MARKKU HAKALA

Revising the actin disassembly machinery

The role of GMF and twinfilin in turnover of dendritic actin arrays

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ACADEMIC DISSERTATION

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Preface

This thesis has taught me a lot. This is especially for the reason that I was able to collaborate with so many fantastic scientists along the way. If you have courage to read further, you will find out that this study utilized approaches like mutagenesis, biochemistry, cell biology, MD simulations and genetics, among others. At this point it's (probably) safe to admit that I didn't know much of many of these topics and methods when I started my studies. It's also (again, probably) safe to say that nowadays I know a lot more. With all these different techniques, there was also a new expert joining my projects either by collaborating with me or by supervising me, and I couldn't be more thankful for all of you who have helped me during my studies.

When I started PhD studies, I had two projects to choose from. From researchers who had been involved in these projects, I received very encouraging advices: "stay away from them if you ever want to graduate!" Luckily, my supervisor had a second opinion. So, I started working on with twinfilin. Probably the project would have been quite awful without the great guidance I received from Pekka. I can't imagine anyone who would be more enthusiastic about the project, and who would more eager be ready to talk about results and problems. So, thank you, Pekka, for taking me to your lab and for believing me for all these years. The project proved to be very exciting, as you promised. This was a long road and, I think, pretty much everything we guessed about the functions of twinfilin along the way were incorrect. But on the other hand, it was much more exciting this way.

Beside Pekka, there was a continuous support from the whole Lappalainen lab. It has been a privilege to solve mysteries of actin dynamics with a group of so talented and intelligent researchers. As a PhD student, I have learnt so much from the broad expertise in the group. For few former lab members, I need to give a special, huge thanks, for their help and support. First, from Hongxia and Yosuke I learned basically everything I know about lipids and protein-lipid interactions. Thus, your help and advices were very crucial for me to succeed. I'm sure I will need many of those skills in the future as well. Thank you for both of you for your time and patience with my endless questions. I'm happy to know you have been successful in your careers after leaving Pekka's lab, and quite sure our paths will cross again at some point.

Especially in the beginning of my thesis work, and already during my master's, I had long and profound discussions with Minna. I was very lucky to have you in our "office", as I probably learned more of actin, actin dynamics, cell migration and other actin-related and non-related thingies, by discussing with you than I would have learnt by reading any of the books. I admire your willingness to discuss about science and to get excited about unanswered questions. This is a skill I am planning to borrow from you in my new adventures. Thanks for taking me into your GMF project, you did great job to introduce me to Drosophila genre. I even started to like those little creatures. I'm sure that if you hold on to this excitement towards science, mentoring and teaching you will be very successful in your career and life as well. Thank you for all your help, support and guidance!

It's really hard to find correct words to my fellow PhD students of Lappalainen lab. I couldn't wish for better peer support during this long road. I have always been able to count on you both in good and in bad times. Pekka might think our coffee breaks are too long (hmm…) and they are too frequent (well…), but for me they kept me going and actually helped to solve many problems and questions. Jaakko, your expertise in imaging and interest to improve both your skills and also to share your knowledge is admirable. Also. I appreciate your efforts to get me climbing or to

gym more often. Hope you keep on asking. Kasia, I really miss your dark humour and our endless discussions about different ways to sell kidneys for money. It's always good to have a good backup plan. I was very happy to know you have found a job and there's probably no need to sell any of body parts at this point. Reena, I admire your passion to search and learn new things, whether they are science related or non-related. Whenever I needed to work late in the lab, I could count on that you were keeping me company. I'm sure we'll find that slot for the tennis, eventually, and I'm looking forward for all those bicycle trips we have planned to do in Switzerland, Italy, and France. Tommi, your honesty and openness have been crucial for our group dynamics. If there is anyone who I can rely on to ask for help, it is you. Not because you always promise to do it, but because if you promise to do it, I can trust your word. Thank you for all of you for these great moments. And thanks for all those gin-and-tonics, too. I'm quite sure there will be many more to come.

For all current and former members of Lappalainen lab: Elena, Kostya, Yaming, Geri, Mirva, Johan, Lina, Shrikant, Sari, Anna-Liisa (I'm sure I'm missing someone), thank you for each and every one of you for your great support and advices whenever I needed them. We all come from so many different background and cultures, and I have learnt so much from all of you. I hope we stay in touch also in a future, wherever it may lead us. Take care all of you! I also want to thank members of Vartiainen lab for your help and support, and especially for the supply of reagents whenever I have needed. Last but not least, I want to thank my former students Mitro and Mari, who were helping me a lot in lipid and mammalian cell projects, respectively. I'm sure you both have great futures ahead of you, whatever you will decide after your own PhD studies.

I want to thank also my collaborators in Helsinki and Paris for their great support during this PhD. From Ilpo Vattulainen and his group at Department of Physics, especially from Maria and Giray, I learned everything I know from membrane biophysics and simulations. Have to say, that learning curve was steep. It was great to have you and your expertise in the paper. Next time if I decide to collaborate with MD simulation experts I at least can speak their language already in beginning. I also want to acknowledge Romet-Lemonne/Jegou lab at Institute Jacques Monod. Guillaume, Antoine and Hugo, huge thanks for you all for all that hard work. I hope I can visit your institute at some point and bring you a collection of wine to celebrate our achievements. My special thanks go to the whole ProLipids community. Past six years in the Centre of Excellence in Biomembrane research taught me a lot, and also change my career plans and research interest from the actin field more towards the membrane research. I'm very grateful for this experience and for all the people in Lappalainen, Vattulainen and Ikonen groups. Thank you!

This study would not have been possible without excellent microscopy core facilities in Viikki and Meilahti. I want to thank especially Kimmo, Mika, Marko and Harri in Viikki Light microscopy unit, as well as Mikko and Kirstin at Biomedicum imaging unit for all your help and support along the way. There are also bunch of students, post-docs and group leaders in both Viikki and Meilahti who have been ready to chat with me and giving me instructions whenever I have needed them. I want to thank especially Juha Saarikangas and Ville Paavilainen for your comments and suggestions for my projects, and advices in search for post-doctoral positions. Special thanks to Leo Almeida-Souza and An-Sofie Lenaerts for your hospitality in Cambridge and your countless advices of what I should take into account when moving abroad. And your honest view on Brexitland.

I was lucky to have so many nice people around me during my undergraduate and PhD studies. I will probably forget to mention half of you, and I apologize for that. Anni, Tea, Pauliina, Aino Tuomas K, Markus S, Tuomas A, Kalle M, Tuomas O, Elli, thanks for all of you for those great memories and support, all the coffee, all the bird watching, bicycle fixing, nice gigs and music

festivals and whatever there has been in past years. Thanks to Ewelina, Elina, Mridul, Siggi, Darshan, Behnam, Kul, Isabel, Otto, Lydia and many others in ILS and/or DPBM for sharing those good and bad times of PhD life. Tapanila climbing crew: Nalle, Johanna and Sergio, thanks for keeping me alive. I hope we have many nice climbing trips ahead of us.

Many, many thanks for all those great moments for Kerjääjät band. Playing with you was so much fun and helped me to shift my thoughts from research to something completely different at least once a week. So, big thanks to Otso, Johanna, Antti, Lauri, Valtteri, Martta, Anna, Juan, Mari, Tadeu, and Jorge for keeping up with my fluctuating tempo, all those nice tours in Finland, UK and Germany, and for countless bad jokes. Hope I get change to see you playing many times in coming years with new people on board. Big thanks to the Pukkila gang, Marja, Hanna, Tomi, Henriikka and Toni (yes, I count Askola here as well) for staying in my life since primary school. Even though we meet nowadays very (too) seldom, it always feels there is a special connection between us. Thanks for all those lovely moments. I hope, and I know there will be many more to come.

To my family, Sanja, Olavi and Ilkka, I first of all owe you an apologize for all those excessively long working days. Sanja, things haven't always been too easy for us and combining two PhD thesis and two kids has been quite a task. But we managed, and I know we manage many other things as well after this. Olavi and Ilkka, te kaksi olette minulle tärkeimpiä ihmisiä maailmassa ja minä odotan innolla tulevia vuosia ja uusia seikkailuja teidän kanssa. To my parents, Eino and Sirkka, en voi kylliksi kiittää kaikesta tuesta jonka olen saanut teiltä. Kiitos! Eiköhän nämä opinnot nyt olleet tässä eli ei tarvitse enää kysellä milloin minä oikeastaan valmistun. Thanks to my brothers and sisters as well for everything. Outi, Pirjo, Toivo, Janne, Erkki, Pekka and Mikko, I'm lucky to have all of you as people who I can really count on in every turn and everytime when I need any help. Thank you!

I would like to end by acknowledging those who helped me to get this thesis over the line. Beside my supervisor, professor Kari Keinänen was a familiar face to me in last few months. Docent Maria Vartiainen fulfilled my thesis committee with Kari, and I thank you both for your great comments and suggestions in our meetings. I got lots of valuable comments and suggestions for my thesis from Associate Professor Diana Toivola and Professor Jari Ylänne. I thank you for the time and effort you gave to the thesis, which, based on your emails, accumulated heavily on evenings and weekends.

I want to express my deepest thanks to my opponent, Associate Professor Alexis Gautreau, who seemingly spent his holiday on reading my thesis. Thank you for accepting my invitation and putting the effort on this examination. Finally, I hope you all enjoy the show as much as I do. And, of course, I hope enjoy reading the thesis.

Abstract

Polymerization of actin filaments against cellular membranes and contraction of actomyosin fibers generate pushing and pulling forces for cell migration, endocytosis, cell division, and maintenance of cell morphology, as well as for intracellular motility and morphogenesis of organelles. Thus, the actin cytoskeleton is a fundamental cellular component in development, immune responses, and in several other aspects of physiology. Moreover, the actin cytoskeleton is hijacked by viruses and pathogens during the infection process. Owing to their central role in above-mentioned cellular processes, actin and actin-binding proteins have been in the limelight of cancer research.

Actin is a globular protein, which can polymerize into filaments and depolymerize back to monomers. Dozens of actin binding proteins regulate actin dynamics in cells. Whereas regulation of actin filament nucleation and filament elongation are relatively well understood, the disassembly is far more enigmatic topic. ADF/cofilin is the key actin disassembly factor. It belongs to a family of six actin depolymerizing homology (ADF-H) domain proteins, which all interact with actin or actin-related proteins. However, apart from ADF/cofilin, biochemical and cellular functions of members of this protein family have remained elusive.

In this work, I studied the cellular and biochemical roles of two ADF-H domain proteins, glia maturation factor (GMF) and twinfilin. I show that they both promote the disassembly of dendritic actin networks in cells, but by distinct mechanisms. GMF, which binds actin-related proteins (Arp) in the Arp2/3 complex, debranches dendritic actin networks *in vitro*. The data presented here show that GMF regulates the dynamics of lamellipodial, dendritic actin network in *Drosophila* cells and promotes collective border cell migration in vivo. Moreover, *Drosophila* GMF display a strong genetic interaction in cells and *in vivo* with another actin-regulatory protein, actin-interacting protein 1 (Aip1), indicating that they facilitate actin disassembly in a synergistic manner.

Twinfilin interacts with actin monomers and actin filament barbed ends to inhibit actin polymerization. Moreover, it binds heterodimeric Capping Protein (CP) and membrane phosphatidylinositol phosphates (PIPs), which inhibit the actin-binding function of twinfilin. However, the molecular mechanism of this interaction has remained unknown. Thus, in the second part of the thesis I utilized a combination of mutagenesis and biochemistry, supplemented with molecular dynamics simulations, to reveal how PIPs inhibit twinfilin. Interestingly, twinfilin interacts with PIPs with a two-step mechanism. First, the CP-interaction motif in the carboxy-terminal tail of twinfilin anchors the protein to plasma membrane. Subsequently, the actin-binding interface interacts with lipids, leading to inhibition of both the CP- and actin-binding activities of twinfilin.

Cellular functions of twinfilin have remained elusive despite extensive studies in past decades. In the third part of the thesis, I generated mouse twinfilin knockout cell lines and showed that twinfilin regulates both actin and CP turnover in lamellipodia. Surprisingly, twinfilin promotes CP dynamics in cells and in vitro by uncapping filament barbed ends, thus providing an explanation why the localization of CP in cells is restricted to the very distal edge of lamellipodia. Moreover, twinfilin itself does not accelerate filament depolymerization after uncapping, but instead allows filaments to disassemble after removal of CP from actin filament barbed ends, explaining the diminished filament disassembly rates in twinfilin-deficient cells.

Together, the work presented here highlights the important roles of twinfilin and GMF in regulation of lamellipodial actin networks. Their distinct roles in actin disassembly show that actin turnover in dendritic arrays is maintained by several functionally different proteins which, in concert, facilitate the turnover of branched actin filament networks in cells.

Tiivistelmä

Aktiinisäikeiden solun kalvorakenteita vasten tuottama työntövoima sekä aktiini- ja myosiinikimppujen synnyttämä kimppujen supistumisvoima ylläpitävät muun muassa solujen liikkumista, kalvoliikennettä, jakautumista sekä solujen muodon ja rakenteen säilymistä. Näin ollen solujen aktiinitukiranka on välttämätön muun muassa yksilönkehityksessä ja immuunipuolustusjärjestelmässä. Useat virukset ja taudinaiheuttajat hyödyntävät aktiinikoneistoa päästäkseen soluun sisälle. Lisäksi aktiinitukirangan merkitys edellä mainituissa solubiologisissa tapahtumissa on tuonut aktiinin ja aktiinitukirankaa säätelevät proteiinit syöpätutkimuksen valokeilaan.

Aktiini on pallomainen proteiini, joka kykenee pidentymään pitkiksi säikeiksi ja purkautumaan yksittäisiksi monomeereiksi. Aktiinisäikeiden pidentyminen ja sen säätelijät tunnetaan varsin hyvin. Sen sijaan purkautumiseen osallistuvat proteiinit ja niiden rooli on huonommin tiedossa. Useat tutkimukset viime vuosikymmeninä ovat osoittaneet, että ADF/kofiliinilla on keskeinen rooli aktiinisäikeiden purkautumisen säätelyssä. ADF/kofiliini kuuluu kuuden proteiinin muodostamaan proteiiniperheeseen, jotka kaikki sitoutuvat joko aktiiniin tai aktiinin kaltaisiin proteiineihin. Toisin kuin ADF/kofiliinin, muiden tämän perheen jäsenten biokemialliset ja solubiologiset toiminnot ovat huonosti ymmärrettyjä.

Tässä tutkielmassa tutkin kahden tämän perheen proteiinin, GMF:n ja twinfiliinin, solubiologisia ja biokemiallisia toimintoja. Näytän, että ne molemmat osallistuvat haaroittuneiden aktiinisäieverkostojen purkautumiseen omilla hyvin erilaisilla tavoilla. GMF, joka sitoutuu aktiinin kaltaiseen proteiiniin (Arp) Arp2/3-kompleksissa, purkaa aktiinisäieverkostojen haaroja. Tämän tutkielman tulokset osoittavat, että GMF säätelee solun levyjalan aktiinisäikeiden kierrätystä ja on tärkeässä roolissa rajasolujen liikkumisessa banaanikärpäsen munakammion kehittymisen aikana. Lisäksi GMF:n ja toisen aktiinia säätelevän proteiinin, Aip1:n, geeniluennan samanaikainen hiljentäminen johti aktiinisäikeiden kertymiseen sekä viljellyissä soluissa että kärpäsen munakammioissa.

Aiemmin on osoitettu, että twinfiliini sitoutuu sekä yksittäisiin aktiinimonomeereihin että aktiinisäikeiden nopeasti kasvaviin pluspäihin, estäen näin säikeiden pidentymistä. Tämän lisäksi twinfiliini sitoutuu aktiinisäikeiden pluspäihin sitoutuvaan CP-tulppaproteiiniin ja solukalvon PIP-lipideihin. PIP-lipidit estävät twinfiliinin sitoutumisen aktiiniin, mutta tämän säätelyn tarkka mekanismi on ollut tuntematon. Tässä työssä käytimme puhdistettuja proteiineja biokemiallisissa kokeissa sekä hyödynsimme tietokonemallinnusta selvittääksemme, miten twinfiliini sitoutuu PIP-lipideihin. Tuloksemme osoittavat, että twinfiliini sitoutuu lipideihin kaksiosaisella mekanismilla. Aluksi twinfiliinin häntä ankkuroi proteiinin solukalvoon, minkä jälkeen loppu proteiini aktiininsitoumisalueineen sitoutuu kalvoon. Näin ollen twinfiliinin kyky sitoa aktiinia ja CP:ia estyvät sen sitoutuessa solukalvon PIP-lipideihin.

Twinfiliinin tarkka rooli aktiinisäikeiden säätelyssä soluissa on jäänyt toistaiseksi epäselväksi. Tässä tutkielmassa käytin hiiren soluja, joista olin estänyt twinfiliinin geenin ilmentymisen mutaatiolla, ja vertasin näitä soluja villityyppisiin soluihin. Näin osoitan, että twinfiliini säätelee sekä aktiinisäikeiden että CP:n dynamiikkaa solujen levyjaloissa. Twinfiliini poistaa CP:n aktiinisäikeiden plus-päistä ja siten edistää aktiinisäikeiden purkautumista soluissa. Tämä havainto selittää sen, miksi CP paikantuu solujen levyjalassa aivan solukalvon lähelle ja sen, miksi CP:n dynamiikka on huomattavasti nopeampaa soluissa kuin mitä sen biokemialliset ominaisuudet ennustavat. Tulokset selittävät myös sen, miksi aktiinisäikeiden lyhentyminen on hitaampaa soluissa, joista twinfiliinin ilmentyminen on estetty mutaatiolla.

Tämä väitöskirjatyö osoittaa, että twinfiliinillä ja GMF:llä on tärkeä rooli solujen aktiiniverkostojen säätelyssä. Niiden hyvin erilaiset roolit aktiinisäikeiden purkautumisen säätelyssä osoittavat, että aktiinisäikeiden kierrätystä ylläpitää soluissa useat proteiinit yhteistyössä toistensa kanssa.

Abbreviations

ABP1	actin-binding protein 1
ADF	actin depolymerizing factor
ADF-H	actin depolymerizing factor homology
ADP	adenosine diphosphate
ADP-Pi	adenosine diphosphate-phosphate
Aip1	actin-interacting protein 1
Arp	actin related protein
ATP	adenosine triphosphate
BAR	bin-amphiphysis-rsv
CAP	cyclase-associated protein
CME	clathrin-mediated endocytosis
CP	heterodimeric Capping Protein αβ
C-terminal	carboxy-terminal
DPH	1,6-diphenyl-1,3,5-hexatriene
Drebrin	developmentally regulated brain protein
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescence protein
Ena/VASP	enabled/vasodilator-stimulated phosphoprotein
ERM	ezrin-radixin-moesin
F-actin	filamentous actin
FH2	formin-homology 2 (domain)
FMNL	formin-like (family of formin proteins)
FRAP	fluorescence recovey after photobleaching
G-actin	globular (monomeric) actin

GMF	glia maturation factor
Hsp70	heat shock protein 70
mDia	mammalian diaphanous-related formin
NPF	nucleation promoting factors
N-WASP	neural Wiscott-Aldrich syndrome proteins
PIP	phosphatidylinositol phosphate
SCAR	suppressor of cAMP receptor
WASH	Wiscott-Aldrich syndrome protein and SCAR homolog
WASP	Wiscott-Aldrich syndrome proteins
WAVE	WASP and verprolin homology
WDS	WISH/DIP/SPIN90

Contents

List of original publications

This thesis work is based on the following original articles, which are referred in the text by their roman numerals

I. Poukkula M, **Hakala M**, Pentinmikko N, Sweeney MO, Jansen S, Mattila J, Hietakangas V, Goode BL, Lappalainen P. GMF promotes leading-edge dynamics and collective cell migration in vivo. *Curr Biol*. 2014, 24:2533-40

II. **Hakala M**, Kalimeri M, Enkavi G, Vattulainen I, Lappalainen P. Molecular mechanism for inhibition of twinfilin by phosphoinositides. *J Biol Chem*. 2018, 293:4818-4829

III. **Hakala M**, Wioland H, Tolonen M, Jegou A, Romet-Lemonne G, Lappalainen P. Twinfilin uncaps filament barbed ends to promote turnover of actin filament networks. *BioRxiv* 2019

Contribution of the author to each publication

I. M.H. performed *GMF*, *Aip1* and *p16* depletions in S2R+ cells, as well as imaging and image analysis for figure 3. M.H. purified mGMF-γ for experiments in figure 1.

II. M.H. performed all experiments except molecular dynamics simulations in figure 4 and supplemental figures S3-S7. M.H., M.K., G.E., and P.L. wrote the manuscript with contribution from other authors.

III. M.H. performed all experiments except single filament assays in figures 3, 6 and 7. M.H. and P.L. wrote the manuscript with contribution from other authors.

1. Review of the literature

1.1. The actin cytoskeleton

Since actin was discovered in the early 1940's in the laboratory of Albert Szent-Györgyi in series of publications that eventually led to purification of the protein by Brúnó F. Straub [reviewed in (Bugyi and Kellermayer, 2019)], it has become evident how integral cellular component actin is throughout the evolution with its homologues existing also in archaea and bacteria (Cabeen and Jacobs-Wagner, 2010; Velle and Fritz-Laylin, 2019). Actin is a critical player in cell motility, vesicle transport and endocytosis, cell division, maintenance of cell polarity and shape, as well as in muscle cell contraction, just to name few. Recently, the potential of actin has spread beyond cells, with its properties emerging as solutions for electrical connections (Galland et al., 2013) and parallel-computation systems (Nicolau et al., 2016).

Vertebrates express six isoforms of actin. These include skeletal and cardiac muscle α-actin isoforms, smooth muscle α- and γ-isoforms, and non-muscle β- and γ-isoforms (Dominguez and Holmes, 2011). These isoforms differ only on few amino acids, especially in the N-terminal region of proteins (Herman, 1993). *Drosophila* has six actin genes, which encode two isoforms of cytoplasmic actin, larval muscle actin, and adult muscle actin (Fyrberg et al., 1981, 1980). Structurally, actin belongs to a superfamily of sugar kinases, hexokinases, heat shock protein 70 (Hsp70), actin-related proteins (Arp) and prokaryotic actin-like proteins, which all contain a common ~375-amino-acid polypeptide folding into two α/β-domains (Dominguez and Holmes, 2011). These domains can be further divided into subdomains 1-4 (Kabsch et al., 1990).

The most fundamental property of actin is its ability to convert between a monomeric, globular actin (G-actin) (Kabsch et al., 1990) and a filamentous actin (F-actin) (Fujii et al., 2010; Holmes et al., 1990) states. Additionally, actin binds divalent cations, and nucleotides adenosine diphosphate (ADP) or adenosine triphosphate (ATP). Actin also act as an ATPase (Korn, 1982), giving rise to its property to polymerize into F-actin state and depolymerize to the G-actin state. ATPase activity of actin is prominent only in the F-actin state, where irreversible ATP hydrolysis (Carlier et al., 1988) acts as an indicator of filament aging and triggers depolymerization when F-actin is in the ADP state. The hydrolysis of ATP is rapid process with a halftime of ~2 seconds (Blanchoin and Pollard, 2002), whereas the dissociation of phosphate group from the newly formed ADP-Pi transition state is relatively slow (Carlier and Pantaloni, 1986). Thus, it is thought that most subunits in F-actin are in the ADP-Pi form, which resembles the ATP-actin form from the

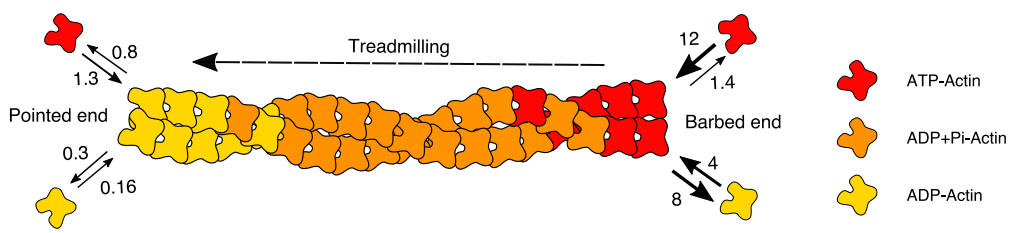

Figure 1. Actin filament assembly, disassembly and treadmilling. Association constants have unit of μM-1s-1 and dissociation constants unit of s-1. Direction of treadmilling of actin subunits in the filament is indicated with a dotted arrow. Adapted from Pollard & Borisy (2003) Cell. 112:453-465.

biochemical point of view (Pollard and Borisy, 2003). Notably, the kinetics of ATP hydrolysis and phosphate dissociation from ADP-Pi-actin in cells are not known.

Myosins are a large family of actin-dependent molecular motors that interact with actin and utilize energy from the ATP-hydrolysis to generate contractile movement (Sellers, 2000). Myosin-decorated F-actin resembles an arrowhead shape in electron micrographs (Moore et al., 1970), giving rise to the nomenclature of barbed and pointed ends for different ends of F-actin. Due the irreversible ATP hydrolysis, and different association and dissociation rate constant at barbed and pointed ends for ATP- and ADP-G-actin (Pollard, 1986), filaments polymerize at barbed ends and depolymerizes at pointed ends at the steady state conditions (figure 1). This leads to slow treadmilling of actin along the filament from barbed ends to pointed ends (Fujiwara et al., 2002). However, the spontaneous depolymerization and polymerization of F-actin are far too slow for the actin cytoskeleton to function in its many cellular assignments. Thus, several actin binding proteins, consisting over 60 classes of proteins, regulate the physiological behaviour of actin (Pollard and Borisy, 2003).

1.2. Actin filament populations in mammalian non-muscle cells

Mammalian non-muscle cells have several different actin filament populations (figure 2) that provide different types of forces to enable various functions of cells (Lehtimäki et al., 2017; Svitkina, 2018b). A pushing force is produced by the coordinated polymerization of actin filaments against cellular membranes. It is generated by polymerization of either branched filament networks or parallel, linear actin filament bundles. A pulling or contractile force is provided via sliding of motor protein myosin II along thick antiparallel actin bundles. The third force, the resistance, maintains the cellular shape via cross-linking branched actin filament networks to cellular membranes (Svitkina, 2018a).

1.2.1. Dendritic actin networks

Branched or dendritic actin networks are present at the leading edge of migrating cells, where the structure is called lamellipodium (Abercrombie et al., 1970), at the sites of endocytosis (Collins et al., 2011), and in specialized cellular structures, such as in the growth cone of neuronal axons (Svitkina, 2018a). The basic structure of the actin cytoskeleton in these above-mentioned processes is highly similar. Lamellipodium, a protrusive, actin-rich structure that serves as a major cellular element in cell migration and navigation, is filled with branched actin filaments (Small et al., 1978). These filaments are attached to sides of each other with a 70° angle between barbed ends of two filaments (Svitkina et al., 1997). Importantly, barbed ends in the lamellipodial actin network are facing the plasma membrane, explaining why polymerization of filaments generates protrusive force for cell migration, which is the main function of lamellipodia in motile cells (Small et al., 1978). During endocytosis, branched network of actin provides force to overcome the membrane tension barrier in the endocytic vesicle formation (Boulant et al., 2011).

1.2.2. Parallel actin filament bundles

Cells have various parallel actin bundles depending on the cell type and environment. Individual filaments in these structures are cross-linked to each other and attached to the plasma membrane

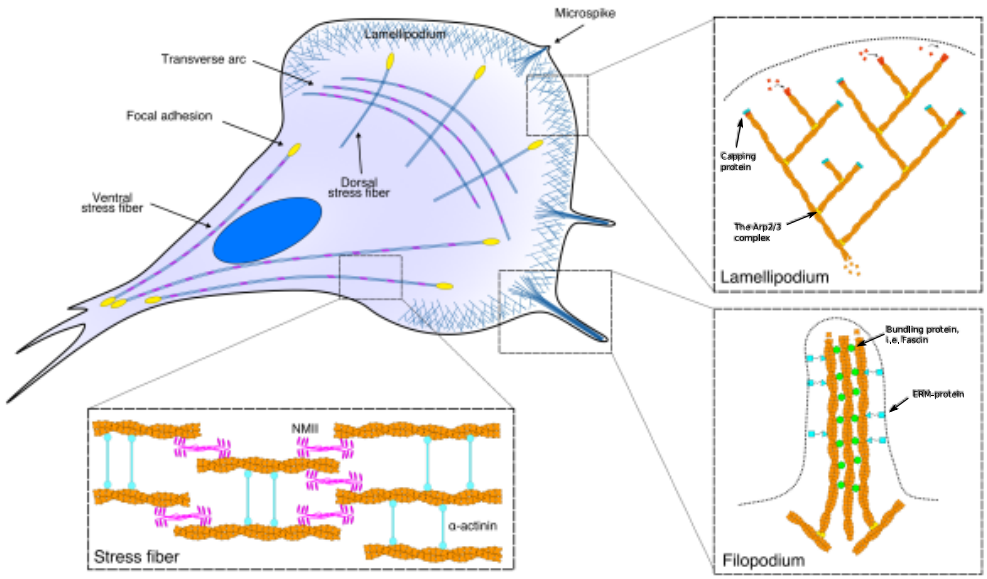

Figure 2. Actin filament populations in motile, non-muscle mammalian cells. Lamellipodium consists short, branched network of filaments. In filopodium, filaments are long and parallel, whereas in stress fibers actin filaments display an anti-parallel orientation.

by ezrin-radixin-moesin (ERM) proteins, enabling the generation of protrusive force against the membrane (Vignjevic et al., 2006; Yamashiro-Matsumura and Matsumura, 1986). Microvilli and stereocilia are relatively stable protrusive structures in the intestinal epithelium and inner ear sensory hair cells, respectively. Actin filaments in both of these structures are tightly bundled to each other. Microvilli contain ~30 parallel actin filaments, whereas in stereocilia the density of filaments is much higher and can reach to over 300 filaments (Mooseker and Tilney, 1975; Tilney et al., 1980). Filopodia are the most thoroughly studied cellular protrusions formed by parallel actin bundles. They are finger-like, long and unbranched protrusions rising from the lamellipodium, and contain bundled actin filaments that elongate through the entire filopodium length (Small et al., 1978). The main function of filopodia is to sense environment, and generate new attachments to the extra-cellular matrix through focal adhesions (Lehtimäki et al., 2017; Mattila and Lappalainen, 2008; Svitkina, 2018a). Filopodia undergo dynamic transition to microspikes and retraction fibers. These transitions are guided by protrusions and retractions of the surrounding lamellipodium (Svitkina et al., 2003).

1.2.3. Actin stress fibers

Stress fibers are bundles of 10-30 cross-linked anti-parallel actin filaments. When coupled with non-muscle myosin-II motor protein, they provide contractile force for cell motility. Stress fibers share features of muscle sarcomeres in that non-muscle myosin II forms discontinuous, filamentous stacks, which somewhat resemble myosin stacks of muscle cells (Fenix et al., 2016; Hu et al., 2017). Additionally, α-actinin and tropomyosins, which bundle and decorate filaments, respectively, show similar periodic pattern in stress fibers compared to muscle sarcomeres (Langanger et al., 1986; Lazarides, 1976). Based on their morphology and protein content, stress

fibers are classified to dorsal stress fibers, transverse arcs, ventral stress fibers, and perinuclear actin cap (Small et al., 1998; Tojkander et al., 2012).

Dorsal stress fibers are anchored to focal adhesions, which are multiprotein complexes facilitating the connection between cell and extracellular matrix (Burridge et al., 1988). However, dorsal stress fibers do not contain myosin II, and thus do not contract. Instead, they appear to act as precursor for other, contractile stress fiber populations (Hotulainen and Lappalainen, 2006; Tojkander et al., 2011). In contrast to dorsal stress fibers, transverse arcs contain a periodic pattern of α-actinin and myosin II. However, they are not attached to focal adhesions and thus do not directly produce contractile force to the extracellular environment. During cell migration, transverse arcs display a retrograde flow from cell periphery towards the center of the cell (Hotulainen and Lappalainen, 2006; Small et al., 1998; Tojkander et al., 2015). Ventral stress fibers are considered as mature stress fibers, which possess periodic arrangement of α-actinin and myosin II, and are attached to extracellular environment through focal adhesions (Burridge, 2017). Hence, they can transmit the contractile force from stress fibers to the extracellular matrix. The assembly of ventral stress fibers was shown to be partially regulated by mechanical tension (Tojkander et al., 2018). The perinuclear actin cap is composed of stress fibers that elongate from the leading edge towards the cell rear. They are connected to the extracellular matrix through focal adhesions and to a nuclear envelope through a linker of the nucleoskeleton and cytoskeleton complex (LINC) (Khatau et al., 2009). The exact role of the perinuclear actin cap have remained unclear. They are thought to transmit mechanical stimuli between the extracellular matrix and the nucleus, guide a nuclear orientation during cell migration and constrain the nucleus upon cell shape changes (Khatau et al., 2009; Maninova et al., 2017; Shiu et al., 2018).

1.2.4. Cortical actin network

The actin cortex at cell periphery is a relatively disordered network of actin filaments and non-muscle myosin-II (Bray and White, 1988). Due presence of myosin-II, the cortical actin network has contractile properties, and it regulates the cell surface tension for example during cell division (Chugh et al., 2017; Stewart et al., 2011). At least in motile fibroblasts, the cell cortex seems to contain different populations of actin filament bundles and branched actin networks, whose ratio might change over time. Thus, these different actin populations provide different mechanical properties for the cortex (Eghiaian et al., 2015) and maintain the cell polarity during migration (Ramalingam et al., 2015). During certain modes of cell migrations, the cortical actin network has important function in formation of cell surface blebs (Bovellan et al., 2014; Paluch and Raz, 2013). However, the precise role and underlying molecular mechanisms of the cortical actomyosin network dynamics in above-mentioned processes are still incompletely understood.

1.3. Actin in action

Considering the amount of different actin populations in mammalian cells, it is not surprising that they are involved in wide variety of cellular processes. Perhaps the best characterized example is muscle contraction, where the powerstroke of myosin motor protein pulls actin filaments to provide force for body movements (Sweeney and Hammers, 2018). In non-muscle cells, the actin cytoskeleton promotes cell division (Mangione and Gould, 2019), endosome recycling (Simonetti and Cullen, 2019), organelle dynamics (Venkatesh et al., 2019), and developmental processes such as meiosis (Uraji et al., 2018). Actin monomers and filaments are present also in the

nucleus, where they act as components of chromatin remodelling machinery, regulate activities of transcription factors, and participate in DNA repair (Kyheröinen and Vartiainen, 2019; Virtanen and Vartiainen, 2017). Here, I discuss in more detail two fundamental cellular processes where actin is involved: cell migration and endocytosis.

1.3.1. Cell migration

Cell migration is one of the most fundamental physiological processes during development, immune response, wound healing and maintenance of tissue morphology. However, uncontrolled cell migration is also one of the hallmarks of cancer (Hanahan and Weinberg, 2011, 2000). To migrate, cells need force to translocate their body mass, organelles and cytoplasm towards the direction of migration, and generate attachments to their environment to transform cellular forces to movement.

Depending on the cell type and environment, cells utilize different strategies to migrate through tissues. The migration modality depends on a variety of determinants of extracellular matrix, including dimension, stiffness, orientation, and its components. Moreover, cellular determinants, such as cell-cell and cell-matrix contacts, cytoskeleton polarity, cell stiffness and ability to provide pericellular polarity, are key aspects controlling which migration mode cells utilize. In two-dimensional environment, cells migrate utilizing adhesion-independent amoeboid or adhesion-dependent mesenchymal migration mode either as individual cells or as a collective group of cells. (Friedl and Wolf, 2010). In more complex three-dimensional environment, such as in tissue or in extra-cellular matrix, cells migrate in amoeboid, mesenchymal, lobopodial, and collective mode (Yamada and Sixt, 2019).

Mesenchymal migration is an adhesion-dependent migration mode, where cells generate leading edge protrusions, mainly lamellipodia and filopodia in two-dimensional migration, through polymerization of actin filaments against the plasma membrane (Schaks et al., 2019). To translocate, cells need to pull their cytoplasm and organelles towards the direction of migration. This process requires force provided by the actomyosin bundles, and generates traction force, which is proportional to properties of extra cellular matrix, strength of cell-matrix adhesion, and the pulling force of stress fibres. Thus, different cell types, such as fibroblast and neutrophils, generate different amounts of traction forces to translocate their cytoplasm (Beningo et al., 2001; Smith et al., 2007). To convert the pulling force to motion, cells need to attach to their environment. This is achieved most commonly through focal-adhesions, which generate connections between cells and extracellular matrix (Changede and Sheetz, 2017).

In contrast to mesenchymal cell migration mode, the amoeboid migration is not dependent on adhesions between cells and extracellular matrix. The exact mechanism of amoeboid migration remains enigmatic, and few alternative models have been suggested. Cells lacking specific attachments to extracellular matrix can exhibit rounded protrusions, often referred as membrane blebs, towards the direction of migration by utilizing their contractile actin cortex (Paluch and Raz, 2013; Petrie and Yamada, 2012). A bleb-like protrusions can provide poorly-adhesive "structural" anchoring, also called "elbowing", for actomyosin contraction and cell rear retraction (Charras and Paluch, 2008). A recent study suggested an alternative model for amoeboid migration mode, where macrophage cells lacking adhesions to the extracellular environment use membrane trafficking from the cell rear towards the cell front. This membrane trafficking is coupled with increased endocytosis at the back of the cell (O'Neill et al., 2018) Several cell types, such as various mesenchymal cells, leukocytes and tumor cells, can undergo transition between mesenchymal and

amoeboid migration modes (Lämmermann et al., 2008; Liu et al., 2015; Ruprecht et al., 2015; Wolf et al., 2003). Thus, it seems the cell migration mode is not strictly cell type dependent, but is determined both by features of migrating cells as well as by the composition and architecture of the extracellular environment.

Collective cell migration is a special mode of cell motility, where a group of moving cells generate connections and affect one another at least during some of the time. The degree of cell connections and interplay during collective migration varies significantly depending on the cell type (Rørth, 2009). Collective cell migration is important in developmental processes, where large tissue structures are re-shaped, but also during the regeneration of epithelial tissues and in the epidermal wound closure. Additionally, collective cell invasion is common feature of several cancer types (Montell, 2008; Rørth, 2009). In certain types of collective cell migration, such as in vascular sprouting, cells at the leading front of the group specialize into "leader cells", whereas other cells act as "followers". Leader cells sense the environmental cues and generate protrusions towards the direction of migration, whereas follower cells form tight cell-cell connections between each other and leader cells through cadherin proteins. However, in many other types of collective migration, the position of individual cells is not as strictly determined (Friedl and Gilmour, 2009; Haeger et al., 2015). A popular and well-studied model system for collective cell migration is a border cell migration in *Drosophila* ovary. Border cells are a small cluster of about 8 cells that delaminate at the anterior epithelium of a *Drosophila* egg chamber and migrate in between nurse cells to the posterior side of egg chamber near the oocyte. There, border cells form a structure called micropyle, which is important for fertilization of the egg (Montell, 2003; Rørth, 2009). During migration, the border cell cluster generates long actin-dependent lamellipodia-like protrusions (Verkhusha et al., 1999) and cell-cell contacts with surrounding nurse cells through E-cadherin adhesion proteins (Niewiadomska et al., 1999). Finally, border cells utilize myosin-dependent tractions to squeeze their way between nurse cells (Geisbrecht and Montell, 2002). The precise regulation of the actin dynamics and cellular protrusions during border cell migration has remained largely elusive.

1.3.2. Endocytosis

In endocytosis, small membrane vesicles bud off from the plasma membrane and are transported to inner parts of the cell. Endocytosis is used for transportation of several different cargo molecules from the plasma membrane into the cell, and is important for cellular processes such as nutrient uptake, cellular and developmental signalling, and the maintenance of membrane homeostasis (McMahon and Boucrot, 2011; Sigismund et al., 2012). Additionally, several viruses hijack the endocytosis machinery to enter the cell (Helenius, 2018; Mercer et al., 2010). Different endocytosis pathways are named based on the coat protein involved, with clathrin-mediated endocytosis (CME) being the most extensively studied pathway to date (Kaksonen and Roux, 2018). During CME, a vesicle of ~100 nm in diameter is pinched off from the plasma membrane in a process that lasts ~60 s. Dozens of proteins, including clathrin itself, clathrin adaptor proteins and, coat proteins, as well as actin and actin-binding proteins, arrive and leave the clathrin-coated pit in specific sequence and precise timing, which are evolutionarily constrained (Kaksonen and Roux, 2018; Weinberg and Drubin, 2012).

Clathrin and its adaptor proteins are sufficient to generate the initial membrane curvature (Chen et al., 1998; Dannhauser and Ungewickell, 2012; Saleem et al., 2015; Stachowiak et al., 2012). The bin-amphiphysis-rsv (BAR) domain proteins recognize different types of membrane curvatures at the site of endocytosis to further support and enhance membrane bending (David et al., 1996; Kishimoto et al., 2011) and to enhance actin filament polymerization (Almeida-Souza

et al., 2018). In yeasts, CME is depended on force provided by the actin cytoskeleton (Ayscough, 2000; Ayscough et al., 1997; Kübler and Riezman, 1993). In mammalian cells, however, the role of actin is not as clear as in yeasts, since inhibition of actin dynamics does not always lead to a complete inhibition of endocytosis in cultured cells (Fujimoto et al., 2000; Gottlieb et al., 1993). However, actin seems to be necessary for mammalian cell endocytosis to overcome the increased membrane tension barrier (Boulant et al., 2011), and inhibition of actin dynamics was shown to interrupt several stages of CME, including the clathrin pit formation and vesicle internalization (Yarar et al., 2005).

In CME, actin filaments polymerize to surround the endocytic invagination (Collins et al., 2011; Mulholland et al., 1994). Filaments are branched (Collins et al., 2011) and consist of several actin binding proteins found also in lamellipodia (Kaksonen et al., 2006), suggesting that endocytic and lamellipodial actin networks share both structural similarities and similar regulation mechanisms. In both yeast and mammalian cells, actin filaments start to polymerize at the sites of endocytosis only after the clathrin coat has already formed (Kaksonen et al., 2005; Merrifield et al., 2002; Taylor et al., 2011). Thus, it seems that actin is not important in early stages of clathrin coat formation, but is instead involved in the last steps of endocytosis events, namely vesicle budding and membrane scission (Kaksonen and Roux, 2018). Actin polymerizes at the base of the endocytic pit with filament barbed ends oriented against the plasma membrane and drives the endocytic internalization (Akamatsu et al., 2020; Kaksonen et al., 2003; Picco et al., 2015).

Apart from endocytic invagination, actin has a fundamental role also in endosome recycling. Canonically, the cargo is transported first from endocytic sites to early endosomes, where they are sorted back to the plasma membrane, to the endosomal recycling complex or to late endosomes and further to lysosomes (Simonetti and Cullen, 2019). Several studies show that actin cables and dendritic actin patches are present throughout the endosome recycling pathway (Gauthier et al., 2007; Huckaba et al., 2004; Nakagawa and Miyamoto, 1998; Pol et al., 1997). Actin is linked to multiple processes, such as vesicle fusion, endosome biogenesis and cargo sorting (Kjeken et al., 2004; Morel et al., 2009; Muriel et al., 2016; Ohashi et al., 2011). However, the precise role of the actin cytoskeleton in endosome trafficking remains elusive.

Endocytosis and cell migration machineries are linked to each other through recycling of membrane protein complexes involved in cell-cell and cell-matrix contacts. In mesenchymal cell migration depended on integrin-mediated cell-matrix contacts, integrins are recycled through clathrin- and caveolin-mediated endocytosis to the cell interior (De Franceschi et al., 2015). Similarly, cadherin adhesion receptors, which maintain cell-cell contacts during collective cell migration, are recycled through both caveolin- and clathrin-mediated endocytosis pathways (Brüser and Bogdan, 2017; Cadwell et al., 2016). Indeed, N-cadherin-containing adherent junctions undergo continuous treadmilling during collective cell migration, which is supported by the intake of N-cadherin at the rear of the cell through endocytosis (Peglion et al., 2014). These examples highlight the complexity of functions of the actin cytoskeleton that should be considered when dissecting the possible phenotypes of individual actin regulators in cell migration and endocytosis.

1.4. Regulation of actin dynamics

Actin interacts with a vast amount of proteins. In fact, it is involved in more protein-protein interactions than any other known protein (Dominguez and Holmes, 2011), highlighting the complexity of its regulation. However, only nine actin-regulating proteins are conserved during

evolution from the protozoan parasites, such as *Leismania* or *Trypanosoma*, to yeasts, *Dictyostelia* and animals. These proteins include formins, the actin-related protein (Arp)2/3 complex, profilin, actin-depolymerizing factor (ADF)/cofilin, twinfilin, actin-interacting protein 1 (Aip1), cyclase-associated protein (CAP), heterodimeric Capping Protein (CP), and coronin (De Melo et al., 2008). Moreover, in the reconstituted motility system only few proteins of either *Listeria* or *Shigella*, namely the Arp2/3 complex and its activator, ADF/cofilin, profilin, and CP, are required to generate the actin-dependent motility (Loisel et al., 1999). These above-mentioned proteins form a core group of actin regulators that facilitate most fundamental phases of actin dynamics: filament nucleation, branching and capping, as well as polymerization, depolymerization, and severing of filaments.

1.4.1. Filament nucleation and branching

Nucleation of new actin filaments is a critical to define the architecture of the actin cytoskeleton. To simplify very complex regulatory mechanism, I will focus here on two main classes of actin nucleators: the Arp2/3 complex, which nucleates branched actin networks, and formins, which nucleate linear actin filament (Pollard, 2007).

The Arp2/3 complex, a multiprotein complex consisting of two actin related proteins and five other subunits (Machesky et al., 1994), facilitates filament branching by binding to the side of a "mother filament" where it nucleates a new "daughter filament" (Mullins et al., 1998; Welch et al., 1998) (figure 3 A). The angle between "mother" and "daughter" filament in the Arp2/3-nucleated actin network is ~70° (Mullins et al., 1998; Svitkina et al., 1997). The Arp2/3 complex localizes to the lamellipodial region in motile cells (Machesky et al., 1997; Welch et al., 1997), and depletion or inhibition of the Arp2/3 complex in yeast, mammalian and *Drosophila* cells leads to loss of dendritic actin filament networks (Bailly et al., 2001; Rogers et al., 2003; Winter et al., 1997). Moreover, the Arp2/3 complex nucleates endocytic dendritic actin filament networks (Benesch et al., 2005; Picco et al., 2015; Taylor et al., 2011). The Arp2/3 complex is inherently inactive and can be activated by nucleation promoting factors (NPFs), a diverse group of proteins with a distinct cellular localization pattern (figure 3). These different NPFs seem to serve as organelle-specific activators of actin assembly. The most studied families of NPFs are Wiscott-Aldrich syndrome proteins (WASP) and WASP- family verprolin homologue proteins (WAVE), which activate the Arp2/3 complex at the plasma membrane (Blanchoin et al., 2000a; Machesky et al., 1999; Machesky and Insall, 1998; Winter et al., 1999). Moreover, Wiscott-Aldrich syndrome protein and SCAR homolog (WASH) family of NPFs activate the Arp2/3 complex on the surface of endosomes (Derivery et al., 2009; Gomez and Billadeau, 2009). The importance of NPFs is highlighted in several studies, where depletion or inhibition of WASP, N-WASP and WAVE proteins impaired various cellular processes, such as lamellipodia formation, endocytosis, dorsal and peripheral membrane ruffling, and cell motility (Agathon et al., 2003; Innocenti et al., 2005; Legg et al., 2007; Steffen et al., 2006; Suetsugu et al., 2003). NPFs are further regulated by Rho GTPases and membrane phosphoinositides. These topics are discussed in following chapters. Beside NPFs, the Arp2/3 complex is regulated also by other proteins, such as cortactin and arpin. Arpin inhibits the activity of the Arp2/3 complex and regulates the directional persistence of cell migration (Dang et al., 2013). In contrast, cortactin promotes nucleation of new actin filaments by activating and stabilizing the Arp2/3 complex (Uruno et al., 2001; Weaver et al., 2001).

Formins are a conserved family of proteins, which contain unique formin homology domain (FH2) and several other domains (Chesarone et al., 2010; Zigmond, 2004). Purified FH2 domain catalyses nucleation of actin filaments from purified actin monomers *in vitro* (Pruyne et al., 2002;

Sagot et al., 2002). In cells, formins promote nucleation of linear actin filaments independently from the Arp2/3 complex (Evangelista et al., 2002; Sagot et al., 2002; Watanabe et al., 1999) (figure 3 B). Compared to the Arp2/3 complex, formins are a diverse group of proteins with at least two genes in fungi, 15 in mammals and over 20 in plants (Chesarone et al., 2010). Thus, it is not a surprise that formins contribute to many cellular processes, such as cytokinesis (Castrillon and Wasserman, 1994), stress fiber assembly (Hotulainen and Lappalainen, 2006), lamellipodia and filopodia formation (Kage et al., 2017; Schirenbeck et al., 2005; Yang et al., 2007), focal adhesion formation (Kobielak et al., 2004), and vesicle transport (Gasman et al., 2003).

Formins and the Arp2/3 complex with its activators balance each other in nucleation of different actin filament arrays in cells. In yeast, inhibition of the Arp2/3 complex leads to increase in formin-nucleated actin filament populations, whereas inhibition of formins have the opposite effect (Burke et al., 2014). However, recently it was shown that also the Arp2/3 complex can nucleate linear actin filaments when bound to WISH/DIP/SPIN90 (WDS) proteins (Balzer et al., 2019, 2018), whereas FMNL formins are shown to enhance the lamellipodial protrusion force by nucleating and elongating filaments (Kage et al., 2017). These observations indicate that the linear and branched actin arrays could be nucleated by both formins and the Arp2/3 complex in an orchestrated fashion.

1.4.2. Regulation of filament elongation

The control of filament elongation is crucial for maintaining the architecture and functions of actin networks. Many proteins contribute to filament elongation by either enhancing or inhibiting filament polymerization.

Enabled/vasodilator-stimulated phosphoproteins (Ena/VASP) localize to lamellipodia and filopodia tips in animal cells (Rottner et al., 1999) and promote filament elongation both *in vitro* and in cells (Bear et al., 2002) (figure 3 C). Thus, they for example regulate the length and dynamics of filopodia (Barzik et al., 2014) and protrusion of lamellipodia (Dimchev et al., 2017) The ability of Ena/VASP to enhance filament elongation was suggested to rise from its ability to protect filament barbed ends from CP, which inhibits filament elongation (Barzik et al., 2005). Later, it was shown that Ena/VASP enhances elongation of filaments by directly interacting with actin filament barbed ends and actin monomers. Thus, Ena/VASP proteins processively add actin monomers to growing barbed ends (Breitsprecher et al., 2011, 2008) and remain associated to barbed end during filament elongation (Winkelman et al., 2014). In addition to actin filament nucleation, formins also enhance filament elongation by both protecting barbed end from capping, and by permitting addition of actin monomers to the growing filament end (Zigmond et al., 2003) (figure 3 B). Similar to Ena/VASP, formins remain associated with barbed ends during filament elongation (Kovar and Pollard, 2004).

Profilin is one of the first actin-binding proteins described (Carlsson et al., 1977), and since then multiple interaction partners, including Ena/VASP proteins, WAVE complex, formins, and several other proteins containing proline-rich domains or motifs, are described for profilin (Krishnan and Moens, 2009; Witke, 2004). Profilin interacts with ATP-actin monomers and enhance filament elongation (Carlsson et al., 1977; Pantaloni and Carlier, 1993), as well as accelerates nucleotide exchange in actin monomer from ADP to ATP (Goldschmidt-Clermont et al., 1992). Later, cyclase-associated protein (CAP), a multidomain protein which binds both ADP- and ATP-actin monomers, as well as profilin-ATP-actin complex, was found to catalyse nucleotide exchange in actin monomers. Moreover, due its higher affinity to substrate ADP-actin monomers than profilin,

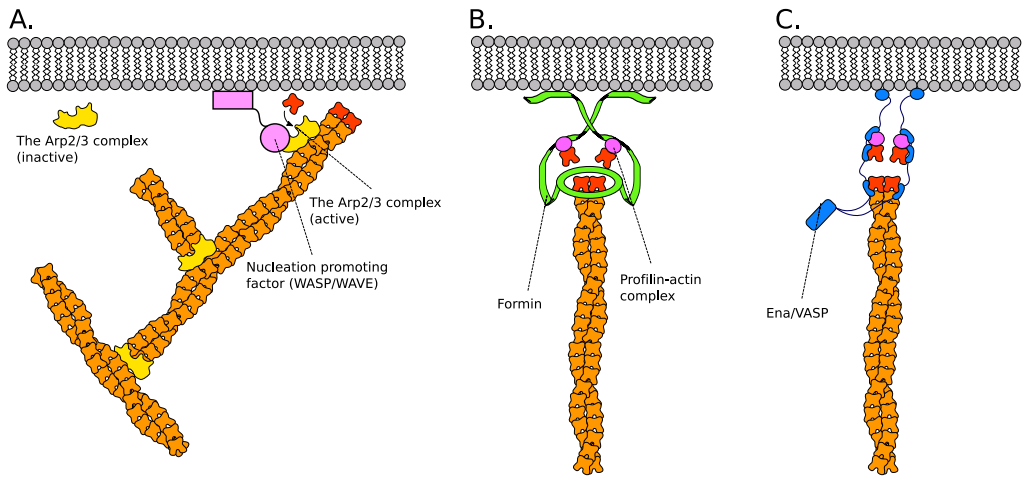

Figure 3. Nucleation of and elongation of actin filaments. (A) The Arp2/3 complex, which is activated near plasma membrane by NPFs, promotes nucleation of new branches to pre-existing actin filaments. (B) Formins nucleate linear actin filaments. Both formins and (C) Enabled/vasodilator-stimulated phosphoproteins (Ena/VASP) enhance elongation of linear actin filaments by promoting addition of profilin-actin complexes to the growing barbed ends of filaments.

CAP is thought to be more efficient nucleotide exchange factor than profilin (Balcer et al., 2003; Kotila et al., 2018; Mattila et al., 2004; Moriyama and Yahara, 2002). Profilin is required for the processive function of formin (Romero et al., 2004). It was shown that profilin promotes formin- and Ena/VASP-mediated actin filament assembly by delivering ATP-actin monomers these nucleation and elongation factors over the Arp2/3 complex (Rotty et al., 2015; Suarez et al., 2015).

The capping of filament barbed ends is crucial to maintain the architecture of short, branched nature of dendritic actin networks. Several proteins function as barbed end cappers, with the heterodimeric Capping Proteins (CPs) being the most prominent ones. CP binds to the barbed end of filament with nanomolar affinity and inhibits both filament polymerization and depolymerization (Schafer et al., 1996). CP, which is a heterodimer of α and β subunits and a homolog of a striated muscle cell CapZ, is abundant in all cell types and species with a concentration of 1-2 μM (Edwards et al., 2014). In cells, CP localizes to the edge of lamellipodia (Mejillano et al., 2004; Rogers et al., 2003; Schafer et al., 1998) and to endocytic actin structures (Kaksonen et al., 2005; Kim et al., 2004). Moreover, the depletion of CP disrupts the lamellipodial actin network morphology and protrusions, as well as cell migration (Iwasa and Mullins, 2007; Mejillano et al., 2004; Rogers et al., 2003; Sinnar et al., 2014). CP is very dynamic component of dendritic actin networks in cells (Iwasa and Mullins, 2007; Lai et al., 2008; Miyoshi et al., 2006) and its association to barbed ends is regulated by several proteins (Edwards et al., 2014). A protein V-1, also called myotrophin, binds CP and inhibits its association to actin filaments by binding directly to the actin filament binding site in CP (Bhattacharya et al., 2006; Taoka et al., 2003). Since V-1 is present in cells in micromolar concentration, it was suggested that most of CP in cells would remain bound to V-1 (Shekhar et al., 2016). Capping Protein Interaction (CPI) motif proteins, such as CARMILs, bind CP and release it from V-1 protein through an allosteric competition to promote filament capping (Edwards et al., 2015; Fujiwara et al., 2014). Additionally, CPI-motif proteins enhance dissociation of CP from actin filament barbed end *in vitro* (Fujiwara et al., 2010; Uruno et al., 2006). However, their role in promotion of CP dissociation from filament barbed ends in cells

has not been shown. Interestingly, CP displays much more rapid dynamics in cells compared to *in vitro* studies (Iwasa and Mullins, 2007; Lai et al., 2008; Miyoshi et al., 2006; Schafer et al., 1996), suggesting that additional factors regulating the CP dynamics in cells might exist.

As described above, CP competes in binding to filament barbed ends with both formins and Ena/VASP (Barzik et al., 2005; Zigmond, 2004). However, CP and mammalian diaphanous-related formin (mDia) 1 can simultaneously bind to actin filament barbed ends *in vitro*, and form a decision complex for filament capping and elongation (Bombardier et al., 2015). At least in yeast CP protects barbed ends in dendritic actin networks from formins, and thus maintains the identity of branched networks nucleated by the Arp2/3 complex (Billault-Chaumartin and Martin, 2019). Thus, the precisely regulated balance between local concentrations and activities of the Arp2/3 complex, formins, Ena/VASP and CP appears to be essential for the maintenance of the complex architecture of cellular actin filament arrays. Further studies are required to understand the effect of this complex interplay on regulation of balance between different actin populations *in vitro* and in cells.

1.4.3. Actin filament disassembly

The amount of polymerization-competent actin monomers defines the ability of actin filaments to elongate in processes described above. However, since the concentration of actin in cells is limited, efficient elongation needs to be coupled with filament disassembly to maintain the pool of polymerization-competent actin monomers. Several actin binding proteins facilitate filament disassembly by enhancing filament severing and depolymerization from pointed and barbed ends.

The key component of actin filament disassembly machinery is actin depolymerizing factor (ADF)/cofilin, which promotes disassembly of actin filament structures *in vivo* (Lappalainen and Drubin, 1997). ADF/cofilin binds actin filaments in cooperative manner with one ADF/cofilin interacting with one actin subunit in filament, which eventually leads to filament domains to be decorated by ADF/cofilin (Cao et al., 2006; De La Cruz, 2005; Suarez et al., 2011; Wioland et al., 2017). The binding of ADF/cofilin to filament induces torsional stress in filament by changing the flexibility and bending of filament (McGough et al., 1997; Prochniewicza et al., 2005; Wioland et al., 2018), which leads to severing of filament from the border of bare and ADF/cofilin decorated filament domains (Andrianantoandro and Pollard, 2006; Pavlov et al., 2007; Wioland et al., 2018). Additionally, ADF/cofilin-decorated filaments depolymerize from both barbed and pointed ends (Wioland et al., 2017). ADF/cofilins prefer to bind to ADP-actin instead of ADP-Pi- or ATP-actin, and in fact, binding of ADF/cofilin to actin filament enhances the release of the bound phosphate (Blanchoin et al., 2000b; Suarez et al., 2011). Thus, ATP hydrolysis and phosphate dissociation in the filament seems to serve as a 'clock' for ADF/cofilin mediated filament disassembly. Several biochemical studies have shown that actin-interacting protein 1 (Aip1) and coronin enhance the severing of ADF/cofilin-decorated actin filament and might serve as a trigger for fast filament disassembly (Gandhi et al., 2009; Gressin et al., 2015; Hayakawa et al., 2019; Jansen et al., 2015; Nadkarni and Brieher, 2014) (figure 4).

Recently, it was shown that cyclase-associated protein (CAP) promotes rapid depolymerization of ADF/cofilin-decorated actin filaments from their pointed ends (Kotila et al., 2019; Shekhar et al., 2019) (figure 4). Thus, ADF/cofilin seems to serve as a 'marker' for disassembly-competent filaments, and additional factors, such as Aip1 and CAP are required to trigger the rapid disassembly of filaments. The synergistical effect of ADF/cofilin, coronin, Aip1 and CAP in disassembly of actin filaments is also highlighted by studies done on mammalian and *Drosophila* cells, and in yeast.

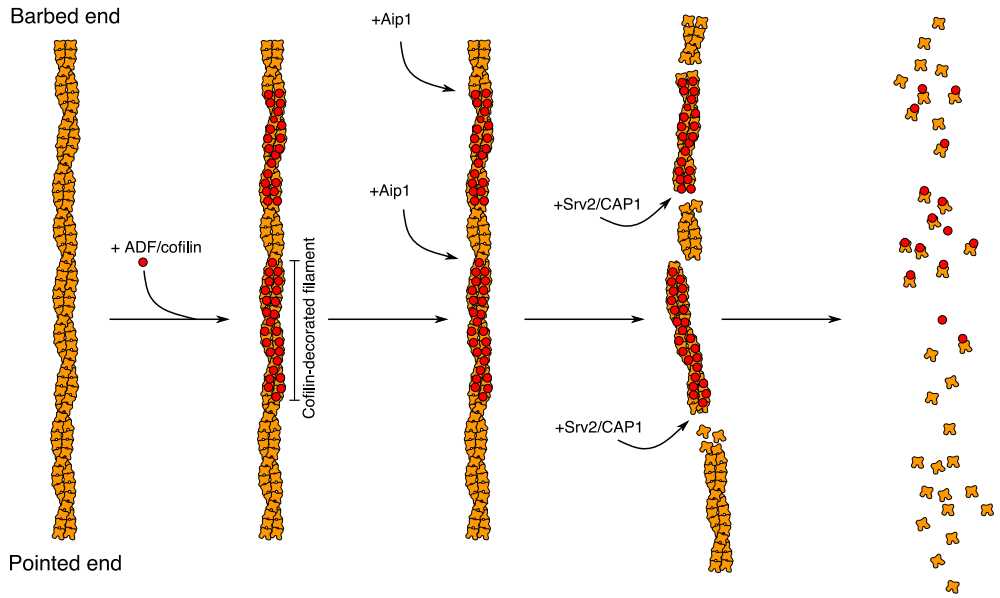

Figure 4. Actin filament disassembly. ADF/cofilin-decorated actin filaments are severed from pointed end side of the ADF/cofilin-decorated filament domain spontaneously and from barbed end side of decorated domain by Aip1. Free barbed ends of filaments depolymerize rapidly through spontaneous manner. ADF/cofilin-decorated filaments are depolymerized from their pointed end by CAP.

Depletion or inhibition of these proteins leads to accumulation of actin filaments, defects of the normal cell and actin cytoskeleton morphology, and reduced cell migration and endocytosis (Arber et al., 1998; Bertling et al., 2004; Hotulainen et al., 2005; Lin et al., 2010; Rogers et al., 2003). In summary, biochemical, cell biological and *in vivo* studies highlight how different actin disassembly factors work in concert, but with distinct roles, to maintain the normal actin filament turnover and cellular morphology.

1.4.4. Membrane phosphoinositides regulate actin dynamics

Membrane phosphatidylinositol phosphates (PIPs or phosphoinositides) have a key role in regulation of the actin cytoskeleton dynamics through a large amount of actin-binding proteins (Saarikangas et al., 2010; Senju and Lappalainen, 2019). Mammalian cells have seven phosphoinositide species with distinct subcellular localizations. PI(4,5)P2 is the most abundant phosphoinositide and comprises 0.3-1.5% of total lipid amount in the plasma membrane (Ferrell and Huests, 1984; Tran et al., 1993). Other phosphoinositides, namely PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(3,5)P2 and PI(3,4,5)P3, are present in the plasma membrane in significantly smaller amounts (Saarikangas et al., 2010). However, although the total amount of phosphoinositides in cells is relatively low, their local concentrations might increase through a stimulated synthesis of specific PIP species or through clustering of lipids at the membrane. For example, the local increase in PI(4,5)P2 concentrations were detected in lamellipodial protrusions and during phagocytosis (Botelho et al., 2000; Golub and Caroni, 2005; Ling et al., 2006).

Many proteins facilitating nucleation and polymerization of actin filaments, including N-WASP (Rohatgi et al., 2000), are active only when bound to PIPs (Saarikangas et al., 2010). Moreover, a WAVE2-induced lamellipodia formation was shown to be dependent on interaction of WAVE2

complex with membrane PI(3,4,5)P3 lipids (Oikawa et al., 2004). In contrast, several actin disassembly factors, including ADF/cofilin and CP, are inhibited by phosphoinositides (Kim et al., 2007; Saarikangas et al., 2010; Yonezawa et al., 1990). This inhibition occurs commonly through direct competition between actin and phosphoinositides for the shared binding surface in the actin-binding protein (Kim et al., 2007; Ojala et al., 2001; Yonezawa et al., 1991).

Actin-binding proteins interact with membrane phosphoinositides mainly through electrostatic interactions (Senju et al., 2017; Zhao et al., 2010). Interestingly, the increase in PI(4,5)P2 density of the membrane increases the affinity of actin-binding protein to membrane, which might affect they subcellular localizations and dynamics (Papayannopoulos et al., 2005; Senju et al., 2017). Moreover, the lipid binding domain of mDia1 and mDia2 formins is required for correct localization of these proteins near the plasma membrane in cells (Gorelik et al., 2011; Ramalingam et al., 2010), whereas disruption of Myosin X lipid-binding domain led to incorrect Myosin X localization in cells and impaired filopodia formation (Plantard et al., 2010). Together, these findings suggest that PIPs have important role in regulation of sub-cellular localization of actin-binding proteins.

Phosphoinositides are important regulators both in endocytosis and cell migration. The level of PI(4,5)P2 is changing during vesicle invagination in endocytosis and during lamellipodia protrusion and retraction in cell migration (Golub and Caroni, 2005; Sun et al., 2007). Importantly, acute depletion of PI(4,5)P2 leads to inhibition of clathrin-mediated endocytosis, as well as rapid retraction of lamellipodia (Idevall-Hagren et al., 2012; Zoncu et al., 2007). However, the complex role of phosphoinositides in regulation of actin dynamics and actin dependent processes, such as cell migration and endocytosis, is still incompletely understood.

1.4.5. Rho GTPases as actin regulators

Actin regulating proteins give rise to different actin filament populations. Thus, precise spatiotemporal regulation of different actin regulators is a necessity to control cellular processes, such as leading-edge protrusions and actomyosin contractions during cell migration. An important protein family regulating these processes is Rho GTPases, which consists 20 proteins in humans (Ridley, 2015). The best-known members of this family are RhoA, Rac1 and Cdc42. They all have a distinct effect on the actin cytoskeleton in cells, with activation of Rac1 inducing lamellipodia and membrane ruffle formation (Ridley et al., 1992; Wu et al., 2009), activation of Cdc42 the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995), and activation of RhoA the formation of stress fibers and focal adhesions (Ridley and Hall, 1992). However, Rac1, Cdc42 and RhoA all localize to lamellipodia of motile cells and have a complex relationship with each other (Machacek et al., 2009).

Both Rac1 and Cdc42 induce lamellipodia formation through the Arp2/3 complex nucleation factors WAVE and WASP/N-WASP, respectively (Miki et al., 2000; Miki and Takenawa, 2002; Suetsugu et al., 2003; Takenawa and Suetsugu, 2007). However, they also regulate actin disassembly by inactivating ADF/cofilin through LIM kinases (Oser and Condeelis, 2009; Yang et al., 1998). Thus, these two GTPases seem to regulate actin filament assembly and disassembly by partially overlapping pathways. Depletion of RhoA leads to loss of actin stress fibers, whereas its over-expression leads to diminished lamellipodia protrusions (Heasman et al., 2010). Different spatiotemporal activities of Rac1, Cdc42 and RhoA at leading edge of motile cells indicate that they regulate different stages of protrusion (Machacek et al., 2009). During leading-edge protrusion

phase, both Rac1 and Cdc42 become activated, whereas RhoA is inhibited. Before the retraction phase, RhoA gets activated to enhance retraction (Iseppon et al., 2015; Martin et al., 2016).

Activated RhoA promotes formation of focal adhesions and stress fibers by activating formin mDia1 (Amano et al., 1997; Hotulainen and Lappalainen, 2006), and results in increased contraction by activating myosin II through ROCK (Maekawa et al., 1999; Ridley et al., 2003). Similar to Rac1 and Cdc42, RhoA inhibits filament disassembly through LIM kinases (Maekawa et al., 1999). Interestingly, at the rear of the motile cell, RhoA is more active than Rac1 (Machacek et al., 2009; Schaks et al., 2019). Thus, there seems to be a negative crosstalk regulatory system between Rac1 and RhoA, which maintains both cell polarity and directional movement (Schaks et al., 2019).

Additionally, Rac1 and RhoA participate in regulation of actin dynamics through facilitating the asymmetry of membrane phosphoinositides. They both induce synthesis of PI(4,5)P2 in plasma membrane by activating PIP-kinase I (PIPK-I) that phosphorylates PI(4)P in the 5-position (Chatah and Abrams, 2001; Chong et al., 1994). Rac1 also activates phosphoinositide-3 kinase (PI3K), which in turn facilitates the productions of phosphoinositol-3,4,5-triphosphate [PI(3,4,5)P3] at the leading edge. Interestingly, PI(3,4,5)P3 in turn activates both Rac1 and Cdc42 through positive feedback, which is important for PI(3,4,5)P3-polarity in migrating cells (Weiner et al., 2002). In contrast, RhoA activates PTEN, an antagonist for PI3K, and could thus decrease the amount of PI(3,4,5)P3 at cell rear (Li et al., 2005). These processes further enhance the effect of Rho GTPases on cell polarity during cell migration.

1.4.6. Interplay between the actin cytoskeleton and membrane tension

Animal cells do not have cell wall to maintain their morphology, and thus, plasma membrane and the actin cytoskeleton act together in this fundamental process. The interplay of actin binding proteins and membrane phosphoinositides was discussed above. However, the actin cytoskeleton and plasma membrane are also in a mechanical interplay with each other, that affects both membrane tension and actin network morphology.

Increase in the membrane tension inhibits lamellipodia protrusion rate, whereas decreased membrane tension increases the rate (Raucher and Sheetz, 2000). Moreover, decrease in membrane tension was shown to result in formation of multiple lamellipodia in migrating cells (Lieber et al., 2013; Raucher and Sheetz, 2000). Protrusion of lamellipodia increases the membrane tension, which subsequently activates the exocytosis-mediated flow of lipids to plasma membrane to balance the increased tension (Gauthier et al., 2011). Interestingly, the lamellipodial actin network seems to undergo conformational changes according to the force it needs to overcome (Bieling et al., 2016; Mueller et al., 2017). This has led to the load adaptation model of lamellipodial actin network geometry (Svitkina, 2018b). However, the mechanism regulating these different states remains unknown. Collectively, these studies show that the actin cytoskeleton is controlled by both chemical and mechanical signals, which fine-tune its morphology and functions in different cellular processes.

1.5. The ADF-H domain protein family

Actin depolymerizing factor homology domain (ADF-H) proteins form an evolutionarily conserved protein family, whose members are found in protozoan parasites *Leishmania* and *Trypanozoma*, and eukaryotes such as yeasts, *Drosophila*, *Caenorhabditis elegans*, and animals (De

Melo et al., 2008; Lappalainen et al., 1998; Poukkula et al., 2011). The family consists six classes of proteins in vertebrates: ADF/cofilin, twinfilin, glia maturation factor (GMF), coactosin, actin-binding protein 1 (ABP1) and developmentally regulated brain protein (drebrin) (figure 5). ADF-H domain is a globular protein domain with a molecular weight of 13-19 kDa (Lappalainen et al., 1998). Despite distinct biochemical functions, ADF-H domain in different members of the protein family display high structural similarity (Poukkula et al., 2011).

ADF/cofilins and twinfilins bind both monomeric and filamentous actin, whereas coactosin, Abp1 and drebrin bind only actin filaments. GMF does not bind actin but actin-related protein in the Arp2/3 complex (Luan and Nolen, 2013; Poukkula et al., 2011). ADF-H domains can have two actin binding sites. The regions interacting with actin monomers are referred as "G-actin binding sites". At least in ADF/cofilins and twinfilins the G-actin binding site is conserved (Lappalainen et al., 1997; Paavilainen et al., 2008, 2002). F-actin interactions occurs through both "G-actin binding site" and additional "F-actin binding site" in the ADF-H domain (Lappalainen et al., 1997; Ono et al., 2001). Thus, much larger surface is required for interaction with F-actin than with G-actin. The functions of ADF/cofilins were discussed in chapter 1.4.3. Below, I discuss biochemical functions and cellular roles of GMF and twinfilin in more detail.

1.5.1. Glia-maturation factor

Glia-maturation factor (GMF) consists a single ADF-H domain and is a small protein with molecular weight of 17 kDa. Similar to other members of the ADF-H domain protein family, also GMF is highly conserved during evolution, and its orthologs have been found in yeast, *Dictyostelium*, *C.elegans*, *Drosophila* and mammals (Goode et al., 2018; Poukkula et al., 2011). The gene was duplicated during evolution and mammals have two GMF copies, which were named GMF-β and GMF-γ. The amino acid sequences of these orthologs are ~82% identical to each other (Asai et al., 1998) and their protein structures are highly similar (Goroncy et al., 2009).

Unlike other ADF-H domain proteins, GMF does not bind actin, but instead interacts with Arp2 subunit of the Arp2/3 complex (Gandhi et al., 2010; Luan and Nolen, 2013; Nakano et al., 2010). Two biochemical functions for GMF has been described. It inhibits the nucleation of new actin filaments by the Arp2/3 complex (Gandhi et al., 2010; Nakano et al., 2010) and stimulates debranching of daughter filaments generated by the Arp2/3 complex (Ydenberg et al., 2013).

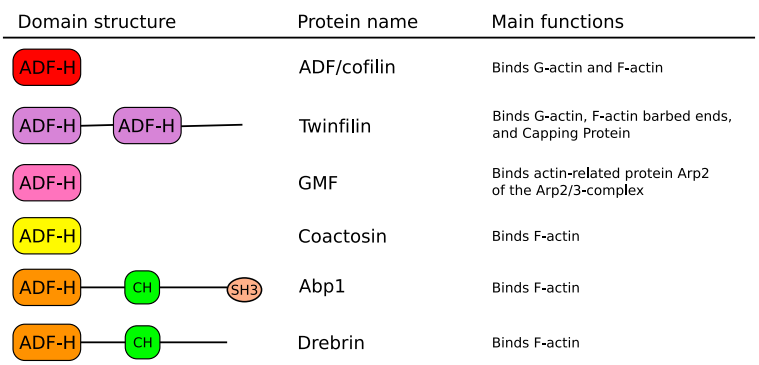

Figure 5. ADF-H domain proteins in vertebrates, their domain structures and main biochemical activities. Adapted from (Poukkula et al., 2011).

Interestingly, GMF utilizes a conserved sites which in other ADF-H domain proteins facilitates the interaction with actin monomers and filaments (Luan and Nolen, 2013; Ydenberg et al., 2013).

In all studied cell types, GMF is enriched in regions of high actin turnover. These include the leading edge of migrating mammalian cells (Aerbajinai et al., 2011; Ikeda et al., 2006; Lippert and Wilkins, 2012), where GMF co-localizes with lamellipodial proteins, such as the Arp2/3 and cortactin (Haynes et al., 2015). Silencing of GMF leads to unstable lamellipodia in neutrophils (Aerbajinai et al., 2011) and decrease in cell migration speed of T-lymphocytes (Lippert and Wilkins, 2012). It regulates directed cell migration also in monocytes (Aerbajinai et al., 2016). Interestingly, GMF regulates the distribution of the Arp2/3 complex and lamellipodia retraction rate in mammalian cells (Haynes et al., 2015), providing a possible explanation for defects in cell migration speed. In budding yeast, GMF localizes to the cortical endocytic actin patches (Gandhi et al., 2010; Nakano et al., 2010). The deletion of GMF alone does not show strong phenotype in yeast, but displays genetic interaction with cofilin in cell growth (Gandhi et al., 2010). Overexpression of GMF leads to re-organization of actin structures both in budding yeast actin patches and in lamellipodia of mammalian cells (Gandhi et al., 2010; Haynes et al., 2015).

GMF was, misleadingly, originally identified as a differentiation factor in brain extract (Lim et al., 1989). Mammalian GMF isoforms are, however, intracellular proteins that are present in several tissues. GMF-β is most abundant in brains, lungs, spleen, colon and thymus, whereas protein levels of GMF-γ are highest in spleen, colon and thymus and significant also in lungs (Ikeda et al., 2006; Inagaki et al., 2004; Tsuiki et al., 2000; Zaheer et al., 1993). GMF-β null mice are viable but show defects in motor skills and learning due to loss of neurons in inferior olive (Lim et al., 2004), suggesting an important role in development or maintenance of central nervous system and neuronal plasticity. This is also supported by the fact that GMF-β is upregulated during axonal regeneration after physical injury or exercise (Bosch et al., 1989; Yin et al., 2018; Zaheer et al., 2006). GMF was also linked to inflammation response and Alzheimer's disease (Kempuraj et al., 2013; Zaheer et al., 2007, 2013). Finally, GMF is linked to development in zebrafish, where the knockout of GMF-γ leads to defects in angiogenic sprouting (Zuo et al., 2013) and to progression and metastasis of serous ovarian and colorectal cancer as well as glioma metastasis and progression (Kuang et al., 2016; Li et al., 2010; Wang et al., 2017). However, the precise mechanism by which GMF contributes to these cellular processes remains elusive.

1.5.2. Twinfilin

Twinfilin genes in human and mouse were first, misleadingly, described as a tyrosine kinases (Beeler et al., 1997, 1994), but were soon defined as molecular weight of 37-40 kDa proteins consisting of two ADF-H domains, which are separated by a short linker and followed by a short carboxy terminal (C-terminal) tail peptide (Goode et al., 1998). Mammals have two genes of twinfilin, namely *twinfilin-1* and *twinfilin-2*, which are approximately 65% homologous with each other (Vartiainen et al., 2003). Moreover, alternative promoter usage generates two splice variants of *twinfilin-2*. Twinfilin-2a and -2b are otherwise identical to each other but have different first 6-8 residues (Nevalainen et al., 2009). Unlike mammals, yeast, *C.elegans*, and *Drosophila* have one twinfilin gene. Yeast twinfilin is approximately 20% identical to mouse twinfilin-1 at the amino acid sequence level (Vartiainen et al., 2000), and *Drosophila* twinfilin display a 26% and 49% sequence identity with yeast twinfilin and mouse twinfilin-1, respectively (Wahlström et al., 2001).

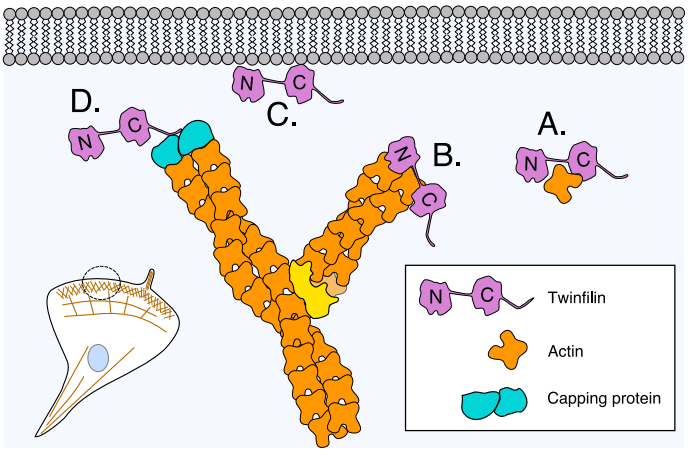

Figure 6. Functions of mammalian twinfilin. Twinfilin interacts and sequesters actin monomers (A), and caps filament barbed ends (B). Association of twinfilin with barbed ends was reported to enhance filament depolymerization. Twinfilin interacts also with plasma membrane phosphoinositides (C), which inhibits its actin-binding function. Twinfilin also interacts with Capping Protein through its C-terminal tail (D).

Yeast, *Drosophila*, and mouse twinfilins sequester actin monomers in 1:1 ratio (figure 6) and inhibit actin filament polymerization (Goode et al., 1998; Vartiainen et al., 2000; Wahlström et al., 2001). Twinfilin has approximately 10-fold higher affinity towards ADP-G-actin than ATP-G-actin. Interestingly, monomer sequestering occurs through the C-terminal ADF-H domain, which has 10-fold higher affinity towards ADP-G-actin that the N-terminal ADF-H domain (Ojala et al., 2002). The ability to sequester actin monomers with 1:1 ration does not explain why twinfilins are composed of two ADF-H domains, and indeed it was revealed that mouse twinfilins interact also with barbed ends of actin filaments with mechanism that requires both ADF-H domains (Helfer et al., 2006; Paavilainen et al., 2007). Similar to actin monomer interaction, twinfilin displays higher affinity towards barbed-ends of actin filaments when the terminal F-actin subunits are in the ADP-state (Helfer et al., 2006). Interactions of twinfilin with barbed-ends of actin filaments leads to decreased actin filament polymerization at least *in vitro* (Helfer et al., 2006). Interestingly, both yeast twinfilin and mouse twinfilin-1 were reported to increase actin filament disassembly from barbed ends *in vitro*. Additionally, yeast twinfilin, but not mouse twinfilin-1, increases filament pointed end depolymerization together with CAP (Hilton et al., 2018; Johnston et al., 2015).

In line with twinfilin's effect on actin filament polymerization and depolymerization *in vitro*, twinfilin null allele in yeast and *Drosophila* leads to defects in the actin filament turnover and actin-dependent cellular processes. In yeast, twinfilin localizes to endocytic actin patches (Falck et al., 2004; Goode et al., 1998), and deletion of twinfilin display a synthetic lethality with certain cofilin mutants (Goode et al., 1998). In *Drosophila*, twinfilin has an important role in development, and twinfilin-null *Drosophila* die at larval stage (Wang et al., 2010). Hypomorphic twinfilin mutant flies are viable, but have an increased amount of filamentous actin in border cells and follicle cells of egg chamber, as well as defects in collective border cell migration, bristle morphology, and synaptic endocytosis (Wahlström et al., 2001; Wang et al., 2010). *Drosophila* twinfilin has also a strong genetic interaction with twinstar, a *Drosophila* homolog of ADF/cofilin, in eye and bristle development (Wahlström et al., 2001).

Unlike any other ADF-H domain protein, twinfilin interacts not only with actin, but also with CP (Palmgren et al., 2001) (figure 6). This interactions is mediated by the C-terminal tail region of twinfilin, and is crucial for the correct localization of twinfilin at least in budding yeast (Falck et al., 2004). The biological role of twinfilin-CP interaction is still unclear. Recently, it was proposed that twinfilin stabilizes CP at the barbed ends of actin filaments by competing in CP-interaction with CARMIL peptide, which has high sequence similarity with the C-terminal tail of twinfilin (Johnston et al., 2018). However, in budding yeast twinfilin localises to cytoplasm and cortical actin patches, and the interaction between twinfilin and CP seems to be crucial for correct subcellular localization of twinfilin rather than for localization of CP (Falck et al., 2004). Moreover, the role of twinfilin for regulation of CP activity in cells remains unknown.

Beside actin and CP, twinfilin interacts with membrane phosphoinositides, especially with PI(4,5) P2 (Palmgren et al., 2001) (figure 6). The interaction with PI(4,5)P2 inhibits the actin-binding functions of twinfilin, and the ability of twinfilin to decelerate actin filament polymerization. However, the mechanism of this interactions, as well as the exact binding site for phosphoinositides in twinfilin, remains unknown. Also, the role of phosphoinositides in regulation of other functions of twinfilin is unclear.

In mammalian cells, both twinfilin-1 and twinfilin-2 localises to the perinuclear region, and to the regions of rapid actin turnover, such as filopodia, lamellipodia, cell-cell contact sites and endosomal actin structures (Helfer et al., 2006; Johnston et al., 2018; Vartiainen et al., 2000, 2003). Similar to *Drosophila* twinfilin, also mammalian twinfilins are linked to regulation of clathrin-mediated endocytosis (Helfer et al., 2006; Pelkmans et al., 2005) and neurite outgrowth (Yamada et al., 2007). The two twinfilin-2 isoforms show tissue-specific localisation pattern. Twinfilin-2a is ubiquitously expressed in all tissue types, whereas twinfilin-2b is expressed mainly in skeletal and heart muscles (Nevalainen et al., 2009). Twinfilin-2a localizes to and regulates the length of cochlear mechanosensory stereocilia, which are thick bundles of actin filaments (Peng et al., 2009; Rzadzinska et al., 2009). In mouse platelets, knockout of twinfilin-2a leads to thickened cortical actin network, as well as restricted inactivation of integrins. Twinfilin-2a deficient platelets showed also increased amounts of active cofilin and profilin, suggesting that twinfilin-2a regulates assembly of actin filaments in platelets (Stritt et al., 2017). At this point, however, the precise cellular role of twinfilin in regulation of actin dynamics or endocytosis remains elusive.

The effect of complete knockout of twinfilin in mammalian cells has not been reported. However, depletion of twinfilin-1 expression with siRNAs caused alterations in the actin cytoskeleton morphology and the loss of actomyosin stress fibres in human breast cancer cells (Bockhorn et al., 2013b). In mouse melanoma cells, silencing of twinfilin expression causes loss of leading edge lamellipodia and an increased amount of filopodia (Johnston et al., 2018). Similar accumulation of actin filaments in twinfilin-deficient mammalian cells compared to yeasts and *Drosophila* has not been reported so far. Thus, the two ubiquitous twinfilin genes in mammals might functionally compensate each other and further studies are required to study their synergistic role in regulation of actin dynamics in mammalian cells.

2. Aims of the study

GMF has been studied extensively during past ten years, and the protein has been linked to several actin-dependent diseases and developmental processes. However, its role in regulation of actin dynamics and in remodelling of actin networks in vivo was previously unknown. Also, the ability of Drosophila GMF to debranch actin filaments was not reported earlier. The aim here was to unravel how Drosophila GMF regulates lamellipodia actin network in cultured cells, and what is its role in collective cell migration and remodelling of actin networks in vivo.

Similar to several other actin-binding proteins (Saarikangas et al., 2010; Senju et al., 2017; Senju and Lappalainen, 2019), the ADF-H domain protein twinfilin interacts with phosphoinositides, and PI(4,5)P2 efficiently inhibits its actin-binding function. However, the mechanism of twinfilin-PI(4,5)P2 interaction was unknown. Thus, the aim of my work was to elucidate how twinfilin interacts with phosphoinositides, to map the binding site for phosphoinositides in twinfilin, and to reveal how phosphoinositides affect other biochemical functions of twinfilin.

As described above, twinfilin has several different biochemical functions with some of them, namely actin monomer sequestration, filament capping and depolymerising functions, arising from its ability to interact with actin through ADF-H domains. On the other hand, twinfilin also interacts with CP through its C-terminal tail. Although work with yeast, Drosophila and mammalian cell cultures suggested that twinfilin regulates actin dynamics in lamellipodia, endosomal actin structures, filopodia and cell-cell contacts, its precise role remains enigmatic. Thus, the third aim of my studies was to address this question by using mammalian cultured cells as a model system.

3. Experimental procedures

The methods I used in this study are listed below. The roman numbering indicates the publication in which the mentioned method was used and is described in more detail.

Method	Publication
Molecular cloning and mutagenesis	I, II, III
Drosophila cell culture	I
Mammalian cell culture	III
Immunofluorescence staining and imaging	I, III
RNA interference	I
Western blot	I, III
Live-cell imaging	I, III
Recombinant protein expression and purification	I, II, III
Preparation of lipid vesicles	II
Lipid co-sedimentation assay	II
Lipid co-flotation assay	II
1,6-diphenyl-1,3,5-hexatriene (DPH) anisotropy assay	II
Actin filament co-sedimentation assay	II
Microscale thermophoresis	II
Gene knockout with CRISPR/Cas9	III
Confocal imaging	III
High-content imaging	III
Fluorescence recovery after photobleaching (FRAP) and photoactivation	III

4. Results and discussion

4.1. Drosophila GMF promotes collective cell migration and turnover of dendritic actin networks (publication I)

Branches within the actin filament networks, generated by the Arp2/3 complex, are remarkably stable in vitro, persisting several minutes (Blanchoin et al., 2000b; Gandhi et al., 2010; Mahaffy and Pollard, 2006). However, in cells the turnover of lamellipodial actin network is an order of magnitude more rapid (Lai et al., 2008; Ponti et al., 2004). Active debranching of filaments might thus be required to maintain the dynamics of branched actin networks. Earlier studies identified GMF as an interaction partner for the Arp2/3 complex. In yeast, GMF inhibits the nucleation and branching activities of the Arp2/3 complex (Nakano et al., 2010) and debranches filaments produced by the Arp2/3 complex (Gandhi et al., 2010). However, the role of GMF in regulation of the branched actin network dynamics during cell migration remains largely unknown. We thus utilized cultured Drosophila S2R+ cells and Drosophila egg chambers to study the cellular and in vivo functions of GMF.

We first examined the localization of GMF in cultured S2R+ cells. By using a polyclonal antibody specific to Drosophila GMF (dGMF), we learned that GMF enriches at the lamellipodia region, as well as in peripheral actin ridges and in the nucleus. Moreover, silencing of dGMF by RNA interference increased the lamellipodia width (publication I, figure 1). Consistent with our results, a subsequent study showed that mammalian GMF-β localizes to lamellipodia in mouse embryonic fibroblasts. Interestingly, the latter study also showed that GMF requires the active Arp2/3 complex for its correct localization to the leading edge of fibroblasts (Haynes et al., 2015). Together with our findings, these results provide the evidence that GMF regulates actin dynamics in lamellipodia.

To study the role of GMF in vivo, we utilized Drosophila egg chambers as a model system. We revealed that dGMF is expressed in the follicular epithelium and enriched in migrative border cells and epithelial polar cells. In border cells, dGMF showed a diffuse cytoplasmic staining (publication I, figure 2). We then generated flies with a dGMF mutant allele gmf1, which lacked the expression of dGMF protein, and compared the migration of border cells in wild type and gmf1 mutant flies during the oogenesis. Although almost all border cells in gmf1 mutants were able to finish the migration by stage 10 of the oogenesis (publication I, figure 2), the speed of the border cell cluster in gmf1 mutant ovaries during the early phase of migration was significantly slower. Additionally, extensions of the leading cell in the border cell cluster was slightly shorter, but more persistent in time (publication I, figure 4), suggesting that the difference in migration speed between control and gmf1 mutants is due to a decrease leading edge protrusion dynamics upon loss of GMF expression. Similar to our results, subsequent study on mammalian fibroblasts showed that expression levels of GMF-β affect the leading edge dynamics, with GMF-β depletion decreasing the rates of both lamellipodia protrusion and retraction (Haynes et al., 2015).

We then examined genetic interactions of GMF with other actin binding proteins by silencing possible candidates in the gmf1 mutant background. Interestingly, silencing of Aip1 in gmf1 mutant background caused almost complete blockage of border cell migration (publication I, figure 2). Moreover, simultaneous silencing of dGMF and Aip1 in border cells resulted in accumulation of the Arp2/3 complex-positive actin filament structures (publication I, figure 2). We then tested, if dGMF and Aip1 would show the genetic interaction also in S2R+ cells. Indeed,

silencing dGMF simultaneously with Aip1 with RNA interference led to a significant increase in the Arp2/3 complex-dependent actin filament accumulations in lamellipodia and lamella of the cell (publication I, figure 3)

Earlier studies with budding yeast identified a genetic interaction between GMF and ADF/cofilin in F-actin disassembly (Nakano et al., 2010) and cell proliferation (Gandhi et al., 2010). Also Aip1 and ADF/cofilin display a genetic interaction in yeast (Clark et al., 2006), suggesting that these proteins regulate actin dynamics in a coordinated fashion or might compensate each other. Indeed, ADF/cofilin induces debranching at least in vitro (Blanchoin et al., 2000b), indicating that the mild phenotype identified for GMF knockout both in yeast and in Drosophila might be partially compensated by ADF/cofilin. Filament debranching by GMF does not lead to disassembly of the entire filament, suggesting that GMF facilitates remodelling of the dendritic actin network by specifically debranching filament networks. Therefore, I suggest that following actin filament debranching by GMF, other filament disassembly factors, such as ADF/cofilin, coronin and Aip1 (Gressin et al., 2015; Jansen et al., 2015), promote rapid filament disassembly of newly formed linear filaments. It is also intriguing to hypothesize that after the debranching of filament by GMF, the newly formed pointed ends, that are free from the Arp2/3 complex (Ydenberg et al., 2013), will be rapidly depolymerized by the actin filament pointed end depolymerization machinery consisting of ADF/cofilin and CAP (Kotila et al., 2019; Shekhar et al., 2019). Interestingly, also GMF and CAP display a genetic interaction in growth assay in yeast, and a simultaneous depletion of GMF, Aip1 and CAP in budding yeast is lethal (Ydenberg et al., 2015). These findings further propose the combinatorial role of several actin-binding proteins in disassembly of the dendritic actin network. However, further studies are required to understand the precise underlying mechanism of this complex actin filament disassembly machinery.

To summarize this part, we have shown here that Drosophila GMF promotes remodelling of the Arp2/3 complex nucleated branched actin networks in cells and in vivo. Consistent with the subsequent study (Haynes et al., 2015), we show that GMF regulates the leading-edge dynamics in migrating cells and facilitates the disassembly of actin filaments in coordinated manner with other actin filament disassembly factors. However, the role of GMF in actin-dependent cellular functions other than cell migration, such as endocytosis, vesicle trafficking and organelle dynamics, remains unknown. Moreover, we revealed that GMF is enriched in the follicular epithelium in Drosophila ovaries, but its role in maintaining the morphology of epithelial cells remains to be studied.

4.2. Twinfilin interacts with membrane phosphoinositides through electrostatic interactions (publication II)

Twinfilin interacts with membrane phosphoinositides, especially with PI(4,5)P2 (Palmgren et al., 2001), and this interaction inhibits twinfilin's ability to prevent actin filament polymerization (Falck et al., 2004). However, the binding site and binding mechanism of twinfilin to membrane phospholipids was not known, and thus we utilized a combined mutagenesis, biochemistry, and molecular dynamics simulation approach to solve this question.

By using a recombinant mouse twinfilin-1 protein and a lipid co-sedimentation assays we revealed that twinfilin prefers phosphoinositides with higher negative charge, namely PI(3,5)P2, PI(4,5) P2 and PI(3,4,5)P3, and binds PI(4,5)P2 with relatively high affinity. Additionally, by performing a co-sedimentation assay with an increasing salt concentration, and by using DPH anisotropy assay (Zhao and Lappalainen, 2012), we learned that twinfilin interacts with membranes through electrostatic interactions (publication II, figure 1). Interestingly, although different actin-binding

proteins interact with membrane phosphoinositides with different affinities and through structurally distinct domains, they all bind to phospholipids through electrostatic interactions (Senju et al., 2017; Zhao et al., 2010). Our results show that twinfilin does not display cooperative binding to the PI(4,5)P2-rich membrane (publication II, figure 1). In contrast to twinfilin, many other actin-binding proteins, such as profilin-1, cofilin-1, mDia2, and N-WASP, PI(4,5)P2 bind cooperatively to membrane in a manner that is depending on the PI(4,5)P2 density (Senju et al., 2017). Thus, twinfilin does not act as a sensor of membrane phosphoinositide density by responding to small increases in PI(4,5)P2 density in a switch-like manner. This suggest that twinfilin has a partially distinct binding mechanism to membrane phosphoinositides compared to other actin-binding proteins.

4.3. Twinfilin binds phosphoinositides through a two-step mechanism (publication II)

Twinfilin consists two ADF-H domains, which facilitate the interaction with actin monomers and actin filament barbed ends. Moreover, a short linker separates these two domains, and a short C-terminal motif follows the ADF-H domains and facilitates the interaction with CP (Poukkula et al., 2011). To elucidate the binding site of twinfilin to PI(4,5)P2-rich membranes, we expressed and purified different domains of twinfilin-1, and studied their affinity to phosphoinositide-rich lipid vesicles with co-sedimentation and co-flotation assays. Surprisingly, we noticed that the C-terminal tail of twinfilin was absolutely necessary for this interaction, and the two ADF-H domains display only relatively weak affinity to PI(4,5)P2-rich membranes (publication II, figure 2). This is in striking contrast with ADF/cofilin, which interacts with PI(4,5)P2-rich membranes through its ADF-H domain (Gorbatyuk et al., 2006; Ojala et al., 2001; Senju et al., 2017; Zhao et al., 2010). In this context it is also important to note that twinfilin binds phosphoinositide-rich membranes with much higher affinity compared to ADF/cofilin. Since ADF/cofilin does not contain similar motif, high affinity of the C-terminal tail to phosphoinositides explains why twinfilin has much higher affinity towards phospholipids compared to ADF/cofilin.

To map the PI(4,5)P2-binding site in the C-terminal tail of twinfilin-1, we utilized a combination of mutagenesis and co-sedimentation assays. We revealed that the tail contains several clusters of positively charged residues, which facilitate the interaction with both PI(4,5)P2-rich membranes and CP (publication II, figure 3, 6). The overlapping binding sites between PI(4,5)P2 and CP in the C-terminal tail of twinfilin suggest that phosphoinositides inhibit also the CP-binding function of twinfilin. However, because also CP binds phosphoinositide-rich membrane with high affinity (Kim et al., 2007), this is difficult to show with experimental methods.

The actin-binding function of twinfilin lies in the two ADF-H domains (Ojala et al., 2002; Paavilainen et al., 2008), whereas our results show that twinfilin binds phosphoinositides mainly through the C-terminal tail. PI(4,5)P2-rich lipid vesicles are, however, able to inhibit the actin-binding function of twinfilin (Palmgren et al., 2001), and thus there seems to be a contradiction between these results. Therefore, we studied by using an actin polymerization assay if the C-terminal tail of twinfilin is necessary for the ability of phosphoinositides to inhibit the actin-binding function of twinfilin. Strikingly, we learned that, whereas both full length twinfilin and protein lacking the tail inhibit actin polymerization, only the full-length protein can be inhibited by lipids. Mutant twinfilin lacking the C-terminal tail inhibits actin polymerization even in the presence of PI(4,5)P2-rich lipid vesicles (publication II, figure 5). We then utilized molecular dynamics simulations to examine the mechanism by which twinfilin associates with membranes. These experiments provided evidence that the C-terminal tail anchors twinfilin on the membrane,

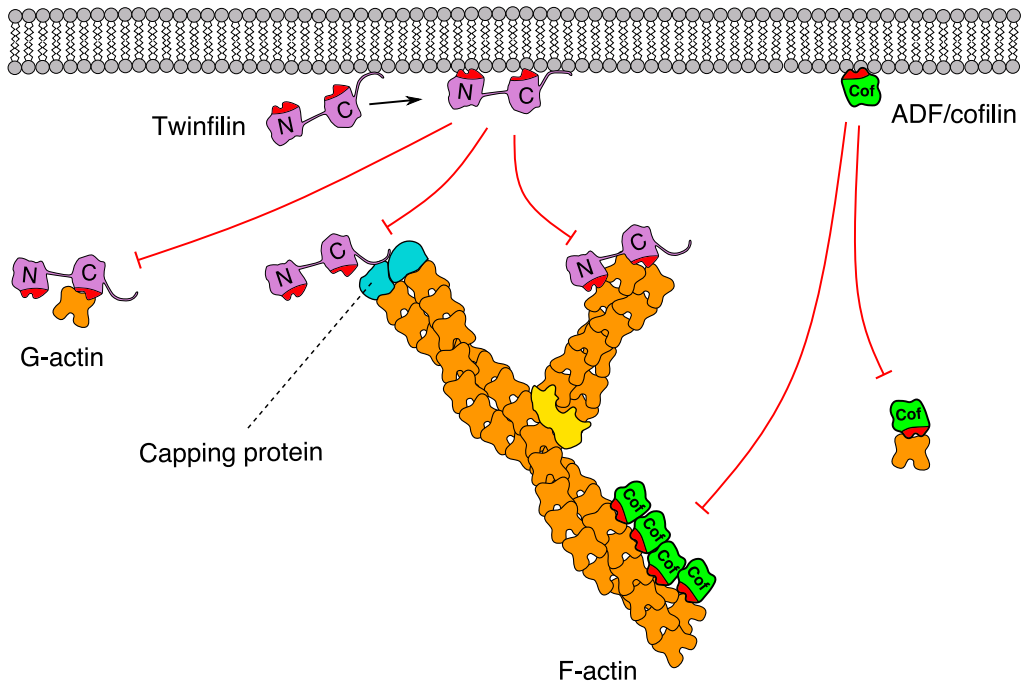

Figure 7.The mechanism of phosphoinositide interaction of twinfilin and ADF/cofilin. Twinfilin interacts with membrane phosphoinositides with two-step mechanism, where high-affinity C-terminal tail region first anchors protein on membrane, and subsequently, the ADP-H domains interacts with lipids. Interaction with phosphoinositides inhibit other biochemical activities of twinfilin. ADF/cofilin interacts with phosphoinositides directly through ADF-H domain with significantly lower affinity compared to twinfilin. Interaction with phosphoinositides inhibit the actin-binding activity of ADF/cofilin.

and that the ADF-H domains subsequently interact with PI(4,5)P2 lipids, leading to inhibition of actin-binding function of twinfilin (publication II, figure 4). Notably, the existence of multiple binding sites for PI(4,5)P2 lipids should lead to cooperative binding. The reason why twinfilin does not display cooperative binding manner to phosphoinositides might rise from its C-terminal tail motif, which possess significantly higher affinity to PI(4,5)P2 lipids compared to its ADF-H domains. Thus, the C-terminal tail could dominate the interaction and the effect of ADF-H domains on binding is negligible.

To summarize this study, we report that twinfilin interacts with phosphoinositide-rich membrane through electrostatic interactions in a two-step mechanism (figure 7). Moreover, our results explain how the actin-binding function of twinfilin is inhibited upon phosphoinositide-interaction. Although the experimental evidence is still missing, it is intriguing to hypothesize that membrane phosphoinositide levels would regulate functions of twinfilin also in cells. This would lead to inhibition of twinfilin in close proximity to membranes, in region where actin filaments are assembled, while twinfilin would be active further away from membrane, in actin filament disassembly region. As described above, PI(4,5)P2 enriched at the leading edge of the cell upon lamellipodial protrusions (Golub and Caroni, 2005), and acute PI(4,5)P2-depletion leads to lamellipodial retraction (Idevall-Hagren et al., 2012). Similar cycles could regulate activity of twinfilin during cell migration. Since twinfilin inhibits actin polymerization at least in vitro, its actin-binding activity could be inhibited during lamellipodia protrusion phase through high density PI(4,5)P2 on membrane. During retraction phase, when actin filaments are not

polymerized, low density of PI(4,5)P2 might trigger activation of twinfilin to sequester actin monomers and cap filament barbed ends. However, further studies are required to uncover the role of phosphoinositides in regulation of twinfilin as well as other actin-binding proteins in a cellular context.

4.4. Mammalian twinfilin is crucial for efficient lamellipodial actin turnover (publication III)

In cells, twinfilin localizes to regions of high actin turnover, such as cortical actin patches in budding yeast (Goode et al., 1998; Palmgren et al., 2001), and lamellipodia, filopodia and cell-cell junctions in mammalian cells (Vartiainen et al., 2003, 2000). However, it remains enigmatic how twinfilin regulates the actin cytoskeleton dynamics in cells. In budding yeast, the knockout of twinfilin leads to increased amount of F-actin in cortical patches (Goode et al., 1998) and in Drosophila the depletion of twinfilin lead to increased amount of F-actin in migrating border cells and in the follicular epithelium (Wang et al., 2010). Earlier studies on mammalian cells have led to somewhat contradictory conclusions on the role of twinfilin in regulation of leading edge dynamics (Bockhorn et al., 2013a; Johnston et al., 2018). We thus utilized CRISPR/Cas9 approach to interrupt the expression of both twinfilin-1 and twinfilin-2 in mouse melanoma B16-F1 cells to uncover the role of twinfilins in regulation of actin turnover in cells. Consistent with earlier studies on Drosophila and budding yeast, we detected an accumulation of F-actin in twinfilin-deficient cells. These accumulations were most abundant in lamellipodia and in the Arp2/3 complex-positive perinuclear actin patches. Additionally, perinuclear accumulations in twinfilin-deficient cells were positive in transferrin after transferrin uptake, indicating that twinfilin regulates actin dynamics also in the endosome recycling pathway (publication III, figure 1, supplementary figure 5). Further on, twinfilin-deficient cells had abnormal lamellipodia morphology, and the lamellipodia protrusion velocity was significantly decreased in knockout cells compared to wild type cells (publication III, figure 1). These lamellipodia phenotypes were consistent with migration defects that were earlier reported both on Drosophila border cells and mouse lymphoma cells upon twinfilin depletion (Meacham et al., 2009; Wang et al., 2010).

The increased F-actin amount in twinfilin-deficient cells could be either due to increased actin filament assembly or decreased disassembly. To solve this question, we utilized photoactivation and fluorescence photobleaching techniques. We revealed that twinfilin-deficient cells display diminished actin filament disassembly rates at their lamellipodia compared to control cells. Moreover, the actin treadmilling rate was significantly slower in twinfilin-deficient cells compared to control cells (publication III, figure 2). These results suggests that twinfilin either regulates filament assembly by capping filament barbed ends (Helfer et al., 2006) or promotes filament depolymerization, as suggested earlier (Hilton et al., 2018; Johnston et al., 2015). To test these possibilities, we utilized single filament imaging approach (Carlier et al., 2014; Jégou et al., 2011b) and learned that at least mouse twinfilin-1 does not enhance filament barbed end depolymerization. Instead, twinfilin inhibited actin depolymerization in concentration-dependent manner. Interestingly, at saturated conditions actin filaments depolymerized from their barbed ends with rate of ~6 subunits/s, whereas in absence of twinfilin-1 the barbed end depolymerization rate was ~10 subunits/s. Our results indicate that twinfilin does not enhance actin filament depolymerization at the barbed ends of actin filaments, but instead allow them to polymerize at least with rate of ~6 subunit/s (publication III, figure 3). Our results are in striking contrast with earlier studies proposing that both yeast and mammalian twinfilin enhances actin filament barbed end depolymerization (Hilton et al., 2018; Johnston et al., 2015). However, in these studies, performed with single filament TIRF approach, where actin filaments crosslinked

to the coverslip at multiple points, the disassembly rate of bare actin filament barbed ends was significantly slower compared to our results and earlier studies with fluorometric approach (0.6-1.4 subunits/s compared to 8-10 subunits/s) (publication III, figure 3)(Pollard, 1986; Pollard and Borisy, 2003). We hypothesize that the differences in results obtained with alternative approaches is due to heavy anchoring of F-actin to glass surface during TIRF imaging (Hilton et al., 2018; Johnston et al., 2015), which is known to affect actin dynamics significantly (Jégou et al., 2011a; Kuhn and Pollard, 2005; Wioland et al., 2019).

To summarize, we show for the first time that twinfilin regulates actin filament disassembly in cells. However, twinfilin itself does not enhance actin filament depolymerization from barbed ends. Thus, it is possible that additional factors are required for rapid filament disassembly from barbed ends, or that twinfilin enhance filament disassembly through previously unknown, indirect approach.

4.5. Twinfilin uncaps filament barbed ends to promote actin disassembly (publication III)

Earlier study showed that twinfilin co-localizes in lamellipodia with CP (Johnston et al., 2018). However, their collective effect on lamellipodial actin dynamics has not been reported. We revealed that twinfilin and CP have distinct localization patterns at the leading edge, where CP localizes to the very distal edge of lamellipodia and twinfilin throughout the entire lamellipodial region (publication III, figure 4). Strikingly however, in twinfilin-deficient cells CP localized throughout entire lamellipodium, suggesting that twinfilin restricts its localization to the distal edge (publication III, figure 5). Interestingly, somewhat similar phenotype was reported earlier in twinfilin-deficient Drosophila S2 cells (Iwasa and Mullins, 2007), indicating that the role of twinfilin to regulate the localization of CP is not restricted only to mammalian cells.

CP is a dynamic component of cellular actin structures and for example in lamellipodia its turnover rates are less than 10 seconds (Lai et al., 2008; Miyoshi et al., 2006). However, in vitro CP binds actin filament barbed ends with sub-nanomolar affinity and with very slow dissociation kinetics ($t1/2 > 30$ min) (Schafer et al., 1996). Thus, the dynamics of capping protein in cells must be accelerated by other proteins, and we hypothesized that twinfilin might, in addition to regulating CP localization, also regulate its dynamics in lamellipodia. We thus utilized fluorescence recovery after photobleaching on EGFP-tagged CP in controls and twinfilin-deficient cells. Strikingly, simultaneous knockout of twinfilin-1 and twinfilin-2 decreased the recovery of EGFP-CP recovery in lamellipodia (publication III, figure 5), indicating that twinfilin promotes CP dynamics in lamellipodia. Importantly, we were able to phenocopy this finding in endosomal actin filament networks (publication III, supplementary figure 6), as well as rescue the CP-dynamics with recombinant expression of mouse twinfilin-1 (publication III, figure 5,7).

We hypothesized that twinfilin enhances CP-dynamics in cells by uncapping filament barbed ends. To examine this question, we then utilized single filament assay, where we polymerized actin filaments from spectrin-actin seeds, capped filament barbed end with CP and observed, if addition of twinfilin-1 with microfluidics would dissociate CP from filament barbed ends. Strikingly, we observed concentration-dependent filament barbed end uncapping after addition of twinfilin-1, indicating that twinfilin uncaps filament barbed ends (publication III, figure 6). We then asked, which biochemical activity of twinfilin is required for the uncapping functions. Surprisingly, mutant twinfilin-1, which does not bind actin, could not promote CP dissociation from barbed ends. Strikingly, mutant twinfilin-1 which does not bind CP displayed elevated uncapping activity

compared to wild type protein, suggesting that the C-terminal tail of twinfilin, which binds CP, tethers CP to close proximity to barbed end of actin filaments (publication III, figure 7). However, when we used wild type twinfilin together with V-1 protein, an inhibitor of CP (Fujiwara et al., 2014; Takeda et al., 2010), we observe ~63-fold increase in filament barbed end uncapping rate compared to V-1 alone (publication III, figure 6). Collectively, our data reveal that twinfilin promotes uncapping of actin filament barbed ends together with V-1.

Twinfilin binds CP through the CPI-like motif in its C-terminal tail region [publication II, (Falck et al., 2004; Johnston et al., 2018)]. An earlier study proposed that twinfilin activates CP by competing with other CPI-motif proteins for binding to CP, and by allosterically competing with V-1 for the CP binding (Johnston et al., 2018). We note that twinfilin may both promote filament uncapping (publication III) and activate CP (Johnston et al., 2018) in cells, because these two functions arise from different domains of twinfilin. However, our EGFP-CP FRAP experiments with transient expression of mutant twinfilins in twinfilin-deficient cells are somewhat contradictory to the capping protein activation model. This is because inhibition of twinfilin-CP interaction through specific point mutations in the C-terminal tail of twinfilin did not significantly affect CP dynamics in cells (publication III, figure 7). Thus, it seems that twinfilin-CP interaction is not necessary for CP activation in lamellipodia, suggesting that other CPI-containing proteins, such as CARMILs (Fujiwara et al., 2014), are the primary CP activation factors in cells, or at least complement the possible activation function of twinfilin. In this context it is important to note that CPI-motifs of CARMILs possess significantly higher affinity to CP and they are much more efficient to compete CP off from V-1 compared to the C-terminal tail motif of twinfilin (Mekel et al., 2020). Since CARMIL-like proteins do not exist in fungi (Liang et al., 2009), it is formally possible that the pro-capping function of twinfilin might be necessary to control the CP activity for example in yeast. However, contradictory to this hypothesis, CP localizes normally to actin patches in twinfilin-null yeast strains (Falck et al., 2004), indicating that it is active also in absence of twinfilin and CARMIL. Additionally, to my knowledge V-1 protein has not been identified in yeast, suggesting that the regulation of CP might be different between yeast and mammalian cells.

To summarize results in publication III, we revealed that twinfilin functions as an essential uncapping factor for CP at barbed ends of actin filaments (see publication III, figure 8 for model). Lack of twinfilin leads to more stable actin filaments due to increased filament capping by CP. Twinfilin itself does not possess filament depolymerization activity, but its association to barbed ends of actin filaments allows filament depolymerization with a moderate speed while simultaneously inhibiting filament elongation. By promoting CP and F-actin turnover, twinfilin is an important component of lamellipodial actin networks to produce protrusive force for cell migration and morphogenesis.

The mechanism by which twinfilin localizes to lamellipodia and endocytic sites in animal cells remains unknown at this point. However, in budding yeast a specific mutations in either actin (Palmgren et al., 2001) or phosphoinositide and CP-binding sites (Falck et al., 2004) disturb the localization of twinfilin to endocytic actin patches. Thus, association of twinfilin with actin filament barbed ends seems to require interaction with both actin and CP. However, further studies are required to reveal the underlying mechanism of twinfilin recruitment to dendritic actin structures in cells, as well as the structural mechanism how twinfilin replaces CP at the barbed end of actin filament.

Moreover, specific roles of twinfilin-1 and twinfilin-2 in regulation of actin and CP dynamics in vitro and in cells remain elusive at this point. Twinfilin-2 localizes to the tips of actin filament bundles at inner ear cochlear stereocilia, and regulate their length (Peng et al., 2009; Rzadzinska

et al., 2009). Additionally, twinfilin-2 was shown to be enriched at the tips of filopodia (Rzadzinska et al., 2009). Because possible biochemical differences between twinfilin-1 and twinfilin-2a/b are incompletely characterized, the mechanism by which twinfilin-2 regulates filopodia and stereocilia length remains unclear. However, since the over-expression of twinfilin-2 in stereocilia led to decreased cilia length, I propose that twinfilin-2 promotes actin filament disassembly in these structures by promoting uncapping of actin filament barbed ends.

5. Concluding remarks and future perspectives

In this study, we elucidated the role of GMF and twinfilin in regulation of dendritic actin networks. We show that GMF regulates lamellipodia dynamics by promoting disassembly of the Arp2/3-positive actin networks, and has thus an important role in collective cell migration in vivo (publication I). Similar to GMF, also twinfilin promotes disassembly of dendritic actin arrays, but through a very distinct mechanism. We show that twinfilin regulates the dynamics of heterodimeric capping protein by uncapping filament barbed ends and subsequently allows actin monomer dissociation from filament barbed ends. Thus, twinfilin also promotes rapid actin disassembly and dynamics of lamellipodia protrusions (publication III). Finally, we revealed the mechanism of twinfilin-phosphoinositide interaction. This explains how the actin-binding function of twinfilin is inhibited by plasma membrane PI(4,5)P2. Moreover, we show that, through direct competition, phosphoinositides most likely inhibit also the CP -binding function of twinfilin (publication II).

The current "textbook" model of actin dynamics in lamellipodia gives a rather simplified view on the topic. Filaments are thought to polymerize at barbed ends near the plasma membrane and depolymerize at pointed ends at the rear of lamellipodia. However, our work here and several other studies have shown that the mechanism of actin filament disassembly is far more complex process, with several proteins with partially overlapping functions maintaining the turnover of dendritic actin network. Our data (publication III) and a study by Wioland and others (Wioland et al., 2017) suggest that filaments are depolymerized from both barbed and pointed ends after the dissociation of CP from the barbed ends. Together, the recently identified actin filament pointed end depolymerization machinery (Kotila et al., 2019; Shekhar et al., 2019), previously established ADF/cofilin-Aip1 disassembly machineries (Gressin et al., 2015; Jansen et al., 2015), and twinfilin-CP interplay can promote rapid actin filament disassembly by severing filaments and promoting their depolymerization from both pointed and barbed ends. Additionally, GMF further enhances actin filament disassembly by debranching dendritic actin networks [publication I, (Haynes et al., 2015)].

Our data together with other studies show that dendritic actin network undergoes "global treadmilling" (Carlier and Shekhar, 2017), where new filaments are nucleated and elongated close to lamellipodia, but filaments are disassembled from multiple points in the network though severing and depolymerization (figure 8). Thus, filament disassembly in cells is very rapid process. However, the global treadmilling model requires that filament disassembly machinery must be "switched off" close to the distal edge of lamellipodia and then "switched on" at correct time. I propose that multiple mechanisms trigger the timing of rapid disassembly. Phosphoinositides, especially PI(4,5)P2, in the plasma membrane inhibit several actin disassembly factors (Saarikangas et al., 2010; Senju and Lappalainen, 2019), including ADF/cofilin (Yonezawa et al., 1990) and twinfilin [(Palmgren et al., 2001), publication II]. Thus, it is possible that these proteins are inactive or partially inactive near the plasma membrane, and bind actin filaments only further away from the membrane. Since these proteins bind phosphoinositides in a concentration-dependent manner, [(Senju et al., 2017), publication II], a decrease in phosphoinositide concentration in the plasma membrane upon lamellipodia retraction (Idevall-Hagren and De Camilli, 2015) might trigger the release of these disassembly factors from the membrane, and begin the disassembly process.

Second trigger for actin filament disassembly might be filament "aging" through hydrolysis of ATP in actin, and subsequent release of the phosphate group from ADP-Pi-actin (Pollard and Borisy, 2003). Both twinfilin and ADF/cofilin have higher affinity towards ADP-actin than ATP-actin (Maciver and Weeds, 1994; Ojala et al., 2002). Additionally, GMF was shown to have

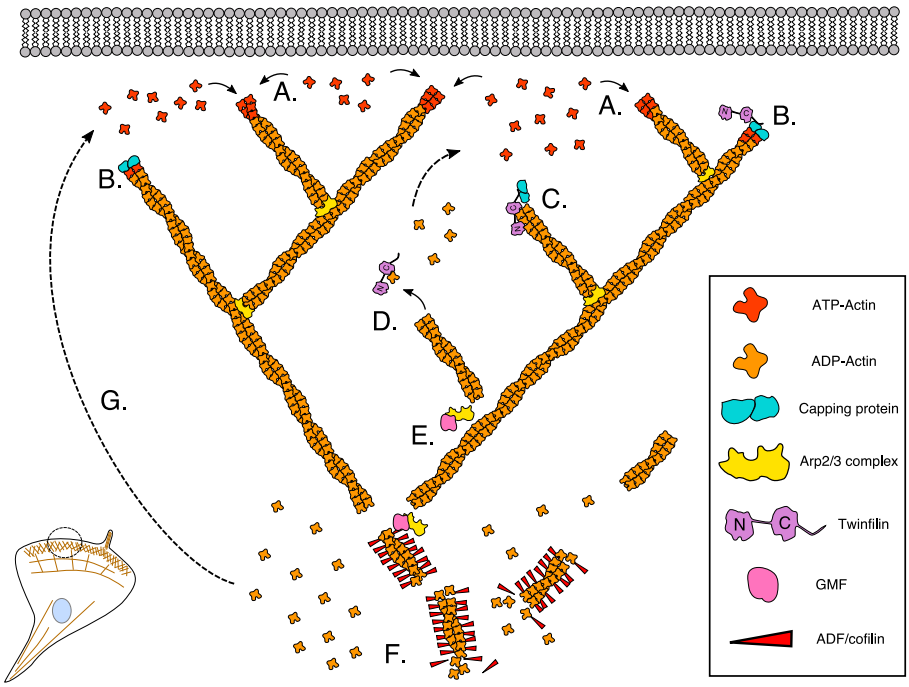

Figure 8. A working model for actin filament turnover in lamellipodia. (A) Filaments are polymerized near the plasma membrane. (B) Capping Protein caps filament barbed ends to funnel actin monomers to growing barbed ends. Twinfilin is recruited to barbed ends by interaction with Capping Protein. (C) Twinfilin uncaps filaments and thus promotes actin disassembly (D) from barbed ends. (E) GMF debranches the network through interaction with the Arp2/3 complex. (F) ADF/cofilin decorated filaments are rapidly disassembled at the rear of lamellipodia by CAP and Aip1. (G) CAP promotes nucleotide exchange in actin monomer to re-charge them for the next round of actin polymerization.

higher affinity towards the ADP-bound Arp2/3-complex (Boczkowska et al., 2013). Thus, it is possible that filament aging serves as a trigger for actin disassembly machinery to interact with "depolymerization-ready" ADP-actin filaments. In this context, it is important to note that ADF/cofilin accelerates the dissociation of phosphate from actin filament from 500 seconds to 30 seconds (Blanchoin et al., 2000b). Thus, the interaction of ADF/cofilin with actin filaments might accelerate filament aging, and further enhance the association of filament disassembly machinery with actin filaments.

The third trigger of actin disassembly, Rho GTPase-mediated inhibition and activation of the disassembly factors is the most complex one. Both ADF/cofilin and GMF can be inactivated through phosphorylation of a serine residue at the amino-terminal region of the proteins (Boczkowska et al., 2013; Van Troys et al., 2008). At least in the case of ADF/cofilin, LIM kinases, which act downstream of RhoA, are the main kinases to phosphorylate and inactivate activities of the protein (Van Troys et al., 2008). On the other hand, certain phosphatases, which dephosphorylate ADF/cofilin, act downstream of Rac1 and Cdc42. Additionally, as discussed above, RhoA acts also through PTEN to regulate the amount of PI(3,4,5)P3 in the plasma membrane (Li et al., 2005). Thus, Rho GTPases are able to regulate actin dynamics through direct phosphorylation and dephosphorylation of actin-binding proteins, as well as through altering the membrane phosphoinositide composition.

Although this study and other recent publications have provided important new insights into actin dynamics, many important open questions concerning the molecular mechanism of actin disassembly machinery regulation remain. Whereas kinases and phosphatases regulating ADF/cofilin are quite well known by now (Van Troys et al., 2008), more work is needed to understand how GMF and twinfilin are regulated by kinase pathways. Additionally, new approaches are required to uncover the complex regulatory mechanism of the entire actin disassembly machinery. One emerging opportunity is to take advantage of the minimal in vitro reconstitution systems to dissect the function of each individual actin regulator in a more complex system, which contains controlled amounts of other actin-binding proteins as well as other factors, such as reconstituted membrane. The combined use of microfluidic single filament assays (Jégou et al., 2011a), in vitro dendritic network growth assays (Manhart et al., 2019), encapsulated cytoskeletal systems (Bashirzadeh and Liu, 2019; Dürre et al., 2018), and complex reconstituted motility approaches (Siton-Mendelson and Bernheim-Groswasser, 2016; Vendel et al., 2019) will shed light on the role of individual proteins in context of other proteins and cellular components. Moreover, new tools, which allow scientist control the amount of proteins in precise manner in cells or in vivo (Li et al., 2019), will supplement the in vitro toolbox, and allow the comparison of results obtained in the test tube and in living organisms.

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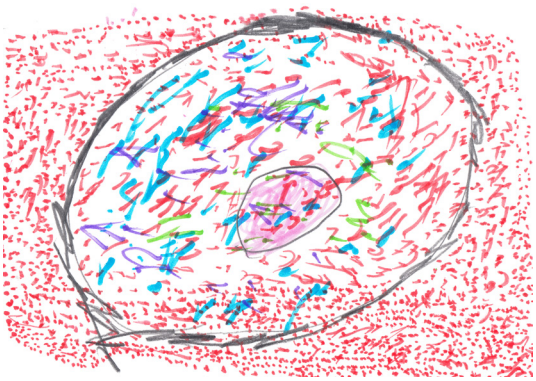

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