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Subcelulární lokalizace a úloha exocyst komplexu v savčích buňkách během cytokineze

Subcellular localization and functional significance of exocyst complex in mammalian cells during cytokinesis

Diplomová práce

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Diploma Thesis

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Prague 2011

Author's Declaration

I declare that the work described in this thesis has been carried out by me unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree.

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Abstrakt

Posledním krokem buněčného cyklu je cytokineze. Během ní dojde k oddělení dvou dceřiných buněk. Tohoto procesu se účastní množství membránových struktur jako jsou endoplazmatické retikulum a *trans*-Golgi komplex. Kromě toho je v posledních letech stále více zdůrazňována úloha recyklačních endozomů. Všechny tyto organely spolu vzájemně komunikují pomocí vnitrobuněčného transportu, který je velice důležitý pro úspěšný průběh cytokineze zejména z pohledu nově vznikající plazmatické membrány oddělujících se dceřinných buněk. Nedávné studie odhalily, že tato membránová dynamika je regulována tzv. malými GTPázami z proteinové nadrodiny Ras. Další proteiny s významnou úlohou během membránového transportu jsou takzvané poutací komplexy, které směrují příslušné vnitrobuněčné komponenty k cílové plazmatické membráně, kde usnadňují vzájemnou fúzi. Nejlépe prozkoumaným poutacím komplexem je exocyst komplex. Jeho přítomnost byla prokázána ve všech typech eukaryotních buněk. Tento proteinový komplex je složený z osmi podjednotek Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 a Exo84. Ačkoliv přesný mechanizmus zůstává neznámý, zdá se, že dochází k interakcím mezi podjednotkami exocyst komplexu a zástupci Ras rodiny proteinů. Je proto možné, že se tyto proteiny podílejí na regulaci transportu vnitrobuněčných váčků směrem k plazmatické membráně a tím i průběh cytokineze (in English).

Klíčová slova: abscise, zaškrcování, telofázní tělísko, vezikulární/membránový transport, vázající komplex, Ras GTPázy

Abstract

Cytokinesis is the last step of cell cycle when two individual daughter cells separate in process called abscission. This process involves various cellular membrane structures such as endoplasmic reticulum or *trans*-Golgi network. Moreover, recent investigation has also highlighted an important role of recycling endosomes. The membrane dynamics appear to be important during cell division especially for the formation of new plasma membrane between two daughter cells. Numerous studies suggest that cytokinesis is tightly linked with highly sophisticated transmembrane shuttle that is controlled by Ras-superfamily members such as Rab and Ral proteins. Moreover, during last years has also been revealed the involvement of tethering factors which mediate the fusion of intracellular vesicles with the target plasma membrane. The best known tethering factor is the evolutionary conserved exocyst complex found in all eukaryotic cells. This protein complex is composed of eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) and was found to interact with members of Ras-superfamily suggesting its involvement in the regulation of cytokinesis. Although the exact mechanism remains shrouded in fog this work suppose the possible interactions among Ras-like proteins and exocyst members which may regulate the vesicle transport from recycling endosomes to the plasma membrane and thus mediate the last steps of cell division.

Key words: abscission, furrow ingression, midbody, vesicle/membrane trafficking, tethering complex, Ras-like GTPases

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Abbreviations

APS	Ammonium persulphate
Arf	ADP-ribosylation factor
BSA	Bovine serum albumin
CHO	Chinese hamster ovary cell line
COS	A cell line derived from kidney cells of the African green monkey
COS-1	A form of COS cell line
D-MEM	Dulbecco's Modified Eagle's Medium
DEPEC	Diethyl dicarbonate
dpm	Disintegrations per minute
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(2-aminoethylether)-N,N,NJ,NJ-tetraacetic acid
EE	Early endosome
ER	Endoplasmic reticulum
g	Gravitational force
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
^{3}H	tritium, a radioactive isotope of hydrogen
HeLa cells	An immortal cell line derived from human epithelial cervical cancer cells taken from a patient Henrietta Lacks
HEPES	N-(2-Hydroxyethyl)-1-piperazine-N'-2-ethanesulphonic acid
HES buffer	A cell lysis buffer composed of HEPES/EDTA/sucrose
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
LE	Late endosome
M	Molar
MAP	Microtubule-associated protein
MDCK cell line	a Madin-Darby canine kidney cell line
mRNA	messenger RNA
MT	Microtubule

Na^+/K^+-ATPase	Sodium-Potassium Adenosine Triphosphatase, or simply known as sodium-potassium pump
Ouabain	1β,3β,5β,11α,14,19-Hexahydroxycard-20(22)-enolide
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween 20
PM	Plasma membrane
PNS	Post-nuclear supernatant
Rab	Ras protein in brain
Ras	Rat Sarcoma
RAS	Genes encode Ras-superfamily-proteins
RE	Recycling endosome
RE-vesicles	Vesicles associated with membrane-trafficking-pathways which is accompanied by recycling endosomes
Rho protein	Small signaling GTPases of Rho subfamily belonging to Ras superfamily
RhoA	Protein-member of Rho subfamily, an effector molecule of ROCK protein
RNAi	RNA interference; its mechanism takes part in controlling gene activity within living cells. Two types of small RNA molecules, concretely microRNA (miRNA) and small interfering RNA (siRNA) are central to RNA interference mechanism
ROCK	Rho-associated kinase
sec genes	genes encode proteins required for vesicle transport in secretory pathways
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA, also known as short interfering RNA or silencing RNA. It is double-stranded RNA molecules which have 20-25 nucleotides in length
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment proteins receptor
TCA	Trichloroacetic acid
TEMED	N,N,NJ,NJ-Tetramethylethylenediamine
TfR	Transferrin Receptor

TGN	*trans*-Golgi network
Tris	Tris-(hydroxymethyl)-aminoethane
Tween 20	Polyethylene glycol sorbitan monolaurate
t-SNARE	a SNARE localized on the target membrane
T-75 flask	a tissue culture treated flask of growth area 75 cm^2 and volume 250 ml
v-SNARE	a SNAREs localized to the membrane of the trafficking vesicle
v/v	volume/volume ratio
w/v	weight/volume ratio

1 Introduction

1.1 Cell division

During cell division, two daughter cells are created from one parent cell following mitosis [1]. This process runs via a series of subsequent stages, as shown in Fig. 1; page 7. The period between two mitotic cell-cycles is known as interphase (not shown in Fig. 1; page 7). During this stage uncondensed chromosome is duplicated forming two copies called sister chromatides, but also other material such as centrosome is geminated.

Mitosis starts in prophase (Fig. 1, a; page 7) when duplicated centrosomes, which are composed of a pair of centrioles, travel around nucleus. This structure is responsible for microtubule nucleation forcing to the spindle poles. The cytoskeleton re-organization allows the microtubules of the mitotic spindle to assemble between the two centrosomes. Prophase passes at prometaphase (Fig. 1, b; page 7) where the nuclear envelope breaks down, allowing the microtubules to connect the replicated and fully condensed chromosomes via kinetochores. Due to the influence of mitotic spindle, the chromosomes are aligned at the equator of the cell (marked as "e" in Fig. 1, c; page 7), in a process termed congression. At metaphase, sister chromatides face opposite poles (marked as "p" in Fig. 1 , c; page 7) and microtubules are oriented with their plus ends to the poles [2]. During anaphase A (Fig. 1, d; page 7) sister chromatides are segregated and moved to the opposite poles under influence of the mitotic spindle. In anaphase B (Fig. 1, e; page 7), the division plane is determined by the spindle which sends a signal to the cell cortex. This has resulted in the location and creation of a contractile ring assembly [1]. Constriction of the contractile ring in late anaphase forms a cleavage furrow which has a very important role during cytokinesis. At telophase (Fig. 1, f; page 7), the two sets of chromosomes are located at the poles and a new nuclear envelope re-assembles around each of them. The contractile ring between daughter cells contracts (furrow ingression), while a barrier between the daughter cells is developing [2]. This "constricted barrier" is called the midbody. A final cut (in other words abscission) occurs to seal the furrow thus creating two independent daughter cells. Each of the cells contains one complement of chromosomes. By the end of telophase, the cytokinesis is completed, the nucleus and cytoplasmatic compartments of each daughter cell then return to interphase, marking the end of mitosis [3].

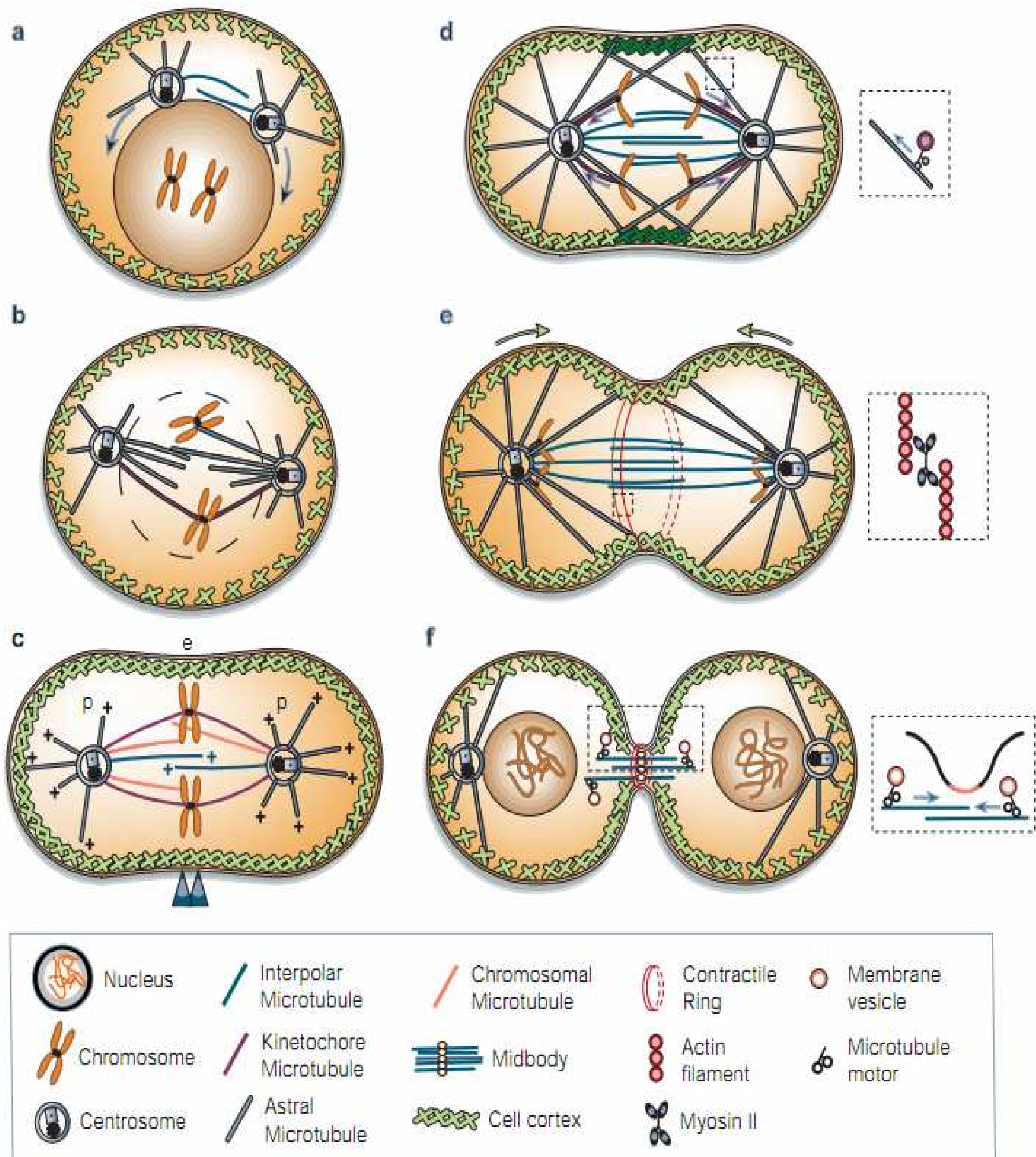


Fig. 1: Mitosis and cytokinesis (modified from [2]): **(a)** Prophase. **(b)** Prometaphase. **(c)** Metaphase. **(d)** Anaphase A. **(e)** Anaphase B. **(f)** Telophase/cytokinesis. Key: „e“ denotes the equator and „p“ marks each pole of the cell.

1.2 Cytokinesis

Cytokinesis was first observed more than 100 years ago and was described as a crucial and well conserved step in cell development and proliferation [4,5]. However, there are numerous biological situations (usually among different cell species) in which nuclear division is followed by a variation on the theme of cytokinesis [1]. For example,

plant cells divide by creating a new membrane compartment between the two daughter nuclei. This structure is known as phragmoplast and is believed to guide the formation of the membrane partition and the new wall [6]. It is formed during late anaphase by the fusion of Golgi-derived vesicles, which are transported along the microtubules to the plane of division [7]. The Golgi-derived vesicles, containing cell wall material, represent blocks for new emerging membrane between two daughter cells. One specialized form of cytokinesis was revealed in *Drosophila melanogaster* and is called cellularisation [8]. The interesting point is, that during early embryogenesis, the zygote undergoes thirteen nuclear divisions without cytokinesis (syncytial divisions) whereas during fourteenth cycle termed cellularisation membrane furrows develop in between the cortical nuclei [8]. In contrast, budding yeast cells division is asymmetric. A bud grows out from the mother cell cortex via polarized cell growth, thus forming actomyosin based-contractile ring at the bud neck. It is known that both, yeast and animal cells employ an actomyosin based-contractile ring to separate dividing cells [9]. For mammalian cytokinesis a formation of actomyosin contractile ring during anaphase is important which consists of actin, myosin II and other structural proteins. It is assembled beneath the plasma membrane, around the equator of the cell; at the mid-point between two nuclei [3]. The constriction of the contractile ring causes an invagination of the plasma membrane forming a cleavage furrow. This structure continues to ingress until the two daughter cells are separated [4].

1.3 The process of animal cytokinesis

The initiation of cytokinesis starts with the establishment of the cleavage site which is determined by an actomyosin-contractile ring assembly during early anaphase [4,10]. Constriction of this contractile ring induces invagination of the cell membrane while a cleavage furrow is ingressing. This leads to the formation of a narrow intracellular bridge linking the two daughter cells. The central region of the intracellular bridge is known as the midbody. Invagination of the intracellular bridge continues until the two daughter cells are pinched apart [4]. Abscission itself is a very complicated process and the mechanisms are currently intensively studied. In any case, recent investigations have highlighted that membrane trafficking plays a crucial role in the abscission [3,11-14]. For illustration see Fig. 2; page 9.

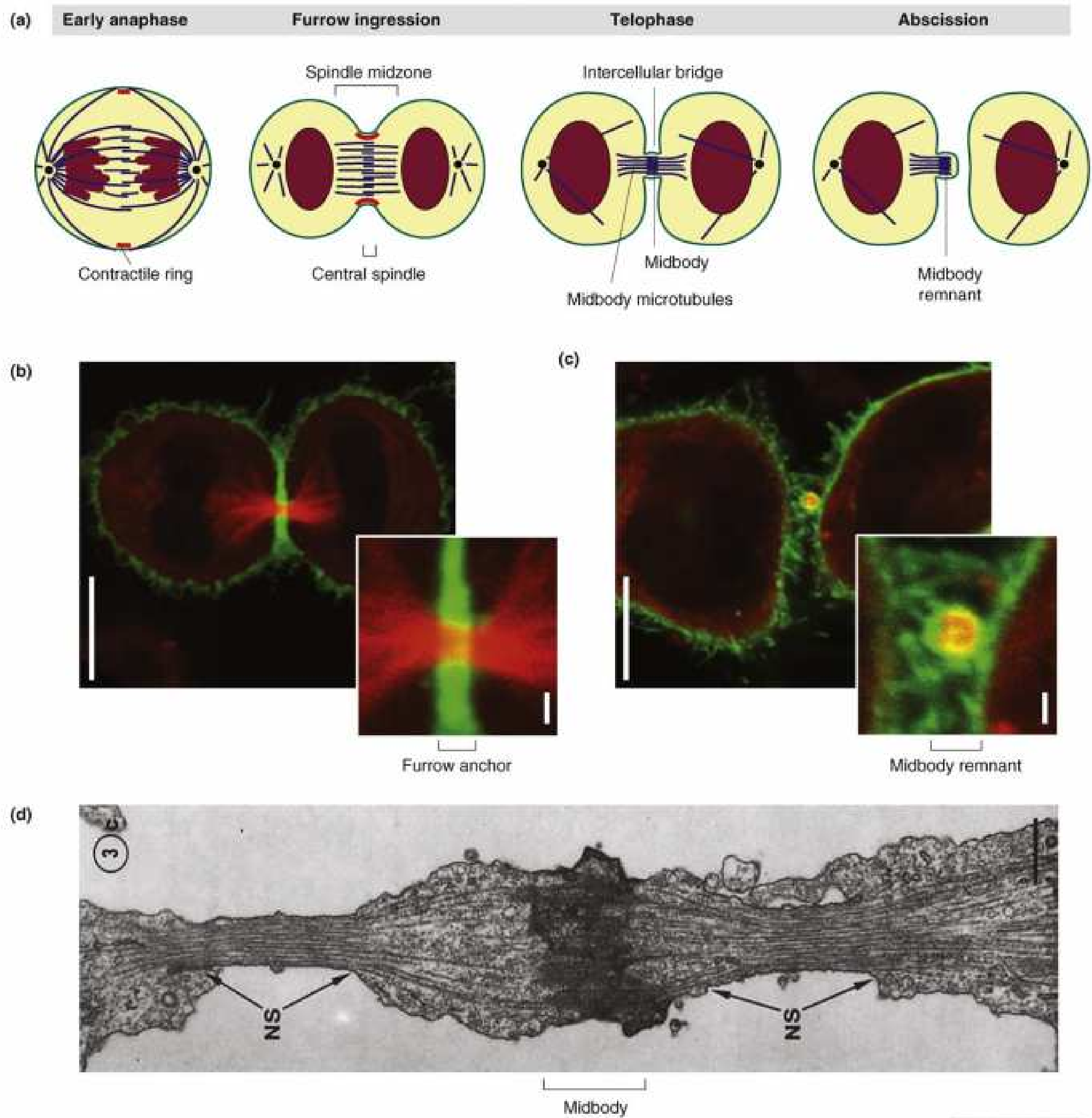


Fig. 2: Cytokinesis stages and the morphology of the intracellular bridge (modified from [15]): **(a)** Cytokinesis stages and morphology of the intracellular bridge, **(b)** Image of live HeLa cell at telophase stage. Midbody microtubules terminate at cytoplasmatic regions close to the nuclei. The ingressed furrow anchors to the midbody. The inset shows an enlarged image of the intracellular bridge, **(c)** the same markers as in (b) but at a post-abscission stage. The midbody remnant between the sister cells contains high levels of tubulin and is covered by plasma membrane, **(d)** electron micrograph of late stage of intracellular bridge. Lateral constriction zones (NS) flank the central electron-dense area of the midbody.

As indicated above, the animal cell cytokinesis is a far more abstruse process. Hence, this highly dynamic mechanism can be broken down into a series of steps based on cell timing and a multitude protein regulation that ensure accurate cell division [4].

1.3.1 Position of the cleavage furrow

The first step of cytokinesis is specification of the cleavage plane position, where the components of the contractile ring are destined to assemble [4]. The location of the contractile ring is specified by the location of mitotic spindle and thereby the position of the cleavage plane during early anaphase [10]. Generally, it could be said that the mitotic spindle is positioned centrally although a special situations can occur in different cell types, because they may adapt the general mechanism of the spindle positioning [16-18]. For example, during embryonic and stem cell division, the spindle is not arranged centrally and the divisions are asymmetrical [19,20]. There is no consensus declared on the cleavage plane specification. However, several models explain which parts of the mitotic spindle define the site of cleavage furrow formation [4]. All that theories are based on spatial and temporal re-arrangement of microtubule structures using diverse mechanisms which are under a control of protein kinases and Rho GTPases [21,22]. For example, it has been proved that inactivation of RhoA GTPase leads to a defect in cytokinesis [1,23-25].

1.3.2 The assembly and constriction of the contractile ring

Once the position of the cleavage furrow has been determined, the next key step is to assemble the contractile ring [1]. The contractile ring is, in large part, composed of actomyosin structures. It has long been acknowledged that actin is the driving force for remodeling cell shape during cell division [3]. More than forty years ago were discovered organized bundles of filaments in the cleavage furrow [26]. A few years later, was revealed that these filaments contain actin [27,28] and are primarily associated with the molecular motor myosin II [1,4,29]. Nowadays, there is evidence that sliding of actin filament by bipolar filaments of myosin II is critical for the progression of cytokinesis [30]. This mechanism is controlled by phosphorylation of myosin II [31] which rapidly increases upon initiation of cytokinesis [32]. Moreover, studies carried out on mutants which cannot be phosphorylated causes cytokinesis defects [33].

It has been long time known that activity of this actomyosin structure causes the cell-shape remodeling but the mechanism has been still unclear and therefore, many hypotheses has dealt with this question. Currently, just only one general contractile-ring

hypothesis is accepted [3,34]. In 2005 was proposed an idea referring to the co-assembly of actin and myosin II filaments into contractile bundle around the equator that leads to the formation of contractile filaments that are drawn together like a „purse string“ by action of myosin II [3,34].

Reshaping of the cell during cytokinesis is complex process and is under control of various molecular mechanisms. A small GTPases from Rho family are main regulators of actin dynamics [35] and during mammalian cytokinesis play the crucial role [36]. These GTPases are accumulated at the future site of furrow ingression [3,37,38] where they bind their specific protein effectors. The two most important effector groups are proteins from formin family and Rho-associated kinases (ROCK) [35]. In the first case, interaction between RhoA and the actin-assembly-factor called formin triggers the formation of the contractile ring [36]. In the second case, RhoA activation results in the activation of ROCK which induces phosphorylation of myosin II. This causes assembly of myosin II into filaments and their subsequent interaction with actin. Thus, the actin-dependent ATPase activity of the motor domain is activated [30] allowing the furrow formation.

During furrow ingression a central spindle is formed. Inside the central spindle, a dense array of antiparallel microtubule bundles is created due to a large number of microtubule bundling factors [15,39,40]. These bundles of microtubules overlap at their plus ends in the midbody region. Simultaneously, the contractile ring contracts via RhoA-depending mechanism [15,38,41]. In simplified words, the formation of central spindle during anaphase is followed by congression and bundling of microtubules which leads to the intracellular bridge assembly [15].

1.3.3 The midbody

Nowadays, the midbody is also known as Flemming body, stembody or midbody-ring. This structure was originally described by Flemming as „Zentralkörper“ [42]. It is formed during the late stages of telophase when the cleavage furrow reaches the point where it cannot be ingressed any further. At this time, a narrow intracellular bridge connects the two new daughter cells. This structure is able to persist several hours (generally from 2 to 3 hours) before the final physical separation [43]. It has been established

that the stable midbody is essential for successful cytokinesis, providing a scaffold that facilitates progression through the final abscission [44-46]. As mentioned in section 1.3.3; page 11 the midbody contains an anti-parallel bundles of microtubules which overlap at the central region of intracellular bridge [15] (for more details see Fig. 2; page 9). Even though exact composition of the mammalian midbody has not been revealed yet some of the components were successfully mapped in 2004, when Skop and co-workers performed proteome analysis of the midbody from purified Chinese hamster ovary cells (CHO) [5]. One hundred and sixty proteins were identified and were classified into five groups. The largest group involves 33 % of proteins that participate in secretory pathway. This suggests that the vesicular transport may play crucial role in cytokinesis. Another 29 % of proteins are actin-associated proteins, 11 % are microtubule-associated proteins, and another 11 % are protein kinases. The remaining 16 % of the midbody proteins are difficult to classify into multiple groups but they may potentially regulate cytokinesis [5]. During 2005, Albertson and co-workers demonstrated a presence of two small GTPases - Arf6 (ADP-ribosylation factor 6) and Rab11 in the midbody and their direct involvement during cell division [11]. This two GTPases are coupled to the exocyst complex which is one of the most important elements in targeting vesicles to the site of abscission. At the same time another group pointed out that in both, yeast and mammalian cells, another small G-protein RalA appears at the site of abscission, where it may promote vesicle tethering to the plasma membrane (PM) via exocyst complex assembling [47].

Another challenge for studying of the midbody structure is its function. It has been proposed that the midbody provides an anchor for the ingressed furrow. Initially, the ingressed furrow still contains remnants of the actomyosin contractile ring, which probably contributes to its mechanical stability [15].

Apart from this, the midbody appears to mediate another two functions. First, it seems to play a role as the docking site for vesicles, which supply the plasma membrane with new material at the site of abscission. Second, the midbody ring seems to function as a diffusion barrier [46]. Previous studies indicated that the midbody structure prevents plasma membrane diffusion across the intercellular bridge [46,48]. This diffusion barrier hold back trans-membrane proteins or proteins anchored to the inner leaflet of the plasma membrane. Proteins anchored to the outer leaflet are not constrained. Diffusion barriers have been also

observed at the bud neck of budding yeasts where a various cytokinesis factors are confined at the site of abscission [49]. However the exact molecular structure of midbody diffusion barriers in higher eukaryotes remains unknown [48].

1.3.4 Abscission and completion of cytokinesis

Cytokinesis terminates with the cleavage of the intracellular bridge in a poorly understood process called abscission [50]. Central to this process is the midbody, which is the target site for membrane delivery and membrane fusion, the driving forces for abscission [46]. This active process is under intensive research. It is generally accepted that abscission might be regulated by diverse mechanisms. The cellular material such as microtubules, actin from contractile ring, and cellular organelles needs to be displaced from the site of abscission. Coinciding with abscission, midbody microtubule bundles adjacent to the one side of the midbody abruptly disassemble [15]. It is very interesting that the disruption of the midbody is asymmetrical. If it depends on any specific signals sent by one of the two daughter cells or if it is stochastic is under deep investigation. Interestingly in some cell types, it has been observed that after cell separation, the midbody remnant can persist in one of the sister cells throughout multiple cell cycles [51]. In other cells it can be degraded by autophagy [52]. Regarding to the actin, disassembly of the contractile ring is also required for successful cytokinesis. When the contractile ring has accomplished the task of cleavage furrow ingression, it must be disassembled, either concurrent with or after the formation of an intracellular bridge [44]. The exact relative timing has not been determined but it is known that disassembly of actin could be controlled by inactivation of RhoA proteins [15].

Differences between cell lines or tissues have been observed and several models of abscission process, which are not mutually exclusive, have been proposed. For more details see Fig. 3; page 14.

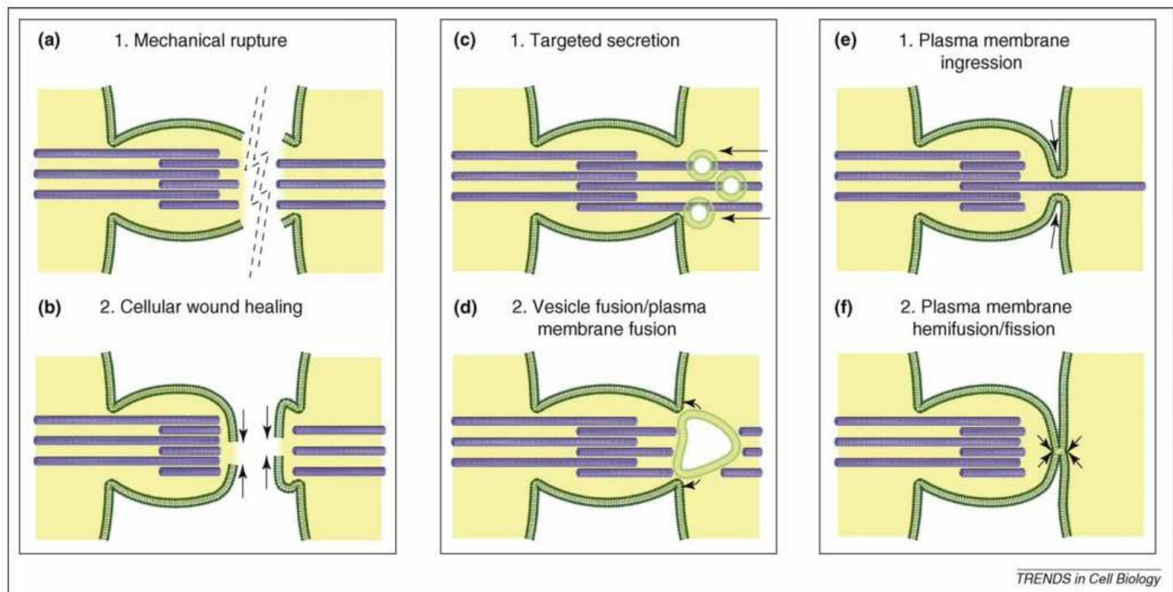


Fig. 3 Proposed models for the mechanism of abscission (modified from [15]): Blue, microtubules; dark green, plasma membrane; light green, vesicles; cream, cytoplasm. The schematic diagrams show the intercellular bridge in **(a, c, e)** early and **(b, d, f)** later stages of abscission. **(a, b) Mechanical rupture model. (a)** Traction forces between sister cells lead to tearing of the intercellular bridge. **(b)** This is followed by resealing of the plasma membrane by cellular wound healing **(c, d) Model of internal vesicles filling the intercellular bridge. (c)** Golgi-derived and endocytic vesicles first accumulate close to the midbody. A key factor for targeting of SNAREs to the midbody is centriolin, which also drives the exocyst complex to the same place [51]. **(d)** Vesicles then fuse with each other and the plasma membrane to support splitting of the sister cells. **(e, f) Constriction of plasma membrane for fission model. (e)** Coordinate disassembly of microtubule bundles adjacent to the midbody and ingression of the plasma membrane of the intercellular bridge. **(f)** This permits direct hemi-fusion and fission of opposing plasma membrane regions.

1.4 Membrane trafficking

1.4.1 Intracellular membrane trafficking pathways

The internal environment of the cells consists of different membrane compartments, called organelles – the nucleus, endoplasmic reticulum (ER), Golgi apparatus (GA), mitochondria, endosomes and lysosomes [53]. Eukaryotic cells have developed very smart mechanism responsible for communication among these organelles. It transports various substances from inner cell towards the surface, as well as for acceptance and subsequent internalization of various nutrients coming from the external environment. This mechanism is called vesicular trafficking.

There are two main vesicular trafficking pathways – the secretory pathway and the endocytic pathway. Briefly, the secretory pathway allows cells to send out newly synthesized macromolecules such as proteins, lipids and carbohydrates, towards the cell surface in such a way that the membrane bound vesicles bud-off from the donor organelle, traffic to the target PM where tether with this compartment membrane and finally dock and fuse [54,55]. The endocytic system has the opposite direction. Material coming from the extracellular environment, as well as some compartments of the PM, is packed into the vesicles and proceeds to the inner cell where vesicles are sorted according to the needs of the cell metabolism. Despite the opposite directions of transport, these two pathways are intricately linked.

Various protein families are known to be implicated in regulation of the membrane dynamics. For example, studies have proved involvement of SNARE proteins [56] (detailed in chapter 1.5.4; page 22). Also GTPases of the Rab subfamily play a key role in many, if not all, membrane traffic steps [57] (detailed in chapter 1.5.3; page 18). Both pathways consist of several organelles differing in chemical structure and thus a distinct function.

1.4.1.1 The secretory pathway

The secretory system or exocytic pathway involves the endoplasmatic reticulum (ER) and the Golgi apparatus (GA) with its *trans*-Golgi network (TGN). The first steps of this pathway are situated to the ER. This membrane compartment consists of many interconnecting membrane tubules and cisternae. Its function is to control a quality of the nascent synthesized polypeptides. Only the correctly folded proteins may continue throughout the TGN [58]. The GA is a place where proteins and lipids are modified then sorted, packed to the membrane vesicles and directed to the PM prior to secretion and exocytosis. Some of them can be retained in the ER or Golgi system. Thus GA serves as a central junction for membrane traffic [57]. In general, TGN is a major sorting centre for cell lipids and proteins at the crossroads of the secretory and endocytic pathways [59].

1.4.1.2 The endosomal pathway

Regarding the endocytic system, it consists of early endosomes (EEs), recycling endosomes (REs), late endosomes (LEs) and lysosomes. These membrane compartments may differ morphologically and have distinct properties [60]. Endocytosis is a diverse set of processes used by cell to internalize regions of the PM simultaneously with lipids, receptors and other transmembrane components as well as small amount of extracellular fluids [61,62]. These cargos have normally several destinations depending on requirements. For example, LE and lysosomes are the place of degradation whereas RE direct the cargo back to the PM [63], [64].

1.5 Membrane trafficking in cytokinesis

1.5.1 The role of membrane trafficking in animal cytokinesis

It has been known that membrane trafficking is central to plant cell division. For animal cells, it has been long thought that cytokinesis is driven "only" by constriction of the actomyosin ring. However, in recent years has been demonstrated that membrane trafficking plays also an essential role in mammalian cytokinesis [3,11,13,36].

The importance of membrane trafficking during cytokinesis lies on several processes. First, during ingression of the cleavage furrow the cell shape is changed and new surface of the PM is created. Second, the delivery and subsequent vesicle fusion are important to shut the intracellular canal between two daughter cells. In other words, membrane trafficking plays crucial role when the intracellular bridge is resolved. The vesicles are thought to accumulate at the furrow, and eventually fuse with the PM. This results in the abscission of the two daughter cells [15]. Third, the membrane traffic is necessary for delivery of various proteins to PM, such as signaling and structural macromolecules, that are required for the proper cytokinesis. Studies have also shown that the lipid and protein composition of the PM at the cleavage furrow is different in this phase of cell cycle [65].

1.5.2 Origin of the membrane delivered to the cleavage furrow

Membrane delivery to cleavage furrow triggers changes in surface area required for abscission. It was believed that this additional membrane is derived from expansion of pre-existing plasma membrane. But Bluemink and co-workers [66] showed that this additional PM is delivered from internal stores. During last decade, the origin of this furrow-membrane has been intensively studied [4]. Interestingly, it has been found that secretory as well as endocytic pathways are involved because both types, the secretory and the endocytic vesicles have been found to accumulate adjacent to the midbody in mammalian cells [11,51,67].

1.5.2.1 Role of the secretory pathway in animal cytokinesis

One theory of the membrane-origin delivered to the cleavage furrow says that the secretory pathway is involved [68]. Further evidence is implicating the Golgi-membrane as an important source of membrane delivered to the midbody during cell division [11]. When the cell enters the mitosis, the GA is disassembled and thus provides the membranes for the invagination of the cleavage furrow [69]. A variety of evidences have also shown that many of Golgi-associated proteins play a key role during cytokinesis. The research, using small interfering RNA (siRNA) screening, uncovered many of these proteins that are crucial for early and late stages of cytokinesis. Almost one quarter of the proteins located in the midbody was Golgi-associated proteins [5].

1.5.2.2 Role of the endosomal pathway in animal cytokinesis

It was discovered that endocytosis-based membrane trafficking is also essential for the successful cytokinesis [13,50,70,71]. Membranes delivered to the cleavage furrow originate from internal stores such as EE, LE and RE [72,73] utilizing endosomal vesicles [11]. Recent studies have highlighted REs function in cytokinesis. It is thought that protein and lipid macromolecules typical for REs may facilitate the process of cytokinesis because these molecules have been found in the cleavage furrow. REs also localized to the so called microtubule-organizing centre, which actively drives the mitotic spindle dynamics and the positioning of the contractile ring [11].

According to previous studies [74], it was thought that endocytosis terminates during mitosis and then is simply resumed after cell division. But variety of evidence has shown that membranes at the cleavage furrow are derived via endocytosis. This trafficking pathway, as an important mechanism for the completion of eukaryotic cytokinesis, was specifically observed in zebrafish embryos [75]. A few years later, endocytosis was observed also in cultured mammalian cells [13,50,71]. The research revealed that at the beginning of mitosis, as the cell rounds up, endocytic recycling considerably slows and thus it was observed a decrease in surface area of the plasma membrane [76]. At the same time, the contractile ring is formed and the furrow ingresses. As cell enters the anaphase, endocytotic recycling resumes its activity. In this ways the cell surface area recovers before abscission. It has been shown that this mechanism is necessary for completion of animal cytokinesis perhaps because of the delivery of cargo required for abscission [74,76].

On the other hand, the exact mechanisms of cytokinesis remains poorly understand. But despite this fact, a variety of endocytic proteins, such as GTPase dynamin, SNARE, and a variety of vesicle-tethering factors or members of Ras superfamily, have been shown to be important for the final events of animal cytokinesis [54,56,77].

1.5.3 The superfamily of Ras-like GTPases

The human Ras superfamily of small guanine nucleotide-binding proteins (often called small G-proteins) has around 200 members which can be divided into five basic families: Ras, Ran, Rho, Rab, and Arf [78]. These proteins regulate wide range of cellular mechanisms. Ras-family members control a signaling pathways, resulting in transcription, resulting in transcription and cellular proliferation, Ran family members play an important role in nuclear envelope formation and the spindle formation, Rho members are responsible for regulation of cell-shape, and cell migration, whereas Rab and Arf proteins are mainly involved in vesicle-associated processes, ranging from vesicle budding to vesicle directing [79].

The small G-proteins are typically between 20-25 kDa in size. In general, their activity is under control of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs and GAPs are multidomain proteins which are regulated by extracellular signals. These proteins control cellular events in time and space [79]. As shows Fig. 4, GEFs promotes GDP release and exchange for GTP. This on-state causes the assembly of protein with its downstream effector molecules. On the other hand, GAPs promote GTP hydrolysis and turn the protein activity off [80].

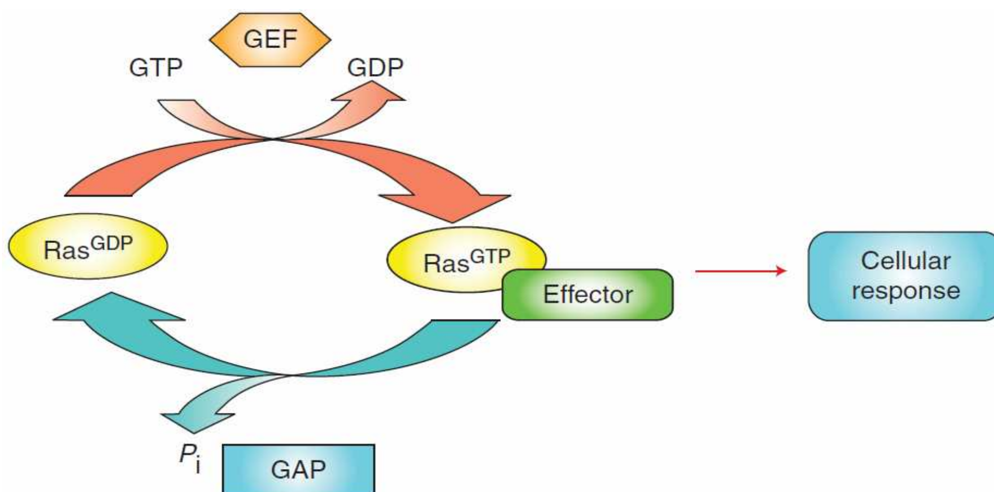


Fig. 4: The GTPase cycle. Protein-molecule from Ras superfamily in this example (modified from [81]): A cycle between inactive GDP-bound state and active, GTP-bound state. Following a specific stimulus, GEFs catalyze the exchange of GDP for GTP, enabling the interaction of GTPases with specific effectors leading to cellular responses. In contrast, GAPs inactivate GTPases by stimulating their intrinsic GTPase activity.

It is very difficult to investigate the mechanism of membrane trafficking step by step. GTPases of Ras, Rab and Arf families were identified in the furrow and midbody [14,82,83]. It is proposed that the GTPases may interact with members of exocyst complex and thereby support membrane trafficking events during cell division.

1.5.3.1 Rab proteins

The proteins of Rab family represents the largest group in the Ras superfamily, consisting of approximately 60 family members in humans [84]. The distinct members of Rab proteins are usually localized to the cytosolic side of intracellular membrane compartments, where they control various processes in vesicular membrane traffic

pathways. It is well established that Rab GTPases are not only restricted to membrane budding and fusion. It also determine the distribution of cellular compartments by regulating the movement of vesicles and organelles along the cytoskeletal filaments [85]. Thus these proteins communicate with different organelles within the secretory and endosomal pathways. In active GTP-bound form, Rab proteins are able to recruit different effector molecules onto membranes and through this control cellular trafficking events such as vesicle formation, actin- and tubulin-dependent movement, cargo sorting and membrane fusion [80,84]. Rab proteins have been found in all eukaryotes. This suggests that interactions between Rab proteins and their general regulators and specific effectors is conserved across evolution, as the sequence determinants of these interactions are conserved in all Rab families [86].

In this context, Rab GTPases play a crucial role as coordinators of vesicular membrane trafficking during cell division. Recent studies pointed out to Rab and Ras members who have been revealed to be involved in cytokinesis events in mammalian cells; specifically on the Rab11a (an isoform of Rab11 founded in human genome), Rab35 and RalA protein [14,82,83].

Rab11 GTPases

Rab11 belongs to the small GTPase family, called Rab family. In humans, it is encoded by RAS family oncogene. Two Rab11 isoforms were found: Rab11a and Rab11b. Rab11a is ubiquitously expressed, while Rab11b is expressed mainly in the heart and brain [87,88]. The Rab11 proteins are involved in intracellular membrane fusion reaction, and are enriched in recycling endosomes and the TGN where are implicated in membrane recycling back to the plasma membrane [89,90]. Moreover, it has been shown traffic from centrosomes into the furrow from both daughter cells [14]. In mammals, it has been shown to co-localize with Sec15 (a subunit in of exocyst complex) [91,92].

Rab35 GTPases

Rab35 GTPases are encoded by RAS family oncogene and belongs to the Rab family. Rab35 is expressed ubiquitously and is localized at the plasma membrane and simultaneously at the endocytotic compartments where it controls recycling pathway [74,82]. Rab35 has been involved in abscission, where might be also responsible for stability of the

intracellular bridge because it seems to control the trafficking of septins (a family of guanine nucleotide-binding proteins which are implicated in filaments remodeling) [82]. Taken these data together, Rab35 seems to be essential for the terminal steps of cytokinesis in eukaryotes [44,82]. In conclusion it can be mentioned that functional impairments of Rabs cause defects during cell division which may cause various human diseases, such as immunodeficiencies and neurological disorders [93,94].

1.5.3.2 Ras proteins

The Ras family members are small monomeric GTP-binding proteins that regulate signal transduction pathways and thus are generally responsible for regulating cell proliferation and differentiation [95]. The mammalian Ras family members could be divided into three groups: H-Ras, K-Ras and N-Ras. It has been shown that members of the Ras family are present in all animal and fungal species, but have not been found in plants [95]. Mutations of these proteins preventing GTP hydrolysis result in persistent signaling and promote uncontrolled cell growth. These types of mutations were found in several human cancers. One of the important members of Ras family involved in cytokinesis steps is Ral protein [83].

Ral GTPases

RalA is a multifunctional small GTPase which plays an important role in apoptosis, cell cycle, cell proliferation and morphology, and oncogenetic transformation, exclusively in mammalian cells [96-98]. Ral is not found in yeast suggesting that its functions are very specific to multicellular organisms. Ral proteins are also involved in controlling intracellular vesicle trafficking. This GTPase was localized in the region of recycling endosomes and also in the cleavage furrow, and thereby later, at the site of abscission. Afterwards, it has been proved that this protein is implicated in trafficking of recycling endosomes which contributes to the completion of cytokinesis [99].

Two different isoforms, RalA and RalB, have been observed. It has been shown that RalA have much more higher affinity for exocyst subunits than RalB does [100]. A number of papers highlighted the interplay between RalA and the exocyst complex [101]. Exocyst complex mediate vesicle tethering and it has been shown that it plays an essential

role during cell division (for more detail see section 1.5.5; page 23). Although the mechanism have been still unknown, it is assumed that RalA may regulate the assembly of vesicle and plasma membrane via interaction with two subunits of exocyst complex, respectively Sec5 and Exo84 [83,102,103].

1.5.4 SNARE proteins

SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins were independently discovered in yeast cells and neurons during the late 1980s [42]. Since then it has been shown that these proteins belongs to a superfamily which is conserved in all eukaryotes. On the basis of their localization, SNARE proteins were initially classified into two groups: t-SNARE (for SNAREs localized on the target membrane) and v-SNAREs (for SNAREs localized to the membrane of the trafficking vesicle) [104,105].

Within the last two decades, research has shown that SNARE proteins are involved in intracellular trafficking. The subcellular localization of SNAREs and their ability to form the so-called SNARE complex (Fig. 5; page 23) may be essential to determining the specificity of intracellular fusion [106]. During membrane trafficking a cargo is packed into nascent transport vesicles that bud off from a donor compartment. This vesicle is then translocated along cytoskeletal tracks, and then dock and fuse with their target organelle. For docking and fusion is important the initial interaction at a distance (tethering), followed by a closer interaction that leads to pairing of vesicle v-SNARE proteins with target membrane t-SNAREs, thereby catalyzing vesicle fusion [107]. Thus SNARE proteins play a central role during final steps of vesicle trafficking. Interestingly, for successful fusion v-SNARE must interact with its appropriate t-SNARE [108]. This ensures not only the wide array of vesicle-mediated transport pathways but also it helps to achieve compartmentalization specificity. This is known as the SNARE hypothesis [104,109].

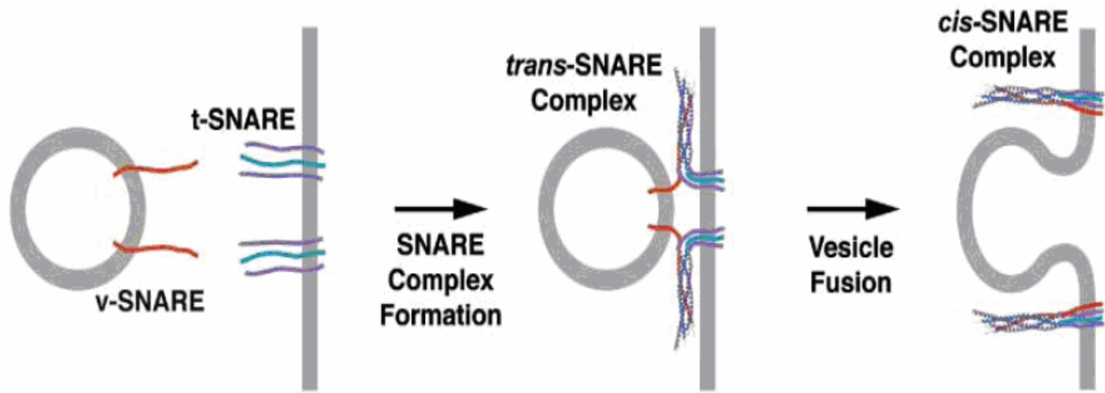


Fig. 5: The function of SNAREs (modified from [54]): A *trans*-SNARE complex assembles when a monomeric v-SNARE on the vesicle binds to an oligomeric t-SNARE on the target membrane, forming a stable four-helix bundle that promotes fusion. The result is a *cis*-SNARE complex in the fused membrane.

1.5.5 Vesicle-tethering factors – the exocyst complex

As mentioned earlier, for docking and fusion of the membrane compartments is also important the initial interaction at a distance - tethering. In all eukaryotes, the donor and target membranes are initially attached by so-called tethering factors. These proteins bring membranes in a suitable approach and thus allow SNAREs to interact together for contemporary fusion of membranes [110] (for more details see Fig. 6; page 25).

One protein group which appears to orchestrate vesicle docking and fusion is known as the exocyst complex [111]. The exocyst complex or simply exocyst is formerly also known as Sec6/8 complex. It is a large octameric complex that is composed of eight subunits Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. The members of exocyst were first identified through the isolation of temperature-sensitive secretory mutants (encoded by *sec* genes) from the yeast *Saccharomyces cerevisiae* [112]. Later, ten *sec* genes (genes encoding proteins required for vesicle transport in secretory pathways) involved in trafficking from GA to the cell surface has been revealed, where six of them belong to the subunits of the exocyst complex [113]. Studies in yeasts have shown that all of these exocyst members are hydrophilic proteins [111,112]. Studies in yeasts have shown that all of these exocyst members are hydrophilic proteins [114]. It was already pointed out that the homologous mammalian complex has similar role in membrane trafficking and that each of that eight subunit shares sequence similarity with the equivalent subunit from yeast [115]. With the ever-evolving and more sophisticated technologies in molecular biology

and biochemistry was possible to obtain crystal structures of several exocyst subunits not only in yeast models (mostly in *S. cerevisiae*) but also in *Drosophila melanogaster*. Overall, the sequence identity among the different subunits in the exocyst is ~ 10%. Moreover, all of them are predicted to have highly helical (~ 40% - 60%) compositions and indeed, each protein consists of several helical bundles [111]. Detailed knowledge of the subunits architecture may help to clarify the assembly and disassembly mechanism, and could also answer the question of how are these events controlled.

As already indicated, the exocyst complex mediates tethering of post-Golgi secretory vesicles to the PM before vesicle fusion [116]. A number of evidence signify that the exocyst is also required for membrane recycling [117,118]. Recycling endosomes are very essential sources of the cargos destined to the PM in many cell types. In addition, exocyst structure play an important role during internalization of the receptors which are recycled back to the cell surface [116]. It was proved that loss of exocyst function blocks recycling machinery resulting in accumulation of the recycling endosomes at the PM [119]. Thus, defects of the exocyst complex lead in vesicular trafficking failure.

Despite intensive research the exact mechanism of vesicle trafficking has been still unknown. A number of regulators which might drive the exocyst-depending machinery have been revealed. Particularly, several small GTPases from Ras-like super-family may directly regulate the exocyst function. For instance, in budding yeast the Sec15 is a down-stream effector of the Rab GTPase Sec4 (which triggers the assembly of the exocyst complex) [120] whereas in higher eukaryotes a downstream effector of the exocyst subunit Sec15 is protein Rab11 (which drives the regulation of vesicle transport from the recycling endosomes to the PM) [91,92]. Another effector molecule which regulate the assembly of the exocyst complex in neuroendocrine cells is Ral GTPase which interact with Sec5 and Exo84 subunits [96]. Finally, in non-polarized MDCK cells (a Madin-Darby canine kidney cell line) has been revealed that subunit Sec10 is a downstream effector of the small GTPase Arf6 (which helps to translocate Sec10 from RE to the PM during cell surface remodeling) [117].

Members of the exocyst family have been implicated in cytokinesis of eukaryotic cells [14,51]. Furthermore, current research have shown that the exocyst components have

been implicated in many other processes such as cell migration, or tumor invasion [121-123]. It has been revealed, that the members play also an important role in remodeling of epithelial polarity, oogenesis, or in polarized cell growth [7,116,118,124]. Moreover, exocyst contributes during neurotransmitter release at mature synapses, a specialized form of regulated exocytosis [124]. Taken this data together, studies *in vivo* have shown diverse functions of the exocyst complex at the tissue and organ levels [116].

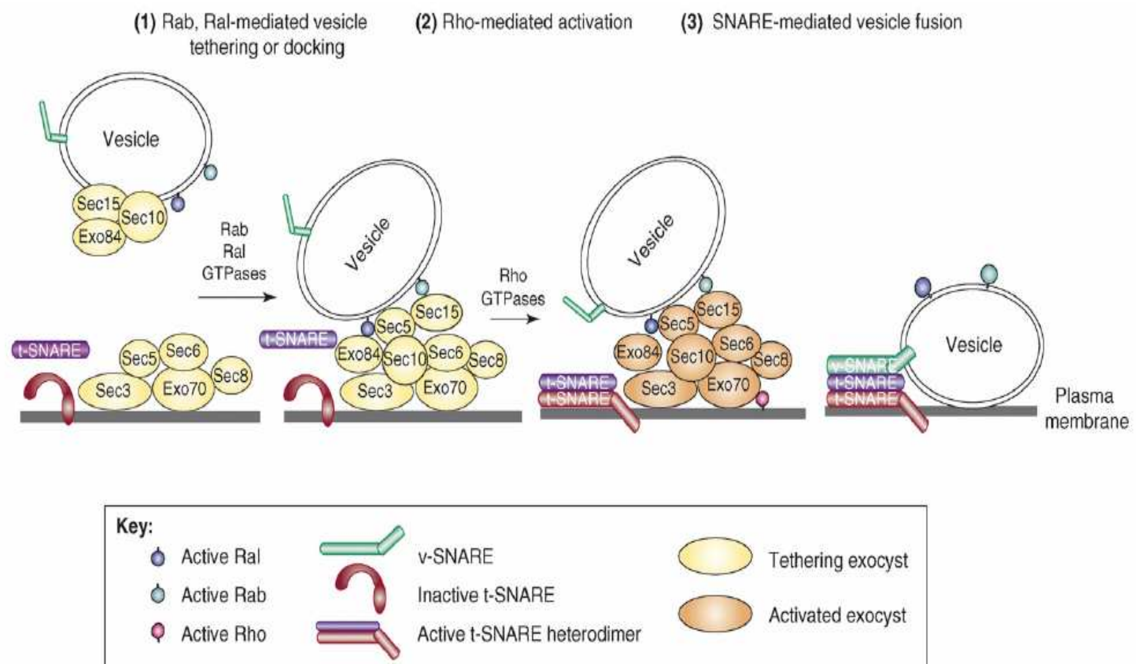


Fig. 6: A three-step model for vesicle docking, exocyst activation and vesicle fusion regulated by small GTPases (modified from [125]): **(1)** The initial vesicle-docking or tethering event is regulated by Rab and Ral GTPases, perhaps by promoting exocyst assembly. The association of particular exocyst subunits with the vesicle or plasma membrane in this diagram is speculative. There is evidence that exocyst assembly is might be regulated by Ral and this function, like that of Rab GTPases, is first required for vesicle tethering rather than fusion [102]. **(2)** This is followed by local activation of the exocyst complex by Rho subfamily GTPases in their active GTP-bound state. Exocyst activation results in a stimulation of downstream fusion activity, probably by promoting assembly of active t-SNARE heterodimers. **(3)** The presence of active t-SNARE dimers results in SNARE-mediated fusion of the secretory vesicles at the site of exocyst activation.

2 Aims

Successful cell division is critical for cell proliferation and development of all eukaryotic organisms. In mammals, cytokinesis relies on temporally and spatially regulated formation of plasma membrane. This formation is carried out by insertion of intracellular membrane compartments into the cleavage site [71]. Studies have shown that the additional membranes are transported from *trans*-Golgi network (TGN) [69]. However, recent investigation has pointed to the vesicles accompanying the recycling pathways which might also play an important role in the construction of a new plasma membrane of the two generating daughter cells. The proteomic composition of the cleavage site is more or less known. Inter alia, the presence of Ras-like GTPases and subunits of exocyst complex was revealed. Deeper investigation uncovered that disruption of any exocyst subunit is accompanied by accumulation of vesicular structures at the midbody site because the vesicles are not able to tether with the plasma membrane. This is the reason for subsequent cytokinesis failure [5,83]. Moreover, another investigation pointed out that membrane structures which are derived from recycling endosomes could be also critical for successful cell division [82] suggesting that the delivery is accompanied by various proteins of Ras-like superfamily. This together suggests that these trafficking pathways might be controlled by interaction between exocyst and Rab11a, Rab35 and RalA proteins; the members of Ras-like superfamily. But how exactly are the vesicular pathways directed to the site of abscission remains unclear as well as how these vesicle-pathways are controlled.

The aim of this work was to investigate the possible interactions of Ras-like GTPases (Rab11a, Rab35 and RalA) and exocyst members (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) which might be the responsible for governing the vesicular-shuttle between recycling endosomes and plasma membrane. Experiments were performed in non-synchronized mammalian HeLa cells using biochemical and cell-biological methods such as ultracentrifugation, siRNA treatment, radioligand-binding assay and immunodetection.

Results of this work extend yet rather limited knowledge of these processes which have been studied before in Chinese hamster ovary cells (CHO) only [83].

3 Materials and methods

3.1 Materials

3.1.1 General reagents

Applied Biosystems, Warrington, UK

- Nuclease-free water – non-DEPEC treated (Product No: AM9930)

Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK

- Dimethyl sulphoxide (DMSO) (Product No: D/4128/PB17)
- Ethanol (Product No: E/0600DF/25)
- Glycine (Product No: BPE381-1)
- N-(2-Hydroxyethyl)-piperazine-N'-2-ethanesulphonic acid (HEPES) (Product No: BPE310-1)
- Sucrose (Product No: S/8560/65)
- Trichloroacetic acid (TCA) (Product No: T/P500/50)
- Tris-base (Product No: BPE152-5)

Invitrogen Ltd., Paisley, UK

- Bovine serum albumine (BSA) (Product No: AM2614)

Kodak, Hemel Hempstead, Hertfordshire, UK

- Kodak Medical X-ray Film, Green / MXG, 18x24 cm (Product No: 100SH)

New England Biolabs (UK) Ltd., Hitchin, Hertfordshre

- Prestained Protein Marker, Broad Range (7-175 kDa) (Product No: P7708S)

Melford Laboratories Ltd., Chelsworth, Ipswich, Suffolk, UK

- Dithiothreitol (DTT) (Product No: MB1015)

La Roche Ltd., Mannheim, DE

- Complete™ Protease Inhibitor Cocktail Tablets (Product No: 04693116001)

Severn Biotech Ltd., Kidderminster, Worcestershire, UK

- 30% (w/v) Acrylamide [Acrylamide to Bis-Acrylamideratio 37.5:1] (Product No: 20-2100-10)

Sigma-Aldrich Ltd., Gillingham, Dorset, UK

- Ammonium persulphate (APS) (Product No: T9281-25ML)
- Bradford reagent (Product No.: B6916)
- Brilliant Blue R (Product No: B7920)
- Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) (Product No: E6758)
- Glycerol (Product No: G8773)
- Luminol (Product No: 123072)
- Methanol (Product No: 322415)
- OptiPrep® Density Gradient Medium (Product No: D1556)
- Sodium dodecyl sulphate (SDS) (Product No: L3771)
- Sodium phosphate monobasic dihydrate ($NaH_2PO_4 . 2H_2O$) (Product No: 71500)
- N,N,NJ,NJ-Tetramethylethylenediamine (TEMED) (Product No: 39339)
- Triton® X-100 (Product No: 93443)
- Tween® 20 (Product No: P9416)
- Urea (Product No: U6504)

VWR International Ltd., Lutterworth, Leicestershire, UK

- Acetic acid (glacial) (Product No: 84528.360)
- di-Potassium hydrogen phosphate (K_2HPO_4) (Product No: 26931.365)
- Sodium chloride (Product No: 27810.295)
- di-Sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) (Product No: 444425M)

3.1.2 Cell culture reagents

All cell culture reagents were purchased from Invitrogen Ltd., Paisley, UK

- Trypsin, 0.05% (1x) with EDTA 4Na, liquid (Product No: 25300062)

- Dulbecco's Modified Eagle Medium (D-MEM) (1x) liquid (High Glucose) containing L-Glutamine, with 4 500 mg/l D-Glucose, without Sodium Pyruvate (Product No: 61965-026)

  - *and supplemented with*:

  - Fetal Bovine Serum (Heat Inactivated), EU Approved (Product No: 10500064)
  - L-Glutamine 200 mM (100x) liquid (Product No: 25030024)
  - Penicillin – Streptomycin (Product No: 15140122)

3.1.3 Dharmacon RNAi (RNA interference) transfection reagents

Thermo Scientific, Epsom, UK

- DharmaFECT 1 Transfection Reagen (Product No: T-2001-04)
- ON-TARGETplus SMARTpool RAB11A (Product No.: NM_004663)
- ON-TARGETplus SMARTpool RAB35 (Product No: NM_006861)
- ON-TARGETplus SMARTpool RALA (Product No: NM_005402)
- ON-TARGETplus SMARTpool GAPDH (Product No: NM_002046)
- ON-TARGETplus Non-targeting Pool (Product No: D-001810-10-20)

3.1.4 Primary and secondary antibodies

Abcam plc., Cambridge, UK

- Mouse anti-EXOC7 (Exo70) antibody (Monoclonal) (Product No.: ab57402)
- Mouse anti-Sec8 antibody (Monoclonal) (Product No.: ab13254)
- Rabbit anti-rSec6 antibody (Polyclonal) (Product No.: ab56979)
- Rabbit anti-Rab11a antibody (Polyclonal) (Product No.: ab78337)

Ambion, Cambridge, UK

- Mouse anti-GAPDH antibody (Monoclonal) (Product No.: AM4302)

BD Biosciences, San Jose, CA, USA

- Mouse anti-RalA antibody (Monoclonal) (Product No.: 610222)
- Sheep anti-EXOC8 (Exo84) antibody (Polyclonal) (Product No.: LS-C3367)

GE Healthcare Global Headquarters, Chalfont, St. Giles, Buckinghamshire, UK

- Amersham ECL Mouse IgG, HRP-Linked Whole Ab (from sheep)
  (Product No.: NXA931)
- Amersham ECL Rabbit IgG, HRP-Linked Whole Ab (from donkey)
  (Product No.: NA934)

Invitrogen Ltd., Paisley, UK

- Mouse anti-Transferrin Receptor (Monoclonal) (Product No.: 13-6800)

Proteintech Group, Inc., Chicago, USA

- Mouse anti-Sec5 antibody (Polyclonal) (Product No.: 12751-1-AP)
- Rabbit anti-EXOC1 (Sec3) antibody (Polyclonal) (Product No.: 11690-1-AP)
- Rabbit anti-Rab35 antibody (Polyclonal) (Product No.: 11329-2-AP)

Santa Cruz biotechnology, INC., Heidelberg, DE

- Goat anti-Sec10 antibody (Polyclonal) (Product No.: sc-30295)
- Mouse anti-EEA1 antibody (Polyclonal) (Product No.: sc-6414)
- Rabbit anti-Na^{+}/ K^{+}-ATPase α antibody (Polyclonal) (Product No.: sc-28800)
- Rabbit anti-Rab5B antibody (Polyclonal) (Product No.: sc-598)
- Rabbit anti-Rab7 antibody (Polyclonal) (Product No.: sc-10767)
- Rabbit anti-Sec15 antibody (Polyclonal) (Product No.: sc-67070)

Sigma-Aldrich Ltd., Gillingham, Dorset, UK

- Amersham ECL Goat IgG, HRP-Linked Whole Ab (from donkey)
  (Product No.: G6638)

Thermo Scientific, Epsom, UK

- Amersham ECL Sheep IgG, HRP-Linked Whole Ab (from donkey)
  (Product No.:SA1-72045)

3.1.5 Radiochemicals

Carl Roth GmbH, Karlsruhe, BRD

- Rotiszint® eco plus – universal scintillation cocktail (Product No: 0016.3)

PerkinElmer Inc., Massachusetts, USA

- Ouabain, [^{3}H(G)]-, 1mCi (37MBq), radiochemical purity is >97% (Product No: NET211001MC)

3.1.6 General solutions

Cell lysis buffer	50 mM HEPES pH = 7.2, 100 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mM EDTA, 1x Complete™ Protease Inhibitor Cocktail Tablet (1 tablet added in 50 ml of cell lysis buffer immediately before use), 0.1% (v/v) Triton X-100 and 1 mM DTT (also added immediately before use)
Coomassie blue distain solution	45% (v/v) methanol, 10% (v/v) acetic acid
Coomassie blue pre-stain solution	40% (v/v) methanol, 10% (v/v) acetic acid
Coomassie blue stain solution	0.25% (w/v) Coomassie Brilliant Blue R in 90 ml methanol: water at a ratio 1:1 and 10 ml glacial acetic acid. This was thoroughly mixed and filtered through Whatman No.2 filter paper
ECL Solution A	100 mM Tris-HCl, pH = 8.5, 2.25 mM Luminol, 0.4 mM *p*-coumaric acid, 1.44% (v/v) DMSO.
ECL Solution B	100 mM Tris-HCl, pH = 8.5, 0.018% (v/v) H_2O_2

HES buffer	1mM EDTA, 225 mM sucrose, 20 mM HEPES, pH = 7.4, Complete™ Protease Inhibitor Cocktail Tablet (one tablet to 50 ml of HES buffer before use)
Incubating media (binding assay)	5 mM NaH_2PO_4, 5 mM $MgC1_2$, 50 mM Tris-HCI, pH = 7.4
Phosphate buffered saline (PBS)	136 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.4
PBS-T	0.01% (v/v) Tween 20 in PBS
SDS-PAGE electrode buffer	25 mM Tris base, 190 mM glycine, 0.1% (w/v) SDS
SDS-PAGE sample buffer (4x)	200 mM Tris-HCl pH = 6.8, 400 mM DTT (added immediately before use), 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, 40% (w/v) glycerol
Stripping buffer	50 mM glycine, 150 mM NaCl, pH = 2.5
Transfer buffer	25 mM Tris-base, 192 mM glycine, 20% (v/v) ethanol
Washing buffer (binding assay)	5 mM Tris-HCl, pH = 7.4, 5 mM $MgCl_2$

3.1.7 Laboratory equipment

Beckman Coulter, Inc., California, USA

- Beckman GPR Centrifuge with rotor S/N, 9E 497 X
- Ultracentrifuge Beckman Optima TM Max with rotor Beckman TLA 100.4
- TLA 100 tubes

BD Plastipak

- Needles 26G (0,45 mm x 16 mm)
- Syringe (1 ml)

Bio-Rad Laboratories, California, USA

- Bio-Rad Mini-PROTEAN 3 apparatus
- Bio-Rad mini Trans-Blot apparatus

BMG LABTECH GmbH, Offenburg, DE

- FLUOstar Optima plate reader

Brandel, Gaithersburg, USA

- Cell-harvester

Hettich Instruments, Beverly, USA

- Benchtop Hettich MICRO 22R centrifuge with r otor 1189-A

Isobiotec, Heidelberg, DE

- Ball-bearing homogenizer with a 12 µm clear

Kodak, Hemel Hempstead, Hertfordshire, UK

- X-Omat Processor

IQ Scientific Instrument, California, USA

- pH meter IQ 140

Prestige Medical, Blackburn, UK

- Autoclave

Wolf Laboratories Limited, York, UK

- Galaxy S incubator

3.2 Mammalian cell culture

3.2.1 Cell culture conditions

Human cervical carcinoma HeLa cells were cultured in T-75 flasks (a tissue culture treated flask of growth area 75 cm^2 and volume 250 ml) and were grown at 37 °C in 5% (v/v) CO_2. A suitable environment ensured Galaxy S incubator from Wolf Laboratories.

Cells were cultured in HeLa complete growth media, which means: D-MEM containing 4 500 mg/l D-Glucose without sodium pyruvate which was supplemented with 10% (v/v) Fetal Calf Serum EU, 2 mM Glutamine, 100 Units/ml Penicillin and 100 µg/ml Streptomycin.

3.2.2 Subculturing procedure

Cells were subcultured at approximately 80% confluence as follows: medium was aspirated and cells rinsed using 2 ml of trypsin/EDTA solution which had been pre-warmed to 37 °C. Cells were than incubated for several min at 37 °C and subsequently, were gently dislodged by tapping the flask. The pre-warmed 8 ml of media were added to the flask with cells and gently mixed. Subsequently, 2 ml of these cells were transferred to a new and sterile T-75 flask containing 13 ml of complete growth media. Afterwards, cells were immediately placed into the incubator and cultured at 37 °C in 5% (v/v) CO_2.

3.3 General laboratory and biological methods

3.3.1 Cell fractionation assay

Before cells collection, all solutions and a ball-bearing homogenizer from Isobiotec, with a 12 µm clearance, were cooled-down. The entire experiment was carried out on ice. Each experiment was conducted in tetraplicates. Parallel fractions, collected at the end of the experiment, were mixed in order to obtain higher volume of the same sample.

HeLa cells of ~ 80% of confluence were collected from six T-75 flasks to a ball bearing homogenizer as follows. The medium was aspirated and the cells were washed by PBS-T two times. Afterwards, cells were collected in 4 ml of HES buffer to a conical tube and centrifuged in a refrigerated S/N, 9E 497 rotor of Beckman GPR Centrifuge at 3 723 g for 5 min at 4 °C . Supernatant was aspired and the pellet was re-suspended in 2 ml of HES buffer. Consequently, cells were homogenized via ten passes through the ball-bearing homogenizer, with a 12 µm clearance. Homogenate was transferred to the conical tube and span again in S/N, 9E 497 rotor of Beckman GPR Centrifuge at 3 723 g for 10 min at 4 °C to pellet nuclei and debris.

Post-nuclear supernatant (PNS) of 1.2 ml was applied to the bottom of Beckman TLA 100 tubes and mixed with 1.2 ml of 60% (w/v) iodixanol (OptiPrep stock solution) to yield a sample containing 30% (w/v) iodixanol. This mixture was overlayed with 1.2 ml of 20% (w/v) iodixanol and on the top was applied 1.2 ml of 10% (w/v) iodixanol. The media of 10% and 20% iodixanol were prepared by dilution of a 60% (w/v) stock solution with HES buffer. Consequently, the density gradient (first described by Boyum, J. in 1968 [126]) was transferred to a Beckman TLA 100.4 rotor in Beckman Optima TM Max Ultracentrifuge and span at 14 300 g for 3 hours at 4 °C.

The fractions, 320 µl each, were collected from the top of the gradient to fifteen micro-centrifuge tubes. In total were collected fifteen fractions, which were numbered from 1 to 15, where number 1 marked the first fraction collected from the top and number 15 marked the last fraction collected from the bottom. Subsequently, 100 µl of TCA was added, vortexed and placed on ice. After 20 min of incubation on ice the samples were transferred into refrigerated 1189-A rotor of a benchtop Hettich MICRO 22R centrifuge and span at 14 300 g for 20 min at 4 °C. After centrifugation, was the supernatant aspirated and the pellet was re-suspended in 80 µl of 1x SDS-PAGE buffer containing 20 mM DTT and 5 M urea.

Thereafter, the gradient fractions were probed for proteins of exocyst subunits and GTPases of interest using SDS-PAGE and Western blot method.

3.3.2 Gene knock-down technigue

Gene knock-down [127] is a method mediated by double-stranded RNA (usually about 20 – 25 base pairs long) which cause drastic decrease in the expression of a targeted gene. Studying the effects of this decrease can reveal the physiological role of the gene product.

Cell preparation

The day before transfection, HeLa cells of 80 – 90% confluence were trypsinized using 2 ml of pre-warmed trypsin/EDTA solution. Immediately, were re-suspended in 10 ml of pre-warmed antibiotics free growth media, containing D-MEM supplemented with 10% (v/v) Fetal Calf Serum EU, 2 mM Glutamine. Cells were subsequently divided 1:5 to reach 50 - 60% confluence. Afterwards, 2 ml of these cells were seeded into each of the wells of a 6-well plate and incubated at 37 °C in 5% (v/v) CO_2 overnight to allow them to attach.

Transfection

Each transfection was carried out with chemically synthesized siRNAs of ON-TARGET SMARTpools from Dharmacon, using a manufacturer's instructions. The following description of the transfection experiment will be described for one well of a 6-well plate. In one well will be used 2 ml of transfection complex and the final siRNA concentration will be 100 nM. Everything was scaled up for number of samples and controls. Each experiment was carried out in duplicates.

All steps of protocol were performed in a laminar flow cell culture box using sterile techniques. RNAse free micro-centrifuge tubes and pipette tips were used. The siRNAs of interest were diluted with siRNA free buffer to get a working concentration of 2 µM with 1x siRNA buffer.

In a RNAse free micro-centrifuge tube (tube A) was diluted 100 µl of 2 µM siRNA in 100 µl of serum-free D-MEM. In RNAse free micro-centrifuge tubes (tube B) were diluted 4 µl of DharmaFECT 1 Transfection Reagent (a lipid transfection reagent which support the efficient siRNA delivery to the cell) in 196 µl of serum-free D-MEM. The reagents in each tube were gently mixed by pipetting up and down several times and

incubated for 5 min at room temperature. Afterward, contents of tube A and tube B were combined to final volume of 400 μl, gently mixed by pipetting up and down several times and incubated at room temperature. Twenty minutes later it was topped up 1600 μl of pre-warmed antibiotics free growth media making total volume of 2 ml of transfection complex containing the siRNA of final concentration of 100 nM.

For a confirmation of the successful siRNA treatment each experiment included at least four different control samples. The untreated cells, i.e. where no siRNA is. In the thesis is labeled ‚-‘. Second control, labeled ‚D’, reflects cells treated with DharmaFECT transfection reagent in the absence any siRNA. In both cases, it is easy to determine the baseline level of target gene, cell viability and phenotype. Third, as a negative control, labeled ‚NT’, is used to distinguish sequence-specific silencing from non-specific effects in the experiment. Neither the mRNA nor protein levels of any known genes in the cell should be affected by this negative control. And finally, a positive control monitors an efficiency of siRNA delivery into the cell. In this case, the siRNA targets a so called housekeeping gene. This gene which is expressed at the cell type must not fluctuate with cell cycle, which is true for example, in a case of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In the experiment is this type of control labelled ‚GAPDH’.

For the experiments HeLa cells of ~ 60% confluences were used, which were cultured in a 6-well plate as mentioned above. Growth medium was aspirated from the well and cells rinsed using 2 ml of transfection complex. Subsequently, the plates were gently rocked back and forth to disperse the transfection complex and incubated at 37 °C in 5% (v/v) CO_2. After 48 hours, cells were collected for further analysis (cell lysis, Bradford protein assay and immunoblotting).

3.3.3 Cell lysis

For crude analysis of protein expression in cells, a lysis buffer was used, which contained 0.1% (v/v) Triton X-100.

On the day of harvest, cells were placed on ice and media removed. Afterwards, cells were washed three times with ice-cold PBS. Subsequently, all of PBS solution was

removed and 80 µl (6 well-plate) of cell lysis buffer was added per each well and then the cells were scraped off, placed to a micro-centrifuge tubes. Subsequently, cells were subject to ten passes through a needle (of 26G size) and left on ice for 20 minutes. Then the needle homogenization was repeated. The broken cells were then spun at 14 300 g in a refrigerated bench-top Hettich MICRO 22R centrifuge for 10 minutes. After spinning, the supernatant was aspirated. These cell lysates were stored at –20 °C and later submitted to Bradford assay and SDS-polyacrylamide gel electrophoresis. Specifically, 5 µl of each sample was utilized for Bradford assay. The rest of sample was mixed with 4x SDS-PAGE sample buffer and to final concentration of 1 µg/µl. Finally, 20 µg of prepared samples were submitted to SDS-PAGE.

3.3.4 Bradford Protein Assay

The total protein concentration was determined in each gene knock-downed sample using Bio-Rad Bradford protein assay reagent [128]. The assays were set up in a 96 well-plate, from Corning COSTAR, according to the manufacturer's instructions.

Calibration

As a standard was used bovine serum albumin (BSA) from Promega. Into each well was gradually applied standard of increasing concentrations as follows: 0 µg/µl → → 1 µg/µl → 2 µg/µl...10 µg/µl of BSA. Subsequently, was adjusted destilled waster to supplement the total volume to 100 µl. Finally, 200 µl of Bradford reagent was added into each well with mixture.

Sample measurement

The assays were set up in duplicates, according to the manufacturer's instructions, i.e. into each well were pipetted 5 µl of sample (supernatant as outlined in section 3.3.3; page 37), 95 µl of distilled water and 200 µl of Bradford solution.

The absorbance was measured at 595 nm using a FLUOstar Optima plate reader (BMG LABTECH, GmbH). Concentrations of proteins were calculated according to a curve derived from the BSA standard values.

3.3.5 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [129] is a general laboratory procedure, which is used for protein separation on the basis of their size, i.e. molecular weight. In this experiment, the proteins were separated in gels containing Tris-HCl, and where the volume of acrylamide ranged from 10% (w/v) to 12 % (w/v) depending on the molecular weight of the protein of interest. This is often known running gel. Later, the running gel was overlayed by so called stacking gel containing Tris-HCl and 5% (w/v) acrylamide. The resolving gels were prepared, using the manufacturer's instructions. The electrophoresis was performed using Bio-Rad Mini-PROTEAN 3 apparatus.

Subcellular-fractionation samples

The protein samples were solubilized in 1x SDS-PAGE sample buffer (as outlined in section 3.3.1; page 34) and incubated in a heat block at 75 °C for 5 minutes. Twenty micrograms of each protein sample (except the first fraction collected from the top) were applied on 10% - 12% gel. In each gel were always used 3 µl of Prestained Protein Marker from Broad Range. The procedure was carried out using 800 ml of SDS-PAGE electrode buffer.

Gene knock-down samples

The protein samples were solubilised in 4x SDS-PAGE sample buffer (as outlined in section 3.3.3; page 37) and incubated in a heat block at 75 °C for 5 minutes. 20 µg of each protein sample were applied on 10% - 12% gel. Also 3 µl of pre-stained Protein Marker from Broad Range were always applied. The procedure was carried out using 800 ml of SDS-PAGE electrode buffer.

Gels were run at a constant voltage of 70 V until the protein sample had passed through the 5% stacking gel. Afterwards, the voltage was increased to 100 V through the running gel. The process of electrophoresis was terminated when the pre-stained broad range marker had separated adequately and when its dye front had reached the bottom of the gel.

3.3.6 Western blotting

Following SDS-PAGE, proteins were transferred onto a nitrocellulose membrane, for immunodetection using Western blot method [130]. The components of Bio-Rad mini Trans-Blot apparatus were pre-washed in transfer buffer. The gels were separated from the glass plates and the stacking gel removed from the running gel.

Afterwards, the transfer system was assembled as follows from cathode to anode: a sponge pad → Whatman 3 mm chromatography paper → Whatman nitrocellulose membrane (0.45 µm pore size) → polyacrylamide gel (from SDS-PAGE and containing the proteins samples) → Whatman 3 mm chromatography paper → a sponge pad. This assembly was enclosed in a cassette and inserted into a mini Trans-Blot cell which was filled with 800 ml of transfer buffer. Finally, the proteins were transferred at room temperature for two hours at a constant current of 200 mA or overnight at 40 mA using Bio-Rad mini Trans-Blot apparatus.

3.3.7 Immunodetection of proteins

Following Western blotting, proteins were visualized by Enhanced Chemical Luminescence (ECL). Nitrocellulose membranes were taken out from the Bio-Rad mini Trans-Blot apparatus and washed with 1x PBS-T stock solution. Consequently, membranes were incubated in 5% (w/v) dried non-fat milk, which was dissolved in PBS-T. The incubation took 30 minutes at room temperature. Membranes were blocked to prevent nonspecific binding of proteins.

Just before the end of the blocking, primary antibodies of interest were prepared as follows. Primary antibodies were diluted in 1% (w/v) dried non-fat milk dissolved in PBS-T. Subsequently, the membranes were incubated in it under agitation, at least 1 hour at room temperature or overnight at 4 °C. Then, the membranes were washed three times in PBS-T over 30 minutes.

As secondary antibodies were used IgG horseradish peroxidase-conjugated antibodies, which were diluted in 5% (w/v) dried non-fat milk/PBS-T. Membranes were

incubated in HRP-linked IgG for one hour at room temperature at rocking platform and washed three times with PBS-T over a 30 minutes period. HRP-Immunolabelled proteins were visualized by the ECL system. ECL reagents A and B were mixed in a 1:1 ratio (the preparation of ECL reagents is outlined in section 3.1.6; page 31). The nitrocellulose membrane was immersed in ECL reagent for 1 min then removed immediately and dried. The Kodak X-ray films were exposed to membrane in a light-proof cassette and developed using an X-Omat Processor (Kodak).

3.3.8 Stripping of nitrocellulose membranes

If it was necessary to continue with detection of proteins with enhanced chemiluminescence (ECL), a nitrocellulose membrane could have been stripped of bound antibodies and reprobed with different antibodies. For stripping of bound antibodies, the membranes were incubated in stripping buffer for 30 minutes at room temperature on rocking platform. Consequently, the membranes were washed with PBS-T and blocked with 5% (w/v) dried non fat milk/PBS-T for 1 hour at room temperature. After verification were the blots incubated in primary antibody, as required.

3.3.9 Coomassie blue staining of SDS polyacrylamide gels

For detection of proteins in a SDS-PAGE gel, the gels were immersed, in Coomassie blue pre-stain solution overnight at room temperature on rocking platform. Afterwards they were transferred into Coomassie blue stain solution for one hour. Coomassie blue stain solution was then removed using Coomassie blue distain solution by agitation for 30 min.

3.3.10 Radioligand binding assay

3.3.10.1 [^{3}H]Ouabain binding assay

Specific ouabain binding has been used as a very reliable plasma membrane marker (ouabain specifically binds the sodium-potassium pump - Na^+/K^+-ATPase and causes its inhibition). The ouabain binding was determined in fractions (labeled from 2 to 15) sedimented from iodixanol density gradient (for more details see section 3.3.1; page 34). For the experiment was used ouabain labeled with radioactive isotop of hydrogen – tritium.

Membrane fractions (50 µl per assay) were incubated with [^{3}H]ouabain (33 nM) in 350 µl of incubating media, for 90 min at 30 °C. In the case of ouabain, the non-specific binding is so low that it can be neglected. The binding reaction was terminated by filtration (on cell-harvester from Brandel) through Whatman GF/C filters using 3x 5 ml of pre-cooled washing buffer. Subsequently, every single Whatman GF/C filter was transferred to scintillation flask and incubated 1 hour in 5 ml of commercial scintillation solution Rotiszint EcoPlus. The radioactivity, which remained on the filters, was assessed by liquid-scintillation counting for 5 minutes by the Radiometry Department of Academy of Science of the Czech Republic.

4 Results

4.1 The subcellular localization

This chapter illustrates the molecular distribution of all exocyst subunits and Ras proteins of interest (Rab11a, Rab35 and RalA) in non-synchronized HeLa cell culture. This was achieved by centrifugation of PNS obtained from HeLa whole-cell lysate. For the experiment 10-20-30% (w/v) iodixanol solution was used (known under commercial name OptiPrep) which represents a suitable non-toxic medium for density gradient.

4.1.1 Mapping of cellular fractionation gradient

Firstly, it was necessary to analyze the density gradient which was prepared by centrifugation in linear OptiPrep gradient. As detailed in chapter 3.3.1 on page 34, the separation of different membrane compartments was achieved using ultracentrifugation in three-step 10-20-30% (w/v) OptiPrep gradients. Afterwards, the fractions were collected from the top to the bottom - labeled from 1 (top) to 15 (bottom), resolved by SDS-PAGE and immunoblots and probed with special markers for early endosomes, late endosomes, recycling endosomes, or plasma membranes.

The area of endosomal fraction was determined using antibodies against Rab5, Rab7, Rab11a and TfR. It is known that protein Rab5 is a very reliable marker of EE, Rab11a is a marker of RE whereas TfR is a marker of RE and simultaneously EE. Antibodies revealing the LE are assigned to a protein Rab7. In this manner was revealed the remnant composition of the fractional gradients, i.e. a region of fractions which are enriched of PM was achieved using Na^{+}/K^{+}-ATPase antibodies. This well-known transmembrane enzyme is located just in PM in all animal cells. Therefore, it was used as a marker of PM. Results of this experiment provides Fig. 7; page 44.

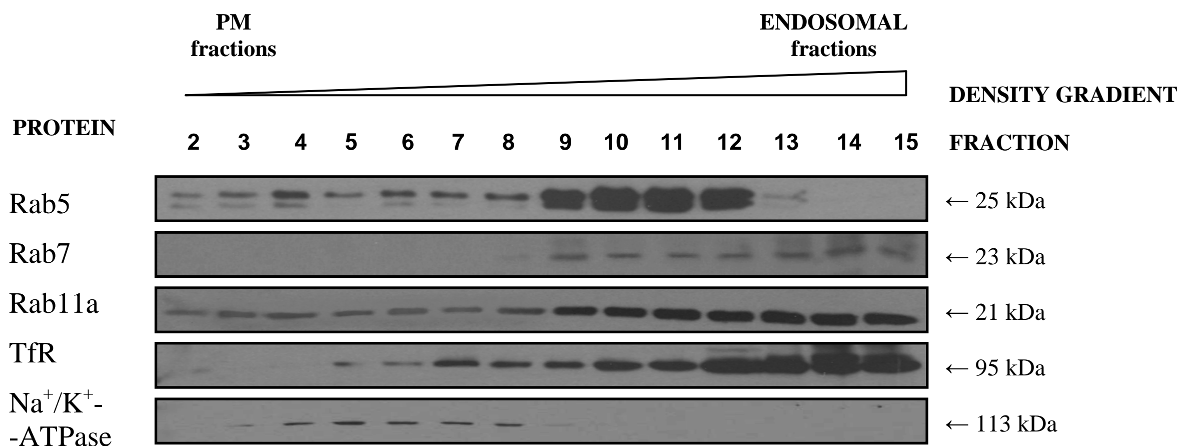


Fig. 7: Cellular fractionation profile of post-nuclear supernatant prepared from non-synchronzed HeLa cells: the non-synchronized HeLa cells were homogenized and centrifuged. The post-nuclear supernatant, which contained the whole cell extracts, was separated and applied on the top of a linear 10-20-30% (w/v) OptiPrep gradient. An equal volume of each fraction was loaded on a 10-12% SDS-PAGE gel. Western blot was used for determination of different proteins: **Rab5** as a marker of the early endosomes (EE), **Rab7** as a marker of the late endosomes (LE), **Rab11a** as a marker of recycling endosomes (RE), **TfR** as a marker for EE and RE, and finally **Na^+/K^+-ATPase** as a marker of the plasma membranes. Numbers ranging from **2** to **15** indicate fractions collected from the top (2) to the bottom (15).

As is evident from Fig. 7, the endosomal fractions are spread between ninth and fifteenth faction. In detail, TfR and Rab11a as markers of RE were detected between ninth and fifteens fraction with the enrichment around fractions twelve and fifteen. Using Rab7 as a marker, the LEs seem to be equally localized between ninth and fifteens fraction. A Rab5 shows, the richest region of EE is settled down between ninth and twelve fractions. Finally as expected, the zone of PM is distributed between third and eighth fractions.

For more precise mapping of the PM area radioligand binding assay was used as outlined in 3.3.10; page 41. For this purpose a poisonous cardiac glycoside known as ouabain was used. This complicated molecule is a very strong ligand, and simultaneously significant inhibitor of the Na^+/K^+-ATPase (a sodium pump). As presumed, the [^{3}H]ouabain binding assay confirmed that the main distribution of PM is located between fourth and eighth fractions, and currently revealed that the highest content of the PM is present in the fifth fraction, as Fig. 8 on page 45 shows.

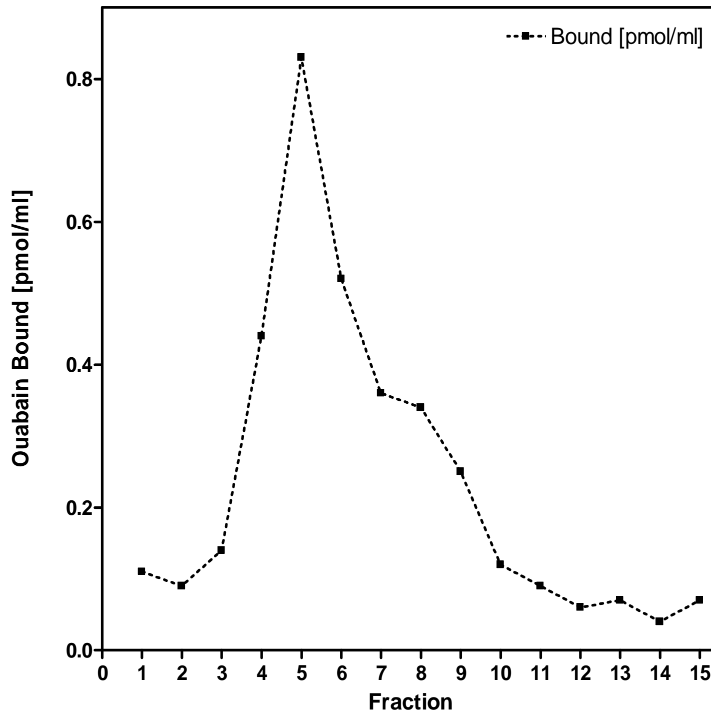


Fig. 8: Distribution of Na^+/K^+-ATPase in density gradient: Each of the fifteen fractions was submitted to radioligand binding assay. In this case, $[^3H]$ouabain plays an important role as a very responsible marker of a plasma membranes. The PMs are higly enriched in Na^+/K^+-ATPase, a binding partner of ouabain. From the results is more than clear that the plasma membranes are mostly distributed between fourth and sixth fracion. The highest measured quantity of the PM is then present in fraction number five.

As was suggested, during centrifugation the plasma membranes float up throughout the gradient to their equilibrium density (approximately to the interface between 10% and 20% OptiPrep) while the cytosolic proteins remain in the highest density medium at the bottom of gradient (30% OptiPrep). Furthermore, the immunoblots revealed that the endosomal fractions are spread out between ninth and fifteenth factions (Fig. 7; page 44). On the other hand, the area of the PM is distributed mainly between fourth and eighth fractions, of which $[^3H]$ouabain binding assay (Fig. 8) shows the highest peak in fraction number five. Regarding to the ninth fraction, a presence of small volume of the PM is also recognizable in here.

4.1.2 Localization of the small GTPases

Recent studies which investigated the GTPases Rab11a, Rab35, and RalA, have shown that all of these proteins could be important regulators of the vesicle transport maintaining the recycling routes. As pointed out in chapter 1.5; page 16, the vesicular shuttle is required for successful cytokinesis. It was also shown that during late stages of cytokinesis, these endosomal proteins occur in abundance on site of ingression. Therefore it would be interesting to determine how the endosomal GTPases such as Rab11a, Rab35 and RalA might orchestrate this process by exchanging membrane material during the termination of the cell cycle. Although it is known that Rab11a, Rab35 and RalA maintained endosomal recycling pathways, the more accurate responsibility is shrouded in fog. For a start, we tried to localize the GTPases mentioned above, in non-synchronized mammalian HeLa cells using a density gradient method.

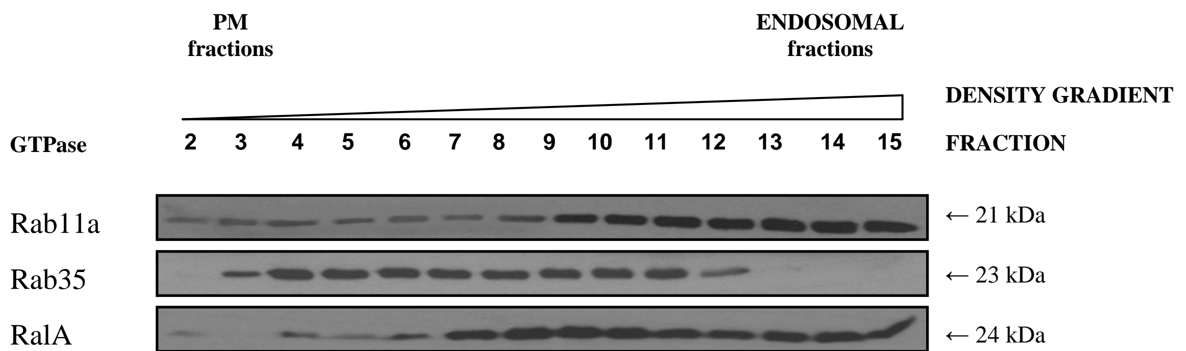


Fig. 9: Fractionation profile of small GTPases acquired from PNS of non-synchronized HeLa cells: HeLa cells were homogenized, and the postnuclear supernatant was separated using a linear 10-20-30% (w/v) OptiPrep gradient. An equal volume of each fraction was loaded on a 10% SDS-PAGE gel. Distribution of different GTPases (shown) was determined by Western blot method. Numbers ranging from **2** to **15** indicates fractions collected from the top (2) to the bottom (15).

Analysis of the fractions is shown in details in the Fig. 9. The localization of Rab35 overlaps with the region of EEs (fractions 12 – 9) but simultaneously also extends to the zone of the plasma membranes (fractions 8 – 3). This might lead to an idea that Rab35 could importantly support other traffic mechanisms (e.g. exocytic pathways) within the framework of membrane trafficking during the whole cell cycle. Because Rab11a is known to regulate the endocytic recycling it is not a big surprise that this protein co-fractionate to the zone of REs (fractions 12 - 15). On the other hand it also overlaps with

the region of EE (fractions 9 – 12) and lightly extends into a territory of PM (fractions 2 - 8). In contrast to previous studies in mammalian Cos-1 cells [131], we revealed that in HeLa cells, RalA is not tied only to the REs (fractions 12 – 15) but its significant location is also situated to the territory of EE (fractions 9 – 12) and slightly in PMs (fractions 4 - 6).

Taken our obtained and already published data [82,93,99] together, we may conclude that the mammalian Rab11a, Rab35 and RalA are localized to the subset of endosomes and partially to the area of PMs. This support the prediction that these proteins undergo cell cycle-dependent re-localization and thus might play role in targeting of subcellar membrane compartments to the plasma membranes [82,83,132].

4.1.3 Localization of the exocyst subunits

As mentioned earlier, a number of evidence signify that the exocyst is required for membrane recycling where plays crucial role in targeting and tethering of the vesicular structures to the PM [117,118]. In parallel, recent investigation of intracellular trafficking determined that the REs are essential for terminal stages of cytokinesis [13,50,70,71]. Therefore, the next center of interest was an ambition to try uncovering a subcellular distribution of exocyst subunits isolated from non-synchronized HeLa cell cultures. As an efficient tool the isopycnic centrifugation in a three-step 10 - 20 - 30% (w/v) OptiPrep gradient was again used as outlined in chapter 3.3.1; page 34.

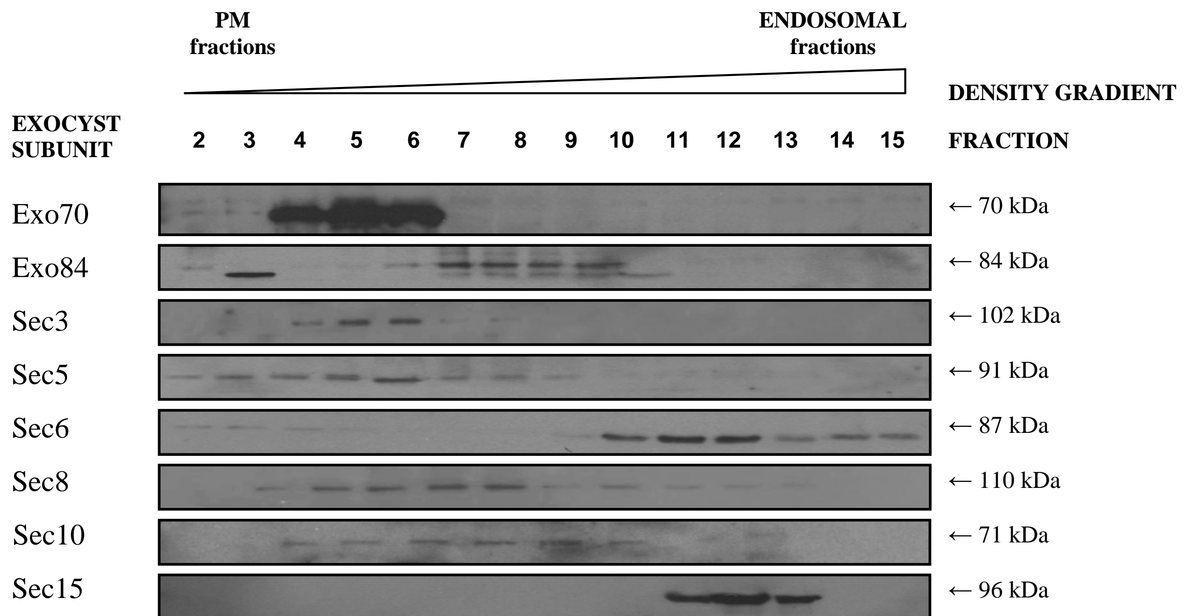


Fig. 10: Fractionation profile of exocyst subunits acquired from PNS of non-synchronized HeLa cells: HeLa cells were homogenized, and the postnuclear supernatant containing whole-cell lysate was separated on a linear 10-20-30% (w/v) OptiPrep gradient. The fractions were collected from the top and an equal volume of each fraction was resolved by 10-12% SDS-PAGE gel. Immunoblots were probed with antibodies specific for all exocyst subunits shown above. Numbers ranging from **2** to **15** indicates fractions collected from the top (2) to the bottom (15).

This investigation exposed surprising results presented in Fig. 10. It shows the - localization of exocyst subunits throughout non-synchronized mammalian HeLa cells. After the first examination, it is obvious that a subunit Exo84 is localized to the area of boarder between EEs and PMs (fractions 7 – 10). The position of Sec8 (fractions 5 – 8) and Sec10 (fractions 4 – 10) is spread over the region of PM but slightly continues to the zone of EEs. We noticed that the settlement of Sec3 and Exo70 overlapped fractions (4 – 6) of PMs as well as Sec5 (fractions 2 – 9) but with the difference that Sec5 slightly continues to the EE area. Surprisingly, subunits Sec15 perfectly coincide with eleventh, twelfth and thirteen fractions, i.e. around the boarder between EEs and REs. Finally, Sec6 (fractions 10 - 13) seems to be primarily situated to the vesicular structures maintaining pathways of early and recycling endosomes in the cell.

In comparison of Fig. 9 and Fig. 10 (pages 46 and 48), we conclude that exocyst subunit Sec15 distribution overlaps with distribution of Rab11a and RalA. Sec6 co-localize with all GTPases but mainly with RalA and Rab11a. On the other hand, Exo84 or Sec10

co-localoze with all types of studied GTPases. The remnant distributions of exocyst members Exo70, Sec3, Sec5, Sec8 and Sec10 for the most overlap the region consistent with Rab35. Does this mean that the Rab35 may affect the delivery of these above-mentioned exocyst subunits (Exo70, Sec3, Sec5, Sec8 and Sec10) to the side of abscission? Might this be also true for example in case of Sec15 and Rab11a or the other proteins? Or actually, does it depend on the common co-localization of the proteins? The answer to this question could provide subsequent experiment where siRNA treatment of the Rab11a, Rab35 and RalA proteins was performed (results detailed in section 4.2).

4.2 The siRNA screening

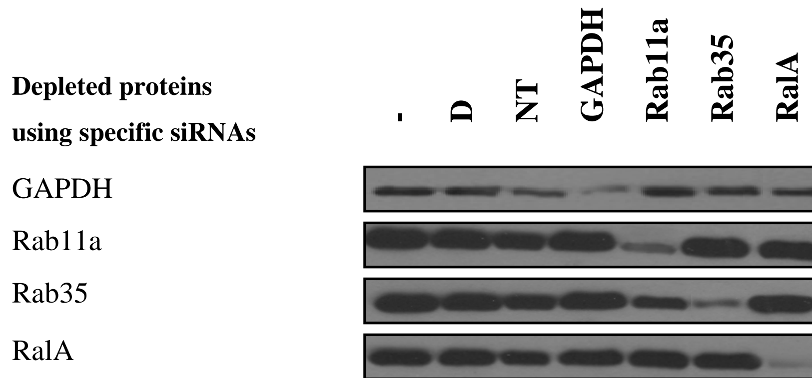
Proteomic study shows the profiling of proteins which are associated with the cleavage site (outlined in 1.3.3; page 12). It was revealed that many different proteins including Rab11a, Rab35 and RalA GTPases as well as exocyst members reside at the abscission site during mammalian cell division. We suggest that the effective delivery and tethering of the RE-vesicular structures into the plasma membrane requires an optimum interplay between different subunits of exocyst complex and its effector GTPases from Ras-like superfamily. However, the exact regulatory mechanisms of these processes remain unknown. Hence, this part of work aims to reveal the mutual interactions among Rab11a, Rab35 and RalA GTPases and the members of exocyst. The protein interactions were most expected between exocyst members and Ras-like GTPases with the most equal cellular localization, i. e. interaction of Rab11a with Sec15; or Rab35 with Exo70, Sec3, Sec5, Sec8 or Sec10 (sections 4.1.2; page 46 and 4.1.3; page 47).

The investigation was achieved by siRNA treatment. This technique of gene knock-down is used as a unique form of post-transcriptional gene silencing where expression of gene of interest is inhibited. In this part of work, we attempted to silence messenger RNA (mRNA) transcripts of Rab11a, Rab35 and RalA GTPases, and depending on that we attempt to examine the behavior of exocyst subunits.

4.2.1 Silencing of the small GTPases and the impact on exocyst members

The top of Fig. 11 on page 51 shows results of immunoblots which were determined by selective treatment of small interfering RNA (siRNA) in non-synchronized HeLa cell cultures. For example, gene knock-down of the cells which were treated with siRNA specific for Rab11a was successful. The same achievements were also acquired with depletion of Rab35, RalA and GAPDH which was used as a positive control. Together, comparing with the controls ‚-', ‚D', ‚NT' and ‚GAPDH', the immunoblots clearly demonstrate that the reduction of proteins (Rab11a, Rab35, RalA and GAPDH) proceeded efficiently.

The effectively depleted samples were submitted to further analysis (detailed in Fig. 11; page 51). In the next part of work we aimed to determine whether depletion of any Rab or Ral proteins might have some effect on any of exocyst subunits. The bottom of Fig. 11 on page 51 maps the immunoblots where specific antibodies for exocyst subunits were used. It shows impacts on the exocyst subunits which are caused by depletion of Rab11a, Rab35 and RalA GTPases.



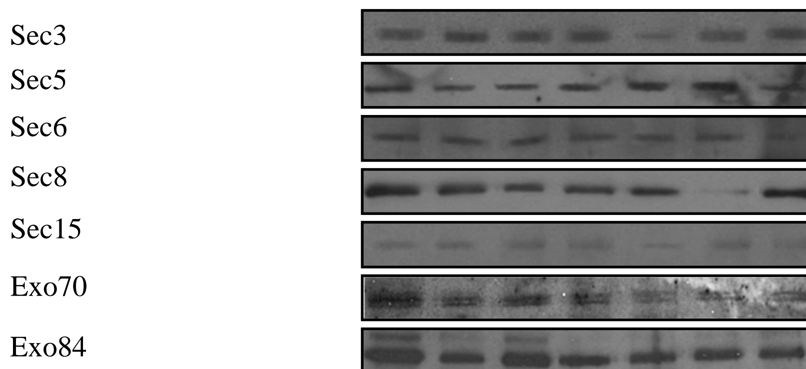


Fig. 11: siRNA depletion of endosomal Rab GTPases and GAPDH protein and its impact on exocyst members: HeLa cells were transected with 100 nM chemically synthesized siRNAs of ON-TARGET SMARTpools specific for the proteins shown. Lysates were prepared as described in section 3.3.3; page 37, and immunobloted with the indicated protein-selective antibodies (the top of Fig. 11). The samples, where the cells were successfully treated with specific siRNAs for Rab11a, Rab35, RalA and GAPDH were immunobloted with the indicated exocyst-subunit-selective antibody. Cellular protein levels were determined by Western blotting following SDS-PAGE (the bottom of Fig. 11). In the figure, a control ‚**-**' correspond to the untreated cells where is no siRNA, a ‚**D**' control reflects cells treated with DharmaFECT transfection reagent in the absence of any siRNA, a negative control ‚**NT**' represents lysate from HeLa cells trasfected with siRNA which have no known target in the cells being used, and as a positive control was used a siRNA treatment of housekeeping gene for ‚**GAPDH**'. The controls reveal the successful selective knock-down of the indicated proteins.

Concretely, Fig. 11 revealed that depletion of Rab11a cause a slight decrease of level of Sec3 subunit. Other results were observed with Rab11a and Sec15 proteins. From previous research of Wu and his co-workers who studied *Drosophila melanogaster* [92] was found that Sec15 is in direct interaction with Rab11a. Thus we presumed that Rab11a depletion would cause absence of Sec15 subunit. Our results have confirmed this assumption. However, surprising result was observed during the knock-down of Rab35

proteins which cause “vanishing” of Sec8 subunit. The observation reveals the fact that Rab35 protein has a significant influence on the Sec8 subunit. Thus would be interesting to investigate whether depletion of Rab35 could cause failures in the proper course of cell division. According to other research on from the laboratory of Prof. Cascone [133], was expected that knock-down of RalA would have negative effect on Sec6 and Sec5 subunits. However, our data shows the ambivalent results, after all, in the case of Sec5 subunit.

In summary, this part of work aimed to determine whether the knock-down of proteins Rab11a, Rab35 and RalA affects any of exocyst subunits. The results indicate possible interactions between Rab11a and Sec3, Rab35 and Sec8 and Rab11a and Sec15 which could have an important role in regulation of membrane trafficking events throughout the cell. Ambiguous results given between RalA and Sec5 or Sec6 lead us to hypothesis that these exocyst subunits may be accessory regulators of distinct vesicular pathways where other small G proteins plays role of crucial regulators

5 Discussion

Membrane trafficking is a crucial but poorly understood process in cytokinesis especially in mammalian systems [11]. In this thesis we report an essential role of the exocyst, as well as its interacting GTPases Rab11a, Rab35 and RalA, in directing the membrane vesicles to the midbody. Our data suggest that despite a general need to complete the formation of newly emerging membrane, the exocyst interacts with Rab11a, Rab35 and RalA GTPases, and thus mediates the delivery of specific sorts of vesicles to the abscission site.

The data represented in this work support the idea that the vesicles derived from recycling endosomes (REs) are critical for the completion of cytokinesis [11,134]. The results also point to a new mechanism responsible for vesicle delivery events. We found that each of the studied GTPases (Rab11a, Rab35 and RalA) is localized in the area of REs; i.e. are localized in the territory of vesicular structures originating in REs. In addition, the parallel studies from our laboratory which have dealt with the distinct phases of cytokinesis (data not shown) revealed the protein redistribution during last steps of cytokinesis. Moreover, several papers also referred to the possible regulation of all the studied GTPases during cell cycle [14,82,83]. Thus we can highlight here that distribution of the Rab11a, Rab35 and RalA GTPases spatially varies depending on the phases of mitosis. We expected that the GTPase re-localization might be coupled with redistribution of exocyst subunits. Taken together, our results suggest a direct role of the Rab11a, Rab35 and RalA GTPases in targeting the RE-derived vesicles to the plasma membrane (i.e. the endosomal pathways regulation) via its interaction with exocyst. This idea is somewhat distinct from the formerly proposed function of the exocyst complex which emphasize its proceeding in secretory pathways [135,136]. On the other hand, some studies have reported the possible cooperation of exocyst protein Sec15 and Rab11 GTPase [91], as well as the localization of Sec10 subunit in the area of REs [117]. Therefore, we presume that in mammalian cells this possible cooperation of Rab11a, Rab35 and RalA GTPases with exocyst members might point to a new particular adaptation of targeting the RE-vesicles. These data together led to theory, that the exocyst may direct the RE-vesicles (which are associated with Rab11a, Rab35 and RalA proteins) to central cytokinetic structures such as abscission site.

In this work, we have confirmed the interaction between endosomal protein Rab11a and the exocyst subunit Sec15 in other mammalian cell type [91,92]. In addition, we have observed the potential influence between Rab11a and Sec3 as well as RalA and Sec5 proteins although the subcellular localization has shown different distribution of these two proteins. This interesting finding leads to an idea that these two proteins might be recruited from different origin to the site of abscission. We also discovered that depletion of Rab35 causes strong decrease of the level of Sec8 subunit. These results have been never described before. Nevertheless, we consider that the interplay between these two proteins point to the fact that this interaction may be crucial for membrane trafficking events throughout the mammalian cell. And finally, in consideration of multivalent results given between RalA and Sec5, or RalA and Sec6, we propose the idea that targeting and delivery of the exocyst subunits may be accompanied by mutually intertwined vesicular pathways in which these interactions may support the membrane dynamics. Therefore, it would be interesting to learn whether at least one of the routes might be truly regulated by above-mentioned GTPases.

The mechanisms governing the mammalian cell division are poorly understood. Nevertheless, several groups have driven attention to REs which may play an important role in the completion of cytokinesis, likely via its transient redistribution to the future site of abscission [71,137]. The specific RE-vesicles may be re-positioned to seal off the abscission or facilitate recruiting of key signaling proteins [137]. Moreover, it is tempting to consider that the exocyst complex may provide the crucial specificity of this process. One possibility is that Rab11a, Rab35 and RalA GTPases, via the exocyst, may direct the delivery of various proteins essential for the terminal stages of cytokinesis [83]. Thus it is suggested that abundance of exocyst along with GTPases (which are distributed in REs and are involved in cytokinesis events) may regulate distinct pools of vesicles which are derived from REs [83]. On the other hand, we have to also consider that all of the studied GTPases Rab11a, Rab35 and RalA itself may have some other function during the cell cleavage.

In summary, this thesis has exposed an essential role of the mammalian exocyst complex which is required for successful completion of cell separation. It has been known that the final cut between two daughter cells is accompanied by targeting vesicles derived from REs [71,137]. We propose here that the trafficking of RE-vesicles is controlled throughout the interplay of exocyst members and Rab11a, Rab35 and RalA GTPases. However, the deeper investigation using more sophisticated molecular methods of fluorescent or time-laps microscopy will be required to uncover more precise interplay between exocyst and its effectors. Therefore, the clarification remains the challenge for the future.

6 Summary and future direction

The aim of this work was to expand the present understanding of the critical role in appropriate interaction between exocyst subunits and Ras-like GTPases; concretely Rab11a, Rab35 and RalA proteins. The mutual communication seems to be crucial for targeting and regulating the recycling pathways which contribute to successful mammalian cell division. The experiments were performed in non-synchronized HeLa cells using various biochemical and cell-biology methods such as density gradient centrifugation, immunoblotting, radioligand binding assay and siRNA screening.

Results obtained may be summarized as follows:

- Results presented in this work support the earlier published data defining the essential role of the mammalian exocyst complex in successful formation of the new plasma membrane and completion of the cell separation of the two daughter cells in the course of cytokinesis.

- It has been known that the final cut between the two daughter cells is accompanied by targeting of vesicles derived from REs to PM. However, we highlight here that the trafficking of RE-vesicles might be controlled throughout the direct interplay of members of exocyst subunits and Rab11a, Rab35 and RalA GTPases. More concretely, we underline the possible interactions between Rab11a GTPase and Sec3; and Rab35 GTPase and Sec8. In addition, we also confirmed interactions between Rab11a GTPase and exocyst subunit Sec15 in other mammalian cell type. **This is new and original finding thoroughly documented in this thesis.**

- The deeper investigation using more sophisticated cell biology and biochemical methods such as fluorescence and "time-laps" microscopy will be required to uncover more precise interplay between protein members of exocyst complex and their regulatory effectors. The clarification of these questions remains the challenge for future research.

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