaip svarbus integrinų pernašos ir sukibimo dinamikos reguliatorius ląstelių migracijos metu [108-110]. Tačiau iki šiol nebuvo aišku, ar Rap1 funkciją tiesiogiai reguliuoja nuo Rab40-CRL5 priklausomas ubikvitinimas. Atsižvelgdami į ankstesnius mūsų duomenis, rodančius, kad Rab40-CRL5 kompleksas sąveikauja su Rap2 [16], siekėme nustatyti, ar Rap1 taip pat yra E3 ubikvitino ligazės komplekso substratas. Aminorūgščių sekų palyginimoji analizė parodė, kad Rap1 ir Rap2 baltymai turi būdingas konservatyvias lizino liekanas, leidžiančias numatyti galimas ubikvitinimo vietas. Siekiant tai patikrinti, atlikome baltymų sąveikos tyrimus. Glutationo-S-transferazės baltymų traukos (angl. *pulldown*) analizė parodė, kad FLAG-Rab40b jungiasi prie Rap1b nuo lizino priklausomu būdu, o lizino taškinės mutacijos reikšmingai sumažino šią sąveiką [171].

Ubikvitinimo tyrimai parodė Rap1b sąveikauja su Rab40b-CRL5 kompleksu ir yra nuo jo priklausomai modifikuojamas (16A,F pav.). Mes taip pat nustatėme, kad Rab40-TKO ląstelėse reikšmingai sumažėja aktyvaus prie GTP prisijungusio Rap1b kiekis, rodantis sutrikusi Rap1b aktyvumą (16G pav.). Imunofluorescencinė analizė parodė, kad Rab40-TKO ląstelėse Rap1b lokalizacija yra pakitusi, jose buvo nustatytas mažesnis viduląstelinės ir bendros Rap1b fluorescencijos santykis, lyginant su kontrolinėmis ląstelėmis (16I–J pav.). Kartu šie rezultatai rodo, kad Rap1 yra Rab40-CRL5 komplekso substratas ir kad jo ubikvitinimas yra svarbus tinkamai aktyvacijai, lokalizacijai ir su migracija susijusioms funkcijoms, įskaitant FA reguliaciją ir kryptingą ląstelių migraciją. Šie duomenys rodo, kad Rab40-CRL5 sukeltas monoubikvitinimas koordinuoja baltymų veikimą ląstelėje, o ne jų skaidymą, ir reguliuoja su migracija susijusių genų ekspresiją.

Vis dėlto reikia pažymėti, kad visi šie rezultatai buvo gauti naudojant vieną krūties naviko ląstelių liniją, augintą dvimatės (2D) kultūros sąlygomis. Kadangi ląstelių elgsena gali skirtis trimatėje (3D) aplinkoje, būtini tolimesni tyrimai siekiant patvirtinti, ar šie mechanizmai veikia ir kitų tipų ląstelėse bei *in vivo* modeliuose.

IŠVADOS

1. AMBRA1 reguliuoja židininių sukibimo vietų dinamiką per Rab40-CRL5 ir E3 ligazės kompleksą, o jo praradimas sutrikdo ląstelės priekio-galo poliškumą. Be to, AMBRA1 reguliuoja genų, susijusių su sukibimu, citoskeleto reorganizacija ir judrumu, ekspresiją, kas patvirtina kad AMBRA1 veikia, kaip dvigubas reguliatorius krūties naviko ląstelėse.
2. Rab40-CRL5 tarpininkaujamas monoubikvitinimas reguliuoja Rap2 lokalizaciją membranoje. Ubikvitinimo sutrikdymas skatina neaktyvios Rap2 formos gamybą, skatina nuo RhoA priklausomą susitraukimą, stabilizuoja židininį sukibimą ir mažina migraciją. Nesant Rap2, ląstelės pasižymi itin dideliu mikrofilamentų susitraukimu bei negebėjimu užtikrinti lamelipodijų judėjimą, o tai rodo, kad ubikvitintas Rap2 veikia kaip RhoA inhibitorius, kas reikalinga koordinuotam citoskeleto persitvarkymui.
3. Rab40-CRL5 tarpininkaujamas monoubikvitinimas reguliuoja Rap1 aktyvumą ir lokalizaciją. Neubikvitintas neaktyvus Rap1 nesikaupia prie ląstelės plazminės membranos ir branduolio apvalkalo, kas būtina, reguliuojant ląstelių migracijia.

BIBLIOGRAPHY

1. Siegel RL, Kratzer TB, Giaquinto AN, Sung H, Jemal A. Cancer statistics, 2025. CA Cancer J Clin [Internet]. 2025 Jan 16; Available from: http://www.ncbi.nlm.nih.gov/pubmed/39817679
2. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023 Jan;73(1):17–48.
3. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. CA Cancer J Clin. 2024 Jan;74(1):12–49.
4. Welch DR, Hurst DR. Defining the Hallmarks of Metastasis. Vol. 79, Cancer Research. American Association for Cancer Research Inc.; 2019. p. 3011–27.
5. Li L, He Y, Zhao M, Jiang J. Collective cell migration: Implications for wound healing and cancer invasion. Vol. 1, Burns and Trauma. Oxford University Press; 2013. p. 21–6.
6. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging Biological Principles of Metastasis. Vol. 168, Cell. Cell Press; 2017. p. 670–91.
7. Wehrle-Haller B, Imhof BA. Actin, microtubules and focal adhesion dynamics during cell migration. Int J Biochem Cell Biol [Internet]. 2003 Jan 1 [cited 2025 Apr 29];35(1):39–50. Available from: https://www.sciencedirect.com/science/article/abs/pii/S1357272502000717?via%3Dihub
8. Gavert N, Ben-Ze'ev A. Epithelial-mesenchymal transition and the invasive potential of tumors. Trends Mol Med. 2008 May;14(5):199–209.
9. Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Vol. 28, Cancer and Metastasis Reviews. 2009. p. 15–33.
10. Charest PG, Firtel RA. Big roles for small GTPases in the control of directed cell movement. Vol. 401, Biochemical Journal. 2007. p. 377–90.
11. Boettner B, Van Aelst L. Control of cell adhesion dynamics by Rap1 signaling. Vol. 21, Current Opinion in Cell Biology. 2009. p. 684–93.
12. Kooistra MRH, Dubé N, Bos JL. Rap1: A key regulator in cell-cell junction formation. J Cell Sci. 2007 Jan 1;120(1):17–22.
13. Rehmann H, Bos JL. Ras Association-Domain Dimers Bring Proteins Together. Vol. 24, Structure. Cell Press; 2016. p. 2039–40.
14. McSherry EA, Brennan K, Hudson L, Hill ADK, Hopkins AM. Breast cancer cell migration is regulated through junctional adhesion molecule-A-mediated activation of Rap1 GTPase. Breast Cancer Research. 2011 Mar 23;13(2).
15. Bos JL, De Bruyn K, Enserink J, Kuiperij B, Rangarajan S, Rehmann H, et al. The role of Rap1 in integrin-mediated cell adhesion [Internet]. Available from: http://smart.embl-heidelberg.de
16. Duncan ED, Han KJ, Trout M, Prekeris R. Ubiquitylation by Rab40b/Cul5 regulates Rap2 localization and activity during cell migration. Journal of Cell Biology. 2022 Apr 4;221(4).
17. Ohba Y, Mochizuki N, Matsuo K, Yamashita S, Nakaya M, Hashimoto Y, et al. Rap2 as a Slowly Responding Molecular Switch in the Rap1 Signaling Cascade. Vol. 20, MOLECULAR AND CELLULAR BIOLOGY. 2000.
18. B1ranger F, Goudt B, Tavitian A, De Gunzburg J. Association of the Ras-antagonistic Rapl/Krev-1 proteins with the Golgi complex (oncogenes/small GTP-binding proteins/membrane association/subceliular localization). Vol. 88, Proc. Nati. Acad. Sci. USA. 1991.
19. Pfeffer SR. Rab GTPases: Master regulators that establish the secretory and endocytic pathways. Vol. 28, Molecular Biology of the Cell. American Society for Cell Biology; 2017. p. 712–5.

20. Cianfanelli V, D'Orazio M, Cecconi F. Ambra1 and beclin 1 interplay in the crosstalk between autophagy and cell proliferation. Cell Cycle. 2015 Apr 1;14(7):959–63.
21. Frias A, Di Leo L, Antoranz A, Nazerai L, Carretta M, Bodemeyer V, et al. Ambra1 modulates the tumor immune microenvironment and response to PD-1 blockade in melanoma. J Immunother Cancer. 2023 Mar 3;11(3).
22. Cianfanelli V, Fuoco C, Lorente M, Salazar M, Quondamatteo F, Gherardini PF, et al. AMBRA1 links autophagy to cell proliferation and tumorigenesis by promoting c-Myc dephosphorylation and degradation. Nat Cell Biol. 2015 Jan 1;17(1):20–30.
23. Ye L, Lin D, Zhang W, Chen S, Zhen Y, Akkouche S, et al. AMBRA1 drives gastric cancer progression through regulation of tumor plasticity. Front Immunol. 2024;15.
24. Schoenherr C, Byron A, Griffith B, Loftus A, Wills JC, Munro AF, et al. The autophagy protein Ambra1 regulates gene expression by supporting novel transcriptional complexes. Journal of Biological Chemistry. 2020 Aug 21;295(34):12045–57.
25. Lauffenburger DA, Horwitz AF. Cell Migration: Review A Physically Integrated Molecular Process. Vol. 84, Cell. 1996.
26. Yamaguchi H, Condeelis J. Regulation of the actin cytoskeleton in cancer cell migration and invasion. Vol. 1773, Biochimica et Biophysica Acta - Molecular Cell Research. 2007. p. 642–52.
27. Berrier AL, Yamada KM. Cell-matrix adhesion. Vol. 213, Journal of Cellular Physiology. 2007. p. 565–73.
28. Jansen KA, Atherton P, Ballestrem C. Mechanotransduction at the cell-matrix interface. Vol. 71, Seminars in Cell and Developmental Biology. Elsevier Ltd; 2017. p. 75–83.
29. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix Elasticity Directs Stem Cell Lineage Specification. Cell. 2006 Aug 25;126(4):677–89.
30. Plessner M, Grosse R. Dynamizing nuclear actin filaments. Vol. 56, Current Opinion in Cell Biology. Elsevier Ltd; 2019. p. 1–6.
31. Svitkina T. The actin cytoskeleton and actin-based motility. Cold Spring Harb Perspect Biol. 2018 Jan 1;10(1).
32. Farooqui R, Fenteany G. Multiple rows of cells behind an epithelial wound edge extend cryptic lamellipodia to collectively drive cell-sheet movement. J Cell Sci. 2005 Jan 1;118(1):51–63.
33. Faix J, Rottner K. The making of filopodia. Vol. 18, Current Opinion in Cell Biology. 2006. p. 18–25.
34. Yang C, Czech L, Gerboth S, Kojima SI, Scita G, Svitkina T. Novel roles of formin mDia2 in lamellipodia and filopodia formation in motile cells. PLoS Biol. 2007 Nov;5(11):2624–45.
35. Schaks M, Giannone G, Rottner K. Actin dynamics in cell migration. Essays Biochem. 2019;63(5):483–95.
36. Weißenbruch K, Mayor R. Actomyosin forces in cell migration: Moving beyond cell body retraction. BioEssays. John Wiley and Sons Inc; 2024.
37. Heissler SM, Manstein DJ. Nonmuscle myosin-2: Mix and match. Vol. 70, Cellular and Molecular Life Sciences. 2013. p. 1–21.
38. Shutova MS, Svitkina TM. Mammalian nonmuscle myosin II comes in three flavors. Biochem Biophys Res Commun. 2018 Nov 25;506(2):394–402.
39. Rottner K, Faix J, Bogdan S, Linder S, Kerkhoff E. Actin assembly mechanisms at a glance. J Cell Sci. 2017;130(20):3427–35.
40. Goode BL, Eskin J, Shekhar S. Mechanisms of actin disassembly and turnover. Vol. 222, Journal of Cell Biology. Rockefeller University Press; 2023.
41. SenGupta S, Parent CA, Bear JE. The principles of directed cell migration. Vol. 22, Nature Reviews Molecular Cell Biology. Nature Research; 2021. p. 529–47.

42. Rodnick-Smith M, Luan Q, Liu SL, Nolen BJ. Role and structural mechanism of WASP-triggered conformational changes in branched actin filament nucleation by Arp2/3 complex. Proc Natl Acad Sci U S A. 2016 Jul 5;113(27):E3834–43.
43. Shekhar S, Kerleau M, Kühn S, Pernier J, Romet-Lemonne G, Jégou A, et al. Formin and capping protein together embrace the actin filament in a ménage à trois. Nat Commun. 2015 Nov 13;6.
44. Molendijk AJ, Ruperti B, Palme K. Small GTPases in vesicle trafficking. Vol. 7, Current Opinion in Plant Biology. 2004. p. 694–700.
45. Ma N, Xu E, Luo Q, Song G. Rac1: A Regulator of Cell Migration and a Potential Target for Cancer Therapy. Vol. 28, Molecules. MDPI; 2023.
46. Fukata Y, Kimura K, Oshiro N, Saya H, Matsuura Y, Kaibuchi K. Association of the Myosin-binding Subunit of Myosin Phosphatase and Moesin: Dual Regulation of Moesin Phosphorylation by Rho-associated Kinase and Myosin Phosphatase [Internet]. Vol. 141, The Journal of Cell Biology. 1998. Available from: http://www.jcb.org
47. Yadav S, Sanjaya KC, Blaskovich MAT, Lu C tai, Lam AK, Nguyen NT. RhoA and Rac1 as Mechanotransduction Mediators in Colorectal Cancer. Adv Biol. 2025;
48. Nguyen LK, Kholodenko BN, von Kriegsheim A. Rac1 and RhoA: Networks, loops and bistability. Vol. 9, Small GTPases. Taylor and Francis Inc.; 2018. p. 316–21.
49. Johnson DI. Cdc42: An Essential Rho-Type GTPase Controlling Eukaryotic Cell Polarity. Vol. 63, MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS. 1999.
50. Ravichandran Y, Hänisch J, Murray K, Roca V, Dingli F, Loew D, et al. The distinct localization of CDC42 isoforms is responsible for their specific functions during migration. Journal of Cell Biology. 2024 May 6;223(3).
51. Bos JL, Rehmann H, Wittinghofer A. GEFs and GAPs: Critical Elements in the Control of Small G Proteins. Vol. 129, Cell. Elsevier B.V.; 2007. p. 865–77.
52. Hornigold K, Baker MJ, Machin PA, Chetwynd SA, Johnsson AK, Pantarelli C, et al. The Rac-GEF Tiam1 controls integrin-dependent neutrophil responses. Front Immunol. 2023;14.
53. Lawson CD, Burridge K. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. Vol. 5, Small GTPases. Taylor and Francis Inc.; 2014.
54. Arthur WT, Burridge K. RhoA Inactivation by p190RhoGAP Regulates Cell Spreading and Migration by Promoting Membrane Protrusion and Polarity. Vol. 12, Molecular Biology of the Cell. 2001.
55. Colón-Bolea P, García-Gómez R, Casar B. Rac1 activation as a potential therapeutic option in metastatic cutaneous melanoma. Vol. 11, Biomolecules. MDPI; 2021.
56. Barz S, Kriegenburg F, Sánchez-Martín P, Kraft C. Small but mighty: Atg8s and Rabs in membrane dynamics during autophagy. Vol. 1868, Biochimica et Biophysica Acta - Molecular Cell Research. Elsevier B.V.; 2021.
57. Yorimitsu T, Sato K, Takeuchi M. Molecular mechanisms of Sar/Arf GTPases in vesicular trafficking in yeast and plants. Vol. 5, Frontiers in Plant Science. Frontiers Research Foundation; 2014.
58. Onodera Y, Nam JM, Hashimoto A, Norman JC, Shirato H, Hashimoto S, et al. Rab5c promotes AMAP1-PRKD2 complex formation to enhance β1 integrin recycling in EGF-induced cancer invasion. Journal of Cell Biology. 2012 Jun 25;197(7):983–96.
59. Wheeler DB, Zoncu R, Root DE, Sabatini DM, Sawyers CL. Identification of an oncogenic RAB protein. Science (1979). 2015 Oct 9;350(6257):211–7.
60. Stenmark H, Olkkonen VM. The Rab GTPase family. Genome Biol [Internet]. 2001;2(5). Available from: http://genomebiology.com/2001/2/5/reviews/3007.1http://genomebiology.com/2001/2/5/reviews/3007

61. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. J Cell Sci. 2002 Jun 15;115(12):2505–15.
62. Fukuda M. Membrane traffic in the secretory pathway: Regulation of secretory vesicle traffic by Rab small GTPases. Vol. 65, Cellular and Molecular Life Sciences. 2008. p. 2801–13.
63. Li G, Marlin MC. Rab family of GTpases. Methods in Molecular Biology. 2015;1298.
64. Xu S, Cao B, Xuan G, Xu S, An Z, Zhu C, et al. Function and regulation of Rab GTPases in cancers. Vol. 40, Cell Biology and Toxicology. Springer Science and Business Media B.V.; 2024.
65. Jiang X, Yang L, Gao Q, Liu Y, Feng X, Ye S, et al. The Role of RAB GTPases and Its Potential in Predicting Immunotherapy Response and Prognosis in Colorectal Cancer. Front Genet. 2022 Jan 28;13.
66. Bertocchi C, Wang Y, Ravasio A, Hara Y, Wu Y, Sailov T, et al. Nanoscale architecture of cadherin-based cell adhesions. Nat Cell Biol. 2017 Jan 1;19(1):28–37.
67. Lo CM, Wang HB, Dembo M, Wang YL. Cell Movement Is Guided by the Rigidity of the Substrate. 2000.
68. Potter DA, Tirnauer JS, Janssen R, Croall DE, Hughes CN, Fiacco KA, et al. Calpain Regulates Actin Remodeling during Cell Spreading [Internet]. Vol. 141, The Journal of Cell Biology. 1998. Available from: http://www.jcb.org
69. Lin YS, Lin YH, Thi MN, Hsiao SC, Chiu WT. STIM1 controls the focal adhesion dynamics and cell migration by regulating SOCE in osteosarcoma. Int J Mol Sci. 2022 Jan 1;23(1).
70. Quadri SK. Cross talk between focal adhesion kinase and cadherins: Role in regulating endothelial barrier function. Microvasc Res. 2012 Jan;83(1):3–11.
71. Hsia DA, Mitra SK, Hauck CR, Streblow DN, Nelson JA, Ilic D, et al. Differential regulation of cell motility and invasion by FAK. Journal of Cell Biology. 2003 Mar 3;160(5):753–67.
72. Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. J Cell Sci. 2005 Mar 1;118(5):843–6.
73. Takai Y, Sasaki T, Matozaki T. Small GTP-Binding Proteins [Internet]. 2001. Available from: http://physrev.physiology.org
74. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Vol. 420, Nature. 2002. p. 629–35.
75. Warner H, Wilson BJ, Caswell PT. Control of adhesion and protrusion in cell migration by Rho GTPases. Vol. 56, Current Opinion in Cell Biology. Elsevier Ltd; 2019. p. 64–70.
76. Vigil D, Cherfils J, Rossman KL, Der CJ. Ras superfamily GEFs and GAPs: Validated and tractable targets for cancer therapy? Vol. 10, Nature Reviews Cancer. 2010. p. 842–57.
77. Goitre L, Trapani E, Trabalzini L, Retta SF. The ras superfamily of small GTPases: The unlocked secrets. Vol. 1120, Methods in Molecular Biology. Humana Press Inc.; 2014. p. 1–18.
78. Johnson DS, Chen YH. Ras family of small GTPases in immunity and inflammation. Vol. 12, Current Opinion in Pharmacology. 2012. p. 458–63.
79. Urade R, Chiu YH, Chiu CC, Wu CY. Small GTPases and Their Regulators: A Leading Road toward Blood Vessel Development in Zebrafish. Vol. 23, International Journal of Molecular Sciences. MDPI; 2022.
80. Downward J. Targeting RAS signalling pathways in cancer therapy. Vol. 3, Nature Reviews Cancer. 2003. p. 11–22.

81. Capella G, Cronauer-Mitra S, Peinado MA, Perucho M. Frequency and Spectrum of Mutations at Codons 12 and 13 of the C-K-ras Gene in Human Tumors. Vol. 93, Environmental Health Perspectives. 1991.
82. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: Weaving a tumorigenic web. Vol. 11, Nature Reviews Cancer. 2011. p. 761–74.
83. Cox AD, Fesik SW, Kimmelman AC, Luo J, Der CJ. Drugging the undruggable RAS: Mission Possible? Vol. 13, Nature Reviews Drug Discovery. Nature Publishing Group; 2014. p. 828–51.
84. Prior IA, Hood FE, Hartley JL. The frequency of ras mutations in cancer. Cancer Res. 2020 Jul 15;80(14):2669–974.
85. Canon J, Rex K, Saiki AY, Mohr C, Cooke K, Bagal D, et al. The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity. Nature. 2019 Nov 7;575(7781): 217–23.
86. Awad MM, Liu S, Rybkin II, Arbour KC, Dilly J, Zhu VW, et al. Acquired Resistance to KRAS G12C Inhibition in Cancer . New England Journal of Medicine. 2021 Jun 24;384(25):2382–93.
87. Rudiman R, Wijaya A, Sribudiani Y, Soedjana HS, Wiraswati HL, Primastari E, et al. Identification of KRAS mutation and HER2 expression in Indonesian colorectal cancer population: a cross-sectional study. Annals of Medicine & Surgery. 2023 May;85(5):1761–8.
88. Deng LL, Deng H Bin, Lu CL, Guo Y, Wang D, Yan CH, et al. Mutations of EGFR or KRAS and expression of chemotherapy-related genes based on small biopsy samples in stage IIIB and IV inoperable non-small cell lung cancer. J Cancer Res Clin Oncol. 2014 Dec 1;140(12):2097–105.
89. Mor A, Wynne JP, Ahearn IM, Dustin ML, Du G, Philips MR. Phospholipase D1 Regulates Lymphocyte Adhesion via Upregulation of Rap1 at the Plasma Membrane. Mol Cell Biol. 2009 Jun 1;29(12):3297–306.
90. Chigaev A, Zwartz G, Graves SW, Dwyer DC, Tsuji H, Foutz TD, et al. α4β1 integrin affinity changes govern cell adhesion. Journal of Biological Chemistry. 2003 Oct 3;278(40):38174–82.
91. Zhang Z, Mitra RS, Henson BS, Datta NS, McCauley LK, Kumar P, et al. Rap1GAP inhibits tumor growth in oropharyngeal squamous cell carcinoma. American Journal of Pathology. 2006;168(2):585–96.
92. Sequera C, Manzano S, Guerrero C, Porras A. How Rap and its GEFs control liver physiology and cancer development. C3G alterations in human hepatocarcinoma. Hepat Oncol. 2018 Jan;5(1).
93. Bailey CL, Kelly P, Casey PJ. Activation of Rap1 promotes prostate cancer metastasis. Cancer Res. 2009 Jun 15;69(12):4962–8.
94. Sawant K, Chen Y, Kotian N, Preuss KM, McDonald JA. Rap1 GTPase promotes coordinated collective cell migration in vivo. Mol Biol Cell. 2018 Nov 1;29(22):2656–73.
95. Looi CK, Hii LW, Ngai SC, Leong CO, Mai CW. The role of ras-associated protein 1 (Rap1) in cancer: Bad actor or good player? Vol. 8, Biomedicines. MDPI AG; 2020.
96. Rio-Vilariño A, Del Puerto-Nevado L, García-Foncillas J, Cebrián A. Ras family of small gtpases in crc: New perspectives for overcoming drug resistance. Vol. 13, Cancers. MDPI; 2021.
97. Bruurs LJM, Bos JL. Mechanisms of Isoform Specific Rap2 Signaling during Enterocytic Brush Border Formation. PLoS One [Internet]. 2014;9(9):106687. Available from: www.plosone.org

98. Di J, Zhao Z, Xia M, Gao K, Chai K, Zhu B, et al. Rap2B drives tumorigenesis and progression of colorectal cancer through intestinal cytoskeleton remodeling. Cell Death Dis. 2025 Dec 1;16(1).
99. Wu J, Du W, Wang X, Wei L, Pan Y, Wu X, et al. Ras-related protein rap2c promotes the migration and invasion of human osteosarcoma cells. Oncol Lett. 2018 Apr 1;15(4):5352–8.
100. Wu F, Lin X, Shan SK, Li F, Xu F, Zhong JY, et al. The Suppression of miR-199a-3p by Promoter Methylation Contributes to Papillary Thyroid Carcinoma Aggressiveness by Targeting RAP2a and DNMT3a. Front Cell Dev Biol. 2020 Dec 7;8.
101. Wu JX, Du WQ, Wang XC, Wei LL, Huo FC, Pan YJ, et al. Rap2a serves as a potential prognostic indicator of renal cell carcinoma and promotes its migration and invasion through up-regulating p-Akt. Sci Rep. 2017 Dec 1;7(1).
102. Shi G, Zhang Z. Rap2B promotes the proliferation and migration of human glioma cells via activation of the ERK pathway. Oncol Lett. 2021 Apr 1;21(4).
103. Peng YG, Zhang ZQ, Chen Y Bin, Huang JA. Rap2b promotes proliferation, migration, and invasion of lung cancer cells. Journal of Receptors and Signal Transduction. 2016 Sep 2;36(5):459–64.
104. de la Vega M, Burrows JF, Johnston JA. Added complexity in Ras and Rho family GTPase function. Small GTPases. 2011;2(4):192–201.
105. Ma Y, Xu J, Huang P, Bai X, Gao H. Ubiquitin-independent, Proteasome-mediated targeted degradation of KRAS in pancreatic adenocarcinoma cells using an engineered ornithine decarboxylase/antizyme system. IUBMB Life. 2019 Jan 1;71(1):57–65.
106. Gao WL, Ye GC, Liu LW, Wei L. The downregulation of Rap1 GTPase-activating protein is associated with a poor prognosis in colorectal cancer and may impact on tumor progression. Oncol Lett. 2018 May 1;15(5):7661–8.
107. Shah S, Brock EJ, Ji K, Mattingly RR. Ras and Rap1: A tale of two GTPases. Vol. 54, Seminars in Cancer Biology. Academic Press; 2019. p. 29–39.
108. Bromberger T, Klapproth S, Rohwedder I, Weber J, Pick R, Mittmann L, et al. Binding of Rap1 and Riam to Talin1 Fine-Tune β2 Integrin Activity During Leukocyte Trafficking. Front Immunol. 2021 Aug 19;12.
109. Dao VT, Dupuy AG, Gavet O, Caron E, de Gunzburg J. Dynamic changes in Rap1 activity are required for cell retraction and spreading during mitosis. J Cell Sci. 2009 Aug 15;122(16):2996–3004.
110. Gaonac'h-Lovejoy V, Boscher C, Delisle C, Gratton JP. Rap1 is Involved in Angiopoietin-1-Induced Cell-Cell Junction Stabilization and Endothelial Cell Sprouting. Cells. 2020 Jan 8;9(1).
111. Lakshmikanthan S, Zieba BJ, Ge ZD, Momotani K, Zheng X, Lund H, et al. Rap1b in smooth muscle and endothelium is required for maintenance of vascular tone and normal blood pressure. Arterioscler Thromb Vasc Biol. 2014;34(7):1486–94.
112. Gérard A, Mertens AEE, Van Der Kammen RA, Collard JG. The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. Journal of Cell Biology. 2007 Mar 12;176(6):863–75.
113. Rappel WJ, Edelstein-Keshet L. Mechanisms of cell polarization. Vol. 3, Current Opinion in Systems Biology. Elsevier Ltd; 2017. p. 43–53.
114. Raaijmakers JH, Bos JL. Specificity in Ras and Rap signaling. Vol. 284, Journal of Biological Chemistry. 2009. p. 10995–9.
115. Mcleod SJ, Li AHY, Lee RL, Burgess AE, Gold MR. The Rap GTPases Regulate B Cell Migration Toward the Chemokine Stromal Cell-Derived Factor-1 (CXCL12): Potential Role for Rap2 in Promoting B Cell Migration 1 [Internet]. Vol. 169, The Journal of Immunology. 2002. Available from: http://journals.aai.org/jimmunol/article-pdf/169/3/1365/1154749/1365.pdf

116. Pöling J, Szibor M, Schimanski S, Ingelmann ME, Rees W, Gajawada P, et al. Induction of smooth muscle cell migration during arteriogenesis is mediated by Rap2. Arterioscler Thromb Vasc Biol. 2011 Oct;31(10):2297–305.
117. Lawson CD, Ridley AJ. Rho GTPase signaling complexes in cell migration and invasion. Vol. 217, Journal of Cell Biology. Rockefeller University Press; 2018. p. 447–57.
118. Arnold TR, Stephenson RE, Miller AL. Rho GTPases and actomyosin: Partners in regulating epithelial cell-cell junction structure and function. Vol. 358, Experimental Cell Research. Elsevier Inc.; 2017. p. 20–30.
119. Bradley WD, Hernández SE, Settleman J, Koleske AJ. Integrin Signaling through Arg Activates p190RhoGAP by Promoting Its Binding to p120RasGAP and Recruitment to the Membrane □ D. Mol Biol Cell [Internet]. 2006;17:4827–36. Available from: http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06
120. McLeod SJ, Shum AJ, Lee RL, Takei F, Gold MR. The Rap GTPases Regulate Integrin-mediated Adhesion, Cell Spreading, Actin Polymerization, and Pyk2 Tyrosine Phosphorylation in B Lymphocytes. Journal of Biological Chemistry. 2004 Mar 26;279(13):12009–19.
121. Pertz O, Hodgson L, Klemke RL, Hahn KM. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature. 2006 Apr 20;440(7087):1069–72.
122. Tune BXJ, Sim MS, Poh CL, Guad R Mac, Woon CK, Hazarika I, et al. Matrix Metalloproteinases in Chemoresistance: Regulatory Roles, Molecular Interactions, and Potential Inhibitors. Vol. 2022, Journal of Oncology. Hindawi Limited; 2022.
123. Jacob A, Prekeris R. The regulation of MMP targeting to invadopodia during cancer metastasis. Vol. 3, Frontiers in Cell and Developmental Biology. Frontiers Media S.A.; 2015.
124. Coppola U, Ristoratore F, Albalat R, D'Aniello S. The evolutionary landscape of the Rab family in chordates. Cellular and Molecular Life Sciences. 2019 Oct 1;76(20):4117–30.
125. Babon JJ, Sabo JK, Zhang JG, Nicola NA, Norton RS. The SOCS Box Encodes a Hierarchy of Affinities for Cullin5: Implications for Ubiquitin Ligase Formation and Cytokine Signalling Suppression. J Mol Biol. 2009 Mar 20;387(1):162–74.
126. Duncan ED, Lencer E, Linklater E, Prekeris R. Methods to Study the Unique SOCS Box Domain of the Rab40 Small GTPase Subfamily. In: Methods in Molecular Biology. Humana Press Inc.; 2021. p. 163–79.
127. Jacob A, Linklater E, Bayless BA, Lyons T, Prekeris R. The role and regulation of Rab40b-Tks5 complex during invadopodia formation and cancer cell invasion. J Cell Sci. 2016;129(23):4341–53.
128. Zacharias M, Brcic L, Eidenhammer S, Popper H. Bulk tumour cell migration in lung carcinomas might be more common than epithelial-mesenchymal transition and be differently regulated. BMC Cancer. 2018 Jul 6;18(1).
129. Li Y, Jia Q, Wang Y, Li F, Jia Z, Wan Y. Rab40b upregulation correlates with the prognosis of gastric cancer by promoting migration, invasion, and metastasis. Medical Oncology. 2015 Apr 1;32(4).
130. Myat MM, Louis D, Mavrommatis A, Collins L, Mattis J, Ledru M, et al. Regulators of cell movement during development and regeneration in Drosophila. Open Biol. 2019 May 1;9(5).
131. Swatek KN, Komander D. Ubiquitin modifications. Vol. 26, Cell Research. Nature Publishing Group; 2016. p. 399–422.
132. Lin S, Lu S, Mulaj M, Fang B, Keeley T, Wan L, et al. Monoubiquitination inhibits the actin bundling activity of fascin. Journal of Biological Chemistry. 2016 Dec 30;291(53):27323–33.

133. Li B, Zhou Q, Wan Q, Qiao X, Chen S, Zhou J, et al. EZH2 K63-polyubiquitination affecting migration in extranodal natural killer/T-cell lymphoma. Clin Epigenetics. 2023 Dec 1;15(1).
134. Hu H, Xi X, Jiang B, Wang K, Wu T, Chen X, et al. RNF187 Facilitates Proliferation and Migration of Human Spermatogonial Stem Cells Through WDR77 Polyubiquitination. Cell Prolif. 2025;
135. Sadowski M, Sarcevic B. Mechanisms of mono- and poly-ubiquitination: Ubiquitination specificity depends on compatibility between the E2 catalytic core and amino acid residues proximal to the lysine. Cell Div. 2010 Aug 13;5.
136. Paudel P, Banos CM, Liu Y, Zhuang Z. Triubiquitin Probes for Identification of Reader and Eraser Proteins of Branched Polyubiquitin Chains. ACS Chem Biol. 2023 Apr 21;18(4):837–47.
137. Liu J, Li Y, Deol KK, Strieter ER, Lett O. Synthesis of Branched Triubiquitin Active-Site Directed Probes HHS Public Access Author manuscript. Org Lett. 2019;21(17):6790–4.
138. Baker R, Lewis SM, Sasaki AT, Wilkerson EM, Locasale JW, Cantley LC, et al. Site-specific monoubiquitination activates Ras by impeding GTPase-activating protein function. Nat Struct Mol Biol. 2013 Jan;20(1):46–52.
139. Deng L, Meng T, Chen L, Wei W, Wang P. The role of ubiquitination in tumorigenesis and targeted drug discovery. Vol. 5, Signal Transduction and Targeted Therapy. Springer Nature; 2020.
140. Hong Z, Liu F, Zhang Z. Ubiquitin modification in the regulation of tumor immunotherapy resistance mechanisms and potential therapeutic targets. Vol. 13, Experimental Hematology and Oncology. BioMed Central Ltd; 2024.
141. Linklater ES, Duncan ED, Han KJ, Kaupinis A, Valius M, Lyons TR, et al. Rab40–cullin5 complex regulates eplin and actin cytoskeleton dynamics during cell migration. Journal of Cell Biology. 2021 Jul 5;220(7).
142. Mcleod SJ, Li AHY, Lee RL, Burgess AE, Gold MR. The Rap GTPases Regulate B Cell Migration Toward the Chemokine Stromal Cell-Derived Factor-1 (CXCL12): Potential Role for Rap2 in Promoting B Cell Migration 1 [Internet]. Vol. 169, The Journal of Immunology. 2002. Available from: http://journals.aai.org/jimmunol/article-pdf/169/3/1365/1154749/1365.pdf
143. Lin KBL, Freeman SA, Gold MR. Rap GTPase-mediated adhesion and migration: A target for limiting the dissemination of B-cell lymphomas? Vol. 4, Cell Adhesion and Migration. Taylor and Francis Inc.; 2010. p. 327–32.
144. Yin G, Huang J, Petela J, Jiang H, Zhang Y, Gong S, et al. Targeting small GTPases: emerging grasps on previously untamable targets, pioneered by KRAS. Vol. 8, Signal Transduction and Targeted Therapy. Springer Nature; 2023.
145. Neumann AJ, Prekeris R. A Rab-bit hole: Rab40 GTPases as new regulators of the actin cytoskeleton and cell migration. Vol. 11, Frontiers in Cell and Developmental Biology. Frontiers Media SA; 2023.
146. Maria Fimia G, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, et al. Ambra1 regulates autophagy and development of the nervous system. Nature. 2007 Jun 28;447(7148):1121–5.
147. Li X, Lyu Y, Li J, Wang X. AMBRA1 and its role as a target for anticancer therapy. Vol. 12, Frontiers in Oncology. Frontiers Media S.A.; 2022.
148. Faienza F, Polverino F, Rajendraprasad G, Milletti G, Hu Z, Colella B, et al. AMBRA1 phosphorylation by CDK1 and PLK1 regulates mitotic spindle orientation. Cellular and Molecular Life Sciences. 2023 Sep 1;80(9).

149. Maiani E, Milletti G, Cecconi F. The pro-autophagic protein AMBRA1 coordinates cell cycle progression by regulating CCND (cyclin D) stability. Vol. 17, Autophagy. Taylor and Francis Ltd.; 2021. p. 4506–8.
150. Cianfanelli V, Nazio F, Cecconi F. Connecting autophagy: AMBRA1 and its network of regulation. Vol. 2, Molecular and Cellular Oncology. Taylor and Francis Ltd.; 2015.
151. Di Bartolomeo S, Corazzari M, Nazio F, Oliverio S, Lisi G, Antonioli M, et al. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. Journal of Cell Biology. 2010 Oct 4;191(1):155–68.
152. Gómez-Virgilio L, Silva-Lucero MDC, Flores-Morelos DS, Gallardo-Nieto J, Lopez-Toledo G, Abarca-Fernandez AM, et al. Autophagy: A Key Regulator of Homeostasis and Disease: An Overview of Molecular Mechanisms and Modulators. Vol. 11, Cells. MDPI; 2022.
153. Antonioli M, Albiero F, Piacentini M, Fimia GM. Temporal regulation of autophagy response by the CULLIN 4-AMBRA1-CULLIN 5 axis. Mol Cell Oncol. 2016 Sep 2;3(5).
154. Simoneschi D, Rona G, Zhou N, Jeong YT, Jiang S, Milletti G, et al. CRL4AMBRA1 is a master regulator of D-type cyclins. Nature. 2021 Apr 29;592(7856):789–93.
155. Maiani E, Milletti G, Nazio F, Holdgaard SG, Bartkova J, Rizza S, et al. AMBRA1 regulates cyclin D to guard S-phase entry and genomic integrity. Nature. 2021 Apr 29;592(7856):799–803.
156. Chaikovsky AC, Li C, Jeng EE, Loebell S, Lee MC, Murray CW, et al. The AMBRA1 E3 ligase adaptor regulates the stability of cyclin D. Nature. 2021 Apr 29;592(7856):794–8.
157. Shanabag A, Armand J, Son E, Yang HW. Targeting CDK4/6 in breast cancer. Experimental and Molecular Medicine. Springer Nature; 2025.
158. Di Leo L, Bodemeyer V, Bosisio FM, Claps G, Carretta M, Rizza S, et al. Loss of Ambra1 promotes melanoma growth and invasion. Nat Commun. 2021 Dec 1;12(1).
159. Di Rienzo M, Romagnoli A, Refolo G, Vescovo T, Ciccosanti F, Zuchegna C, et al. Role of AMBRA1 in mitophagy regulation: emerging evidence in aging-related diseases. Autophagy. Taylor and Francis Ltd.; 2024.
160. Schoenherr C, Byron A, Frame MC. A novel transcriptional signalling pathway mediated by the trafficking protein Ambra1 via scaffolding Atf2 complexes [Internet]. 2020. Available from: http://biorxiv.org/lookup/doi/10.1101/2020.01.08.899328
161. Cosgarea I, McConnell AT, Ewen T, Tang D, Hill DS, Anagnostou M, et al. Melanoma secretion of transforming growth factor-β2 leads to loss of epidermal AMBRA1 threatening epidermal integrity and facilitating tumour ulceration*. British Journal of Dermatology. 2022 Apr 1;186(4):694–704.
162. Nazio F, Po A, Abballe L, Ballabio C, Diomedi Camassei F, Bordi M, et al. Targeting cancer stem cells in medulloblastoma by inhibiting AMBRA1 dual function in autophagy and STAT3 signalling. Acta Neuropathol. 2021 Sep 1;142(3):537–64.
163. Sampath R, Vaeth K, Mikalayeva V, Skeberdis VA, Prekeris R, Han KJ. Rab40 GTPases regulate AMBRA1-mediated transcription and cell migration. J Cell Sci [Internet]. 2025 Apr 1;138(7). Available from: https://journals.biologists.com/jcs/article/138/7/jcs263707/367670/Rab40-GTPases-regulate-AMBRA1-mediated
164. Han KJ, Foster D, Harhaj EW, Dzieciatkowska M, Hansen K, Liu CW. Monoubiquitination of survival motor neuron regulates its cellular localization and Cajal body integrity. Hum Mol Genet. 2016 Apr 1;25(7):1392–405.
165. Krishan A. RAPID FLOW CYTOFLUOROMETRIC ANALYSIS OF MAMMALIAN CELL CYCLE BY PROPIDIUM IODIDE STAINING.
166. Horzum U, Ozdil B, Pesen-Okvur D. Step-by-step quantitative analysis of focal adhesions. MethodsX. 2014;1(1):56–9.

167. Nazio F, Strappazzon F, Antonioli M, Bielli P, Cianfanelli V, Bordi M, et al. MTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. Nat Cell Biol. 2013 Apr;15(4):406–16.
168. Antonioli M, Albiero F, Nazio F, Vescovo T, Perdomo AB, Corazzari M, et al. AMBRA1 interplay with cullin E3 Ubiquitin ligases regulates autophagy dynamics. Dev Cell. 2014 Dec 22;31(6):734–46.
169. Rojas AM, Fuentes G, Rausell A, Valencia A. The Ras protein superfamily: Evolutionary tree and role of conserved amino acids. Vol. 196, Journal of Cell Biology. 2012. p. 189–201.
170. Neumann A, Sampath R, Mayerhofer E, Mikalayeva V, Skeberdis VA, Sarapinienė I, et al. Mono-Ubiquitylation-Dependent Rap2 Activation Regulates Lamellipodia Dynamics During Cell Migration [Internet]. 2025. Available from: http://biorxiv.org/lookup/doi/10.1101/2025.04.25.650668
171. Neumann, A., Sampath, R., Han, K. J., & Prekeris, R. (2025). Rap1 activity and localization is regulated by Rab40-CRL5facilitated mono-ubiquitylation. *microPublication biology*, *2025*, 10.17912/micropub.biology.001629. https://doi.org/10.17912/micropub.biology.001629
172. Hinz, B., Alt, W., Johnen, C., Herzog, V., & Kaiser, H. W. (1999). Quantifying lamella dynamics of cultured cells by SACED, a new computer-assisted motion analysis. *Experimental cell research*, *251*(1), 234–243. https://doi.org/10.1006/excr.1999.4541
173. Katoh K. (2024). Signal Transduction Mechanisms of Focal Adhesions: Src and FAK-Mediated Cell Response. *Frontiers in bioscience (Landmark edition)*, *29*(11), 392.

PUBLICATIONS & CONFERENCES

Main publications related to the dissertation:

1. Neumann, A.; **Sampath, R.**; Mayerhofer, E.; Mikalayeva, V.; Skeberdis, V. A.; Sarapinienė, I.; Prekeris, R. Ubiquitylation-dependent Rap2 activation regulates lamellipodia dynamics during cell migration. *Journal of Cell Science*. Cambridge: The Company of Biologists. ISSN 0021-9533, 2025, vol. 138, p. 1-83. doi: https://doi.org/10.1242/jcs.264375. [Impact factor: 3.6, CiteScore: 7.21].
2. **Sampath, R.,** Vaeth, K.; Mikalayeva, V.; Skeberdis, V. A.; Prekeris, R.; Han, K.-J. Rab40 GTPases regulate AMBRA1-mediated transcription and cell migration. *Journal of Cell Science*. Cambridge: The Company of Biologists. ISSN 0021-9533, 2025, vol. 138, no. 7, p. 1-15. doi: https://doi.org/10.1242/jcs.263707. [Impact factor: 3.6, CiteScore: 7.21]

Other publications related to the dissertation:

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Conference presentations:

1. **Sampath, R.**; Prekeris, R. RAP2 functions in regulating small monomeric proteins and their role in migratory phenotypes in breast cancer // *Health for All 2025 "Healthy Beginnings, Hopeful Futures": Abstract Book: April 4, 2025*. Kaunas: Lithuanian University of Health Sciences. 2025, p. 18–19. Available at: https://hdl.handle.net/20.500.12512/251416.
2. **Sampath R.,** Mikalayeva, V.; Prekeris, R. The role of Rab40 family proteins in regulating Rap1 and Rap2 mechanisms during breast cancer cell migration [Conference winner abstract] // *International Health Sciences Conference for All (IHSC for All) "Precision Medicine": Abstract Book, March 25–26, 2024,* Kaunas: Lithuanian University of Health Sciences, 2024, p. 471–473. Available at: https://smd.lt/uploads/publications/IHSCforAll2024.pdf.
3. **Sampath R.,** Mikalayeva, V.; Prekeris, R. Migration of breast cancer cells is influenced by mutant GTPases of RAB40 and RAP2 // *XVIth International Conference of the Lithuanian Biochemical Society*

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4. **Sampath R.,** Mikalayeva, V.; Prekeris, R. The role of Rab40 and its subfamilies in regulating breast cancer cell mechanisms // *FEBS Open Bio: FEBS 2023 – The 47th FEBS Congress "Together in Bioscience for a Better Future", July 8–12, 2023, Tours, France*. Wiley. ISSN 2211-5463, 2023, vol. 13, suppl. 2, p. 73–73. doi: https://doi.org/10.1002/2211-5463.13646. [Impact factor: 2.8, overall Citescore: 5.136]
5. **Sampath, R.,** Mikalayeva, V.; Prekeris, R. The mechanisms regulating breast cancer cell migration and invasion // *Lithuanian Biochemical Society 2022 Mini-Conference "Biochemistry in the Big Data Age": Programme and Abstract Book, September 30, 2022, Vilnius* / Lithuanian Biochemical Society. Vilnius: Lithuanian Biochemical Society, 2022. ISBN 9786099603018, p. 25–25. Available at: https://hdl.handle.net/20.500.12512/115986.

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