Module: Cell Migration – *2018*

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Structure

Content is the result of lectures, publications, conferences since 2000. Latest revision depicting a simplified version of current knowledge: April 2018 with the support of Mathis Nicolas (CAL, D-Biol, ETH-Z).

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Chapter 5: Chemotaxis in Dictyostelium discoideum

Glossary

Abbreviation	Full name	Class	Details	Chapter Nr.
AC	adenylyl cyclase	enzyme	in Dictyostelium activated by CRAC; enzyme synthesizing cAMP out of ATP; cAMP can relay the cAMP wave outwardly to recruit additional cells. The cells adapt quickly to high cAMP levels. Not required for chemotactic motility of individual cells	5
ActA	Actin assembly-inducing protein	bacterial protein (Listeria): so called: nucleation-promoting factor	Listeria surface protein that mimics N-WASP structurally and functionally; essential for formaiton of actin tails at one end of Listeria; activates ARP2/3 complex of mammalian host cell: The ActA protein is asymmetrically distributed on the bacterial surface. It binds the Arp2/3 complex, and the resulting activation of this complex initiates de novo actin polymerization at the bacterial surface. It has similar to N-WASP an acidic domain binding the Arp2/3 complex and a WH2-like domain, binding monomeric actin. The actin polymerization is further promoted by the binding of VASP, or Vasodilator-stimulated phosphoprotein, which recruits profilin that is bound to ATP-actin monomers, and thereby increases the local concentration of actin monomers (see chapter 3,4). Therefore, actin monomers are brought in close vicinity to active Arp2/3 complex, and enhance the initiation of actin polymerization. The barbed end remains uncapped for a short period, allowing fast growth of the filament. The original Arp2/3 is released after a while, and another Arp2/3 complex binds ActA at the side of an existing filament. This creates a branched network of actin filaments, where the Arp2/3 complex is present throughout the length of the actin tails. Interestingly, ActA appears to have also Arp2/3 independent actin polymerization activities, and it has been suggested that Listeria movement in host cells starts with an Arp2/3 dependent nucleation step, followed by an Arp2/3 independent elongation step	3
Actin filaments	see also F-actin	Cytoskeletal component	Polarized structures (pointed and barbed ends); assemble into a right-handed double helical structure; Actin monomers exist in ATP-bound conformation (T-form) or ADP- bound conformation (D-form)	3
ADF/Cofilin	actin-depolymerizing factor (ADF)	family of actin binding proteins	ADF binds to the side of ADP-actin filaments induces filament severing; also induces pointed-end depolymerization. Both activities lead to an increase in the concentration of monomeric actin at steady state; becomes inactivated by phosphorylation by LIM kinase	1 (Fig. 2), 3
Akt/PKB	PKB - protein kinase B (Akt synonym)	Serine/threonine-specific protein kinase	promotes survival and growth in response to extracellular signals mediated by receptor tyrosine kinases; protein kinase which contains a PH-domain; in Dictyostelium it is involved in maintaining cell polarity and proper chemotaxis; phosphorylates PAKa	5
APC	adenomatous polyposis coli	Microtubule plus end-binding protein	interacts with IQGAP1	1, 3
Arf GTPases	ADP-ribosylation factor GTPAses	Ras superfamily	vesicular transport	2
Arp2/3 complex	complex containing Actin-related protein 2 (Arp2) and Actin-related protein 3 (Arp3)	actin filament nucleator	if activated acts as actin filament nucleator; composed of 7 proteins; induces formation of filaments branching off with 70° angle from a mother actin filament; actin nucleator that remains bound to the pointed end of actin filaments	3
Bombesin		neuro-peptide	composed of 14 AA (amino acids), from frog skin, activates Rac	4
Bradykinin		kinin (a group of polypeptides formed in body tissue in response to injury)	a 9 AA peptide of the kinin group of proteins, that is physiologically and pharmacologically active; causes activation of Cdc42 via a bradykinin receptor in the plasma membrane	4
cAMP	cyclic adenosine monophosphate	cyclic nucleotide	second messenger: in Dictyostelium chemoattractant that activates CAR1	5
CAR1	cAMP receptor 1	transmembrane receptor	ligand for the receptor in Dictyostelium is cAMP; receptor is coupled to a heterotrimeric G-protein; The binding of cAMP regulates five main effectors: PLC, AC, GC, PI3K and PTEN; whereby activation of PI3K is presumed to require the Ras GTP-binding protein in addition.	5
Cdc24	Cell division control protein 24	Rho-GEF	Protein identified genetically as an upstream activator of Cdc42 in budding yeast, GEF for Cdc42.	2
Cdc42	Cell division control protein 42 homolog	Rho-GTPase	small GTP binding and hydrolyzing protein; activates WASP proteins; Cdc42-GTP causes the formation of focal complexes and actin polymerization for filopodia; regulates also plasma membrane polarization and directional movement	1, 2, 3
CLIP-170	cytoplasmic linker protein - 170	Microtubule plus end-binding protein	170 kDa	1, 3
Cobl	Cordon-bleu	actin filament nucleator	promotes nonbundled, unbranched filaments similarly to Spire	3
Cofilin	see ADF/Cofilin			3
CP	Capping protein		by blocking the majority of actin filament barbed ends, capping proteins increase the concentration of monomeric actin at steady state and funnel the flux of actin monomers to the non-capped filaments, which thus can elongate faster	1 (Fig 2), 3
CRAC	cytosolic regulator/activator of adenylyl cyclase	activator of adenylyl cyclase	PH-domain containing protein; activates adenylyl cyclase in the back of a Dictyostelium cell after cAMP sensing (unknown how exactly), resulting in the secretion of cAMP into the medium.	5

Glossary

CRIB domain	Cdc42/RAC interactive binding region	protein domain	GTPase binding domain occurring in many Rho effector proteins; Proteins with CRIB domain bind to Cdc42 and/or Rac in a GTP-dependent manner; can be masked by intramolecular fold (e.g. in WASP)	2
Dbl	diffuse B-cell lymphoma	Mammalian GEF	protein activating Cdc42; Rho-GEF with DH- and juxtapositioned PH-domain. Dbl's N-terminal domain binds to the DH-domain and inhibiting its activity. Dbl with an N-terminal deletion is constitutively active, and the originally discovered oncogenic mutation in Dbl encoded indeed for an N-terminally truncated protein.	2
DH domain	Dbl homology domain	conventional GEF domain for Rho-GTPases	autoinhibited	2
DOCK180	Dedicator of cytokinesis 180 kDa	unconventional Rho-GEF	mediate nucleotide exchange; need second protein such as ELMO1 for exchange reaction in vivo	2
EB1	end-binding protein 1	Microtubule plus end-binding protein	BIM1 in S*accharomyces cerevisiae*	1, 6 (cell-cycle module)
ELMO1	Engulfment and cell motility protein 1	adaptor for unconventional Rho-GEF, cooperates with DOCK180	ELMO1 is important to stabilize the nucleotide-free transition state of Rac; PH-domain of ELMO is involved in targeting DOCK180 to the plasma membrane; ELMO seems to perform similar functions like the PH domain in conventional GEFs	2
F-Actin (see also: actin filaments)	filamentous actin	Cytoskeletal component	composed of 2 actin- protofilaments, with a 55 A spacing, (diameter 7 nm) In Dictyostelium: Assembled at the leading edge of the cell, forming the extending pseudopod. F-Actin synthesis is oriented relative to the chemoattractant (cAMP) source	1, 5
FAK	focal adhesion kinase	kinase in focal adhesions	key kinase in focal adhesions.	1
FGD1	faciogenital dysplasia protein 1	GEF for Cdc42	mutations in FGD1 cause faciogenital dysplasia, also known as Aarskog-Scott syndrome	
formin		family of proteins; actin filament nucleator	contains conserved formin-homology domains (FH1, FH2); actin nucleators that remain bound to the barbed ends (+ end) of actin filaments despite ongoing polymerization. FH1 domain recruits actin monomers through profilin binding, but it does not promote nucleation. FH2 dimer nucleates filament assembly; regulation by Rho-GTPases releases the autoinhibited conformation of formins.	3
GAPs	GTPase-activating proteins	class of proteins with GAP activity		2
GC	guanylyl cyclase	enzyme	in Dictyostelium: Positively regulates Myosin II assembly at the back and the side of the cells	5
GDIs	Guanine nucleotide dissociation inhibitors	class of proteins with common activity	Rho-GDIs extract Rho proteins from membranes and keep them in an inactive cytosolic complex	2
GEF-H1		Mammalian GEF	is enzymatically inactive towards Rho-GDP when bound to microtubules; if microtubule depolymerize, it exhibits GEF activity generating Rho-GTP	4
GEFs	Guanine nucleotide exchange factors	class of proteins with GEF activity	catalyze GDP displacement and stabilize nucleotide-free GTPase	2
IcsA		bacterial protien (Shigella)	is anchored in the outer membrane of Shigella, targeted directly to the old pole of the bacterium and mediates actin tail assembly that propells the bacterium inside mammalian cells. IcsA has been proposed to mimic N-WASP activation by Cdc42-GTP, probably by inducing the conformational changes that release N-WASP from its autoinhibited conformation. IcsA is restricted to one bacterial pole and is absent from the actin tail itself. This suggests that it is not released from the bacterial surface during polymerization, in contrast to what has been shown for N-WASP.	3
Insulin		peptide hormone	composed of 51 AA	4
Intermediate filaments		Cytoskeletal component	No polarity; e.g. cytokeratins, lamins	3
IQGAP1	IQ domain and Ras GTPase-activating-like protein 1	phospho protein, adaptor protein, multifunctional	It was never shown to exhibit GAP activity neither towards Ras nor towards Rho GTPases. IQGAP1 is a ~190 kDa homodimeric protein that is widely expressed among vertebrate cell types from early embryogenesis through adulthood. It is present in S. c. (Cyk1/Iqg1) and S. pombe (RNG2) but apparently not in Drosophila. Rho-specific effector protein (if unphosphorylated) ; plays a role in cell-cell adhesion and migration; can act as a GEF if phosphorylated; a 190 kDa homodimeric protein that is widely expressed among vertebrate cell types; can be autoinhibited. IQGAP1 was identified in 1994 as an IQ domain-containing protein with a region containing sequence similarity to Ras GTPase-activating proteins.	2, 4
IRSp53	Insulin Receptor Substrate of 53kDa	effector protein of Cdc42	can be auto-inhibited, contains an unusual CRIB domain; if bound to Cdc42-GTP the auto-inhibition is relieved and plasma membrane association given.	4
Kar9	Karyogamy protein KAR9	Microtubule plus end-binding protein	in *Saccharomyces cerevisiae*	1, 6 (cell-cycle module)
Leimodin			an actin-binding protein acts with tropomyosin as strong filament nucleator in muscle cells	3
LIM kinase	LIM domain containing kinase	kinase (family)	highly cysteine-rich and contain 2 zinc fingers; get activated/phosphorylated by ROCK and afterwards phosphorylate/inactivate ADF/cofilin	4

Glossary

mDia1	mammalian diaphanous 1	actin nucleator: Formin (sub group: diaphanous)	dimer; relevant here: localizes to mitotic spindle and midbody, plays a role in stress fiber and filopodia formation, phagocytosis; Autoinhibition is achieved through binding of the C-terminal DAD to the N-terminal DID; activated by Rho-GTP; IQGAP1 regulates the localization of mDia1 to the leading edge to cells, but does not activate it. Interacts also with CLIP170.	3
mDia2	mammalian diaphanous 2	actin nucleator: Formin (sub group: diaphanous)	dimeric; regulated by autoinhibition; actin nucleation functions are stimulated by binding to Rho family GTPases such as RhoA-C and Cdc42; FH2 domain dimer associates with barbed end of an actin filament, while FH1 domains recruit profilin-actin; also generates specialized stable microtubules and orients them towards the leading edge	1 (Fig. 2), 3
Microtubule		Cytoskeletal component	Polarized hollow cylinder with a so called GTP-cap constituting the plus-end, about 25 nanometers in diameter; composed of alpha-beta tubulin dimers, which are GTP binding proteins.	3
Myosin II	here: for non-muscle myosin II	actin motor protein	Autoinhibited in non-muscle cells by head-head and head-tail interactions; 2 heavy chains each with 2 light chains; each heavy chain is composed of head, neck and tail domains; activated (not autoinhibited) if myosin light chain is phosphorylated; activated myosin II binds to actin filaments and causes cellular contraction; motor activity of myosin is linked to ATP hydrolysis; head domain binds the filamentous actin, and uses ATP hydrolysis to generate force and to "walk" along the filament towards the barbed (+) end; in Dictyostelium: Assembly of Myosin II increases cortical tension at the sides of the cell, which represses lateral pseudopod extension. Myosin II also promotes contraction at the end of the cell during the movement. Myo II is activated by cGMP, and by p21-activated kinase PKAa (which itself is activated by Akt/PKB).	4, 5
Nck	non-catalytic region of tyrosine kinase adaptor protein	adaptor protein	an mammalian host-cell protein consisting of three SH3 and a single SH2 domain; relevant for vaccinia virus induced actin tail.	3
N-WASP	"Neuronal" WASP	Cdc42 effector protein	contains CRIB domain; involved in actin organization, activator of ARP2/3 complex, can be autoinhibited; if inactive: intramolecular interaction between CRIB domain and VCA-domain exists.	1 (Fig.2), 2, 3, 4
p150	dynactin subunit p150	part of an activator complex for a motor protein	p 150 is part of the dynactin complex, which is an activator (multimeric complex) of the motor protein dynein. P150 (glued) is the largest complex subunit and essential for the function of the complex. The dynactin complex contains Arp1, and actin related protein. Dynactin modulates dynein activity, the microtubule motor protein, transporting organelles along microtubules.	1
PAK	p21-activated kinases	Kinase	highly conserved serine/threonine protein kinases with a CRIB domain; PAKs bind and become activated by Rac-GTP and Cdc42-GTP; PAK phosphorylates and thereby inhibits myosin light chain kinase (MLCK) resulting in lower levels of myosin phosphorylation at the front of the cell	4
PAKa	p21-activated kinase a	PAK, kinase family	Dictyostelium homolog to mammalian PAKs (CRIB domain containing p21-activated kinases); when phosphorylated by Akt/PKB it translocates to posterior part of chemotaxing cells where it promotes myosin II assembly	5
PDE	phosphodiesterase	enzyme	in Dictyostelium exported into the extracellular environment; cleaves extracellular cAMP into AMP and thus eliminates cAMP, thus allowing the cells to become responsive to the next cAMP wave	5
PDGF	platelet-derived growth factor	growth factor	secreted dimeric glycoprotein, induces actin polymerization after binding to corresponding receptor via Rac.	4
PDI	phosphodiesterase inhibitor	inhibitor	in Dictyostelium exported into extracellular environment; inhibits PDE activity; expression is induced by low cAMP levels	5
PH domain	Pleckstrin Homology domain	membrane recruitment domain	domain of 100-120 AA binds to phosphatidylinositides, frequently found in conventional Rho-GEFs where it supports GEF activity. For example present in: Dbl, Akt/PKB, CRAC and PhdA. In Dictyostelium PI3K and PhdA, potentially together with PH-domain containing GEFs for Rac GTP, regulate F-Actin polymerization and pseudopod extension in the direction of the cAMP gradient.	2
PI3Ks	Phosphatidylinositol-3 kinases	group of lipid kinase	phosphorylates PIP2 to PIP3; control PIP3 levels not only in Dictyostelium	5
PIP2	Phosphatidyl inositol (4,5) bisphosphate	a phosphoinositide	In the plasma membrane; interaction with capping protein can lead to the removal of the capping protein from barbed ends	3
PIP3 / $PI(3,4,5)P_3$	Phosphatidyl inositol (3,4,5)-tri phosphate	a phosphoinositide, specific lipid of the phosphatidylinositol family that is class of the phosphatidylglycerides	in many cellular membranes with role in signaling; general, can recruit PH-containing proteins to the site, where it is present. In Dictyostelium it is generated by PI3K after cAMP sensing and leads to the recruitment of CRAC.	2, 5
PLC	Phospholipase C	enzyme	membrane associated enzyme hydrolyzin phospholipids. The PLC pathway is not essential for chemotaxis of Dictyostelium	5

Glossary

Profilin		actin (monomer) binding protein	Profilin (about 19 kd, conserved) enhances the exchange of ADP for ATP to recycle actin monomers during F-actin polymerization. The profilin adds exclusively actin monomers at the barbed end of F-actin. Profilin binds to monomeric actin occupying an actin-actin contact site. Profilin-actin complexes are fed into growing actin polymers by proteins such as formins, N-WASP and VASP.	1(Fig. 2), 3
PTEN	Phosphatase and tensin homolog	tumor suppressor; lipid phosphatase	lipid phosphatase and negative regulator of PI3K pathway by dephosphorylating PIP3; Accumulation of PTEN at the sides and the posterior of the cells antagonizes recruitment of PH proteins to these sites and suppresses lateral pseudopod formation; controls PIP3 levels in Dictyostelium	5
Rab GTPases	"Ras-related proteins in brain" GTPase	distinct subfamily within the Ras superfamily	vesicular transport	2
Rac1	Ras-related C3 botulinum toxin substrate *1*	small GTPase, member of Rho-GTPase subfamily of the Ras superfamily	GTP binding and hydrolyzing protein; the active form is GTP bound Rac1 (Rac1-GTP). Rac1-GTP activates WAVE complex; causes the formation of lamellipodia or ruffles at the leading edge; inhibits myosin light chain phosphorylation/activation via PAK and MLCK	2
Ran GTPase	Ras-related nuclear protein -GTPase	distinct subfamily within the Ras superfamily	regulate nuclear transport and mitotic spindle assembly	2
Ras		small GTPase, founding member of the Ras superfamily	first identified in Harvey and Kirsten rat sarcoma viruses (1967); key regulator of three very different reactions: cell differentiation, cell proliferation and apoptosis; signal transduction	2
Ras GTPases		distinct subfamily within the Ras superfamily	involved in regulation of growth, differentiation and apoptosis	2
Rho GTPase	founding member of subfamily: RhoA	distinct subfamily within the Ras superfamily	regulates organization of cytoskeleton, cell cycle progression, vesicle transport and gene expression; found in all eukaryotic cells; molecular switches that mostly cycle between active GTP-bound state and mostly inactive GDP-bound state	2
RhoA		small GTPase, member of Rho-GTPase subfamily of the Ras superfamily	GTP binding and hydrolyzing protein; if in the GTP bound form it promotes actin-myosin contractility, and the formation of stress fibers and focal adhesions	2
Rho-GAPs	GTPase-activating proteins acting on Rho-GTPases	class of proteins with GAP activity	Rho GAPs activity is encoded in the Rho-GAP domain. Based on the human genome sequence, it is estimated that approximately 80 genes code for Rho-GAPs.	2
ROCK	Rho-associated serine/threonine protein kinase	Serine/threonine protein kinase	ROCK1 and ROCK2; phosphorylate LIM kinases which then phosphorylate and inactivate ADF/cofilin; phosphorylates myosin light chain (MLC) and inactivates MLC phosphatase and thereby promote cellular contractility	4
Septin		Cytoskeletal component	GTP-binding proteins, assemble into homo- and hetero-oligomers and non-polar filaments, predominantly known for their function during cytokinesis	3
Slingshot		phosphatase	phosphatase which dephosphorylates and thereby activates ADF/Cofilin	4
Small GTPases		Ras superfamily	about 21 kDa, GDP and GTP binding protein with enzymatic activity	2
SopE		unconventional GEF (in bacteria)	bacterial protein (Salmonella), promoting bacterial entry into non-phagocytic cells; GEF for Rac and Cdc42	2
Spire			nucleates new actin filaments at a similar rate to formins, but slower than the activated Arp2/3 complex; important roles in membrane transport and cytoskeletal interactions	3
SptP		Secreted effector protein of the bacterium Salmonella	Salmonella protein with GAP activity on Rho-GTPases in mammalian cells	2
Stathmin		regulator protein of microtubule dynamics; phosphoprotein	Microtubule polymerization is inhibited by non-phosphorylated stathmin. One (non-phosphorylated) stathmin molecule destabilizes microtubules by i) sequestering two soluble dimeric alpha, beta-tubulin complexes into a tight complex, reducing thus the polymerization-competent tubulin and by ii) increasing the frequency of microtubule growth-to-shortening rate by directly binding to microtubule ends. Phosphorylated stathmin cannot sequester soluble tubulin dimers and liberates the previously bound alpha, beta-tubulin-complexes, that now can be used for microtubule polymerization.	4
VASP	Vasodilator-stimulated phosphoprotein		binds profilin thus driving actin filament elongation	4
VCA domain	(Verprolin, central, acidic) domain	WASP domain	activates Arp2/3 complex; Verprolin homology region, (also called WH2 domain or WASP homology domain 2) interacts with actin monomers, the central (C) domain and the A-domain (acidic region) bind both to the Arp2/3 complex	3
WASP	Wiskott-Aldrich syndrome protein	Cdc42 effector protein; so called: nucleation-promoting factor	only present in hematopoietic cells; contains CRIB domain; involved in Actin organization; activates Arp2/3 complex; in the dendritic nucleation model, GTP-bound Rho-family GTPases together with PIP2 can activate WASP/WAVE proteins; can be autoinhibited	2, 3, 4
WAVE complex	WASP family Verprolin-homologous protein	so called: nucleation-promoting factor	if itself activated the WAVE complex activates the Arp2/3 complex	3

Glossary

WH2 domain	WASP homology 2 domain	domain	binds an actin monomer, in many actin binding proteins; synonyme of Verprolin homology domain "V" part of the VCA domain..	3
WIP	WASP-interacting protein	WASP-interacting protein	WIP has been shown to interact with N-WASP, profilin, globular and filamentous actin. It stabilizes actin filaments and participates in filopodia and lamellipodia formation in vivo. Here relevant for vaccinia virus induced actin tail.	3
Zizimin		unconventional GEF	mammalian protein, belongs to Dock (Dedicator of Cytokinesis) superfamily of Guanine nucleotide Exchange Factor (GEF) proteins; GEF for Rac and Cdc42 ; implicated in cell-migration and phagocytosis	2

Module: Cell Migration - *2018*

Cell migration

Cell migration is a fundamental process that can be observed both in unicellular organisms, such as amoeba, and in multicellular organisms such as mammals. It refers to the translocation of a cell over time either over a surface or within 3-dimensional structures. During cell migration a cell is crawling which contrasts to cell swimming that occurs in liquid environment. In unicellular organisms and specialized cells of metazoans cell migration allows for gamete pairing, predator evasion and the search for food. Specifically, in multicellular organisms it is essential during organogenesis (e.g. heart), organ homeostasis (e.g. renewal of skin, intestine) and pathologies. For example, gastrulation depends on the migration of cells in groups to constitute a three-layered embryo and has thus morphogenic function. Also, immune cells have to constantly survey and migrate through tissues in order to eliminate invading bacteria and other microorganisms.

Cell migration is a complex process, and every organism has developed a complex network of regulatory controls ensuring a coordinated migration that can be turned on or switched off when needed. Importantly, the deregulation of cell migration contributes to several important pathologies including osteoporosis, chronic inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, mental retardation and cancer. In this later case, metastasizing cancer cells migrate as single cells or in small groups to spread from the initial site of tumour growth, after having acquired an invasive phenotype characterized by the loss of cell-cell interactions and increased cell motility.

The migration cycle

The prototype example for mammalian cell migration is the migration of an individual fibroblast-like cell over a flat surface. Its migration can be conceptualized as a cyclic process in which a cell executes the following steps:

- Protrusion (at the front of the cell, driven by actin polymerization)
- Adhesion (formation and stabilization of cell attachments to the substrate, near the leading edge)
- Contraction and retraction of the trailing edge

Chapter 1: Metazoan migration modes and organization of the cytoskeleton

Remarkable in this chapter:

- Characteristics of mesenchymal (e.g. fibroblast) and amoeboid (e.g. Dictyostelium) migration modes (also migration speeds)
- Structural overview of fibroblast migration over a flat surface (main model in chapters 1, 2, 3 and 4):
    - actin structures (filopodium, lamellipodium, lamella, stress fibers)
    - cell-substrate adhesion: focal adhesion, focal complex, adhesive gradient
    - microtubules (organization, types, function, effect on migration speed)

1 - Introduction

Cell migration is the net advancement of a cell relative to a substrate. Migration can occur in 2- or 3-dimensional (3D) situations, such as single Dictyostelium discoideum cells migrating on the surface of leaves or cancer cells moving through tissues, respectively. Since about 2008 it became evident that two different migration modes can be distinguished: amoeboid and mesenchymal migration (Figure 1).

Amoeboid movement occurs e.g. in single Dictyostelium discoideum cells (chapter 5). In general, amoeboid migration is characterized by rapid protrusion and retraction of protrusions (pseudopods or blebs), which are supported or driven, respectively, by actomyosin cortex contractility, weak cell-substrate interactions (without mature focal adhesions and stress fibers), and a lack of matrix degradation.

Amoeboid migration is extremely fast, with speeds reaching 10–25 μm/min. It was recently classified in two sub-modes:

1. **Pseudopodal mode**: This predominantly in migrating Dictyostelium cells occurring sub-mode is characterized by an actin-rich pseudopod at the leading edge that engages in poorly defined, weak adhesive interaction with the substrate. This mode is also found in other elongated amoeboid cells (e.g. monocytes).
2. **Bleb mode**: Membrane blebbing is the second sub-mode of amoeboid migration (predominant in 3D environment, spatial confinement), it occurs in cells that do exhibit traction on a substrate (yet reduced adhesion compared to fibroblasts) and advance by pushing the cell content forward. Migration-linked membrane blebbing (which is not linked to apoptotic events) occurs in many cell systems, one of which is Dictyostelium. It can also be found in cancer cells, which can switch from an actin polymerization dependent protrusion formation to a bleb-driven motility in 3D matrixes. Membrane blebs are bulbous, cytosol filled plasma membrane extensions at the leading edge that are generated by

hydrostatic pressure increase in the cell and an irregularity in the actin cortex. The pressure increase originates from myosin II dependent cell contraction of the cell cortex distant from the bleb. Blebs are formed at a cortex region with inhomogeneities in the actin cytoskeleton, they are initially free of actin and only subsequently filled with it.

Thus, amoeboid migration exists also in mammals (stem cells, most leukocytes, lymphomas, small-cell lung cancer cells) and is a feature of nonresident cells that move towards various tissues.

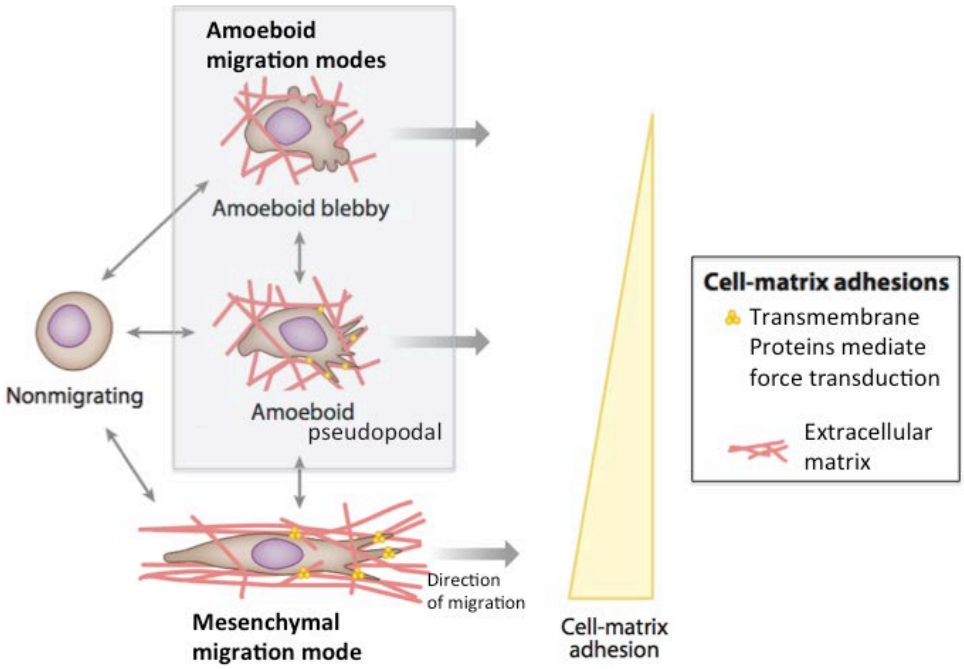

Figure 1: Migration modes of single cells. Characteristics of amoeboid-moving cells include a roundish or ellipsoid morphology with a relatively short trailing edge but a plastic, highly dynamic front edge with bleb-like protrusions (e.g. in primordial germ cells, Dictyostelium) or leading dendrites, filopodia, or pseudopodia (e.g., in dendritic cells, monocytes); prominent deformability of the cell body; weak adhesion toward the substrate; and limited ability to remodel tissue (by proteases) while these cells move. Amoeboid-moving cells apply adaptive adhesive and non-adhesive interactions for speed generation and can readily cross epithelial, endothelial, and basement membrane barriers. Mesenchymal movement generates an elongated cell shape, with long extensions in the forward and rearward directions, strong adhesion (integrin mediated) and traction followed by tissue realignment, and tissue remodeling during migration. Modified from Boekhorst, te, V., et al (2016).

In contrast, mesenchymal migration detectable in fibroblasts and some tumor cells is slower (~ 0.1-1 µm/min) than amoeboid migration and characterized by strong friction, in this case real adhesiveness, to the substrate, as well as proteolysis of the extracellular matrix. This migration is a process in five steps and characterized by actin polymerization, the use of strong integrin mediated cell-substrate adhesions and myosin II dependent contraction of stress fibers anchored at these adhesion sites. F-actin and microtubules influence each other in these five steps:

1) Cell polarization causes localized actin polymerization and thus protrusion formation.
2) New adhesion formation: If the protrusion membrane contacts ligands of the extracellular matrix (ECM), integrins cluster, physically link to the substrate

and build up initially nascent adhesion sites, **focal contacts** (= non-matured adhesion sites).

3) Removal or maturation of the new adhesions: The newly formed focal contacts can be locally degraded by extracellular surface proteases and thus be resolved. Alternatively, these focal contacts can mature into structures known as **focal adhesions**.
4) Cell body contraction: During or shortly after integrin–ECM binding, actin filaments engage with cross-linking amongst themselves and with bundled and activated myosin II to from stress fibers (typically 10-30 bundled actin filaments). Cellular tension is build up on mature focal contacts, the focal adhesions, where myosin II localizes. These newly formed focal adhesions are at the back of the leading edge (at the junction between lamella and lamellipodium, Figure 2) and can be further modified. Signaling induced shortening of membrane-anchored actin stress fibers results in local cell contraction to advance the cell body.
5) Rear detachment: De-adhesion at the trailing cell rear is also contraction dependent and depends on the protease calpain and microtubules that resolve focal adhesions in the back.

We will focus first on mesenchymal migration, namely mammalian integrin-dependent migration – typical for a fibroblast - over a 2-dimensional surface coated with extracellular matrix proteins. This migration is a highly coordinated process building on cell shape changes and the development of traction. Both depend on the dynamics of the cytoskeleton and regulation of cell adhesion. Cell adhesion is mediated via transmembrane integrins that link the chemical and mechanical properties of the ECM (composed of molecules such as fibronectin or collagen) to the actin filament and also to the microtubule system and affect cell physiology on several levels (Figure 2). As intermediate filaments withstand larger deformations than actin filaments or microtubules, they contribute to the cell mechanical effects during migration, an aspect more and more emerging since 2010, but not presented here.

You will learn that Rho-GTPase signaling (chapter 2) strongly influences microtubule and actin dynamics (chapter 3). Further, the internal force development depends on the magnitude of:

i) friction (e.g. if focal adhesions are present or not in a given cell)
ii) actin polymerization in the leading edge (chapter 3), and
iii) cellular contraction (here and chapter 3, 5)

If actin polymerization in the leading edge (ii) is combined with focal adhesions (i) then this results in a protrusive force. Cellular contraction (iii) occurs in fibroblasts mostly via stress fibers anchored in focal adhesions (i) establishing the contractile force. However, in amoeboid migrating cells, cortical contraction (iii) in the rear of the cell causes hydrostatic pressure and frontal bleb formation.

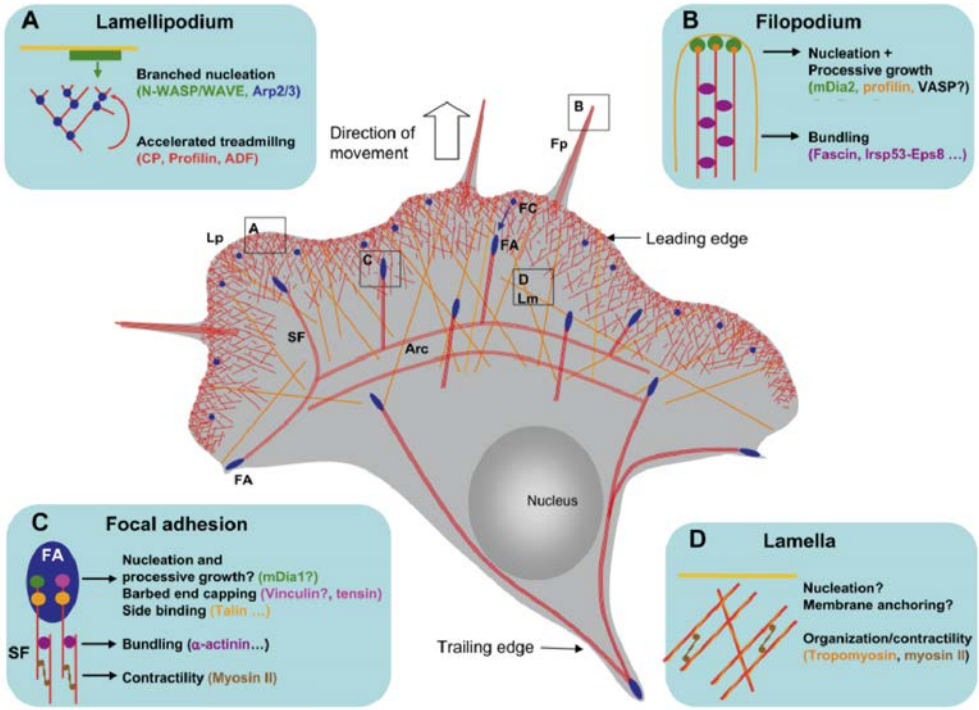

Figure 2: Schematic illustration of the major actin filament dependent structures in a migrating cell: lamellipodium (Lp), lamella (Lm), filopodia (Fp), actin networks (e.g. SF, stress fiber), focal contacts (round small blue symbols, FC), focal adhesions (FA), and the rear of a migrating cell. This cell does not correspond to a specific cell type. The mechanisms by which actin filaments are nucleated and anchored at the plasma membrane are not known. The formation of new FA's is controlled by Rac signaling, and their growth is determined by a Rho-dependent process. Contractile forces behind the leading edge drive movement of the cell body. The turnover of adhesive complexes is regulated by the combined activity of microtubules and regulators that reside in these complexes. In motile cells, traction on the substrate results in net forward movement. For abbreviations of protein names see glossary. Modified from LeClainche (2008).

This chapter highlights principle structures of a migrating cell (fibroblast) and roughly how they are linked. So that a cell can move forward it needs to exhibit friction (links) with the underlying substrate. Thus, we will discuss first the types and dynamics of adhesion sites typical for mesenchymal migration (fibroblast), then the special actin filament structures in newly generated protrusions, and finally integrate this information with the biology of microtubules.

2 - Adhesive complexes

In adhesion sites, specialized regions of the plasma membrane, the link between the immobile extracellular substrate to the cellular cytoskeleton can be tuned. Certain adhesion sites provide traction for force generation and are thus coupled to protrusion formation.

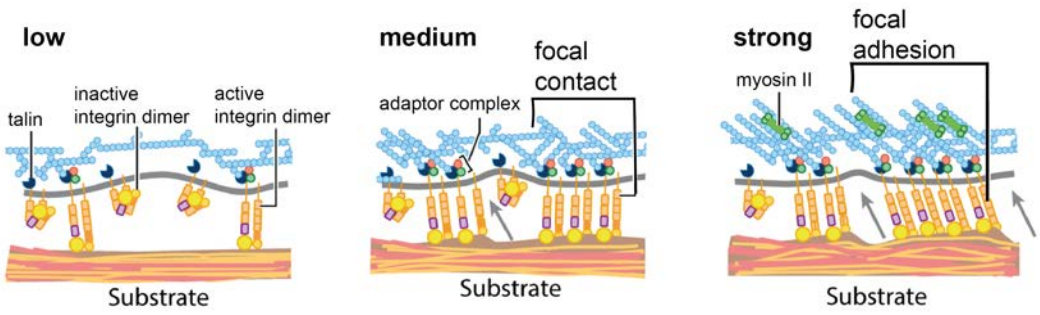

Figure 3: Schematics of adhesion sites with different adhesiveness. Left: Low adhesiveness due to non-clustered integrins diffusely distributed in the plasma membrane (grey line). Middle: Medium adhesiveness due to locally clustered integrins and focalized cortical actin (focal contact). Right: Mature focal adhesions with focalized actin filaments and insertion of contractile stress fibers containing myosin-II (focal adhesion). In focal adhesions, the cytoplasmic domain of integrins and focal adhesion kinase (FAK) lie close to the inner surface of the plasma membrane. The actin layer lies about 40 nm above the cytoplasmic tails of clustered integrin. Modified from: Boekhorst, te et al. (2016).

In adhesion sites, integrins (primary adhesion receptors), actin binding proteins (for example vinculin), actin filaments, and signaling proteins (for example FAK - focal adhesion kinase) cluster together (Figure 3). More than 100 additional adaptors and signaling proteins comprise the cytoplasmic domain of mature adhesion sites forming numerous links, both direct and indirect, between integrins and actin filaments. These dynamic and complex structures thus allow a high degree of regulation with respect to assembly, turnover and signaling activities, thereby ensuring efficient cell migration. Adhesion disassembly occurs both at the cell rear, where it allows rear retraction, and at the cell front, where it accompanies the formation of new protrusions and adhesions.

Heterodimeric integrins, composed of α and β subunits, are transmembrane proteins that are mechanosensors physically linking the ECM to the actin cytoskeleton. Adhesion structures can grow and become stronger when force is applied to them.

Two types of adhesion sites exist (Figures 2 and 3):

- **Focal complexes** (nascent adhesive complexes) are small, dot-like adhesions that are continuously formed at the ventral site of protruding lamellipodia and turned over at the lamellipodium-lamella border. Focal complexes, are intermediate adhesive, undergo maturation and are by this transformed into focal adhesions, which differ in molecular composition from focal complexes.
- **Focal adhesions** (older adhesive complexes) represent the mature and strong adhesive form of focal complexes and serve at least 2 important cellular functions: a) to transmit force or tension at adhesion sites by maintaining strong attachments to the underlying extracellular matrix and b) to act as signaling centers to regulate cell growth, cell survival and gene expression. Focal adhesions are dynamic structures that assemble, grow or disassemble and thus recycle as cells migrate or enter mitosis. They grow and extend centripetally, concomitantly with the formation of actin stress fibers. This transition is linked to a GTPase switch from Rac/Cdc42 to Rho (chapters 2 and 3), and to the engagement of myosin II with actin to from contractile bundles (stress fibers).

The hallmark of a polarized migrating fibroblast is the following asymmetric activity of adhesion sites (adhesion gradient):

- Leading edge: this is the only place where small focal complexes are present, and some focal adhesions. These focal adhesions are immobile relative to the substrate, to exert the necessary traction for migration. They are anchor points for i) the polymerizing actin network that pushes the leading edge membrane forward and ii) the actin bundles that execute the retraction of the rear which localize near the lamellipodium-lamella interface. In contrast to focal adhesions, focal complexes under lamellipodia seem to exert much less traction. It seems that the adhesion turnover in the protrusion front is determined by the molecular composition of the respective adhesion, e.g. adhesions with alpha-actinin do not disassemble.
- In the rear and flanks of the cell: large focal adhesions can be found, but many seem to slide (following inner adhesion proteins) relative to the substrate inwardly with the retracting cell edge and become disassembled. In the cell rear the coupling between actin and integrin is about four times reduced compared to the front of the cell. Thus, anterograde contraction seems to decouple integrins from a group of "inner adhesion" proteins (such as paxillin) and therefore does not lead to increased traction in the rear adhesions. The protease calpain seems to act at this step as it cleaves the scaffold talin (Figure 3) in rear adhesions. In addition, focal adhesions in the rear can be dissolved if microtubule tips "touch" them.

Accordingly, focal adhesions (looking at inner adhesion proteins, not true for integrins) are highly motile in stationary fibroblasts yet stationary in migrating fibroblasts. This suggests the existence of a molecular clutch that couples traction with contractile forces and actin polymerization (Figure 4). In stationary cells, focal adhesions move linearly towards the cell center. This movement is dependent on actin-myosin contractility exerted on central stress fibers.

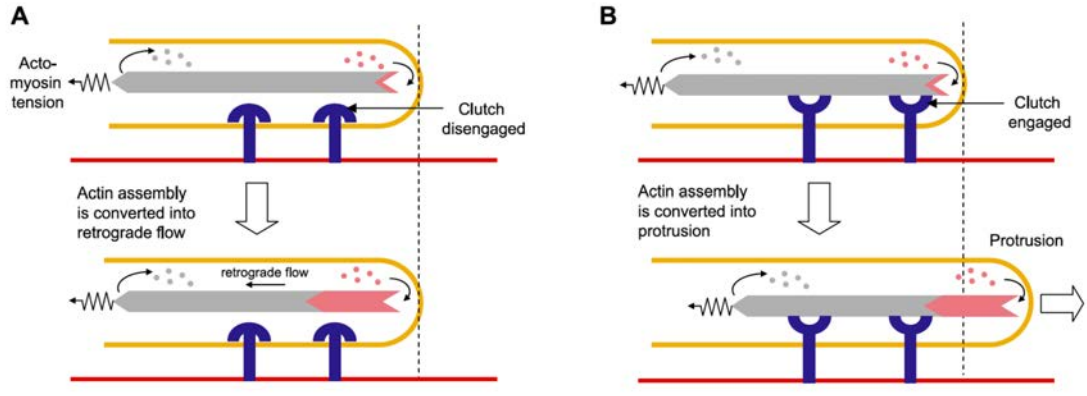

Figure 4: Adhesion acts as a "molecular clutch" to convert the force generated by actin assembly into protrusion. In these panels, the actin network is represented in gray. The newly polymerized actin is represented in pink. A: the molecular clutch is disengaged; there is no connection between adhesions and the actin cytoskeleton. In this situation, no protrusion occurs because actin treadmilling is mainly converted into retrograde flow. B: the molecular clutch is fully engaged. The strong connection between the polymerizing actin network and the substrate results in membrane protrusion. Moreover, this connection enables the conversion of the actomyosin tension into traction of the cell body and retraction of the tail. From LeClainche (2008).

The precise mechanisms that regulate the formation of focal complexes at the cell's leading edge and the release of focal adhesions at the cell's rear remain unclear but are also dependent on Rho-GTPase signaling and microtubules (see chapter 2).

3 - Actin-rich protrusions generated during migration

For a cell to move, it needs to form a protrusion. The formation of these protrusions is dependent on the regulated polymerization of actin into filaments. Briefly, actin filaments are built from polar actin monomers, which move in solution by Brownian diffusion. In the presence of sufficiently high monomer concentrations, free monomers attach to the barbed end of existing filaments (elongation) or form trimeric monomer nuclei (nucleation). This process leads to the assembly of a right-handed double helical structure, consisting of two protofilaments twisted around each other. Actin in filaments is called F-actin, or filamentous actin, and monomers are named G-actin, or globular actin. Actin filaments are indirectly anchored to membranes e.g. via adhesion sites (Figure 3) or ERM proteins (ezrin, radixin and moesin), which organize the actin cortex and membrane domains through their ability to interact with transmembrane proteins and actin filaments (Figure 5). Via such a link the entire plasma membrane can be re-shaped during cell contraction mediated by Rho-GTP and ROCK kinase (chapter 4).

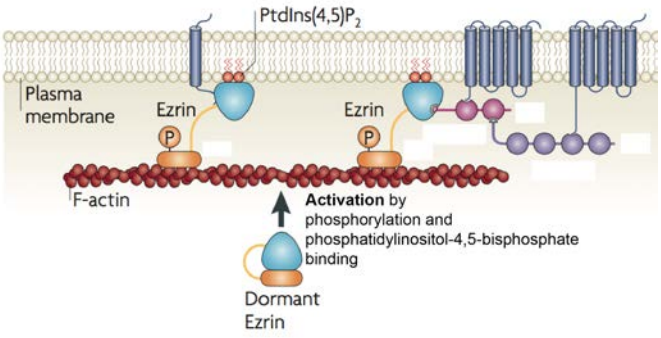

Figure 5: Cortical actin filaments can be anchored to the plasma membrane. Relevant for cell contraction: Autoinhibited ezrin (here: dormant ezrin) can be phosphorylated by ROCK kinase (chapter 4). Modified from Fehon, R. G., et al (2010).

Migrating mammalian cells generate protrusions to start translocation, finger- and sheet-like ones. The finger-like protrusions are termed filopodia and filled with parallel actin filaments (chapters 3 and 4). The sheet-like protrusion forms the leading edge of the cell and is filled with two distinct but overlapping F-actin networks: the lamellipodium, and the lamella (Figures 2 and 6).

Lamellipodium protrusion and retraction, as well as filopodium formation probably serve an exploratory function or could provide rapid responses to extracellular cues. Persistent advancement of the cell however seems to rely predominantly on the underlying lamella. These actin networks, lamellipodium and lamella, can be distinguished by two main criteria: (1) the molecules that differentially localize to them (molecular signatures), (2) the spatial organization of the rates of F-actin assembly/disassembly (kinetic signatures).

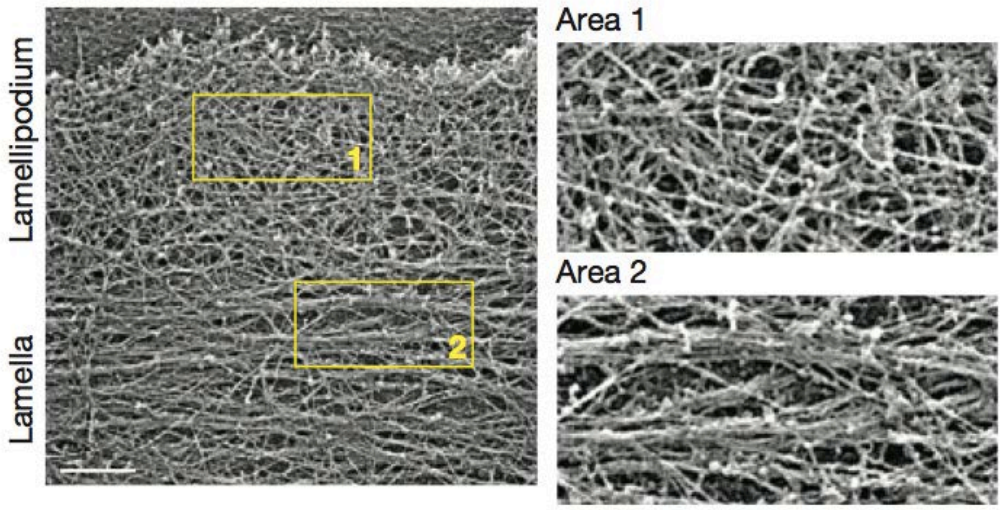

Figure 6: Electron micrograph of a rotary-shadowed PtK1 cell after live-cell extraction. Areas 1 and 2 show actin-filament organization in the lamellipodium and lamella, respectively. Scale bar, 1 μm. From: Burnette et al. (2011).

In the lamellipodium (Figure 2), many actin filaments are rapidly generated and drift cell inward. In other words, the lamellipodium consists of a treadmilling F-actin array with rapid polymerization-dependent retrograde flow (chapter 3). It contains high concentrations of Arp2/3 and ADF/cofilin. The lamellipodial network lies more anterior to the lamellar network. It forms the 2-4 μm-wide leading edge. Its border away from the leading edge is characterized by myosin II-based contractility anchored at sites of stable adhesion. Myosin II mediated contraction will pull lamellipodial filaments rearward. If at the extreme cell edge the lamellipodial attachment with the substrate is broken, myosin II activity causes the newly formed membrane protrusion to move as a peripheral ruffle rearward until it reaches stable adhesion sites. At these sites it becomes disassembled. Thus, if the extreme leading edge remains adhered to the substratum, contraction causes retrograde actin flow. The kinetic signature of the lamellipodium is characterized by fast F-actin polymerization in close proximity to the leading edge, followed by nearly complete filament depolymerization a few micrometers back.

In contrast, the lamella exhibits spatially random punctae of F-actin assembly and disassembly with slow myosin-mediated retrograde flow. It contains myosin II and tropomyosin (which are absent from lamellipodium). The lamella of migrating epithelial cells comprises an area within 3–15 μm from the cell edge. The actin foci cycle aphasically between polymerization and depolymerization. Myosin II–dependent slow retrograde flow (~0.3 μm/min) defines the kinematic signature of the actin filaments in the lamella, which may be mediated by the other signature molecule in this region, tropomyosin.

In the cell body, F-actin undergoes myosin-dependent anterograde (forward) flow, which meets F-actin retrograde flow from the lamella in "the convergence zone". This region is characterized by filament depolymerization but little actin motion.

4 – Organization of microtubules during migration

Microtubules (MTs) also play an important role in cell migration - in a cell type-dependent manner. MTs are polar filaments composed of 13 protofilaments, each consisting of a linear array of α/ß-tubulin dimers. MT filaments are anchored with their slow growing minus-ends in the microtubule organizing center (MTOC) or spindle pole body in budding yeast (which is typically near the nucleus), and the faster growing plus-ends close to the cell cortex.

The above-mentioned cell-type specificity is due to cell-type specific ratios between actin- and microtubule-rich regions in cells. This ratio correlates with the shape of the protrusion, the adhesive and migratory capacity of the cell. In general, in most cell types, migration is altered by the disruption of the microtubule network.

Fibroblasts represent best the "migrating model cell" with little MT but a lot of actin in the protrusion, a central microtubule organizing center (MTOC), stress fibers and adhesion sites. In the very dense actin meshwork of a lamellipodium, only a few highly dynamic "pioneer" microtubules are found that indirectly interact with actin filaments (Figure 6). Bursts of rapid assembly enable pioneer microtubules to intrude deep into the peripheral lamellipodium, where they are destabilized again by the retrograde actin flow, leading to rapid microtubule turnover and constant exploration within the lamellipodium. In contrast, near the nucleus, where the MTs emanate, the concentration of microtubules increases and in general exceeds that of actin filaments.

In general, fast migrating cells have fewer actin stress fiber bundles and focal adhesions when compared to slow migrating cells, which have less focal adhesions. For example, slow migrating astrocytes (typically 0.1 µm/min) have an extremely long microtubule-filled protrusion, hardly any actin-rich lamellipodium but stress fibers anchored in strong focal adhesions, which are localized at the edge of the protrusion. Above described fibroblasts migrate with an average speed of 0.6 µm/min. Cells migrating with intermediate speed are neutrophils (3-6 µm/min, amoeboid migration), which do not have any stress fibers and therefore also lack focal adhesions. Given that MTs are important for promoting the turnover of focal adhesions, neutrophils are less dependent on MTs for directional migration. In contrast, fish epidermal keratocytes are rapidly migrating cells (typically 10 µm/min – still quite slow when compared to bacteria or amoeba, which can move with 60 µm/min and display a high turnover of focal complexes that rarely mature into focal adhesions, a wide flat lamellipodium and no microtubule-rich regions. These cells can migrate without microtubules. Thus, there is a functional link between stress fibers, focal adhesions and microtubules.

Indeed, it seems that the principal effect of microtubules is to reduce cellular contractility as adhesions dissociate upon direct contact with microtubules. Microtubules appear to regulate the turnover of adhesions by targeting them directly and delivering "relaxing signals" to promote their turnover (= disassembly). This occurs in a feedback mode as increasing mechanical stress at adhesion sites signals the polymerization towards these adhesions sites. The precise molecular picture is still missing, but concepts are emerging (chapter 4).

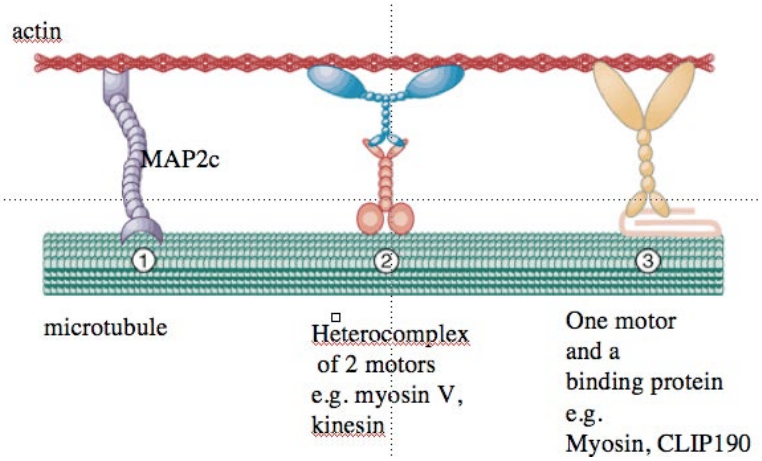

Figure 7: Actin (red) and microtubules (green) can exhibit static or dynamic interactions. Interaction 1 shows a protein that possesses both actin- and microtubule-binding sites and could provide a static crosslink between the two polymers, as hypothesized for MAP2c. Interaction 2 shows a complex between an actin-based motor (blue) and a microtubule-based motor (orange), whereas interaction 3 shows a complex between a motor (yellow) and a binding protein (pink). Both types of interaction could move actin and microtubules relative to one another, as hypothesized for myosin V and kinesin, or myosin VI and Drosophila CLIP-190, respectively. Modified from Rodriguez et al (2003).

Microtubule polarity

The plus ends of growing microtubule are composed of GTP-tubulin. The constant rate of GTP hydrolysis necessitates that microtubules must keep growing to maintain their "GTP-cap". Stalled microtubules rapidly lose their GTP-tubulin cap and are susceptible to collapse. The cycles of microtubule growth and collapse are termed "dynamic instability". Microtubule-associated proteins (MAPs) can stabilize MT and inhibit thus the complete collapse of a filament. In addition, an increasing number of proteins have been identified that localize exclusively to the plus ends of microtubules. The following selection of microtubule plus end-binding proteins (also known as +TIPs, for microtubule plus end tracking proteins) that are presented during this entire course include CLIP-170, APC, Kar9 and EB1, as well as the dynactin subunit p150 (dynein-dynactin complex). Functions of +TIPs include regulation of microtubule plus end dynamics as well as providing microtubule-to-cortex attachment necessary for vesicle delivery, force generation, and signaling of cortical cell polarity. Localization of proteins to microtubule plus ends occurs through one of three ways: kinesin-mediated plus end transport, direct or indirect binding to the microtubule plus end itself.

MT filaments can become enriched in cell regions such as newly formed protrusions. This causes a polarized MT array and is characterized by stabilized MTs. These stabilized MTs are characterized by longer half-lives, increased resistance to MT-depolymerizing drugs such as nocodazole, and the addition of a specialized plus-end cap to abolish shrinking and growth of the MT. Stabilized MTs are further characterized by the accumulation of post-translationally modified tubulin subunits including detyrosinated and acetylated tubulin. These modified MTs seem to recruit special "stable MT"-factors such as kinesins.

The described polarization of stable MTs in migration direction is thought to be the cause for the orientation of the MTOC and the nucleus in migration direction. The dynein-dynactin complex at the plus end of MTs might pull on MTs that are stabilized at the leading edge by the Rho-GTPase Cdc42-GTP (chapters 2 and 3) and APC, thereby inducing the reorientation of the MTOC and Golgi towards the

migration direction. This is thought to facilitate further growth of MTs into the lamellipodium and MT dependent targeted vesicular trafficking.

One discussed function of microtubules is the delivery of new membrane material from the trans-Golgi network to the plasma membrane to allow formation of the new membrane protrusion. This explanation is consistent with the observation that disruption of the Golgi complex — by either brefeldin A or blocking kinesin motor activity — mimics the effects of microtubule disruption on the lamellipodial activity in several cell types. However, microtubule ends lag behind the rapidly protruding cell edge and even their complete loss in such cells does not impede protrusion formation.

5 – References for Figures

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Chapter 2: Rho GTPases

Remarkable in this chapter:

- Structure and regulation of mammalian Rho GTPases (CRIB domain, GEFs, PH domain, phosphatidyl inositol (3,4,5)-tri-phosphate, GAPs)
- Relief of intramolecular autoinhibition of GEFs and effectors during their activation

1 – Introduction

Adenosine and guanosine triphosphates have distinct biological roles. ATP is consumed during phosphorylation reactions and the hydrolysis of ATP is generally used to provide energy required to drive other cellular processes, such as movement of motor proteins. However, GTP hydrolysis seems to be used mostly for the regulation of signaling processes in a large sense. A typical example is the signal transduction by the small GTP-binding protein Ras, which is a key regulator of three very different reactions: cell differentiation, cell proliferation and apoptosis. GTP bound to tubulin-monomers at the plus tip of microtubules can also be hydrolyzed, which is not an example for a role in signal transduction. It is estimated that a eukaryotic cell contains several hundred different guanine nucleotide-binding proteins (GNBPs). These belong to diverse groups like heterotrimeric G-proteins, tubulin, septin, dynamin, peptide elongations factors (EF-G, EF-Tu) and the small GTPases, also termed the Ras superfamily.

The Ras superfamily of GNBPs comprises many important regulators of cellular processes and can be subdivided into 5 major sub-groups, which have different cellular functions. (1) Ras guanosine triphosphatases (GTPases) are involved in the regulation of growth, differentiation and apoptosis; (2,3) Rab and ARF GTPases function mainly in vesicular transport; (4) Ran GTPase regulates nuclear transport and mitotic spindle assembly; and (5) Rho GTPases regulate the organization of the cytoskeleton, cell cycle progression and gene expression.

GTPases are highly conserved molecular switches, which use a simple chemical mechanism to control many different cellular processes common to all eukaryotes, such as cellular morphogenesis, movement and cell division. They cycle between two different conformational states, GTP-bound and GDP-bound, and are able to hydrolyze GTP to GDP through an intrinsic GTPase activity. This is discussed in the next section.

Rho GTPases are found in all eukaryotic cells. This chapter focuses on the structural features and the regulation of Rho GTPases, while their function in the context of cell migration will be discussed in the subsequent chapters.

2 – The GTPase cycle and prenylation

At least 22 mammalian genes encoding Rho GTPases have been described, with RhoA, Rac1 and Cdc42 being the best-studied members (Figure 1). In contrast, the genome of budding yeast appears to encode only 5 Rho GTPases. Even plants have them, yet all contained in a single subfamily, named Rho-like GTPase from plants (ROPs), which seems to be older than the divergence of fungal and animal Rac, Rho, and Cdc42. Remarkably, the Cdc42 and Rho subfamilies, characteristic for metazoa and fungi, are absent in Dictyostelium (chapter 5).
In general, Rho GTPases act as molecular switches that cycle between an active GTP-bound state and a mostly inactive GDP-bound state (Figure 2), with the exception of the members of the Rnd sub-group which appear to be constitutively bound to GTP. Only GTP-bound GTPases are able to interact with their effector molecules to mediate various cellular responses.

Rho GTPase	Official symbol (human)[b]	Localization	Post-translational modifications
RhoA	RHOA	PM and cytosol [16]	GG and PH [23,105]
RhoB	RHOB	PM and endosomes [16]	GG, F and P [105]
RhoC	RHOC	PM and cytosol [16]	GG [105]
RhoD	RHOD	PM and endosomes [97]	GG[c]
Rif	RHOF	PM [8]	GG[c]
Rnd1	RND1	PM [98]	F[c]
Rnd2	RND2	endosomes and cytosol? [99]	F[c]
Rnd3/RhoE	RND3	PM, Golgi and cytosol [13]	F and PH [14,106]
RhoH/TTF	RHOH	?	GG[c]
Rac1	RAC1	PM [18]	GG [107]
Rac2	RAC2	PM and cytosol (Knaus, 1991)	GG [107]
Rac3	RAC3	PM and endomembranes [100]	GG [100]
RhoG	RHOG	PM and endosomes [101]	GG[c]
Cdc42	CDC42	PM and Golgi [63]	GG[c]
TC10	RhoQ	PM and perinuclear [9]	F and P[c]
TCL	RHOJ	PM and endosomes [76]	F and P[c]
Wrch1	RHOV	PM and endomembranes [102]	P [102]
Chp/Wrch2	RHOU	PM and endomembranes [103]	P [103]
RhoBTB1	RHOBTB1	Vesicular[d] [3]	None known
RhoBTB2	RHOBTB2	Vesicular[d] [3]	None known
Miro1	RHOT1	Mitochondria [104]	None known
Miro2	RHOT2	Mitochondria [104]	None known

Figure 1: Localization and posttranslational modifications of mammalian Rho GTP-binding proteins. Abbreviations: c, predicted; PM, plasma membrane; GG, geranylgeranylation; F, farnesylation; P, palmitoylation; PH, phosphorylation. From Ridley (2006).

Diverse signals induce the intrinsically slow conversion of the GDP-bound GTPase into the GTP-bound form. This activation of Rho GTPases involves the following two steps: dissociation of protein-bound GDP and the subsequent loading of GTP. Guanine nucleotide exchange factors (GEFs) catalyze the GDP displacement and stabilize the nucleotide-free GTPase intermediate, which leads to accelerated GTP-loading. Effector proteins are characterized by the fact that they bind preferentially to a GTP-bound GTPase and thereby eliciting a conformation change in the effector protein and thus downstream events. Rho-signaling stops by the hydrolysis of protein bound-GTP to GDP, an irreversible step in cells. This GTPase reaction is intrinsically very slow and is accelerated by GTPase-activating proteins (GAPs). The activation of the GTPase switch occurs via the activation of GEFs in response to a signal that is in most cases triggered by an extracellular stimulus, although internal cues can also lead to GEF activation.

Another important biochemical feature of most Rho proteins is their post-translational modification by lipids. The majority of Ras and Rho family proteins terminate with a C-terminal CAAX (C=Cys, A=aliphatic, X=any amino acid) tetrapeptide sequence. The CAAX motif is the recognition sequence for farnesyltransferase and geranylgeranyltransferase I, which catalyze the covalent addition of a farnesyl or geranylgeranyl isoprenoid to the cysteine residue of the CAAX motif, respectively. Afterwards the AAX is removed and the cysteine

methylated. These modifications are important to promote the membrane association that is critical for the biological activities of Rho GTPases.

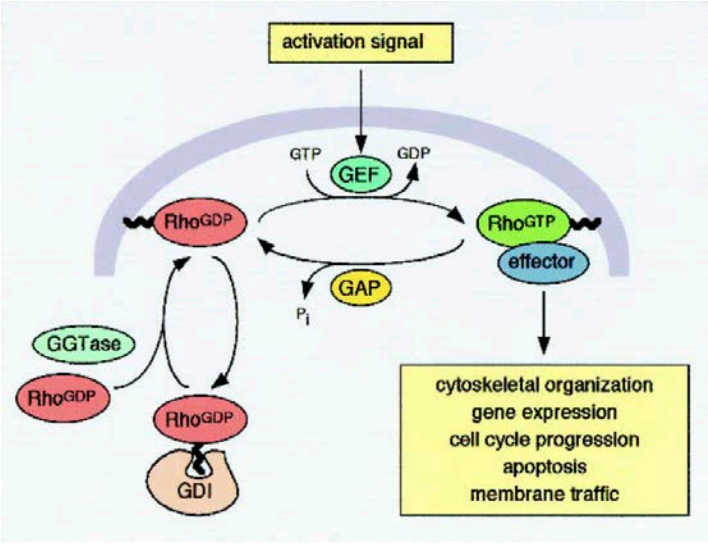

Figure 2: The Rho GTPase switch. From Schmidt and Hall (2002).

In addition to GEFs and GAPs, Rho GTPases are regulated by a third class of proteins, the guanine nucleotide dissociation inhibitors (GDIs). They mask the prenyl modification and promote the cytosolic sequestration of the GTPases. This allows the recycling of Rho GTPases between different membrane compartments of the cell. At the same time GDIs block the release of the bound nucleotide. In addition, since about 2009 the unconventional modes of regulation of Rho GTPases became more and more apparent: i) Their expression can be modulated by microRNAs. ii) Palmitoylation of the GTPases and nuclear localization signals (NLSs) can additionally to the prenylation determine their subcellular localization. iii) Post-translational covalent modifications, including phosphorylation, transglutamination, AMPylation and SUMOylation dictate their ratio of GDP versus GTP-bound state. And iv) even ubiquitin dependent degradation is observed to reduce that amount of GTP-bound GTPases. Although the physiological role of these modifications is not yet fully established, it seems that they establish a second layer of regulation of the Rho GTPase activity, which can be mimicked by bacterial factors (see below). The first (conventional) regulation layer includes the GEFs, GAPs and GDIs.

3 – The G box or guanine nucleotide binding motif

Ras superfamily GTPases share a conserved GDP/GTP-binding motif, which consists of five elements (G1-G5) (Figure 3a):
G1: GXXXXGKS/T (also called the P-loop)
G2: T
G3: DXXG
G4: NKXD
G5: SAK
Together, these elements make up the 20 kDa G-domain or G box and provide the nucleotide-binding activity. The G box has a conserved structure and biochemistry that is shared by all Ras superfamily proteins, as well as the Gα subunits of heterotrimeric G-proteins. The G-domain fold consists of a mixed six-stranded β sheet and five helices located on both sides. Nucleotide binding mainly

occurs through the interactions of the nucleotide base with the NKXD motif, and of the β, γ-phosphates with the P-loop.

The GDP- and the GTP-bound states have similar conformations, with the exception of two regions, called the switch I and switch II regions. These switch regions show an increased flexibility in structural studies. Importantly, while GDP-bound proteins show large structural variations, the GTP-bound forms of different Ras-family GTP-binding proteins are very similar. The trigger for the conformational change is most likely universal, and it can best be described as a loaded-spring mechanism. In the triphosphate-bound form, there are two hydrogen bonds from the γ-phosphate oxygen to the main chain NH groups of the invariant Thr and Gly residues in the switch I and II regions, respectively (Figure 3b). The release of the γ-phosphate after GTP hydrolysis allows the two switch regions to relax into the GDP-specific conformation. The GTPase thus undergoes a characteristic conformational change.

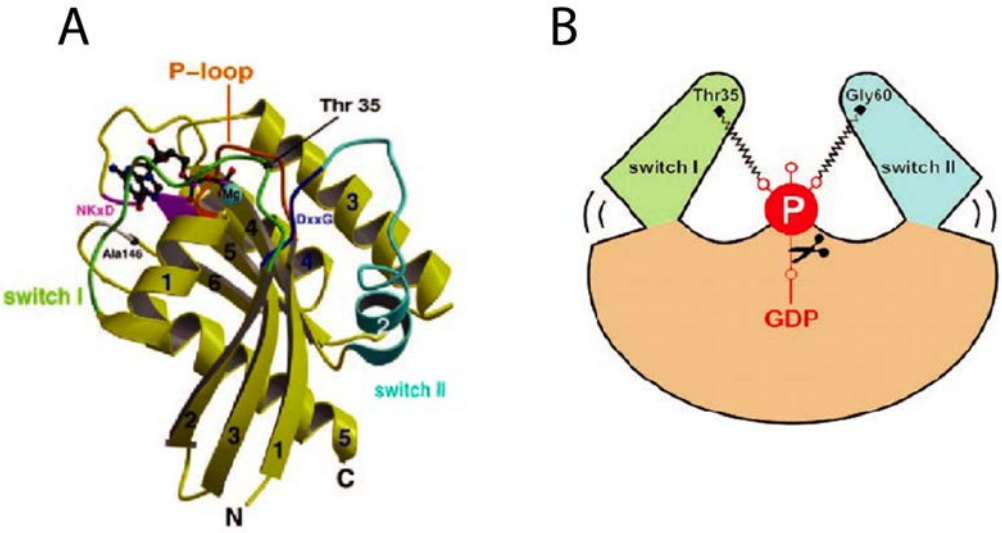

Figure 3: (A) Structure of guanine-nucleotide binding proteins. The Mg^{2+} contributes to the tight binding of nucleotides. (B) Schematic diagram of the universal switch mechanism. From Vetter and Wittinghofer (2001).

4 – The functional domains of Rho GTPases

The core domain structure of Rho GTPases is shown in Figure 4. The effector domain (switch I) changes conformation as a function of the nucleotide-status of the GTPase and is usually required for binding to downstream targets. A unique feature that distinguishes the Rho family from other Ras-related GTPases is the insert region, which may confer Rho-specific signaling events; Rho-specific effector proteins such as IQGAP1 (chapter 4) have been shown to bind to it. Note that some Rho GTPases have an N-terminal extension beyond the core structure, and/or the length of the C-terminal hypervariable region can vary between different GTPases. The CAAX box is a general signal for the prenylation and membrane targeting of the GTPases.

There are a number of protein mutants in the Ras superfamily GTPases that lock

the GTPases either in a GTP- or GDP-like conformation and these are therefore often used as experimental tools. Most of these mutations were originally identified in Ras, but since they affect mostly conserved amino acid positions, the corresponding mutants can be generated in many different members of the Ras superfamily. The mutations described in the following section are numbered according to Ras, Rac and Cdc42. Rho has an N-terminal extension of 2 amino acids. Therefore, the respective mutations in Rho would be G14V, T19N, respectively.

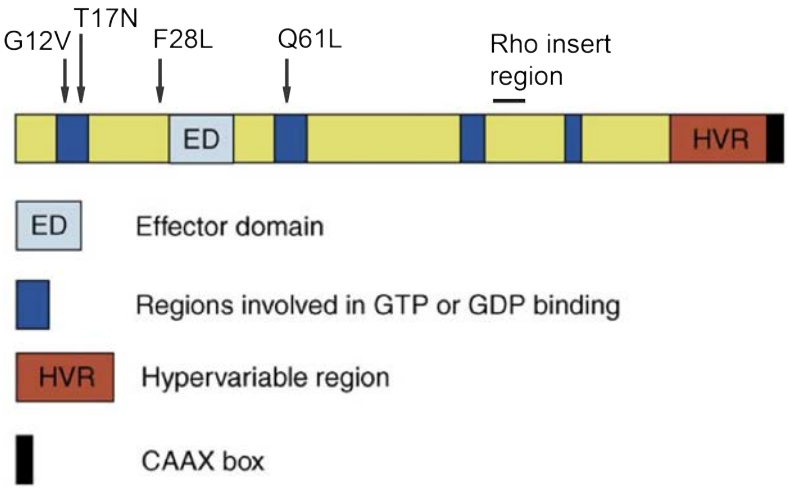

Figure 4: Schematic structure of a typical Rho GTPase like Cdc42. Commonly used mutants are indicated. The position of the G box domains G1-G5 is indicated. Modified from Ridley (2006).

Activating mutations:
The G12V and Q61L mutant proteins are unable to hydrolyze GTP even in the presence of a GAP. This leads to the constitutive association of these mutated Ras GTPases with their effectors and therefore to continuous signaling. Given that the cellular GTP levels are significantly higher than the GDP levels, a non-physiological excess of activated GTPase and consequently increased downstream signaling is generated in cells harboring such a mutant protein.

Dominant-negative mutation:
The T17N amino acid change results in a mutant GTPase that is in the nucleotide-free conformation. GEFs exhibit high affinity to these proteins. Overexpression of a T17N-GTPase in cells therefore leads to the sequestration of all cellular GEFs that would normally be required to activate the endogenous GTPase. Thus, any activation signal would no longer be able to cause the formation of a GTP-bound GTPase, and the T17N mutation therefore has a dominant-negative effect.

5 – The GEF reaction

Guanine nucleotide release from GNBPs is slow, and GEFs accelerate it by several orders of magnitude. The mechanism of GEF action involves a series of fast reaction steps: the GNBP-GDP complex binds to GEF forming a GNBP-GDP-GEF complex. Due to the GEF activity the GDP leaves this trimeric complex leading to a GNBP-GEF nucleotide-free complex, which is stable in the absence of a nucleotide (Figure 5a).

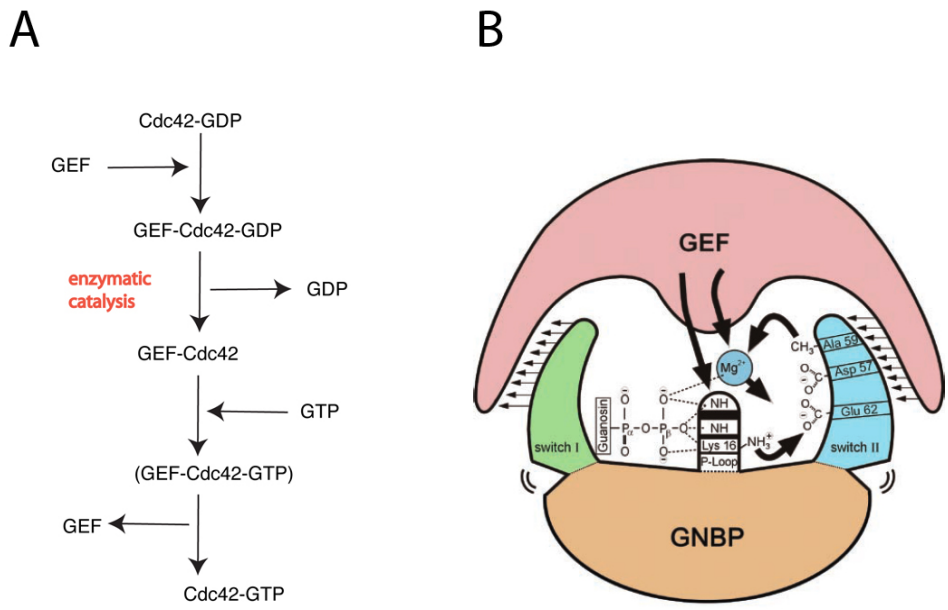

Figure 5: The GEF reaction. (A) A schematic representation of the relevant reaction steps. (B) A diagram of the mechanistic principles underlying a GEF action. Amino acid numbers in the GTPase refer to Ras. From (A) R. Kroschewski and (B) Vetter and Wittinghofer (2001).

The GEF therefore catalyzes the displacement of GDP and stabilizes the nucleotide-free state. The rebinding of nucleotide reverses this series of reactions. Since the cellular concentrations of GTP are significantly higher than the ones of GDP, it is predominantly GTP that binds to the nucleotide-free GNBP. Importantly, the binding of GTP is not catalyzed by the GEF. The exchange reactions are in principle fast and fully reversible, so that the GEF merely acts as a catalyst to increase the rate at which the equilibrium between the GDP- and GTP-bound forms of the protein is reached. The position of the equilibrium is dictated by the relative affinities of the GNBP for GDP and GTP, the intracellular concentrations of the nucleotides and the affinities and concentrations of other proteins, such as effectors, which pull the equilibrium towards the GTP-bound form.

While GTPases within a particular sub-family use similar GEFs, the GEFs used by the different sub-families of GNBPs are structurally distinct. However, the action of all GEFs is based on some common mechanistic principles (Figure 5b). The GEFs interact with the switch I and II regions. They insert residues close to the P-loop, and thereby create structural changes that are blocking the binding of the phosphates. During the GEF reaction, the Mg^{2+} ion is pushed out of its position by elements of the GNBP itself, i.e., the Ala^{59}, and by residues of the GEF. Residues of the P-loop are disturbed, and its lysine is reoriented toward an invariant carboxylate from the switch II region, either Asp^{57} or the highly conserved Glu^{62}. In what might be called a push-and-pull mechanism, switch I is pushed out of its normal position, whereas switch II is pulled toward the nucleotide-binding site. This leads to a dramatic decrease in affinity of the GNBP for GDP.

6 – Conventional GEFs

The first mammalian GEF, Dbl, was isolated in 1985 based on an oncogenic

mutation, and was found to contain a region of sequence similarity to Cdc24, a protein identified genetically as an upstream activator of Cdc42 in budding yeast. It was subsequently shown that a conserved domain in Dbl and Cdc24, called the Dbl homology (DH) domain, harbors GEF activity. Based on the analysis of complete genome sequences, six DH domain-containing GEFs have been identified in budding yeast, and approximately 60 DH domain-containing GEFs in human. Some GEFs are very specific for certain GTPases, while other GEFs are able to activate several different GTPases and are thus promiscuous. For example, Dbl activates Cdc42 and Rac; Vav activates Rac and Rho; and Fgd1 is specific for Cdc42.

In addition, a second shared domain, called the pleckstrin homology (PH) domain, is found in almost all DH domain family members. The PH domain is invariably located immediately C-terminal to the DH domain, and this invariant topography suggests a functional interdependence between the two structural modules. The PH domain binds to phosphatidylinositides, and this lipid-binding has been shown in some cases to positively affect the catalytic activity of the DH domain.

GEFs control GTPase signaling in combination with GAPs and GDIs, but in many cases they seem to play the most important role (Figure 2). Therefore, the GEFs themselves have to be tightly regulated. Despite the observation that different GEFs can have different mechanisms of activation, some general principles have emerged for GEF regulation including the following ones:

- Exposure of the DH domain by relief of an intramolecular inhibitory fold (see below), e.g. in response to phosphorylation
- Signal-induced interaction with other proteins, leading to the exposition of the DH domain independently of the relief of autoinhibitory sequences
- Alteration of intracellular localization.

Many GEFs contain a regulatory domain that keeps the GEF in an inactive, closed state, through an intramolecular interaction, and thereby blocks GEF activity. It has been shown that this interaction can be relieved by posttranslational modification (e.g. phosphorylation), binding to other proteins, or interaction of the PH domain with phosphoinositides (Figure 6).

Many of the cellular functions ascribed to Rho GTPases depend on the spatial control of activation. It is therefore likely that the subcellular localization of GEFs is a key component in the regulation of their activity. Indeed, in many cases GEF activation seems to be intimately linked with their intracellular relocalization. Apart from allosterically regulating the DH domain, the PH domain has been suggested to mediate the translocation of GEFs to membranes and to cytoskeletal structures.

How to understand the physiological importance of the generally weak (10 μM) and promiscuous phosphoinositide binding seen with up to 90% of the PH domains? It is assumed that in response to cell stimulation, the phosphorylation (or any other modification) of the GEF could cause its oligomerization. The cooperative increase in the strength of the interactions could result in a high affinity interaction between the oligomerized GEF and the membrane. Fact is that phosphatidyl-inositol(3,4,5)-tri-phosphate in cholesterol-rich membrane domains is critical for the activation of Rac1 likely via a PH domain containing GEF (chapter 5).

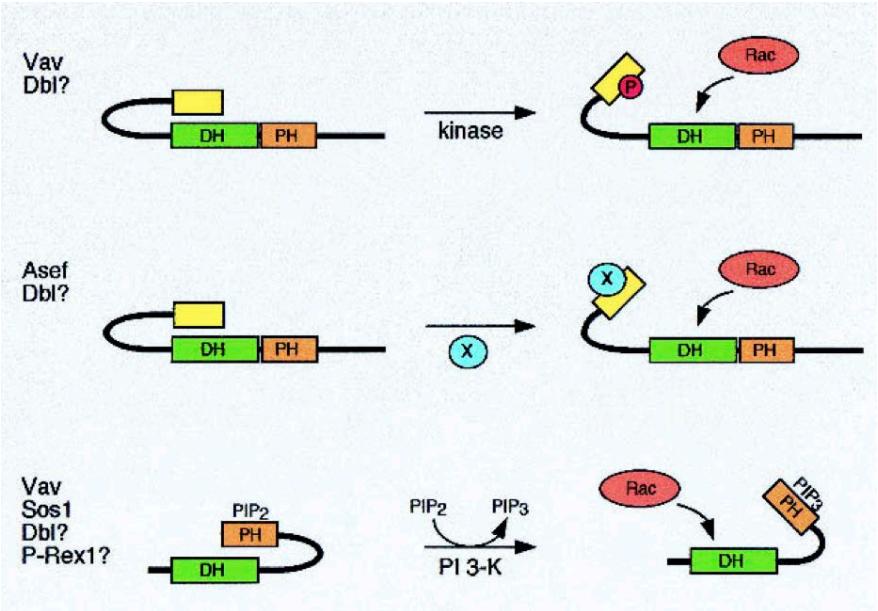

Figure 6: GEF activation through relief of intramolecular inhibitory sequences. From Schmidt and Hall (2002).

The affinity of individual interactions is not changed, but membrane recruitment results from regulated increases in avidity. For example, if the K_d for binding of a monomer to the membrane surface were in the millimolar range, the effective K_d for tetramer binding would be in the picomolar range. In fact, oligomerization of Dbl has been demonstrated to occur *in vitro* by a DH domain-dependent mechanism and has also been linked to the ability of Dbl to transform cells.

7 – Unconventional GEFs

In addition to the conventional DH domain-containing GEFs, recent work has uncovered two main groups of unconventional GEFs. The first group includes DOCK180 and Zizimin and the second the bacterial protein SopE.

DOCK180 has a docker domain, which was shown to bind GTP and to be necessary and sufficient to mediate nucleotide exchange *in vitro*. However, it appears that a second protein such as ELMO1 is required for the exchange reaction to occur *in vivo*. One model proposes that ELMO1 is important to stabilize the nucleotide-free transition state together with DOCK180. As the experiments did not monitor directly GDP displacement from Rac but its GTP loading, there is no direct analysis of the typical GEF catalytic step, the GDP displacement (Figure 7).

In addition, there is evidence that the PH-domain of ELMO is involved in targeting DOCK180 to the plasma membrane. Therefore, the PH domain of ELMO seems to perform functions that are similar to the role of the PH domain that is found in combination with the DH domain in conventional GEFs.

In contrast, the catalytic activity of SopE, a bacterial GEF specific for Rac and Cdc42, is encoded in a loop of 4 amino acids. Therefore, it is an example of convergent evolution where proteins have no sequence-similarity to mammalian

host factors but display mimicry at the functional level. Specifically, the bacterium Salmonella has developed an efficient strategy to promote infection of mammalian host cells by manipulating the cellular Rho GTPase system in a reversible manner. First, the bacterium injects via its type III secretion apparatus the bacterial SopE protein into the host cell. There SopE causes the activation of these GTPases leading to massive rearrangements of the actin cytoskeleton and membrane ruffling, thereby facilitating bacterial infection of host cells. Once safely inside the cell, Salmonella also has the ability to restore the normal cellular status and inactivate Rac and Cdc42 signaling.

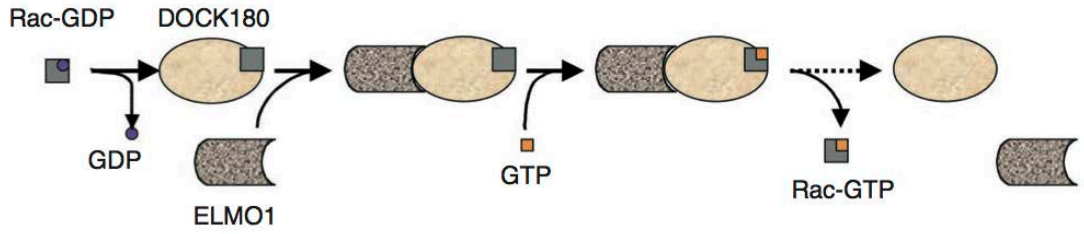

Figure 7: Proposed mechanisms of function of the unconventional GEF composed of DOCK180 and ELMO1. From Braga (2002).

8 – GAP proteins and the GTPase reaction

The intrinsic GTPase reaction of most GNBPs is slow (e.g. the time for 50% of GTP-hydrolysis on a RhoGTPAse is 1700 sec and in presence of a GAP 15 sec) and would not be suitable for most biological signal transduction processes, which are very dynamic and require complete inactivation of the GTPase within minutes after GTP loading. As with GEFs, the structures of GAPs for various families of GNBPs are different, but there are some common principles underlying the mechanism of GAP action (Figure 8).

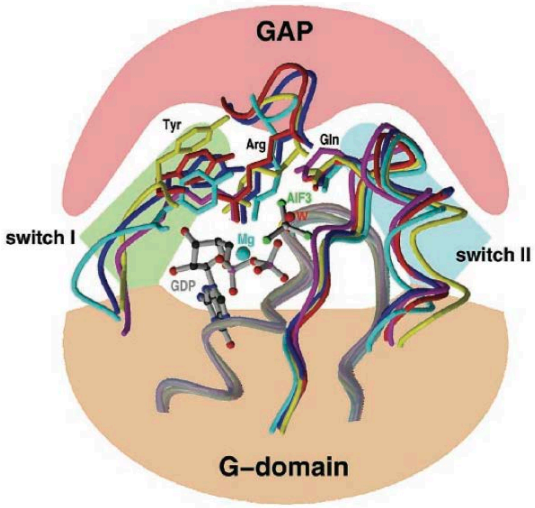

Figure 8: A diagram of the mechanistic principles underlying GAP action. The structures of the Ras-RasGAP (yellow), Rho-RhoGAP (red), Rac-ExoS (cyan), and Gia-RGS (magenta) complexes, which have all been solved in the presence of GDP and aluminum fluoride, which occupies the position of the gamma-phosphate of GTP. The catalytic water is labeled with a red "W". From

Vetter and Wittinghofer (2001).

Rho-GAPs are like the Rho-GEFs multi-domain proteins, and their GAP activity is encoded in the Rho-GAP domain. The active sites of many GNBPs show a conserved glutamine residue that is positioned near the ϒ-phosphate of the nucleotide. This glutamine, together with the arginine supplied by a structure of the GAP called the arginine finger, seem to stabilize the transition state of the GTP hydrolysis. This transition state involves also a water molecule. This mode of GAP-assisted GTP hydrolysis probably applies to all Rho-GAPs, but not to all GNBPs, and multiple mechanisms exist that are independent of the Gln and Arg residues.

One remarkable exception is found again in the bacterium Salmonella mentioned in the GEF section above. When Salmonella injected SpoE into the host cell to invade it, it also injected SptP, a bacterial protein with GAP activity. The GAP domain of SptP also contains a catalytically active arginine, but it is different from typical Rho GAPs in that it does not contain a complete arginine finger. Recent evidence suggests that similar amounts of SopE and SptP are injected into the host cell, but that SopE is rapidly degraded through a proteasome-dependent pathway whereas SptP is more stable. This ensures that the initial activation of actin polymerization is reversed once the bacterium has entered the host cell. Thus, SptP is a second example for a convergent evolution, where proteins that have no sequence-similarity to mammalian host factors display mimicry at the functional level.
Although GEFs have been proposed to be the key regulators of Rho activity, it has become increasingly clear that GAPs also contribute significantly to Rho signaling. For example, it has been demonstrated that the inhibition of a certain Rho-GAP activity is sufficient to promote the accumulation of Rho-GTP *in vivo*. This shows that not only a GEF-activity is essential for the presence of Rho-GTP.
In fact, you know about two examples of GAP proteins whose activities are tightly regulated, and which have been discussed in previous modules of this course. One is the bipartite GAP Bub2/Bfa1 that is involved in the regulation of mitotic exit in budding yeast (phosphorylation of Bfa1 by Polo kinase blocks GAP activity, chapter 5 of the cell division module). And the other is the bipartite GAP Tsc1/Tsc2 which activates TOR signaling (phosphorylation promotes complex formation and thereby GAP activation, chapter 3 of the cell growth module).

9 – Rho effectors and the CRIB domain

One of the key functions of Rho GTPases is the regulation of the actin cytoskeleton, but they also participate in the regulation of gene transcription, G1 cell-cycle control, cytokinesis, microtubule and septin dynamics, and vesicular transport – aspects that constitute cell polarity. In agreement with this list of activities, a large number of potential target proteins have been identified (Figure 9). Effectors for GTP-binding proteins are operationally defined as molecules interacting more tightly with the GTP-bound than with the corresponding GDP-bound form of a particular GTPase. Most effector proteins undergo a conformational change upon binding to the GNBP (Figure 10). This is an example of an allosteric regulation, as the binding site between GNBP and effector protein differs from the site on the effector protein, where the functional domain(s) lie (the regulatory site of an allosteric protein is physically distinct from its active site).

Potential effector protein	Type of protein	Functions	Selectivity of Rho GTPase binding			GTPase-binding motif
ROKα, ROKβ	Ser/Thr kinase	Actin/myosin	Rho			RKH
PKN/ PRK1, PRK2	Ser/Thr kinase	Unknown	Rho			REM
Citron kinase (citron)	Ser/Thr kinase	Cytokinesis	Rho			
p70 S6 kinase	Ser/Thr kinase	Translation regulation		Rac	Cdc42?	
Mlk2, 3	Ser/Thr kinase	JNK		Rac	Cdc42	CRIB
MEKK1, 4	Ser/Thr kinase	JNK		Rac	Cdc42	CRIB for MEKK4
PAK1, 2, 3	Ser/Thr kinase	JNK/actin		Rac	Cdc42	CRIB
PAK4	Ser/Thr kinase	Actin			Cdc42	CRIB
MRCKα, MRCKβ	Ser/Thr kinase	Actin			Cdc42	CRIB
Ack1, 2	Tyr kinase	Unknown			Cdc42	CRIB
MBS	Phosphatase subunit	MLC inactivation	Rho			
PI-4-P5K	Lipid kinase	PIP_2 levels/actin	Rho^{*+}	Rac^{*}		
PI3K	Lipid kinase	PIP_3 levels		Rac	Cdc42	
DAG kinase	Lipid kinase	PA levels	Rho^{+}	Rac^{*}		
PLD	Lipase	PA levels	Rho	Rac	Cdc42	
PLC-β2	Lipase	DAG/IP_3 levels		Rac	Cdc42	
Rhophilin	Scaffold	Unknown	Rho			REM
Rhotekin	Scaffold	Unknown	Rho			REM
Kinectin	Scaffold	Kinesin binding	Rho			RKH
Dia1, Dia2	Scaffold	Actin organization	Rho			
WASP, N-WASP	Scaffold	Actin organization			Cdc42	CRIB
WAVE/ Scar	Scaffold	Actin organization		Rac^{+}		
POSH	Scaffold	Unknown		Rac		
POR-1	Scaffold	Actin organization		Rac		
p140Sra-1	Scaffold	Actin organization		Rac		
$p67^{phox}$	Scaffold	NADPH oxidase		Rac		
MSE55, BORGs	Scaffold	Unknown			Cdc42	CRIB
IQGAP1,2	Scaffold	Actin/cell–cell contacts		Rac	Cdc42	
CIP-4	Scaffold	Unknown			Cdc42	

Figure 9: Potential effector proteins for Rho, Rac and Cdc42 GTPases. From Bishop and Hall (2000).

The binding site of the effector proteins on a GTPase involves the switch I region, and often also the switch II region of the GTPase, but can vary substantially. However, one well-characterized binding domain in many Rho effector proteins is the Cdc42/RAC interactive binding region, also called the CRIB domain. Proteins with a CRIB motif bind to Cdc42 and/or Rac in a GTP-dependent manner. CRIB containing proteins can have very different biological activities, examples include Ser/Thr kinases (PAKs which are p21-activated kinases), proteins without enzymatic activity (WASP, N-WASP and IQGAP), and lipid kinases (PI3K, Phosphoinositid-3-Kinase). Two prominent Cdc42 effector proteins containing a CRIB domain are WASP and N-WASP, which will be discussed further in chapter 3 and 4 of this module in the context of the regulation of actin dynamics. Importantly, the binding specificities can vary between different CRIB domain: e.g. IQGAP1 is able to bind both to Cdc42 and Rac, while WASP/N-WASP binds only to Cdc42.

The most common mechanism of effector activation by Rho GTPases appears to be the disruption of intramolecular autoinhibitory interactions, leading to the exposition and activation of functional protein domains within the effector proteins (Figure 10). For example, it has been shown in several cases that the CRIB domain binds to a functional domain of effector proteins, keeping them in a closed and thus inactive configuration. Upon binding of the GTP-bound GTPase to the CRIB domain competition occurs, and the effector protein opens up, and thereby renders the functional domain (e.g. VCA domain of WASP/N-WASP; chapter 3) accessible, which is now able to exert its biological activity (binding and thus activation of ARP2/3 complex, chapter 3). Subsequently, the bound GTP is hydrolyzed to GDP, and the resulting inactive GTPase dissociates from the effector protein, which returns into its closed, inactive configuration.

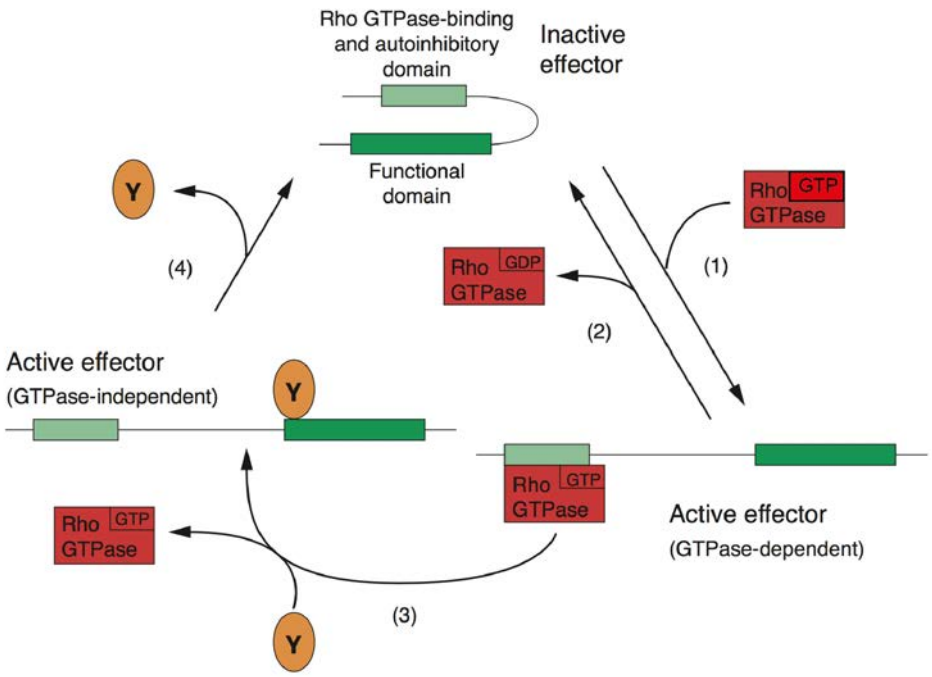

Figure 10: A general model for the activation of effector proteins by GTP-bound Rho GTPases. The effector remains active until GTP hydrolysis occurs (2) or until modification Y is removed (4). This has for example been demonstrated for PAK and N-WASP, two proteins involved in the regulation of the actin cytoskeleton. Modified from Bishop and Hall (2000).

10 – Analysis of Rho GTPase function *in vivo*

Several approaches have been used to investigate the function of Rho GTPases in animal cells.

- Overexpression of dominant-negative Rho mutants
- Down-regulation of Rho GTPases by RNA interference
- Inactivation/activation of Rho GTPases by bacterial toxins

Bacteria produce an array of cytotoxins, some of which have evolved to directly target Rho GTPases in mammalian host cells. Many cytotoxins are enzymes that modify their eukaryotic targets, often irreversibly, in a catalytic manner, a fact that is responsible for their high potency (Figure 11).

The irreversible inhibition of Rho GTPases by cytotoxins is achieved through covalent modification by glucosylation (e.g. Toxin A and Toxin B of Clostridium difficile), N-acetylglucosaminylation (e.g. α-Toxin of Clostridium novyi), ADP-ribosylation (e.g. C3 ADP ribosyltransferase of Clostridium botulinum, frequently used to efficiently inactivate Rho A, B and C in tissue culture cells), or proteolytic cleavage (for example by YopT of Yersinia enterocolitica). While most bacterial toxins have an inhibitory effect on Rho GTPases, the CNF1 deamidating-factor of E. coli is able to activate them. The mentioned toxins seem to be released from the bacteria into the environment, yet they can exert modifications of intracellular proteins, thus they have to translocate the plasma membrane by yet unknown means.

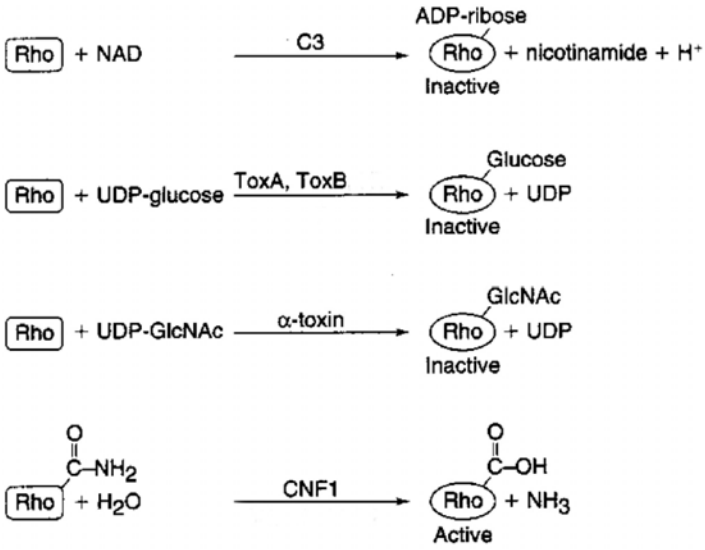

Figure 11: Chemical reactions of selected bacterial toxins. From Aktories (1997).

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Chapter 3: Actin filaments and nucleators, important compounds for cell migration

Remarkable in this chapter:

- Regulation and effects of profilin, coflin/ADF, capping protein on actin filaments
- Second force generating principle (in the broad lamellipodium): actin polymerization exerts force pushing the plasma membrane forward.
- Relief of intramolecular autoinhibition of WASP by phosphatidylinositol 4,5 bisphosphate and Cdc42-GTP
- Several types of actin nucleators (Arp2/3 complex, formin, Spire, Cordon-bleu, Leiomodin)
- Energy consumption (continued) during the activation of Arp2/3 complex and the lifetime of an actin filament
- WH2 domain, an ancient actin monomer-binding motif, in several types of molecules: WASP-family members, novel actin nucleators (Spire, Cordon-bleu, Leiomodin)

1 – Introduction

The cytoskeleton, a cellular scaffold found in the cytoplasm of eukaryotic and prokaryotic cells, is a dynamic structure that plays an important role in intracellular transport, cell division and cell movement, as well as the maintenance of cell shape.

Eukaryotic cytoskeletal components include actin filaments (also called microfilaments), intermediate filaments, microtubules and septins. Whereas the intermediate filaments display no polarity, both the actin filaments (composed of ATP-binding actin monomers) and microtubules (composed of GTP-binding tubulin α,β-dimers) are polarized structures. Septins, also GTP-binding proteins, assemble into homo- and hetero-oligomers and non-polar filaments, predominantly known for their function during cytokinesis.

The discovery of bacterial cytoskeletal elements was surprising, as the cell wall was thought to define the shape of the cell and since these cells are small, they could rely on passive transport of molecules by diffusion. The cytoskeleton in bacteria for the interested ones:

- The actin homologs MreB and Mbl are filament-forming ATPases involved in shape control (discovered 2001).
- Crescentin in the vibroid-shaped cells of *Caulobacter crescentus*, the only bacterial relative to eukaryotic intermediate filament proteins. (discovered 2003).
- FtsZ, like tubulin, forms filaments in a GTP-dependent manner (discovered 1998).

The actin family is a diverse and evolutionarily ancient group of proteins. Actin, Hsp70 proteins and sugar kinases belong to a protein superfamily

defined by a similar protein fold. ATP hydrolysis or phosphate transfer is in these proteins coupled to a large conformational change. Most, if not all, eukaryotic cells also contain actin-related proteins (ARPs). Some of these ARPs play well-characterized roles in cytoskeletal processes, including actin polymerization (Arp2/3, see below), and the cytoplasmic dynein motor activity (Arp1, a component of the dynactin complex).

This chapter concentrates on the molecular regulation of actin dynamics in mammalian cells. We aim to address the questions: How is rapid actin polymerization maintained at steady state in the lamellipodium while the cell moves? And, how is actin assembly constantly initiated in a site-directed manner to account for directional migration? Which are the nucleating molecules? Actin polymerization provides the main force that drives protrusion formation and thus locomotion of cells including fibroblasts, leukocytes and amoeba (chapter 5). It also underlies the movement of neuronal growth cones, cytoplasmic endosomes, bacteria in cells.

2 – Molecular basis of actin treadmilling

Actin monomers assemble into a right-handed double helical structure, two protofilaments that constitute an actin filament. Each actin monomer has an intrinsically asymmetric structure. This gives polymerized actin filaments a "polarity", with the ends referred to as the "pointed" (or minus) and "barbed" (or plus) ends. While monomers are added to both sides of a filament, they do so with different rates (Figure 1). Actin monomers can exist in an ATP-bound conformation (T-form), or an ADP-bound conformation (D-form).

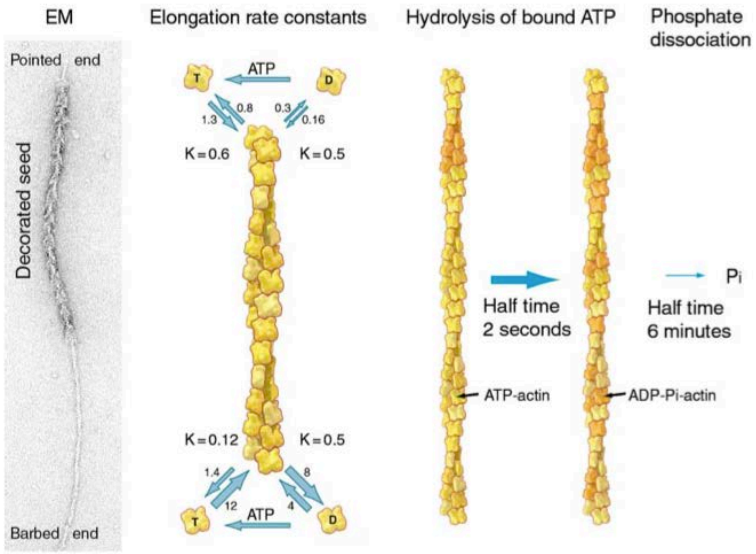

Figure 1: The kinetics of actin filament elongation. From Pollard and Borisy (2003).

The critical concentration of actin monomers is the concentration, above which actin monomers will polymerize, and below which actin filaments will depolymerize. The polymerization rate depends largely on the concentration of the monomer pool. A balance is eventually reached

between the filament length and the free monomer concentration, and the filaments enter a steady-state phase. In this state, the filaments undergo a constant polymerization and depolymerization process, while maintaining a constant length. This process is referred to as "treadmilling".

In vitro, treadmilling is very slow, and is limited either by the dissociation of ADP-actin at the pointed end or the association of ATP-actin at the barbed end, depending on which reaction is slower. In vitro the treadmilling rate of pure actin preparations is estimated to be around 0.05μm/min.

However, keratocytes and motile bacteria can move with velocities of 10μm/min, more than 200x faster than pure actin filaments undergoing treadmilling in vitro. Thus, how can the discrepancy between in vitro and in vivo velocity rates be explained?

3 – Regulation of actin treadmilling

Obviously, filament treadmilling needs to be regulated by additional factors in order to explain the characteristics of actin-based motility in vivo. In order to achieve higher treadmilling rates, the dissociation rate of ADP-actin at the pointed end and the association rate of ATP-actin at the barbed end have to be increased. In addition, ADF/cofilin, a protein influencing the pointed end depolymerization rates plays a role (see below).

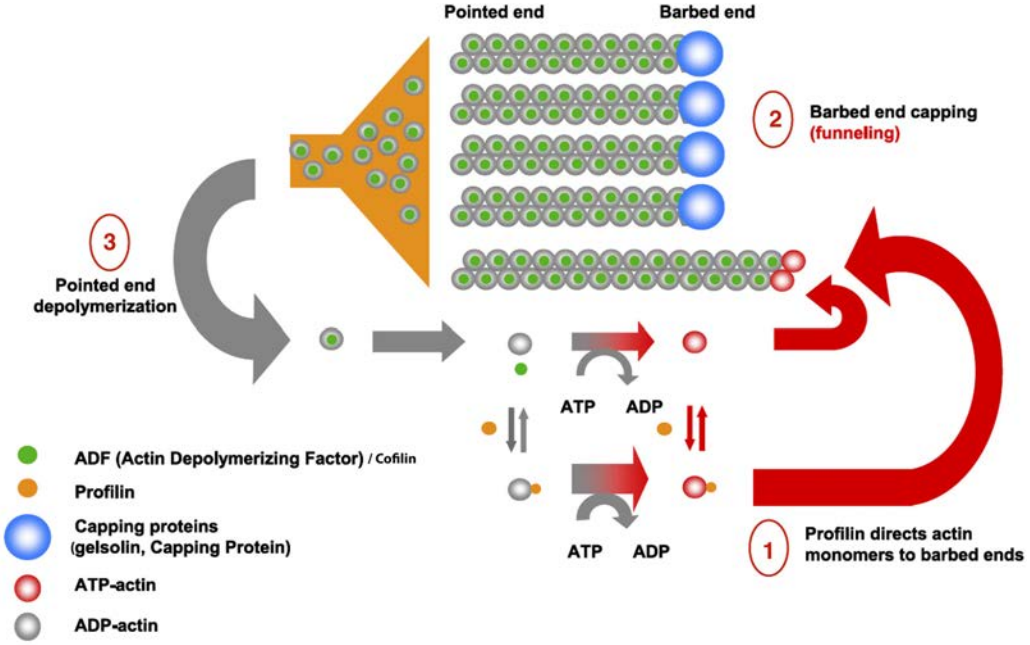

Figure 2: Regulation of actin treadmilling. *1*) Profilin enhances the exchange of ADP for ATP to recycle actin monomers. The profilin-actin complex assembles exclusively at the barbed end. *2*) By blocking the majority of actin filament barbed ends, capping proteins increase the concentration of monomeric actin at steady state and funnel the flux of actin monomers to the noncapped filaments, which individually grow faster. *3*) ADF binds to the side of ADP-actin filaments and induces pointed-end depolymerization to increase the concentration of monomeric actin at steady state. Modified from LeClainche and Carlier (2008).

How do cells maintain a pool of unpolymerized actin?
A higher polymerization rate at the barbed end necessitates an increase of association rate on the barbed end of actin filaments for ATP-actin, and/or an increased critical concentration of ATP-actin (i.e. at steady state the equilibrium concentration of the pool of unassembled subunits). In fact, the monomeric actin concentrations measured in cells can actually be as high as 100 µM, implying that most monomers are sequestered by actin monomer binding proteins.

In general, the elongation of actin filaments can be regulated at the level of the monomers, and/or at the level of the actin filaments themselves. The cells make use of both mechanisms to control actin polymerization. First, some proteins bind monomeric actin and modify at the same time its polymerization competence. Second, capping protein binds to filament ends thus preventing monomer addition to the filaments.

<u>Actin monomer-binding proteins</u>
The two main monomer-binding proteins in vertebrate cells are profilin and thymosin. Only profilin is conserved in all eukaryotes and is also found in fungi and protozoa. Thymosin binds preferentially to ATP-actin and prevents the monomers from polymerizing. Thymosin-ATP-actin complexes do not bind to filaments, in contrast to profilin. The role of thymosin is therefore to provide a reservoir of monomeric actin, which can be assembled into a filament after thymosin has been displaced by profilin.

Profilin exhibits several important properties (Figure 2):
A) It regulates the nucleotide-status of actin by stimulating the exchange of ADP for ATP.
B) The entire profilin-actin-ATP complex is then added to the barbed end of filaments and subsequently profilin is released from the elongated filament.
C) In addition, due to a high affinity of profilin-actin-ATP complex for actin barbed ends, profilin stimulates the polymerization of actin filaments, and thus affects the rate of actin elongation by raising the association rate on the barbed end of actin filaments for ATP-actin.
D) Profilin competes with thymosin in the binding to ATP-actin, since they bind similar sites on ATP-actin. Profilin can therefore shuttle actin monomers away from thymosin onto barbed filament ends, where they polymerize. Therefore, profilin maintains a pool of ATP-actin that is ready to elongate any free barbed end and promotes barbed end polymerization. The specificity to barbed end assembly gives directionality to filament polymerization.

<u>Capping protein</u>
If all the barbed ends of cellular actin filaments were polymerization competent, the pool of free actin monomers would be rapidly depleted by filament elongation. One factor that plays an important role in the regulation of filament elongation is capping protein (Figure 2). It binds very tightly to the barbed ends and has a very slow dissociation rate ($t_{1/2}$: 1000s), implying that filaments are not able to elongate anymore during their lifetime unless they are actively uncapped or severed. Taking into consideration the estimated concentrations of free ends and capping

protein, it has been suggested that most of the free ends are capped in cells. Filaments in which the barbed end is capped are not able to undergo treadmilling. As a result, the filament gets slowly degraded from the pointed end, which provides new actin monomers. Together with profilin and thymosin, capping protein ensures the maintenance of a pool of ATP-actin monomers and provides thus the building blocks for the elongation of the limited number of uncapped filaments. Capping of barbed ends increases C_{ss}, because k_{off}^{P} stays the same while the effective k_{on}^{B} gets smaller since there are fewer filaments with a free barbed end. As a result, the limited number of non-capped filaments grows faster.

Taken together, capping protein makes at least two important contributions to actin-driven motility: (1) it limits the length of filaments (short filaments can generate more force); and (2) it biases the treadmilling towards a restricted number of filaments. Given that there is no significant elongation occurring at the pointed end, no capping is needed. However, based on the cellular concentrations of Arp2/3 (see below), it seems likely that most pointed ends are also capped.

ADF/cofilin

ADF/cofilin, two names for the same protein (Figure 2), is conserved in all eukaryotes and has two biochemical effects: (1) It enhances de-polymerization of actin filaments by severing them and creating more pointed ends, and (2) increases the rate of subunit dissociation from pointed ends (Figure 2). It binds preferentially to polymerized ADP-actin subunits. Binding of ADF/cofilin to actin filaments has been shown to induce structural changes, and thereby promote subunit dissociation. Thus, ADF can perform its function only after the time-dependent hydrolysis of ATP-actin and the subsequent dissociation of the phosphate.

ADF/cofilin and profilin synergize in enhancing in vivo treadmilling. ADF/cofilin accelerates pointed-end depolymerization, thereby increasing C_{ss}. On the other hand, profilin catalyzes the regeneration of T-form actin monomers, and targets ATP-actin specifically to barbed ends. Both actions together increase the processivity of the treadmilling by a factor of 125 in vitro. Treadmilling will not be observed for the majority of filaments in vivo, as their barbed ends are capped, the concept applies to the small number of filaments that are pushing the membrane forward at the leading edge of the cell.

4 - How do cells create actin filaments with free barbed ends?

In order for filaments to elongate and drive cell motility, the cell has to ensure that a sufficient number of elongation-competent barbed ends become available at the right time and place. The cell uses three different mechanisms to create free ends: (1) uncapping of filaments, (2) severing of filaments and (3) *de novo* nucleation of actin filaments.

Uncapping and severing
It has been shown that the two most abundant barbed-end capping proteins, capping protein and gelsolin, can be removed from barbed ends of filaments by interaction with phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) at the plasma membrane. This may help to direct the growth of new filaments towards the leading edge of a migrating cell.

Severing of existing filaments into multiple smaller ones can produce new free ends, and it has been demonstrated that the activation of gelsolin, which can cap and sever, is a major mechanism of inducing actin polymerization in some cell types. ADF/cofilin also exhibits severing activity. It is thought that the mechanism of filament severing mainly leads to the formation of short actin oligomers that are subsequently depolymerized into ADP-actin monomers.

De novo nucleation by the Arp2/3 complex
Pure actin monomers can self-assemble into filaments, but only with low efficiency. The rate-limiting step is the formation of a trimeric nucleus via a dimeric intermediate that is formed with an approximate K_d of 100'000 µM. Actin-dimers and -trimers are very unstable, but once a trimer is formed it can be elongated (and stabilized) by a diffusion-limited process involving the addition of a forth monomer. Therefore, complete de novo formation of filaments in the absence of additional factors is unlikely to occur in vivo, and additional factors are needed to promote the nucleation of actin filaments.

One of the key actin filament nucleators is the Arp2/3 complex, composed of 7 proteins. It induces the formation of branched filaments, which display a 70° angle, by a side branching mechanism: The new filament is formed by i) the attachment of the ATP-bound inactive Arp2/3 complex to the side of an existing actin filament and followed by ii) the activation of it by a WASP family member, which causes ATP hydrolysis in Arp2 and a confirmation change in the complex allowing subsequent actin polymerization (Figure 3).

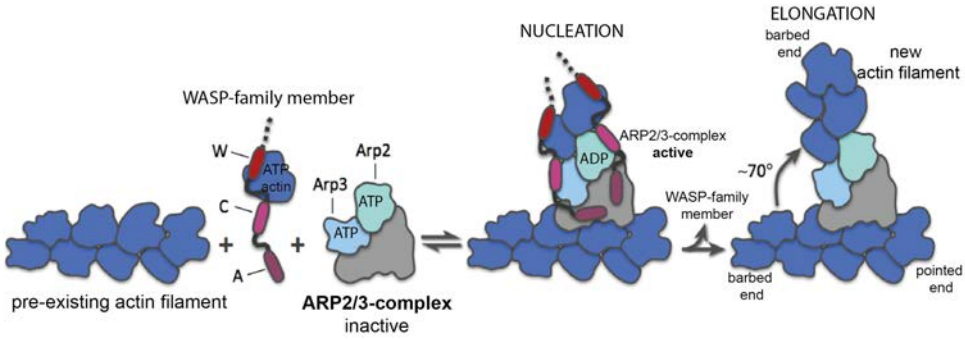

Figure 3: How the APR2/3-complex nucleates a new actin filament. Individual Arp2/3 complex binds the sides of a preexisting actin filament and seeding a new branch after activation by a WASP-family member. Modified from Dominguez, R. (2016).

The Arp2/3 complex needs to be activated in order to efficiently nucleate new filaments by bringing the Arp2 and Arp3 proteins, two actin-related

proteins that bind ATP (only Arp2 hydrolyzes it to ADP during the complex activation step), into a conformation that mimics the normal actin dimer configuration. The main activators are the WASP/WAVE proteins (see below). The synergism of WASP and pre-existing actin filaments leads to an autocatalytic reaction that results upon energy (ATP) consumption in an active complex, biases the initiation of new filaments to the sides of existing filaments and leads to the formation of a highly branched actin meshwork.

Incorporated Arp2 and 3 within the complex mimic the barbed end of a filament, and the complex localizes to the pointed end of the newly growing filament branch. Indeed, recent structural work based on electron tomography and computational docking revealed that the arrangement of Arp2 and Arp3 incorporated in a filament is different than in the inactive soluble complex (Figure 3). In the branch, Arp2 and Arp3 form a short-pitch helix dimer and thus contribute the first two subunits of the daughter actin filament.

All seven Arp2/3 complex subunits make some contact with the mother filament, and the total area of surface that is buried at this contact site is substantial. Of note: In fact, the Arp2/3 complex is the only known actin nucleator that produces branched actin filaments.

5 - The dendritic nucleation model

We will now focus on the signal responsive machinery to fine-tune the nucleation of actin filaments which establishes a directed protrusion formation: The dendritic nucleation model. It proposes that the activated ARP2/3 complex binds to the side of a pre-existing filament initiating there a lateral branch.

The dendritic nucleation model is a conceptual model and based on only 4 of the more than 60 actin-binding proteins (profilin, Arp2/3, capping protein and ADF/cofilin). It is sufficient to reconstitute actin-based motility of pathogenic, intracellular bacteria in vitro. The key components and reactions that form the dendritic nucleation model can be summarized as follows (see Figure 4):

(1) Extracellular signals activate receptors.

(2) The associated signal transduction pathways produce GTP-bound Rho-family GTPases, which

(3) together with $PI(4,5)P_2$ will activate WASP/WAVE proteins.

(4) WASP/WAVE proteins activate Arp2/3 complex and thus bring it together with an actin monomer on the side of a preexisting filament to form a branch.

(5) Rapid growth occurs at the barbed end of the new branch,

(6) and pushes the membrane forward.

(7) Capping protein terminates growth within a second or two.

(8) Filaments age by hydrolysis of ATP bound to each actin subunit (white subunits turn yellow) followed by dissociation of the phosphate (yellow subunits turn red).

(9) ADF/cofilin promotes phosphate dissociation, severs ADP-actin filaments and promotes dissociation of ADP-actin from filament ends.

(10) Profilin catalyzes the exchange of ADP for ATP (turning red subunits white),

(11) thereby refilling the pool of ATP-actin bound to profilin, ready to elongate barbed ends as they become available.

(12) Rho-family GTPases also activate PAK and LIM kinases, which phosphorylate ADF/cofilin. This tends to slow down the turnover of the filaments.

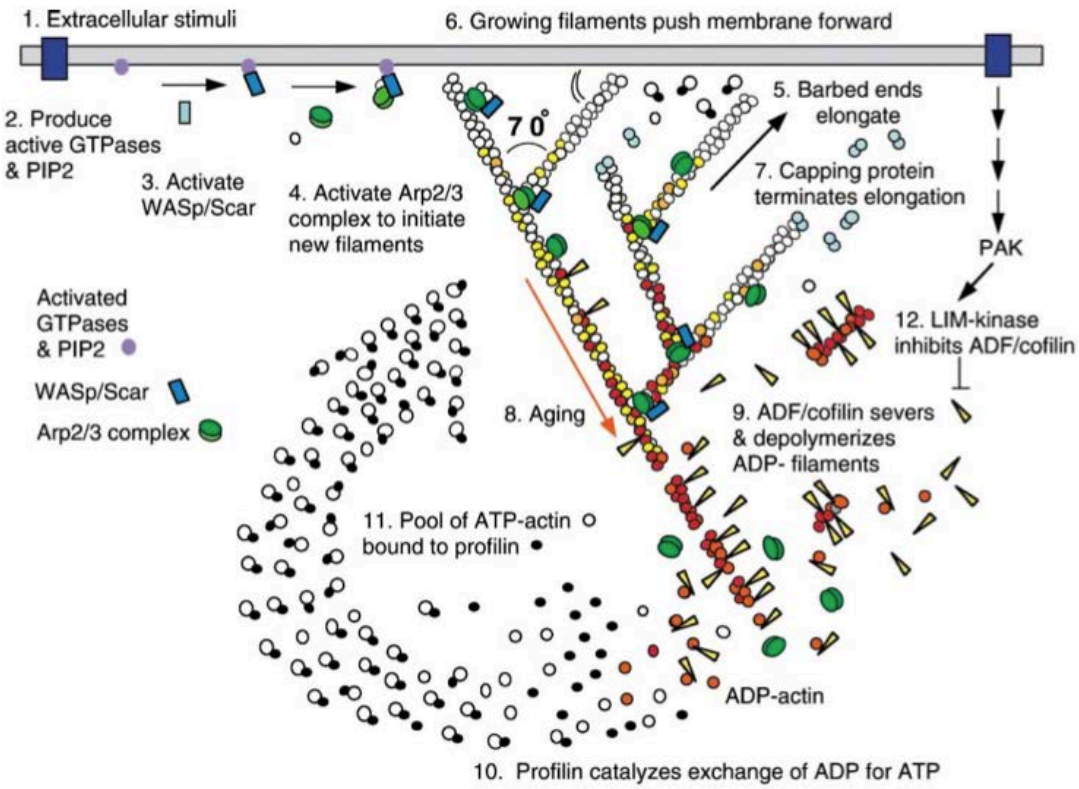

Figure 4: The dendritic nucleation model. From Pollard and Borisy (2003).

6 - How is the activity of the Arp2/3 complex regulated?

External signals can induce assembly of the cortical actin meshwork. Multiple signaling pathways involving receptor tyrosine kinases, seven-helix transmembrane receptors and integrins converge on Rho GTPases. They in turn activate the WASP proteins and WAVE-complex, respectively (chapter 4). The WASP proteins (N-WASP, WASP) and members of the WAVE-complex are highly conserved from protozoa to animals, and they are the key activators of the Arp2/3 complex. In general, Cdc42 activates WASP proteins, while the WAVE complex is downstream of Rac (chapter 4).

Exemplarily, the WASP proteins are composed of several functional domains that are able to interact with a large number of different ligands (Figure 5). The C-terminal VCA domain activates the Arp2/3 complex. The Arp2/3 complex undergoes conformational changes upon binding to the VCA domain, and adopts a compact, actin-dimer-like conformation. Subsequently, a stable trimer is formed in combination with a proximal actin monomer (proximity as it was bound to WASP), and a new actin filament is ready to be elongated.

The Verprolin homology region (V) or also termed WH2 domain acts with the same molecular principle in "novel" actin nucleators (see below).

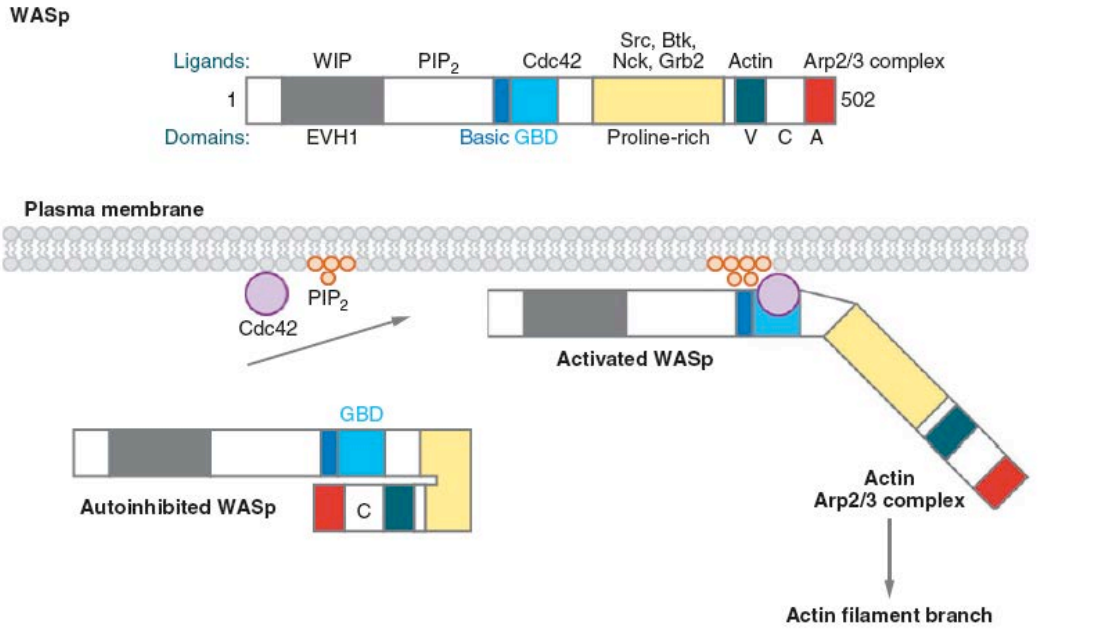

Figure 5: The domain organization and regulation of WASP. Cooperative binding of Phosphatidylinositol 4,5 bisphosphate (PIP2) and Cdc42-GTP to WASP relieves its autoinhibitory conformation. The G protein binding domain (GBD) includes the CRIB domain (see chapter 2). From Pollard (2007).

The WASP proteins exist in a closed, auto-inhibited conformation, in which N-terminal sequences (including especially the CRIB domain) block the activity of the VCA domain. The binding of a Rho GTPase, often combined with interactions between WASP and phosphoinositides, induce a structural

change and liberate the VCA domain, which is then able to activate the Arp2/3 complex.

Given that the Arp2/3 complex is integrated into the actin network, and that capping proteins terminate the polymerization relatively soon, a continuous supply of activated Arp2/3 is required to maintain high rates of actin filament nucleation. This might explain the observation that filament elongation stops quickly after the removal of activation stimuli such as chemotactic signals.

There are mechanisms used by pathogenic bacteria to control the host machineries to infect cells. At the end of this chapter you will learn about ActA, another activator of the Arp2/3 complex.

7 - Nucleation of actin filaments by Formins

Pointed-end associated nucleators such as the Arp2/3 complex promote the formation of actin meshworks, but they do not produce long filaments since the free barbed ends are very quickly bound and blocked by capping proteins. Long actin filaments are however important for many cellular processes, including the formation of the contractile ring during cytokinesis, the formation of yeast actin cables directing vesicular traffic towards the bud site, and the formation of filopodia. All formins have *per definition* the conserved formin-homology (FH) domains, FH1 and FH2. Formins are actin nucleators that remain bound to the barbed ends of actin filaments (in contrast to the ARP2/3 complex, that is at which end of the actin filament?); several formins act however rather as capping proteins as their nucleation activity is low. For example, Cdc12 (fission yeast) is a strong capper, whereas mDia1 (mammals) and Bni1 (budding yeast) are weak cappers.

Today about 15 mammalian formins are known, which fall into 7 different subclasses based on FH2 sequence divergence: Dia (Diaphanous), FRL (formin-related proteins in leukocytes), DAAM (Dishevelled-associated activators of morphogenesis), FHOD (formin-homology domain proteins), FMN (Formin), Delphilin, and INF (inverted-formin).

Formins remain processively associated with the elongating barbed ends. In vivo they work together with profilin-bound ATP-actin. In the absence of profilin, formins nucleate filaments and cap barbed ends more or less tightly, rather than promoting elongation. However, in presence of profilin-bound ATP-actin the elongation rate can be doubled.

Formins are multidomain proteins that function as homodimers. The dimerization has been shown to involve the dimerization domain (DD). The dimer of FH2-domains (Formin homology domain 2) forms a stable but flexible "donut" around the growing end of the filament (Figure 6). The FH2 dimer exists in equilibrium between a closed state (blocking filament elongation) and an open state (promoting filament elongation). The

transition from the closed to the open state is thought to involve the movement of the FH2 dimer towards the barbed end. Profilin is able to bind both to actin and the FH1 domain, and thereby increases the barbed-end elongation rate of formins.

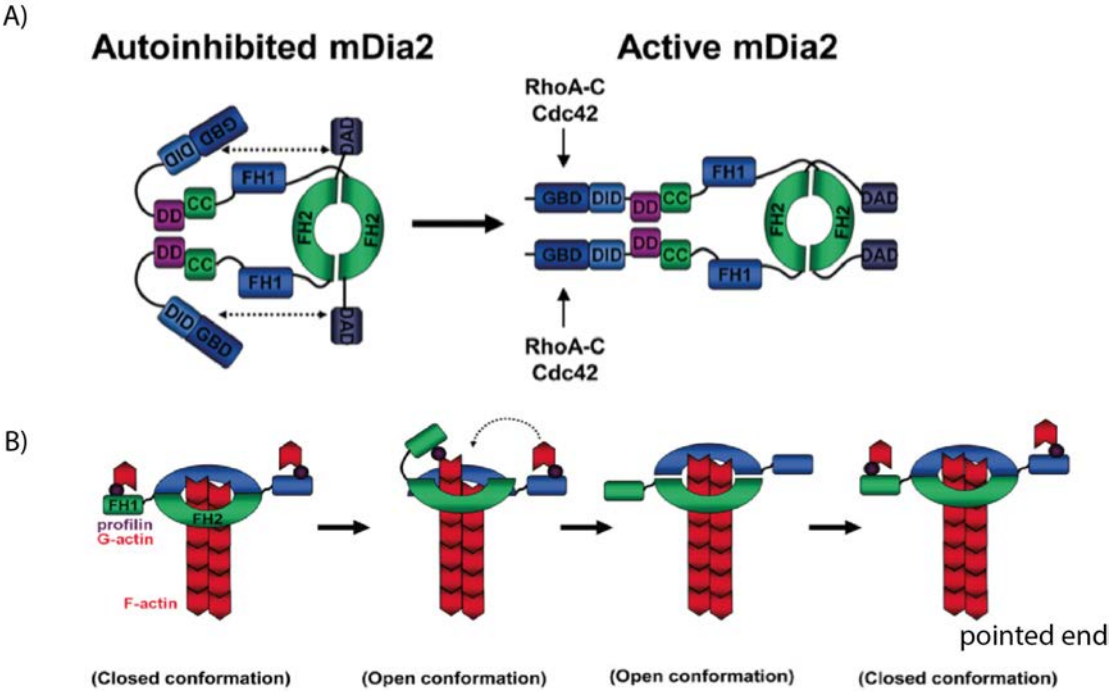

Figure 6: The domain organization of mDia2 and elongation model of formin-mediated actin polymerization (A, B). Diaphanous-related formins like mDia2 are dimeric and regulated by autoinhibition. Their actin nucleation functions are stimulated by binding to Rho family GTPases, such as RhoA-C and Cdc42. CC, coiled-coil; DAD, diaphanous-autoinhibitory-domain; DID, diaphanous-inhibitory-domain; DD, dimerization-domain; FH, formin-homology; FSI, formin-spire-interaction; GBD, GTPase-binding-domain; PDZ, PSD95-DlgA-ZO1; W, WH2 domain. B) An FH2 dimer associates with the barbed end of an actin filament (red), while the FH1 domains recruit profilin-actin (1, closed conformation). The FH1 domain delivers profilin-actin to the barbed end, and this is either preceded by or follows the FH2 domain stepping towards the barbed end (2, open conformation). The second FH2 repeats this process (3, open conformation). The formin closed conformation prevents capping by other factors (4). Adapted from Campellone et al. (2010).

Most capping proteins are soluble; however, formins operate at the plasma membrane in a signal-responsive fashion. A signal activates Rho-GTPases, which activate the FH2 domain by binding to it, and thereby cause the targeting of the activated formin to the membrane. Thus, similarly to WASP proteins, the function of formins is regulated by an induced relief of an autoinhibition (Figure 6A). Recent data suggests that some formins might function more as elongation factors and work in coordination with Spire (see below) or other formins.

Remarkably, formins do not only influence the actin cytoskeleton but are needed for proper turnover of cell-substrate adhesion sites and the stabilization of microtubules. For the very interested ones: In fact, the domain known to predominantly interact with microtubules or microtubule-interacting proteins (like EB1, APC, CLIP170 - see chapter 4) is the FH2 domain. For example, mDia1 and mDia2 regulate microtubule stability

alone or in combination with microtubule-interacting proteins independent of their activity on actin. The association of formins with microtubules is needed for spindle positioning, centrosome orientation, cell migration, cytokinesis and cell elongation and often associated with actin alignment. Research is ongoing in this direction.

8 – Novel actin filament nucleators

Spire represents a third type of actin nucleator, which is metazoan specific. It was originally found in D. melanogaster to be involved in egg and embryo development, but in the meanwhile two mammalian orthologs (Spire 1, 2) are also known. Spire nucleates new filaments at a rate similar to the formins, but slower than the activated Arp2/3 complex. It was suggested that Spire stabilizes and remains associated with the pointed end of the new filament, generating a barbed end free to elongate. Spire contains a cluster of four WASP homology 2 (WH2) domains, each of which binds an actin monomer. The C-terminal WH2 domain might cap the pointed end of the filament, while the three other WH2 domains seem to bind consecutively actin monomers thus generating one linear polymer of four adjacent actin monomers. Laterally to this first polymer a second one seems to form, facilitating barbed end elongation and promoting the formation of a new filament. Spire associates with and bundles microtubules, implying that it mediates actin-microtubule interactions. Taken together, the data suggest that Spire plays important roles in membrane transport and cytoskeletal interactions. Which exactly remains to be seen.

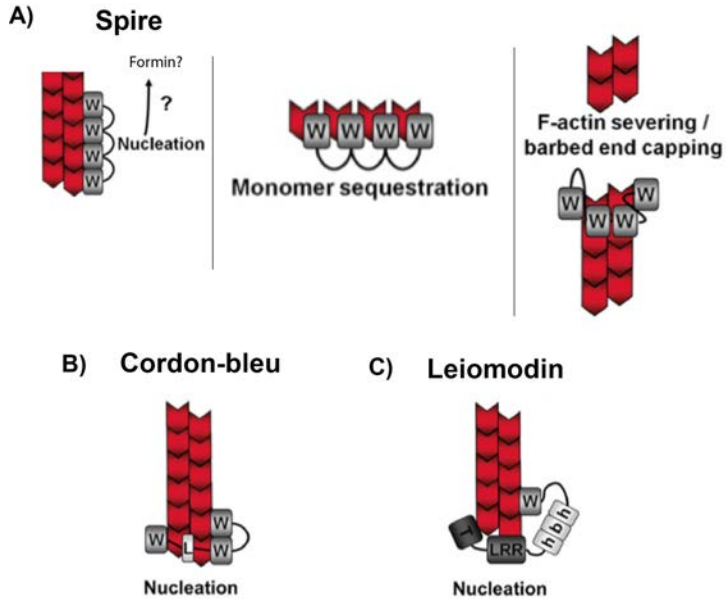

Figure 7: WH2 domain-containing actin nucleators and models for polymerization. A comparison of Spire, Cordon-bleu and Leiomodin concerning their interactions with actin. Spire is a versatile regulator of actin dynamics that can nucleate actin filaments (which might be further elongated by formins, as denoted by the question mark (?)), cap and sever existing filaments, and sequester monomers. Cordon-bleu and Leiomodin-family nucleators use multiple monomer-binding sequences to assemble trimeric actin nuclei and may remain associated with their pointed ends. It is proposed that Leiomodin stabilizes a trimeric actin nucleus and tropomyosin (T) enhances nucleation and mediates the

localization of Leiomodin to filament pointed ends; LRR, leucine-rich-repeats, h-b-h, helix-basic-helix, W, WH2. Red: actin monomers, the pointy tip indicates the pointed end. Modified from: Campellone et al (2010).

Rat cordon-bleu (Cobl) is a brain-enriched protein using its three C-terminal WH2 domains for actin binding. Cobl is present in all vertebrates but absent from e.g. *S. cerevisiae*. It promotes nonbundled, unbranched filaments similarly to Spire (Figure 7).
In 2008, yet another new actin nucleator was discovered: Leiomodin, an actin-binding protein acts with tropomyosin as strong filament nucleator in muscle cells. This complex localizes to the middle of muscle sarcomeres and seems to be responsible for the nucleation of tropomyosin-decorated filaments in muscles. Leiomodin-tropomyosin caps similar to the ARP2/3 complex and Spire the pointed end of actin filaments (Figure 7).

9 – Growing filaments: a force pushing the membrane

Actin polymerization produces mechanical energy that could deform a plasma membrane and generate a membrane protrusion. Until recently the accepted model illustrating this process is the Brownian ratchet. The model explains how a polymerizing filament could rectify the Brownian motion (thermal motions) of an object to produce unidirectional force.

The persistence length of the filament is the typical length at which the filament starts to bend spontaneously under the effect of thermal fluctuations. Actin filaments have a persistence length of about 15 μm, and microtubules have a persistence length in the range of 1–6 mm. Microtubules are rigid, whereas actin filaments are deformable behaving like elastic springs (Figure 8).

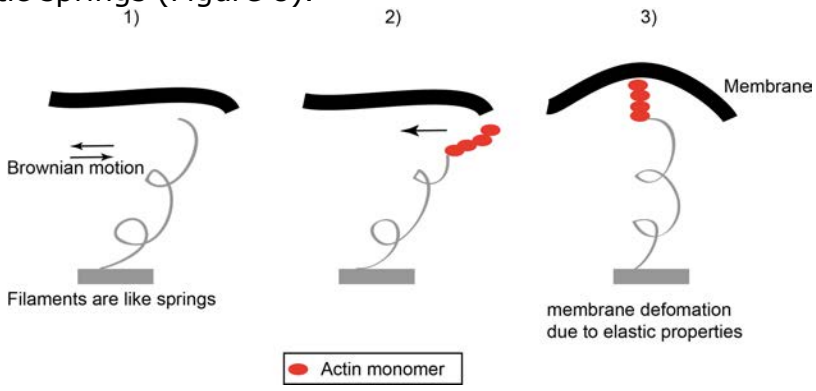

Figure 8: The elastic Brownian ratchet model. Adopted from Mogliner and Oster Biophysical Journal (71) 1996, by Ruth Kroschewski.

Thermal fluctuations displace a filament from the membrane, allowing additional monomers to be added to the end of the filament. The elastic force of the filament tries to return to its original position, and thereby deforms the membrane and pushes it forward. As a consequence, the filaments have to be short to be able to generate this force and anchored by some mechanism.

This model and the view that all barbed ends are oriented towards the displacing plasma membrane was 2010 challenged by *in vitro* data demonstrating that barbed ends that encountered a wall oriented away from it (for interested ones: see comprehensive summary: Cécile Sykes, Julie Plastino, Nature 2010 vol. 464 (7287) pp. 365-6).
In any case: Polymerizing filaments can only cause the membrane to protrude if they are "anchored"; i.e. a retrograde counter force is present that "resists" (usually provided by transmembrane receptors of the integrin family within focal adhesions).

10 – Actin tails cause motility of pathogens in mammalian cells

An overview over three different strategies used by pathogens to exploit the host cell's machinery to generate actin-based motility is provided in Figure 9. Listeria expresses the N-WASP mimicking protein ActA asymmetrically on its surface; the IcsA protein of Shigella mimics the Cdc42-dependent activation of cellular N-WASP, and Vaccinia virus triggers the Scr-family tyrosine kinase signaling network, leading to the phosphorylation of the viral A36R protein, and the subsequent recruitment and activation of N-WASP in complex with Nck and WIP. While all the pathogens appear to make use of the Cdc42 pathway and the downstream Arp2/3 complex, the different pathogens enter this signaling pathway at very specific steps. Remarkably, 2013 it was found that a mammalian formin is recruited by N-WASP to elongate the actin tail of cell-associated enveloped vaccinia virus, extending thus the types by pathogens used mammalian actin nucleators beyond the Arp2/3 complex.

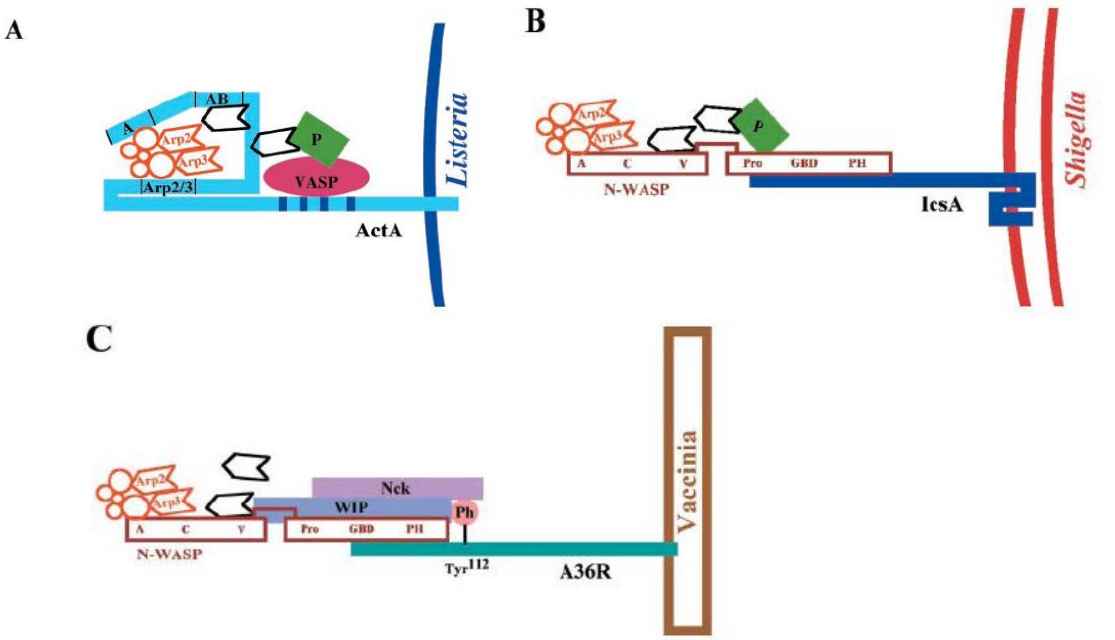

Figure 9: Comparison of the different mechanisms used by Listeria (a), Shigella (b) and Vaccinia virus (c) to promote actin-tail formation. Modified from Goldberg (2001)

After entry of motile bacteria into the cytoplasm of a host cell, the pathogens assemble an actin tail. While the nucleation promoting factor, WASP, remains attached to the bacteria, the Arp2/3 complex will distribute in general as part of the branch points in the actin meshwork throughout the whole actin comet tail. As a consequence, the growing barbed ends localize to the bacteria, while the depolymerizing pointed ends localize to the end of the comet tail. The "elastic Brownian ratchet" model can also be used to explain the movement of bacteria in vivo and in vitro.
Remarkably, it is possible to reconstitute and thus dissect the actin tail of e.g. Listeria monocytogenes in vitro using chemically inert polystyrene beads (mimicking the bacterial body), that are coated with the Arp2/3 activator ActA. Such a synthetic system is called a biomimetic system, if these grafted beads are surrounded by a defined medium that supports motility. The minimal motility medium supporting the propulsion of N-WASP-coated beads is composed of Arp2/3 complex, profilin, capping protein and ADF/cofilin and F-actin.

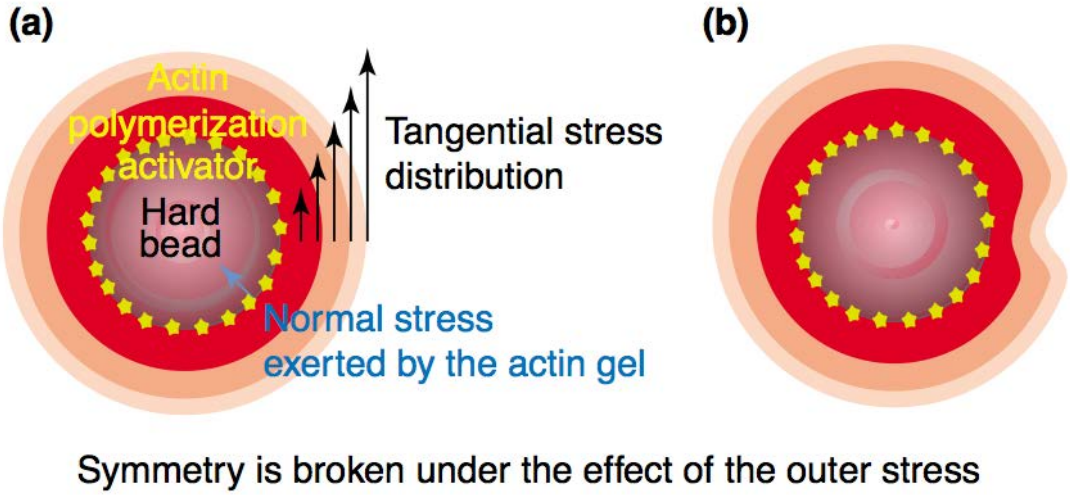

Figure 10: A model for spontaneous symmetry breaking on beads coated with an actin-polymerizing factor. Newly synthesized actin filaments are depicted in red, older ones in fading red tones. From Plastino and Sykes (2005).

Surprisingly, despite the homogenous distribution of the activating proteins on the bead surface, an actin tail can be formed that pushes the bead forward. Initially a cloud of filamentous actin forms around the coated beads. But subsequently, this cloud transforms into one actin tail that extends exclusively from one pole of the bead. This demonstrates that beads homogeneously coated with an actin-polymerizing factor are able to spontaneously break symmetry in vitro.

The observed symmetry breaking appears to be due to release of elastic energy in the actin gel. Thus, one can interpret these results in the following way (Figure 10): Repetitive actin layers are synthesized at the surface of the bead. Therefore, already existing layers will be progressively pushed outwards by the new layers that form directly on the surface of the bead. Eventually, the outside layer will become so stretched that it breaks at one particular point. This break is propagated into the inner actin layers and renders the actin coat asymmetric. Later this initial asymmetry will be

amplified, leading to the formation of a polarized actin tail and bead movement.

Further experiments investigating the mechanical properties of actin tails are currently being carried out, assessing the role of various actin-binding proteins during bead propulsion. The actin gel continuously undergoes deformations during the growth of the comet tail. Depending on the protein composition in the motility medium, deformations arise from either gel elasticity or monomer diffusion through the actin comet. Thus, these findings demonstrate that actin-based movement is also governed by the mechanical properties of the actin network, which are fine-tuned by proteins involved in actin dynamics and assembly.

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Chapter 4: Coordinated regulation of actin and microtubules by Rho GTPases

Remarkable in this chapter (strong links to chapters 1, 2 and 3):

- Molecular understanding of how Rho GTPases link actin and microtubule dynamics:
    - molecular interactions between Rho GTPases, ROCK, PAK, Cofilin, N-WASP (autoinhibited), WAVE (autoinhibited), Formin (Diaphanous, autoinhibited), Myosin II (autoinhibited), Stathmin, GEF-H1 (microtubules)
    - stress fibers are anchored in mature focal adhesions
    - IQGAP1, a key example for Rho GTPase-dependent conformational change and molecular crosstalk between actin and microtubules
    - Effects of these molecular interactions on actin structures and microtubule organization (when and where in a migrating fibroblast, early and late phase of migration): interdependence between adhesion, actin and microtubules
- Two ways to form a filopodium.
- One force generating principle (to be continued): stress fiber contraction is network shrinkage
- Energy consumption (to be continued) during myosin II activity.

1 - Introduction

GTPases of the Ras superfamily act as molecular switches to control a wide range of essential biochemical pathways in all eukaryotic cells. The Ras and the Rho families are of special interest since they couple intracellular signal transduction pathways to changes in the external environment. The Rho gene was identified in 1985, but only in 1992 the first insights into the cellular function of Rho GTPases were gained. Constitutively activated (GTPase deficient) mutants of Rho and Rac were found to induce the assembly of contractile actin and myosin filaments (stress fibers) and actin-rich surface protrusions (lamellipodia), respectively, when introduced into fibroblasts. Later, Cdc42 was shown to promote the formation of actin-rich, finger-like membrane extensions (filopodia). Subsequent work has shown that Rho, Rac and Cdc42 link different signals to the assembly of distinct filamentous actin structures in a wide variety of mammalian cell types as well as in yeast, flies and worms. In addition to those linked to the actin cytoskeleton, Rho GTPases have been linked to many other processes, such as the regulation of adhesion, gene transcription, G1 cell cycle progression, microtubule dynamics, regulation of vesicular transport and a variety of enzymatic activities ranging from an NADPH oxidase in phagocytes to a glucan synthase in yeast.

Consistent with this assorted list of cellular activities, diverse membrane receptors and upstream regulators can activate Rho GTPases, a huge list of cellular target proteins can interact with an individual GTPase, and extensive cross-talk and cooperation exists between GTPase-regulated signal

transduction pathways. Unfortunately, not all target proteins contain a diagnostic sequence motif useful in database searches. Still, for RhoA, Rac1 and Cdc42, the three best-characterized members of the family, over 60 targets have so far been identified experimentally. This chapter will describe some of the most important functions of Rho GTPases in the regulation of the actin and microtubule cytoskeleton and discuss IQGAP1 as a remarkable scaffold.

2 – Signals causing the activation of Rho GTPases

Cdc42, Rac1 and RhoA function as downstream components of signaling pathways initiated by ligand-stimulated G protein-coupled serpentine receptors, receptor tyrosine kinases or integrins. Some relevant examples are listed below. Certainly not all signals and plasma membrane receptors causing the activation of Rho GTPases are known.

- In fibroblasts, extracellular stimuli have been shown to activate the Rho GTPase cascade at different points. Addition of LPA (lysophosphatidic acid, a phospholipid derivative) to quiescent fibroblasts induces the formation of actin stress fibers.

- Important growth factors such as PDGF, insulin, or bombesin, stimulate polymerization of actin in many cell types to induce lamellipodia formation and surface membrane ruffling.

- Bradykinin causes the activation of Cdc42 via a bradykinin receptor in the plasma membrane.

The bradykinin, LPA, and bombesin receptors belong to the seven-transmembrane-domain heterotrimeric G protein-coupled receptor family. PDGF and insulin receptors belong to the group of receptor tyrosine kinases that mediate signaling from outside into the cell.

Last but not least, the extracellular matrix component fibronectin can cause the activation of integrin receptors and thus influences Rho mediated signaling.

3- Rho GTPases and actin dynamics

Through a series of parallel and serial signal transduction pathways, the members of the Rho subfamily of GTPases control a plethora of cellular activities. In particular, the different GTPases have distinct effects on the cellular actin cytoskeleton, and consequently on cell migration (Figure 1):

- **Cdc42-GTP** causes the formation of focal complexes (immature substrate-cell connection points, chapter 1) and actin polymerization for filopodia, which are thin, finger-like cell protrusions that contain parallel actin bundles and act as mechanosensors in the exploration of the extracellular environment. Cdc42 also regulates plasma membrane polarization and directional movement.

- **Rac-GTP** promotes the formation of focal complexes, and actin polymerization causing lamellipodia, which are curtain-like extensions that consists of thin protrusive actin sheets at the leading end of migrating cells.

- **Rho-GTP** promotes actin-myosin contractility, and the formation of stress fibers and focal adhesions (chapter 1), thereby regulating cell shape, attachment and motility.

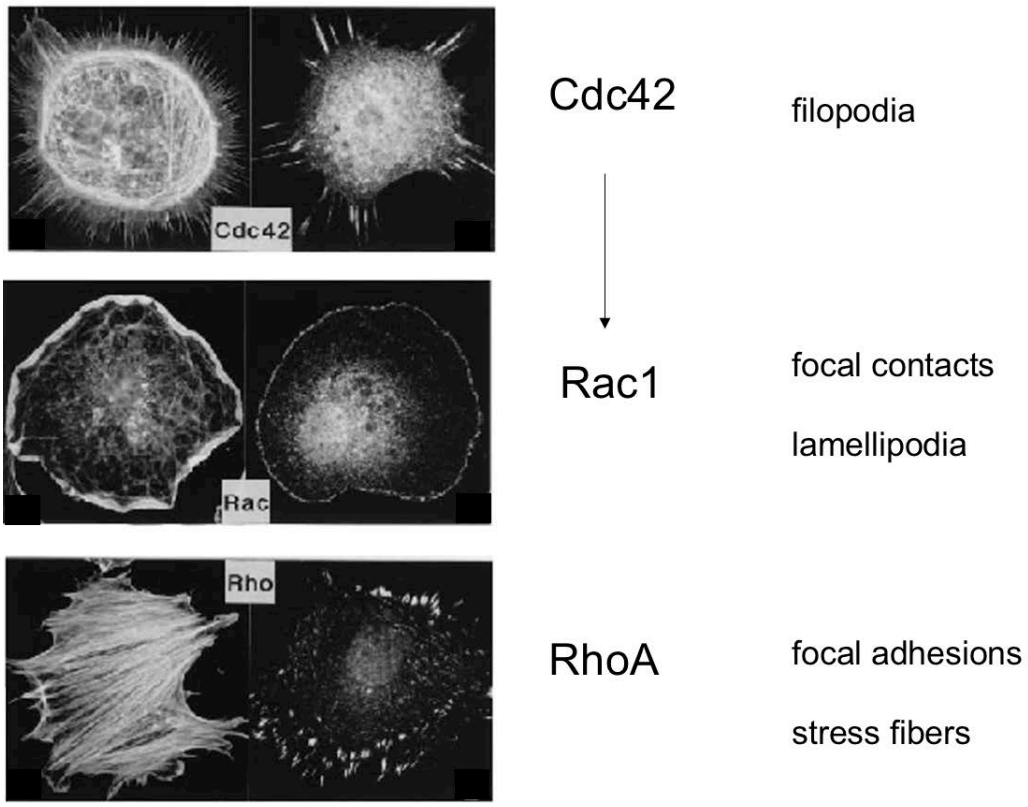

Figure 1: Rho GTPases have distinct effects on the actin cytoskeleton. The individual GTPase can be activated by different experimental means. Cdc42: overexpression of its specific GEF Fgd1; Rac: transfection of the constitutively active mutant Rac G12V; Rho: addition of lysophosphatidic acid (LPA). The panels on the left are stained with actin, the panel on the right with vinculin, which is a marker for focal complexes or focal adhesions. The arrow indicates that Cdc42 activation causes subsequently the activation of Rac. Modified from Hall (1998).

It was shown that the activation of Cdc42 leads to the sequential activation of Rac and finally Rho - however in a cell type dependent manner. This suggested that there is a coordinated, hierarchical control of cell motility by the different members of the Rho GTPase family. In the following, some key effector proteins (underlined) of these GTPases will be presented that specifically affect actin arrangements. The detailed interplay of the actin-binding proteins to control of actin dynamics will be discussed here and in chapter 3.

Arp2/3 and formins

Rho GTPases, in particular Rac and Cdc42, are able to induce the formation of two different types of actin arrangements: the highly crosslinked meshwork found for example in lamellipodia, and the long parallel actin bundles that constitute filopodia.

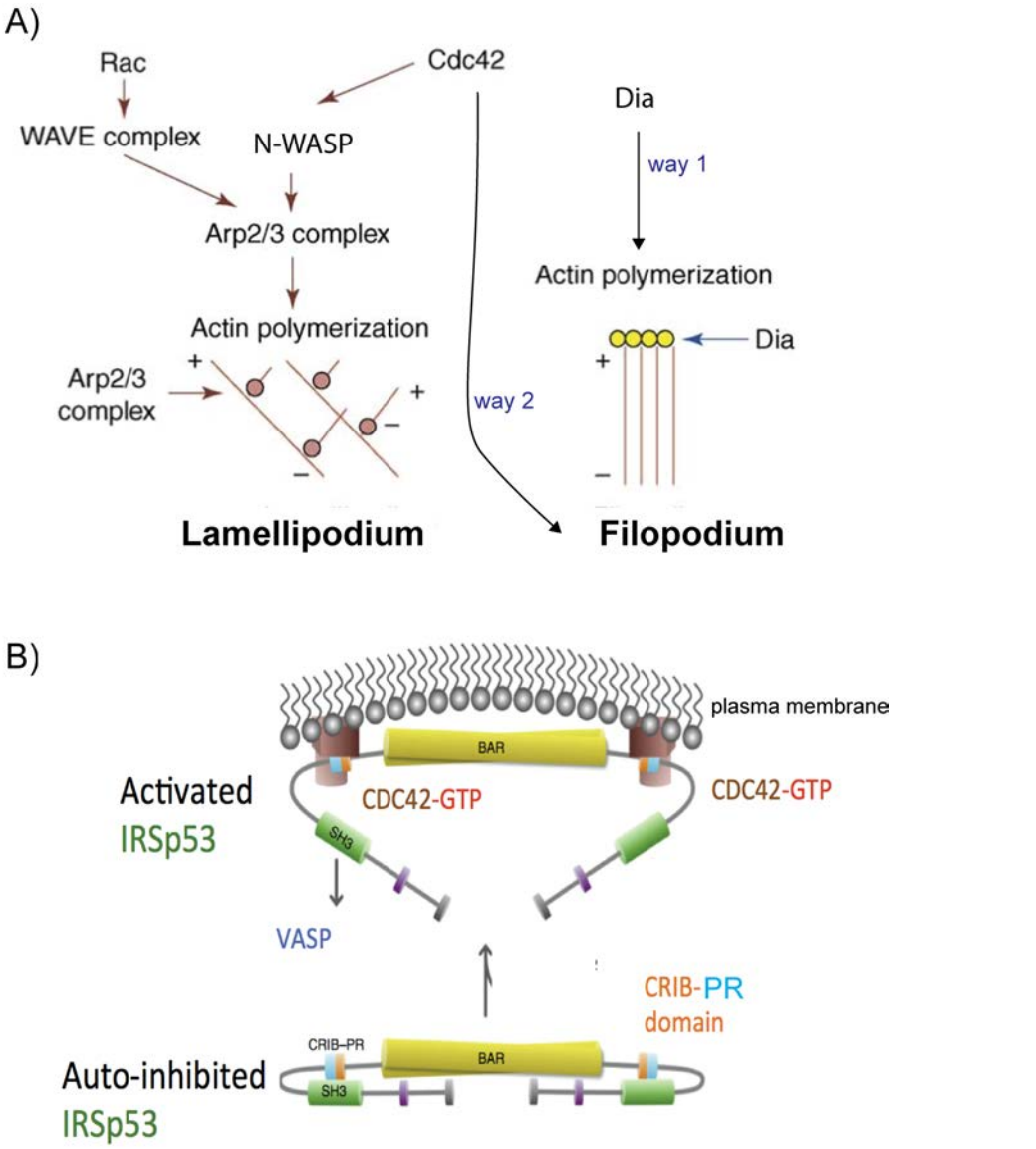

Figure 2: Rho GTPases, actin nucleators and formation of two actin structures: lamellipodium, filopodium. A) Overview. B) Detail of way2 showing how Cdc42-GTP (brown) helps to relieve the autoinhibition of IRSp53, which results in the clustering of VASP and subsequently the emergence of filopodia from the leading edge of a lamellipodium. Dia stands for Diaphanous, which is a formin. BAR (Bin–Amphiphysin–Rvs) domain binds to phosphatidylinositol 4,5-bisphosphate-rich membrane inducing negative curvature in it. Upon Cdc42-GTP binding to the (unusual) CRIB domain (CRIB-PR) of IRSp53, the autoinhibition of IRSp53 is relieved and VASP can help in the emergence of a filopodium. Adapted from Ridley (2006) and Kast (2014).

Two different Rho effector groups induce these two distinct actin structures (Figure 2):

- WASP and the WAVE complex both can activate the Arp2/3 complex, which promotes lamellipodia formation.
- Formins (inactive in autoinhibited state), such as Diaphanous, trigger parallel actin bundles as in filopodia.

WASP (only present in hematopoietic cells) and its ubiquitous counterpart N-WASP contain a CRIB domain, and they are inactive while being in an auto-inhibited conformation (chapter 3).

ADF/cofilin

Rho proteins can also affect actin polymerization by regulating ADF (actin depolymerizing factor)/cofilin. ADF/cofilin exhibits actin filament depolymerizing activity and severing activity. Although its activity is required for cell motility, it is not clear how the relative contributions of these two activities are balanced. On one hand, the activation of Rho GTPases induces actin polymerization via Arp2/3 and formins and it stabilizes the filaments by inhibiting the depolymerizing activity of ADF/cofilin. On the other hand, the activity of ADF/cofilin is required for the generation of new polymerization-competent ends by filament severing, and also provides (via pointed-end depolymerization) the actin monomers necessary for filament extension at the leading edge. Therefore, the activity of ADF/cofilin has to be very precisely regulated during lamellipodia formation.

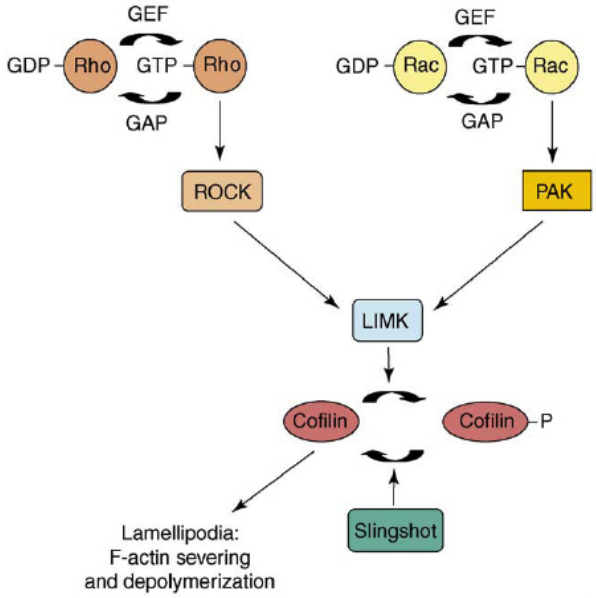

Figure 3: Regulation of cofilin by Rho and Rac. ROCK1/2 and PAK1-3 can phosphorylate and activate LIMKs (LIMK1 and LIMK2), which in turn phosphorylate and thus inactivate ADF/cofilin. Cofilin is dephosphorylated by the phosphatase Slingshot. Unphosphorylated cofilin stimulates severing and depolymerization of filamentous actin (F-actin) in lamellipodia. From Ridley (2006).

Rho-GTPases activate two different kinases (Figure 3):

- ROCK, a Rho-associated serine/threonine protein kinase, which exists in two isoforms called ROCK1 and ROCK2.
- PAKs (PAK1-3), p21-activated kinases, which are a highly conserved family of serine/threonine protein kinases with a CRIB domain. PAKs bind and become activated by Rac-GTP and Cdc42-GTP.

Both ROCK and the PAKs

- have been implicated in diverse cellular functions in addition to cytoskeletal organization, including modulation of gene expression, and control of cell division, and have been linked to cell transformation and a number of pathological conditions.
- phosphorylate and activate LIM kinases (containing LIM domains). LIM kinases directly phosphorylate ADF/cofilin and thereby inactivate it.

The inhibitory phosphorylation of ADF/cofilin is counteracted by several protein phosphatases, including the phosphatase Slingshot.

Myosin II

Conventional myosin II consists of two heavy and two regulatory light chains and is (in higher eukaryotes) activated by phosphorylation of the regulatory light chain. In non-muscle cells myosin II exists in two conformations. One in which the tail domain of the heavy chain interacts with the head domain. Myosins in this conformation cannot self-assemble into myosin filaments (autoinhibited). If, however the myosin light chain is phosphorylated, then the back-folded conformation opens up so that the myosin tail is elongated and, in addition, the actin binding site in the myosin head domain becomes exposed. Myosins in this conformation spontaneously self-assemble into bipolar filaments of up to 20 molecules, that allow cellular contraction by a mechanism analogous to muscle contraction. Myosin II is a motor protein and its activity is linked to ATP hydrolysis (energy consumption).

In addition to the effect on LIM kinase, the Rho-dependent kinase ROCK also promotes contractility and thus influences cell migration. Activated ROCK increases myosin activity by two mechanisms:

a) phosphorylation of myosin light chain (MLC), and
b) by inactivation of MLC phosphatase (Figure 4).

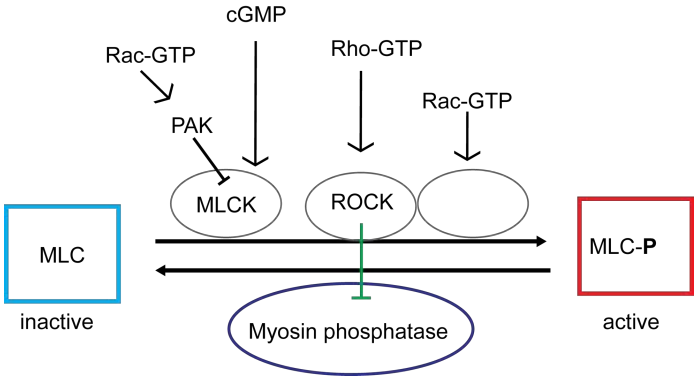

Figure 4: Multiple MLC (myosin light chain) kinases and a single myosin phosphatase are regulated by a variety of upstream molecules. Note that a positive regulator of MLC phosphorylation also promotes MLC phosphorylation by inhibiting myosin phosphatase (indicated by green). MLCK, myosin light chain kinase; ROCK, Rho-associated protein kinase (ROCK); PAK, p21-activated protein kinase; additional proteins not relevant for our module are also involved (empty circle); cGMP see chapter 5. Modified from Matsumura (2005).

Both mechanisms lead to an increase in MLC phosphorylation, which allows formation of myosin filaments and the interaction of myosin II with actin, which results in the generation of contractile forces. These forces promote the crosslinking of actin filaments, and thus contribute to the establishment of the force required for contraction of the cell body and the back end of the cell.

This downstream effect of Rho has to be inhibited at the leading edge of the cell, since it is incompatible with the formation of membrane protrusions. It

has been suggested that Rac, via its downstream PAK kinases, might phosphorylate MLCK, the myosin light chain kinase, causing its inhibition, which then reduces the levels of myosin phosphorylation at the front of the cell.

3 – Rho GTPases linking actin and microtubule dynamics

To support directional migration, actin dynamics and myosin contractility need to be precisely regulated in a spatially and temporally appropriate manner.

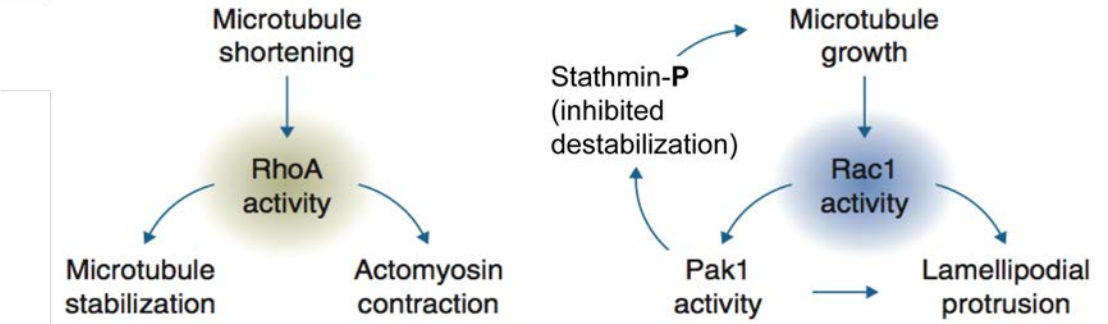

Figure 5: Rho GTPases and cytoskeletal filaments can regulate each other. Microtubule disassembly stimulates RhoA activity (left). Rho stabilizes microtubules through the formin mDia and also results in actomyosin contraction through stimulation of Rho kinase, resulting in myosin light chain phosphorylation. Microtubule growth stimulates Rac1 activity (right), which mediates actin polymerization and lamellipodial protrusion, and promotes further microtubule growth through activation of the Pak1-Stathmin signaling pathway to generate a positive feedback loop. Modified from Rodriguez et al. (2003).

We just saw that Rho GTPases cause the actin cytoskeleton to be arranged into specific structures: Cdc42-GTP causes the formation of filopodia, Rac-GTP causes the formation of lamellipodia or ruffles at the leading edge. Both induce also the formation of focal complexes. Rho-GTP causes stress fibers and the formation of focal adhesions from which stress fibers emanate. Stress fibers assemble from the actin-filament network at the lamellipodium/lamella boundary. The transition from focal complexes to focal adhesions is stimulated by the activation of Rho. It was shown that microtubule dynamics has a direct impact on the activity of Rho and Rac. Thus, Rho GTPases and cytoskeletal elements can regulate each other via several different pathways. Some relevant functional links are summarized schematically in Figure 5.

Rho-GTPase activity influences microtubules, actin and adhesion:
- Activation of RhoA causes the activation of the RhoA effector mDia (mouse diaphanous, chapter 3), which was shown to generate specialized stable microtubules and orients them towards the leading edge. At the same time, RhoA-GTP stimulates actin-myosin contractility via a kinase cascade involving ROCK, leading to the phosphorylation of the regulatory light chain of myosin II. Increased actin-myosin contractility results in the bundling of actin filaments that form stress fibers, which in turn cluster integrins and

their associated proteins into focal adhesion.

- Stathmin is an important phosphorylation-controlled regulator of microtubule (MT) dynamics and plays a crucial role in cell division and cell proliferation. Microtubule polymerization is inhibited by non-phosphorylated stathmin. One (non-phosphorylated) stathmin molecule destabilizes microtubules by i) sequestering two soluble dimeric alpha, beta-tubulin complexes into a tight complex, reducing thus the polymerization-competent tubulin and by ii) increasing the frequency of microtubule growth-to-shortening rate by directly binding to microtubule ends. Phosphorylated stathmin cannot sequester soluble tubulin dimers and liberates the previously bound alpha, beta-tubulin-complexes, that now can be used for microtubule polymerization. Stimulation of HeLa cells with EGF (epidermal growth factor) results in phosphorylation of stathmin in a Rac1- and Pak-dependent manner (Figure 5): i) Constitutively active Rac1-Q61L promotes Pak-dependent microtubule growth and turnover. And ii) Pak also mediates lamellipodial actin polymerization and retrograde flow downstream of Rac1-Q61L. Thus, a coordinated regulation of microtubules and actin dynamics occurs in the leading edge of migrating cells.

<u>Microtubules in turn modulate Rho GTPases and thereby control actin dynamics:</u>
- Microtubule growth induces activation of Rac, which in turn promotes the formation of lamellipodia.
- Disassembly of MT results in the activation of Rho. Recently, GEFs were identified that interact with microtubules and could form a regulated link between microtubules and actin. E.g. GEF-H1 was shown to be enzymatically inactive towards Rho when bound to microtubules. However, upon microtubule depolymerization, GEF-H1 generates Rho-GTP, which leads to the activation of myosin II, stress fiber assembly and regulated gene expression.

<u>Rac and Rho activities are interconnected in a reciprocal fashion.</u>

- The formation of the Rac dependent focal complexes requires contraction of an actin-myosin network that is independent of ROCK. A decrease of activated Rac causes loss of focal complexes and the growth of focal adhesions.
- Integrin signaling causes a biphasic Rho response. In a newly formed protrusion (early, 10 min) a Rho-GAP becomes activated mostly due to new beta1 integrin signaling. This prevents the accumulation of Rho-GTP that would inhibit the Rac dependent formation of an actin network. In a second phase about 45 minutes later, a Rho-GEF is being activated at that location in response to non-beta1 integrin signaling leading to Rho-GTP generation. Rho-GTP causes then the formation of the needed stable adhesion and stress fiber to promote cell translocation. Also retracting microtubules cause a local increase of Rho-GTP, as GEF H1 activity is turned on, if not bound to microtubules.

A key element in cell migration is the reciprocal regulation of Rac and Rho. Rac is active at the cell's leading edge where new protrusions and initial adhesions are formed. Rho stabilizes adhesions and causes cellular tension.

4 - IQGAP1 in migration

IQGAP1 serves as a scaffold linking actin to microtubule dynamics during cell migration, and thus ideally suited to elucidate the interplay of the two cytoskeletal systems during migration. IQGAP1 plays a pivotal role in the control of cell-cell adhesion and migration, two processes that are both based on cell polarization.

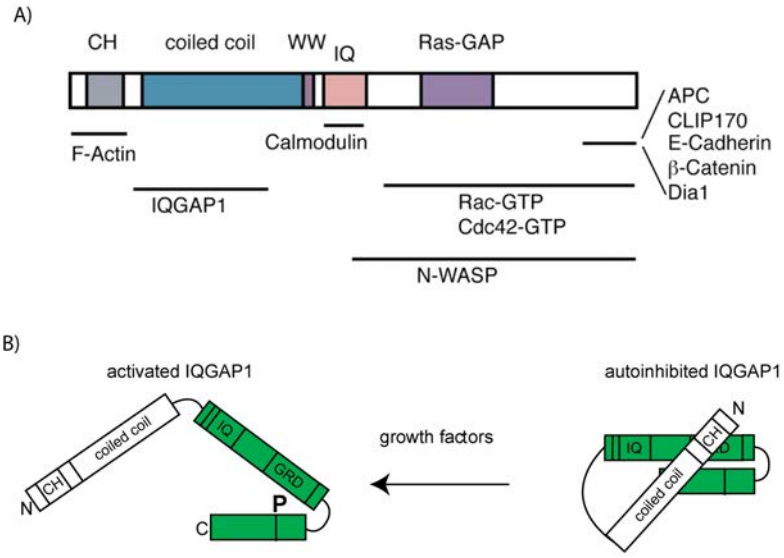

Figure 6: a) Domain structure of human IQGAP1 showing interaction sites of in the text mentioned binding partners. Domains: calponin homology domain (CH), poly proline protein-protein domain containing two conserved Trp (W) residues (WW), IQ motif (IQ), Ras GTPase-activating protein like domain (Ras-GAP). IQGAP1 was also shown to regulate E-cadherin dependent cell-cell contacts and actin polymerization. B) Phosphorylated IQGAP1 is in an open conformation. The C-terminal half (green) can bind to N-WASP and activate it. Ruth Kroschewski.

It is best known as an effector of the Rho- GTPases Rac1 and Cdc42, however it might also possess GEF activity if phosphorylated and bound to an unknown protein. In this way, phosphorylated IQGAP1 seems to act as a GEF and non-phosphorylated IQGAP as an effector (Figure 6B). Phosphorylation leads to a relief of the autoinhibited conformation of IQGAP1. The c-terminal half of activated IQGAP1 interacts with the CRIB domain of N-WASP and promotes thus Arp2/3 mediated actin polymerization.

Several sequentially arranged domains enable IQGAP1 to bind directly to a spectrum of cytoskeletal, adhesion and regulatory proteins (Figure 6). Like many of its associating proteins, IQGAP1 preferentially accumulates at the dynamic actin-rich cell cortex, such as newly formed leading edges and cell-cell junctions.

Cortical capture sites

Links between the plus ends of microtubules and peripheral actin-rich regions are essential for the establishment of cell polarity and directional migration. Cortical capture sites are defined by the local accumulation of

Cdc42-GTP or Rac-GTP, which reflect the local activation and recruitment of their relevant GEFs in response to an external migration signal. Some cells exhibit also an intrinsic mechanism that seems to stochastically generate local accumulation of Cdc42-GTP or Rac-GTP, which drives then movement into a randomly chosen direction (chapter 5).

Cdc42/Rac-GTP forms a tripartite complex with IQGAP1 and APC (chapter 1). Depletion of APC or IQGAP1 inhibits actin meshwork formation in the leading edge interdependently and leads to reduced cell migration. An important implication here is that the +TIP protein APC seems to connect via IQGAP1 to the actin cytoskeleton in the leading edge.

In fibroblasts, activated Rac1/Cdc42 forms also a tripartite complex with IQGAP1 and CLIP-170 (chapter 1), enhancing the interaction of IQGAP1 with CLIP-170 and capturing CLIP-170 at the leading edge and the base of filopodia. It was thus proposed, that the IQGAP1-CLIP-170 complex is targeted to the cortical capture sites, leading to formation of polarized and stabilized microtubules and enhancing cell polarization. MT stabilization follows the localized (= polarized) induction of actin polymerization and is thus secondary.

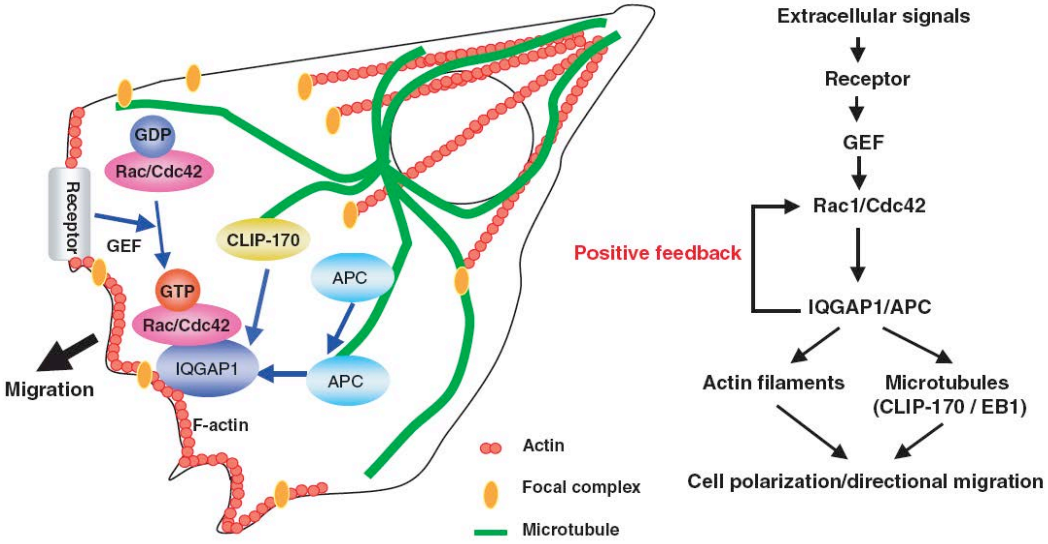

Figure 7: Role of the IQGAP1-APC complex in cell polarization and migration. Extracellular signals activate Rac1 and Cdc42 through their receptors and certain GEFs at leading edges. Activated Rac1 and Cdc42 induce the polymerization of actin filaments through their effectors and also mark spots where IQGAP1 crosslinks actin filaments. There, APC is recruited through IQGAP1 to actin filaments. IQGAP1 catches the plus-ends of microtubules through CLIP-170. APC then stabilizes microtubules. IQGAP1 can also activate N-WASP independently of Cdc42-GTP and therefore polarize actin polymerization. From Noritake et al (2005).

Polarizing actin and microtubules during migration

Rac1 and Cdc42 are activated by extracellular signals through receptors and GEFs at the leading edge. Rac1-GTP and Cdc42–GTP induce the polymerization of actin filaments through various effectors. Remarkably, IQGAP1 itself is also able to cause actin polymerization at the leading edge of cells by activating N-WASP through binding to its CRIB region; a mechanism similar to Cdc42-GTP (chapter 3).

Cdc42-GTP/Rac-GTP-IQGAP1 seems to anchor the plus ends of microtubules through CLIP-170 and APC. By this, the cortical actin sites become reinforced and MTs stabilized and oriented (Figure 7).

In addition, IQGAP1 not only positions the plus ends of microtubules but also the activity of the formin Diaphanous 1 (Dia1). Again, the removal of an autoinhibition, this time triggered by Rho-GTP, causes the activation of the formin and thus unbranched actin polymerization (chapter 3). A very C-terminal region of IQGAP1 interacts with the active form of Dia1 (chapter 3, Figure 6a). However, in contrast to the N-WASP interaction, this binding does not affect the actin polymerization activity of Dia1. In this case it is only the recruitment of an active formin to an IQGAP1-rich location that occurs. Thus, IQGAP1 influences two potent actin nucleator systems and microtubules.

IQGAP1, a scaffolding GEF?

It remains to be established how MT organization and actin polymerization are coordinated at the leading edge. As indicated before Dia1, CLIP170 and APC bind all to the same region in IQGAP1 (Figure 6). It is thus unlikely that all these three proteins are simultaneously bound to IQGAP1. How are these interactions regulated?

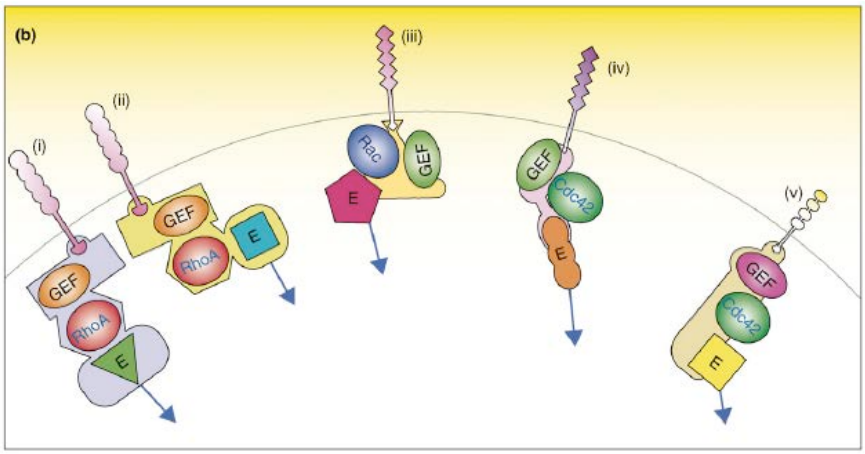

Figure 8: Scaffolds enable signals to be temporally and spatially regulated. (i,ii) Binding to a different scaffold would enable one receptor to signal through the same GEF and GTPase to different effectors. (iii, iv) The same Rho-GEF could activate a different GTPase and thus different effectors by interacting with a different receptor–scaffold complex. (iv,v) Through different receptor–scaffold–Rho-GEF combinations, the same GTPase can be activated by signals from different stimuli and activate different downstream effectors. Effects downstream of a particular effector molecule (blue arrows) are spatially restricted by the presence of scaffolding molecules. Scaffolding proteins are represented as irregular shaped objects. From Garcia-Mata R et Burridge K. (2007).

One possibility is a cell-type specific abundance of various proteins relative to one another that determines which reaction to take. Alternatively, the diverse IQGAP1 conformations might locally funnel signaling activities as it is proposed for scaffolding GEF's (Figure 8 and chapter 2). By this mechanism, several different functions of IQGAP1 could be simultaneously used in a single cell.

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Chapter 5: Chemotaxis in Dictyostelium discoideum

Remarkable in this chapter:

- *Amoeboid migration (two types differing in major force generation mode)*
- *Directional sensing*
- *Phosphatidylinositol (3,4,5)-tri-phosphate induced reactions*
- *Cdc42 and members of the Rho-subfamily are absent in Dictyostelium discoideum; Rac-related members are present.*

1 - Introduction

Cells can sense the presence of extracellular signals, either diffusible molecules or molecules fixed to the substratum, and regulate their behavior with respect to the concentration gradient of this signal. The process of directed cell migration in response to a chemical signal is called chemotaxis, and it can either occur towards an attractant or away from a repellent. Chemotaxis is found in prokaryotes and eukaryotes and plays for example an important role in nutrient sourcing of prokaryotes, the formation of multicellular structures in protozoa, the tracking of bacterial infections by neutrophils, and the organization of metazoan embryos. In general, the cells can use two types of signals to orient their movement: either the spatial concentration gradient of the attractant/repellent, or the temporal signals generated when the cells move in a static gradient. Threshold concentrations for detection of chemoattractants are typically in the range of 10^{-6} to 10^{-8} Molar, with repellents often having a higher threshold. There are many different types of chemical signals. For example, bacteria are attracted by aspartate and repelled by nickel ions. cAMP (cyclic AMP) is a chemoattractant for Dictyostelium discoideum cells, and neutrophils can be attracted by N-formylated peptides. There are cases in which the same substance attracts some cells while repelling others. Netrin for example attracts neurons but is a repellent for oligodendrocyte precursor cells in the embryonic spinal cord.

Prokaryotic cells are too small to use the spatial gradient and make use of the temporal component. Bacterial chemotaxis is regulated by a signaling pathway that involves 6 proteins, which regulate the clockwise or counterclockwise rotation of the flagellum. Bacteria undergo a "random walk" that results from the alternation of forward movements and tumbling movements. In the presence of a static concentration gradient, bacteria reduce their tumbling frequency and the movement in one particular direction is prolonged. If the movement happens towards increasing signal concentration, then the tumbling frequency is kept low and the movement continues. However, if the signal concentration decreases, the tumbling frequency will increase again and give the cell the opportunity to randomly change direction. Bacterial chemotaxis should therefore be called kinesis rather than taxis, since it is a non-directional change in cellular activity in response to a stimulus, which generates a movement that only appears to be similar to a directed motion.

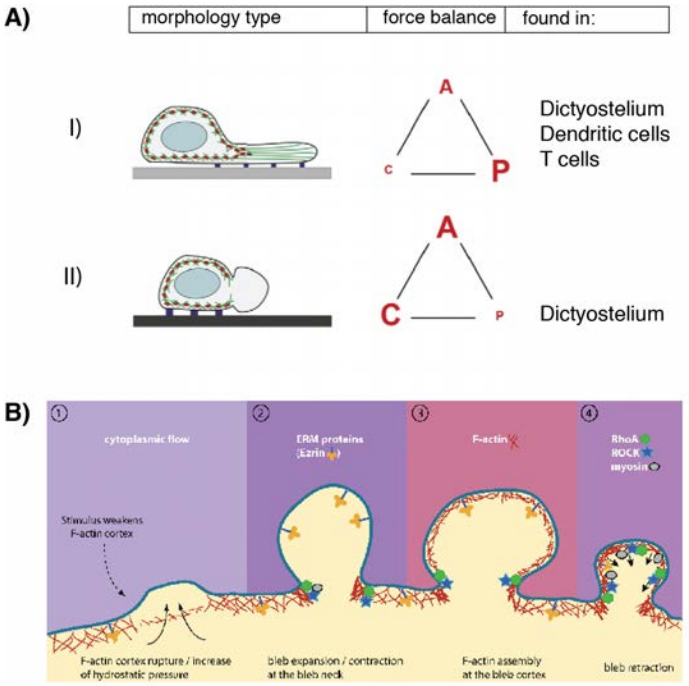

Figure 1: The two migration sub-modes of amoeboid migration (I, II) differ mechanically. Dictyostelium can exhibit both modes. A) The three major force generating aspects in cell migration are adhesion (A), contraction (C) and polymer-network expansion (P). Cell forward locomotion results from their balanced interplay (indicated by the red-lettered triangles). Any cell movement requires adhesion to transduce internal contractile forces onto the substratum. (I) The predominant migration form of Dictyostelium: Actin polymerization (green) 'pushes' the membrane forward. It produces traction under weak adhesion points (blue). Myosin II (red ellipses)-based contraction moves the cell content in direction of the protrusion. (II) The rarer migration form of Dictyostelium: membrane blebbing due to myosin driven hydrostatic pressure. B) Molecular requirements for bleb formation and retraction. Blebbing is initiated by extracellular triggers **OR** internal inhomogeneity (two different opinions are present), observed is a localized reduction of the cortical actin meshwork (1). Local disruption of the cortex–membrane interaction leads to the rapid formation of a bulky plasma membrane protrusion promoted by the cytoplasmic hydrostatic pressure. The expanding bleb membrane is not coupled to an actin cortex but is coated by actin–membrane cross-linker proteins (ERM, 2, chapter 1). Actin is then polymerized at the bleb cortex (3) by unknown mechanisms leading to a halt in bleb expansion (static phase). Increased actin filament assembly, recruitment of myosin to the bleb lumen, and local activity of RhoA-ROCK generate contractility that consequently retracts the bleb. A) modified from: Lämmermann T, Sixt M. (2009), B) from Fackler O. T., Grosse R. (2008).

Eukaryotic cells, measuring on average 10-20 µm in length, are big enough to use both, the temporal as well as the spatial information to orient their direction of migration. They have developed mechanisms that enable them to detect and internally amplify the difference of chemoattractant concentration between the ends of the cell. Therefore, they are able to respond strongly to concentration differences as small as 2% between the front and the back of the cell. The concentration difference is amplified as it is translated by the cell into a steep intracellular gradient of signaling components, leading to an asymmetric cellular response and thus to directed cell migration. This chapter describes the molecular mechanisms underlying chemotaxis in the amoeba Dictyostelium discoideum, which has been one of the key model systems used for the investigation of this process.

2 – The model system Dictyostelium

As mentioned in Chapter 1, Dictyostelium discoideum advances by an amoeboid mode of migration. Remarkably, Dictyostelium cells can exhibit several types of amoeboid motility; they can produce pseudopods, as well as membrane blebs (Figure 1A see I and II respectively). The type, where weak adhesion sites are present, stress fiber are absent, and actin polymerization drives protrusion formation, is the predominant form found in experiments (Figure 1 A) I). Remarkably, the adhesion receptors are not really identified. There is apparently only one Dictyostelium protein present which has similarities to the beta chain of integrins (2006). In general, the chemotacting cells extend the cell membrane in the form of pseudopods at the leading edge, while suppressing pseudopod formation elsewhere in the cell. The rear of the cells is then retracted at the trailing or posterior end.

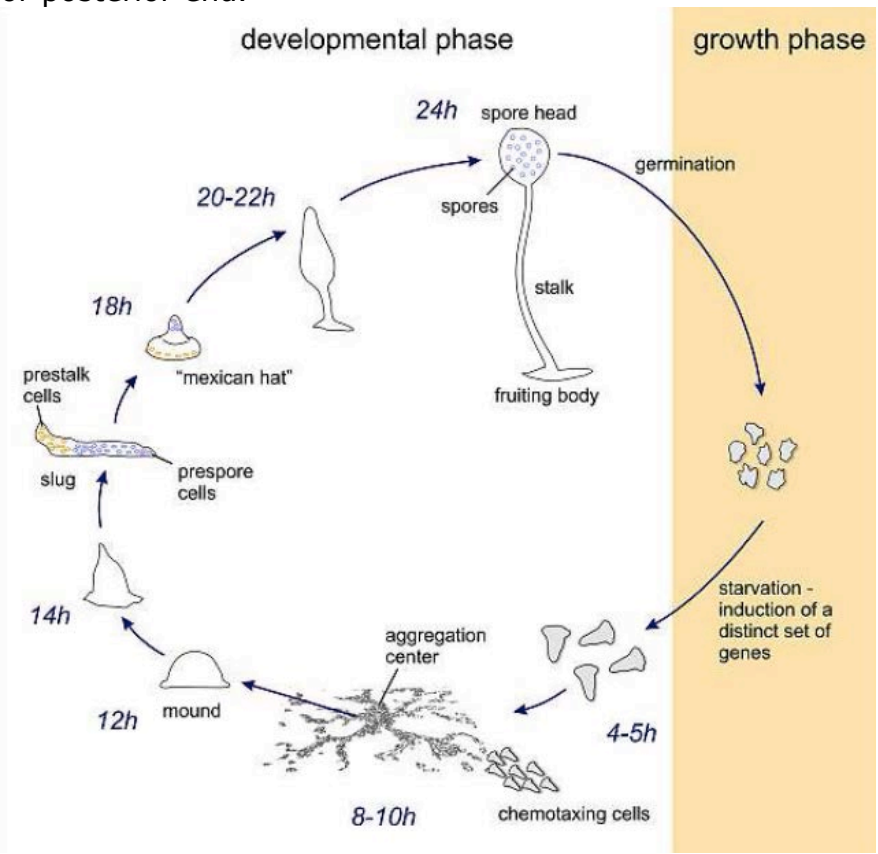

Figure 2: Lifecycle of Dictyostelium discoideum. (1) In favorable nutrient conditions, the cells grow and divide as single cells (growth phase). (2) Aggregate formation: when food is depleted, the amoeboid cells migrate toward an aggregation center, where hundreds of cells concentrate in response to the chemical attractant cAMP, which is present at nM concentrations. (3) Slug formation: the individual cells form a multicellular aggregate, containing approximately 10^6 cells, that elongates and forms a migrating slug. Importantly, precursor-cells such as prestalk and prespore cells start to differentiate, and sort within the slug along the anterior-posterior axis. cAMP reaches mM concentrations at this stage. (4) Culmination: the aggregate migrates up to 20 days until it finds appropriate external conditions, before settling down and developing a stalked asexual fruiting body: some cells dry up and form a supportive stalk, while others form spores. (5) Spores are then released, dispersed by the wind and new amoeba can hatch. Next to each stage of development, the corresponding time (in hours) after the beginning of starvation is shown. From Bargorda et al (2006).

The social amoeba Dictyostelium discoideum is one of the key model systems used for analyzing the molecular mechanisms underlying chemotaxis. Dictyostelium grow as individual cells, which undergo a vegetative division cycle. During that stage, they display some chemotactic responses. They are attracted towards the bacteria they eat by bacterial products such as folic acid, and they are repelled by other amoeba cells and disperse as widely as possible.

However, upon depletion of nutrients they initiate a highly complex, developmental program leading to the generation of a multicellular organism (Figure 2). This process involves the chemotaxis of single cells towards an aggregation center and culminates in the formation of a terminally differentiated fruiting body containing spores. This development can be separated broadly into two distinct phases: (1) chemotaxis and aggregation in response to the morphogen cAMP, leading to the formation of a multicellular organism, and (2) different stages of cyto-differentiation within the multicellular organism, including cell sorting and multi-cellular morphogenesis.

The cells within the developing cell aggregate secrete cAMP. Chemotaxis directs the moving cells toward the highest cAMP concentration, thereby forming a multicellular aggregate. Cells within the aggregate differentiate into non-terminally differentiated prespore and prestalk populations (Figure 3). The aggregate undergoes morphogenesis and the different cells sort within the developing aggregate. Prespore cells of the slug posterior give rise to the terminally differentiated spores of the mature fruiting body, and prestalk populations differentiate into various stalk structures.

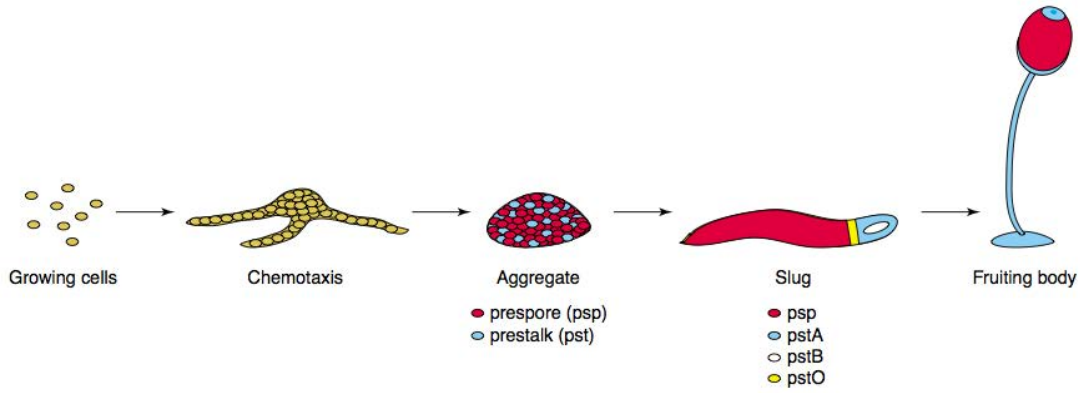

Figure 3: The major stages of Dictyostelium development. Three different types of prestalk cells are present in the aggregate as a homogenous population. At the slug stage, the posterior is primarily comprised of prespore (red). The anterior prestalk contains three different zones, with pstA cells (blue) comprising the major group. From Kimmel and Firtel (2004).

3 – cAMP signaling in Dictyostelium

In the absence of chemoattractants, Dictyostelium cells appear round and apolar. Once they become exposed to a chemoattractant gradient, they quickly polarize and start to migrate towards the signal source. Dictyostelium cells exhibit cell motility in the absence of stimulation. Dictyostelium pseudopods are periodically extending, self-organizing structures. The chemotactic behavior of neutrophils is very similar to the one of aggregating Dictyostelium cells. However, neutrophils are immobile until they are exposed to a chemoattractant. Aggregation of Dictyostelium is driven by the periodic cAMP secretion from a small number of cells in the aggregation center. This cAMP signal is subsequently detected by the surrounding cells, which initiate themselves the production of cAMP and thereby relay the cAMP signal to the cells that are even further away (Figure 4).

The extracellular cAMP in the nM concentration range binds to the high affinity receptor CAR1 (cAMP receptor 1) and induces the aggregation-stage specific signaling pathways. This leads to the generation of phosphatidyl inositol (3,4,5)-tri-phosphate ($PI(3,4,5)P_3$) at the cell front, which in turn recruits CRAC, a PH-domain containing cytosolic regulator/activator of adenylyl cyclase (AC). By mechanisms not fully understood, CRAC somehow diffuses through the cell and activates AC at the back of the cell. This results in the secretion of cAMP into the medium, and propagation of the signal to the neighboring cell.

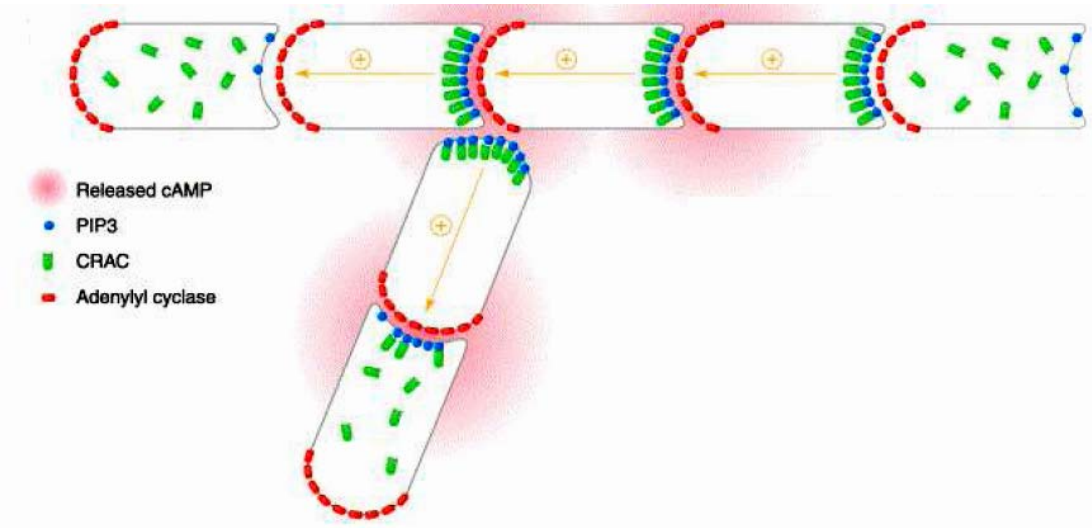

Figure 4: Signal relay in Dictyostelium cells. The cells are moving from left to right, the cAMP pulse is propagated from right to left. From Dormann and Weijer (2003).

Once the cAMP concentrations reach a particular level, the cells adapt and become insensitive to the extracellular cAMP levels. Sensitivity to cAMP is restored after a period of low extracellular cAMP levels, which results from the activation of the cAMP-induced extracellular phosphodiesterase (PDE). Low cAMP levels permit the expression of the phosphodiesterase inhibitor (PDI), and its secretion into the extracellular environment, where it blocks

PDE activity allowing a new accumulation of extracellular cAMP. These cycles of activation and adaptation result in cAMP pulses of roughly every 6 minutes. The cAMP oscillations are essential to relay the chemotactic gradient, ensure the directionality of the chemotactic movement, and also induce the expression of aggregation-specific genes.

4 – Directional sensing versus polarization

Localized and temporally controlled actin polymerization is crucial for chemotaxis. Actin at the front of the cells promotes pseudopod formation. Myosin II is assembled at the sides of the Dictyostelium cell to suppress lateral pseudopod formation, and at the rear of the cell to trigger the retraction of the trailing edge. Once a cell is polarized, the leading edge of the cell is more sensitive to chemoattractants than other regions, as the molecular machinery is already in place. This localized sensitivity focuses the activity of the actin cytoskeleton. However, it reduces the region of the cell contributing to the chemoattractant sensing, and therefore reduces the ability of the cell to respond to rapid changes in gradient direction. In contrast, an unpolarized cell maintains equal sensitivity around its entire perimeter.

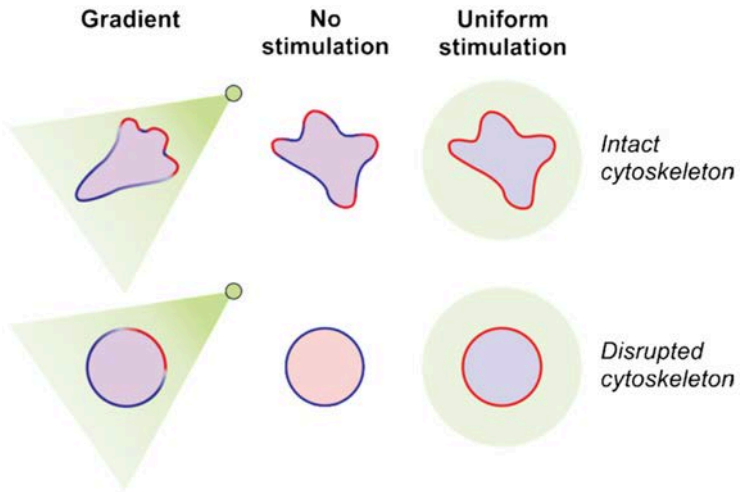

Figure 5: Spatiotemporal regulation of "front" (red) and "back" (blue) proteins with chemoattractant stimulation (green). The cytoskeleton and stimulation act synergistically to translocate cytoplasmic "front" proteins to the plasma membrane. Upon chemoattractant stimulation "front" and "back" proteins translocate relative to the respective "no stimulation" condition within 10–30 s but then return to the starting state after about 30-60 s. Evidence for directional sensing: In a round cell with a disrupted cytoskeleton, the "front" proteins localize to the high side of the gradient in a "crescent", whereas the "back" proteins have opposing localization, the cell is polarized as it sensed the gradient, yet the cell does not form a protrusion. Chemoattractants also trigger the activation of some proteins at the front or the back of a cell without affecting localization of those proteins. From Artemenko (2014).

It has been shown that cells are able to detect an extracellular gradient and to trigger the asymmetric accumulation of signaling molecules in the absence of morphological cell polarization; this process is referred to as directional sensing and links the cellular response constantly to the

external gradient (Figure 5). Consistently, amoeba or neutrophils treated with Latrunculin A, an inhibitor of actin polymerization, become depolarized and immobile, but they can still detect gradients and display highly polarized distributions of specific signaling components.
The local excitation and global inhibition model is able to explain two key features of chemotaxis: i) the adaptation to the chemoattractant, and ii) the sensing of spatial gradients. The model is based on a fast, short range, self-enhancing positive signal and a slow, long-range, freely diffusible negative signal (Figure 6). The combination of these two opposing signals, which derive both from the same original stimulus, has been proposed to be a general feature of many pattern formation processes. However, it does not easily explain the spontaneous polarization and directed movement that is observed in cells that are exposed to uniform concentrations of chemoattractants.

Adaptation: During the early stages of the response, activation is high, and inhibition is low, leading to strong intracellular signaling. At later stages, the activator and the inhibitor reach their steady-state levels and establish thus a new balance, and the cellular response returns at this point to its pre-stimulus level. The adapted cells can respond further if receptor occupancy is increased again.

Directional sensing: Assuming that the level of inhibition is defined by the average receptor occupancy, whereas activation correlates with local receptor occupancy, the cell will experience a net activation at the front (exposed to the highest concentrations of the chemoattractant), a balance between activation and inhibition at the center, and a net inhibition at the rear. This leads to a persistent, asymmetric cellular response.

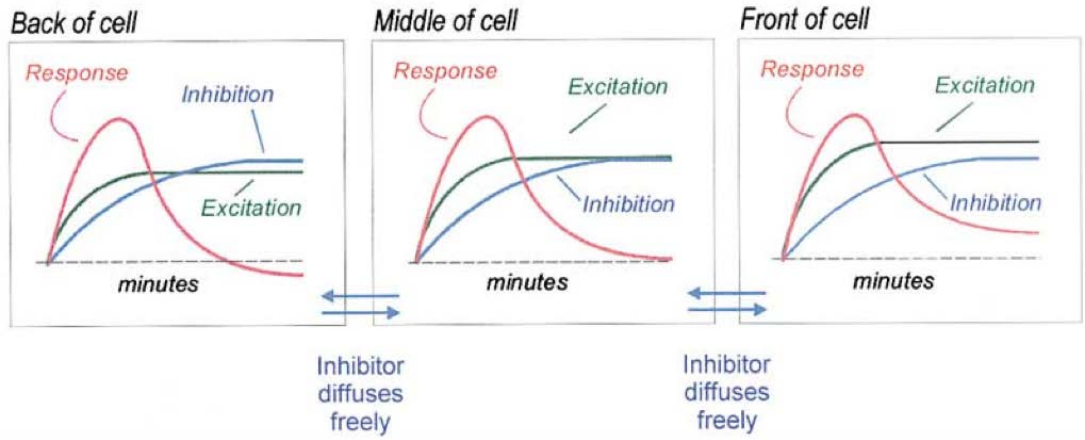

Figure 6: The local excitation and global inhibition model of directional sensing. From Iijima et *al.* (2002).

5 – Signaling networks in chemotaxis

The first response of a cell to a uniform concentration of a chemoattractant is the accumulation of $PI(3,4,5)P_3$ at the plasma membrane. Local $PI(3,4,5)P_3$ levels are controlled by the class I PI-3 kinases (PI3Ks) and the $PI(3,4,5)P_3$ 3-phosphatase PTEN (phosphatase and tensin homolog).

The localization of these two enzymes is reciprocally regulated in response to chemotactic stimulation. In unstimulated cells, the PI3Ks are mostly cytosolic, while PTEN associates with the plasma membrane. Upon stimulation of the cells with a uniform concentration of chemoattractant, PI3Ks are transiently targeted to the plasma membrane, while PTEN becomes cytosolic (Figure 7).

After approximately 60 seconds, the cells adapt and return to their previous state with cytosolic PI3Ks and membrane-localized PTEN, which removes the previously produced PI(3,4,5)P_3. Chemoattractants also induce two phases of actin polymerization: a rapid early peak followed by a later second peak. The heterotrimeric G-proteins, that transduce the presence of the stimulation into the cell interior, remain constantly activated as long as the chemoattractant is present.

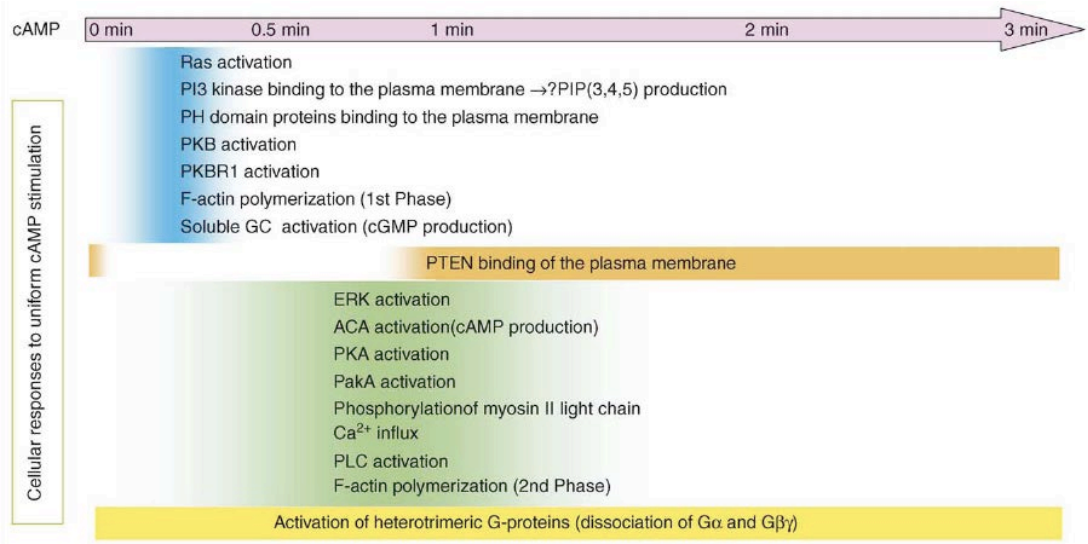

Figure 7: Uniform chemoattractant-stimulation triggers the activation of multiple signaling networks. Following the timescale represented by the pink arrow, most responses are transient and can be classed as early (10–30 seconds, blue shading) or late (30–90 seconds, green shading). From Franca-Koh et al (2006).

However, in a steep chemoattractant gradient, the PI3Ks accumulate at the leading edge of the cell, leading to a persistent enrichment of PI(3,4,5)P_3 at the leading edge, while PTEN is restricted to the plasma membrane of the lateral and trailing edge of the cell (Figure 8). PTEN thereby restricts the diffusion of PI(3,4,5)P_3 away from the leading edge. The importance of locally controlled PI(3,4,5)P_3 levels for chemotaxis are demonstrated by the fact that cells lacking PTEN randomly extend multiple pseudopodia around the entire cell perimeter.

The response of a cell to a chemoattractant gradient can be roughly divided into two phases. The first phase lasts for several seconds and leads to the activation of the gradient machinery and a relatively uniform localization of PI(3,4,5)P_3 along the plasma membrane. During the second phase, the difference in receptor occupancy is amplified, leading to the highly asymmetric distribution of PI(3,4,5)P_3. These patches of PI(3,4,5)P_3

have been shown to be the region of the plasma membrane where pseudopods are preferentially synthesized and extended.

The chemoattractant receptors and their associated heterotrimeric G-proteins maintain a uniform distribution along the cell membrane in stimulated cells. This suggests that the signal amplification occurs downstream of the G-proteins. Although it is not completely understood how the shallow chemoattractant gradient is translated into this highly asymmetric localization of downstream factors, it is becoming clear that two positive feedback loops play a major role in this process. The first feedback loop is actin independent and involves Ras and PI3Ks, while the second feedback loop involves Rho GTPases and actin.

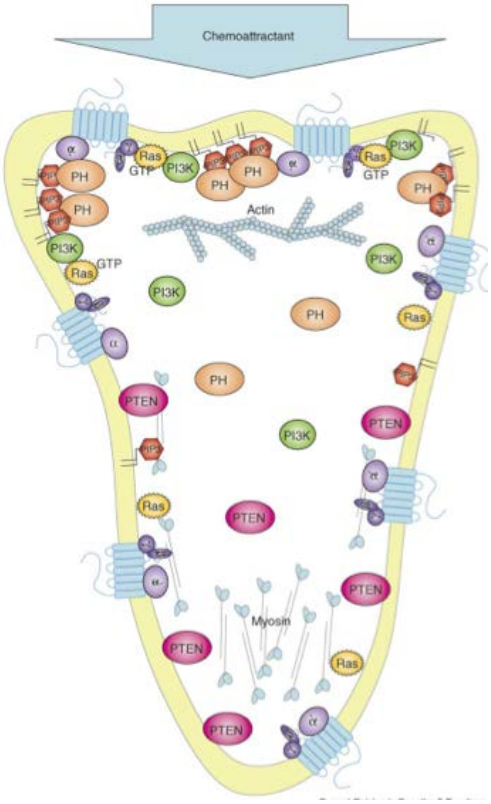

Figure 8: Schematic representation of the spatial localization of key components implicated in chemotaxis of Dictyostelium. Seven-transmembrane chemoattractant receptors (CAR) and heterotrimeric G-proteins (α, β and γ) are evenly distributed along the perimeter of the cell. The receptor occupancy reflects the extracellular gradient of chemoattractant. The small G-protein Ras displays uniform localization along the cell cortex, but its activated form (Ras GTP) is predominantly found at the leading edge. PI3K, PI(3,4,5)P_3, PH-domain containing proteins and F-actin are enriched at the leading edge, whereas PTEN and myosin localize to the lateral sides and posterior of chemotaxing cells. From Charest and Firtel (2006).

Strong PI(3,4,5)P3 asymmetry by positive feedback loops

Cdc42 and Rho subfamily members, characteristic of metazoa and fungi, are absent in the Dictyostelium genome. Rac-like Rho GTPases are however involved in an actin dependent positive feedback loop. The activity of RacB in Dictyostelium parallels the actin polymerization waves

described above. Consistently with the presence of a feedback loop, Rac was shown to be both upstream and downstream of $PI(3,4,5)P_3$ generation (Figure 9).

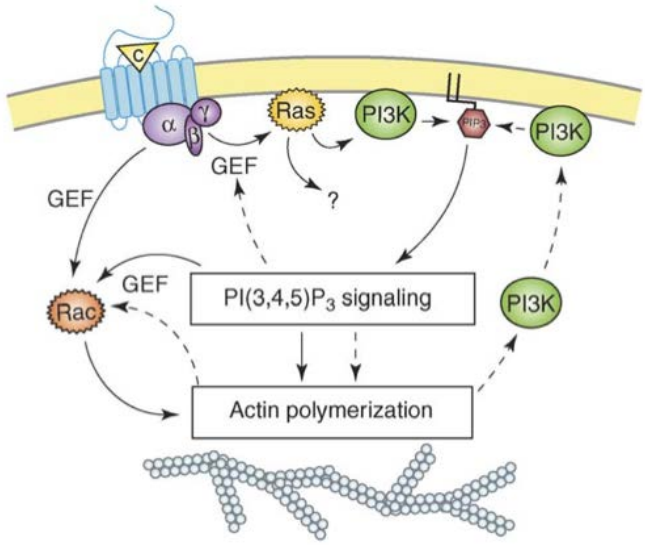

Figure 9: Positive feedback loops amplify the chemoattractant signal to produce a strong $PI(3,4,5)P_3$ response. The small G-proteins Rac and Ras are activated by heterotrimeric G-proteins. Ras activates a membrane-bound pool of PI3K, thereby inducing $PI(3,4,5)P_3$ production. $PI(3,4,5)P_3$ signaling then leads to increased F-actin polymerization, and both events participate in enhancing the Rac activity by promoting the localized activation of RacGEFs. $PI(3,4,5)P_3$ is also believed to increase Ras activation. F-actin assembly then promotes the translocation of cytoplasmic PI3K to the cell cortex, which increases the localized PI3K activity, leading to robust $PI(3,4,5)P_3$ production. The solid lines represent the first signaling events, whereas the dashed lines stand for positive feedback signaling. From Charest and Firtel (2006).

Pre-existing plasma membrane pools of Ras and PI3Ks participate in a positive feedback loop that establishes asymmetric $PI(3,4,5)P_3$ distribution that is enhanced as follows: $PI(3,4,5)P_3$ accumulation at the leading edge recruits PH-containing proteins. Typical PH containing proteins are Rho-GEFs, which might act as regulators of WAVE-complex/WASP dependent actin polymerization. Indeed, the specific intracellular localization of Dictyostelium RacGEF1 was recently shown to be required for Rac activation, and therefore probably also for localized actin polymerization. Actin polymerization was shown to increase the translocation of PI3K to the membrane. Thus, Rac indirectly causes $PI(3,4,5)P_3$ accumulation at the plasma membrane by promoting actin polymerization.

6 – Regulation of Myosin II

In addition to the regulation of actin polymerization by the PI3K/PTEN pathway, the cells also have to tightly regulate the activity of myosin II, which provides the power required to retract the rear of the cell and prevents the formation of lateral pseudopods. The synthesis of cGMP is an essential event triggered downstream of the heterotrimeric G-proteins. Increased levels of cGMP activate myosin-light-chain kinase (MLCK), which phosphorylates MLC and thereby increases the motor activity of myosin II (chapter 4). There are some differences with respect to the molecular mechanisms controlling the activity of myosin II in neutrophils. The

regulation of myosin appears to be downstream of the Rho GTPases, which activate ROCK and subsequently MLCK, leading also to the phosphorylation of MLC. In addition, ROCK has been shown to also inhibit the counteracting MLC phosphatase.

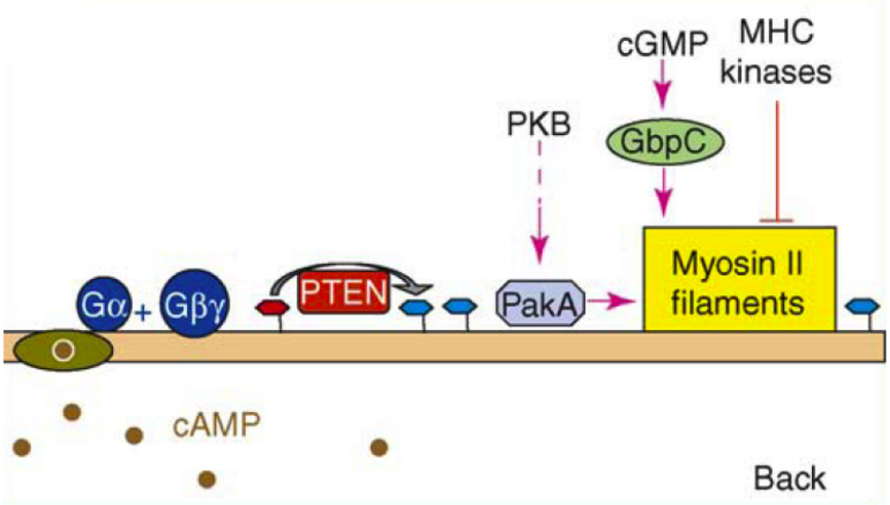

Figure 10: Activation of pathways specific for the rear of chemotactic Dictyostelium cells. This diagram shows the plasma membrane at the back end of the cell, where cAmp levels are not high enough to trigger a sufficient activation of the heterotrimeric G protein signaling. PTEN remains membrane-bound and degrades PIP3 (red to blue symbols), whereas myosin II is assembled into contractile filaments that suppress pseudopod formation and promote retraction of the cell's rear. PakA activity is promoted by Akt/PKB, and the cGMP binding protein GbpC. It promotes the assembly and activity of myosin II, which is antagonized by myosin heavy chain kinases present at the front. From Franca-Koh et al (2006).

Studies performed with Dictyostelium have established that Akt/PKB is involved in maintaining cell polarity as well as proper chemotaxis. The PH-domain containing protein kinase Akt/PKB associates with the leading edge of Dictyostelium cells. Cells lacking Akt/PKB are less polar and show significant defects in their ability to localize PAKa, a homologue of mammalian CRIB domain containing p21-activated kinases, and myosin II to their posterior. Since active PAKa is required for myosin II assembly in the rear of moving cells, it was proposed that the role of Akt/PKB, once it has been translocated to and activated at the leading edge, consists in phosphorylating PAKa. Phosphorylated PAKa translocates then to the posterior part of chemotaxing cells where it promotes myosin II assembly (Figure 10).

7 – References for Figures

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