Chapter 1: Integrating Cytoskeletal Dynamics during Cell Migration

Introduction

Cell migration:

- Netto advancement of a cell relative to a substrate
- In 2-dimensional (Dictyostelium discoideum migrating in leave surface) or 3-dimensional situations (cancer cells moving through tissue)
- Two migration modes:
  - Amoeboid
  - Mesenchymal

The most primitive form of migration is **amoeboid movement**. It occurs in single *Dictyostelium discoideum* cells. Amoeboid migration occurs in cells without mature focal adhesions and stress fibers (weak adhesion) and in rounded cells. There are two submodels:

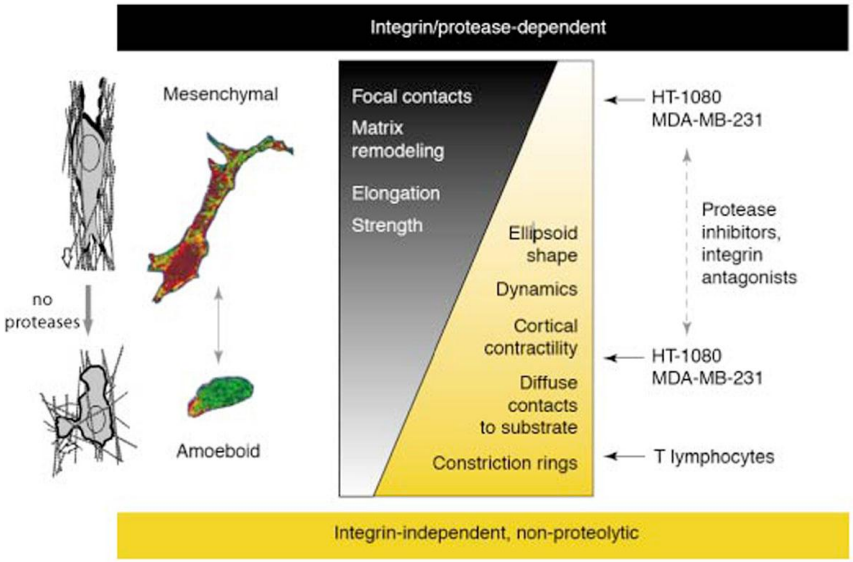

Figure 1: Amoeboid vs. mesenchymal migration of single cells. Migrating mesenchymal HT1080 fibrosarcoma cells and MDB-MB-231 breast cancer cells are spindle-shaped cells that use integrins and proteases for adhesive and proteolytic interactions with the extracellular matrix (ECM) substrate. On the other hand, T lymphocytes maintain migration by integrin-independent mechanisms and do not induce pericellular proteolysis. Between both extremes, cells may also display mixed phenotypes. Abrogating integrin and protease function can result in the reversible reprogamming of the migration type and the conversion from mesenchymal to amoeboid migration pattern. On the very left, cells are depicted surrounded by matrix fibers.

- **Membrane blebbing**: In cells that do exhibit traction on a substrate (strongly reduced adhesion, little matrix rigidity) but advance by pushing the cell content forward. It occurs in many cells such as *Dictyostelium discoideum* and can also be found in cancer cells (switch from actin polymerization dependent protrusion formation to blebbing-driven motility in 3D). Blebs are bulbous, cytosol filled plasma membrane extensions at the leading edge, generated due to hydrostatic pressure increase in the cell. Blebs are formed at a cortex region with inhomogeneities in the actin cytoskeleton and are initially free of actin.

- **Actin-rich filopodia** at the leading edge are found in elongated amoeboid cells. The filopodia engage in a poorly defined, weak adhesive interaction with the substrate (occurring in migrating *Dictyostelium* cells).

Amoeboid migration exists in mammals (stem cells, leukocytes, lymphomas, small-cell lung cancer cells) and is a feature of nonresident cells moving towards tissues.

Mesenchymal migration is a five step process characterized by a strong integrin mediated cell substrate adhesion and myosin II dependent cell contraction (thus stress fiber dependent). F-actin and microtubules are involved in all steps:

1. Cell polarization -> local actin polymerization -> protrusion formation
2. Protrusion membrane contacts ligands of ECM -> integrins cluster and link to substrate -> building up of adhesion sites (initially focal contacts)
3. ECM underlying focalized actin locally degraded (surface proteases) -> focal contacts mature (into focal adhesions) or decay
4. After integrin-ECM binding, actin filaments cross-link with myosin II -> stress fibers (10-30 bundled actin filaments); cellular tension on mature focal adhesions, where myosin II localizes; newly formed focal adhesions serve as back of the leading edge (junction between lamella and lamellipodium) and can be further modified; signaling induced shortening of membrane-anchored stress fibers -> local cell contraction -> advancement of cell body
5. De-adhesion at trailing cell rear is contraction dependent, depends on protease calpain; in 3D, focal contact formation and turnover occurs within 10 to 120 min resulting in a slow migration of 0.1-2 μm/min; other cells subsequently follow the pioneer cell guided by ECM strands

Mammalian integrin-dependent migration over an inert surface: Highly coordinated process, involving cell shape changes and traction depending on cytoskeleton and cell adhesion regulation. Cell adhesion is mediated via transmembrane integrins linking the chemical and mechanical properties of the ECM that is composed of fibronectin, collagen and the actin and microtubule filament system that is connected to the integrin-containing adhesion sites. Migration is regulated:

- Rho GTPase signaling -> actin and microtubule dynamics
- Force development
- Cell adhesion

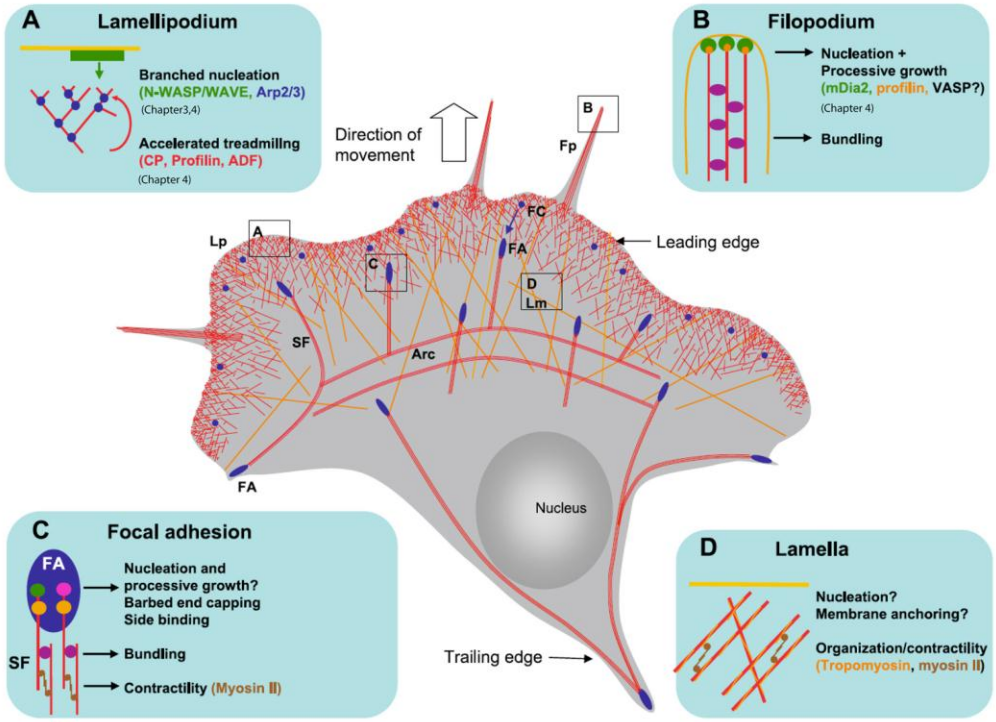

Figure 2: Major actin dependent structures in a migrating cell, lamellipodium (Lp), lamella (Lm), filopodia (Fp), actin networks, focal contacts (round small blue), focal adhesions (FA), and the rear of a migrating cell. New FAs are formed with the control of Rac signaling; their growth is determined 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 mictrotubules and regulators that reside in these complexes. In motile cells, traction on the substrate results in net forward movement. CP, capping protein.

Adhesive Complexes

Adhesion site: Specialized region of the plasma membrane, link between immobile extracellular substrate and cellular cytoskeleton. Certain adhesion sites provide traction -> force generation and are coupled to protrusion as they increase probability of its stabilization and regulate the rate of formation.

In adhesion sites, **integrins** (primary receptors), **actin binding proteins** (e.g.

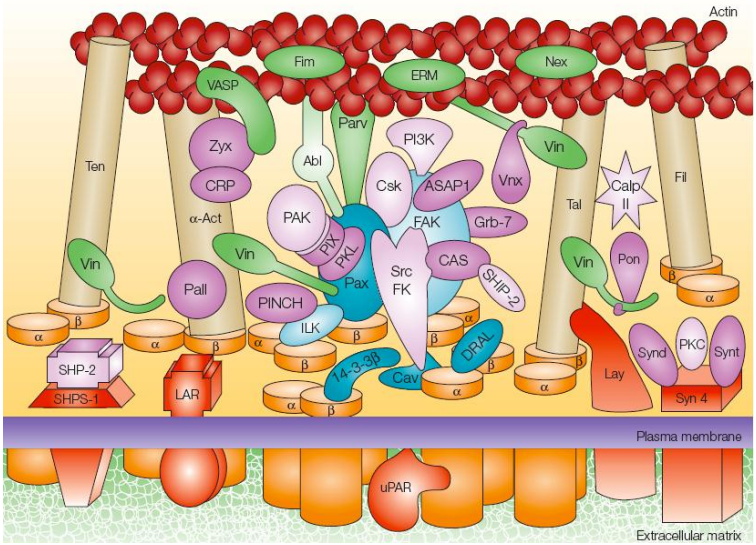

Figure 3: Main molecular domains of cell-matrix adhesions. Orange cylinders -> integrins, red -> additional membrane-associated molecules (Syn4). Protein interacting with actin and integrin (scaffolds) contain actinin), talin -> golden rods. Blue -> FAK, paxillin, integrin-linked kinase (ILK), caveolin). Green -> VASP, Vin. Adaptors, purple -> zyxin, PIX. Enzymes, lighter shades -> PAK, PI3K, Src FK, Csk, PKC.

vinculin), **actin filaments**, and **signaling proteins** (e.g. FAK, focal adhesion kinase) cluster together. Over 100 additional adaptor and signaling proteins comprise the cytoplasmic domain of mature adhesion sites forming numerous links, both direct and indirect, between integrins and actin filaments -> high degree of regulation -> efficient cell migration.

Adhesion disassembly occurs both at cell rear (retraction) and at cell front (formation of new protrusions and adhesions).

Integrins are heterodimeric, composed of α and β subunits, transmembrane proteins, mechanosensors, and physically link ECM to actin cytoskeleton. Under force application, adhesion structures can grow and become stronger. There are two types of adhesion sites (Figure 2):

- **Focal complexes**: Nascent adhesive complexes, small (< 1 µm), dot-like, continuosly formed and turned over under protruding lamellipodia, maturation -> transforming into focal adhesions
- **Focal adhesions**: Older adhesive complexes, even larger elongated structure in cell rear, mature form, dynamic structures, recycle as cells migrate or enter mitosis, grow and extend centripetally, concomitantly with the formation of actin stress fibers, transition linked to GTPase switch from Rac/Cdc42 to Rho and to engagement of myosin II with actin to form contractile bundles (stress fibers). Focal adhesions have two cellular functions:
    - Transmission of force or tension at adhesion sites by maintaining strong attachments to underlying ECM
    - Signaling centers to regulate cell growth, survival and gene expression

Hallmarks of polarized migrating cells:

- **Leading edge**: Small focal complexes, some focal adhesions (fix, traction for migration); strengthening into larger focal adhesions due do tension; anchorage points for actin bundles, retraction of rear localizing near to the lamellipodium-lamella interface (Figure 5); focal complexes under lamellipodia excert much less traction; adhesion turnover determined by molecular composition
- **Rear and flanks of cell**: Large focal adhesions, slide, then disassemble; coupling between integrin and actin is four time reduced compared to front, anterograde contraction decouples integrins from a group of inner adhesion proteins and therefore do not lead to increased traction in the rear adhesions; protease calpain acts by cleaving scaffold talin; calpain is an ERK and therefore Ras regulated

Focal adhesions are highly motile in stationary fibroblasts but stationary in migrating fibroblasts -> molecular clutch coupling traction with contractile forces and actin polymerization. In stationary cells,

focal adhesions move linearly towards the cell center, dependent on actin-myosin contractility exerted on central stress fibers.

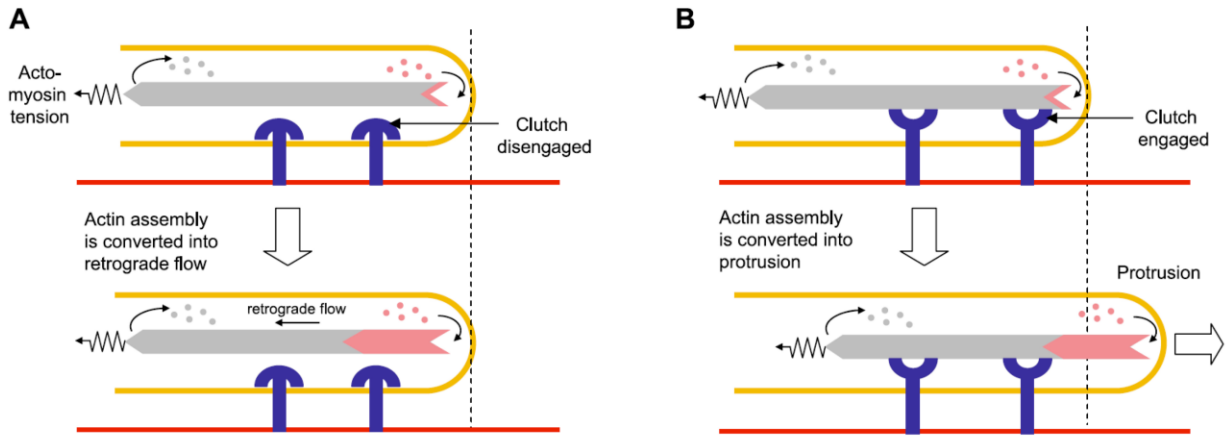

Figure 4: Actin network in grey, newly synthesized actin in pink. Adhesion as molecular clutch, convert actin assembly force into protrusion. (A) Molecular clutch disengaged; no connection between adhesions and actin cytoskeleton. No protrusion as actin treadmilling converted into retrograde flow. (B) Molecular clutch engaged, strong connection between polymerizing actin network and substrate -> membrane protrusion. Connection enables conversion of the acto-myosin tension into traction of the cell body and retraction of the tail.

Actin-Rich Protrusions Generated During Migration

Movement needs **protrusions**. Formation is dependent on regulated polymerization of actin into filaments:

- Actin filaments built from actin monomers
- Sufficiently high monomer concentration -> monomers attach to barbed end of existing filaments (elongation), or form trimeric monomer nuclei (nucleation)
- Right-handed double helical structure, two protofilaments gently twisted around each other with 7nm spacing
- Actin filaments -> F-actin, or filamentous actin
- Monomers -> G-actin, or globular actin
- Most filaments are directly or indirectly anchored in adhesion sites

Migrating mammalian cells generate protrusions to start translocation:

- Finger-like: **Filopodia**, parallel actin filaments
- Sheet-like: **lamellipodium** and **lamella**, leading edge, two distinct F-actin networks

Lamellipodium protrusion and retraction (23s periodicity) and filopodium formation serve an exploratory function and are a rapid response to extracellular cues. Persistent advancement relies on underlying lamella. Lamellipodium and lamella can be distinguished by two main criteria:

1. Molecules diferentially localize to them (molecular signature)

2. Spatial organization of the rates of F-actin assembly and disassembly (kinetic signature)

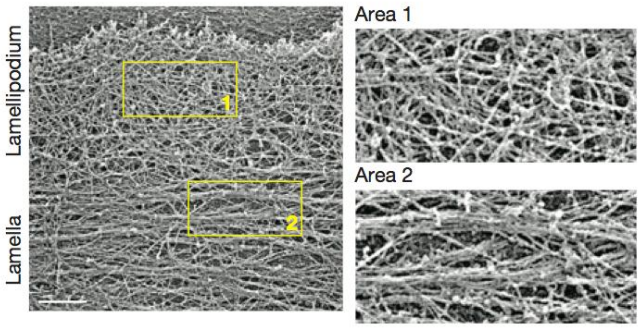

Figure 5: EM of rotary-shadowed PtK1 cell, areas 1 and 2 show f-actin organization in lamellipodium and lamella. Scale bar = 1 µm.

Lamellipodium: Actin filaments rapidly generated and drift cell inward -> treadmilling F-actin array, retrograde flow, high concentrations of Arp2/3 and ADF/cofillin, anterior to lamellar network, 2-4 µm wide leading edge, border characterized by myosin II based contractility anchored at sites of stable adhesion, pulls lamellipodial filaments rearward; if attachment with substrate at extreme cell edge is broken, myosin II activity causes protrusion to move as peripheral ruffle rearward until it reaches stable adhesion sites; there, disassembly occurs; thus, if extreme leading edge remains adhered to the substrate, contraction causes retrograde actin flow; kinetic signature of lamellipodium is characterized by fast F-actin polymerization close to the leading edge, followed by nearly complete depolymerization a few µm back.

Lamella: Spatially random punctae of F-actin assembly and disassembly, slow myosin mediated retrograde flow; contains myosin II and tropomyosin (absent in lamellipodia); area within 3-15 µm from the cell edge, actin foci cycle aphasically between polymerization and depolymerization; myosin II dependent slow retrograde flow (about 0.3 µm/min) defines kinematic signature of actin filaments (might be mediated by tropomyosin).

In the cell body, F-actin undergoes myosin dependent anterograde 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.

Organization of Microtubules During Migration

Microtubules (MT):

- Important role in migration
- Polar filaments, 13 protofilaments, each consisting of a linear array of α/β tubulin dimers
- MT filaments anchored with slow growing minus ends in the microtubule organizing center (MTOC) or spindle pole body in yeast, near nucleus
- Faster growing plus ends are close to the cell cortex
- In most cells, migration is altered by disruption of the microtubule cytoskeleton

Fibroblasts: Best model, little MT, a lot of actin in the protrusion, central MTOC, stress fibers and adhesion sites. In the dynamic dense actin meshwork of a lamellipodium, only a few highly dynamic pioneer MTs are found that indirectly interact with actin filaments. Bursts of rapid assembly enable these MTs to intrude deep into the peripheral lamellipodium, where they are destabilized by retrograde actin flow, leading to rapid MT turnover and constant exploration within the lamellipodium. Near the nucleus, the concentration of microtubules increases and in general exceeds that of actin filaments.

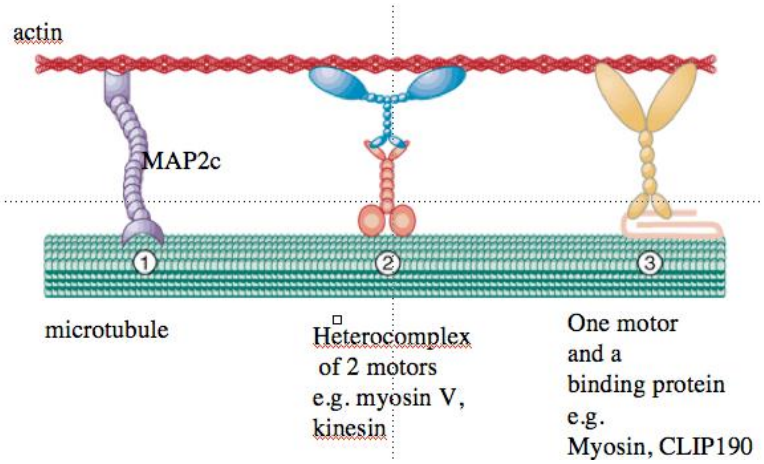

Figure 6: Actin (red) and MTs (green) exhibit static or dynamic interactions. (1) Protein that possesses both actin- and MT-binding sites, static crosslink between the two polymers (MAP2c). (2) Actin-based motor (blue) and a MT-based motor (orange). (3) Motor (yellow) and binding protein (pink). Both types of interaction could move actin and MTs relative to one another, as hypothesized for myosin V and kinesin, or myosin VI and CLIP-190 (*Drosophila*).

Generally, fast migrating cells have fewer actin stress bundles compared to slow migrating cells, which have less focal adhesions. E.g. slow astrocytes (0.1 µm/min) have an extremely long MT-filled protrusion. Fibroblasts migrate with an average speed of 0.6 µm/min. Intermediate speed migration (amoeboid) in neutrophils (3-6 µm/min), no stress fibers, lack of focal adhesions. MTs are important for promoting turnover of focal adhesions, therefore neutrophils are less dependent on MTs. Fish epidermal keratinocytes are rapidly migrating cells (10 µm/min), high turnover of focal complexes (rarely mature into focal adhesions), wide flat lamellipodium and no MT rich regions. Migrate without MTs. Functional link between stress fibers, focal adhesions and MTs. Bacteria and amoeba -> 60mm/min.

Principal effect of MTs is to reduce cellular contractility as adhesions dissociate upon direct contact with MTs. MTs appear to regulate turnover of adhesions by targeting them directly and delivering relaxing signals to promote their disassembly. Feedback mode if increasing mechanical stress at adhesion sites occurs signals the polymerization towards these adhesion sites.

Microtubule Polarity

Plus ends of growing MTs are composed of GTP-tubulin. Constant GTP hydrolysis necessitates that MTs must keep growing to maintain their GTP-cap. Stalled MTs rapidly lose their cap and are susceptible to collapse. Cycles of MT growth and collapse -> dynamic instability. MT associated proteins (MAPs) can stabilize MTs and inhibit complete collapse. Plus-end binding proteins (+TIPs):

- CLIP-170 (cytoplasmic linker protein 170kD)
- APC (Adenomatous polyposis coli, Kar9 in *Saccharomyces cerevisiae*)
- EB1 (end-binding protein 1, BIM1 in S.c.)
- p150 (dynein-dynactin complex)

Functions of +TIPs:

- Regulation of MT plus end dynamics
- Providing MT-to-cortex attachment (necessary for vesicle delivery, force generation and signaling of cortical cell polarity)

Localization of proteins to MT plus ends occurs through one of three ways:

- Kinesin-mediated plus end transport
- Direct or indirect binding to the MT plus end itself

MT filaments can become enriched in cell regions such as newly formed protrusions -> polarized MT array, stabilized MTs. These are characterized by longer half-lives, increased resistance to MT-depolymerizing drugs (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. Dynein-dynactin complex at the plus end of MTs might pull on stabilized MTs at the leading edge by the Rho GTPase Cdc42-GTP and APC, thereby inducing the reorientation of the MTOC and Golgi towards the migration direction. This may facilitate further growth of MTs into the lamellipodium and MT dependent targeted vesicular trafficking.

One function of MTs is the delivery of new membrane material from the TGN to the plasma membrane to allow formation of the protrusions. Consistent with observations that disruption of the Golgi complex (brefeldin A or blocking kinesin motor activity) mimics the effects of MT disruption on the lamellipodial activity in several cell types. MT ends lag behind the rapidly protruding cell edge and even their complete loss in such cells does not impede protrusion formation.

Chapter 2: Rho GTPases

Introduction

ATP:

- Consumed during phosphorylation reactions
- Hydrolysis used to provide energy required to drive other cellular processes (motor proteins)

GTP:

- Hydrolysis mostly used for regulation of signaling transduction by small GTP-binding Ras, key regulator of
  - Cell differentiation
  - Cell proliferation
  - Apoptosis
- Hydrolysis at tip of plus end of MT

Guanine nucleotide binding proteins (GNBPs), belong to diverse groups (heterotrimeric G-proteins, tubulin, septin, dynamin, EF-G, EF-Tu, small GTPases, Ras superfamily, 21kD.

Ras GNBPs are regulators, 5 major subgroups:

- Ras guanosine tripohosphatases (GTPases) -> growth, differentiation, apoptosis
- Rab GTPases -> vesicular transport
- ARF GTPases -> vesicular transport
- Ran GTPases -> nuclear transport and mitotic spindle assemply
- Rho GTPases -> cytoskeleton organization, cell cycle progression, gene expression

GTPases are highly conserved, molecular switches, controlling cellular morphogenesis, movement and cell division. They cycle between GTP-bound and GDP-bound state.

The GTPase Cycle and Prenylation

RhoA, Rac1, Cdc42 -> best-studied Rho GTPases (at least 22 mammalian genes encoding for Rho GTPases). Budding yeast ->

Rho GTPase	Official symbol (human)[b]	Localization	Post-translational modifications
RhoA	RHOA	PM and cytosol [16]	GG and PH [23,106]
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 [9]	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]
Ode4E	CDC42	PM and Golgi [99]	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 7: Rho GTP binding proteins (human). PM -> plasma membrane, GG -> geranylgeranylation, F -> farnesylation. P -> palmitoylation, PH -> phosphorylation.

only about 5 Rho GTPases.

Switch, active GTP-bound state, inactive GDP-bound state (Rnd subgroup constitutively binds GTP). Only GTP-bound GTPases are able to interact with their effector molecules. Intrinsic conversion of GDP-bound form to the GTP-bound form is slow. Activation of GTPases needs the dissociation of protein-bound GDP and loading of GTP.

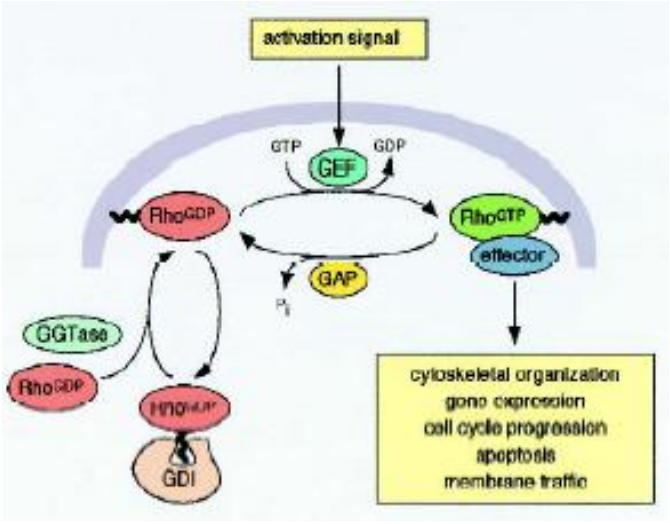

Figure 8: The Rho GTPase switch.

Guanine nucleotide exchange factors (GEFs) catalyze the GDP displacement and stabilize the nucleotide-free GTPase intermediate which leads to accelerated GTP-loading. Effector proteins bind to the GTPase form and elicit downstream events. Signaling involves the hydrolysis of GTP to GDP (irreversible). GTPase reaction is intrinsically very slow, and is accelerated by GTPase-activating proteins (GAPs).

Rho proteins are often posttranscriptionally modified by lipids -> C-terminal CAAX tetrapeptide sequence (Cys, aliphatic, any), recognition sequence for farnesyltransferase and geranylgeranyltransferase I, which cytalyze covalent addition of farnesyl or geranlygeranyl isoprenoid to cysteine residue, AAX removed afterwards, and cysteine methylated. Important for promoting membrane association (critical for biological activity).

Rho GTPases are also regulated by guanine nucleotide dissociation inhibitors (GDIs), not only GEFs and GAPs. GDIs mask prenyl modification and promote cytosolic sequestration -> recycling of Rho GTPases between different membrane compartments. GDIs also block the release of nucleotide. RhoGTPases can also be regulated by direct phorphorylation, transglutamination and ubiquitination -> second layer of regulation of activity, mimicked by bacterial factors. First regulation layer: GEFs, GAPs, GDIs.

The G Box or Guanine Nucleotide Binding Motif

Ras superfamily GTPases share a conserved GDP/GTP binding motif of five elements:

- G1: GXXXXGKS/T (P-loop)
- G2: T
- G3: DXXG
- G4: NKXD
- G5: SAK

-> 20 kDa G-domain or G box, nucleotide binding activity. Conserved structure, shared by all Ras proteins, and by the Gα subunits of G-proteins. 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. GDP- and GTP-bound forms have similar conformations, with the exception of two regions -> switch I and switch II. They have an increased flexibility. GDP-bound form shows large structural variations, GTP-bound not. Trigger for conformational change most likely universal, loaded-spring mechanism. In triphosphate-bound state, 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. The release of γ-phosphate after GTP hydrolysis allows the two switch regions to relax -> conformational change.

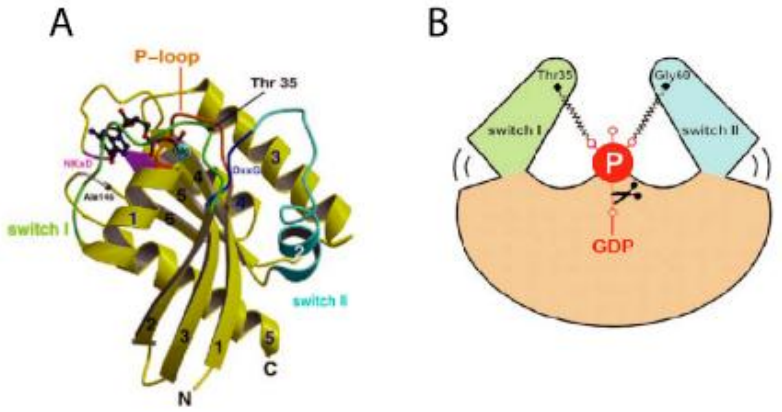

Figure 9: (A) Structure of guanine-nucleotide binding proteins. The Mg^{2+} contributes to the tight binding of nucleotides. (B) Schematic diagram of the universal switch mechanism.

The Functional Domains of Rho GTPases

- **Effector domain**: Changes conformation dependent on nucleotide-status of the GTPase, and is usually required for binding downstream targets
- **Insert region**: Distinguishes Rho GTPases from other Ras related GTPases, confers Rho specific signaling events, binds Rho specific effectors (such as IQGAP1)
- Some Rho GTPases have an N-terminal extension beyond the core structure and/or varying length of the C-terminal hypervariable region
- **CAAX box**: General signal for prenylation and membrane targeting

Many Ras superfamily mutants exist, which either lock GTPases in a GTP- or GDP-like conformation -> experimental tools.

- **Activating mutants**:
  - G12V and Q61 mutant proteins are unable to hydrolyze GTP -> constitutive association with effectors -> continuous signaling.

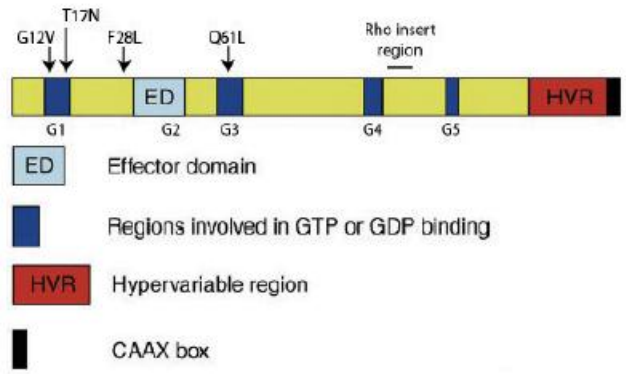

Figure 10: Structure of a Rho GTPase (Cdc42). Used mutants and G box domains are indicated.

- F28L mutants are fast-cycling and have low nucleotide affinity. Cellular GTP levels higher than GDP -> excess of activated GTPase -> increased downstream signaling.

- **Dominant-negative mutation:**
  - T17N mutants have a nucleotide-free conformation. High GEF affinity -> sequestration of cellular GEFs normally required for activation of endogenous GTPase -> activation signal no longer able to cause formation of a GTP-bound GTPase -> dominant-negative effect.

The GEF Reaction

GEFs accelerate guanine nucleotide release:

- GNBP-GDP complex binds GEF -> GNBP-GDP-GEF complex
- GEF activity -> GDP leaves trimeric complex
- GNBP-GEF nucleotide-free stable complex (in absence of nucleotides)

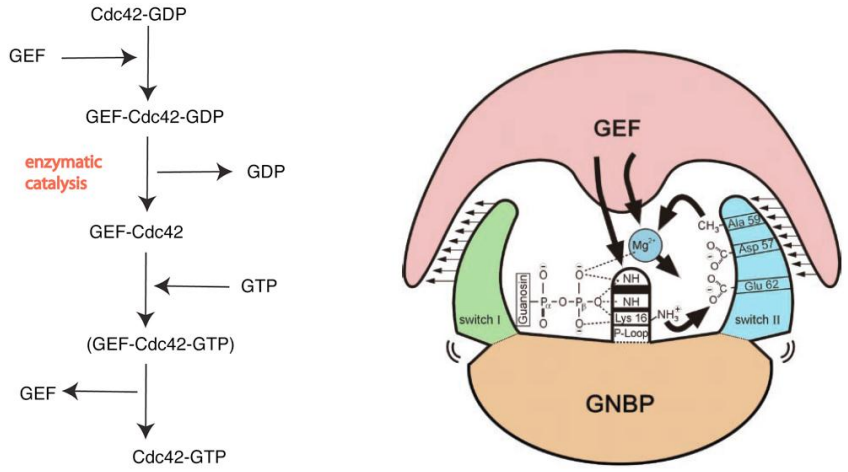

Figure 11: (A) Representation of relevant reaction steps. (B) Diagram of mechanistic principles underlying GEF action. Amino acid numbers refer to Ras GTPase.

The cellular concentrations of GTP are higher than GDP and therefore GTP binds nucleotide-free GNBP (not catalyzed by GEF). GEF acts as catalyst to increase the rate at which equilibrium between GDP- and GTP-bound forms is reached. Position of equilibrium is dictated by affinities of GNBP for GDP and GTP, their intracellular concentrations and the concentration and affinity of other proteins (effectors) -> pulls equilibrium towards GTP-bound form.

Action of GEFs based on common mechanistic principles:

- Interaction with switch I and II regions
- Insertion of residues close to P-loop -> structural changes -> block phosphate binding
- Mg^{2+} ion pushed out of its position by elements of GNBP (Ala^{59}) and GEF residues
- P-loop residues disturbed, lysine reoriented towards invariant carboxylate from switch II (Asp^{57} or Glu^{62}
- Push-and-pull mechanism: Switch I pushed out of normal position, switch II pushed towards nucleotide-binding site -> decrease in affinity of GNBP for GDP

Conventional GEFs

Dbl was the first mammalian GEF identified. Shares sequence similarity with Cdc24 (upstream activator of Cdc42 in budding yeast) -> conserved **Dbl homology domain** (**DH**) constitutes GEF activity. Six DH domain containing GEFs in yeast, 60 in humans. Some GEFs are specific for one GTPase, while others are not. E.g. Dbl activates Cdc42 and Rac, Vav activates Rac and Rho and Fgd1 is specific for Cdc42.

Second shared domain called **pleckstrin homology domain** (**PH**), is found in most DH family members. The PH domain is C-terminal to the DH domain -> interdependence. The 100-120 amino acid domain binds to phosphatidylinositides, which has been shown to positively affect catalytic activity of DH domain in some cases.

GEFs themselves are tightly controlled:

- Exposure of DH domain by relief of intramolecular inhibitory fold (response to phosphorylation)
- Signal-induced interaction with other proteins -> exposition of DH domain
- Alteration of intracellular localization

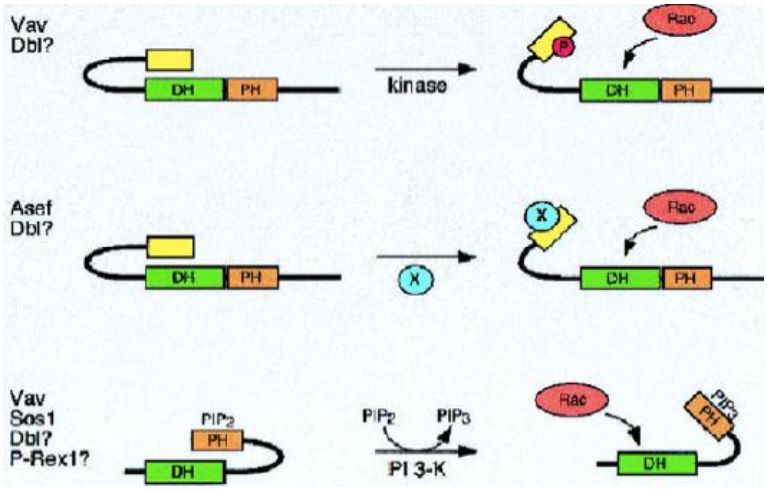

Figure 12: GEF activation through relief on intramolecular inhibitory sequences.

Many GEFs contain regulatory domain that keeps them inactive through an intramolecular interaction. This interaction can be relieved by posttranslational modifications, binding to other proteins or interaction of the PH domain with phosphoinositides. Consistently, N-terminal deletion of Dbl makes it constitutively active.

Subcellular localization of GEFs seems to play an important role. In many GEFs, activation seems to be linked with their intracellular relocalization. PH domain seems to mediate translocation of GEFs to membranes and cytoskeletal structures.

Cell stimulation -> modification of GEF (e.g. phosphorylation) -> oligomerization, increase in strength of interactions -> high affinity interaction between oligomerized GEF and membrane (through phosphoinositide binding, which normally is weak). Affinity of individual interaction not changed, but membrane recruitment results from increased avidity (K_d for monomer -> millimolar range, K_d for tetramer -> picomolar range). Oligomerization of Dbl occurs *in vitro* by a DH domain dependent mechanism. The inactivation mechanisms of GEFs are still unclear. Dephosphorylation, disruption of protein-protein or protein-lipid interactions might play a role. Several proteins have been suggested to act as GEF inhibitors.

Unconventional GEFs

Two groups:

- DOCK180 and zizimin
- Bacterial protein SopE

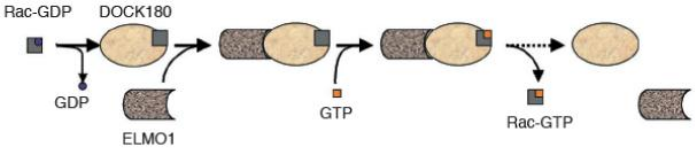

Figure 13: Proposed mechanism of the unconventional GEF composed of DOCK180 and ELMO1.

DOCK180 has a docker domain which binds GTP and is necessary and sufficient to mediate nucleotide exchange *in vitro*. *In vivo*, a second protein, ELMO1, is required for the exchange reaction as well. ELMO1 is important to stabilize (together with DOCK180) the nucleotide-free transition state. The PH domain of ELMO is involved in targeting DOCK180 to the plasma membrane (similar to role of PH domains of conventional GEFs).

Catalytic activity of **SopE**, a bacterial GEF specific for Cdc42 is encoded in a loop of 4 amino acids.

GAP Proteins and the GTPase Reaction

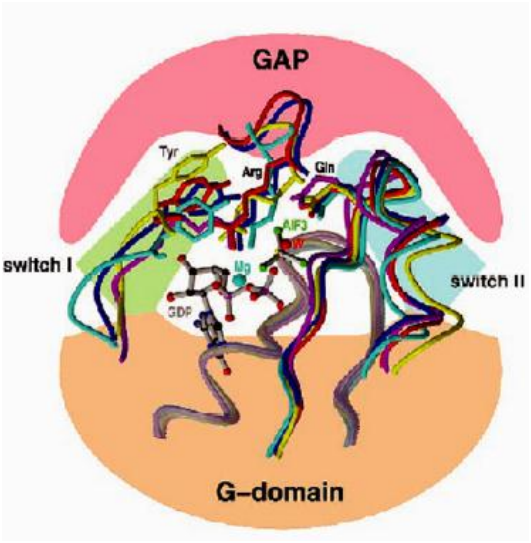

Figure 14: Ras GAP (yellow), Rho GAP (red), Rac-ExoS (cyan), Gia-RGS (magenta) complexes.

Intrinsic GTPase reaction is slow, GAPs accelerate the reaction (e.g. RhoGTPase normally 1700s, with GAP 15 sec). Rho GAPs are multi-domain proteins, and their activity is encoded in the Rho-GAP domain. Active site mostly shows a conserved glutamine residue positioned near the γ-phosphate of the nucleotide. Together with arginine supplied by an arginine finger, this seems to stabilize transition state of GTP hydrolysis which involves a water molecule. There are multiple mechanisms independent of Gln and Arg as well. In humans, about 80 genes encode for Rho GAPs. Inhibition of Rho GAP activity is sufficient to promote the activation of Rho *in vivo*. Examples are Bub2/Bfa1 and Tsc1/Tsc2.

Rho Effectors and the CRIB Domain

Rho GTPases have many different functions: Regulation of actin cytoskeleton, gene transcription, G1 cell cycle control, cytokinesis, microtubule and septin dynamics, and vesicular transport. A large number of potential targets have been identified. Effectors are defined as molecules interacting more tightly with the GTP-bound than GDP-bound form. Most effector proteins undergo a conformational change upon binding to GNBP -> allosteric regulation.

Binding site of effectors involves switch I and often switch II as well. One important effector domain is called **Cdc42/RAC interactive binding** region, or **CRIB**. CRIB binds Cdc42 and/or Rac in a GTP-dependent

manner. Some proteins containing CRIB: Ser/Thr kinases, WASP, N-WASP, IQGAP, PI3K… Binding specificities can vary between different CRIB domains. IQGAP1 binds both Cdc42 and Rac, while WASP/N-WASP only binds Cdc42.

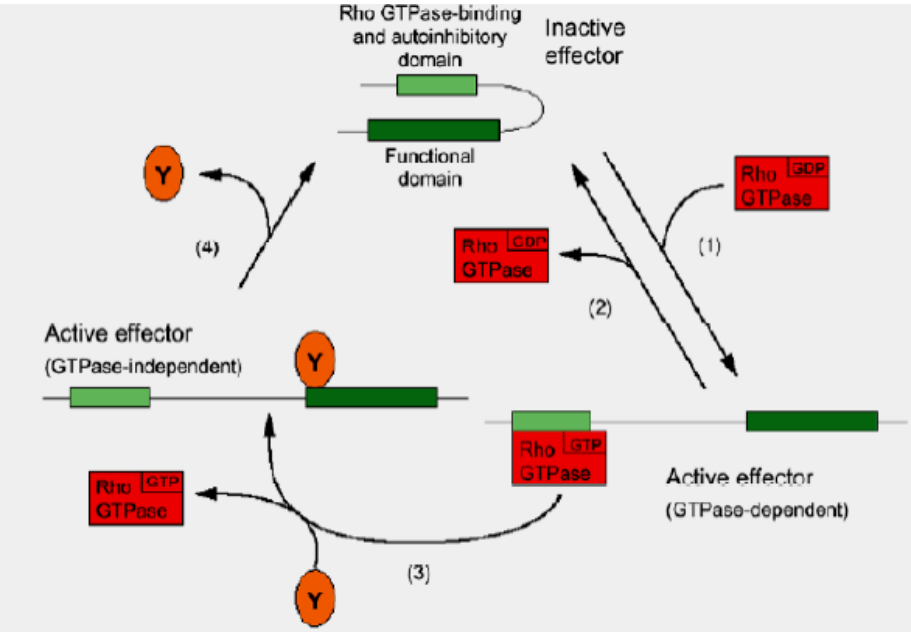

Figure 15: Activation of effector proteins by Rho GTPases (e.g. demonstrated for PAK and N-WASP).

Mechanism of effector activation by Rho GTPases seems to be disruption of intramolecular autoinhibitory interactions -> exposition and activation of functional protein domains within effector. CRIB domain can bind a functional domain of effector proteins, keeping them in an inactive state. Upon binding of GTP-bound GTPase to the CRIB, competition occurs, and effector opens up, rendering the functional domain accessible. Bound GTP is hydrolyzed, GDP-bound GTPase dissociates from effector that can return to its closed state.

Analysis of Rho GTPase Function *in vivo*

Approaches:

- Overexpression of dominant-negative Rho mutants
- Donwregulation of Rho GTPases by RNAi
- Inactivation of Rho GTPases by bacterial toxins

Some bacterial cytotoxins evolved directly to target Rho GTPases in mammalian host cells. Many are enzymes that modify eukaryotic targets (irreversibly) in a catalytic manner -> high potency.

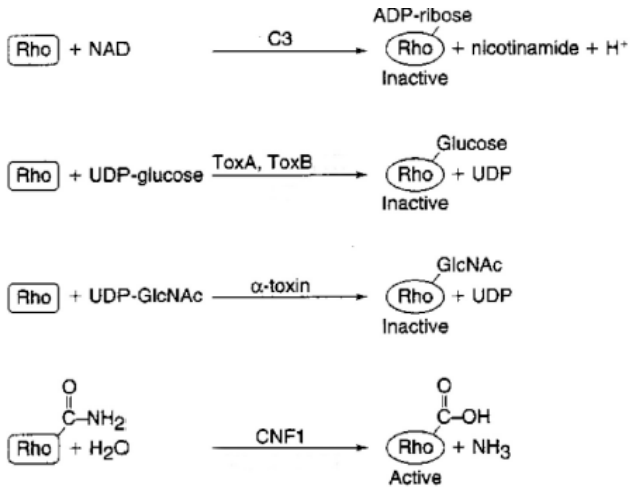

Figure 16: Chemical reactions of selected bacterial toxins.

Irreversible inhibition is achieved through covalent modification by glucosylation, N-acetylglucosaminylation, ADP-ribosylation or proteolytic cleavage. Most toxins inhibit Rho GTPases, but CNF1 deamidating-factor of *E. coli* is able to activate them.

Unresolved experimental challenge in the analysis of *in vivo* activation patterns of Rho GTPases. Overexpression of used high affinity effector domains interfere with endogenous GTPase cycles, and modify normal steady state equilibrium of GDP-GTPase to GTP-GTPase:

- Intracellular localization of WASP derived CRIB-GFP fusion protein provides indication with respect to where Cdc42 has been activated.
- Fluorescence resonance energy transfer, FRET, probing the activation of GTPases. Intramolecular FRET probe for Ras activation comprises Ras and Ras-binding domain (RBD) of Raf sandwiched between a pair of GFP mutants, YFP and CFP. For plasma membrane localization, the probe is fused to farnesylation consensus site. Upon stimulation, GDP on Ras exchanged for GTP, interaction of Ras-GTP with RBD occurs, fusion protein folds and brings two fluorophores together, FRET fluorescence detectable. Ras activity measured by FRET, where GTPase is fused to YFP and GTPase binding domain to CFP.
- Antibodies specific for conformations, via generation of single chain variable fragments (scFvs). Minimalist antibodies -> variable domain of regular antibody heavy chain linked by a flexible polypeptide linker to a light chain variable domain followed by various tags. Gene encoding for a selected scvF is known, direct expression tagged with e.g. GFP.

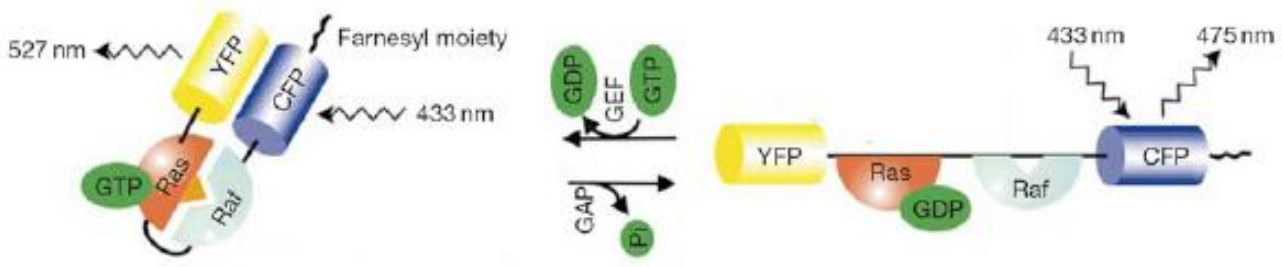

Figure 17: Basic structure of a GFP-based FRET probe for Ras GTPase.

Chapter 3: Rho GTPases, Key Regulators of Actin and Microtubules

Introduction

Constitutively activated 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), when introduced into fibroblasts. Cdc42 was shown to promote formation of actin-rich, finger-like membrane extensions (filopodia). Rho, Rac and Cdc42 link different signals to the assembly of distinct filamentous actin structures in a wide variety of mammalian cells, as well as in yeast, flies and worms. Besides actin cytoskeleton regulation, Rho GTPases are also linked to regulation of adhesion, gene transcription, G1

cell cycle progression, microtubule dynamics, regulation of vesicular transport and a variety of enzymatic activities (from NADPH oxidase in phagocytes to glucan synthase in yeast).

Diverse membrane receptors and upstream regulators activate Rho GTPases and a huge list of cellular target proteins can interact with an individual GTPase. For RhoA, Rac1 and Cdc42, over 60 targets have been experimentally identified so far.

Signals Causing the Activation of Rho GTPases

G-protein coupled serpentine receptors, receptor tyrosine kinases or integrins can activate Cdc42, Rac1 and RhoA:

- Fibroblasts: Addition of LPA (lisophosphatidic acid) induces the formation of actin stress fibers. This Rho-dependent response can be blocked by microinjection of C3 transferases
- Growth factors (PDGF, insulin, bombesin) stimulate polymerization of actin to induce lamellipodia formation and surface membrane ruffling that can be inhibited by overexpression of dominant-negative Rac-N17
- Bradykinin causes activation of Cdc42 via a bradykinin receptor in the plasma membrane

Bradykinin, LPA and bombesin receptors -> seven transmembrane domain heterotrimeric G-protein coupled receptors. PDFG and insulin receptors -> Receptor tyrosine kinases. The ECM component fibronectin causes the activation of integrin receptors and thus influences Rho mediated signaling.

Rho GTPases and Actin Dynamics

- **Cdc42-GTP** causes formation of focal complexes and actin polymerization for **filopodia**; Cdc42 also regulates plasma membrane polarization and directional movement
- **Rac-GTP** promotes formation of focal complexes and actin polymerization causing **lamellipodia**
- **Rho-GTP** promotes actin-myosin contractility, and the formation of **stress fibers** and focal adhesions

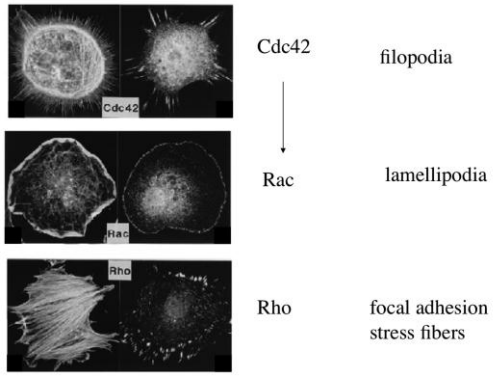

Figure 18: Cdc42 -> overexpression of GEF Fgd1; Rac -> Constitutively active mutant Rac G12V; Rho -> LPA addition; left stained with actin, right with vinculin (marker for focal complexes/adhesions. Cdc42 activates Rac.

Activation of Cdc42 leads to the sequential activation of Rac and finally Rho -> coordinated, hierarchical control of cell motility.

Arp2/3 and **formins**: Rac and Cdc42 are able to induce formation of two actin rearrangements, namely lamellipodia (highly crosslinked meshwork) and filopodia (long parallel bundles). They are induced by two distinct Rho effector groups:

- **WASP** and **Scar/WAVE** activate the Arp2/3 complex, which promotes **lamellipodia** formation
- **Formins**, such as Diaphanous, trigger parallel actin bundles as in **filopodia**

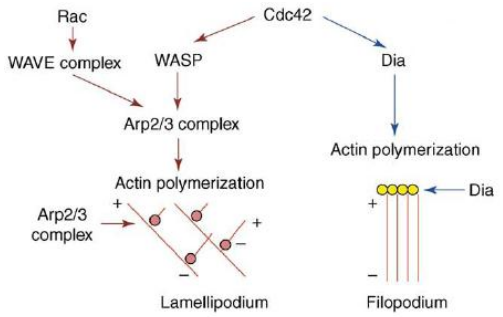

Figure 19: Rho GTPases, actin nucleators and actin dynamics.

WASP, N-WASP and Scar/WAVE all contain a CRIB domain, and are inactive while being in an auto-inhibited conformation.

ADF/Cofilin: Rho proteins regulate ADF/cofilin (actin depolymerizing factor). ADF/cofilin exhibits actin filament depolymerizing and severing activity. Activation of Rho GTPases induces actin polymerization via Arp2/3 and formins, and stabilizes filaments by inhibiting depolymerization by ADF/cofilin. On the other hand, ADF/cofilin activity is needed for the generation of new polymerization-competend ends by filament severing and provides actin monomers (by depolymerization) for filament extension at the leading edge.

Rho GTPases activate two different kinases:

- **ROCK**: Rho-associated serine/threonine protein kinase, exists in two isoforms, ROCK1 and ROCK2
- **PAKs** (1-3): p21 activated kinases, highly conserved family of ser/thr protein kinases with CRIB domain, they bind and become activated by Rac-GTP and Cdc42-GTP

Both ROCK and PAKs:

- Diverse cellular functions in addition to cytoskeletal organization, e.g. modulation of gene expression, control of cell division, and have been linked to cell transformation and pathologies
- Phosphorylate and activate LIM kinases, which have LIM domains, highly conserved cysteine-rich structures containing 2 zinc fingers, and directly phosphorylate ADF/cofilin and inactivate it

Inhibitory phosphorylation of ADF/cofilin is counteracted by several phosphatases, including recently described Slingshot.

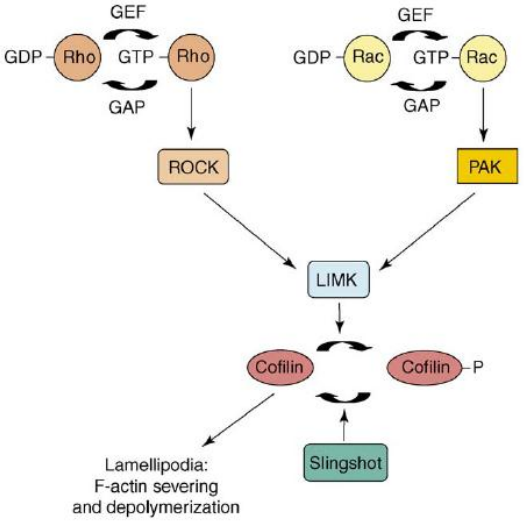

Figure 20: Regulation of cofilin by Rho and Rac. ROCK1/2 and PAK1-3 phosphorylate and activate LIMKs (1 and 2), which in turn phosphorylate and inactivate ADF/cofilin. Cofilin is dephosphorylated by the phosphatase Slingshot. Unphosphorylated cofilin stimulates severing and depolymerization of F-actin in lamellipodia.

Myosin II: Consists of two heavy and two regulatory light chains, and is activated by phosphorylation of the light chain. In non-muscle cells, myosin II exists in two

conformations:

- Tail domain of heavy chain folded and interacting with head -> no self-assembly to filaments
- Myosin light chain is phosphorylated, the backfolded conformations opens up, the myosin tail is elongated and in addition the actin binding site in the myosin head domain becomes exposed -> spontaneous self-assembly into bipolar filaments of up to 20 molecules for cellular contraction

ROCK also promotes contractility and thus influences cell migration. Activated ROCK increases myosin activity by:

- Phosphorylation of myosin light chain (MLC)
- Inactivation of MLC phosphatase

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

This downstream effect of Rho has to be inhibited at the leading edge, since it is incompatible with formation of membrane protrusions. Rac, via downstream PAK kinases, might phosphorylate MLCK (MLC kinase), causing its inhibition, which reduces levels of myosin phosphorylation at the front of the cell.

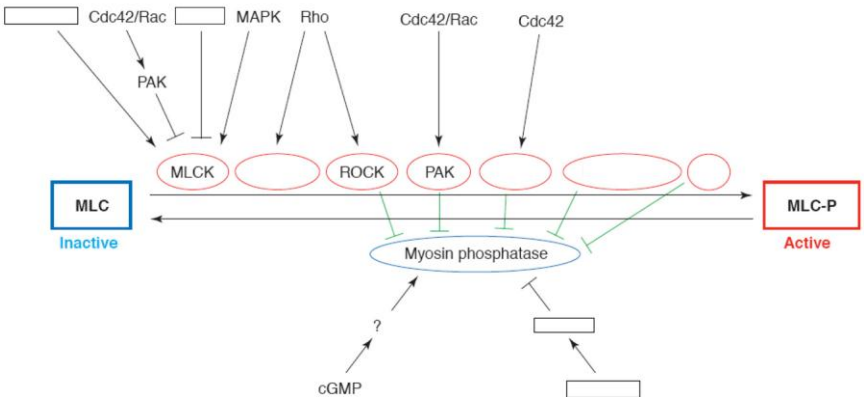

Figure 21: Multiple MLC kinases and a single phosphatase are regulated by a variety of upstream molecules. Positive regulators of MLC phosphorylation also promote phosphorylation by inhibiting myosin phosphatase.

Rho GTPases Linking Actin and Microtubule Dynamics

Migration needs spatial and temporal regulation of actin and myosin. Rho GTPases -> actin cytoskeleton arrangements:

- Cdc42-GTP -> filopodia, focal complexes
- Rac-GTP -> lamellipodia or ruffles at leading edge, focal complexes
- Rho-GTP -> Stress fibers, focal adhesions

Stress fibers assemble from the actin-filament network behind the lamellipodium boundary. Transition from focal complexes to adhesions is stimulated by Rho activation. MT dynamics has direct impact on activity of Rho and Rac.

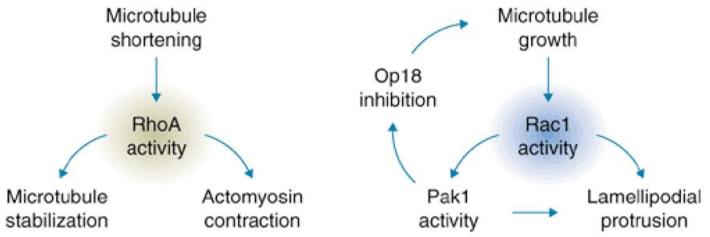

Figure 22: MT disassembly stimulates RhoA activity. Rho stabilizes MT through the formin mDia and also results in actomyosin contraction through stimulation of Rho kinase, resulting in myosin light chain phosphorylation. MT growth stimulates Rac1 activity which mediates actin polymerization and lamellipodial protrusion, and promotes further MT growth through activation of the Pak1-Op18/stathmin signaling pathway to generate a positive feedback loop.

Rho GTPase activity influences MTs, actin and adhesions:

- RhoA activation causes activation of mDia (formin) that generates the specialized stable MTs and orient them towards leading edge. RhoA-GTP also stimulates actin-myosin contractility via ROCK cascade, leading to MLC phosphorylation. Increased contractility results in bundling of actin filaments that form stress fibers, which then cluster integrins and their associated proteins into focal adhesions.
- Op18/stathmin is an important phosphorylation-controlled regulator of MT dynamics. It plays a crucial role in cell division and proliferation. MT polymerization is inhibited by non-phosphorylated stathmin. Two stathmin molecules form a tight complex with one tubulin monomer, which therefore can no longer contribute to MT polymerization. Phosphorylation of stathmin relieves this inhibition. Stimulation with EGF results in phosphorylation of stathmin in a Rac1- and Pak-dependent manner:
    - Constitutively active Rac1-Q61L promotes Pak-dependent MT growth and turnover
    - Pak mediates lamellipodial actin polymerization and retrograde flow downstream of Rac1-Q61L

-> Coordinated regulation of MT and actin dynamics occurs in leading edge of migrating cells.

MTs in turn modulate Rho GTPases and thereby control actin dynamics:

- MT growth induces Rac activation, which in turn promotes lamellipodia
- Disassembly of MT results in activation of Rho; GEFs can interact with MT and can form a regulated link between MT and actin, e.g. GEF-H1 was shown to be enzymatically inactive towards Rho when bound to MT (upon depolymerization, GEF-H1 generates Rho-GTP, which leads to activation of myosin II, stress fiber assembly and regulated gene expression)

Rac and Rho activities are interconnected in a reciprocal fashion:

- Rac dependent focal complexes require contraction of actin-myosin network (independent of ROCK); decrease of activated Rac -> loss of focal complexes and growth of adhesions

- Integrin-signaling causes biphasic Rho response
    - Initially (10-30 min) Rho GAP becomes activated mostly due to β1-integrin signaling; this prevents the accumulation of Rho-GTP that would inhibit the Rac dependent formation of an actin network
    - In a second phase (45 min), Rho-GEF gets activated in response to non-β1 integrins leading to Rho-GTP generation; Rho-GTP causes the formation of the needed stable adhesion and stress fibers to promote cell translation

Reciprocal regulation of Rac and Rho -> key element in cell migration. Rac active at leading edge -> new protrusions and initial adhesions. Rho stabilizes adhesions and causes cellular tension.

IQGAP1 in Migration

IQGAP1: Scaffold, linking actin to MT, pivotal role in control of cell-cell adhesion and migration, processes based on cell polarization. Known as an effector of the Rho GTPases Rac1 and Cdc42, however it might also possess GEF activity if phosphorylated and bound to unknown protein. So, phosphorylated IQGAP1 seems to act as a GEF and non-phosphorylated IQGAP as an effector.

It was identified as an IQ domain-containing protein with a region containing sequence similarity to Ras GTPase-activating proteins. It was never shown to exhibit GAP activity. IQGAP1 is a 190 kDa homodimeric protein widely expressed among vertebrate cell types from embryogenesis to adulthood. Present in *S.c.* (Cyk1/Iqg1) and *S.p.* (RNG2) but not in *Drosophila*. Sequentially arranged domains enable IQGAP1 to bind a spectrum of cytoskeletal, adhesion and regulatory proteins. It accumulates at the dynamic actin-rich cell cortex, such as newly formed leading edges and cell-cell junctions.

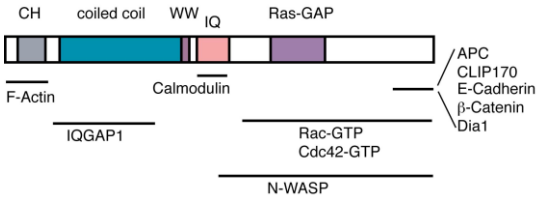

Figure 23: IQGAP1 (human) domain structure. Calponin homology domain (CH), poly proline protein-protein domain containing two conserved Trp residues (WW), IQ motif, Ras GTPase activating protein like domain (Ras-GAP). It was shown to regulate E-cadherin dependent cell-cell contacts.

Cortical capture sites: Links between the plus ends of MTs 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 seem to exhibit an intrinsic mechanism that seems to stochastically generate local accumulation of these GTPases, which drives movement into a randomly chosen direction.

In fibroblasts, activated Rac1/Cdc42 forms a tripartite complex with IQGAP1 and CLIP-170 enhancing interaction of these two and capturing CLIP-170 at the leading edge and the base of filopodia. IQGAP1-

CLIP-170 complex is targeted to the cortical capture sites, leading to formation of polarized and stabilized MT and enhancing cell polarization. MT stabilization follows the localized induction of actin polymerization and is thus secondary.

In addition, Cdc42/Rac-GTP forms a tripartite complex with IQGAP1 and APC. 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 +TIP protein APC seems to connect via IQGAP1 to the actin cytoskeleton in the leading edge.

Polarizing actin and MT 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 by binding to its CRIB region; a mechanism similar to Cdc42-GTP. Thus, IQGAP1 seems to anchor APC to cortical actin sites and also captures the plus end of MT through CLIP-170. By this, the cortical actin sites become reinforced and MTs stabilized. Alternatively, high levels of IQGAP1 influence cell motility positively by increasing the levels of active Cdc42 and Rac1 at the leading edge of migrating cells as they bind to IQGAP1 and become sequestered. IQGAP1 not only positions the plus ends of MT but also the activity of Dia1 (formin). The removal of an autoinhibition, triggered by Rho-GTP, causes the activation of the formin and thus unbranched actin polymerization. A very C-terminal region of IQGAP1 interacts with the active form of Dia1. 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. Thus, IQGAP1 influences two potent actin nucleator systems and MT.

IQGAP1, a scaffolding GEF?

Dia1, CLIP-170 and APC all bind to the same region in IQGAP1. It is thus unlikely that all three proteins are simultaneously bound to IQGAP1. One possibility for

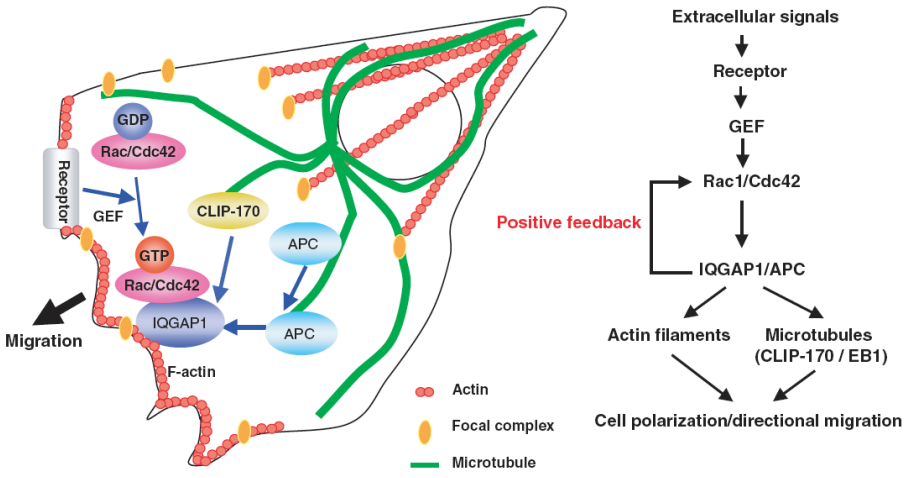

Figure 24: Role of IQGAP1-APC complex. Signals activate Rac1 and Cdc42 through their receptors and certain GEFs at leading edges. Activated Rac1 and Cdc42 induce the polymerization of actin filaments through their effectors and also mark spots where IQGAP1 crosslinks actin filaments. There, APC is recruited through IQGAP1 to actin filaments. IQGAP1 catches the plus-ends of MT through CLIP-170. APC then stabilizes MT. IQGAP1 can also activate N-WASP independent of Cdc42-GTP and therefore polarize actin polymerization.

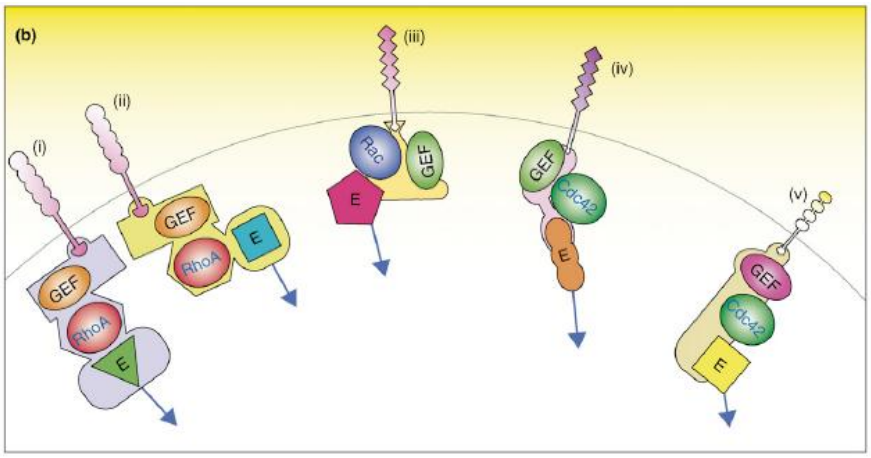

Figure 25: (i, ii) Binding to a different scaffold would enable one receptor to signal through the same GEF and GTPase to different effectors. (iii, iv) The same Rho-GEF could activate a different GTPase and thus different effectors by interacting with a different receptor-scaffold complex. (v, vi) Through 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.

regulation 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 GEFs. By this mechanism, several different functions of IQGAP1 could be simultaneously used in a single cell.

Chapter 4: Regulated Actin Assembly Controls Migration

Introduction

Cytoskeleton: Cellular scaffold, dynamic structure, functions in intracellular transport, cell division and movement and maintenance of cell shape. In eukaryotes, components include actin filaments, intermediate filaments, microtubules and septins. Whereas microtubules and actin filaments are polarized, intermediate filaments are not. Septins (also GTP binding) assemble into homo- and heterooligomers and non-polar filaments (function during cytokinesis).

In bacteria, shape control is mediated by actin homologs MreB and Mbl. Crescentin (*Caulobacter crescentus*) is the only relative to eukaryotic intermediate filaments found in bacteria. FtsZ forms filaments like tubulin.

Actin family: Diverse, ancient group, actin, Hsp70 and sugar kinases belong to a protein family defined by a similar protein fold, where ATP hydrolysis or phosphate transfer causes a large conformational change. Cells additionally contain actin-related proteins (ARPs), and some play well-characterized roles in cytoskeletal processes (actin polymerization -> Arp2/3, cytoplasmic dynein motor activity -> Arp1).

Molecular Basis of Actin Treadmilling

Actin monomer assembly -> right-handed double helical structure, two protofilaments constitute one actin filament. Each monomer has an intrinsically asymmetric structure, a polarity, with one **pointed** (or

minus) and one **barbed** (or **plus**) end. Monomers can be added to both sides of a filament, but at different rates. Monomers can exist in an ATP-bound (T-form) or an ADP-bound conformation (D-form).

Critical concentration: Concentration of monomers above which actin monomers will polymerize and below which actin filaments will depolymerize. Polymerization rate depends on monomer concentration.

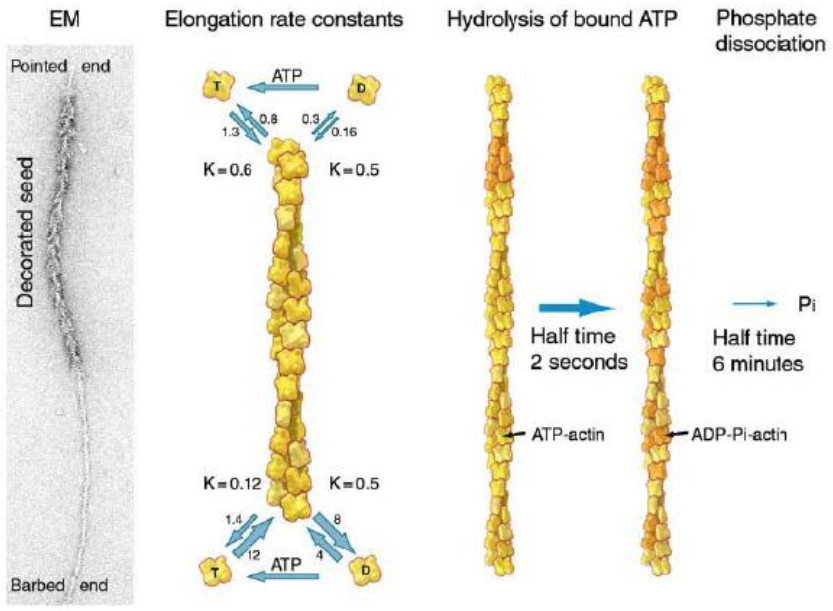

Figure 26: The kinetics of actin filament elongation.

A balance is reached between filament length and free monomers, and filaments enter a steady-state phase, undergoing constant de- and polymerization while maintaining a constant length -> treadmilling. On average, the filaments polymerize at their barbed ends and depolymerize at the pointed ends. ATP is constantly hydrolyzed and the system operates far from a thermodynamic equilibrium. Adding additional actin monomers and filaments will not change the concentration of free actin monomers, but leads to an increase in the total length of the actin filaments.

Important actin monomer concentrations:

- C_{SS}: Steady state concentration (=above 0.12 µM and well below 0.6 µM)
- C_C^B: Critical concentration at the barbed end (= 0.12 µM)
- C_C^P: Critical concentration at the pointed end (= 0.6 µM)
- -> ATP constantly added to barbed end
- $C_{ss} = k_{off}/k_{on} = (1.4s^{-1}+0.8s^{-1})/(12\mu M^{-1}s^{-1}+1.3\mu M^{-1}s^{-1}) = 0.16\mu M$

ADP-actin dissociates faster than ATP-actin from the barbed ends. Both ADP- and ATP-actin dissociate slower from the pointed ends than barbed ends. After T-form monomers have been incorporated into the barbed end, they hydrolyze ATP ($t_{1/2}$=2 sec) and phosphate dissociates ($t_{1/2}$=6 min) -> timer, indicating age of the filament, reduces binding strength between neighboring subunits and leads to dissociation of the D-form at the pointed end. Taken together -> steady state, ATP-actin associates at the barbed end and ADP-actin dissociates from the pointed end -> treadmilling. Length of filament stays the

same, but filament is getting displaced. If pointed end dissociation is blocked, no growth at the barbed end occurs (because C_{SS} would be equal to C_C^B -> no tradmilling).

In vitro, treadmilling is very slow and limited by dissociation of ADP-actin at pointed end or association of ATP-actin at barbed end. Dissociation at the pointed end -> $0.3s^{-1}$, association at barbed end -> $k_{on}^B*(C_{SS}-C_C^B) = 0.48s^{-1}$. Treadmilling rate -> 0.05 µm/min.

But keratinocytes and motile bacteria can move much faster (200x, 10 µm/min). Explain discrepancy?

Regulation of Actin Treadmilling

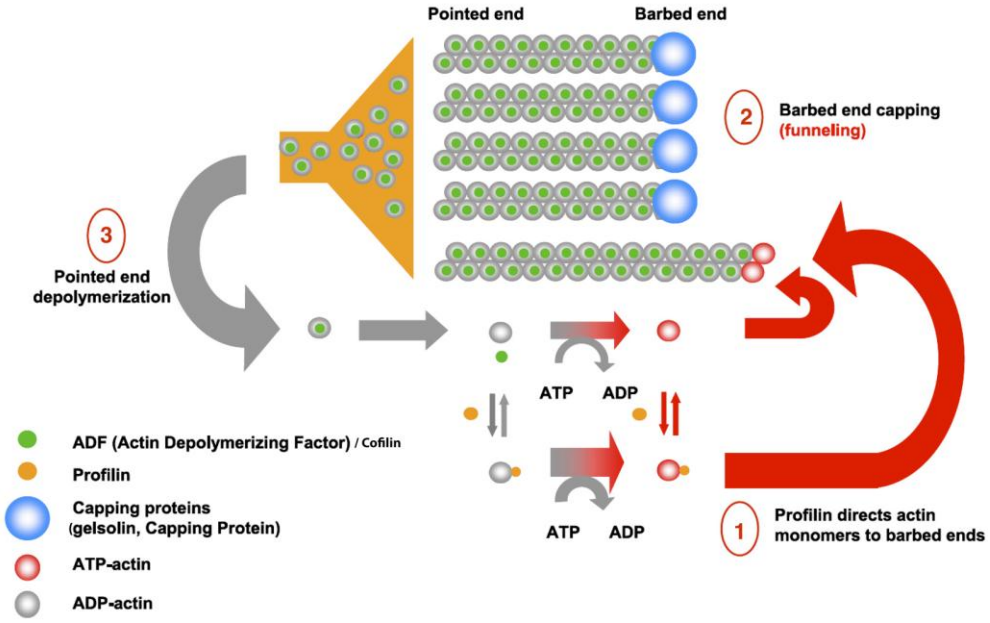

Figure 27: 1) Profilin enhances the exchange of ADP for ATP to recycle actin monomers. Profilin-actin complex assembles exclusively at the barbed end. 2) Blocking majority of actin filament barbed ends, capping proteins increase the concentration of monomeric actin at steady state and funnel the flux 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.

Higher treadmilling rastes need an increased dissociation rate of ADP-actin at the pointed end and increased association rate of ATP-actin at the barbed end. ADF/cofilin influences the pointed end depolymerization rates.

How do cells maintain a pool of unpolymerized actin? Monomeric actin concentrations measured in cells can be as high as 100 µM, so most monomers are sequestered by actin monomer binding proteins. Elongation of filaments can be controlled at the level of monomers and/or filaments. First, proteins bind monomeric actin and modify its polymerization competence and second, capping proteins bind filaments thus preventing monomer addition.

Actin monomer binding proteins: Profilin and thymosin. **Profilin** is conserved in all eukaryotes and found in fungi and protozoa. **Thymosin** binds preferentially to ATP-actin and prevents monomers from polymerizing. Thymosin-ATP-actin complexes do not bind to filaments, in contrast to profilin. Thymosin therefore provides a reservoir of monomeric actin, which can be assembled into a filament after thymosin has been displaced by profilin.

Profilin:

- Regulates nucleotide-status of actin by stimulating exchange of ADP for ATP
- Profilin-actin-ATP complex is added to barbed ends, profilin released
- High affinity of profilin-actin-ATP complex for barbed ends -> stimulation of polymerization of filaments, affecting the rate of actin elongation by raising k_{ON}^{B} of ATP-actin
- Competes with thymosin in the binding of ATP-actin, profilin can shuttle actin monomers away from thymosin onto barbed filament ends, where they polymerize; profilin maintains a pool of ATP-actin that is ready to elongate free barbed ends and promotes their polymerization; the specificity to barbed end assembly gives directionality to filament polymerization

Capping protein: If all barbed ends were polymerization competent, the pool of free monomers would be rapidly depleted. Capping protein binds to barbed ends tightly and has a very slow dissociation rate ($t_{1/2}$: 1000s) which means that filaments can no longer elongate unless they are actively uncapped or severed. In a cell, most of the ends are capped. These filaments cannot undergo treadmilling and are therefore slowly degraded from the pointed end, providing new monomers. Together with profilin and thymosin, capping protein ensures the maintenance of a pool of ATP-actin monomers for the elongation of uncapped filaments. Capping increases C_{SS} because k_{off}^{P} stays the same while the effective k_{on}^{B} gets smaller (fewer filaments with free barbed end). Thus, the limited non-capped filaments grow faster. Capping protein contributes to actin-driven motility:

- It limits the length of filaments (short filaments -> generate more force)
- It biases the treadmilling towards a restricted number of filaments

At the pointed end, no significant elongation takes place, so no capping is actually needed there. However, it has been shown that (based on the cellular concentrations of Arp2/3) most are capped.

ADF/cofilin: Two names -> same protein. Conserved, two biological effects:

- Enhances depolymerization of actin filaments by severing them and creating more pointed ends
- Increases the rate of subunit dissociation from pointed ends

It preferentially binds polymerized ADP-actin subunits, which has been shown to induce structural changes, thereby promoting subunit dissociation. Thus, ADF can perform its function only after the time-dependent hydrolysis of ATP-actin and the subsequent dissociation of phosphate.

ADF/cofilin and profilin synergize in enhancing treadmilling. ADF/cofilin also accelerates pointed-end depolymerization, thereby increasing C_{SS}. Profilin catalyzes the regeneration of T-form actin monomers, and targets ATP-actin to barbed ends. Both actions together increase processivity of treadmilling by a factor of 125 *in vitro*. This concept only applies to the small number of uncapped filaments in the cell.

How do Cells Create Actin Filaments with Free Barbed Ends?

Mechanisms for creating free ends: **Uncapping**, **severing** and ***de novo*** **nucleation** of actin filaments.

Uncapping and severing: Barbed-end capping proteins, **capping protein** and **gelsolin**, can be removed by interaction with phosphatidylinositol 4,5-bisphosphate (**PI(4,5)P_2**) at the plasma membrane -> direct growth towards leading edge of migrating cell.

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

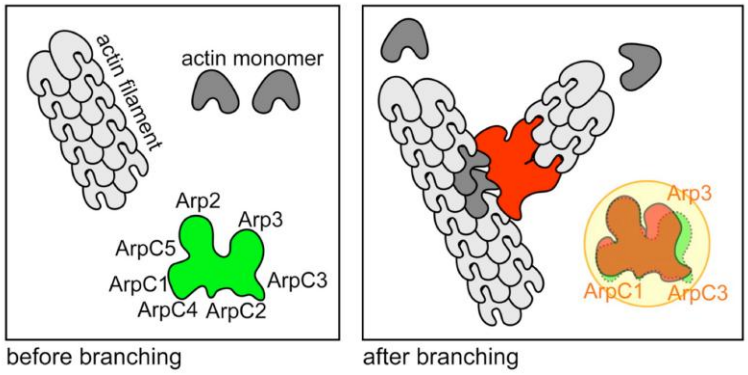

Figure 28: Conformational changes in both mother filament and the Arp2/3 complex upon branch formation. The yellow circle shows a comparison of the inactive, soluble Arp2/3 (green) and the complex in the branch (red).

De novo **nucleoation by Arp2/3**: Actin monomers can assemble into filaments, but with low efficiency. Rate-limiting step is formation of trimeric nucleus via dimeric intermediate (K_d: 100'000 µM). Dimers and trimers are instable, but once trimer is formed it can be elongated and stabilized involving addition of a forth monomer. Additional factors for nucleation are needed. The Arp2/3 complex is a key nucleator, inducing the formation of branched filaments, which display a 70° angle. The new filament is formed by attachment of the Arp2/3 complex (composed of 7 proteins) to the side of an existing filament.

Arp2/3 has to be activated in order to efficiently nucleate new filaments, by bringing Arp2 and Arp3 (actin-related proteins) into a conformation mimicking the normal actin dimer configuration. Main activators are WASP/Scar. Already existing filaments also contribute to the activation of Arp2/3 complex. Synergism of WASP and actin filaments leads to autocatalytic reaction which biases the initiation of new filaments to the sides of existing filaments, and leads to the formation of a highly branched actin meshwork. Incorporated Arp2/3 mimics barbed ends of a filament and localizes to the pointed end of the newly growing filament branch. In the branch, Arp2 and Arp3 form a short-pitch helix dimer and contribute the first two subunits to the daughter filament. Moreover, the actin subunits contacting Arp2/3 in the mother filament alter significantly. All seven Arp2/3 complex subunits make contact with the mother filament, and the total area of surface that is buried at this contact site is substantial.

The Dendritic Nucleation Model

Establishment of a directed protrusion happens as described in the dendritic nucleation model. It proposes that the activated Arp2/3 complex binds the side of a pre-existing filament initiating a lateral branch. Kinetic and structural data however support the barbed end branching mechanism, where the activated Arp2/3-VCA-G-actin complex binds at the barbed-end tip of a filament (not its side). The dendritic nucleation model is 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*. Key components and reactions:

1. Extracellular signals activate receptors
2. Associated signal transduction pathways produce GTP-bound Rho-family GTPases which
3. Together with PI(4,5)P_2 will activate WASP/Scar proteins
4. WASP/Scar activates Arp2/3 complex, brings it together with actin monomer on side of preexisting filaments to form a branch
5. Rapid growth occurs at the barbed end of the new branch
6. Forward pushing of the membrane
7. Capping protein terminates growth within a second or two
8. Filaments age by ATP hydrolysis -> phosphate release (white -> yellow -> red, see figure)
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 (red -> white)
11. Refilling the pool of ATP-actin bound to profilin, ready to elongate barbed ends when available
12. Rho-family GTPases activate PAK and LIM kinases, which phosphorylate ADF/cofilin -> slows down turnover of filaments

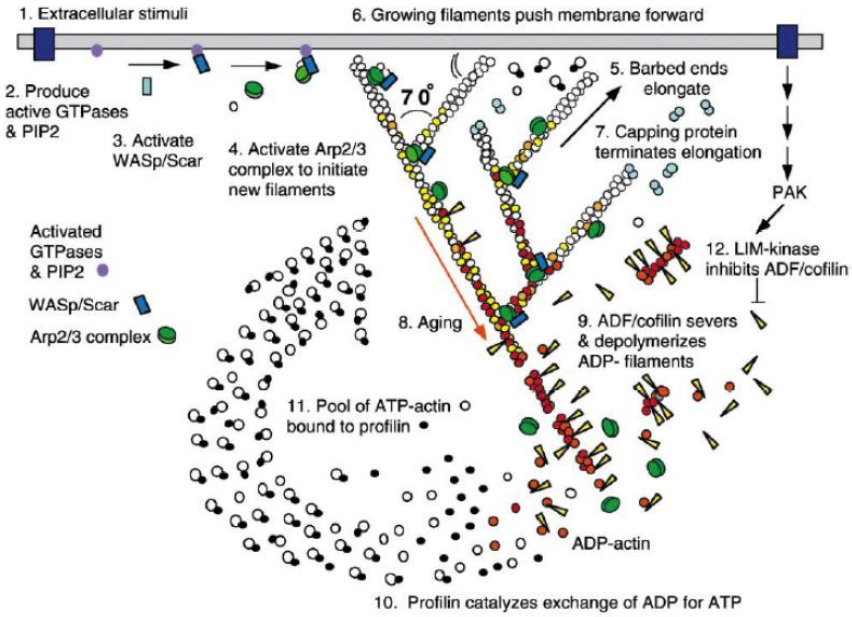

Figure 29: Dendritic nucleation model.

How is the Activity of the Arp2/3 Complex Regulated?

External signals -> receptor tyrosine kinases, seven-helix transmembrane receptors, integrins -> Rho GTPases -> WASP/Scar -> activation of Arp2/3. WASP/Scar proteins are composed of several functional domains. The C-terminal VCA domain activates Arp2/3: **V**erprolin homology region (interacts with monomers), **C**-domain (central domain), and **A**-domain (acidic region, both binding Arp2/3 complex). Upon binding to the VCA domain, Arp2/3 undergoes conformational changes and adopts a compact, actin-dimer-like conformation. Subsequently, a stable trimer is formed in combination with an actin monomer, and a new actin filament is ready to be elongated. The VCA domain (or WH2) acts with the same molecular principle in novel actin nucleators.

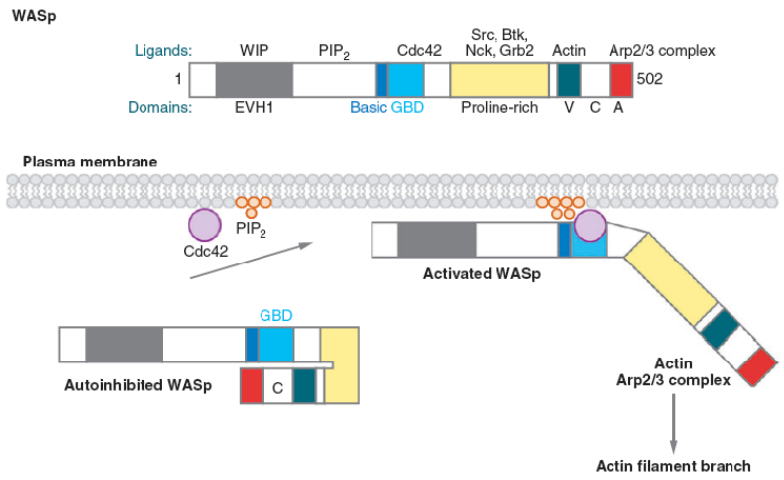

Figure 30: Domain organization and regulation of WASP. G protein binding domain (GPB) includes the CRIB domain).

WASP/Scar exists in a closed, auto-inhibited conformation, in which N-terminal sequences block activity of VCA. Binding of a Rho GTPase in combination with interactions with phosphoinositides, induce a structural change and liberate VCA, which is then able to activate Arp2/3. Generally, Cdc42 activates WASP, while Scar/WAVE is downstream of Rac.

Continuous supply of Arp2/3 is necessary to maintain high rates of nucleation (as capping proteins terminate polymerization soon). Filament elongation stops quickly after removal of activation stimuli.

Nucleation of Actin Filaments by Formins

Arp2/3 promotes formation of actin meshworks, but does not produce long filaments (fast blocking by capping proteins). Long actin filaments are important for many cellular processes, including the formation of contractile ring during cytokinesis, the formation of yeast actin cables directing vesicular traffic towards the bud site and the formation of filopodia. **Formins** are actin nucleators bound to the barbed ends; several act rather as capping proteins as their nucleation activity is low. Cdc12 (fission yeast) is a strong capper, mDia1 (mammals) and Bni1 (budding yeast) are weak cappers.

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

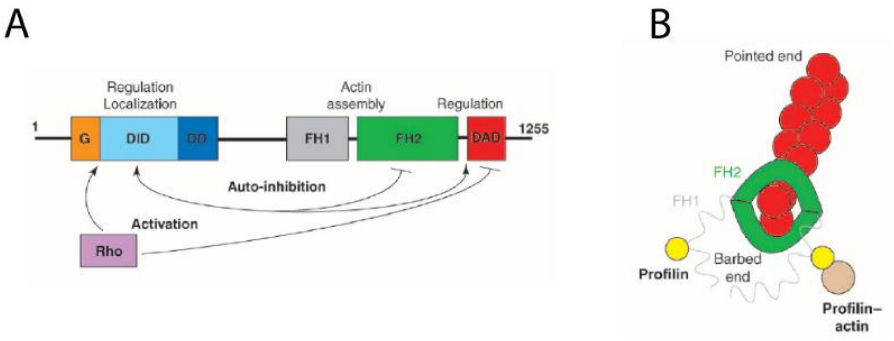

Figure 31: Domain organization, regulation and structure of formins. (a,b) mDia protein-mediated actin-filament assembly is controlled by intramolecular interactions mediated by the Dia-inhibitory domain (DID) and Dia-autoregulatory domain (DAD). To activate mDia proteins, activated GTPasees bind to Armadillo-repeat structures within the GTPase-binding domain (G)-DID region and sterically interfere with DAD binding.

Formins are multidomain proteins and homodimers. Dimerization involves the dimerization domain (DD). Dimer of FH2-domains (formin homology domain 2) forms a stable but flexible donut around growing end of filaments. FH2 exists in equilibrium between a closed state (blocking elongation) and an open state (promoting elongation). Transition is thought to involve movement of FH2 dimer towards the barbed end. Profilin is able to bind both actin and FH1 domain, and thereby increases the barbed-end elongation rate of formins.

Most capping proteins are soluble, but formins operate at the plasma membrane. A signal activates Rho-GTPases, which activate the FH2 domain, and therefore cause (by binding) targeting of the activated formin to the membrane. Formin function is regulated by an induced relief of autoinhibition. It is suggested that some formins might function more as elongation factors and work in coordination with the Arp2/3 complex, Spire, or other formins. Remarkably, formins not only influence actin but are needed for proper turnover of cell-matrix adhesion sites and the stabilization of MTs.

Novel Actin Filament Nucleators

Spire is another actin nucleator specific to metazoans. It nucleates new filaments at a rate similar to formins, but slower that Arp2/3 complex. Spire stabilizes and remains associated with pointed end of a new filament, generating a barbed end free to elongate. Spire contains a

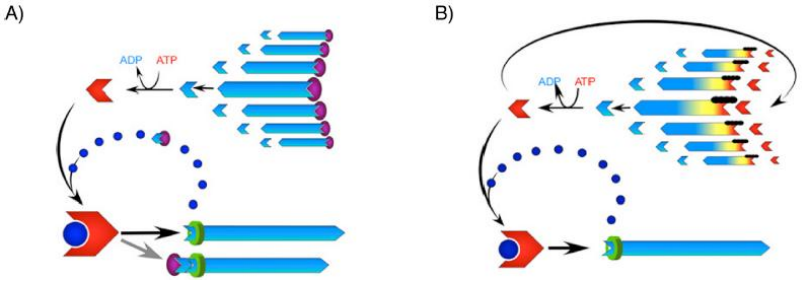

Figure 32: Synergy between Spire, formin and profilin. When barbed ends are capped (A), rapid growth of formin-bound filaments is arrested after a few seconds. When barbed ends are bound to Spire (B), processive assembly is sustained. In this example, formin is present in minute amounts as compared to the pool of filaments in the medium and serves as an indicator, but does not contribute to the steady state. ATP-actin (red), ADP-Pi-actin (yellow), ADP-actin (pale blue), profilin (dark blue), capping protein (purple), formin (green), Spire (black).

cluster of 4 WASP homology 2 domains (WH2), each binding one actin monomer. C-terminal WH2 might cap the pointed end of the filament, while the 3 other WH2 domains seem to bind actin monomers thus generating one linear polymer of 4 adjacent actin monomers. Laterally, a second polymer forms, facilitating barbed end elongation and promoting formation of a new filament.

Spire crosslinks MTs and actin. Spire protects growing barbed ends from capping protein and thereby ensures the constant elongation of the filament, while formins might function as elongation factors.

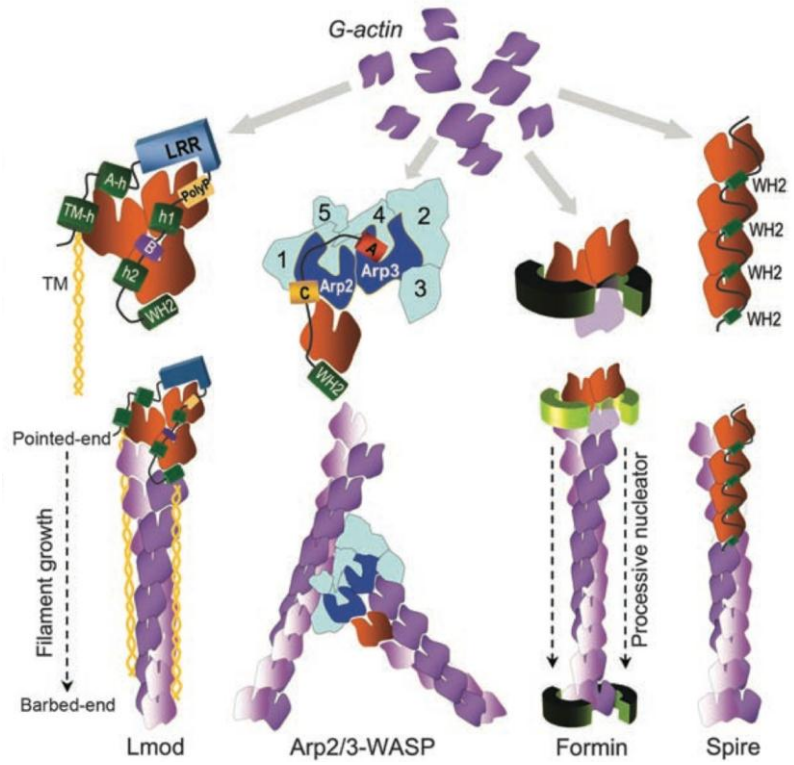

Figure 33: Comparison of models for actin filament nucleation. Leiomodin (Lmod) stabilizes a trimeric actin nucleus and tropomyosin (TM) enhances nucleation and mediates the localization of Lmod to filament pointed ends. Arp2/3 forms a trimeric seed with an actin monomer bound to WH2 of a nucleation-promoting factor. Formin dimers form nuclei and remain associated with the barbed end of the growing filament. Spire stabilizes a nucleus consisting of four actin subunits along a long-pitch filament strand.

Rat cordon-bleu (Cobl) is a brain-enriched protein using its three C-terminal WH2 domains for actin binding and is present in all vertebrates but absent from *S.c.* It promotes nonbundled, unbranched filaments similarly to Spire.

Leiomodin is another actin nucleator which acts with tropomyosin as strong filament nucleator in muscle cells. The complex localizes to the middle of muscle sarcomeres and is responsible for the nucleation of tropomyosin-decorated filaments in muscles. Leiomodin-tropomyosin caps similar to Arp2/3.

Growing Filaments - A Force Pushing the Membrane

Actin polymerization produces mechanical energy that could deform a plasma membrane and generate protrusions. The Brownian ratchet model explains how a polymerizing filament could rectify the Brownian motion 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 fluctuation. Actin filaments have a persistence of about 15 µm, and MTs in the range of 1-6 mm. MTs are rigid, whereas actin filaments are deformable like springs.

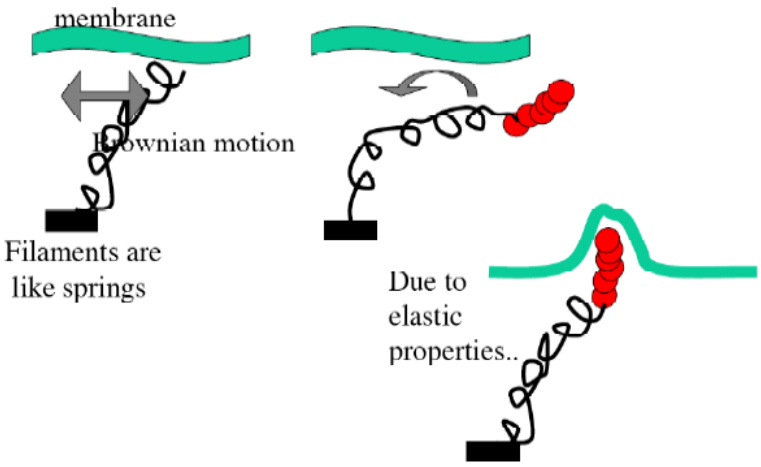

Figure 34: Elastic Brownian ratchet model.

Thermal fluctuations displace a filament from the membrane, allowing additional monomers to be added to the end of the filament. Elastic force 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 and pushes it forward was recently challenged by *in vitro* studies demonstrating that barbed ends that encountered a wall oriented away from it.

Chapter 5: Chemotaxis in *Dictyostelium discoideum*

Introduction

Chemotaxis: Directed cell migration in response to a chemical signal, either towards an attractant or away from a repellent. It functions in nutrient sourcing of prokaryotes, tracking of bacterial infections by neutrophils and organization of metazoan embryos. Two types of signals: either spatial concentration gradient or temporal signals generated when in a static gradient. Threshold concentration for detection: 10^{-6}-10^{-8} Molar, repellents often having higher thresholds. Different signals, bacteria attracted by aspartate and repelled by nickel, *Dictyostelium* attracted by cAMP, neutrophils attracted by N-formylated peptides. Netrin attracts neurons and repells oligodendritic precursor cells.

Prokaryotic cells are too small to use the spatial gradient and therefore make use of the temporal component. Bacterial chemotaxis involves 6 proteins, which regulate clock- or counterclockwise rotation of the flagellum. Bacteria undergo a random walk that results from the alternation of forward and tumbling movements. Encountering a static concentration gradient, bacteria reduce tumbling frequency and movement in one direction is prolonged. If the movement happens towards increasing signal concentration, tumbling frequency is kept low and movement continues. If signal concentration decreases, tumbling frequency increases, giving the cell possibility to randomly change direction.

Therefore, bacterial chemotaxis should rather be called kinesis, since it is a non-directional change in cellular activity in response to a stimulus, which generates a movement.

Eukaryotic cells (10-20 µm) are big enough to use temporal and spatial information. They are able to detect and internally amplify the difference of chemoattractant concentration between the ends of the cell (differences as small as 2% are recognized). The differences are translated into a steep intracellular gradient of signaling components, leading to an asymmetric cellular response and to directed cell migration.

The Model System *Dictyostelium*

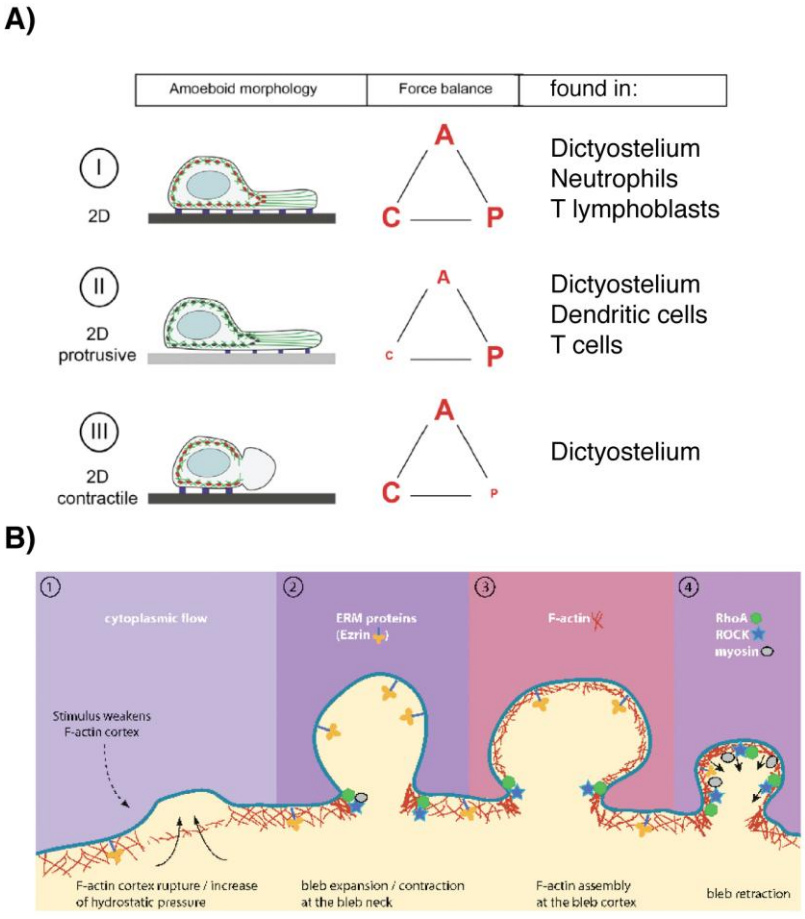

Figure 35: Three different migration modes of *Dictyostelium*. (A) Force-relationship between adhesion (A), contraction (C) and polymer-network expansion (P) determines the amoeboid phenotype, the three major forces in cell migration. Cell forward locomotion results from their balanced interplay (indicated by the red-lettered triangles). Amoeboid cell movement on twodimensional (2D) surfaces requires adhesions to transduce internal contractile forces onto the substratum. (I) A polymerizing network (green, mostly actin) pushes the membrane forward. Myosin II (red ellipses) based contraction behind the leading edge produces traction underneath the adhesion points (blue). On high adhesive surfaces (black thick line), actomyosin contraction at the back is required to detach the cell. Rear end contraction is not necessary when cells migrate on low adhesive substrates (grey thick line). (II) When the contractile function of myosin II is defect (black ellipses), actin polymerization alone can produce traction under adhesion points. Migration without contraction postulates cell retraction either by membrane tension or by polymer-network disassembly. (B) Molecular requirements for bleb formation and retraction. Blebbing is initiated by extracellular triggers or internal inhomogeneity, causing in localized destabilization or depolymerization of the cortical actin meshwork. (1) Local disruption of 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 of the ERM family such as ezrin. (2) Actin is subsequently polymerized at the bleb cortex by mechanisms still unknow, 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.

Dictyostelium discoideum advances by an amoeboid mode of migration. Remarkably, these cells can exhibit several types of amoeboid motility: they can use filopodial spikes and pseudopods as well as membrane blebs. Weak adhesion sites, absence of stress fibers and protrusion formation driven by actin polymerization is the predominant migration form found. Generally, 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.

Dictyostelium grow as individual cells, which undergo a vegetative division cycle. During that state, they display chemotactic responses. They are attracted toward 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.

Upon depletion of nutrients they initiate a highly complex developmental program, leading to the generation of a multicellular organism. The process involves chemotaxis of single cells towards an aggregation center, and culminates in the formation of a terminally differentiated fruiting body containing spores. Two phases:

- Chemotaxis and aggregation in response to cAMP (morphogen) -> multicellular organism
- Different stages of cyto-differentiation within it (cell sorting, multi-cellular morphogenesis

Cells within the aggregate secrete cAMP. By chemotaxis, moving cells move towards highest concentration of cAMP, forming a multicellular aggregate. Cells then differentiate into non-terminally prespores and prestalk populations. Morphogenesis and sorting of cells occur, prespore cells give rise to spores of the mature fruiting body and prestalk population differentiate into various stalk structures.

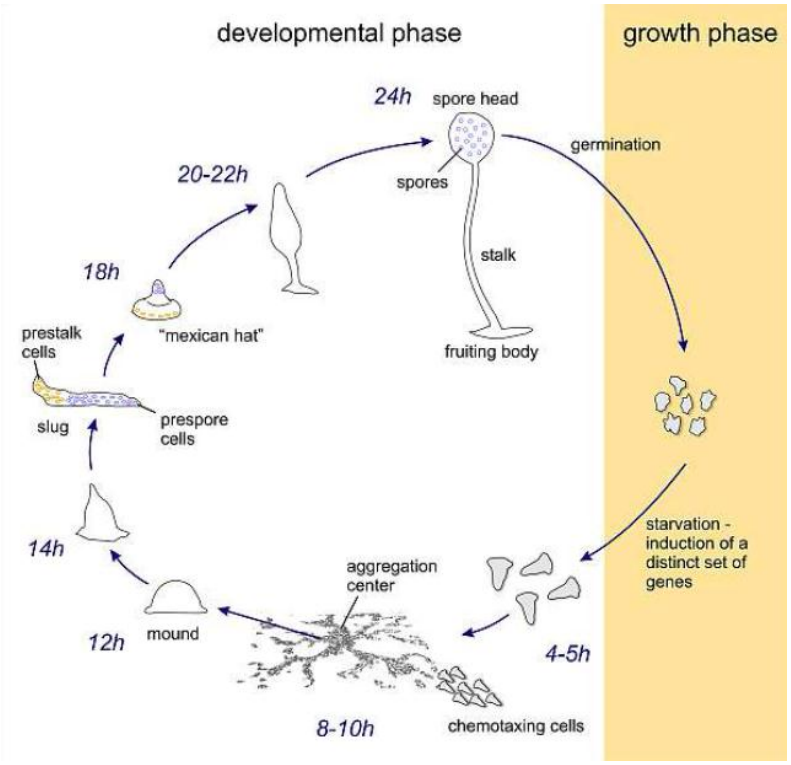

Figure 36: Lifecycle of *Dictyostelium discoideum*. (1) In favorable nutrient conditions, the cells grow and divide as single cells (growth phase). (2) Aggregate formation: When food is depleted, the amoeboid cells migrate toward an aggregation center, where hundreds of cells concentrate in response 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 animals, and new amoeba can hatch. Next to each stage of development, the corresponding time (in hours) after the beginning of starvation is shown.

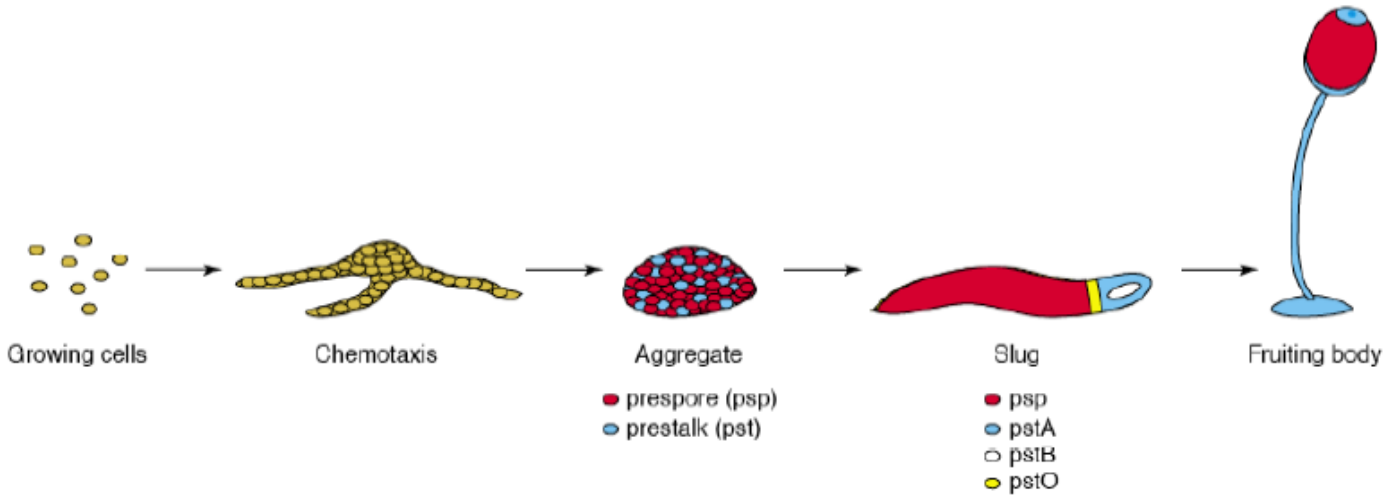

Figure 37: Major stages of *Dictyostelium* development. Three different types of prestalk cells are present in the aggregate as a homogenous population. At the slug stage, the posterior is primarily comprised of prespore (red). The anterior prestalk contains three different zones, with pstA cells (blues) comprising the major group.

cAMP Signaling in *Dictyostelium*

Without chemoattractants, cells appear round and apolar. Upon exposure to a chemoattractant gradient, they quickly polarize and start migrating towards the signal source. Cells exhibit cell motility in the absence of a stimulation. Pseudopods are periodically extending, self-organizing structures. Chemotactic behavior of neutrophils is close to *Dictyostelium* cells. But neutrophils are immobile till exposed to a signal. Aggregation of *Dictyostelium* is driven by the periodic cAMP secretion of few cells in the aggregation center. The signal is detected by surrounding cells, which initiate cAMP production and relay cAMP signal to cells even further away. Extracellular cAMP (nM) binds to high affinity receptor CAR1 and induces aggregation-stage specific signaling. This generates a phosphatidylinositol (3,4,5)-triphosphate ($PI(3,4,5)P_3$) at the cell front, which in turn recruits CRAC, a PH-domain containing cytosolic regulator of adenylyl cyclase (AC). CRAC somehow diffuses through the cel and activates AC at the back of the cell. This leads to the secretion of cAMP into the medium, and propagation of the signal to the neighboring cell. Once cAMP reaches a particular level, cells adapt and become insensitive. Sensitivity is restored

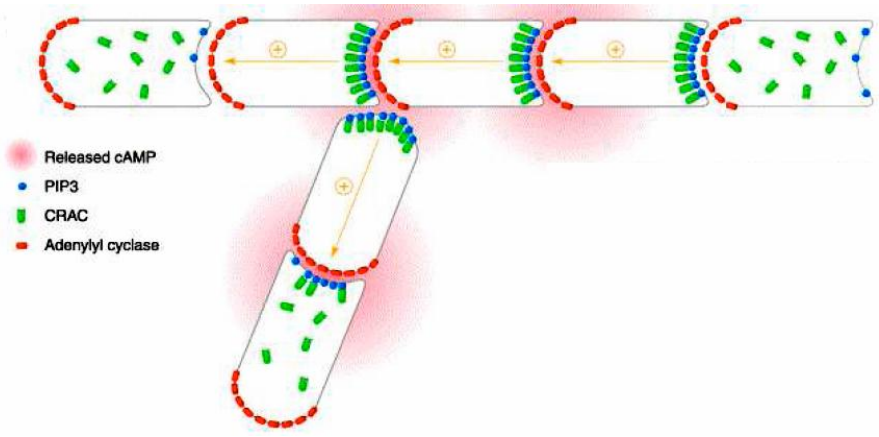

Figure 38: Signal relay in *Dictoystelium* cells. Cells are moving from left to right, cAMP pulse is propagated from right to left.

after a period of low extracellular cAMP resulting from the activation of the cAMP-induced extracellular phosphodieseterase (PDE). Low cAMP levels allow expression of the phosphodieserase inhibitor (PDI) and its secretion into the extracellular environment, where it blocks PDE activity allowing a new accumulation of extracellular cAMP. Cycles of activation and adaption result in cAMP pulses of roughly every 6 minutes. The oscillations are essential to relay the chemotactit gradient, ensure the directionality of movement and induce expression of aggregation-specific genes.

Directional Sensing versus Polarization

Actin in the front of the cells promotes pseudopod formation. Myosin II is assembled at the sides to suppress lateral pseudopod formation and at the rear of the cell to trigger retraction of trailing edge. Once polarized, the leading edge is more sensitive to chemoattractants than other regions (molecular machinery already in place). This localizes actin cytoskeleton activity and also reduces the region of the cell contributing to sensing, therefore reducing the ability of the cell to respond to rapid changes in gradient direction. Unpolarized cells maintain equal sensitivity around the entire perimeter.

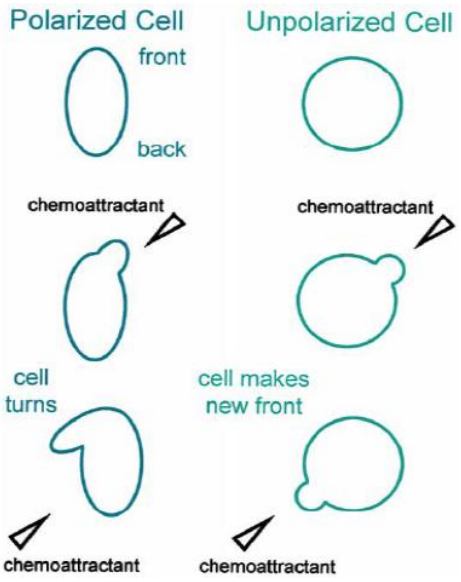

Figure 39: Polarization and directional sensing. Since the ends of polarized cells differ in their sensitivity towards a chemotactic signal and in their arrangement of the actin cytoskeleton, the cell will turn if the source of the gradient is moved.

Even unpolarized cells are able to detect a gradient and trigger asymmetric accumulation of signaling molecules -> directional sensing links cellular response constantly to the external gradient. Amoeba or neutrophils treated with Latrunculin A (actin polymerization inhibitor) become depolarized and immobile, but can still detect gradients and display polarized distribution of specific signaling factors.

Model to explain adaptation to chemoattractant and sensing of spatial gradients: Fast, short range, self-enhancing positive signal and a slow, long-range, freely diffusible negative signal. The combination of these signals (coming from the same original stimulus) seems to be a general feature of many pattern formation processes. It does however not easily explain the spontaneous polarization and directed movement that is observed in cells that are exposed to uniform concentrations of chemoattractant.

Adaptation: During early stages of response, activation is high and inhibition low, leading to strong intracellular signaling. Later, activator and inhibitor reach their steady-state levels and establish a new

balance, the cellular response returns at this point to its pre-stimulus level. Adapted cells can respond further if receptor occupancy is increased again.

Directional sensing: Assuming 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, a balance between activation and inhibition at the center, and a net inhibition at the rear. This leads to a persistent, asymmetric cellular response.

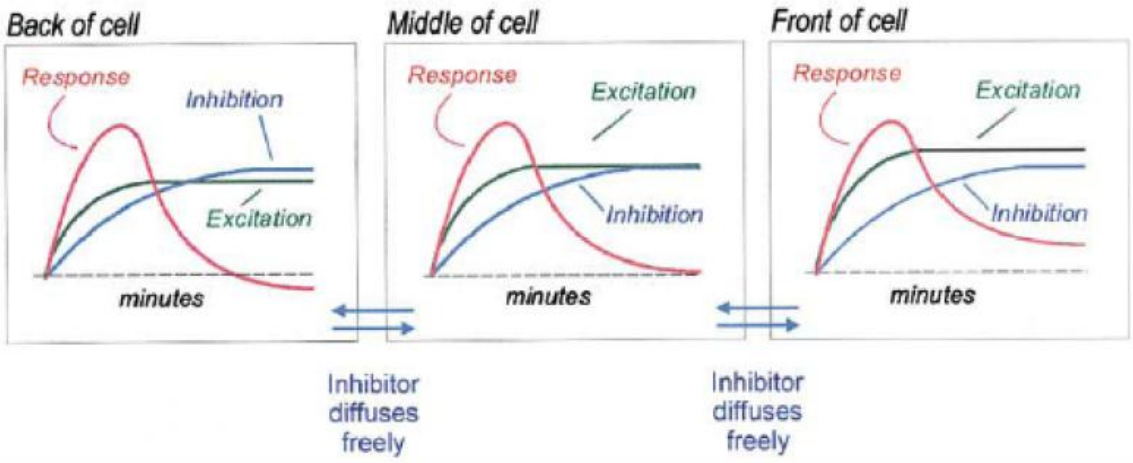

Figure 40: The local excitation and global inhibition model of directional sensing.

Signaling Networks in Chemotaxis

First response to a **uniform** concentration of chemoattractant is the accumulation of PI(3,4,5)P_3 at the plasma membrane. Local levels are controlled by the class I PI-3 kinases (PI3Ks) and the PI(3,4,5)P_3 3-phosphatase PTEN. Localization of these two enzymes is reciprocally regulated in response to chemotactic stimulation. In unstimulated cells, PI3Ks are mostly cytosolic, while PTEN associates with plasma membrane. Upon stimulation with uniform chemoattractant, PI3Ks are transiently targeted to the plasma membrane, while PTEN becomes cytosolic.

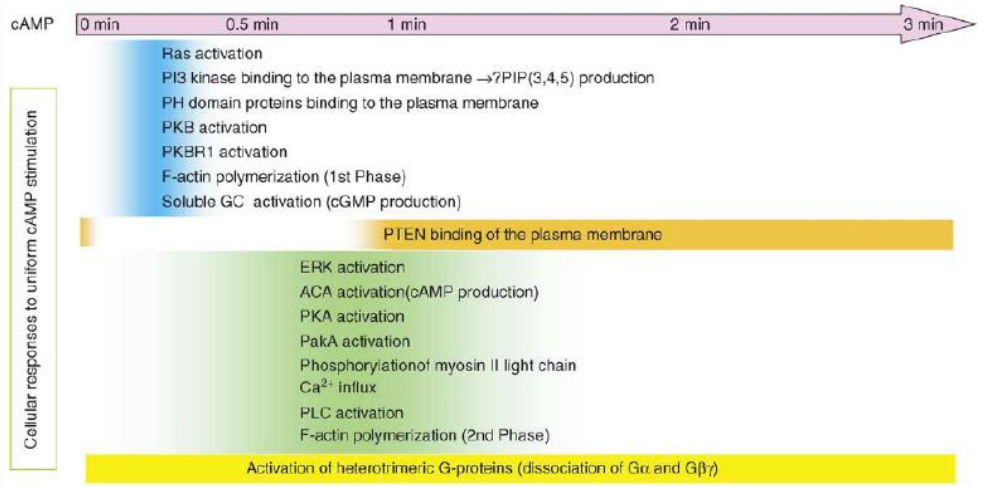

Figure 41: Uniform chemoattractant-stimulation triggers the activation of multiple signaling networks. Following the timescale, most responses are transient and can be classed as early (10-30 sec) or late (30-90 sec).

After about 60sec, the cells adapt and return to their previous state with

cytosolic PI3Ks and membrane bound 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. Heterotrimeric G-proteins transduce the presence of stimulation into the cell interior and remain constantly activated as long as chemoattractant is present.

In a **steep** gradient, 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 lateral and trailing edge. PTEN restricts diffusion of $PI(3,4,5)P_3$ away from the leading edge. 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 to a gradient can be divided into two phases:

- First phase lasts several seconds, activates gradient machinery, uniform $PI(3,4,5)P_3$ localization
- Second phase, difference in receptor occupancy amplified, asymmetric $PI(3,4,5)P_3$ distribution

Patches of $PI(3,4,5)P_3$ have been shown to be the region of plasma membrane where pseudopods are preferentially synthesized and extended.

Chemoattractant receptors and associated heterotrimeric G-proteins maintain uniform distribution all the time. Signal amplification occurs downstream of the G-proteins. It seems 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.

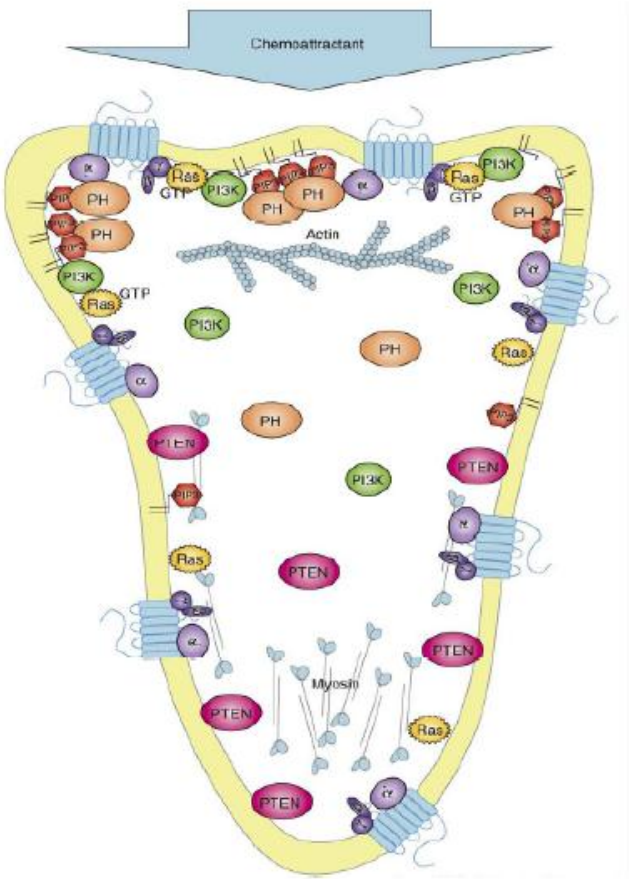

Figure 42: Representation of the spatial localization of key components implicated in chemotaxis of *Dictyostelium*. Seven transmembrane chemoattractant receptors (CAR) and heterotrimeric G-proteins (α, β, γ) are evenly distributed along the perimeter of the cell. The receptor occupancy reflects the extracellular gradient of chemoattractant. The small G-protein Ras displays uniform localization along the cell cortex, but its activated form (Ras-GTP) is predomionantly found at the leading edge, whereas PTEN and myosin localize to the lateral sides and posterior of chemotaxing cells.

Actin-independent positive feedback loop: The gradient-induced relocalization of PI3Ks and PTEN is not dependent on their catalytic activity, actin polymerization or total $PI(3,4,5)P_3$ levels. This suggests that

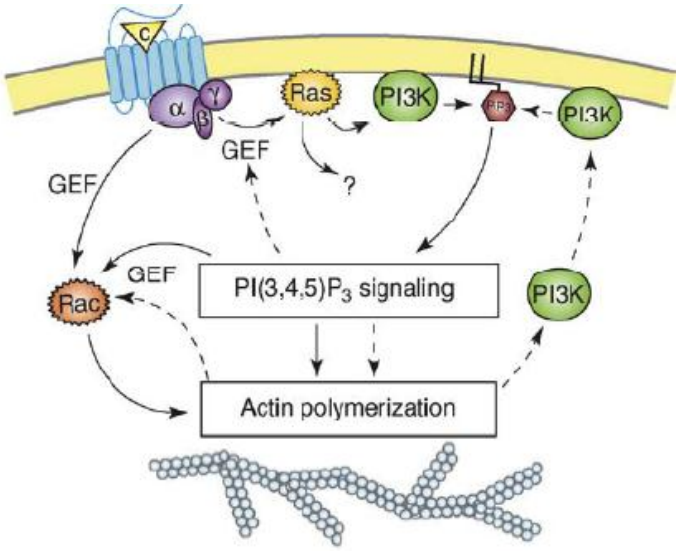

Figure 43: Positive feedback loops amplify chemoattractant signal to produca a strong PI(3,4,5)P_3 response. Rac and Ras are activated by G-proteins. Ras activates a membrane-bound pool of PI3K, thereby inducing PI(3,4,5)P_3 production. PI(3,4,5)P_3 signaling then leads to increased F-actin polymerization, 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 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.

the pre-existing membrane pool of PI3Ks is sufficient to produce enough PI(3,4,5)P_3 to recruit the relevant downstream target proteins, such as PH-domain containing proteins. Ras is uspstream of PI(3,4,5)P_3 since it is required for PI3K activity, but also downstream of PI(3,4,5)P_3 since increased Ras activity is observed in PTEN$^-$ cells. This is consistent with a model in which the pre-existing plasma membrane pools of Ras and PI3Ks participate in a positive feedback loop establishing asymmetric PI(3,4,5)P_3 distribution. However, this asymmetry is further enhanced by a positive feedback loop involving the actin cytoskeleton.

Actin-dependent positive feedback loop: Cdc42 and Rho subfamily members, characteristic of metazoa and fungi, are absent in *Dictyostelium*. Rac-like Rho GTPases are involved in an actin dependent positive feedback loop. The activity of RacB in *Dictyostelium* parallels the actin polymerization waves described. Consistently with the presence of a feedback loop, Rac was shown to be both upstream and downstream of PI(3,4,5)P_3. PI(3,4,5)P_3 accumulation at the leading edge recruits PH-containing proteins, typically Rho-GEFs, which might act as regulators of Scar/WASP dependent actin polymerization. Specific intracellular localization of RacGEF1 was 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. Rac therefore indirectly causes PI(3,4,5)P_3 accumulation at the plasma membrane by promoting actin polymerization.

Regulation of actin polymerization is likely to involve the PI(3,4,5)P_3 and Rho GTPase dependent activation of the Arp2/3 complex via the WASP and Scar/WAVE proteins. *Dictyostelium* cells with lowered levels of WASP are unable to aggregate. Scar/WASP might also play a role in positive feedback loop, given that they are regulated by PI(3,4,5)P_3.

Regulation of Myosin II

Cells have to tightly regulate myosin II which provides the power to retract the rear of the cell and prevents formation of lateral pseudopods. Synthesis of cGMP is an essential event triggered downstream of the heterotrimerig G-proteins. Increased levels of cGMP activate myosin light chain kinase (MLCK), which phosphorylates MLC and thereby increases the motor activity of myosin II. There are differences to mechanisms controlling myosin in neutrophils. Myosin regulation is downstream of Rho GTPases, which activate ROCK and MLCK, leading also to the phosphorylation of MLC. Additionally, ROCK inhibits counteracting MLC phosphatase.

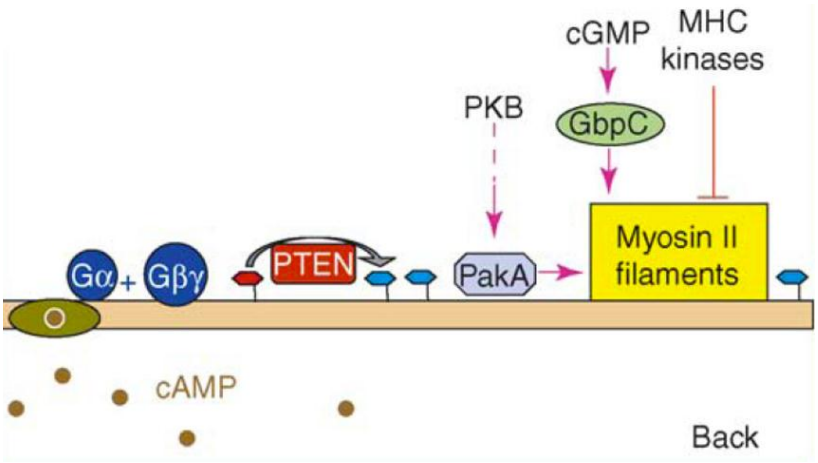

Figure 44: Activation of pathways specific for the rear of chemotactic *Dictyostelium* cells. Diagram shows plasma membrane at the back end, where cAMP levels are not high enough to trigger a sufficient activation of the heterotrimeric G protein signaling. PTEN remains membrane-bound and degrades PIP3 (red to blue), whereas myosin II is assembled into contractile filaments that suppress pseudopod formation and promote retraction of the cells 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.

Studies with *Dictyostelium* 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 leading edge. Cells lacking Akt/PKB are less polar and show significant defects in ability to localize PAKa, a homologue of mammalian CRIB domain containing p21 activated kinases, and myosin II to their posterior. Active PAKa is required for myosin II assembly in the rear of moving cells. It is suggested that the role of Akt/PKB, once translocated and activated at the leading edge, consists in phosphorylating PAKa, which then translocates to the posterior part of chemotactic cells where it promotes myosin II assembly.

Signaling Components from *Dictyostelium*

cAMP: Chemoattractant activating CAR1

CAR1: cAMP receptor, coupled to a heterotrimeric G-protein; binding of cAMP regulates five main effectors: PLC, AC, GC, PI3K and PTEN; activation of PI3K is presumed to require Ras GTP-binding protein

PLC: Phospholipase C; PLC pathway is not essential for chemotaxis

CRAC: PH-domain containing protein, CRAC moves from the leading edge to the posterior, activates AC

AC: Adenylyl cyclase is activated by CRAC; ACA is the best-studied AC gene, and is responsible for synthesis of cAMP that relays the cAMP wave outwardly to recruit additional cells, cells adapt quickly to high cAMP levels, not strictly required for chemotaxis

PDE: Phosphodiesterase, exported into the extracellular environment, degrades extracellular cAMP, allowing cells to become responsive to the next cAMP wave

PDI: Inhibitor of PDE, exported into the extracellular environment, induced by low cAMP levels

PI3K: Lipid kinase, phosphorylates PI(4,5)P_2 and PI(3,4,5)P_3

PI(3,4,5)P_3: Recruits a subset of PH-proteins to the leading edge

PH-proteins: Pleckstrin-homology domain containing proteins, Adk/PKB, CRAC and PhdA; PI3K and PhdA; PI3K and PhdA, potentially together with PH-domain containing GEFs for Rac-GTP binding proteins regulate F-actin polymerization and pseudopod extension in the direction of cAMP gradient

PTEN: Phosphatase and tensin homologue, negative regulator of PI3K pathway by dephosphorylating PI(3,4,5)P_3; accumulates at the sides and the posterior of the cells antagonizing recruitment of PH proteins to these sites and suppressing lateral pseudopod formation

GC: Guanylyl cyclase, positively regulates myosin II assembly in the back and the side of the cells

F-actin: At leading edge, forming extending pseudopod; synthesis oriented relative to cAMP source

Myosin II: Assembly of myosin II increases cortical tension at the sides of the cell, repressing lateral pseudopod extension; it also promotes contraction at the end of the cell during the movement; myosin II is activated by cGMP and by p21-activated kinase PKAa (itself activated by Akt/PKB

Molecular Basis of Nematode Sperm Motility

There are also forms of motility that occur in the absence of actin. In sperm cells of nematodes (which lack actin), major sperm protein (MSP) takes over its role. These cells illustrate the machinery of migration in a basic form, and migrate in the absence of a flagellum by a form of crawling similar to amoeboid migration. They extend a persistent protrusion that attaches to the substrate and pulls along the cell body. MSP also assembles into two-stranded polymers, but shows no sequence or structural similarity to actin. MSP filaments have no intrinsic polarity, and polymerization of both ends is controlled by additional factors.

Activation of nematode sperm during copulation leads to rapid assembly of MSP filaments, that then crosslink along advancing front. Within 6 minutes, cells extend filament-packed pseudopod, which pulls organelle-packed cell along (70 μm/min). Filaments assemble at the leading edge and disassemble at the back, providing subunits that can be recycled for reassembly at the front of the meshwork -> constant treadmilling of MSP polymers from front to rear, close connection between cytoskeletal rearrangements

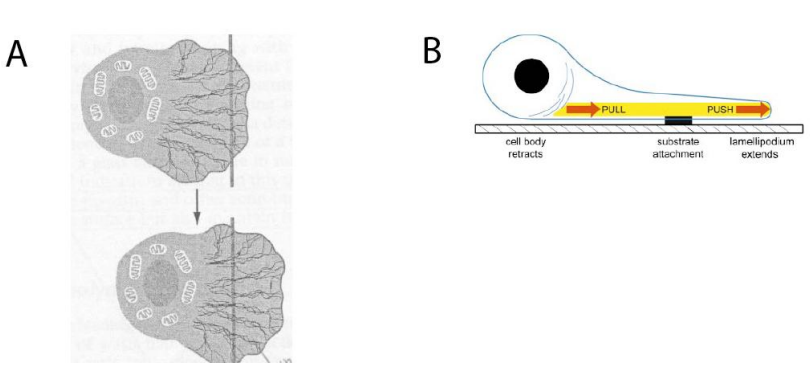

Figure 45: Moving nematode sperm (A) and proposed push-and-pull model (B). Assembly and bundling of MSP filaments into fiber complexes (yellow) pushes the membrane at the leading edge forward. At the same time a second force, which is associated with disassembly of the fiber complexes at the base of the lamellipodium, pulls the cell body forward. In this model, attachments where the cytoskeleton is linked to the membrane and the membrane anchored to the substratum establish traction and separate mechanically the forces produced at opposite ends of the fiber complexes. Thus, rather than canceling each other, these forces can be exerted independently against the substratum.

and cell motility. Major difference between MSP and actin -> MSP does not directly bind and hydrolyze ATP, but ATP seems to be required indirectly to reconstitute a protrusion in cell-free sperm extracts.

Chapter 6: Actin-Based Motility of Intracellular Pathogens

Introduction

Success of microbial pathogens (bacteria, viruses or other microbiological organisms) requires:

- Colonizing mammalian host
- Avoiding host defense mechanism
- Replication
- Exit infected organism to spread to a new, uninfected host

Human body is protected by skin, but some organs (lung, intestine) are only covered with one cell layer. This is exploited by some bacteria, which enter host cells by penetrating through monolayer of epithelial cells in the intestine. Invasive bacteria induce their own phagocytosis into cells normally non-phagocytic. Examples are *Shigella*, *Lysteria* and *Rickettsia*. They use actin-based motility to move and spread. Actin may also play a role by avoiding recognition by ubiquitin degradation system. Vaccinia virus used the induction of actin-tails for its near range dissemination.

Infection Routes

- Pathogens access cytoplasm of host cells by inducing their phagocytic uptake (into vacuole)
- Lysis of vacuole -> release into cytoplasm
- Recruitment of actin remodeling host cell proteins -> polymerize actin monomers into actin tails
- Actin tail assembly provides force to propel organism into adjacent cells

- Passage occurs via membrane protrusion (bacterium pushes out against membrane)
- Protrusion engulfed by membrane of neighboring cell -> bacteria enclosed by double membrane
- Lysis of double membrane vacuole, released into cytoplasm

Shigella, *Listeria* and *Rickettsia* use similar process, but molecular details are different, and involve either proteins that mimic cellular actin nucleation promoting factor, or proteins that activate cellular actin nucleation promoting factors such as WASP and N-WASP. Arp2/3 is involved in all pathways. Two general strategies to enter host cells:

- Zipper mechanism: Bacteria adhere to non-phagocytic cell (e.g. epithelia) cause modest changes in host cytoskeleton and are then taken up
- Trigger mechanism: Not dependent on initial adhesion, but directly inject effector proteins into the host cells, causing massive cytoskeletal changes and subsequent phagocytosis

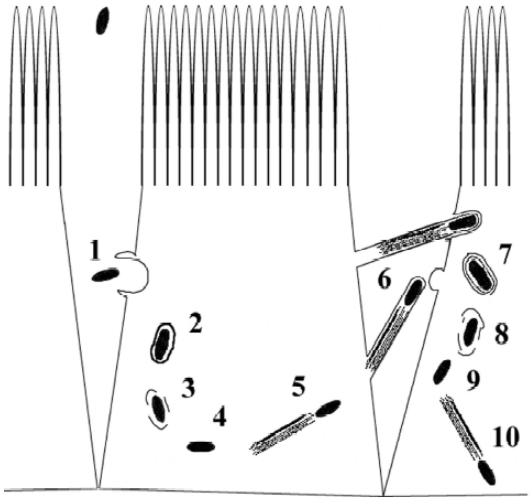

Figure 46: Pathogenesis of the enteroinvasive bacterium *Shigella*. Process depicted is also representative of bacteria such as *Listeria* and *Rickettsia*.

Zipper mechanism: 3 successive steps are necessary for invasion by *Listeria*, using zipper mechanism:

1. Contact with host cell, adherence via interaction of bacterial ligand with receptor of host cell
2. Signaling triggered by transiently formed ligand-receptor complex -> formation of phagocytic cup to engulf bacterial cell, accompanied by actin polymerization and membrane extension
3. Phagocytic cup closure and retraction into cytosol -> complete entry into host cell

The bacterium expresses a protein on the surface that mimics an ECM component, a cell-cell adherence protein or an extracellular ligand (e.g. growth factor). Bacterial protein binds receptor, which leads to its clustering and activates a downstream signaling cascade. The cascade leads to engulfment of bacterium involving actin polymerization and membrane extension. In *Listeria*, internalin A (InlA) binds E-cadherin which constitutes cell-cell contacts and interacts with actin via β- and α-catenin. InlA-dependent actin polymerization requires Rac activity. The second protein, InlB, interacts with receptor tyrosine kinase Met, a growth factor receptor for HGF. Activation of Met leads to dimerization and autophosphorylation, and subsequent recruitment of several host factors. Downstream signaling cascade leads to actin polymerization via Rac and Arp2/3. Bacterial ligands in this case only mimic host ligand functionally but not structurally -> functional mimicry.

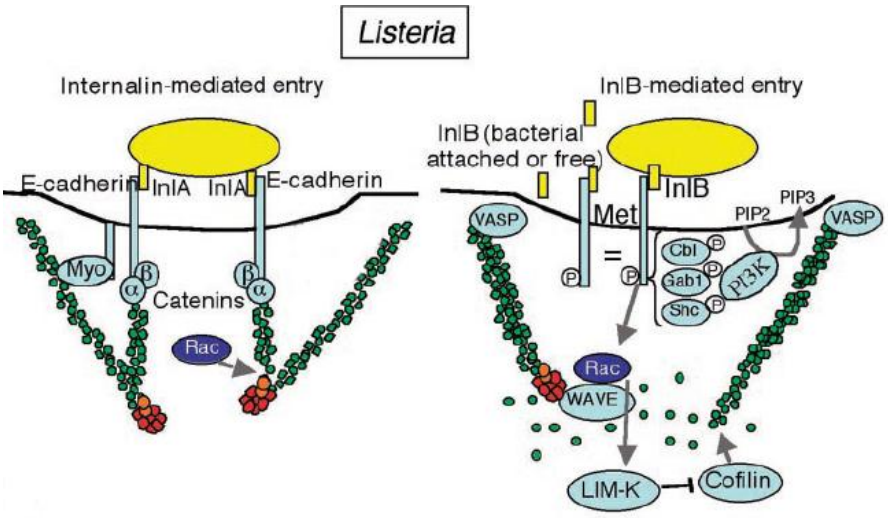

Figure 47: Zipper mechanism for cell entry, used e.g. by *Listeria*, legend in next figure.

LIM kinase and cofilin also contribute to regulation of actin dynamics during invasion. During early stages, cofilin activity is kept low by LIM kinase. Later, cofilin levels increase; severing and depolymerizing activity promotes filament disassembly and retraction of phagocytic cup, terminating pathogen-induced signaling events. *Listeria* bound to Met was shown to enter via CME and septins support bacterial internalization.

Trigger mechanism: Both *Shigella* and *Salmonella* use this mechanism. Type III secretory system (TTSS) plays a central role. Components of the TTSS adhere to host cell receptors, e.g. CD44 in the case of *Shigella*. Adherence (receptor not known) activates TTSS translocator complex, leading to injection of bacterial components through a pore formed by Sip (*Salmonella*) or Ipa (*Shigella*) proteins. Together with injected proteins, these factors mediate downstream events leading to actin rearrangements that provoke macropinocytosis like processes.

In the early stage of ***Shigella*** entry, VirA destabilizes MTs and triggers actin polymerization via activation of Cdc42/Rac, WASP and Arp2/3. IpaC, a component of the translocation system, contributes to initiation

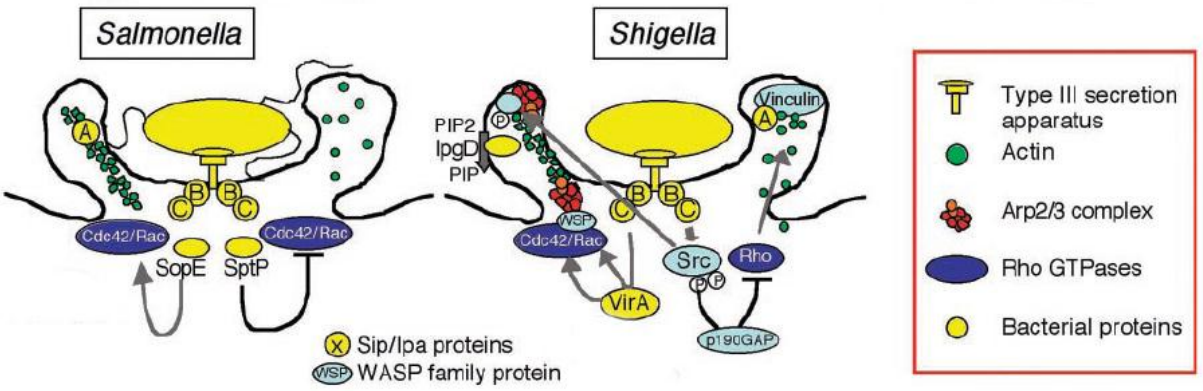

Figure 48: Trigger mechanism for cell entry used e.g. by *Salmonella* and *Shigella*.

of actin polymerization (mechanism not understood). Actin dynamics are further enhanced by activation of Src kinase, which inhibits Rho GTPase. Later, the IpaA protein binds vinculin, key protein in formation of cell-adherens plaques, and induces actin depolymerization.

Salmonella manipulates cellular Rho GTPase in a reversible manner. First, bacteria inject SopE, which has a GEF activity towards Rac and Cdc42. Activation of these leads to massive rearrangements of the actin cytoskeleton and membrane ruffling, facilitating bacterial infection. Once inside the cell, *Salmonella* has the ability to restore normal cellular status and inactivate Rac and Cdc42 signaling. It makes use of SptP, a bacterial factor with GAP activity. GAP domain also contains catalytically active arginine, different from typical Rho GAPs in that it does not contain a complete arginine finger. It seems that similar amounts of SopE and SptP are injected, but that SopE is rapidly degraded (proteasome-dependent) and SptP is more stable. This ensures the reversion of initial actin polymerization once bacterium has entered. SopE and SptP, produced by convergent evolution, have no sequence similarity to mammalian host factors but copy function -> functional mimicry.

Invasive Strategies: Intestine contains epithelial and M cells. Last take up and translocate bacteria, expose them to macrophages and other cells that endocytose bacteria, which take advantages of this route to cross the epithelial barrier (e.g. *Listeria*). Translocation also happens by direct entry into the common epithelial cells or capture by dendritic cells, which crawl between cells and transiently extend pseudopods into intestinal lumen. Once translocated, *Listeria* survives in macrophages by escaping from vesicles into the cytoplasm and evade being killed in lysosomes. Here, bacteria move at rates of up to 22 µm/min. Actin-driven cell-to-cell transmission is important for local spread. Systemic dissemination occurs inside circulating macrophages and dendritic cells -> meningitis, fetal death, and diarrhea.

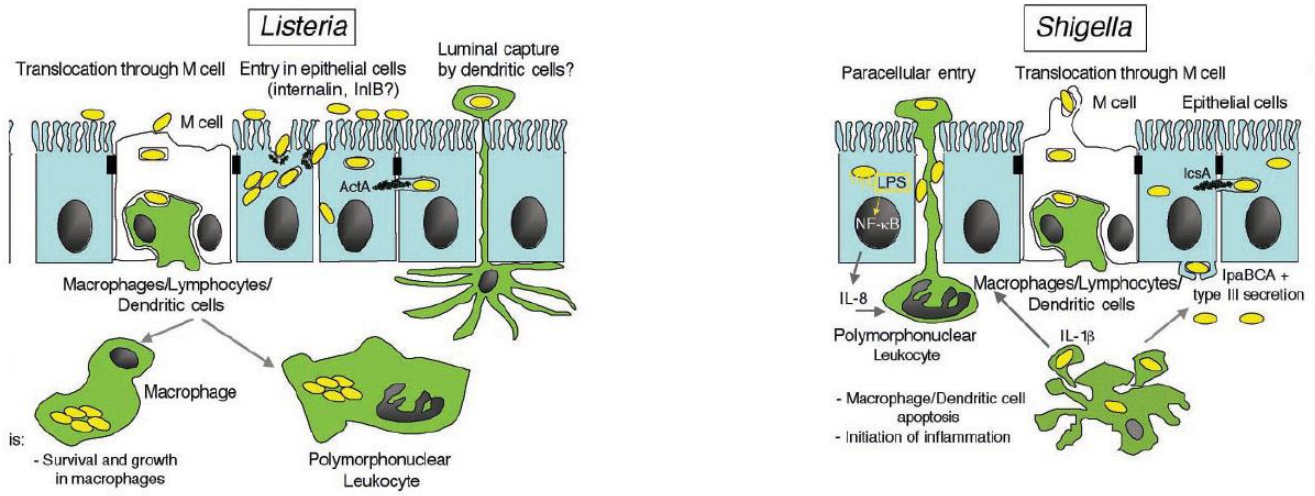

Figure 49: Invasive strategies of *Lysteria* and *Shigella*.

Shigella invades and spreads through the lining of the human colon, causing fever and diarrhea in older patients and children. Bacteria are translocated by M cells through the epithelial barrier and are unable to directly disrupt the epithelial monolayer. Bacteria cause apoptosis in macrophages to avoid their own destruction. Additionally, they cause mucosal inflammation by triggering the release of IL-1β and IL-8, which disrupts epithelial impermeability and facilitates paracellular entry through epithelium. *Shigella* can move till up to 26 µm/min. Because main mode of transmission is based on cell-to-cell spread, infectious process usually stays at the mucosal level rather than becoming systemic.

Intracellular Movement

Actin tails formed by *Listeria* and *Shigella* are composed of short, crosslinked actin filaments that rapidly turn over. This behavior of actin is similar to the one observed in lamellipodia at the leading edge of motile cells. Actin tails formed by *Rickettsia* are similar to actin in filopodia (long, unbranched). Formation of actin tails by bacteria provides a very powerful experimental system to identify and characterize cellular host factors that control actin dynamics.

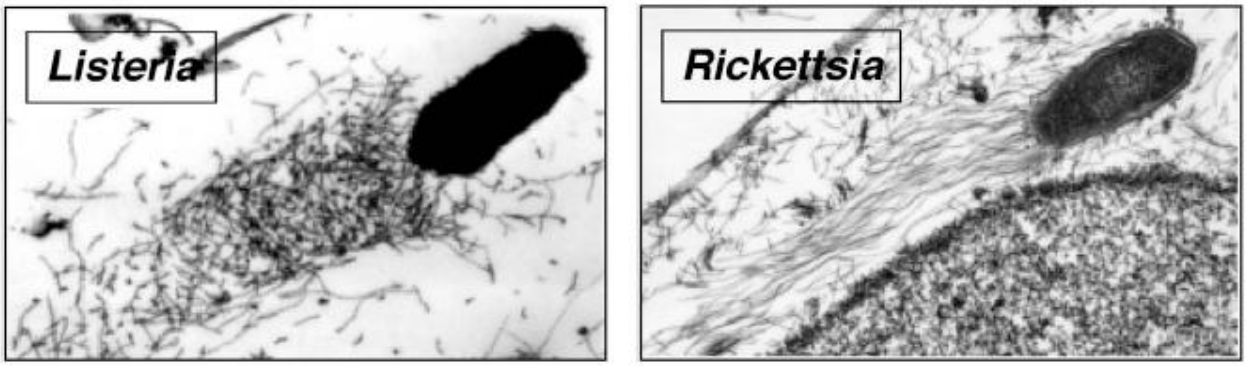

Figure 50: Electron micrographs of actin tails.

Listeria: Actin tails consist of multiple short filaments that are crosslinked within a non-parallel network. Disruption in the surface protein ActA leads to strains unable to assemble actin tails or spread from cell to cell. Purified ActA linked to surface of beads enables formation of actin tails in the appropriate medium containing ATP, Arp2/3, ADF/cofilin, profilin and capping protein, demonstrating that ActA is required and sufficient to induce actin-based motility. ActA is structurally and functionally similar to members of the WASP family. It can trigger actin polymerization in the absence of WASP and seems to mimic the activation of the Arp2/3 complex by WASP.

ActA is asymmetrically distributed on bacterial surface. It binds Arp2/3 and the resulting activation of this complex initiaties *de novo* actin polymerization at the bacterial surface. Polymerization is further promoted by VASP, which recruits profilin that is bound to ATP-actin monomers, and thereby increases

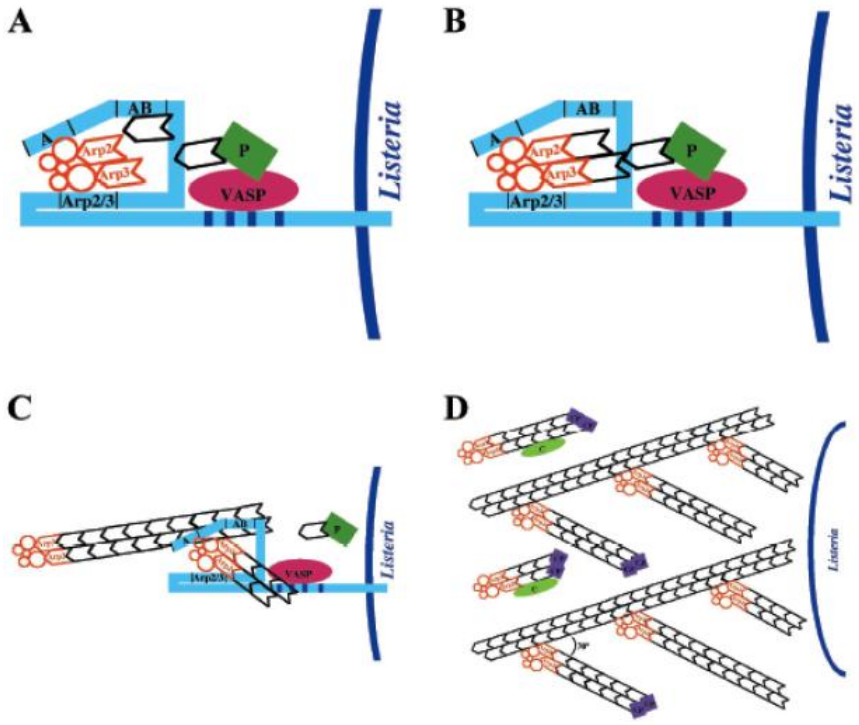

Figure 51: Model of actin tail assembly by *Lysteria.* ActA (light blue) is anchored with the 26 C-terminal aa in the bacterial membrane. It binds Arp2/3 via its Arp2/3 binding domain and possibly the acidic domain (A), and an actin monomer via its actin-binding domain (AB). The proline-rich repeat is required for VASP binding. VASP recruits profilin (green). Growth of actin filament is terminated by capping protein (violet). At a distance, from the bacterial surface, filaments debranch and, with the assistance of the actin-severing protein cofilin (light green), depolymerize, thereby maintaining a local pool of actin monomers.

local concentration of actin monomers. Therefore, actin monomers are brought in close vicinity to active Arp2/3 and enhance the initiation of actin polymerization. Barbed end remains uncapped for a short period, allowing fast growth of filament. Original Arp2/3 is released, and another Arp2/3 binds ActA at the side of an existing filament, creating a branched network of actin, where Arp2/3 is present throughout the length of actin tails. ActA seems to have Arp2/3 independent actin polymerization activities as well. Movement on host cells probably starts with an Arp2/3 dependent nucleation step, followed by an Arp2/3 independent elongation step.

Shigella: Forms an actin tail morphologically similar to *Listeria* one, containing short, bundled actin cables. IcsA is required and sufficient to mediate actin tail assembly. IcsA is anchored in the outer membrane and targeted directly to old pole of bacterium. It mimics N-WASP activation by Cdc42-GTP, inducing conformational changes that release N-WASP from its autoinhibited conformation. Expression of N-WASP in the host cell is required for *Shigella* infection, in N-WASP$^{-/-}$ cells, or cells overexpressing dominant-negative mutants of N-WASP, no actin tails are assembled by *Shigella*. The fact that *Shigella* fails to infect cells exclusively expressing WASP shows its specificity. Remarkably, IcsA is restricted to one bacterial pole, and is absent from the actin tail itself, suggesting that it is not released from bacterial surface, in contrast to what has been shown for N-WASP.

Rickettsia: Obligate intracellular bacteria targeting the microvascular endothelium. Infection leads to increased microvascular permeability leading to cerebral and pulmonary edema. Entry to host cell is

followed by rapid escape into cytoplasm. Members of the spotted-fever group also show intracellular movement based on actin polymerization (5-8 µm/min), which is significantly slower than *Listeria*. Actin tail is only generated on the rickettsial pole, propelling the bacteria across the cytoplasm and into neighboring cells. RickA protein works as WASP analogue. In contrast to *Listeria*, the actin is composed of parallel actin bundles and not a branched actin network, with Arp2/3 only localizing to the bacteria-tail interface and not to the length of the actin tail. RickA *in vitro* is able to crosslink actin, which means that other bacteria or host factors must play a role in forming unbranched arrays of actin filaments observed in the tails. VASP might play a role, since it has shown to compete with capping proteins at barbed ends, thereby increasing length of actin filaments.

Vaccinia virus: Double-stranded DNA virus, poxviridae, natural host unknown, vaccine against smallpox (caused by variola virus) -> eradication in 1980. Replication cycle:

- Attachment of virions to cell surface; two different forms, intracellular mature virus (IMV) and extracellular enveloped virus (EEV), differ in structure and surface proteins;
- Majority is endocytosed in a pH-dependent manner
- Small fraction fuses directly with plasma membrane
- Delivery of virus core into cytoplasm
- Transported on MTs from the cell periphery to central regions
- Cascade of early, intermediate and late viral gene transcription
- Assembly of new virions
- Four types of virions during whole cycle: IMV, intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and EEV

IMVs most abundant form, retained in cells until cell death, then released by cell lysis. IMVs are stable and robust, and well suited to transmit infection within infected organs and between hosts. Possibly, IMVs can exit host by budding mechanism.

IEVs (intermediate between IMV and CEV/EEV) are formed by wrapping of IMVs with two additional lipid bilayers (from Golgi or endosomal

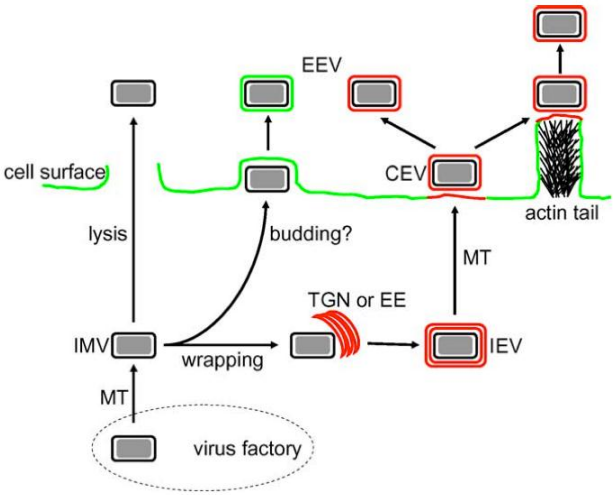

Figure 52: Release mechanisms of vaccinia virions. IMV either released from cell upon lysis or transported via MT to site near MTOC where they are wrapped by intracellular membranes from TGN or EE. IMV may also bud from membrane. IEVs are transported via MT to cell surface, fuse. From then on, CEV exposed by exocytosis. CEV either induce polymerization of actin tail or released as EEV.

membranes). Via MTs, they are transported to the host membrane, where they bud from the cell shedding one membrane. The double membrane virions remain bound to the membrane and are called CEVs. These can induce formation of actin tails within the host cell. Virions at the tips of actin tails move with a speed of 2.8 µm/min away from the cell and are propelled by this mechanism to surrounding cells. Alternatively, the surface virion may be released as EEV mediating the long-range dissemination of virus.

In contrast to *Listeria* and *Shigella*, vaccinia induces local signaling involving the Src family kinases at the plasma membrane. The EEV-specific protein A36R is phosphorylated asymmetrically on the virus envelope. This mimics Src-family tyrosine kinases in the molecular induction of actin polymerization, and is thought to promote recruitment of a preformed complex. consisting of the cellular proteins Nck (adaptor, consisting of three SH3 and a single SH2 domain), WIP (WASP interacting protein) and N-WASP. WIP interacts with N-WASP, profilin, and globular and filamentous actin. It stabilizes actin filaments and participates in filopodia and lamellipodia formation. The complex accumulates beneath CEV at the plasma membrane and promotes actin polymerization in host cell. This results in the formation of an actin tail and subsequent propulsion of the virus away from the cell. Thus, actin tail is not produced to allow intracellular movement of the virus, but is an intracellular structure.

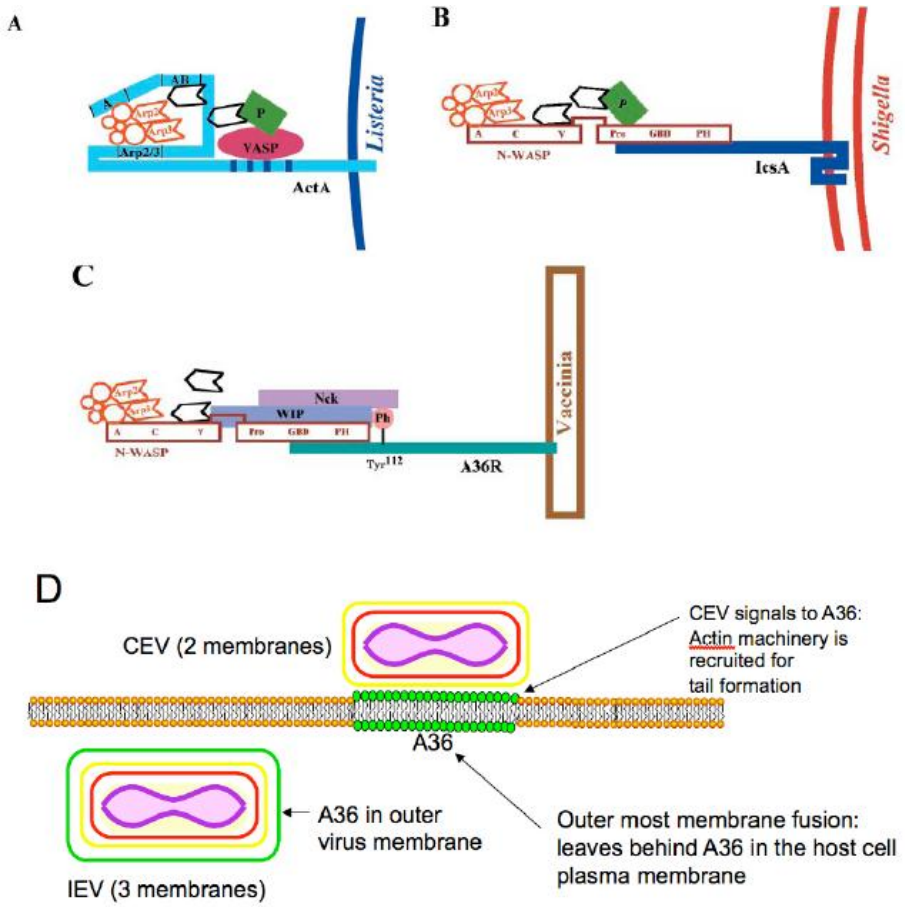

Figure 53: Comparison of the different mechanisms used by *Listeri* (a), *Shigella* (b) and vaccinia virus (c) to promote actin-tail formation. (d) Illustration of the membranes involved in the exiting fusion step forming CEV.

Summary:

- *Listeria* expresses the N-WASP mimicking protein ActA asymmetrically on its surface

- *Shigella* IcsA protein mimics Cdc42-dependent activation of cellular N-WASP
- Vaccinia triggers the Src-family tyrosine kinase signaling pathway leading to phosphorylation of A36R protein -> subsequent recruitment and activation of N-WASP (complex with Nck and WIP)
- All pathogens appear to use Cdc42 pathway and downstream Arp2/3 complex, but enter at different steps

Xenopus egg ectracts: Endogenous vesicles can nucleate actin tails and undergo actin-based motility in a Cdc42-dependent manner. This tail formation requires tyrosine-phosphorylation, N-WASP and Arp2/3. Interestingly, actin-based motility of vesicles has also been observed *in vivo*.

Listeria -> *Shigella* -> vaccinia -> mammalian vesicles: Complexity to form actin tail increases, evolutionary tendency. Increasing complexity means increasing chance to interfere with mechanism or control needs to be more stringent in more complex systems.

Motility and Spontaneous Symmetry Breaking

After entry of mobile bacteria into cytoplasm, WASP remains attached to the bacteria and Arp2/3 will distribute throughout the actin tail (branch points). So the growing barbed ends localize to the bacteria, while the depolymerizing pointed ends localize to the end of the tail. The elastic Brownian ratchet model described can also be used to explain the movement of bacteria.

In vitro, it is possible to reconstitute the actin tail using chemically inert polystyrene beads (mimicking bacterial body), that are coated with the Apr2/3 activator ActA -> biomimetic system. Minimal motility medium supporting the propulsion of N-WASP-coated beads is composed of Arp2/3 complex, profilin, capping protein, ADF/cofilin and F-actin. Despite the homogenous distribution of activating proteins on bead surface, an actin tail pushing the bead forward is assembled.

- Cloud of F-actin forms around coated beads
- Cloud transforms into actin tail extending exclusively from one pole of the bead
- Spontaneous symmetry break *in vitro*

This break seems to be due to release of elastic energy in the actin gel. Interpretation: Repetitive actin layers are synthesized at the bead surface. Therefore, already existing layers will be progressively pushed outwards by the new layers that form directly on the surface. 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.

During tail growth, the actin gel continuously undergoes deformations. Depending on the protein composition in the motility medium, deformations arise from either gel elasticity or monomer diffusion through the actin comet. These findings demonstrate that actin-based movement is governed by the mechanical properties of the actin network, which are fine-tuned by proteins involved in actin dynamics and assembly.

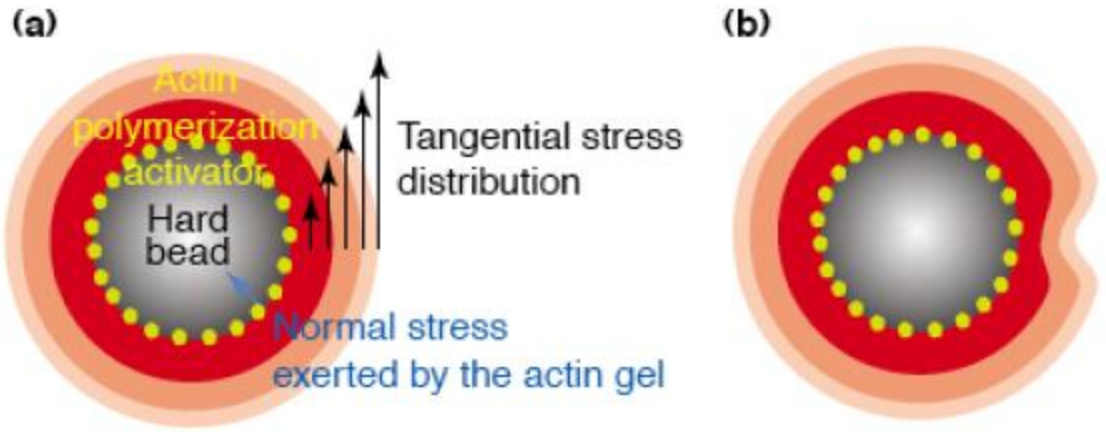

Figure 54: Model for spontaneous symmetry breaking on beads coated with an actin polymerizing factor. Newly synthesized actin filaments are depicted in red, older ones in fading red tones.