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# Regulation of the invasion suppressor Arpin by Tankyrases

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# Régulation du supprimeur d'invasion Arpin par les Tankyrases

Thèse de doctorat de l'Université Paris-Saclay et  
de Lomonosov Moscow State University  
préparée à l'École Polytechnique

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et innovation (Interfaces)  
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La migration cellulaire intervient dans des nombreux processus cruciaux comme l'embryogenèse, la réponse immunitaire, l'homéostasie des tissus et la cicatrisation des plaies. Les aberrations dans la migration cellulaire augmentent le risque de développement des processus pathologiques tels que le cancer, les maladies vasculaires, l'ostéoporose, les maladies inflammatoires chroniques et d'autres. Il existe généralement deux types de migration cellulaire : la migration cellulaire individuelle et collective. La migration cellulaire collective est typique pour les groupements multicellulaires cohésives. La migration individuelle est caractéristique aux cellules amiboïdes ou mésenchymateuses. Les cellules qui sont fortement attachées au substrat dont le cytosquelette est contractile migrent en mode mésenchymateux. A la première étape de leur migration, les cellules forment de grandes protrusions à leur bord appelées les lamellipodes et de petites protrusions en forme de doigt appelées les filopodes.

La migration qui implique la protrusion réunit plusieurs processus subcellulaires coordonnés qui peuvent être classés en cinq types : la protrusion du bord cellulaire, l'interaction de la cellule avec la matrice extracellulaire et la formation d'adhésions focales, la protéolyse focalisée, la contraction de l'arrière de la cellule par l'actomyosine et le détachement du bord arrière.

Les protrusions au bord devant sont faites par des structures appelées les lamellipodes et les filopodes. Les lamellipodes et les filopodes sont des structures à la base d'actine bien étudiées, assemblées au bord des cellules mobiles, ils sont d'une importance cruciale pour la mobilité des cellules. Les lamellipodes sont des structures tridimensionnelles temporelles qui génèrent de la force pour pousser la membrane cellulaire et provoquer ainsi la migration cellulaire. Au cours de la migration, le lamellipode reste stable suffisamment longtemps pour former de nouvelles adhésions focales avec la matrice extracellulaire et ainsi permettre à la cellule d'ancrer la protrusion. Le lamellipode est formé du réseau d'actine branchée qui est assemblé par le complexe Arp2/3.

Le complexe Arp2/3 conservé au cours de l'évolution joue un rôle central dans la nucléation des réseaux de filaments d'actine branchée qui permet la migration cellulaire, l'endocytose et d'autres processus. Le complexe des protéines liées à l'actine 2 et 3 (Arp2/3) est l'un des principaux nucléateurs de l'actine. Le complexe Arp2/3 induit directement la formation d'un réseau d'actine branchée au bord des cellules mobiles en réponse à la signalisation extracellulaire, générant ainsi la force permettant la formation des lamellipodes et des invadopodes. Le complexe Arp2/3

comprend les protéines liées à l'actine Arp2 et Arp3 et cinq sous-unités supplémentaires ARPC1 (p40), ARPC2 (p35/p34), ARPC3 (p21/p18), ARPC4 (p20/p19) et ARPC5 (p16/p15). Les sous-unités Arp2 et Arp3 jouent un rôle essentiel dans la construction des réseaux de filaments d'actine. Grâce à leur homologie avec les monomères d'actine, les sous-unités Arp2 et Arp3 peuvent former un pseudo-dimère d'actine qui peut se lier à la première G-actine liée à l'ATP.

Ainsi, Arp2 et Arp3 forment le noyau pour l'élongation ultérieure du nouveau filament d'actine. Etant donné que les sous-unités Arp2 et Arp3 peuvent agir en tant que monomères d'actine et former un pseudo-dimère d'actine, les sous-unités Arp2 et Arp3 doivent être situées à proximité l'une de l'autre pour former le noyau pour l'élongation. La première structure cristalline du complexe Arp2/3 a révélé un gap entre les sous-unités Arp2 et Arp3, qui fait référence à un état ouvert et donc inactif du complexe Arp2/3. En soi, le complexe Arp2/3 est stabilisé dans l'état conformationnel ouvert. La liaison des facteurs favorisant la nucléation (NPF), portant les domaines VCA, induit des réarrangements conformationnels dans le complexe Arp2/3 : les sous-unités Arp2 et Arp3 se déplacent vers le centre de la fente, le rapprochement ultérieur des sous-unités Arp2 et Arp3 forme un pseudo-dimère et met le complexe Arp2/3 dans son état fermé/actif. Ainsi, les NPFs déclenchent l'assemblage d'actine dirigé par le complexe Arp2/3.

Le complexe Arp2/3 génère le réseau de filaments d'actine branchée via la formation d'une jonction dite de branche entre deux filaments d'actine. On sait que les NPFs jouent un rôle crucial dans la formation de jonctions de branches. Les NPFs agissent à plusieurs étapes: ils activent le complexe Arp2/3, facilitent sa liaison au filament préexistant (mère) d'actine, augmentent le taux d'association du complexe Arp2/3 avec le filament mère et apportent le premier monomère d'actine lié à l'ATP à la jonction de branche. Par la suite, le nouveau filament (fille) s'allonge à un angle de 70 degrés par rapport au filament existant dans une orientation en Y. Les NPFs les mieux caractérisés sont les domaines VCA C-terminaux des protéines de la famille WASP (protéine du syndrome de Wiskott–Aldrich) : WASP, N-WASP (WASP neuronal), complexe WASH, complexes WHAMM et WAVE1-3. Le domaine VCA est constitué de trois motifs courts : motif d'homologie Verprolin également connu sous le nom de WASP homologie 2 (WH2) domaines (V), central (C), acide (A). Le motif V lie la G-actine et délivre ainsi une sous-unité initiale au filament fille. Le motif C lie à la fois le complexe G-actine et Arp2/3 au VCA. Le motif A ne lie que le complexe Arp2/3. Ainsi, l'incorporation de motifs CA dans le complexe Arp2/3 permet aux monomères d'actine de se lier à la jonction des branches. De plus, Arp2 fournit une hydrolyse ATP

rapide sur le monomère d'actine lié par VCA qui initie la polymérisation d'actine. Ainsi, les domaines VCA des protéines de la famille WASP activent le complexe Arp2/3 par formation du complexe Arp2-Arp3-noyau d'élongation sur le filament d'actine préexistante. Par la suite, l'attachement de domaines VCA entraîne des changements de conformation dans le complexe Arp2/3.

Etant donné la capacité de réorganisation des filaments d'actine, l'activité du complexe Arp2/3 doit être étroitement contrôlée. Un moyen qui permet de réguler l'activité du complexe Arp2/3 c'est la liaison des inhibiteurs. Les inhibiteurs sont les protéines qui convertissent le complexe Arp2/3 dans son état inactif et bloquent ainsi la nucléation de l'actine. A l'heure actuelle, il y a peu d'inhibiteurs connus du complexe Arp2/3. Il semble que dans chaque partie de la cellule où se produit la réorganisation de l'actine, il existe deux molécules antagonistes qui régulent l'activité du complexe Arp2/3.

Jusqu'à récemment, il n'était pas clairement défini comment le complexe Arp2/3 peut être directement inactivé dans le lamellipode. Arpin est un nouvel inhibiteur du complexe Arp2/3 au niveau du lamellipode qui a été identifié lors d'une recherche bioinformatique des protéines présentant l'homologie avec le motif VCA des NPFs. Arpin est une protéine relativement petite (25 kDa) à la queue C-terminale allongée. L'extrémité C-terminale d'Arpin contient un motif acide (mais il lui manque des motifs V et C) similaire au motif acide des NPFs qui interagissent avec le complexe Arp2/3. Il a été vérifié si le motif A seul est suffisant pour l'inactivation du complexe Arp2/3. En effet, le motif acide seul inactive le complexe Arp2/3, mais moins efficacement que Arpin dans son intégrité. En revanche, Arpin dépourvu de motif acide a perdu la capacité d'inhiber le complexe Arp2/3.

Il était prédit que Arpin pouvait se lier au complexe Arp2/3 via deux sites de liaison comme le domaine VCA des FNP. Nous avons utilisé la microscopie électronique à une seule particule (single particle electron microscopy) pour obtenir une reconstruction 3D du complexe Arp2/3 lié à Arpin à une résolution de 25 Å. Nous avons montré que la liaison de Arpin entraîne la conformation ouverte du complexe Arp2/3. Nous avons confirmé qu'il existe deux sites de liaison sur le complexe Arp2/3 pour Arpin : l'un à l'arrière de la sous-unité Arp3 et le second est situé entre les sous-unités Arp2 et ARPC1. La distance entre le complexe Arp2/3 et Arpin (5 nm) permet de penser que Arpin interagit avec son partenaire via sa queue acide C-terminale non structurée.

Ensuite, grâce à l'analyse pull-down (pull-down assay), nous avons identifié des nouveaux partenaires de liaison de Arpin, Tankyrase1 et Tankyrase2. Les tankyrases sont des protéines de la famille des poly (ADP-ribose) polyméras (PARP) localisées dans les télomères qui se lient aux protéines de liaison à l'ADN télomériques, TRF1 et TRF2, tandis que TRF2 protège les extrémités des télomères et régule négativement la longueur des télomères. La surexpression de tankyrases provoque l'élongation des télomères dans les cellules cancéreuses grâce à la libération de TRF1 par les télomères. Tankyrase a deux homologues : 142 kDa Tankyrase1 et 130 kDa Tankyrase2 qui sont exprimés de manière omniprésente dans les lignées cellulaires de mammifères. Les tankyrases ont 85% d'acides aminés identiques et sont supposées d'avoir les mêmes fonctions. Cependant, contrairement à la tankyrase-1, la surexpression de Tankyrase-2 provoque l'apoptose précipitée.

Les tankyrases interagissent avec un large spectre de protéines et arbitre la poly (ADP-ribosyl) de ces protéines, régulant ainsi les processus cellulaires essentiels. Les tankyrases lient les télomères, les centrosomes, l'appareil de Golgi, la NuMA (protéine de l'appareil mitotique nucléaire), SH3BP2 et d'autres protéines. Une mutation qui supprime la liaison de SH3BP2 à aux tankyrases provoque un chérubisme chez l'homme. Les tankyrases sont également impliquées dans la régulation de la longueur des télomères, la séparation des télomères sœurs, la mitose et le métabolisme du glucose. Les tankyrases se sont avérées d'être les cibles de signalisation de la protéine kinase MAPK (mitogen-activated protein kinase) dans le Golgi.

Il est intéressant de noter que les Tankyrases et le complexe Arp2/3 possèdent des séquences d'acides aminés qui se chevauchent au niveau des sites de liaison de Arpin. Par conséquent, nous avons démontré une compétition entre le domaine ARC4 de Tankyrase1 et le complexe Arp2/3 d'une manière dose dépendante. Pour comprendre les principes de l'interaction Tankyrases-Arpin, nous avons créé une forme mutée d'Arpin (ArpinG218D) qui a perdu la capacité d'interagir avec les Tankyrases, mais pas avec le complexe Arp2/3 in vitro. Notons cependant que ArpinG218D n'a pas été capable d'inhiber le complexe Arp2/3 in vivo, ce qui suggère que la Tankyrase pourrait être nécessaire pour l'interaction du complexe Arpin-Arp2/3.

Il était connu auparavant que l'efficacité de la migration cellulaire dépend de la vitesse de la cellule et de la capacité de conserver la direction lors de la migration. Arpin a été identifié

comme un facteur qui pousse les cellules mobiles à changer leur direction de migration : les microinjections d'Arpin dans les kératocytes de poisson dont la migration est très persistant et directionnel ont forcé ces cellules à tourner. Ainsi, la diminution de la persistance de migration des cellules a été identifiée comme une caractéristique clé d'Arpin. Nous avons présenté une analyse de la persistance de migration directionnelle de cellules exprimant Arpin sauvage (ArpinWT) ou Arpin muté (ArpinG218D) en parallèle avec la déplétion d'Arpin endogène. Les cellules présentant une surexpression de ArpinG218D ont montré une persistance de migration directionnelle supérieure à celle des cellules surexprimantes ArpinWT. Nous avons suggéré que Arpin muté (Arpin G218D) n'avait aucune activité inhibitrice in vivo, ce qui correspond à l'absence de l'interaction du complexe Arpin G218D - Arp2/3 in vivo.

Nous avons suggéré que le mutant ArpinG218D n'était pas actif et qu'il ne pouvait pas inactiver le complexe Arp2/3 puisqu'il n'était pas présent au lamellipode. Nous avons comparé la quantité de protéines ArpinWT et ArpinG218D dans la fraction membranaire des cellules en migration. Une différence significative (44%) de la quantité d'ArpinWT et d'Arpin G218D était cohérente avec notre hypothèse.

Les tankyrases sont des cibles thérapeutiques dans divers cancers, mais il n'existe actuellement aucun modèle structurel pour ces protéines volumineuses et flexibles. Dans le présent travail, nous avons obtenu pour la première fois deux reconstructions 3D de Tankyrase1 et Tankyrase1 liée à Arpin en utilisant la microscopie électronique à une seule particule. La résolution obtenue (25 Å) était suffisante pour détecter un changement de conformation spectaculaire dans les domaines SAM et PARP de Tankyrase lors de la liaison des molécules d'Arpin. Dans notre reconstruction, trois Arpins étaient liés aux domaines ARC1-2 et ARC4 de Tankyrase1. ARC5 s'est avéré d'être la partie la plus flexible du cluster ARC.

Toutes ces données réunies nous ont permis de proposer un modèle de régulation de l'activité d'Arpin par les Tankyrases. Selon notre modèle, les Tankyrases se lient à Arpin dans le cytoplasme, ce qui provoque des modifications dans l'état conformationnel des Tankyrases. De plus, les Tankyrases rapprochent Arpin à la membrane dans le lamellipode. En passant les signaux extracellulaires, Rac GTPase active Arpin, qui inactive séquentiellement le complexe Arp2/3, tandis que les Tankyrases sont libérés.

Étant donné que les complexes Tankyrases et Arp2/3 partagent le même motif de liaison et sont en compétition pour les interactions avec Arpin, nous supposons la présence de protéines



intermédiaires capables de sentir le changement de conformation des Tankyrases en présence d'Arpin et de réguler ensuite la délivrance d'Arpin à la membrane dans le lamellipode de la cellule.

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## Acronyms

ABP	Actin binding proteins
Arp2/3	Actin-related protein 2 and 3
AP	Adaptor protein
ANK	Ankyrin
ARC	Ankyrin repeat cluster
ARD	Ankyrin Repeats Domain
CC	Coiled-Coil
Crn1	Coronin 1
DH	Db1 homology domain
Aip1	Actin interacting protein 1
Ena/VASP	Enabled/vasodilator-stimulated phosphoprotein
ECM	Extracellular matrix
FAB	Filamentous actin binding
FGF	Fibroblast growth factor
FAK	Focal adhesion kinase
FH1	Formin Homology 1
FH2	Formin Homology 2
GAB	Globular actin binding
GFR	Growth factor receptors
GBD	GTPase binding domain
GAP	GTPase-activating proteins
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
IRSp5	Insulin Receptor Substrate of 53 kDa
JMY	Junction mediating and regulatory protein)
MMP	Matrix metalloproteinase
NMMII	Non-muscle myosin II

NPFs	Nucleation Promoting Factors
GEF	Guanine nucleotide exchange factors
NuMA	Nuclear mitotic apparatus protein
PI3K	Phosphatidyl inositol-3-kinase
PI4P5K	Phosphatidyl inositol-4-phosphate 5-kinase
PIP <sub>2</sub>	Phosphatidyl inositol (4,5) bis phosphate
PIP <sub>3</sub>	Phosphatidyl inositol (3,4,5) tris phosphate
PH	Pleckstrin homology domain PH
PARP	Poly(ADP-ribose)polymerase
SAXS	Small-angle X-ray scattering
TEM	Transmission Electron Microscopy
TNKS1	Tankyrase-1
TNKS2	Tankyrase-2
TNBC	Triple-negative breast cancer
WASP	Wiskott-Aldrich syndrome protein
WH2	WASP homology 2
WIP	WASP-interacting protein
PAK	p21-activated kinase

# **Introduction**

## **1. Cell migration**

Cell migration promotes a variety of crucial processes like embryogenesis, immune cell trafficking, tissue homeostasis and wound healing. Aberrations in cell migration facilitate the pathological processes like cancer, vascular disease, osteoporosis, chronic inflammatory diseases and others.

Cell migration is a cell polarity-based process. Polarized actomyosin-driven shape change of the cell body is a basic process for all types of migration (Lan et al. 2016). Central features of cell migration are also the formation of cell membrane protrusions and cell adhesions. Cell membrane protrusions are usually the result of intense actin polymerization at the leading edge of migrating cells in response to a variety of extracellular signals. Cell adhesion and attachment to extracellular matrix (ECM) is critical for the cell migration. These processes stabilize cell protrusions and form traction sites for cell migration. Cell adhesion is mediated by transmembrane glycoprotein adhesion receptors like integrins (Buck and Horwitz 1987).

### **1.1 Cell polarity**

The ability of cells to dynamically polarize toward extracellular signals is critical to migration. To migrate, the cell has to polarize and define a leading edge and a cell rear.

Cells are getting spatially asymmetrical due to the chemical gradients (Wang 2009). Cells analyze the extracellular information coming from the all sides and identify the chemical gradient. Due to the gradient of extracellular signals, membrane receptors are getting occupied asymmetrically on the cell surface. This spatial asymmetry causes intracellular gradient of polarity effectors. For example, the front-rear polarity was identified in motile cells: a high concentration of actin at the cell leading edge and high concentration of myosin at the cell rear along with a



distinct localization of PI3K, PIP3 and small GTPases such as Cdc42, Rac and RhoA. In general, the polarity proteins include three groups of proteins: the Par complex which consist of Par proteins and protein kinase C, the Scrib complex and the Crb complex.

Cells stimulate the activity of actin nucleators and other proteins involved in the reorganization of cytoskeleton (Parent and Devreotes 1999). Extracellular signals inducing cell motility (or motility factors) include almost all growth factors including basic fibroblast growth factor (FGF), hepatocyte growth factor (scatter factor), vascular endothelial growth factor and epidermal growth factor. Motility factors bind to their receptors on the cell surface and induce stimulatory signals to reorganize the cytoskeleton at the leading edge resulting in the cell migration (Anand-apte et al. 1997).

## **1.2 Types of cell migration**

Generally, two types of cell migration exist: collective and individual cell migration. Collective migration is common for cohesive multicellular units (Friedl and Gilmour 2004). Individual migration is typical for amoeboid or mesenchymal cells.

Amoeboid migration is rounded, blebby migration which is common for the cells that do not adhere to the substrate. These cell migrate by making blebs. Blebs are the protrusions of the cell membrane that are the result of the detachment of the cell membranes from the actin cortex or by the breaks in actin cortex due to the contractility of actin–myosin networks. Under these circumstances cytosol flows out and protrudes cell membrane (Charras and Paluch 2008).

Cells with the strong attachment to the substrate and cytoskeletal contractility migrate in a mesenchymal mode. At the first step of this migration cells make large sheet-like protrusions at its leading edge called lamellipodia and finger-like small protrusions called filopodia.

There are also several intermediate modes. Cells can migrate by pulling the membrane on and further move in a propulsive migration mode. More elongated amoeboid cells can form filopodia or pseudopodia at its leading edge and move slightly contacting substrate.

### **1.3 The basic machinery of actin-based protrusions**

Protrusion-based migration involves several coordinated subcellular processes (Burrige and Wennerberg 2004; Lauffenburger and Horwitz 1996). These processes can be classified into five activities: Protrusion of the leading edge, interaction of cell with the extracellular matrix and formation of focal contacts, focalized proteolysis, cell rear contraction by actomyosin and detachment of the trailing edge (Friedl and Wolf 2003).

#### **1.3.1 Membrane protrusions formation at the leading edge**

In response to extracellular signals, branched actin networks start to polymerize at the leading edge of cells. The growing branched actin network provides a force to protrude the membrane. Formation of membrane protrusions called lamellipodia and filopodia at the cell leading edge enables a cell to protrude its membrane and establish new contacts with its environment (Condeelis 1993).

#### **1.3.2 Cell-extracellular matrix interactions**

If membrane protrusions are not able to adhere to the substratum, they fold back on themselves, forming membrane ruffles that do not support cell migration. Focal adhesions ensure the attachment of the cell to the ECM through an activation and clusterization of integral receptors at the cell membrane.

A large family of cell adhesion receptors, integrins, mediates cell contacts with many ECM molecules. The  $\beta$  subunit cytoplasmic domains of integrins activate signaling proteins and thus

provide extracellular signals inside the cell. The intracellular tail of integrins interacts with alpha-actinin, focal adhesion kinase, talin 1 and other proteins. These proteins further recruit regulatory molecules like GTPases and actin-binding proteins to focal contacts.

The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration at this step. There are distinct types of cell-matrix adhesions: 1) classical focal adhesions located at the termini of stress fibers, that provide long-term cell anchorage, and colocalized with integrins (Hotulainen and Lappalainen 2006). Talin is a cytoplasmic protein that binds the intracellular part of integrin  $\beta$  subunits and actin filaments. Thus talin acts as an integrin-cytoskeletal linker (Calderwood et al. 1999). Stress fibres are contractile actomyosin bundles. Actin bundle is a structure of parallel or antiparallel aligned actin filaments crosslinked by the actin-bundling protein alpha-actinin. Actin filament crosslinking proteins are the proteins with multiple actin-binding domains, but mostly they contain two domains that are separated by a long flexible linker, which allows a perpendicular arrangement of actin filaments (Winder 2005). 2) Nascent focal complexes associated with lamellipodia and filopodia that support protrusion and traction at the leading edge. Focal complexes are signaled by Rac1 or Cdc42 and may transform into long-term focal adhesions (Kaverina, Krylyshkina, and Small 2002). It was found that focal complex formation and Arp2/3 complex mediated actin polymerization at the leading edge are coupled through an integrin-associated protein vinculin (Galbraith, Yamada, and Sheetz 2002). Once coupled to adhesion complexes, the actin cytoskeleton generates the force to translocate the cell forward. 3) fibrillar adhesions located at the central area of the cell and colocalized with matrix fibrils like fibronectin.

### **1.3.3 Focalized proteolysis**

Proteolysis of ECM serves to remove excess components and assembly of ECM. These processes play the key role in ECM synthesis. Focalized proteolysis is a process of ECM degradation, that involves several classes of proteolytic enzymes. The metalloproteinases like matrix metalloproteinase (MMPs) family of proteins are known to be the important regulators of ECM remodelling via proteolysis. MMPs like membrane-type 1 MMP and MMP 2 are able to

activate pro-MMPs and cleave ECM macromolecules like collagen into shorter pieces that undergo subsequent degradation.

MMPs are the zinc- and calcium-dependent proteases that share the conserved zinc-binding motif in their catalytic binding site. MMPs have a N-terminal signal peptide for secretion, a pro domain and a C-terminal catalytic domain. In inactive state pro-MMPs catalytic domain is sequestered by prodomain. Dissociation of the prodomain from the catalytic site activates the cleavage. The localization, activation and activity of MMPs are regulated by their interactions with other proteins, proteoglycan core proteins and/or their glycosaminoglycan chains, as well as other molecules (Hadler-Olsen et al. 2011).

#### **1.3.4 Contraction of the cell rear**

Actomyosin contraction induces tension through stress fibers and induces the detachment of contact points at the trailing edge of the cell and tightening the cell body toward the leading edge (W.-T. Chen 1981). Stress fibres are mostly consist of actin filaments and non-muscle myosin II. ATP-driven movement of myosin II motor domain supply the force for stress fibers formation at the cell rear. Small GTPase Rho contribute to the actomyosin-based contraction of the cell rear. Rho GTPases activate Rho kinase. Rho kinases consist of catalytic, Rho-binding, coil-coiled and plekstrin homology domains. Rho kinase is calcium and calmodulin-dependent kinases that phosphorylate light chain of myosin II at its Ser19 and/or Thr18 residues and thus promotes actomyosin contraction. Myosin phosphatase is a contraction regulating protein that dephosphorylates light chain of myosin II and thus ceases the actomyosin-based contraction.

It was found that Rho kinases contribute to the actomyosin contraction either by direct activation of myosin or by inactivation of myosin phosphatases. MLC is phosphorylated at its Ser19 and/or Thr18 residues by MLC kinase, which is a (Ca<sup>2+</sup>-calmodulin)-dependent kinase.

### **1.3.5 Detachment of the trailing edge**

Focal complexes formed at the cell leading edge undergo several cycles of assembly and disassembly. It was shown that MMP-based proteolysis of ECM induces distinct integrin signals that leads to the calpain-mediated cleavage (Carragher et al. 1999). Calpain protease cleaves focal contact components like talin and cytoplasmic tail of  $\beta 1$  and  $\beta 3$  integrins. Calpain is essential for cytoskeletal organization during cell motility, apoptosis, cell proliferation and hemostasis. Calpain family of proteases are able to regulate the dynamics of integrin-mediated focal adhesions, focal complexes and actin-based membrane protrusions. Calpain is a calcium-regulated cysteine protease that consist of large (80kDa) and small regulatory (28kDa) subunits. There are two types of calpains:  $\mu$ -calpain and m-calpain that are activated by micromolar and millimolar concentrations of calcium respectively. Calpain colocalizes with focal adhesions and integrin clusters and cleaves many focal adhesions proteins including  $\beta 3$  integrin, spectrin and talin.

Calpains are also known to modulate signalling molecules like focal adhesion kinase (FAK), protein kinase C and Rho family GTPases (Franco 2005). FAKs cause the disassembly of focal adhesions. It was shown that calpains act downstream of microtubules to mediate adhesion complexes disassembly (Bhatt et al. 2002).

## **1.4 Cell membrane protrusions**

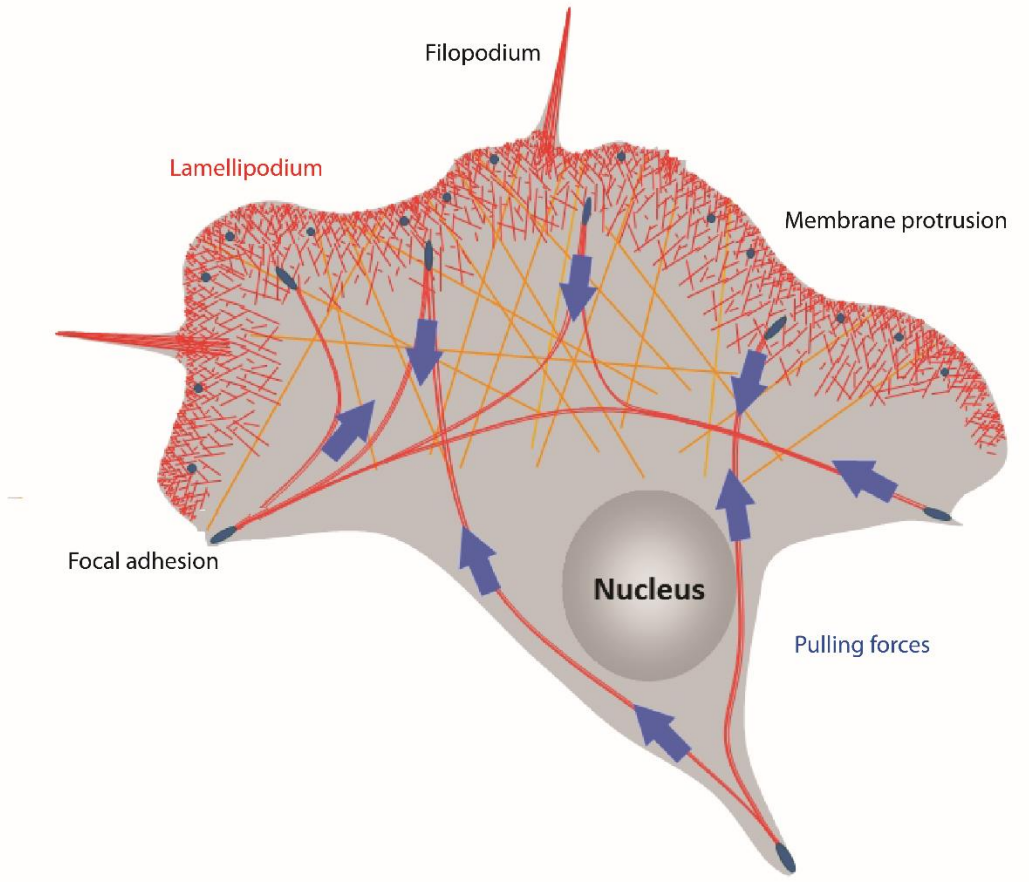
Lamellipodia and filopodia are well investigated actin-based structures assembled at the leading edge of motile cells and crucially important for direct cell motility.

### **1.4.1 Filopodia**

Filopodia consist of parallel bundles of actin filaments that are formed by the reorganization of lamellipodial actin filaments. Lamellipodial actin filaments first increase in length and then become aligned and cross-linked. This required elongation of actin filaments might be caused either by actin assembly acceleration or by barbed ends capping delay. Reorganization of lamellipodial branched actin filaments into filopodial parallel F-actins is crucial for processes like tumor metastasis and chemotaxis (Hansen and Mullins 2010; Svitkina et al. 2002).

### **1.4.2 Lamellipodia**

Lamellipodia are the three dimensional temporal structures that generate force to protrude cell membrane and thus facilitate cell migration. During protrusion-based migration lamellipodium stays long enough to form new focal adhesions with extracellular matrix and thus cell anchors the protrusion (Lan et al. 2016; *T. D. Pollard and Borisy 2003*). The lamellipodium is formed by branched actin networks that are assembled by Arp2/3 complex (Fig.1).



**Figure 1. Schematic illustration of the leading edge in motile cell.** Modified from (Clainche and Carlier 2008).

The leading edge of a migrating cell consists of membrane protrusions lamellipodium and filopodia. Lamellipodium is formed by Arp2/3 complex-dependent branched actin network.

In theory, cell membrane has to stretch a lot to make protrusions like lamellipodia. However, it was shown experimentally that cell membrane can physically stretch only for 2-3%. The expansion of the cell membrane can be explained by following models: 1) Fountain flow model says that membrane precursor vesicles fuse with the anterior cell membrane to supply membrane (exocytosis), and membrane is taken up at the rear (endocytosis). 2) The membrane unfolding model is the utilization of the membrane folds, folding and unfolding of the membranes. 3) Caterpillar flow model says that the cell membrane moves circularly in the order of the ventral, anterior, dorsal, and rear regions. In this case, the cell membrane may turn over everywhere (Tanaka et al. 2017).

### **1.4.3 Invadopodia and podosomes**

Podosomes and invadopodia are special types of adhesion that mediate invasion of cancer cells. Both invadopodia and podosomes consist of actin-rich core, which distinguishes them from other matrix contacts, and are regulated by a multitude of signalling pathways including Rho GTPases, cortactin, N-WASP, adaptor proteins Tks4 and Tks5, tyrosine kinase Src, MT1-MMP and microtubule-dependent transport (Linder, Wiesner, and Himmel 2011). N-WASP as a regulator of Arp2/3 complex was found to be important in the formation of invadopodia and podosomes (Nürnberg, Kitzing, and Grosse 2011).

Though the similarity between invadopodia and podosomes, there are some differences in their functionality. Invadopodia are known to attach the ECM and form stable contacts for hours, while podosomes preferably form short protrusions that retract rapidly (Murphy and Courtneidge 2011).



## 1.5 Signalling of cell migration

Growth factors and insulin promote actin polymerization at the plasma membrane of different cell types and thus induce the formation of protrusions.

Chemokine receptors and growth-factors receptors activate PI4P5K (phosphatidylinositol-4-phosphate 5-kinase) that generate PIP<sub>2</sub> (phosphatidyl inositol (4,5) bis phosphate) before PI3K (phosphatidylinositol-3-kinase) can generate PIP<sub>3</sub> (phosphatidyl inositol (3,4,5) tris phosphate).

PI4P5K generates PIP<sub>2</sub> which is involved in regulation of actin assembly and cell growth. PIP<sub>2</sub> releases G-actin from profilin-GTP-bound actin complex that is a signal for actin assembly. PIP<sub>2</sub> also regulates the interaction of alpha-actinin and a number of actin-capping proteins with actin filaments. PI3K is a family of intracellular lipid kinases associated with tyrosine-kinase receptors. PI3K family of kinases consists of three classes I, II and III. Classes I and II are known to generate both PIP<sub>2</sub> and PIP<sub>3</sub> (Jean and Kiger 2014). PIP<sub>2</sub> and PIP<sub>3</sub> are established regulators of actin polymerization. PIP<sub>2</sub> is responsible for the restriction of actin polymerization in the cortex (Insall et al. 2001). PIPs are known to be the activators of Rho family of GTPases. PIP<sub>3</sub> acts as a second messenger that induces local actin polymerization. PIP<sub>3</sub> controls the spatial sensing and determination of place of actin polymerization in the cell (Haugh et al. 2000). Concentration of PIP<sub>3</sub> is coupled to the actin polymerization signaling, increase of PIP<sub>3</sub> level stimulation actin assembly. Moreover, the spatial distribution of PIP<sub>3</sub> overlaps the distribution of actin filaments polymerization.

### 1.5.1 Rho family GTPases

Spreading and migration of the cells are mediated by Rho family GTPases, growth factors or integrin-dependent attachments of the cell to ECM. Rho family of small GTPases play a critical role in cell polarization, actin cytoskeleton assembly and its dynamics (Nobes et al. 1995). Later Rho family GTPases were found to be associated cell cycle progression, cell survival, neurogenesis

and immune response. All Rho family GTPases contain C-terminus hypervariable region that is isoprenylated for association with the membrane.

The most studied Rho GTPases are RhoA, Rac1 and Cdc42 proteins, while there are 22 mammalian Rho GTPases. All three proteins are found to be spatially asymmetric during the motility process. Rac1 and Cdc42 are activated at the leading edge of the cell, while Rho is active at the cell rear. Rho and Rac mediate signal transduction pathways between the transmembrane receptors like GFR (growth factor receptors) and control the polymerization of actin filaments. Rho GTPases are known to tune not only actin cytoskeleton dynamics and cell polarity, but also vesicle trafficking, endocytosis, oncogenesis, gene transcription and differentiation (Burrige and Wennerberg 2004).

GTPases cycle between GDP-bound inactive or GTP-bound active forms. This GTPase cycling between active and inactive states is crucial for cell growth and development. Rho GTPase activity is stimulated by guanine nucleotide exchange factors (GEF) that exchange GDP to GTP. GEFs facilitate dissociation of GDP from GTPases and consequent attachment of GTP. In contrast, GTPase-activating proteins (GAP) catalyze GTP hydrolysis and thereby inhibit Rho GTPases. Guanine nucleotide dissociation inhibitors (GDIs) remove inactive GTPases from the membrane and insulate them in the cytosol.

It is known that GEFs bear a catalytic Db1 homology domain (DH) that is adjoined to the Pleckstrin homology domain (PH) that can bind some phosphoinositol lipids and proteins. Interestingly, it was shown in fibroblasts that Rho GTPases may participate in a linear activating signalling cascade: Cdc42 activation causes activation of Rac and it subsequently stimulates Rho's activity (Ridley et al. 1992). Moreover, Rac antagonizes Rho and induces cell spreading.

### **1.5.2 Rac GTPase**

Rac-like subfamily of Rho family GTPases stimulate the formation of lamellipodia and ruffles (Ridley et al. 1992). Moreover, Rac induces focal complexes formation. Rac1 is important for the cell polarity identifying.

This subfamily includes GTPases Rac1, Rac2 and Rac3.. Rac1 is widespread, while location of Rac2 and Rac3 is confined by hematopoietic and neural tissues. As was mentioned already, Racis activated by guanine nucleotide exchange factors. It was shown that nucleotide exchange factor Tiam1 specifically activates Rac, but not the other Rho-like GTPases. Activation of Rac by Tiam1 induces an epithelial-like morphology with functional cadherin-based adhesions and inhibits migration of fibroblasts (Sander et al. 1999).

### **1.5.3 Rho GTPase**

Rho-induced synthesis of PIP<sub>2</sub> is necessary for focal adhesions and stress-fibres formation. Rho undergoes post-translational modifications upon which methylation of Cys190 and proteolytic removal of the C-terminal three residues. These modifications are necessary for the Rho translocation to the cell membrane in response to extracellular signaling (Ren et al. 1996).

GTP-bound Rho activates downstream effectors like Rho-kinase and PIP5K. Rho-kinase plays important role in actomyosin contraction at the cell rear during cell migration.

### **1.5.4 Cdc42**

Cdc42 is an essential regulator of cell polarity. The main function of Cdc42 is a direction sensing. Inhibition of Cdc42 excluded the chemotaxis of cells toward the extracellular signals. Without Cdc42 cell can be polarized, but it loses the ability to move toward the chemotactic gradient. Cdc42 initiates cell polarization via cell-polarity Par proteins. Cdc42 is also responsible for the centrosome and Golgi reorientation as well as microtubule network polarization (Etienne-Manneville 2004).

There are two ways to activate and recruit Cdc42 at the leading edge: PI3K pathway and G-protein signaling pathway. Epidermal growth factor may also activate Cdc42 through receptor tyrosine kinase. Cdc42 is dispensable for filopodia formation. Cdc42 binds and stimulates WASP activity and thus activates the Arp2/3 complex.

## **1.6 Aberrations in cell motility that contribute to metastasis of cancer cells**

Metastasis is an invasive migration of transformed cells into surrounding tissues via blood vessels or lymphatic system and formation of colonies at the secondary sites in a host organism. A critical step for a process of metastasis is the invasion of transformed cells. Actin assembly dynamics is necessary for cancer-cell invasion. It is known that Arp2/3 complex regulation in cancer cells is vastly modified. WAVE complex is involved in cancer cell invasion. For example, WAVE3 is necessary for lamellipodium formation in breast cancer cells. Moreover, subunits of WAVE complex are overexpressed in different types of cancer (Molinie and Gautreau 2018). However, it was established that CYFIP1 suppresses tumor invasion (Silva et al. 2009). In normal untransformed cells N-WASP contributes to endocytosis, while in cancer cells, N-WASP is involved in the formation of specific structures called invadopodia. It was shown that WAVE complex knockdown promotes N-WASP-dependent cancer cell invasion (Tang et al. 2013).

Cancer cell motility undergoes the same steps mentioned above as the non-transformed cells. Expression of ECM-degrading proteins is increased to stimulate the dissemination and metastasis.

Rho GTPases are known to be associated with development of cancer cells. Surprisingly, both loss- and gain-of-function mutations were found in genes encoding Rac1, GEFs that regulate Rac activity and Rac downstream p21-activated kinases PAK: PAK1, PAK4 and PAK5 (Bustelo 2018).

The basic machinery of cell motility in cancer cells seems to be unchanged in parallel with regulatory imbalance that is represented by the absence of stop-signals leading to the continuous migration. Cancer cells during the metastasis process migrate either by the ‘individual cell migration’ way which is more common for the early stages of cancer, or by the ‘collective migration’ way (Friedl and Gilmour 2004). Type of migration also depends on the type of cancer. Individual cell migration is represented by either mesenchymal or ameboid modes of migration. These modes of migration are interchangeable, cells can switch between ameboid and mesenchymal modes of migration due to the cooperation of Rac and Rho GTPases (Sanz-Moreno et al. 2008).

The basic migration machinery of cancer cells also requires the formation of actin-based protrusions and thus the Arp2/3 complex activity. Arp2/3 complex hence was found to be a crucial player in the cancer cell invasion and migration of different cancer cell types (Yang 2013). Arp2/3 complex is overexpressed in different types of cancer and this overexpression is associated with poor prognosis. In some cases, Arp2/3 complex overexpression was associated with WAVE overexpression, whereas the subunits of WAVE complex showed the inhibitory activity regarding the metastasis of epithelial cancer, prostate cancer and others. It was shown recently that the down regulation of the new inhibitor of Arp2/3 complex called Arpin was associated with poor prognosis (Lomakina et al. 2016).

## 2. Actin cytoskeleton dynamics provides a major driving force for cell motility

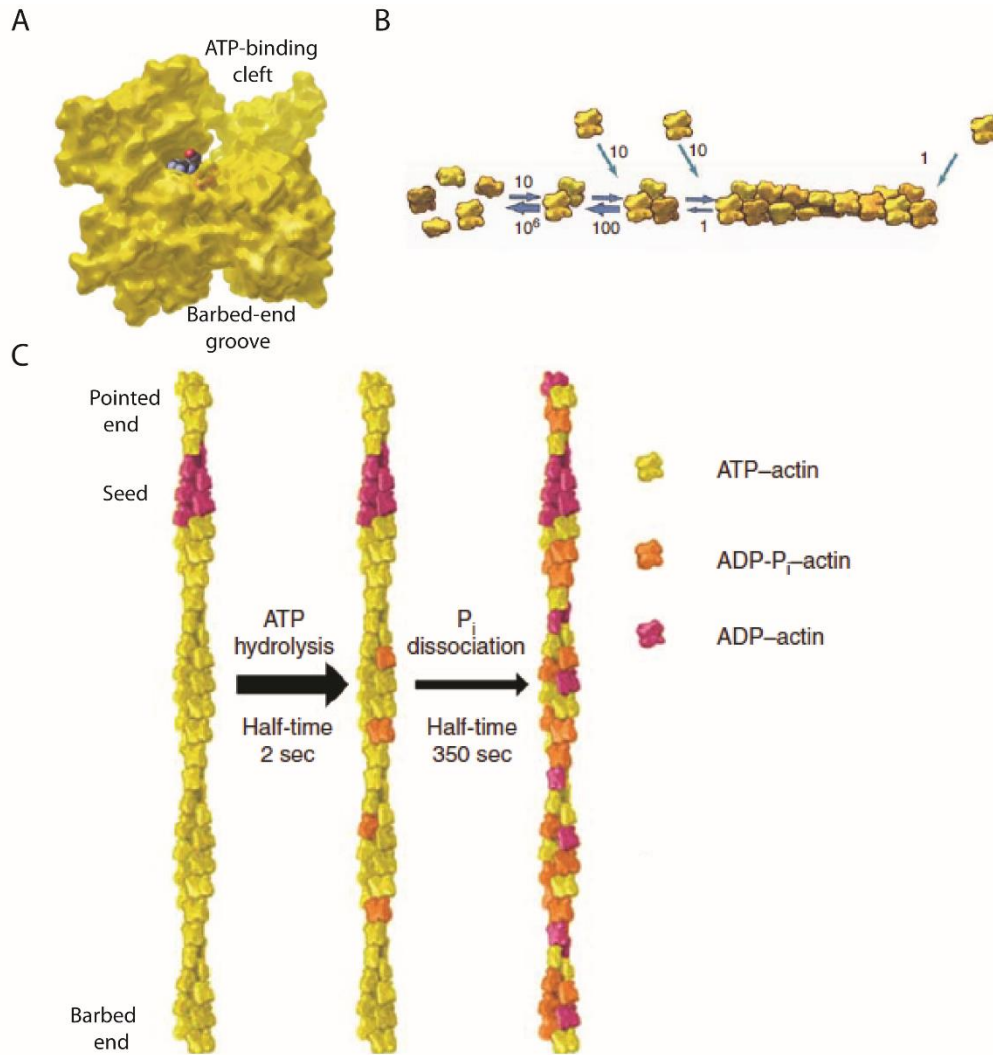
### 2.1 Actin cytoskeleton

Actin cytoskeleton has been well established to contribute to cell migration. Reorganization of actin filaments at the leading edge generates force for the formation of cell membrane protrusions like filopodia and lamellipodia (T. D. Pollard and Borisy 2003).

Actin cytoskeleton (also known as microfilaments) consists of actin and crosslinking proteins. Actin is one of the most spread and well-conserved (less than 5% difference in different species) proteins in many eukaryotic cells. Actin as a part of cytoskeleton plays important role in processes like muscle contraction, cytokinesis, cell adhesion and migration, apoptosis, endocytosis, immune response and other. There are 6 isoforms of actin, each encoded by its own gene. Four isoforms  $\alpha_{\text{skeletal}}$ -actin,  $\alpha_{\text{cardiac}}$ -actin,  $\alpha_{\text{smooth}}$ -actin, and  $\gamma_{\text{smooth}}$ -actin, are expressed primarily in skeletal, cardiac, and smooth muscle. The remaining two isoforms,  $\beta_{\text{cyto}}$ -actin and  $\delta_{\text{cyto}}$ -actin are ubiquitously expressed (Perrin and Ervasti 2010).

Actin is a protein with 42 kDa mass and has a globular shape called G-actin. At the critical concentration of G-actin, monomers of actin start to polymerize spontaneously into a thin 8 nm filament with a double helical structure called F-actin. Monomeric G-actin should be noncovalently bound to ATP at its ATP-binding cleft (Fig.2A). During actin filament polymerization ATP bound to G-actin is getting hydrolyzed into ADP and phosphate  $P_i$ . This reaction goes through the intermediate step when F-actin is bound to ADP- $P_i$  (Korn, Carlier, and Pantaloni 1987). ATP-bound monomers of actin preferably associate with barbed end of actin filament. During the elongation of F-actin, ATP bound to previously embedded actin hydrolyzes,  $P_i$  releases and ADP-bound actin disassembles from the filament (Fig.2C). Released ADP-actin undergoes nucleotide exchange and ATP-bound monomers of actin can be reused for polymerization of F-actin. Equilibrium where monomer disassembly from the minus end and polymerization at the plus end is balanced and sustained by a critical concentration of actin monomers in the cell is known as ‘actin treadmilling’.

Polymerization usually begins with a formation of G-actin trimer called nucleus and a subsequent attachment of G-actin to the growing filament. During spontaneous polymerization of actin, one end of F-actin grows faster than the opposite one. G-actin attaches to the plus end 10x faster than to the minus end (Fig.2B). Fast-growing end is called plus end, slow-growing end is called minus end. F-actin is also considered to be polarized due to the fact that all the microfilaments are located in the same direction: fast-growing ends toward the cell membrane (T. D. Pollard and Borisy 2003). When concentration of actin monomers is relatively low, F-actin starts to depolymerize. Thus actin microfilaments are the highly dynamic structures that can assemble and disassemble according to the concentration of G-actin.



**Figure 2. Structure of actin filament.** Modified from (T. D. Pollard 2016)

**A)** Space-filling model of actin monomer showing nucleotide-binding cleft and barbed-end groove. **B)** Mechanism of nucleation and elongation of actin filament. Monomers of actin may spontaneously form a trimer. Trimer or nucleus enables the attachment of G-actins with high rate. **C)** Aging of actin filament. Over time ATP bound to actin is hydrolyzed randomly to ADP and  $P_i$ . Subsequently,  $P_i$  is slowly getting released and ADP-actin rapidly dissociates from the filament.



The most important physiological function of actin filaments in cells is to produce force for the above-mentioned cellular processes. Polymerization of branched actin network provide the force to make membrane protrusions like lamellipodia. It also provides the force for formation of membrane invaginations during endocytosis (J. W. Pollard 2009; Tojkander, Gateva, and Lappalainen 2012).

Actin filaments dynamics is strongly regulated *in vivo* by distinct signaling pathways involving different regulatory proteins called actin binding proteins (ABP). Those are capping proteins, actin monomers sequestering proteins, F-actin crosslinking proteins, actin filaments severing proteins, nucleators of actin filaments assembly and others.

## **2.2 Actin binding proteins regulating actin dynamics**

Rapid reorganization of actin filament network in response to extracellular and intracellular stimuli requires pool of available for incorporation ATP-bound G-actins. *In vivo* the availability of ATP-bound G-actins is tightly regulated by actin binding proteins.

Profilin is a small protein that bind ADP-bound monomeric actin, exchanges ADP on it to ATP and then delivers actin to the barbed end of F-actin. In its inactive state, profilin binds to membrane lipid PIP<sub>2</sub>. Profilin also binds the barbed end of actin filament. Profilin binds actin monomers and contributes to barbed end elongation, but not the pointed end elongation. It was shown that low concentration of profilin blocks the actin assembly end elongation. However, profilin can promote the disassembly of aged actin filaments in a concentration dependent manner (Jégou et al. 2011). CAP protein also exchange ADP to ATP on G-actin.

Proteins, that are responsible for ATP-bound actin monomers delivery to the growing end of actin filament or to Arp2/3 complex, are: twinfilin, Srv2/CAP, profilin, verprolin/WIP and WASP family of proteins.

For the fast actin assembling it is critical to reserve actin monomers that can be released later for rapid F-actins growth. Thymosin  $\beta$ 4 prevents incorporation of G-actin molecules into actin filaments. Thymosin  $\beta$ 4 is the most abundant G-actin sequestering protein: it binds

monomeric actin and forms a G-actin/thymosin  $\beta$ 4 complex thus blocking actin filaments assembly (Pantaloni 1993).

Capping proteins control the length of the actin filaments by blocking the addition of the new monomers to the barbed end. Capping protein gelsolin severs actin filaments and thus increases actin dynamics. There are also pointed end cappers that block actin filaments disassembly and thus contribute to the high-speed actin filament elongation. Thus cell motility requires capping proteins because they keep the steady-state pool of actin monomers and thereby control the rate of actin filaments growth (Cooper and Sept 2008).

### **2.2.1 Elongators of actin filaments.**

There are also elongating proteins from formin and SPIRE family that contribute to the lamellipodium and filopodium formation at the leading edge. Formins both nucleate unbranched actin and act as the elongation factors that processively associate with growing barbed ends. Formins protect barbed ends of F-actin from capping thus contribute to the actin filaments elongation. Formins are characterized by conserved Formin Homology 1 (FH1) and Formin Homology 2 (FH2) domains. FH2 domain forms a unique stable and flexible tethered dimer that *de novo* nucleates actin filaments assembly. FH2 domain continuously attaches to the barbed end of actin thus protecting it from capping and accelerates unbranched actin filaments elongation. FH1 domain further stimulates the elongation binding profilin-G-actin and enables its delivery to the barbed end (Chesarone and Goode 2010). The source of energy for such processive association of formins via its FH2 domain with actin filaments remains controversial.

Cordon-Bleu (COBL) is a member of SPIRE family and has four V-motifs that bind G-actin form a novel single strand nucleus for 'barbed end' actin filament elongation.

Another group of proteins is an Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of proteins that promotes filopodial actin filaments assembly and contribute to its reorganisation into filopodial actin bundles. Ena/VASP is a capping protein that protects barbed end of actin filaments from capping (Hansen and Mullins 2010). Ena/VASP proteins

promote formation of both lamellipodial and filopodial actin networks. It was shown that deletion of VASP protein in fibroblasts suppressed filopodia formation (Bear et al. 2002).

Ena/VASP enable the binding of both profilin-G-actin complex and filamentous actin via its GAB (globular actin binding) and FAB (filamentous actin binding) domains. Profilin-G-actin complexes are not able for neither spontaneous actin assembly nor autonomous actin filament elongation. But profilin-actin complexes are known to be bound by both Ena/VASP and formins in a processive manner that enables the barbed ends elongation. JMY is another protein that has one Arp2/3 complex binding C-motif and thus enables Arp2/3 complex activation.

### **2.2.2 Nucleators of actin filaments**

A critical part of actin filament assembly is the *de novo* nucleation of filament (E. D. Goley et al. 2010). *De novo* F-actin assembly is an energetically unfavourable, rate-limiting process that is characterized by a strong kinetic barrier. Formation of the nucleus from 3 actin monomers is needed for the subsequent elongation of the filament. ABPs are the proteins that overcome this kinetic barrier and ensure the rapid formation of the nucleus. Proteins that facilitate the *de novo* formation of actin filaments are called nucleators. Arp2/3 complex and formins are the best characterized nucleators that play prominent role in cell motility.

### **3. The Arp2/3 complex is a major nucleator of actin filaments at the leading edge**

As was mentioned before, Actin-related protein 2 and 3 (Arp2/3) complex is one of the major actin nucleators. The Arp2/3 complex directly induces the formation of branched actin network at the leading edge of motile cells in response to extracellular signalling, thus generating the force responsible for lamellipodia and invadopodia formation (T. D. Pollard 2007; Svitkina and Borisy 1999).

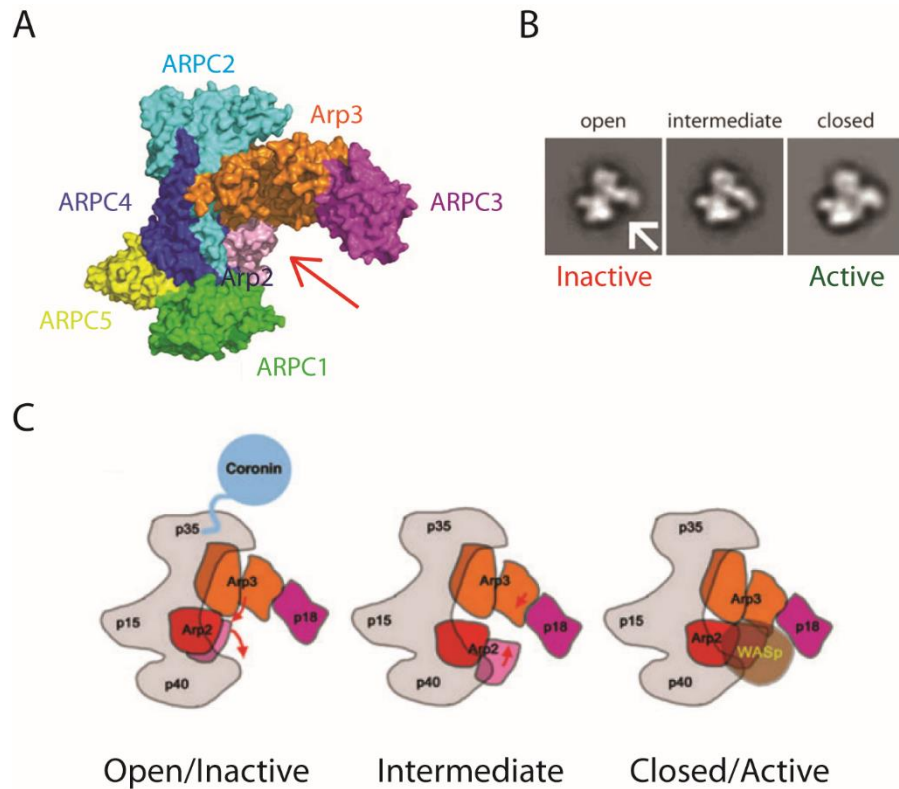
Crystal structure of bovine Arp2/3 complex was reported in 2001 at 2 Å resolution. The Arp2/3 complex consists of highly evolutionary conserved actin-related proteins Arp2 and Arp3 and five additional subunits ARPC1 (p40), ARPC2 (p35/p34), ARPC3 (p21/p18), ARPC4 (p20/p19) and ARPC5 (p16/p15) (Machesky et al. 1994). Subunits Arp2 and Arp3 play pivotal role in the Arp2/3 complex mediated actin filament assembly. Due to its homology with monomers of actin, Arp2 and Arp3 subunits may converge and form an actin pseudo-dimer that attracts the first ATP-bound G-actin. Thus Arp2 and Arp3 make the nucleus for subsequent elongation of the new actin filament.

#### **3.1 Conformational states of the Arp2/3 complex**

Since Arp2 and Arp3 subunits may act as actin monomers and form actin pseudo-dimer, Arp2 and Arp3 subunits should be located close to each other to form the nucleus. However, the crystal structure of the Arp2/3 complex showed a giant cleft between subunits Arp2 and Arp3 (Fig.3A). This cleft impedes the formation of actin pseudo-dimer and this conformational state of the Arp2/3 complex is called 'inactive'.

Using Electron Microscopy and Single Particle Analysis, it was found that the Arp2/3 complex can exist in several conformational states (Rodal et al. 2005). By itself the Arp2/3 complex is stabilized in the open conformational state. Binding of Nucleation Promoting Factors (NPFs), bearing VCA-domains, induces conformational rearrangements in the Arp2/3 complex: subunits Arp2 and Arp3 move toward the center of the cleft, subsequent closure of subunits Arp2

and Arp3 forms an actin pseudo-dimer and shifts the Arp2/3 complex in its closed/active state (Fig.3C). Thus, NPFs trigger the Arp2/3 complex mediated actin assembly (Fig.3B).



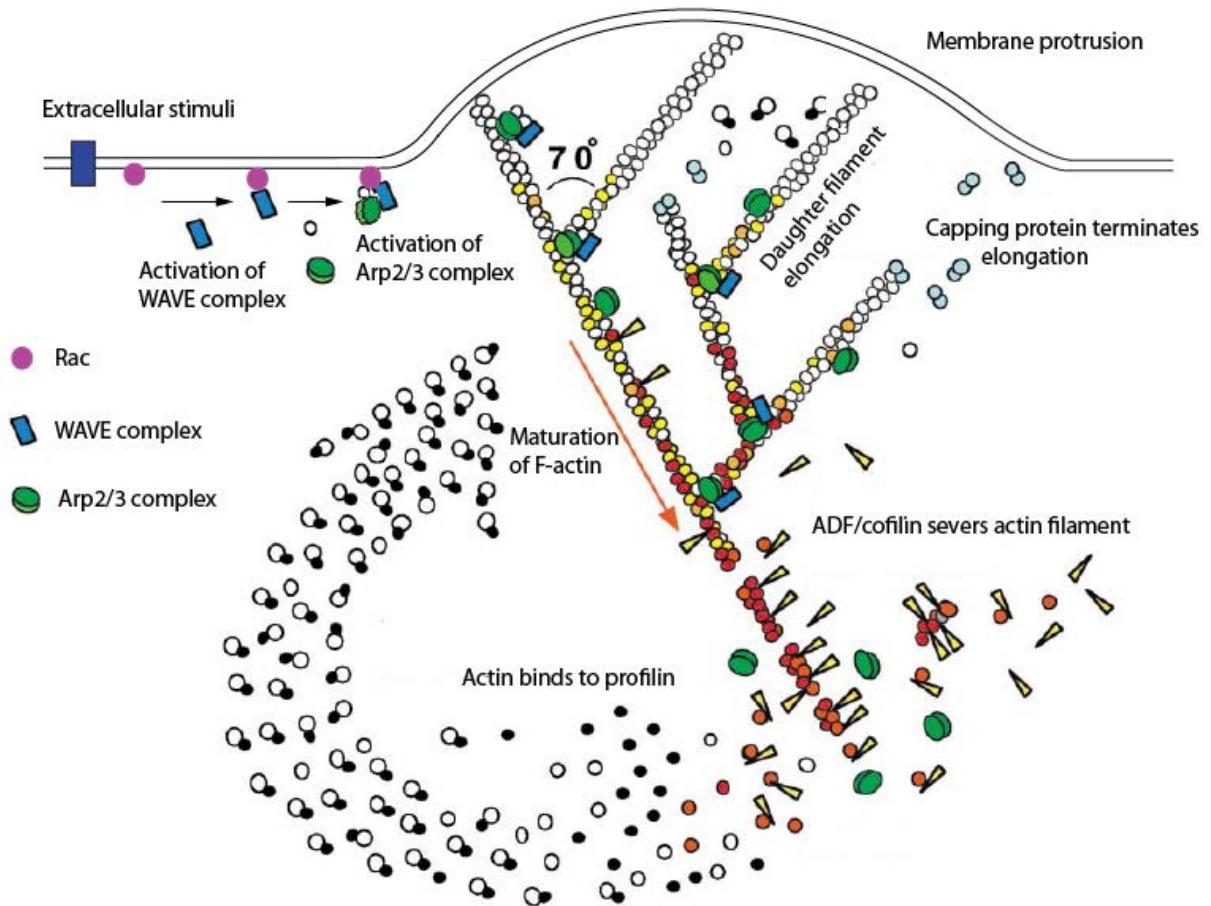
**Figure 3. Open and closed conformational states of the Arp2/3 complex.** Modified from (Rodal et al. 2005)

**A)** Crystal structure of the Arp2/3 complex at 2 Å resolution. Red arrow points the cleft between subunits Arp2 and Arp3. **B)** 2D projections of the Arp2/3 complex in its open/inactive, closed/active and intermediate states. **C)** Scheme illustrating the activation of the Arp2/3 complex by Nucleation Promoting Factor WASP. Coronin as an inhibitor of the Arp2/3 complex stabilizes its inactive/open state. WASP attaches the complex and brings subunits Arp2 and Arp3 together. Red arrows show conformational changes in the Arp2/3 complex during its activation. Moreover, WASP stabilizes the closed conformational state of the Arp2/3 complex.

### 3.2 Mechanism of branched actin networks formation in lamellipodia via the Arp2/3 complex

Arp2/3 complex generates the branched actin filaments network via formation of so called branch junction between two actin filaments (Pfaendtner et al. 2011). NPFs are known to play a pivotal role in the branch junction formation. NPFs act at multiple steps: it activates Arp2/3 complex and facilitates its binding to the side of pre-existing (mother) actin filament, increases the association rate of Arp2/3 complex with the mother filament and brings the first ATP-bound monomer of actin to the branch junction (B. A. Smith et al. 2013). Subsequently, new (daughter) filament is elongating at an angle of 70 degrees from existing filament in a Y-branch orientation (Fig.4).

Arp2/3 complex requires tight regulation *in vivo* due to the importance of branched actin network assembly that is critical for complex cellular processes like lamellipodia and invadopodia formation as well as endocytosis involvement.



**Figure 4. Activity of Arp2/3 complex at the leading edge of motile cell.** Modified from (T. D. Pollard 2007)

At the leading edge Arp2/3 complex is activated by WAVE complex in response to the extracellular stimuli. Activated Arp2/3 complex binds pre-existent actin filament and facilitates the growth of daughter filament. Daughter filament grows until it capped by capping proteins. ADF/cofilins cut matured actin filament and thus reveal G-actin. Consequently, G-actins are getting recruited by profilins.

### 3.2.1 Model of the branch junction

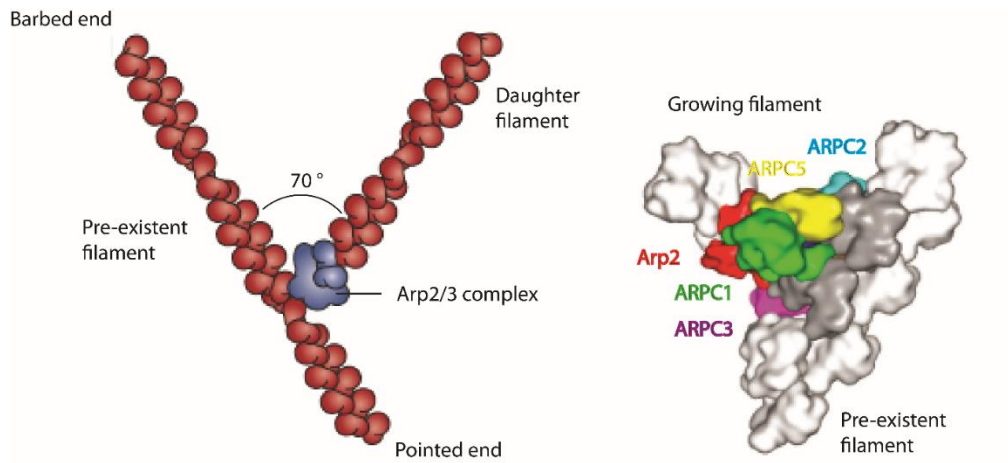


Electron microscopy three dimensional reconstruction of the branch junction was derived with a quite low resolution (2.6 nm), but crystal structures were well fitted into this reconstruction (Rouiller et al. 2008). This reconstruction helped to decipher the molecular mechanisms of the Arp2/3 complex-actin filament interaction. Model of the branch junction showed that all seven subunits of the Arp2/3 complex are involved in the branch junction assembly. Moreover, surprisingly, all seven subunits attach mother filament, that could be explained by the high significance of branch junction stability.

During the subsequent analysis of the branch junction it was established that Arp2/3 complex contact five monomers of actin from the pre-existing filament (E. D. Goley et al. 2010).

### **3.2.2 Contribution of Arp2/3 complex subunits into the formation of branch junction**

The fact is that not only Arp2 and Arp3 subunits play pivotal role in the branched actin network assembly. It was found that loss of ARPC1 subunit dramatically decreases the ability of Arp2/3 complex to bind VCA-domain and nucleation activity of Arp2/3 complex (Pang 2004). Subunits ARPC1, ARPC2, ARPC4 and ARPC5 make a supportive platform for Arp2 and Arp3. Moreover, ARPC2 and ARPC4 form a heterodimer ARPC2/ARPC4 that is essential for the Arp2/3 complex functionality, and that is known to be a core structure for the attachment of Arp2/3 complex to the pre-existing filament. According to the yeast two-hybrid analysis ARPC3 can bind VCA-domain, thus ARPC3 is also significant for the Arp2/3 complex activity.



**Figure 5. The actin branch junction.**

Modified from (Erin D Goley and Welch 2006) and (Rouiller et al. 2008) consequently. Arp2/3 complex binds pre-existent (mother) actin filament, forms so-called branch junction and contributes to the growth of daughter F-actin in a Y-shape manner at a  $70^\circ$  angle. On the 3D reconstruction we observe that all subunits of Arp2/3 complex are involved in branch junction formation.

### **3.3 Cortactin contributes to the regulation Arp2/3 complex**

Not only NPFs regulate Arp2/3 complex activity. Cortactin promotes and stabilizes Arp2/3-induced branched actin filaments formation.

Cortactin like NPFs has A-motif that attracts Arp2/3 complex, but instead of a G-actin binding V-motif, cortactin has an actin filament binding motif. Interestingly the A-motif of cortactin is functionally and biochemically different from the NPF A-motif. Cortactin is relatively weak activator of Arp2/3 complex, because it lacks actin monomer binding site. However, in the presence of active N-WASP, cortactin dramatically stimulates Arp2/3-induced branched actin formation compared to branching with VCA-domain or cortactin alone. Thus cortactin and VCA-domains synergistically promote actin branching. Moreover, cortactin potentially inhibits the debranching of actin filaments (Weaver et al. 2001). Cortactin is known to bind preferably ATP- or ADP-P<sub>i</sub>- bound actin thus protecting new added monomers from P<sub>i</sub> release. In contrast, cofilin/ADP has higher affinity to ADP-bound actin monomers and stimulates P<sub>i</sub> detachment. Cooperated activity of cortactin and cofilin balances the actin dynamics at the leading edge of migrating cells.

### **3.4 Nucleation of new actin filaments for the Arp2/3 complex activity**

As was mentioned before, the Arp2/3 complex requires the presence of pre-existing actin filaments. It was found so far that new actin filaments can be generated either by severing the existing filaments via ADF/Cofilins or can be assembled *de novo*.

The Arp2/3 complex requires the presence of already existing actin filaments. There are three potential mechanisms that can explain the origin of the initial actin filament for the Arp2/3

complex activity in lamellipodia. The first possible mechanism is a severing by cofilins that gives new actin filaments for Arp2/3 complex priming (Fig.4A).

The second way is a JMY protein activity. JMY (junction mediating and regulatory protein) has three V-motifs and one C-motif. As was mentioned before several V-motifs are also found in SPIRE family of proteins. JMY can bind actin monomers via its V-motifs, form an actin nucleus and thus contribute to the elongation of F-actin (Zuchero et al. 2009) (Fig.4B). However it is very unlikely that JMY could be a universal provider of primary actin filaments for Arp2/3 complex. JMY is participated in cell migration of only some cell lines, decreasing E-cadherin protein stability and cell–cell adhesion, thereby indirectly increasing cell migration.

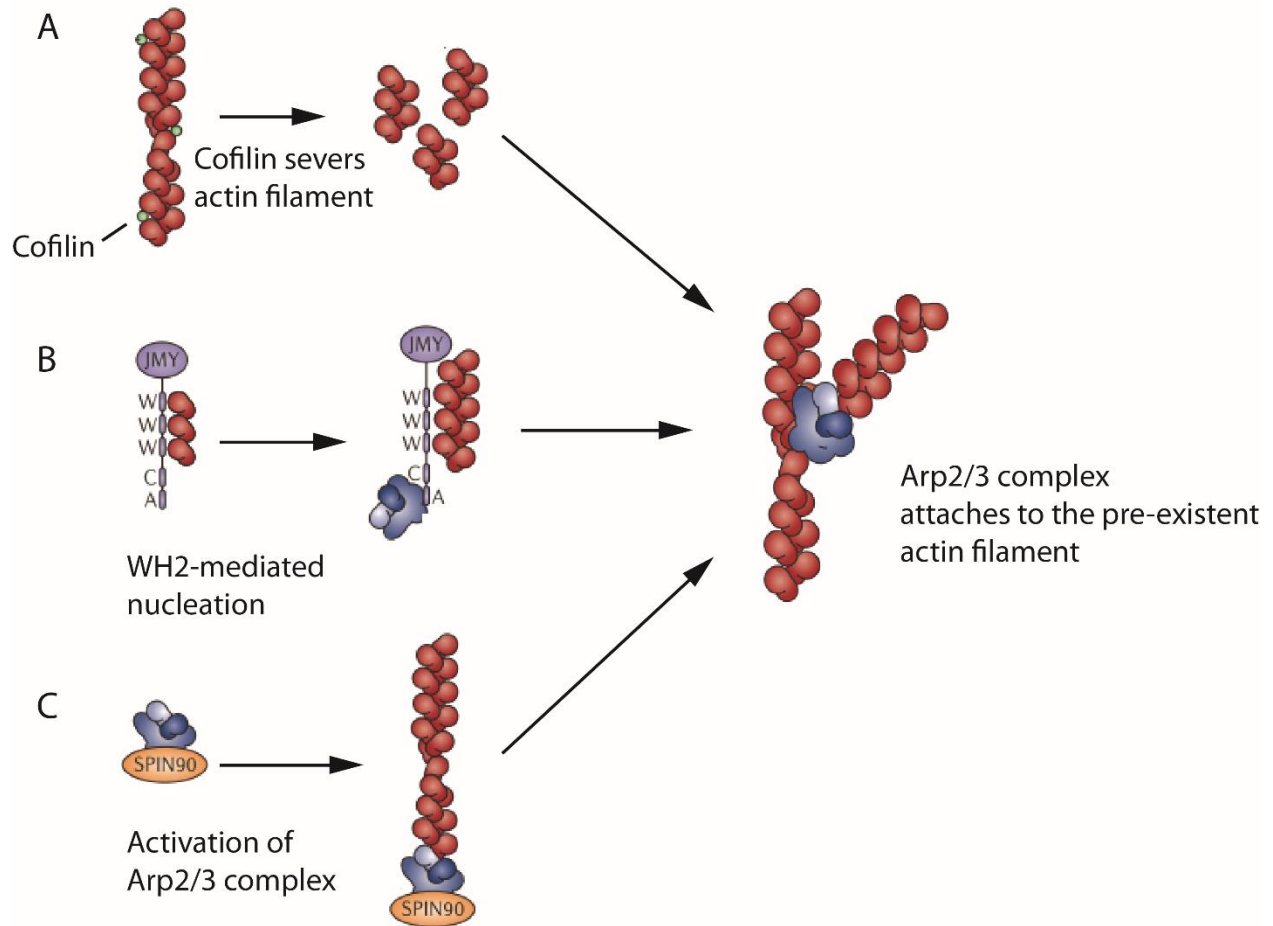
The last possible way is to promote Arp2/3 complex activity without any pre-existing filaments. Dip1 protein (also known as NCK interacting protein with SH3 domain, SPIN90 or WISH) can activate Arp2/3 complex in non-NPF-like mode. Without any pre-existing filament, Dip1 brings Arp2 and Arp3 subunits together, thus Arp2 and Arp3 mimic to the actin dimer and can initiate the polymerization of actin filament (Krause and Gautreau 2014; Wagner et al. 2014) (Fig.4C).

ADF/cofilin group of proteins includes cofilin and actin depolymerizing factor protein ADF. Cofilin was originally found as a protein that enables actin filaments formation (COFILamentous structures of actIN). Cofilin is highly similar to ADF and they share mostly the same biochemical properties, thus these proteins were considered to compose single group of proteins called ADF/cofilin. All eukaryotes express proteins from the ADF/cofilin family. In mammals three forms are expressed: ADF or destrin, cofilin-1 and cofilin-2. Cofilin-1 is expressed in higher concentration and it has been studied more profoundly (Goroncy et al. 2009).

Cofilin is known for its actin depolymerizing and severing abilities. In its inactive form cofilin is bound to the membrane via  $PIP_2$  and due to the extracellular signals,  $PIP_2$  is getting hydrolyzed, cofilin gets released and attach to actin filaments. Cofilin has its highest affinity for ADP-actin monomers that are accumulated in the aged region of the actin filament. Cofilin binds to the two neighboring actin monomers along the whole aged filament and causes significant twist

of actin filament. Due to this twist actin filament becomes fragile and finally it falls apart into short fragments. Moreover, cofilin accelerates the dissociation of  $P_i$  from ADP- $P_i$ -bound actin in filament and thus promotes debranching of actin filament (Blanchoin and Pollard 1999).

Thereby, Cofilin severs actin filaments making more ends and thus accelerating actin filament dissociation. Pointed ends of new short fragments rapidly disassemble and G-actin are released. This turnover of actin is highly important for actin dynamics and thus to cell locomotion and other processes.



**Figure 6. Generation of pre-existent mother filament *de novo* for priming of the Arp2/3 complex.** Modified from (Krause and Gautreau 2014)

**A)** Cofilin may sever actin filament and provide multiple number of actin filaments that will encounter Arp2/3 complex. **B)** JMY protein linear actin filament via its WH2 domains and also activates Arp2/3 complex. **C)** SPIN90 brings Arp2 and Arp3 subunits together without pre-existent mother filament.

## **4. Activation of the Arp2/3 complex via Nucleation Promoting Factors**

As was mentioned above, tertiary structures of Arp2 and Arp3 subunits were homologous to the actin monomers and these subunits could be possible sites for G-actins during the nucleation. However, the crystal structure couldn't explain how nucleation of actin filaments happens because of the big cleft between subunits Arp2 and Arp3. Afterwards with the help of Electron Microscopy it was shown that Arp2/3 complex fluctuates between open (inactive) and closed (active) conformational states (Rodal et al. 2005).

Without NPFs Arp2 and Arp3 are spatially separated and cannot nucleate the daughter filament. To form the branch junction Arp2/3 complex has to be activated by so called Nucleation Promoting Factors (NPFs). Further Arp2 and Arp3 subunits of the activated Arp2/3 complex bind ATP-bound actin monomer. It is known that the slowest step in a spontaneous actin polymerization is a formation of actin dimer. It is believed that in the presence of NPFs, subunits Arp2 and Arp3 overcome kinetic barrier to nucleation by mimicking to the actin dimer and binding the first actin monomer of new actin filament.

This theory was confirmed by Electron Microscopy and Single Particle Analysis showed that NPFs induce conformational changes of originally inactive Arp2/3 complex so that Arp2 and Arp3 close in and form a nucleus for ATP-bound actin connection (Rodal et al. 2005).

### **4.1 Structure and activity of Nucleation Promoting Factors**

Arp2/3 complex activity is regulated by NPFs, also called activators of Arp2/3 complex, and inactivators. The WASP family proteins are the biggest class of NPFs, which in mammals includes WASP, N-WASP, WASH, WHAMM, WAVE 1 to 3 and already mentioned JMY. Distinct NPFs activate Arp2/3 complex regulating different processes and respectively have different structural features. In general, WASP-family proteins share the well-conserved C-termini and dissimilar N-termini. Unique N-terminus domains regulate the activity of WASP family proteins, intracellular localization and interaction with other proteins.

As was mentioned before, the best characterized NPFs are the C-terminus VCA domains of the proteins of the WASP (Wiskott–Aldrich syndrome protein) family: WASP, N-WASP (neuronal WASP), WASH complex, WHAMM and WAVE1-3 complexes. VCA domain consists of three short motifs: Verprolin homology motif that also known as WASP homology 2 (WH2) domains (V), central (C), acidic (A). V-motif binds G-actin and thus delivers an initial subunit to the daughter filament, C-motif binds both G-actin and Arp2/3 complex with VCA, A-motif binds Arp2/3 complex only. Thus, the incorporation of CA-motifs into Arp2/3 complex allows the actin monomers attraction to the branch junction. Further Arp2 provides rapid ATP-hydrolysis on the VCA-attracted actin monomer that initiates actin polymerization. Thus VCA-domains of WASP family proteins activate Arp2/3 complex by formation of Arp2-Arp3-nucleus complex on the pre-existing filamentous actin (Dayel and Mullins 2004). Subsequently, VCA-domains attachment causes conformational changes in Arp2/3 complex.

Motifs C and A together supply most of the energy to bind Arp2/3 complex and change conformational state of the complex. Interestingly, C-motif binds actin monomer with higher affinity than it binds Arp2/3 complex. It was found that Arp2/3 complex can associate one or two NPFs. VCA binding sites are preferably located nearby subunits Arp2 and Arp3. For example, Arp2/3 complex simultaneously binds two WAVES: one WAVE contacts Arp3, the second one contacts ARPC1 and Arp2 (Padrick et al. 2011).

Moreover, VCA-domains coordinate the association of activated G-actin-bound Arp2/3 complex with pre-existing filamentous actin to initiate daughter filament assembly.

#### **4.2 Models for Arp2/3 complex activation via NPFs**

A model for Arp2/3 complex activation via NPFs initially was proposed by Kelly et. al. (Kelly et al. 2006). According to this model VCA-domain first primes Arp2/3 complex by inducing conformational changes in complex, and then attracts monomer of actin thus making a nucleus. Later cross-linking study showed that actin-monomer recruitment by NPF induced conformational changes in Arp2/3 complex leading to the nucleation of branched actin (Hetrick et al. 2014).



Recently a new NPF-Arp2/3 binding model was proposed (Luan et al. 2018). Data based on the chemical cross-linking and mass-spectrometry, structure-based mutational analysis and *in silico* assay showed that C-motif binds to the Arp2 and Arp3 subunits while A-motif attaches to the back side of Arp3. This model proposes that one NPF binding site is on the Arp3 and the second one embraces subunits Arp2 and ARPC1 at the bottom part instead of Arp2/ARPC1 interface. All together the data confirmed the Arp3 tail release model and explain the nature of NPFs-mediated conformational changes in Arp2/3 complex.

### **4.3 Regulation of Nucleation Promoting Factors**

WASP family proteins are known to have similar C-terminal VCA, but they are characterized by distinct N-terminal parts that are responsible for WASP family proteins activation, intracellular localization and protein-protein interactions. Several NPFs are known to be activated by Rho-family GTPases and thus link Arp2/3 complex with extracellular signaling. It was recently found that WASP-family proteins can also act as polymerases and stimulate an elongation of uncapped actin filaments. In the absence of profilins, V-motif is enough to expedite the growth of F-actin (Bieling et al. 2017).

#### **4.3.1 WASP and N-WASP**

Besides the VCA-domain, WASP protein has also other different N-terminal regulatory regions: WASP homology domain WH1, a basic region, GTPase binding domain (GBD) and a proline rich region.

WASP and N-WASP proteins have high sequence homology, but N-WASP has specific regions in its N-terminus and modified VCA-domain. Instead of having one V-motif, N-WASP has two verprolin homology motifs (VV-motif) that increases the binding affinity to the Arp2/3 complex. Thus, to avoid the abnormalities N-WASP is supposed to be tightly regulated by cell.

N-termini of WASP and N-WASP proteins consist of 16 residues sequence motif called a CRIB that enables Cdc42 binding. WASP and N-WASP bind Rho family GTPases via GBD. GBD includes CRIB motif and surrounding sequences.

It is known that VCA-domains of both inactive WASP and N-WASP are autoinhibited by contact with GBD. Interaction of Rho family GTPase Cdc42 with GBD abolishes an autoinhibition due to the dramatic conformational change in WASP and N-WASP that release VCA domain. Thus, after Cdc42 connection WASP family proteins are capable of Arp2/3 complex activation (Kim et al. 2000). N-WASP is also can be activated by PIP<sub>2</sub>, that binds to the N-WASP synergically with Cdc42. WASP autoinhibition is also modulated by phosphorylation of the GBD at tyrosine 291.

N-WASP is phosphorylated by Src family tyrosine kinases. Src family tyrosine kinase can activate N-WASP by phosphorylation of its tyrosine and thus inducing Arp2/3 complex mediated actin polymerization. However, Src family tyrosine kinase is able to degrade N-WASP through ubiquitination. N-WASP activation and degradation is balanced in the cell in response to the extracellular conditions (Suetsugu et al. 2002).

WASP-interacting protein (WIP) is known to accelerate the Arp2/3 complex activity via binding to the WH1. WIP also protect WASP from calpain-induced degradation. Moreover, WIP binding is crucially important for the expression of WASP *in vivo* (Burianek and Soderling 2013).

Not only Cdc42, PIP<sub>2</sub> and Src kinase can activate N-WASP, but also WASP interacting SH2 protein WISH and the adaptor protein via its SH3 domains. WISH binds to the proline rich region of N-WASP and thus enhances the Arp2/3 complex activation even without Cdc42 binding.

#### **4.3.2. WASH**

WASH is a SCAR and WASP homolog that is also known as WASHC1. WASH is localized at the surface of endosomes where it is responsible for branched actin network formation. The general activity of WASH is an endosomal transport.

WASH has N-terminal proline rich region and two WASH homology domains. WASH localizes in filopodia and lamellipodia, where it interacts with Arp2/3 complex. WASH also colocalizes with EEA1 and transferrin in endosomes. It is suggested that WASH does not have autoinhibition that is typical for WASP and N-WASP.

WASH exists within a pentameric complex called WASH Regulatory complex or SHRC. SHRC consists of Strumpellin, FAM21, SWIP and CCDC53. FAM21 is known to be important for the localization of SHRC on the endosomes. Interestingly, it is highly suggested that SHRC may inhibit WAVE until its activation. SHRC may be activated by Rho.

The mechanism of WASH complex assembly is poorly understood. It is known that the degradation of one of the SHRC subunits causes degradation of all the others. All the subunits require binding to their partners in order to obtain its native stable state (Derivery and Gautreau 2010a). The assembly factor that could manage the assembly of SHRC was remaining unknown until recently: it was found that HSBP1 promotes the assembly of SHRC (Visweshwaran et al. 2018).

### **4.3.3 WHAMM**

WHAMM is a WASP homolog associated with actin, membranes and microtubules. Unlike other NPFs, WHAMM regulates endoplasmic reticulum to Golgi transport. WHAMM localizes to the cis-Golgi apparatus and tubulo-vesicular membrane transport intermediates (Campellone et al. 2009).

WHAMM has two V-motifs (VVCA), proline rich domain, coil-coiled region and specific N-terminal region that is not found in other proteins from WASP family. This specific region contributes to the attachment of WHAMM to the Golgi apparatus. VVCA-domain of WHAMM is usually sequestered by interaction of its coil-coiled region with microtubules. Thus WHAMM

activity is regulated by the microtubule cytoskeleton. Moreover, an important role of WHAMM in an autophagosome genesis was reported.

## 5. WAVE complex is an activator of Arp2/3 complex in lamellipodia

WAVE is a well-studied activator of Arp2/3 complex that is localized at the lamellipodium tip of motile cell. WAVE is a WASP-family verprolin-homologous protein that has three isoforms WAVE1, WAVE2 and WAVE3 (also known as SCAR1-SCAR3). WAVE2 is expressed ubiquitously in mammals, whereas WAVE1 and WAVE3 expression is enriched in brain. WAVE2 is responsible mostly for the activation of Arp2/3 complex at the lamellipodium tip.

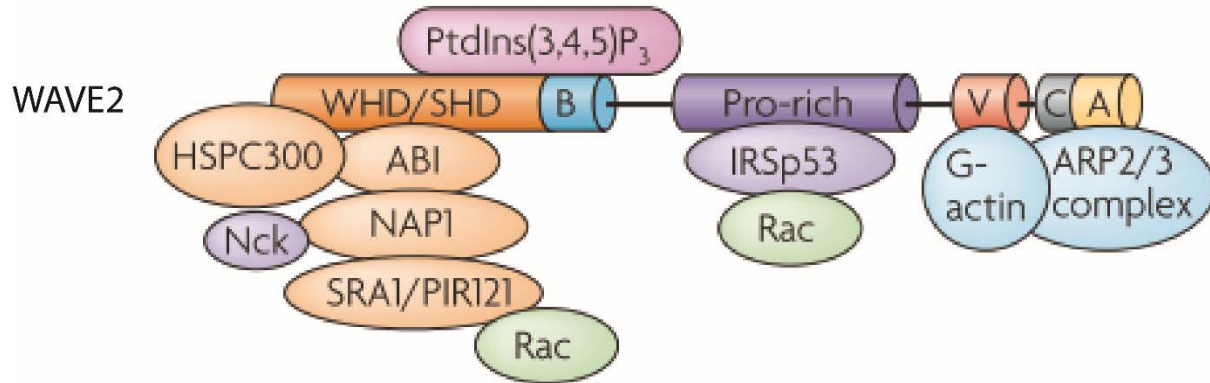
### 5.1 Structure of WAVE complex

While C-terminus of WAVE bears VCA-domain, N-terminus region of WAVE consists of WAVE-homology domain WHD and a basic region. Basic region of WAVE2 binds to PIP<sub>3</sub>, that is important for WAVE2 localization at the leading edge. WHD is a coiled-coil region in all WAVEs and is responsible for heterocomplex formation. It was established that Rac-GTP and phospholipids are necessary for WAVE complex activity and localization at the lamellipodia. Central part of WAVE a Pro-rich region that plays role in modulation of WAVE complex activity (Takenawa and Suetsugu 2007).

In cells WAVE proteins always form a heteropentameric WAVE complex by consistent interaction with four additional proteins: Sra (known paralogous proteins are Sra1/CyFIP1 and PIR121/CyFIP2), Nck-associated protein Nap (known paralogous proteins are Nap1/NcKAP1/Hem2 and Hem1/NcKAP2), Abelson-interacting protein Abi1 (known paralogous proteins are Abi1/Nap1BP, Abi2/ArgBP, Abi3/NESH), and Brk1 (also known as HSPC300) (Derivery and Gautreau 2010b; Takenawa and Suetsugu 2007) (Fig.7). Functionally, WAVE complex is similar to the SHRC mentioned above. According to the Electron Microscopy data WAVE and WASH complexes share quite similar structural topology (Jia et al. 2010).

It is barely known how the WAVE complex is getting assembled *in vivo*. The protein Nudel that is important for lamellipodial formation was found as an assembly factor of WAVE complex (Wu et al. 2012). It was shown that Nudel binds to Sra1 and prevents Sra1-Nap1-Abi-Brk1

complex from degradation. Interestingly, Nudel is required only at the early steps of WAVE complex assembly. It barely binds the mature WAVE complex and suppresses Arp2/3 complex-mediated branched actin polymerization at the lamellipodium tip. Brk1 is known to form trimer, nevertheless only one Brk1 is required for WAVE complex formation (Derivery et al. 2008).



**Figure 7. Structure of WAVE complex and its binding partners.** Modified from (Takenawa and Suetsugu 2007)

N-terminus of WAVE2 consists of WAVE-homology protein WHD and a basic region B, that binds PIP<sub>3</sub>. WHD binds four additional proteins of the WAVE complex: HSPC300, ABI, NAP1 and Sra1/PIR121.

C-terminus of WAVE contains VCA domain that can activate Arp2/3 complex.

Pro-rich region of WAVE2 is known to bind both IRSp53 and Rac to modulate WAVE complex activity.

## 5.2 Regulation of WAVE complex' activity

C-terminus VCA region in WAVE complex is known to be inhibited by contact with Sra1, which contribute to maintaining the WAVE in an inactive state (Z. Chen et al. 2010). To activate Arp2/3 complex WAVE has to be stimulated by the small GTPase Rac, kinases and phosphatidylinositols. Small GTPase Rac1 binds to WAVE complex via Sra1 protein and may release the sequestered VCA-domain.

Moreover, a molecule called IRSp53 (Insulin Receptor Substrate of 53 kDa) has been proposed to modulate WAVE2 activity (Suetsugu et al. 2006). It was shown *in vitro* that Rac1 is able to bind to the Pro-rich region of WAVE2 through IRSp53 molecule and release VCA-domain (Krause and Gautreau 2014).

The phosphorylation of WAVE proteins has been reported to play a regulatory role (Lebensohn and Kirschner 2009). VCA-domain has five Casein Kinase 2 (CK2) phosphorylation sites. These are serines 482, 484, 488, 489, and 497. Phosphorylation of Ser488, Ser489 and Ser497 increase the negative charge of VCA-domain thus increasing the affinity of WAVE2 complex to the Arp2/3 complex. Phosphorylation of Ser482 and Ser484 inhibits the activation of the Arp2/3 complex by VCA domain (Pocha and Cory 2009).

WAVE2 activity and localization in lamellipodia is also regulated by PIP3 (Oikawa et al. 2004). In response to the chemoattractant gradient, cells induce PIP3 production at the leading edge to establish cell polarity. PIP3 gradient at the membrane initiates actin reorganization and thus polarized cell moving. PIP3 binds to the basic domain of WAVE2 complex and recruits WAVE2 complex to the polarized membrane. PIP3 binds to the basic domain of WAVE2 complex.



## **6. Inhibition of the Arp2/3 complex**

Due to the ability of actin filaments reorganization Arp2/3 complex functionality must be tightly controlled. One way to regulate the Arp2/3 complex activity is a binding of inhibitors. Inhibitors are the proteins that convert Arp2/3 complex into inactive state and thus block the actin nucleation. At the present time only few of the Arp2/3 complex inhibitors are known. It seems like in each part of the cell where actin reorganization occurs, there are two antagonistic molecules that regulate Arp2/3 complex activity.

### **6.1 Coronin**

One of the well knowns inactivators of Arp2/3 complex is a protein called Coronin. Coronin 1 is a best characterized protein from the Coronin family. Coronin 1 (Crn1) is a leading edge protein which has three domains: N-terminal  $\beta$ -propeller domain consists of six  $\beta$ -propeller-like WD-repeats and binds actin, middle domain and C-terminal coiled-coil (CC) domain that is responsible for dimerization of coronins and also regulates Arp2/3 complex activity. Crn1 binds by the  $\beta$ -propeller domain at ARPC2 subunit of Arp2/3 complex and induces a standard open conformation of Arp2/3 complex thus initiating dissociation of branch junction. Coronin1 also stabilizes Arp2/3 complex in inactive conformational state (Rodal et al. 2005).

### **6.2 GMF**

GMF belongs to the ADF-H domain family of actin regulatory proteins as well as ADF-cofilins, Abp1/Drebrin, Twinfilin and Coactosin. GMF is a highly conserved 17 kDa protein that contains ADF-H domain. ADF-H domain is a globular protein module that was found in all the members of ADF-H family of proteins. Despite the fact that ADF-H domain is responsible for binding with both F-actin and G-actin, GMF directly binds the Arp2/3 complex. The reason why GMF acts unlike the other proteins from ADF-H family is that GMF has a homology to the C-

motif of VCA-domain and while ADF-H family proteins contribute to the disassembly of aged filamentous actin, GMF binds directly to the Arp2/3 complex.

GMF binds near ARPC2 subunit and induces the standard open conformation of Arp2/3 complex. It was found that GMF also promotes an atypical open conformation of Arp2/3 complex characterized by a substantial shift of the ARPC3 subunit toward Arp3. Knowing that GMF and Crn1 have distinct binding sites on the Arp2/3 complex, it was shown that these inhibitors can synergize in inhibiting Arp2/3 complex (Sokolova et al. 2017).

The mechanism of Arp2/3 complex inactivation via GMF differs from the other inhibitors: GMF binds Arp2/3 complex and prevents Arp2/3 complex binding with actin. Thus GMF inhibits activity of the Arp2/3 complex and facilitates the dissociation of daughter filament from the pre-existing F-actin.

Moreover, it was shown that GMF has low affinity to ATP-bound Arp2/3 complex comparing to the ADP-bound Arp2/3, while it does not bind neither ADP-bound G-actin nor ATP-bound G-actin. GMF inhibits WAVE-activated Arp2/3 complex, but does not influence on the N-WASP-activated Arp2/3 complex activity (Boczkowska, Rebowski, and Dominguez 2013). Similar to ADF/cofilins, GMF has conserved N-terminal serine residues at the positions 2 and 4. Phosphorylation of Ser2 may regulate the activity of GMF. Thus GMF acts in a similar way with ADF/cofilins.

### **6.3 PICK1 and Gadkin**

PICK1 and Gadkin (also known as AP1AR and c-BAR) are another types of inactivators that mimic to the VCA domains of NPFs and directly compete for the Arp2/3 complex binding sites. Gadkin and PICK1 inhibit Arp2/3 complex activation by sequestering it from the NPFs. Gadkin is a trans-Golgi network/endosomally localized adaptor protein (AP)-1-associated adaptor protein. Gadkin attaches to the Arp2/3 complex via its acidic cluster motif reminiscent the A-motif

of NPFs (Maritzen et al. 2012). Gadinin inhibits Arp2/3 complex at the endosomes, while PICK1 inactivates Arp2/3 complex at the clathrin-coated pits.

PICK1 is a PDZ and BAR-domain containing protein that is involved in endocytosis in neurons. PICK1 binds both filamentous actin and Arp2/3 complex and inhibits N-WASP-mediated activation of Arp2/3 complex which required for the vesicle trafficking and the development of neuronal tissue (Rocca et al. 2008).

## **7. Arpin is an inactivator of Arp2/3 complex in lamellipodia that counteracts the WAVE complex**

Until recently it was not clear how Arp2/3 complex can be directly inactivated in lamellipodia (Irene Dang et al. 2013). Arpin is a novel inhibitor of Arp2/3 complex at the lamellipodium tip that was found using a bioinformatic search for the proteins with a homology to VCA motif of NPFs (Fig.8A). Arpin has an acidic motif (but it lacks V- and C-motifs) similar to the acidic motif of NPFs that interact with Arp2/3 complex.

It was tested whether A-motif alone is sufficient for the Arp2/3 complex inactivation. Indeed, separated acidic motif still inactivates the Arp2/3 complex, but less effectively than the full-length Arpin. In contrast, Arpin with lack of acidic motif was deprived of the ability to inhibit Arp2/3 complex.

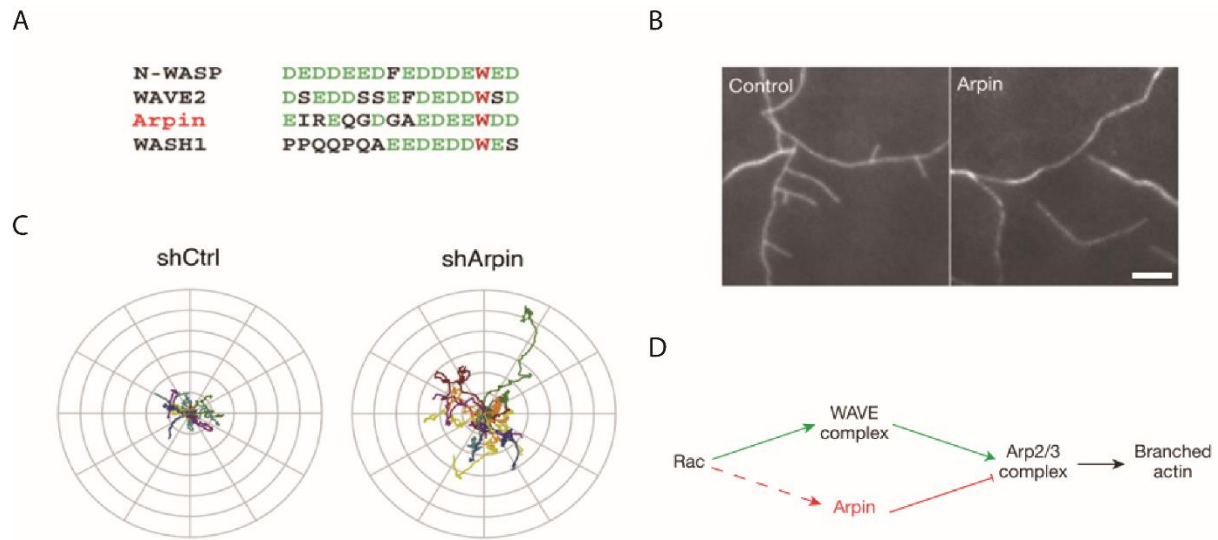
## 7.1 Activity of Arpin *in vivo*

TIRF microscopy demonstrated that Arpin decreases the amount of the branch junctions (Fig.8B). Arpin impairs actin polymerization in the presence of activated Arp2/3 complex in a dose-dependent manner. Arpin competes the NPFs for Arp2/3 binding and thus controls actin filaments dynamics at the leading edge of the cell *in vivo*.

It was identified that Arpin does not prevent the formation of lamellipodium, but suppresses the already existent one. Consequent Arpin depletion confirmed this statement: shArpin increased the speed of the protrusion formation. Moreover, knockdown of Arpin increased the directionality of cell trajectories and increased the speed of motility (Fig.8C). Microinjections of Arpin in fish keratocytes, known for its high directional persistence, induced these cells to turn. Thus Arpin is able to regulate the directional persistence of the cells.

It was shown that Arpin is recruited and activated by GTPase Rac1 at the lamellipodium tip, where Rac1 also stimulates WAVE complex. Co-existence of positive Rac-WAVE-Arp2/3 and negative Rac-Arpin-Arp2/3 regulatory circuits generates an «incoherent feedforward» loop showing a paradoxical regulatory activity of Rac1 (Fig.8D).

Although Arpin shortens lamellipodia lifetime and decreases the directional persistence of the cells, it is not involved in chemotaxis and directed cell migration (Irène Dang et al. 2017). Thus, taking into account the important role of Arpin in actin dynamics regulation, Arpin has to be itself strongly tuned by the cell.



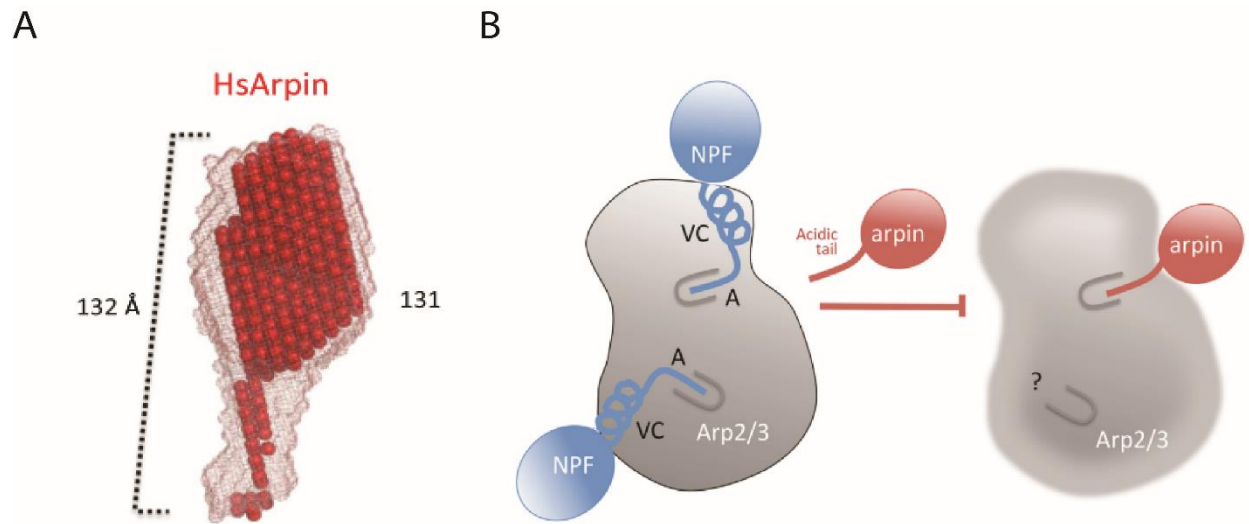
**Figure 8. Arpin inhibits Arp2/3 complex at the lamellipodium tip.** Modified from (Irene Dang et al. 2013)

- A)** Arpin has a homology with C-terminus A-motif of NPFs, but it lacks V<sub>1</sub>-C-motifs.
- B)** TIRF microscopy showed that in the presence of Arpin branched actin assembly reduces.
- C)** Arpin depletion increases the directionality of mammalian cells.
- D)** Arpin is activated by the same GTPases Rac that activates WAVE complex and thus Arpin is involved in “incoherent feed-forward loop”.

## 7.2 Structure of Arpin

Arpin is highly conserved protein found in mammals, reptiles and amoeba. Due to the mobility of C-terminus the crystal structure of Arpin was not derived. Combination of small-angle X-ray scattering (SAXS) and X-ray crystallography showed that Arpin is an elongated globular core with unstructured linear C-terminal tail (Fig.9A). This linear part is responsible for Arp2/3 binding (Fetics et al. 2016). Moreover, it was shown that A-motif of Arpin might compete with NPFs for the Arp2/3 complex binding (Fig.9B).

To depict the molecular mechanisms of Arpin-Arp2/3 complex binding, using Transmission Electron Microscopy (TEM) and Single Particle Analysis a three-dimensional reconstruction of Arpin bound to the Arp2/3 complex was derived. Two potential Arpin binding sites on the Arp2/3 complex were identified: one site is located near subunits Arp3 and ARPC2 and the second one is located near subunits Arp2 and Arp3. Consistent with the SAXS and X-ray crystallography data, Arpin was located at the distance of approximately 5 nm from the Arp2/3 complex. This distance is suggested to be the C-terminus tail of Arpin (Sokolova et al. 2017).



**Figure 9. Structure of Arpin.** Modified from (Fetics et al. 2016)**A)** Ab Initio Model of Arpin derived based on SAXS data.**B)** Scheme of Arpin-Actin-Arp2/3 complex binding. Arpin binds to Arp2/3 complex via its acidic tail and Arpin might compete with NPFs for binding with Arp2/3 complex.

## **Objectives**



Cell migration is required for such essential physiological processes like embryogenesis, wound healing and immune response. Cell migration is also a prominent property of pathological process metastasis. During the migration motile cell undergoes several steps, including the formation of membrane protrusions called lamellipodia and filopodia that allow the cell to extend its whole body forward. Lamellipodia is a giant sheet-like membrane protrusion that is formed by the growing branched actin filament network.

Since branched actin formation is such an important and complicated process, it should be well-tuned by the cell. The major molecular machine that facilitates the branched actin filaments assembly is an Arp2/3 complex. Arp2/3 complex consists of seven subunits: two actin monomer reminiscent proteins Arp2 and Arp3 that form a platform for G-actin and make a nucleus for growth of «daughter» filament and five additional subunits ARPC1-5. Arp2/3 complex is intrinsically inactive due to the gap between subunits Arp2 and Arp3 that prevents the binding of the first G-actin in the growing filament.

Nucleation Promoting Factors (NPFs) are the activators of Arp2/3 complex which bring Arp2 and Arp3 subunits together and attract the first actin monomer in the nucleus. NPFs are characterized by the presence of VCA-domain that enables binding with both Arp2/3 complex and G-actin. It is known that different WASP family proteins work at the distinct parts of the cell, for example WASH complex activate Arp2/3 complex at the endosomes, while WAVE complex regulates Arp2/3 complex activity at the lamellipodial tip of motile cell. It was found that activators are known to be counteracted by inhibitors of Arp2/3 complex. Inhibitors like Cororin, GMF and Gadkin can prevent NPFs binding to the Arp2/3 complex and facilitate disassembly of the actin filament.

For a while it was not established whether any inactivator counteracts WAVE complex at the lamellipodium tip. However, an inactivator of the Arp2/3 complex at the lamellipodium is required for the tight regulation of the branched actin assembly. Recently, through the bioinformatics research for the proteins containing VCA region, inactivator of Arp2/3 complex at the leading edge of the cell was found and called Arpin. Arpin binds Arp2/3 complex at two distinct

binding sites and causes its standard inactive conformational state. The activity of Arpin was analyzed *in vivo* and Arpin was identified as a turning factor of cells. Surprisingly, Arpin itself requires activating signals from small GTPase Rac in a similar manner to the WAVE complex. This finding lead to the suggestion of Arpin as a part of «incoherent feed-forward» loop. Arpin activity thus should be itself regulated by cell.

The goal of my thesis was to decipher the molecular mechanisms of regulation of Arpin activity. Before my arrival to the lab, Tankyrases were identified as Arpin partners by the host group. My goal was to analyze the importance of Tankyrase1/2 for Arpin activity *in vivo* and the effect of Tankyrase1/2 binding on the cell migration *in vivo*. During the analysis of cell migration, we demonstrated that Arpin with lack of Tankyrase1/2 binding is no longer active. To identify the molecular mechanisms of Arpin-Tankyrase1/2 binding, we performed the first EM three-dimensional reconstruction of Tankyrase-1 and Tankyrase-1 bound to Arpin. My next goal was to explain the observed information. We demonstrated that lack of Tankyrase1/2 binding decreases the amount of Arpin in the membrane fraction.

## **Results**



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## Structural basis of Arp2/3 complex inhibition by GMF, Coronin, and Arpin

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### Abstract

The evolutionarily conserved Arp2/3 complex plays a central role in nucleating the branched actin filament arrays that drive cell migration, endocytosis, and other processes. To better understand Arp2/3 complex regulation, we used single particle electron microscopy to compare the structures of Arp2/3 complex bound to three different inhibitory ligands: GMF, Coronin, and Arpin. Although the three inhibitors have distinct binding sites on Arp2/3 complex, they each induced an ‘open’ nucleation-inactive conformation. Coronin promoted a standard (previously described) open conformation of Arp2/3 complex, with the N-terminal  $\beta$ -propeller domain of Coronin positioned near the p35/ARPC2 subunit of Arp2/3 complex. GMF induced two distinct open conformations of Arp2/3 complex, which correlated with two suggested binding sites for GMF. Further, GMF synergized with Coronin in inhibiting actin nucleation by Arp2/3 complex. Arpin, which uses VCA-related acidic (A) motifs to interact with the Arp2/3 complex, induced the standard open conformation, and two new masses appeared at positions near Arp2 and Arp3. Further, Arpin showed additive inhibitory effects on Arp2/3 complex with Coronin and GMF.

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Together, these data suggest that Arp2/3 complex conformation is highly polymorphic and that its activities can be controlled combinatorially by different inhibitory ligands.

## Keywords

actin nucleation; single particle EM; yeast; conformation

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## INTRODUCTION

Dynamic reorganization of the actin cytoskeleton is vital to cell and tissue morphogenesis, and to processes such as cell motility, endocytosis, inflammatory responses, wound healing, neuronal activity, and intracellular transport [1–3]. Central to these rearrangements of the actin cytoskeleton is the *de novo* formation of new filaments by actin nucleation mechanisms. One of the most important and conserved nucleators of new actin filaments is the actin-related protein (Arp)2/3 complex, which specifically assembles branched filament arrays [4–6]. Due to the potency of its nucleation capabilities, the Arp2/3 complex must be tightly controlled *in vivo*. This is achieved by several known strategies. First, strong nucleation by Arp2/3 complex requires its association with a stimulatory co-factor or nucleation-promoting factor (NPF), the best characterized of which are the WASP/WAVE family proteins [7]. Second, nucleation by Arp2/3 complex can be inhibited by some binding partners, including Coronin [8–11], Glia Maturation factor (GMF) [12–14], Gadin [15], and Arpin [16]. While significant advances have been made in defining the structural basis of Arp2/3 activation both in solution and on actin filaments [17–20], there remains limited understanding of how Arp2/3 complex is inhibited.

Unbound (ligand-free) yeast and bovine Arp2/3 complexes exist in equilibrium between two distinct conformational states, open (inactive) and closed (primed for nucleation) [20–22]. Interactions with NPFs can shift the conformational distribution of Arp2/3 complex molecules toward the closed state [20, 21]. These interactions also enhance Arp2/3 complex binding to the side of an existing (mother) filament, leading to nucleation of a new (daughter) filament at a 70° angle [19, 23–27]. Importantly, even in the absence of NPFs, Arp2/3 complex has some inherent nucleation activity, and possibly for this reason cells additionally express inhibitory ligands of Arp2/3 complex such as Coronin, GMF, and Arpin.

The Arp2/3 complex has a conserved architecture, consisting of two actin-related proteins (Arp2 and Arp3) and five additional subunits [4]: ARPC1 (p40), ARPC2 (p35), ARPC3 (p21), ARPC4 (p19), ARPC5 (p15). Different subunits in the complex contribute in different ways to Arp2/3 complex function and regulation, including NPF binding [20] and/or participation in joining mother and daughter filaments [19]. Many NPFs, including WASP, Scar/WAVE, WASH proteins, possess a short segment at their C-terminus called the ‘VCA’ domain, which consists of a WH2 or ‘V’ (WASp-homology 2 or Verprolin homology) domain, a ‘C’ motif (Central), and an ‘A’ motif (Acidic). The V portion of VCA binds Gactin, while the CA portion binds Arp2/3 complex. Free VCA is unstructured, but may acquire secondary structure upon contacting Arp2/3 complex [28]. Each Arp2/3 complex can bind two VCA

domains via separate surfaces [28–30]. Electron tomography at actin filament branch junctions suggests that Arp2/3 complex undergoes additional conformational changes during daughter filament nucleation. In the final branch site, Arp2/3 complex forms a stable link between the side of the mother filament and the pointed end of the daughter filament [19].

Among the three above-mentioned inhibitors of Arp2/3 complex, the first identified was Coronin [8]. Subsequently, *S. cerevisiae* Coronin (Crn1) was shown to bind near the p35/ARPC2 subunit and dramatically shift the distribution of Arp2/3 complex toward the open/inactive state [21]. Mammalian homologs of Coronin similarly inhibit Arp2/3 complex [9, 10], presumably through a similar mechanism. The second Arp2/3 inhibitor identified was GMF (18 kDa), which is conserved from yeast to humans and has a fold similar to ADF/cofilin proteins [31]. However, rather than binding to actin like ADF/cofilin, GMF binds directly to Arp2/3 complex via interactions with the Arp2 and p40/ARPC1 subunits [12–14]. Through these interactions, GMF inhibits actin nucleation by Arp2/3 complex, and catalyzes the dissociation of daughter filaments from mother filaments, i.e., debranching [13, 32, 33]. A co-crystal structure of GMF-bound Arp2/3 complex revealed that one of the GMF binding sites to be located on the Arp2 and ARPC1/p40 subunits [14]. However, binding saturation analysis, chemical crosslinking, and molecular modeling all suggested that there may be a second, weaker binding site that involves interactions with Arp3 [13]. The third Arp2/3 inhibitor identified was Arpin, which possesses an A-motif that competitively blocks the stimulatory effects of VCA to control leading edge actin network dynamics and cell migration in vivo [16]. Together, these observations have raised new questions about the structural and mechanistic basis of these inhibitors, including: (1) How does each inhibitor affect Arp2/3 complex conformation? (2) How many GMF- and Arpin-binding sites are on the Arp2/3 complex, and what is the relationship between where they bind and how they affect Arp2/3 conformation? (3) Can pairs of inhibitors synergize in blocking nucleation by Arp2/3 complex?

Here we used single particle electron microscopy to determine 2D and 3D structures of *S. cerevisiae* Arp2/3 complex bound separately to GMF and Arpin, and compare them to our previously defined Coronin-bound structure [21]. This analysis has revealed key similarities and differences in the interactions of the different inhibitors. Further, we demonstrate that Coronin and GMF synergize in suppressing actin nucleation by Arp2/3 complex, and that Arpin exhibits compounded effects on Arp2/3 complex in the presence of Coronin or GMF.

## RESULTS

### Effects of Gmf1, Arpin, and GST-Crn1 on Arp2/3 complex conformation

As a first step toward understanding the structural basis for Arp2/3 inhibition by GMF, Arpin, and Coronin, we used single particle EM analysis to generate 2D projection images of free and ligand-bound Arp2/3 complexes. The Arp2/3 complex is known to adhere to EM grids by a flat surface, making 2D projections a useful analysis. For this work, we used *S. cerevisiae* Arp2/3 complex, which is more amenable to conformational analysis by EM than

bovine Arp2/3 [21], the yeast homologs of GMF (Gmf1) and Coronin (Crn1), and human Arpin. By using yeast Arp2/3 complex, we were able to integrate a C-terminal 3HA-TEV tag, isolate the Arp2/3 complex on HA-antibody-coated beads, load different ligands (Gmf1, GST-Crn1, and Arpin), and TEV protease release the Arp2/3-ligand complexes for EM imaging. This reduced the background of free ligand on the EM grids. Importantly, human Arpin was able to inhibit actin nucleation by yeast Arp2/3 complex (Fig. S1), similar to its effects on mammalian Arp2/3 complex [16].

Consistent with our previous studies [21], we observed that free yeast Arp2/3 complex adopted different conformations. We used hierarchical ascendant classification in IMAGIC5 software followed by summing the particles into 50 classes, as described in Methods. Approximately 60% of the particles in Arp2/3 complex alone samples had a cleft between the Arp2 and Arp3 subunits, classifying them as the 'open' (inactive) conformation, whereas the remaining ~40% of particles lacked a visible cleft, classifying them as the 'closed' (primed for nucleation) conformation (Fig. 1A and 1B). Addition of each inhibitor skewed Arp2/3 conformational distribution toward the open/inactive state (Fig. 1B). These results suggest that the three inhibitors induce related conformational changes in Arp2/3 complex.

### **GST-Crn1 position on Arp2/3 complex**

Next, we attempted to locate GST-Crn1 on the surface of Arp2/3 complex by comparing 2D projection images for free and ligand-bound Arp2/3 complexes (Fig. 1C). Our previous work showed that binding of untagged Crn1 monomer to Arp2/3 complex produces a single new mass located near the p35/ARPC2 subunit [21], agreeing with two-hybrid interactions between the C-terminus of Crn1 and p35/ARPC2 [8]. This ~5 nm diameter single new mass presumably corresponds to the globular N-terminal beta-propeller domain (~40 kDa) of Crn1. In contrast, the C-terminus of Crn1, which contacts the p35/ARPC2 subunit, was not visible, possibly because it lacks sufficient mass and/or tertiary structure. In the current experiments, using artificially dimerized GST-Crn1, we were able to expand on our previous findings. We detected two masses of a similar size near the p35/ARPC2 subunit (Fig. 1C, arrows), in the majority of cases (14 out of 20 class averages), consistent with the presence of two Crn1 beta-propeller domains linked together by a smaller GST dimer, which possibly contributes to these masses. The distance between each of the two large masses and the surface of the Arp2/3 complex was 10–15 nm, similar to the distance we previously observed for the single mass using untagged Crn1 [21].

Free Crn1 in solution readily oligomerizes via homophilic interactions of its C-terminal coiled coil domain [34], which is a conserved feature in Coronins from other species [35, 36]. However, in our previous work we never observed more than a single Crn1 molecule bound to Arp2/3 complex [21], and here using artificially dimerized GST-Crn1 we instead observed two molecules bound to each complex in most cases. The simplest interpretation is that one Crn1 molecule in the GST-Crn1 dimer is bound to Arp2/3 complex while the other is free, as depicted in our cartoon representation (Fig. 1C, right). Together, these observations support the view of a single Crn1-binding site on Arp2/3 complex, mediated by the Crn1 coiled-coil domain, and suggest further that Crn1 may bind Arp2/3 complex as

a monomer because coiled-coil-dependent oligomerization and coiled-coil-dependent Arp2/3 binding are mutually exclusive [8, 34].

### Two distinct 3D structures of Gmf1-bound Arp2/3 complex

Crn1 (GST-tagged) and untagged Arpin each induced a 'standard' open structure, characterized by a visible and pronounced p21/ARPC3 subunit in 2D projections, as described for untagged Crn1 [21]. However, untagged Gmf1 induced two separate open conformations (Fig. 2A): (1) a 'standard' open conformation, and (2) a new open conformation, in which the p21/ARPC3 subunit has undergone a major shift in its position as highlighted by the difference map between these two conformations (Fig. 2A, bottom row, orange arrowheads). We solved the 3D structures of each Gmf1-induced open conformation (Fig. 2C & 2D). Tilted pairs of images (2400 individual particles) were processed to obtain 100 class averages (representative classes in Fig. S5A), from which we solved the 3D structures. To aid in the interpretation, we derived a 3D map of the co-crystal structure of GMF-bound Arp2/3 complex [14] filtered at 25 Å resolution (Fig. 2B). This crystal structure includes an intact Arp2 subunit, whereas previous structures lacked a portion of Arp2 [14]. Importantly, this structure was never used here for refining the 3D reconstructions, and is shown only for comparative purposes.

Our 3D reconstructions of the two open conformations of Arp2/3 complex induced by GMF (Fig. 2B & 2C) confirmed the major structural changes observed in our 2D projections (Fig. 2A). In the Gmf1-bound new open conformation there was a major rearrangement of the p21/ARPC3 subunit, which pivots to a new position behind Arp3, explaining why it was not visible in our 2D projections. In the 3D reconstruction of the Gmf1-bound standard open conformation, there was a more modest shift of the p21/ARPC3 subunit in the same direction. In both Gmf1-bound open conformations, there was substantial rotation of the p35/ARPC2 subunit, perhaps accompanying the mentioned shift in the p21/ARPC3 subunit. Indeed, mutational studies of p35/ARPC2 have suggested that this subunit binds directly to mother filament sides and mediates both inhibitor- and activator-induced structural rearrangements of other subunits in the complex [14, 21, 37]. In each 3D reconstruction, we also observed at least one new mass roughly the size of Gmf1 (Fig. 2C & 2D, pink masses; also see difference maps in Fig. S3). In the standard open conformation, a clear new mass appeared in the same position as GMF in the co-crystal (grey shading in Fig. 2B). In the new open conformation (Fig. 2D), this mass on Arp2 and p40/ARPC1 was absent, but a new mass appeared in a distinct location, on Arp3. Interestingly, the standard open conformation induced by GMF showed a less prominent new mass at the second location (Fig. S3C). This observation raises the possibility that only a subset of the particles analyzed to obtain this structure had a GMF bound at the second site. All together, these data suggest that GMF induces two distinct open conformations in Arp2/3 complex, likely arising from interactions of GMF at distinct binding sites on Arp2/3 complex.

In addition, we attempted to map the Gmf1 binding site(s) on Arp2/3 complex by analyzing 2D projections of Gmf1-GFP-bound Arp2/3 complexes. This revealed variable positions for GFP tag (25 kDa) (Fig. S2A), but it was difficult to locate the Gmf1 portion of Gmf1-GFP because of its smaller mass (17 kDa) and because it directly contacts the complex. The



Gmf1-GFP fusion used includes a 13 residue-long linker connecting Gmf1 and GFP, which would allow for a gap of 3–5 nm between GFP and Gmf1 on Arp2/3 complex. Consistent with this linker flexibility, we anticipated seeing GFP masses at heterogenous distances from its binding site(s). However, instead we observed GFP always ~10 nm from the putative Gmf1-binding site on Arp2/3 (Fig. S2A), and the reason for this consistent distance remains unclear. To map a potential Gmf1 binding site(s) from which these GFP molecules stem, we combined all 2D projections, superimposed the aligned Arp2/3 complexes onto each other, mapped the GFP locations, and drew circles with a 10 nm radius (Fig. S2B); this accounts for the size of GMF, the maximal length of the linker, and the size of the GFP. Our analysis was consistent with the existence of two binding sites, one on the Arp2 subunit and one on the Arp3 subunit (Fig. S2B, open circles in cartoon). The mapping also agrees well with the crystal structure of GMF-bound Arp2/3 complex [14], in which GMF directly contacts the ARPC1/p40 subunit, and with chemical crosslinking analysis, which identified a potential second GMF-binding site on Arp3 [13].

### **GMF and Coronin synergize in inhibiting actin nucleation by Arp2/3 complex**

Given that Coronin and GMF are conserved from yeast to mammals and appear to have separate binding sites on Arp2/3 complex, we asked whether these two proteins can work together to inhibit Arp2/3 complex-mediated actin nucleation. To test this, we used the VCA domain of yeast WASP (Las17) to activate yeast Arp2/3 complex in bulk fluorescence assays in the presence and absence of different concentrations of Gmf1 and/or Crn1. 250 nM Crn1 and 100 nM Gmf1 each modestly inhibited Arp2/3-dependent actin nucleation, but together abolished VCA-Arp2/3 complex activity (Fig. 3A). Further, at a fixed concentration of Crn1 that has modest inhibitory effects (250 nM), Gmf1 further inhibited nucleation in a concentration-dependent manner (Fig. 3B). Combined inhibitory effects were also observed when full-length Las17 was used instead of VCA domain to activate Arp2/3 complex (Fig. 3C); however, this required higher concentrations of the inhibitors, consistent with Las17 being a much stronger NPF than VCA [38, 39]. We also observed that the inhibitory effects of Gmf1 and Crn1 on Arp2/3 complex appear to be synergistic rather than additive. This raises the possibility that binding of one inhibitor could positively affect interactions of the other. Consistent with this view, 2D-classification analysis showed that the fraction of Arp2/3 particles with visible Crn1 bound increases by 3-fold in the presence of Gmf1 (Fig. 3D).

### **The structure of Arpin-bound Arp2/3 complex**

Next, we asked where Arpin binds on the surface of Arp2/3 complex in relationship to GMF and Coronin, since each inhibitor induces an open conformation (Fig. 1B). The structure of Arpin (25 kDa) was recently solved by small-angle X-ray scattering (SAXS) and shown to be comprised of an N-terminal non-spherical globular domain connected by a linear tail (20 residues) to a short acidic (A) motif at its C-terminus [16, 40]. We generated 2D projection classes for Arpin-bound Arp2/3 complexes (Fig. 4A, upper panel), and compared them to ligand-free complex (Fig. 1A). This revealed that Arpin-bound complexes have either one or two new masses in each representative class average (yellow and white arrows,

respectively, Fig. 4A, upper panel). In most of the class averages, there was a single new mass (yellow arrows, Fig. 4A, upper panel), while in some there were two new masses (white arrows, Fig. 4A, upper panel).

As a fully extended polypeptide chain, the linear tail of Arpin would span 3–6 nm, and indeed, we detected additional masses (~1.5 nm in diameter) located about 3 nm from the surface of Arp2/3 complex. Using the same strategy by which we mapped Gmf1-GFP binding sites on Arp2/3 complex, we identified two potential Arpin-binding sites, one located near the interface of the Arp3 and ARPC2/p35 subunits, and the other near the interface of Arp2 and Arp3 subunits (cartoon in Fig. 4A, open circles). Unlike Gmf1-GFP, Arpin was untagged; however, its globular core provided sufficient mass to serve as the tag.

To help clarify the positions of Arpin on Arp2/3 complex, we solved the 3D structure of Arpin-bound Arp2/3 complex (Fig. 4B) and compared it to ligand-free Arp2/3 complex (Fig. 4C). This revealed two main difference peaks, both located near the Arp2 subunit (pink shading in Fig. 4B; also see difference maps in Fig. S3B). These two new masses likely correspond to globular domains of two Arpin molecules. Taking into account the linear tails connecting these masses to the Arp2/3-interacting A motifs, the positions of these masses are consistent with predicted binding of the A motifs of Arpin to Arp2 and Arp3. Binding of Arpin may be stronger to Arp2 than Arp3, because all of our 2D projections showed an additional mass at this location, whereas some lacked an additional mass at the second location. We also note that Arpin binding appears to induce changes in the position of the p35/ARPC2 subunit, shifting it counter-clockwise (Fig. S3B).

## DISCUSSION

The Arp2/3 complex is a conserved nucleator of branched actin filament networks. A number of studies have addressed the structural changes in Arp2/3 complex that accompany its co-activation by NPFs [20, 28, 41] and mother filaments [19]. Although Arp2/3 complex alone (no NPF) has some nucleation activity, strong nucleation requires binding of two VCA molecules to distinct surfaces on Arp2/3 complex [28–30]. At the suggested high-affinity site, the ‘A’ motif of VCA binds to the interface between Arp2 and ARPC1/p40 subunits [28], while the C motif binds Arp2 [30]. At the suggested low-affinity site, both the A and C motifs bind to the back of Arp3 [30]. The V motifs (or WH2 domains) of each VCA molecule recruit actin monomers, a critical step in nucleation, which become the first two conventional subunits of the daughter filament. Thus, nucleation appears to involve formation of a pseudo-symmetrical complex with two VCAs binding opposite sides of Arp2/3 [28]. In contrast to this detailed understanding of Arp2/3 activation, there have been few structural studies to date addressing Arp2/3 inhibition [13, 21], and only one that has provided the 3D structure of the complex bound to an inhibitory protein [14].

In this study, we used single particle electron microscopy to obtain 2D and/or 3D models of Arp2/3 complex bound to its three established inhibitors: GMF, GST-Crn1, and Arpin. Our results suggest that the inhibitors bind to distinct surfaces on Arp2/3 complex, and that

GMF and Arpin each have two binding sites on the complex, analogous to VCA. Coronin appears to bind to the ARPC2/p35 subunit. Arpin apparently binds to the two VCA-interacting surfaces on Arp2/3 complex, consistent with its inhibitory effects being dependent on its A motifs. GMF binds to the interface between Arp2 and ARPC1/p40 subunits, and possibly to a second site on Arp3. Despite these differences in where they bind, each inhibitor induces one or more 'open' conformations in the Arp2/3 complex. Importantly, this suggests that diverse regulatory inputs can produce structurally and functionally related effects on Arp2/3 complex. Further, it raises the possibility of multiple inhibitors interacting simultaneously with the Arp2/3 complex, and indeed, GMF and Coronin acted synergistically to inhibit Arp2/3 complex in vitro (Fig. 3), and Arpin combinatorially inhibited Arp2/3 complex with GMF or Coronin (Fig. 4D). Thus, having multiple inhibitors with non-overlapping binding sites on Arp2/3 complex may provide cells with increased versatility by which to spatially and temporally tune actin nucleation activity. Another important implication of these results is that they suggest Arp2/3 complex may serve as a coincidence detector, receiving and integrating inhibitory signals from multiple pathways. This idea is particularly intriguing because the WASP/WAVE family of Arp2/3-activating proteins are also coincidence detectors, but instead receive and integrate multiple stimulatory signals [42].

#### **GMF induces a novel open conformation in Arp2/3 complex**

We solved two distinct 3D structures of GMF-bound Arp2/3 complex (Fig. 2C & 2D). One structure adopts the 'standard open' conformation and has a new mass (the approximate size of GMF) at a location consistent with the GMF-Arp2/3 co-crystal structure [12]. The second structure has a novel open conformation and lacks a new mass at the first GMF binding site, but has additional mass at a second site. This second site is on Arp3, which agrees with previous anisotropy, binding saturation analysis, and chemical cross-linking experiments [13]. Given that the majority of GMF-bound particles (~85%) fell into the first category, i.e., the standard open conformation, and fewer fell into the second category, i.e., the novel open conformation, GMF may have higher affinity for the first binding site [14]. These results have important implications for the GMF mechanism. The new open conformation induced by GMF is highly incompatible with mother filament binding, and even the standard open conformation induced by GMF may interfere with filament side binding. Structural analysis of branch junctions suggests that most if not all subunits in the Arp2/3 complex interact with the mother filament to some degree [19], with subunits ARPC1, ARPC2 and ARPC4 making the most extensive interactions. In the novel open conformation induced by GMF, there are massive structural rearrangements, including a major shift of ARPC3 away from Arp2 and toward Arp3, which may render the complex less capable of binding the mother filament. Some of these structural changes may underlie GMF's ability to catalyze daughter filament dissociation from mother filaments [12, 13]. However, the structures we have solved here are for ligand-bound Arp2/3 complex in solution (without actin), and Arp2/3 complex found in actin filament branch junctions has a distinct conformation. Further, while GMF induces debranching, Crn1 and Arpin do not, yet all three induce open conformations. Therefore, it is not yet clear how our findings relate to GMF's debranching function.

### **Arpin binds two sites on Arp2/3 complex and induces the standard open conformation**

Arpin (25 kDa) contains an Acidic (A) motif, which is required for its ability to inhibit Arp2/3 complex [40], and thus has been proposed to inhibit Arp2/3 complex by blocking VCA interactions. Our structural data are consistent with Arpin competing with VCA, but further reveal that Arpin binding influences the conformation of Arp2/3 complex, thus inhibiting nucleation in a manner not anticipated by a purely competitive binding model. Our 3D structure of Arpin-bound Arp2/3 complex shows that Arpin induces a standard open/inactive conformation, and supports the view that there are two Arpin binding sites on Arp2/3 complex (Fig. 4A & 4B; Fig. S3B), possibly corresponding to the two VCA binding sites. One Arpin density (presumably its globular core) contacts Arp2, while the other Arpin density sits closer to the backside of Arp3 where VCA interacts [27, 28]. These observations are also consistent with Arpin's inhibitory effects being less potent (micromolar range  $IC_{50}$ ) compared to GMF and Coronin, even on mammalian Arp2/3 complex [40]. Effective inhibition may require binding of two Arpin molecules to block both VCA interactions. This scheme may enable cells to use Arpin to fine-tune VCA effects on Arp2/3 complex at the leading edge and/or allow Arpin to function in concert with other inhibitors to more fully block Arp2/3 activity.

### **Conclusions**

Previous studies have shown that activation of the Arp2/3 complex is accompanied by structural rearrangements that close the gap between its Arp2 and Arp3 subunits, bringing them into closer register to stimulate daughter filament nucleation [4, 5]. These conformational changes induced by NPF binding are thought to 'prime' the Arp2/3 complex for nucleation, but additional steps are needed, including a second set of conformational changes that are suggested to occur when the primed complex interacts with the side of the mother filament [19, 43]. By comparison, there has been limited understanding of how inhibitors affect the structure of Arp2/3 complex. We previously showed that yeast and bovine Arp2/3 complexes (without ligands) each exist in an equilibrium between open and closed conformations [21], and that NPF binding stabilizes the closed state, whereas the inhibitor Coronin stabilizes the open state. In this study, we have shown that second inhibitor, Arpin, induces a related open/inhibitory conformation, and that a third inhibitor, GMF, induces both the standard open conformation and a strikingly different new open conformation. Together, these observations suggest that different inhibitors with different binding sites can induce similar open conformations, and that the Arp2/3 complex is capable of adopting a wide range of conformational/functional states. Indeed, this concept is supported by molecular dynamics simulations studies [44, 45]. Integration of this new information on inhibitors with existing knowledge of activators should provide a more complete view of how the activities of the Arp2/3 complex are locally controlled and finetuned by the concerted effects of multiple cellular ligands.

## MATERIALS AND METHODS

### Protein purification

Yeast Arp2/3 complex, full-length Las17 (WASP) and GST-VCA from Las17 were each purified as described [21]. Wild-type yeast GMF (Gmf1) and Gmf1-GFP were expressed as GST-fusion proteins in *E. coli* and purified by glutathione affinity chromatography, then treated with PreScission Protease (GE Healthcare) to remove the GST tag. Gmf1 proteins were further purified on a SOURCE 15Q column and then on a Superdex 200 column equilibrated in HEK buffer (20 mM Hepes pH 7.5, 1 mM EGTA, 50 mM KCl). GST-Crn1 was purified as described [46], and the GST tag was intentionally not removed. Multi-tagged human GST-6His-Arpin was expressed in *E. coli*, purified using Nickel affinity chromatography, and digested with TEV protease to remove both tags. Arpin was further purified and concentrated on a Resource Q column (GE healthcare), and dialyzed against buffer (20 mM Tris pH 7.5, 30 mM NaCl, 1 mM DTT). All proteins were flash frozen in aliquots in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use.

### Actin Assembly Kinetics

Gel-filtered monomeric skeletal muscle actin in G-buffer (10 mM Tris pH 7.5, 0.2 mM  $\text{CaCl}_2$ , 0.2 mM DTT, 0.2 mM ATP) was cleared by ultracentrifugation at 90,000 rpm for 1 h at  $4^{\circ}\text{C}$  in a TLA100 rotor (Beckman Coulter; Indianapolis, IN). Each reaction (60  $\mu\text{l}$ ) contained 2  $\mu\text{M}$  G-actin (5% pyrene labeled). To initiate polymerization, G-actin was converted to  $\text{Mg}^{2+}$ -ATP-actin, and then 2 min later, 42  $\mu\text{l}$  G-actin was added to 15  $\mu\text{l}$  of the indicated proteins/buffer, and mixed with 3  $\mu\text{l}$  initiation mix (40 mM  $\text{MgCl}_2$ , 10 mM ATP, 1 M KCl). Fluorescence was monitored in a spectrophotometer (Photon Technologies International) at excitation and emission wavelengths of 365 and 407 nm, respectively.

### Electron microscopy and image analysis

Arp2/3 complexes bound to Gmf1, GST-Crn1, or Arpin (gels of purified proteins in Fig. S6) were prepared by incubating for 30–60 min a ten-fold excess of ligand with immobilized HA-tagged Arp2/3 complex still attached to anti-HA antibody-coated beads, then washing the beads and releasing the complexes by TEV protease digestion for 30 min at room temperature [47]. The concentrations of Arp2/3 complex and regulators used were optimized in previous studies for their effects in functional assays and for single particle EM [13, 21, 47]. Freshly eluted complexes were applied to carbon-coated glow-discharged copper EM grids, then negatively stained with 0.75% Uranyl formate for 30 sec, air-dried, and analyzed in a JEOL 2100 electron microscope at 200 kV at low-dose conditions (Fig. S4). For random conical tilt method, the grids were tilted at 45 degree angles, and images captured with a Ultrascan 1000XP CCD (Gatan) at 40,000 $\times$  magnification and 1.5–2.8  $\mu\text{m}$  underfocus. To obtain a tilt pair of images, the same field of view was imaged without tilting. In this manner, pairs of corresponded images were obtained for each sample: 1500 pairs for Arp2/3 complex alone, 2400 Arp2/3 complex with Gmf1, and 2500 for Arp2/3 complex with Arpin. The corresponded tilted and untilted views of each complex were collected manually in BOXER [48]. A total of 1250 untilted views of GST-Crn1-bound Arp2/3

particles were collected using BOXER. Correction for the contrast transfer function (CTF) of the microscope was done in EMAN2.1 [48].

Untilted particles were processed using the IMAGIC5 package [49] as described in [21]. Briefly, all particles were filtered, normalized and subject to reference-free classification, followed by several rounds of multi-reference alignment. The final dataset had 50 classes, which were assigned to different conformations (open or closed), based on the existence of the cleft between Arp2 and Arp3 subunits. Particles attributed to each conformation, were extracted from the dataset, using IMAGIC5 command `msa-extract` and corresponded numbers were plotted on the graph. Difference maps between Arp2/3 complex structures with and without bound inhibitors were calculated after scaling each projection structure to give the same variance and zero mean. The Fourier ring correlation, calculated between projection structures that each included one half of the data, was used to estimate the resolution of the projection structures. The resolution of all structures was estimated at 25 Å, corresponding to approximately the first zero of the CTF of the electron microscope.

The preliminary 3D reconstructions of Arp2/3 complex bound to Gmf1 and bound to Arpin were generated in EMAN2.1 [48], using the Random Conical Tilt method and C1 symmetry. Subsequent refinement of the 3D structures was done in FREALIGN [50]. Statistical analysis revealed that single particles possess different orientations (Fig. S5D, S5E & S5F). For solving the 3D structure of Arpin-bound Arp2/3 complex (Fig. 4B), over 1000 additional untitled particles were added to the data set before refinement in FREALIGN. The resulting 3D structures of the ligand-bound Arp2/3 complexes had a resolution of 23–28 Å, estimated by the Fourier Shell Correlation method (Fig. S5G & S5H). 3D difference mapping was performed with UCSF Chimera [51], in which we used the crystal structure of GMF-bound Arp2/3 complex (pdb code 4JD2), and calculated its 3D structure at 20 Å resolution using command `molmap`, then calculated difference maps between Arp2/3 complexes with and without different inhibitors using command `vop subtract`.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

<b>EM</b>	electron microscopy
<b>NPF</b>	nucleation-promoting factor
<b>FSC</b>	Fourier Shell Correlation

<b>CTF</b>	contrast transfer function
<b>RCT</b>	random conical tilt
<b>GMF</b>	glia maturation factor

## LITERATURE

1. Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 2003; 112:453–465. [PubMed: 12600310]
2. Kaksonen M, Toret CP, Drubin DG. Harnessing actin dynamics for clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol*. 2006; 7:404–414. [PubMed: 16723976]
3. Rotty JD, Wu C, Bear JE. New insights into the regulation and cellular functions of the ARP2/3 complex. *Nat Rev Mol Cell Biol*. 2013; 14:7–12. [PubMed: 23212475]
4. Pollard TD. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annu Rev Biophys Biomol Struct*. 2007; 36:451–477. [PubMed: 17477841]
5. Goley ED, Welch MD. The ARP2/3 complex: an actin nucleator comes of age. *Nat Rev Mol Cell Biol*. 2006; 7:713–726. [PubMed: 16990851]
6. Firat-Karalar EN, Welch MD. New mechanisms and functions of actin nucleation. *Curr Opin Cell Biol*. 2011; 23:4–13. [PubMed: 21093244]
7. Higgs HN, Pollard TD. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu Rev Biochem*. 2001; 70:649–676. [PubMed: 11395419]
8. Humphries CL, Balcer HI, D'Agostino JL, Winsor B, Drubin DG, Barnes G, et al. Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin. *J Cell Biol*. 2002; 159:993–1004. [PubMed: 12499356]
9. Cai L, Makhov AM, Schafer DA, Bear JE. Coronin 1B antagonizes cortactin and remodels Arp2/3-containing actin branches in lamellipodia. *Cell*. 2008; 134:828–842. [PubMed: 18775315]
10. Foger N, Rangell L, Danilenko DM, Chan AC. Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. *Science*. 2006; 313:839–842. [PubMed: 16902139]
11. Liu SL, Needham KM, May JR, Nolen BJ. Mechanism of a concentration-dependent switch between activation and inhibition of Arp2/3 complex by coronin. *J Biol Chem*. 2011; 286:17039–17046. [PubMed: 21454476]
12. Gandhi M, Smith BA, Bovellan M, Paavilainen V, Daugherty-Clarke K, Gelles J, et al. GMF is a cofilin homolog that binds Arp2/3 complex to stimulate filament debranching and inhibit actin nucleation. *Curr Biol*. 2010; 20:861–867. [PubMed: 20362448]
13. Ydenberg CA, Padrick SB, Sweeney MO, Gandhi M, Sokolova O, Goode BL. GMF severs actin-Arp2/3 complex branch junctions by a cofilin-like mechanism. *Curr Biol*. 2013; 23:1037–1045. [PubMed: 23727094]
14. Luan Q, Nolen BJ. Structural basis for regulation of Arp2/3 complex by GMF. *Nat Struct Mol Biol*. 2013; 20:1062–1068. [PubMed: 23893131]
15. Maritzen T, Zech T, Schmidt MR, Krause E, Machesky LM, Haucke V. Gadkin negatively regulates cell spreading and motility via sequestration of the actin-nucleating ARP2/3 complex. *Proc Natl Acad Sci U S A*. 2012; 109:10382–10387. [PubMed: 22689987]
16. Dang I, Gorelik R, Sousa-Blin C, Derivery E, Guerin C, Linkner J, et al. Inhibitory signalling to the Arp2/3 complex steers cell migration. *Nature*. 2013; 503:281–284. [PubMed: 24132237]
17. Volkmann N, Amann KJ, Stoilova-McPhie S, Egile C, Winter DC, Hazelwood L, et al. Structure of Arp2/3 complex in its activated state and in actin filament branch junctions. *Science*. 2001; 293:2456–2459. [PubMed: 11533442]
18. Egile C, Rouiller I, Xu XP, Volkmann N, Li R, Hanein D. Mechanism of filament nucleation and branch stability revealed by the structure of the Arp2/3 complex at actin branch junctions. *PLoS Biol*. 2005; 3:e383. [PubMed: 16262445]



19. Rouiller I, Xu XP, Amann KJ, Egile C, Nickell S, Nicastro D, et al. The structural basis of actin filament branching by the Arp2/3 complex. *J Cell Biol.* 2008; 180:887–895. [PubMed: 18316411]
20. Xu XP, Rouiller I, Slaughter BD, Egile C, Kim E, Unruh JR, et al. Three-dimensional reconstructions of Arp2/3 complex with bound nucleation promoting factors. *Embo J.* 2012; 31:236–247. [PubMed: 21934650]
21. Rodal AA, Sokolova O, Robins DB, Daugherty KM, Hippenmeyer S, Riezman H, et al. Conformational changes in the Arp2/3 complex leading to actin nucleation. *Nat Struct Mol Biol.* 2005; 12:26–31. [PubMed: 15592479]
22. Martin AC, Xu XP, Rouiller I, Kaksonen M, Sun Y, Belmont L, et al. Effects of Arp2 and Arp3 nucleotide-binding pocket mutations on Arp2/3 complex function. *J Cell Biol.* 2005; 168:315–328. [PubMed: 15657399]
23. Mullins RD, Heuser JA, Pollard TD. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc Natl Acad Sci U S A.* 1998; 95:6181–6186. [PubMed: 9600938]
24. Blanchoin L, Amann KJ, Higgs HN, Marchand JB, Kaiser DA, Pollard TD. Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASP/Scar proteins. *Nature.* 2000; 404:1007–1011. [PubMed: 10801131]
25. Amann KJ, Pollard TD. The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. *Nat Cell Biol.* 2001; 3:306–310. [PubMed: 11231582]
26. Smith BA, Padrick SB, Doolittle LK, Daugherty-Clarke K, Correa IR Jr, Xu MQ, et al. Three-color single molecule imaging shows WASP detachment from Arp2/3 complex triggers actin filament branch formation. *Elife.* 2013; 2:e01008. [PubMed: 24015360]
27. Smith BA, Gelles J, Goode BL. Single-molecule studies of actin assembly and disassembly factors. *Methods Enzymol.* 2014; 540:95–117. [PubMed: 24630103]
28. Boczkowska M, Rebowski G, Kast DJ, Dominguez R. Structural analysis of the transitional state of Arp2/3 complex activation by two actin-bound WCAs. *Nature communications.* 2014; 5:3308.
29. Padrick SB, Doolittle LK, Brautigam CA, King DS, Rosen MK. Arp2/3 complex is bound and activated by two WASP proteins. *Proceedings of the National Academy of Sciences of the United States of America.* 2011; 108:E472–E479. [PubMed: 21676863]
30. Ti SC, Jurgenson CT, Nolen BJ, Pollard TD. Structural and biochemical characterization of two binding sites for nucleation-promoting factor WASp-VCA on Arp2/3 complex. *Proceedings of the National Academy of Sciences of the United States of America.* 2011; 108:E463–E471. [PubMed: 21676862]
31. Goroncy AK, Koshiba S, Tochio N, Tomizawa T, Sato M, Inoue M, et al. NMR solution structures of actin depolymerizing factor homology domains. *Protein Sci.* 2009; 18:2384–2392. [PubMed: 19768801]
32. Poukkula M, Hakala M, Penttimikko N, Sweeney MO, Jansen S, Mattila J, et al. GMF promotes leading-edge dynamics and collective cell migration in vivo. *Curr Biol.* 2014; 24:2533–2540. [PubMed: 25308079]
33. Haynes EM, Asokan SB, King SJ, Johnson HE, Haugh JM, Bear JE. GMFbeta controls branched actin content and lamellipodial retraction in fibroblasts. *J Cell Biol.* 2015; 209:803–812. [PubMed: 26101216]
34. Goode BL, Wong JJ, Butty AC, Peter M, McCormack AL, Yates JR, et al. Coronin promotes the rapid assembly and cross-linking of actin filaments and may link the actin and microtubule cytoskeletons in yeast. *The Journal of cell biology.* 1999; 144:83–98. [PubMed: 9885246]
35. Spoerl Z, Stumpf M, Noegel AA, Hasse A. Oligomerization, F-actin interaction, and membrane association of the ubiquitous mammalian coronin 3 are mediated by its carboxyl terminus. *J Biol Chem.* 2002; 277:48858–48867. [PubMed: 12377779]
36. Gatfield J, Albrecht I, Zanolari B, Steinmetz MO, Pieters J. Association of the leukocyte plasma membrane with the actin cytoskeleton through coiled coil-mediated trimeric coronin 1 molecules. *Mol Biol Cell.* 2005; 16:2786–2798. [PubMed: 15800061]
37. Goley ED, Rammohan A, Znameroski EA, Firat-Karalar EN, Sept D, Welch MD. An actinfilament-binding interface on the Arp2/3 complex is critical for nucleation and branch stability. *Proc Natl Acad Sci U S A.* 2010; 107:8159–8164. [PubMed: 20404198]
38. Rodal AA, Manning AL, Goode BL, Drubin DG. Negative regulation of yeast WASp by two SH3 domain-containing proteins. *Curr Biol.* 2003; 13:1000–1008. [PubMed: 12814545]



39. Sun Y, Martin AC, Drubin DG. Endocytic internalization in budding yeast requires coordinated actin nucleation and myosin motor activity. *Dev Cell*. 2006; 11:33–46. [PubMed: 16824951]
40. Fetics S, Thureau A, Campanacci V, Aumont-Nicaise M, Dang I, Gautreau A, et al. Hybrid Structural Analysis of the Arp2/3 Regulator Arpin Identifies Its Acidic Tail as a Primary Binding Epitope. *Structure*. 2016; 24:252–260. [PubMed: 26774128]
41. Goley ED, Rodenbusch SE, Martin AC, Welch MD. Critical conformational changes in the Arp2/3 complex are induced by nucleotide and nucleation promoting factor. *Molecular cell*. 2004; 16:269–279. [PubMed: 15494313]
42. Padrick SB, Rosen MK. Physical mechanisms of signal integration by WASP family proteins. *Annu Rev Biochem*. 2010; 79:707–735. [PubMed: 20533885]
43. Cai L, Bear JE. Peering deeply inside the branch. *J Cell Biol*. 2008; 180:853–855. [PubMed: 18316414]
44. Dalhaimer P, Pollard TD. Molecular dynamics simulations of Arp2/3 complex activation. *Biophys J*. 2010; 99:2568–2576. [PubMed: 20959098]
45. Pfaendtner J, Voth GA. Molecular dynamics simulation and coarse-grained analysis of the Arp2/3 complex. *Biophys J*. 2008; 95:5324–5333. [PubMed: 18805923]
46. Gandhi M, Achard V, Blanchoin L, Goode BL. Coronin switches roles in actin disassembly depending on the nucleotide state of actin. *Mol Cell*. 2009; 34:364–374. [PubMed: 19450534]
47. Ydenberg CA, Smith BA, Breitsprecher D, Gelles J, Goode BL. Cease-fire at the leading edge: new perspectives on actin filament branching, debranching, and cross-linking. *Cytoskeleton*. 2011; 68:596–602. [PubMed: 22002930]
48. Ludtke SJ, Baldwin PR, Chiu W. EMAN: Semiautomated software for high-resolution singleparticle reconstructions. *J Struct Biol*. 1999; 128:82–97. [PubMed: 10600563]
49. van Heel M, Harauz G, Orlova EV, Schmidt R, Schatz M. A new generation of the IMAGIC image processing system. *J Struct Biol*. 1996; 116:17–24. [PubMed: 8742718]
50. Grigorieff N. FREALIGN: high-resolution refinement of single particle structures. *J Struct Biol*. 2007; 157:117–125. [PubMed: 16828314]
51. Goddard TD, Huang CC, Ferrin TE. Visualizing density maps with UCSF Chimera. *J Struct Biol*. 2007; 157:281–287. [PubMed: 16963278]

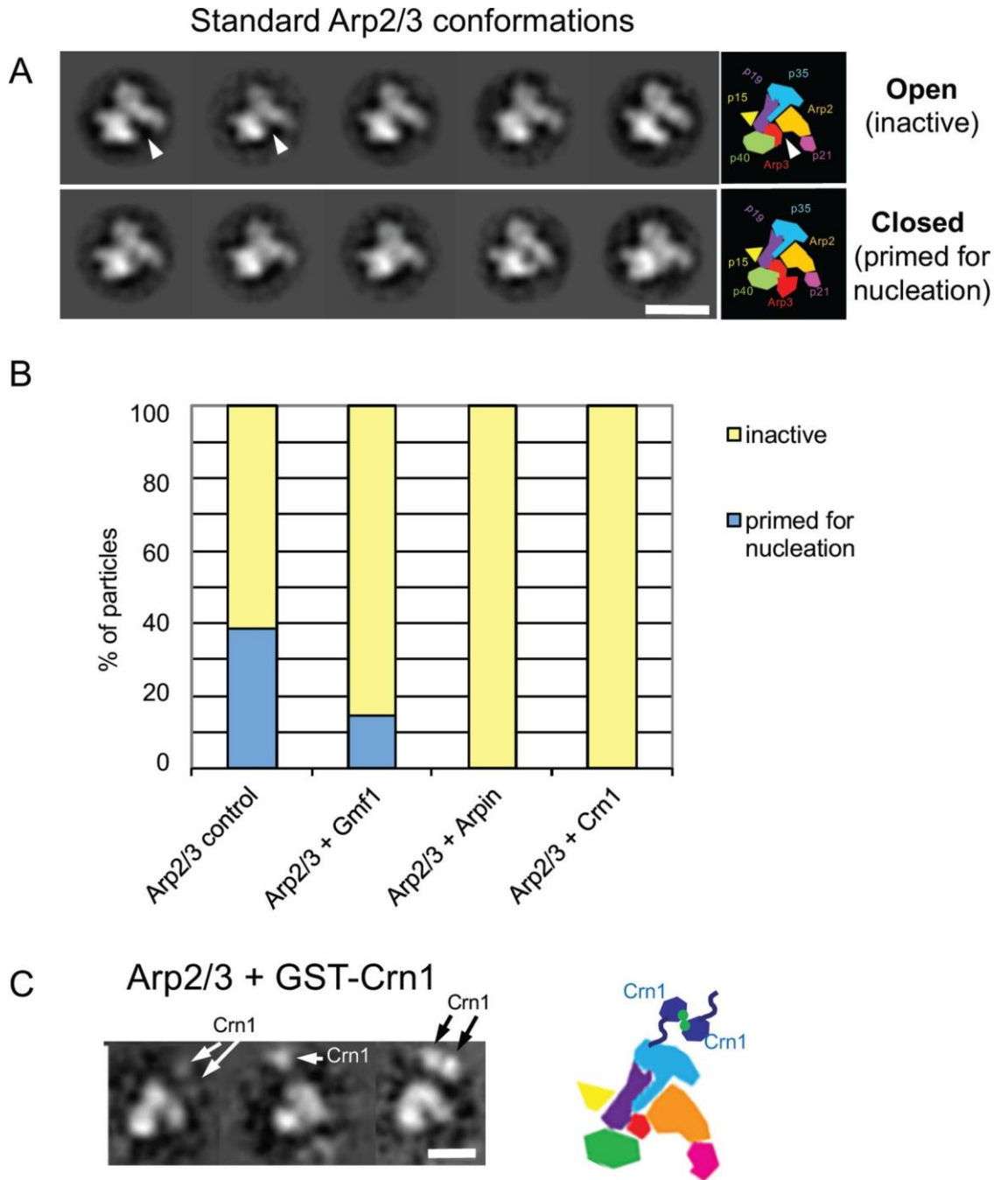
### Research highlights

Coronin, GMF, and Arpin each induce related open conformations in Arp2/3 complex

GMF binding induces two distinct inhibitory states of Arp2/3 complex

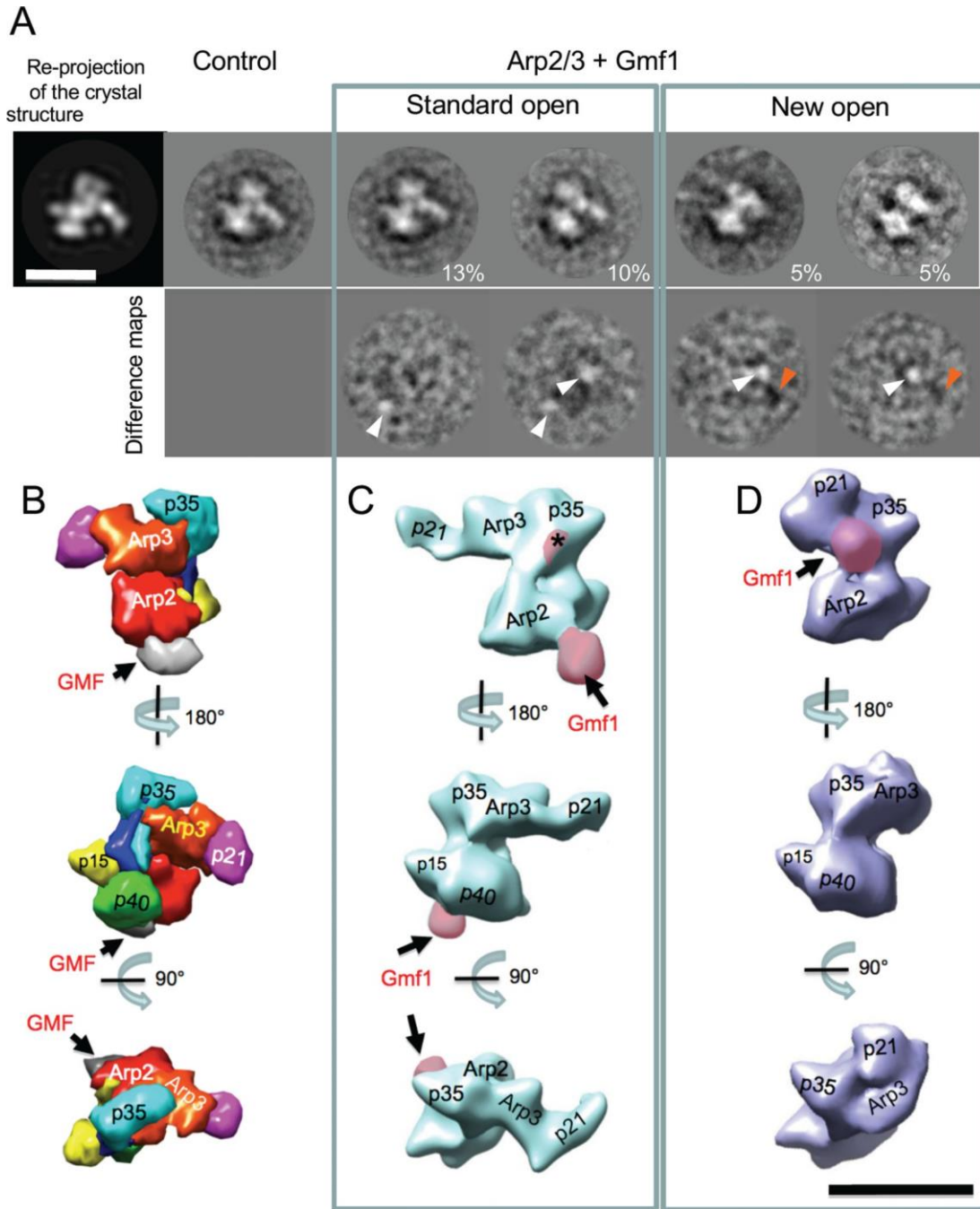
Coronin, GMF, and Arpin combinatorially inhibit Arp2/3 complex activity

Arpin has two separate binding sites on Arp2/3 complex Arp2 and Arp3



**Figure 1. Distribution of Arp2/3 complex conformations in ligand-bound and unbound states**  
 (A) Two-dimensional projections of ‘standard’ open and closed conformations of ligand-free Arp2/3 complex. Upper row, representative class averages of open conformation. Bottom row, representative class averages of closed conformation. Scale bar, 10 nm. Arrowheads point to the clef between Arp2 and Arp3 in the open conformation. Cartoon panels in each row show the 7 subunits of Arp2/3 complex with color-coding as in all other figures: Arp2, red; Arp3, orange, ARPC1/p40, green, ARPC2/p35, blue; ARPC3/p21, light purple; ARPC4/p19, dark purple; ARPC5/p15, yellow. (B) Percentage of Arp2/3 complex particles in the open (inactive) versus closed (primed for nucleation) conformations

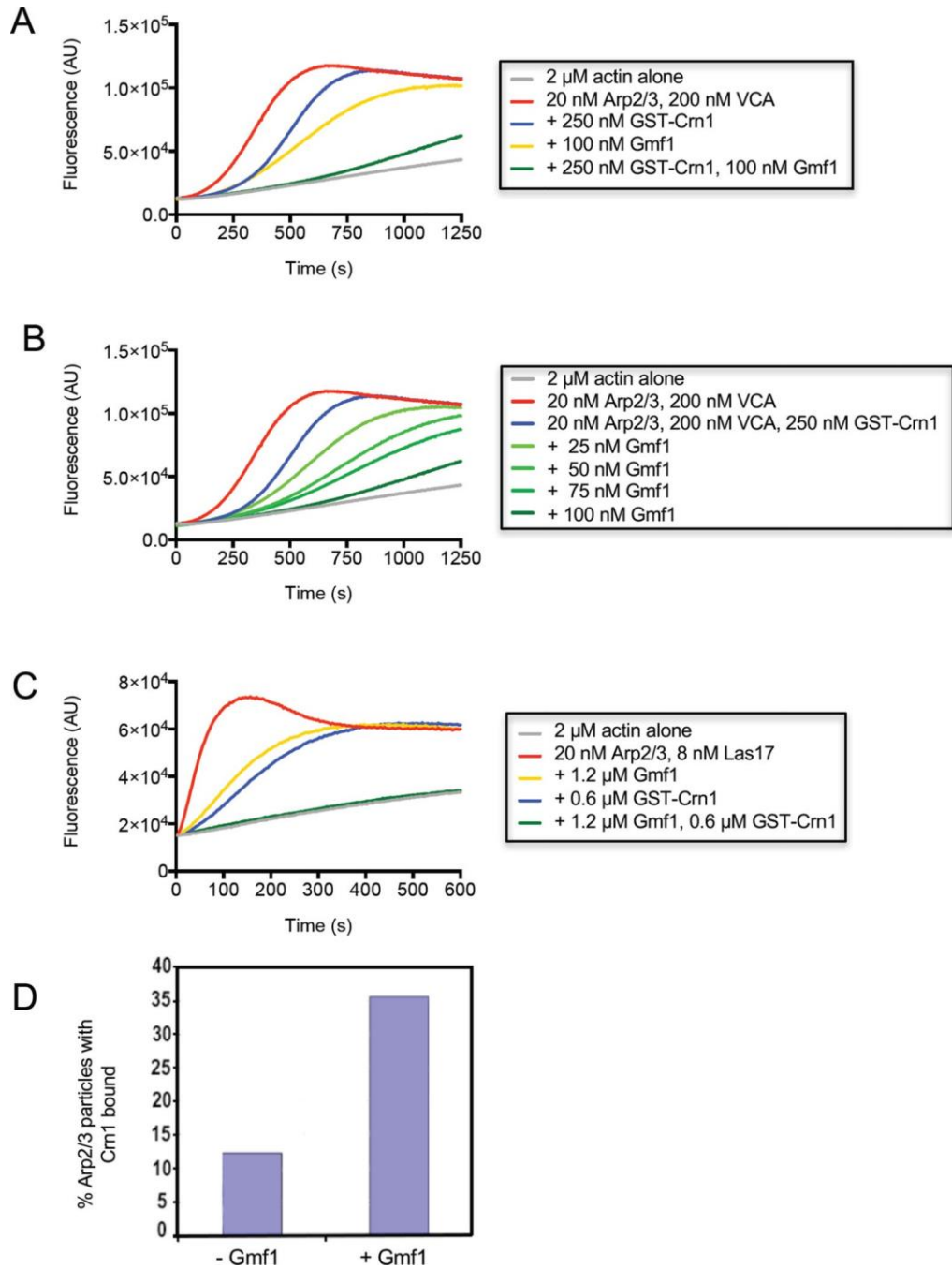
(Arp2/3, n = 262; Arp2/3+Gmf1, n = 731; Arp2/3+Arpin, n = 1130; Arp2/3+Crn1, n = 362).  
(C) Twodimensional projections of GST-Crn1 bound to Arp2/3 complex (left). Arrows highlight the visible new masses, which likely represent the globular  $\beta$ -propeller domains of a Crn1 dimer. Scale bar, 10 nm. Cartoon (right) depicts proposed arrangement of two Crn1 molecules (dark blue) dimerized by GST (green dots), with one Crn1 molecule in the dimer interacting with the p35/ARPC2 subunit of Arp2/3 complex.



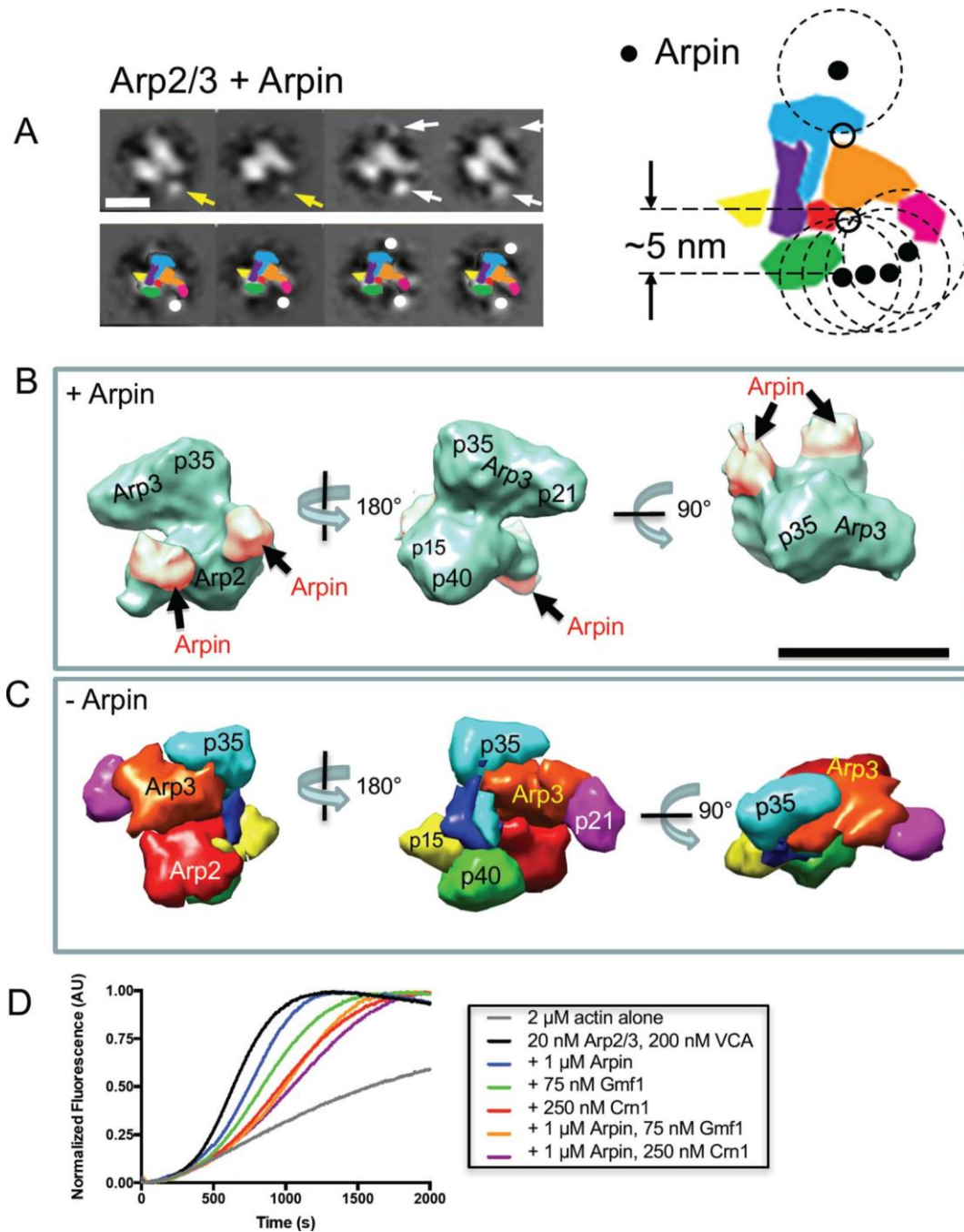
**Figure 2. Three-dimensional structures of Gmf1-bound Arp2/3 complexes**

(A) Two-dimensional projections of Gmf1-bound Arp2/3 complex. Upper row, from left to the right: re-projection of the crystal structure of Arp2/3-GMF complex [14], free Arp2/3 2D projection in the same orientation, two representative 2D projections of Arp2/3-Gmf1 in standard open conformation, and two 2D projections of Arp2/3-Gmf1 in new open conformation. Numbers on the right reflect % of the particles in each class. Bottom row: difference maps, generated by subtracting the 2D projection of free Arp2/3 from Arp2/3Gmf1 projections, respectively. White arrowheads highlight adding of the new mass, orange arrowheads highlight loss of the mass. (B) Crystal structure of GMF-bound

Arp2/3 complex [14] filtered to 25 Å resolution using UCSF Chimera [51]. Arp2/3 complex subunits, colorcoded and labeled as in Figure 1A. Arrows highlight the position of GMF (grey) bound to Arp2 and p40/ARPC1 subunits. (C,D) Three-dimensional reconstructions of Gmf1-bound Arp2/3 complex in the standard open conformation (C) and the new open conformation (D). Additional densities, approximately the size of Gmf1, are shaded in pink and labeled with arrows. Asterisk in (C) marks a new mass at the proposed second binding site for GMF on Arp3. Three separate views of Arp2/3 complex shown in B-D are aligned to each other for comparison. Scale bar, 10 nm.



**Figure 3. Gmf1 and Crn1 synergize in inhibiting Arp2/3-mediated actin assembly in vitro (A, B)** Bulk actin polymerization assays containing G-actin (2  $\mu$ M, 5% pyrene-labeled) and the indicated concentrations of Arp2/3 complex, VCA, Gmf1, and Crn1. (C) Bulk actin polymerization assays, performed as in A and B, except using full-length Las17 (yeast WASP) instead of VCA. (D) Percentage of Arp2/3 complex particles with Crn1 bound in the presence and absence of Gmf1 (- Gmf1, n = 366; + Gmf1, n = 398).



**Figure 4. Three-dimensional structure of Arpin-bound Arp2/3 complex**

(A) Two-dimensional projections of Arpin-bound Arp2/3 complex. Arrows highlight new masses, which likely represent the globular core domains of Arpin [40]. Yellow arrows indicate the presence of a single new mass, while white arrows indicate two new masses. Scale bar, 10 nm. Lower panel includes corresponding cartoons of each class average. The larger cartoon on the right shows Arp2/3 complex with superimposed positions of Arpin (filled circles). Dashed circles have a 5 nm radius, corresponding to the estimated length connecting the center of Arpin globular domain and its C-terminal acidic (A) motif. The circles were used to map potential Arpin-binding sites on Arp2/3 complex (open circles).

(B) Three-dimensional reconstruction of Arpin-bound Arp2/3 complex viewed from different angles. Additional density, attributed to Arpin, is shaded pink. (C) Crystal structure of Arp2/3 complex [14] filtered to 25 Å resolution using UCSF Chimera [51], with subunits color-coded and labeled. Views are aligned with those in B for comparison. Scale bar, 10 nm. (D) Bulk actin polymerization assays containing G-actin (2 μM, 5% pyrene-labeled) and the indicated concentrations of Arp2/3 complex, VCA, Arpin, Gmf1, and Crn1.





# The Arp2/3 inhibitory protein Arpin must bind to Tankyrase to restrict cell migration

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Running title: Role of the Arpin-Tankyrase interaction

During cell migration, protrusion of the leading edge is driven by the small GTPase Rac1 and the polymerization of Arp2/3-dependent branched actin networks it triggers. Migration persistence and protrusion lifetime are negatively regulated by the Arp2/3 inhibitory protein Arpin. To understand Arpin regulation, we looked for interacting partners and identified both Tankyrases 1 and 2 using a yeast two hybrid screen and immunoprecipitations using full-length Arpin as a bait. Arpin binds to ankyrin repeats of Tankyrases through a site on its acidic tail, which overlaps with the Arp2/3 binding site. Arpin binding induces a conformational change in full-length Tankyrase 1, as revealed by transmission electron microscopy and negative staining. An Arpin mutation that abolishes binding to Tankyrases impaired membrane translocation of Arpin, its interaction with the Arp2/3 complex and its function in inhibiting migration persistence. Arpin activation thus requires its sequential binding to, first, Tankyrase, and, then, to the Arp2/3 complex.

## **Introduction**

Cell migration is crucial during embryogenesis and during adulthood for immune functions and wound healing, for example. Cell migration depends on various types of membrane protrusions. Most protrusions are driven by cortical actin polymerization (Ridley, 2011). The Arp2/3 complex is the machinery that generates branched actin networks at the leading edge of migrating cells. These actin networks power the formation of membrane protrusions, called lamellipodia. The activity of the Arp2/3 complex is tightly regulated by positive and negative regulators (Molinie and Gautreau, 2018). Lamellipodial protrusions exquisitely depend on the activity of the small GTPase Rac1, which activates the WAVE complex, an Arp2/3 activator at the plasma membrane (Steffen et al., 2014). We identified a conserved Arp2/3 inhibitory protein, Arpin, which antagonizes the WAVE complex at the plasma membrane (Dang et al., 2013). Arpin inhibits cell migration by decreasing directional persistence of cell migration (Krause and Gautreau, 2014; Gorelik and Gautreau, 2015).

Arpin is structurally organized around a folded domain with an extended C-terminal acidic tail protruding from this core (Fetics et al., 2016). Through its acidic tail, Arpin competes with VCA containing Arp2/3 activators (Dang et al., 2013). Arpin occupies two binding sites on the Arp2/3 complex and maintain it in an inactive conformation (Sokolova et al., 2017; Popinako et al., 2017). Recombinant purified Arpin is a constitutive Arp2/3 inhibitory protein in in vitro assays, but appears regulated in the cell. Arpin does not prevent lamellipodia from forming, but rather induce their retraction after their initiation, thereby controlling protrusion lifetime (Dang et al., 2013). Arpin ability to interact with the Arp2/3 complex depends on Rac1 activity, as the result of

a yet unknown signaling pathway, since no direct interaction was detected between the two purified proteins.

Understanding Arpin regulation in the cell is thus a major challenge, which we addressed by looking for interacting proteins. Here we report that Tankyrases are essential partners for Arpin to perform its function. Tankyrases are overexpressed in many cancers and have become attractive drug targets to down regulate Wnt signaling (Mariotti et al., 2017). Drug-mediated Tankyrase inhibition impairs migration and invasion of various tumor cell lines (Bao et al., 2012; Tian et al., 2014; Lupo et al., 2016; Li et al., 2018). In contrast, Arpin is down regulated in breast and gastric cancers of poor prognosis in line with its inhibitory role on cell migration (Lomakina et al., 2016; Liu et al., 2016; Li et al., 2017). Here we decided to analyze the role of the Arpin-Tankyrase interaction in untransformed cells to avoid the further complexity of Arpin and Tankyrase deregulation in cancer.

## **Results and discussion**

### **Arpin binds to Tankyrases**

To identify proteins that bind to Arpin, we first immunoprecipitated Arpin from a stable 293 cell line expressing a Protein C (PC) tagged version of Arpin through its epitope tag. Arpin was efficiently immunoprecipitated from the lysate prepared from cells expressing tagged Arpin, but not from a control stable cell line transfected with the empty plasmid. Silver staining was used to identify potential partner proteins (Fig.1A). In a single step immunoprecipitation, many bands were detected, including Ig light and heavy chains. However, two specific bands between the 120 and 150 kDa markers were detected when Arpin was immunoprecipitated, but not in the control lane. These two proteins were identified by LC-MS/MS as Tankyrase1 (TNKS1, 142 kDa) and Tankyrase2 (TNKS2, 127 kDa). With the expectation to identify additional potential partners of the Arpin protein, we performed a yeast two hybrid screen of a library containing random primed human placenta cDNAs, with full-length Arpin as a bait. More than  $10^8$  clones were analyzed by yeast mating. Out of the 187 clones selected, 177 corresponded to either TNKS1 or TNKS2 (Fig.1B). The remaining 10 clones were comparatively of low confidence, as they corresponded to out-of-frame fusions or to DNA sequences, which were not annotated as protein encoding genes. These two approaches thus point at Tankyrases as major Arpin partners.

The Tankyrases are homologous proteins composed of three domains. From the N- to the C-terminus, these proteins contains ankyrin repeats organized into Ankyrin Repeat Clusters (ARCs), a Sterile Alpha Motif (SAM), which mediates oligomerization, and a C-terminal Poly ADP Ribosyl Polymerase (PARP) catalytic domain. All yeast two-hybrid clones interacting with full-length Arpin mapped to the N-terminal region composed of ankyrin repeats. ARCs recognize a consensus motif (octapeptide RXXGXGXX) identified through the screening of a peptide library

(Guettler et al., 2011). We found the consensus motif conserved in the acidic tail of Arpin from several vertebrate species (Fig 1C).

To test for a direct interaction, we produced full-length Arpin and the ARC4 of TNKS2, which has been previously crystallized (Guettler et al., 2011). When the two proteins were mixed, a new molecular species was detected by Size Exclusion Chromatography – Multi Angle Light Scattering (SEC-MALS). As expected, this species displays a mass corresponding to a 1:1 complex (Fig.1D). We then investigated a possible binding competition between the Arp2/3 complex and Tankyrases for Arpin binding. GST-Arpin pull-down with lysates from mouse embryonic fibroblasts (MEF) retrieved the Arp2/3 complex and both Tankyrases. Adding an excess of purified ARC4 displaced not only Tankyrases, but also the Arp2/3 complex, in line with their overlapping binding sites in the acidic tail.

Tankyrases are pleiotropic regulators of various cellular functions, including telomere maintenance, Wnt signaling, insulin-dependent glucose uptake (Riffell et al., 2012). Their functions usually require binding to substrates through ARC domains and poly ADP ribosylation (PARylation) through their catalytic PARP domain. Usually but not always, the fate of PARylated proteins is to be degraded by the ubiquitin-proteasome pathway, because the E3 ubiquitin ligase RNF146 recognizes PARylated proteins (Zhang et al., 2011). Tankyrases also PARylates themselves, which results in their turnover. To investigate whether Arpin might be similarly regulated, we inactivated tankyrases with the XAV939 inhibitor. As expected, this treatment resulted in increased levels of both Tankyrases. Levels of Arpin were, however, not modified by the XAV939 treatment (Fig.S1). Even though Arpin does not appear to turn over as a result of Tankyrase activity, we sought to detect its potential PARylation. To this end, we used the WWE domain of RNF146 as a recognition module to pull down PARylated proteins (Zhang et al., 2011). In the pull-down, Tankyrase, but not Arpin, is retrieved (Fig.S1).

Together these experiments indicate that Arpin is a direct Tankyrase partner, but is likely not subjected to PARylation mediated degradation, in contrast with most Tankyrase binding partners. Mcl-1L and GDP Mannose 4,6 Dehydratase are similar to Arpin, since they are Tankyrase binding partners, which are not PARylated (Bae et al., 2003; Bisht et al., 2012).

### **A mutation that impairs Tankyrase binding inactivates Arpin**

To address the role of the Tankyrase interaction, we then looked for a mutation in the Arpin acidic tail that would specifically impair Tankyrase binding. We replaced the G218 from the consensus ARC binding site by a D residue, since Arp2/3 binding requires acidic residues. Biotinylated ArpinA peptides corresponding to the acidic tail, i.e. the last 20 C-terminal residues of Arpin, were synthesized and used in pull-down assays using streptavidin beads. Purified ARC4 was efficiently retrieved by the WT peptide, but not by the G218D peptide (Fig.2A). Using Isothermal Titration Calorimetry (ITC), we detected an interaction between ArpinA and ARC4 characterized by a  $K_d$  of  $0.4 \mu\text{M}$ , a relatively high affinity for monovalent binding to a single ARC

domain in agreement with the perfect match of the Arpin tail to the consensus motif (Guettler et al., 2011). In sharp contrast, binding of the G218D peptide to ARC4 was undetectable using ITC.

Purified Arp2/3 complex bound as efficiently to WT and G218D ArpinA peptides (Fig.2C). We then attempted to compete Arp2/3 binding with an excess of ARC4. Whereas ARC4 completely displaced Arp2/3 from the WT ArpinA peptide, it had a minimal residual effect on the G218D ArpinA peptide (Fig.2D). Together these in vitro experiments show that Arp2/3 and Tankyrase binding to the acidic tail are competing for overlapping binding sites and that the G218D mutation of the Arpin tail allows to specifically impair Tankyrase binding. We thus introduced the G218D mutation into full-length Arpin and performed cell migration assays expecting to find increased Arp2/3 binding and thus increased inhibition of cell migration with the G218D mutation.

We used the immortalized, but untransformed, cell line MCF10A, derived from human breast, to isolate stable cell lines expressing GFP, GFP-Arpin or GFP-Arpin G218D. These lines were obtained by homologous recombination at the AAVS1 locus to obtain similar level of expression of WT and mutant Arpin. Western blot analysis indicated that the exogenous Arpins were overexpressed compared to the endogenous (Fig.3A). Interestingly, overexpression of WT Arpin, but not of G218D Arpin, increased levels of endogenous Arpin. We depleted endogenous Arpin using a siRNA that targets the 3'UTR of the Arpin mRNA, which is absent from the transgene. We performed videomicroscopy with these 3 cell lines with or without endogenous Arpin and tracked single cells for 24 h. As expected, Arpin depletion induced GFP expressing cells to explore a larger territory than controls (Fig.3B). This effect is due to the role of Arpin in regulating migration persistence (Dang et al., 2013; Gorelik and Gautreau, 2015). WT Arpin expression fully rescued the phenotype, but did not further decrease cell exploration or migration persistence compared to the control situation despite its overexpression (Fig.3C).

Surprisingly, G218D Arpin was inactive. G218D Arpin was completely unable to rescue the phenotype associated with Arpin depletion (Fig.3B). Not only G218D Arpin was inactive, but it also slightly affected migration persistence when endogenous Arpin was expressed (Fig.3C), suggesting that G218D exerts a dominant-negative influence over endogenous Arpin. To understand why G218D Arpin was inactive, we analyzed Arpin interaction with Tankyrase and Arp2/3 using co-immunoprecipitation. As expected, G218D Arpin was unable to interact with tankyrases, but G218D Arpin was also unable to bind to the Arp2/3 complex. This result is in line with the phenotypic analysis, but at odds with in vitro analyses of interactions. The simplest possible explanation to account for this conundrum is that the two interactions are not in competition in the cell, but rather that Tankyrase binding is a prerequisite for Arpin to inhibit the Arp2/3 complex.

## **A conformational change in Tankyrase promotes membrane translocation of Arpin**

To get insights into possible mechanisms, we sought to analyze the Arpin-Tankyrase interaction by transmission electron microscopy (EM). We derived a stable 293 cell line expressing full-length His-PC-tagged TNKS1. TNKS1 was partially purified using PC immunoprecipitation followed by native elution using  $\text{Ca}^{2+}$  chelation (Fig.S2). Samples were applied onto EM grids and negatively stained. To learn to recognize Tankyrase particles among contaminants, His tagged TNKS1 was labeled with 5 nm nanogold coupled to Ni-NTA (Fig.S2). For 3D reconstructions, we used unlabeled images. Preliminary reconstruction has been accomplished in EMAN2.1 using 120 unlabeled EM images of TNKS. The final data set contained 6500 2D projections. The 2D projections were sorted into 75 class averages (Fig.4A). The average dimensions of 2D particles were 100 x 165 Å. Out of 75 class averages, the 50 best classes, containing 4500 particles, were selected and used to refine a 3D reconstruction using RELION5.0 (Scheres, 2012). Due to partial purification and conformational heterogeneity of particles, the 3D reconstruction (Fig.4B) has a limited resolution of 25 Å (Fig.S3). With or without nanogold labeling, we saw no evidence of Tankyrase oligomerization and polymerization, which are mediated by the SAM domain, presumably because of the low Tankyrase concentration in our samples (Mariotti et al., 2016; Riccio et al., 2016).

We then incubated the TNKS1 containing fraction with an excess of Arpin. Similarly, we acquired 2500 single particles, sorted them into 40 class averages, and performed a 3D reconstruction, which had a resolution of 23 Å. The 3D structures of TNKS1 with or without Arpin were markedly different (Fig.4B). First, the 3D structure of TNKS1 incubated with Arpin unambiguously revealed 3 additional densities. Two of these densities had a diameter of 25 Å, similar to what we found in our previous study of Arpin bound to the Arp2/3 complex (Sokolova et al., 2017). One Arpin density, the lowest in figure 4, had a larger diameter of 30 Å. We favor the possibility that this density corresponds to two Arpin molecules, since the 5 ARCs are expected to bind 4 Arpin molecules, due to the inactivity of ARC3 (Guettler et al., 2011). However, we cannot formally rule out that this larger density is due to an increased conformational flexibility of a single Arpin molecule. A measured distance of 4.5-5.0 nm between Arpin molecules and Tankyrase is also compatible with the length of the extended acidic tail of Arpin, as seen for Arp2/3 binding (Fetics et al., 2016; Sokolova et al., 2017).

The three domains of Tankyrases were previously crystallized (Guettler et al., 2011; Morrone et al., 2012; Mariotti et al., 2016; Riccio et al., 2016; Qiu et al., 2014). However, full-length tankyrases were never crystallized, most likely because of their flexibility. Our 3D reconstructions are therefore the first structural models of a full-length Tankyrase. We were able to dock the crystal structures of ARC, SAM and PARP domains into both 3D envelopes. In free TNKS1, the fit was good for the ARC and SAM domains and limited for the PARP domain, which appeared V-shaped in the electron density (Fig.S3). When TNKS1 was bound to Arpin, the fit was also good to the ARC and SAM domains (Fig.4C), but the ARCs were significantly reorganized (Fig.4D). A conformational change was previously reported when ARCs bound to Axin (Eisemann



et al., 2016). Axin, however, is a bivalent ligand. Here the conformational change is induced by the binding of a monovalent ligand, Arpin.

Since Arpin binds to the Arp2/3 complex at the plasma membrane (Dang et al., 2013), we then asked whether Tankyrase binding would be required to translocate Arpin to membrane. Using biochemical fractionation of our MCF10A cell lines, we found that endogenous Arpin is mostly a cytosolic protein, with a comparatively small amount bound to membranes (Fig.5A). Exogenous GFP-tagged WT and G218D Arpin proteins behaved like endogenous Arpin, being cytosolic for the most part. The minor membrane pool of Arpin was further reduced the G218D mutation. It is unclear whether the residual amount of membrane bound G218D Arpin is real. It might be due to cytosolic contamination of the membrane pellet, which cannot be washed in the procedure, since membrane amounts are increased when more concentrated extracts are used. Because Arpin binding to the Arp2/3 complex depends on Rac1, we examined the Arpin-Tankyrase interaction in *Rac1* KO MEFs (Steffen et al., 2013). Arpin bound to Tankyrases in a Rac1 independent manner (Fig.5B), in line with the suggestion that Arpin binding to Tankyrase precedes Rac1-dependent Arp2/3 inhibition.

### **Model of Arpin regulation**

Our in vitro binding assays clearly indicate that Arpin cannot bind simultaneously to Tankyrase and Arp2/3, because they compete for overlapping binding sites. Since the G218D mutation that specifically impairs Tankyrase binding prevents Arp2/3 binding in the cell and that Arpin binding to Tankyrases does not require Rac1 function, our working model is that Tankyrase binding is a required step before Rac1-dependent Arp2/3 binding (Fig.5C). We found that Tankyrase promotes membrane association of Arpin. But how Arpin binds to the plasma membrane and how Tankyrase promotes membrane association of Arpin are yet unresolved questions. The conformational change of Tankyrase induced by Arpin binding that we document here can suggest possible molecular scenarios, which can be tested in future studies. For example, the conformation of Tankyrase bound to Arpin could recognize a membrane protein and deliver Arpin at the plasma membrane. Tankyrase binding, occupying the Arp2/3 binding site, might also prevent Arpin from premature activation in the cytosol. In conclusion, we identified Tankyrases as essential protein partners for Arpin to exert its function of limiting cell migration through the inhibition of the Arp2/3 complex at the plasma membrane.

## MATERIALS AND METHODS

### Plasmids, siRNAs and antibodies

To produce recombinant proteins in *E.coli*, ARC4 from TNKS2 was cloned into pQE30 (Qiagen). Arpin and the WWE domain of RNF146 (amino-acids 100-175) were produced as GST fusion proteins from a modified pGEX vector containing a TEV protease cleavage site after the GST moiety. For expression in MCF10A cells, full length ORFs encoding Arpin WT and G218D were cloned in the custom-made plasmid MXS AAVSL1 SA2A Puro bGHpA PGK rtTA3 bGHpA mEGFP Blue SV40pA AAVS1R between FseI and AscI sites. For expression in HEK293 Flp-In cells, human Arpin and TNKS1 ORFs were cloned in pcDNA5 His PC TEV Blue between FseI and AscI sites. For the yeast two-hybrid screen, full length human Arpin was cloned in pB27 in fusion with the LexA DNA binding domain. A random primed cDNA library from human placenta was screened by Hybrigenics using a mating protocol and 2 mM 3-aminotriazole to reduce background.

The non-targeting control siRNA is 5'-AAUUCUCCGAACGUGUCACGUUU-3', the Arpin siRNA, 5'-GGAAAGAAGAGAAUGAUUUUU-3', targets the 3'UTR, which is absent from expressing constructs. SiRNAs were bought from Sigma-Aldrich.

Rabbit polyclonal antibodies obtained and purified using full-length Arpin were previously described (Dang et al., 2013). The following commercial antibodies were used: TNKS1/2 pAb (H-350, Santa Cruz Biotechnology), ArpC2 pAb (Millipore) and Cortactin mAb (clone 4F11, Millipore), ArpC5 mAb (clone 323H3, Synaptic Systems). PC mAb (clone HPC4, Sigma), GFP mAb (clone 7.1 and 13.1, Roche). EEA1 pAb (2411S, Cell Signaling Technology), RhoGDI pAb (sc-360, Santa Cruz Biotechnology).

### Cell culture, transfection and live imaging

MCF10A cells were maintained in DMEM/F12 medium (Life Technologies) supplemented with 5% horse serum (Sigma), 100 ng/ml cholera toxin (Sigma), 20 ng/ml epidermal growth factor (Sigma), 0.01 mg/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma) and 100 U/ml penicillin/streptomycin (Life Technologies). To integrate plasmids at the AAVS1 locus (González et al., 2014), the MXS plasmid was transfected with two TALEN expressing plasmids (Addgene #59025 and 59026) using Lipofectamine 3000 (Thermo Fisher Scientific). Cells were selected with 1 µg/ml of Puromycin. Individual clones were picked with cloning rings. Expression of GFP fusion protein was induced with 2 µg/ml of doxycycline. HEK293 Flp-In stable cell lines expressing Arpin and TNKS1 were obtained as previously described (Derivery and Gautreau, 2010). Rac1 KO MEFs were previously described in (Steffen et al., 2013).

Knockdown of endogenous Arpin in MCF10A lines expressing GFP tagged proteins was obtained by a double transfection of 140 pmol of siRNAs (Sigma) using lipofectamine RNAiMax (Life Technologies). Briefly, 100,000 cells were reverse transfected when seeded into wells of a 6-well dish. Two days later, cells were forward transfected and induced with doxycycline. Two days later, cells were seeded onto glass bottomed  $\mu$ -Slide (Ibidi) coated with 20  $\mu$ g/ml of fibronectin. Imaging was performed on an Axio Observer microscope (Zeiss) equipped with a Plan-Apochromat 20x/0.80 air objective, a Hamamatsu camera C10600 Orca-R<sup>2</sup> and a Pecon Zeiss incubator XL multi S1 RED LS (Heating Unit XL S, Temp module, CO<sub>2</sub> module, Heating Insert PS and CO<sub>2</sub> cover). Pictures were taken every 10 min for 24 h. Cell migration was analyzed as described (Gorelik and Gautreau, 2014).

### **Analysis of cell migration and statistics**

Single cell trajectories were obtained by tracking cells with Image J and analyzed using the DiPer software (Gorelik and Gautreau, 2014) to obtain directional autocorrelation (without gaps). The area under the curve (AUC) of the autocorrelation function was measured for each cell, individually, using the GraphPad Prism software. Multiple comparison was performed using parametric ANOVA followed by Post Hoc test (Name of the Post Hoc test). A representative experiment is plotted and results are expressed as means and standard errors of the mean (sem) with respect to the number of cells (n). Four levels of statistical significance were distinguished: \* P<0.05; \*\* P<0.01; \*\*\* P<0.001, \*\*\*\* P<0.0001.

### **Protein purification and characterization of interactions**

GST-Arpin WT and G218D were purified from *E. coli* BL21\* strain (Life Technologies) using standard purification protocols, dialyzed against storage buffer (50 mM Hepes pH 7.5, 100mM NaCl, 2 mM  $\beta$ -mercaptoethanol), frozen in liquid nitrogen and stored at -80°C. When indicated, Arpin was cleaved off GST using the TEV protease. Arpin bound to Glutathione sepharose 4B beads was cleaved with overnight incubation at 4°C using His-tagged TEV protease in 50 mM Tris pH 7.5, 2 mM  $\beta$ -mercaptoethanol, 100 mM NaCl, 5 mM MgCl<sub>2</sub>. TEV was removed by incubation with Ni<sup>2+</sup> beads (GE Healthcare). Arpin was further purified by size exclusion chromatography on a Superdex-200 column (GE Healthcare) and concentrated on Vivaspin filters. Both Arpin proteins have an amino-terminal extension of 10 amino-acids (GAMAHMGRP) after TEV cleavage.

TNKS1, tagged with His and PC tags, was purified from the stable 293 cell line expressing it grown in spinner cultures as described (Derivery and Gautreau, 2010). Cell pellets (2 ml, roughly 10<sup>9</sup> cells) were lysed in 8 ml RIPA buffer (50mM Hepes, pH 7,5, 150mM NaCl, 1% NP-40, 0.5% DOC, 0,1% SDS, 1mM CaCl<sub>2</sub>) supplemented with the EDTA free protease inhibitor cocktail (Roche). Clarified lysates were incubated with 120  $\mu$ l anti-Protein C resin (Sigma-Aldrich) 2 h,

rocking at 4° C. After an incubation matrix was washed twice in RIPA buffer and once in XB buffer (50mM Hepes, pH 7.5, 150mM NaCl). Affinity matrix was incubated with 50 µl of elution buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 5mM EGTA) for 1 h at 4° C.

Purified Arp2/3 complex, from porcine brain, was purchased from Cytoskeleton and was resuspended in 20 mM Tris pH 7.5, 25 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.1 mM ATP, 1.0% (v/v) dextran and 5% (v/v) sucrose. The biotinylated peptides corresponding to the last 20 amino acids of Arpin (ArpinA WT and G218D, purity >95%) were synthesized by ProteoGenix and resuspended in PBS pH 7.2.

For SEC-MALS, purified proteins were separated in a 15 ml KW-803 column (Shodex) run on a Shimadzu HPLC system. MALS, QELS and RI measurements were achieved with a MiniDawn Treos, a WyattQELS and an Optilab T-rEX (all from Wyatt technology), respectively. Mass calculations were performed with the ASTRA VI software (Wyatt Technology) using a  $dn/dc$  value of 0.183 mL.g<sup>-1</sup>.

ITC experiments were performed with an ITC200 isothermal titration calorimeter from MicroCal (Malvern). Experiments were carried out at 20°C. The microcalorimeter cell (0.2 ml) containing purified ARC4 at 30 µM received 20 injections of 2 µl of Arpin WT or G218D at 300µM at intervals of 180s while stirring at 700 rpm. Experimental data were fitted to theoretical titration curves with the ORIGIN software supplied by MicroCal.

In vitro interactions with biotinylated peptides were performed by associating first peptides to High Capacity Streptavidin Agarose beads (Thermo Fisher Scientific) in PBS pH 7.2 for 1 hour at room temperature. Then beads were washed in the optimized interacting buffer (10 mM Hepes pH 7.7, 50mM KCl, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>) and allowed to interact with either purified ARC4 or Arp2/3 complex for 1 hour at 4°C with rocking. Beads were quickly washed twice, and analyzed by SDS-PAGE and stained with Coomassie.

### **Co-immunoprecipitation and GST pull-down**

Stable 293 cells expressing His-PC-Arpin or the empty plasmid as a control were lysed in (50 mM Hepes pH7.7, 150 mM NaCl, 1mM CaCl<sub>2</sub>, 1% NP40, 0.5% Na Deoxycholate, 0.1% SDS 1% supplemented with protease inhibitor cocktail, Roche). Clarified lysates were incubated with 10 µl of HPC4 coupled beads (Sigma) for 3 h at 4°C. After 5 washes in the same buffer, beads were analyzed by SDS-PAGE. For Tankyrase identification, tryptic peptides were analyzed by NanoLC-MS/MS analyses using a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) coupled to the EASY nLC II high performance liquid chromatography system (Proxeon, Thermo Scientific). Peptide separation was performed on a reverse phase C18 column (Nikkyo Technos). NanoLC-MS/MS experiments were conducted in a Data Dependent acquisition method by selecting the 20 most intense precursors for CID fragmentation and analysis in the LTQ. Data were processed with

the Proteome Discoverer 1.3 software and protein identification was performed using the Swissprot database and MASCOT search engine (Matrix science).

For endogenous co-immunoprecipitation of the Arp2/3 complex with Arpin, MEF cells were lysed in (50 mM KCl, 10 mM Hepes pH 7.7, 1 mM MgCl<sub>2</sub>, 1% NP40, 0.5% NaDeoxycholate, 0.1% SDS). Clarified extracts were incubated for 2 h with magnetic beads previously coupled to 10 µg of non-immune rabbit IgG or 10 µg of affinity purified Arpin antibodies (tosyl-activated dynabeads, Life Technologies). Beads were incubated with extracts for 2 h at 4°C, washed 5 times in the same buffer and analyzed by Western blot.

HeLa cell pellets were lysed in (50 mM Hepes pH7.7, 150 mM NaCl, 1mM CaCl<sub>2</sub>, 1% NP40, 0.5% Na Deoxycholate, 0.1% SDS 1% supplemented with protease inhibitor cocktail, Roche). 20 µg of GST fusion protein and 20 µl of Glutathione Sepharose 4B Beads (GE Healthcare) were incubated with 1 ml of HeLa cell extract for 2 h at 4°C. When indicated, purified ARC4 protein is added into the mixture to compete interaction. Beads were washed 5 times in the same buffer and analyzed by Western blot.

### **Biochemical fractionation and Western blots**

For cell fractionation, cells were disrupted in 1 ml of 50 mM Hepes pH7.7, 150 mM NaCl, 1mM CaCl<sub>2</sub> buffer using nitrogen cavitation (500 psi for 20 min, Parr instruments). Debris and nuclei were pelleted by centrifugation at 2500 rpm for 5 minutes at 4°C. The supernatant is the total cell fraction, which is then fractionated into a cytosolic supernatant by ultracentrifugation at 150,000 x g for 60 min at 4°C (rotor TLA120, Optima Beckman) and a tight glassy pellet. The membrane fraction is obtained by resuspending the pellet in 100 µl of RIPA buffer and dissolving it for 60 min with rocking. The insoluble material was removed by ultracentrifugation at 150,000 x g for 60 min at 4°C.

SDS-PAGE was performed using NuPAGE 4-12% Bis-Tris gels (Life Technologies). For Western blots, proteins were transferred using the iBlot system (Life Technologies) and developed using HRP-coupled antibodies, Supersignal kit (Pierce) and a LAS-3000 imager (Fujifilm) or AP-coupled antibodies and NBT/BCIP as substrates (Promega).

### **Electron microscopy and Single Particle Analysis**

Freshly eluted His PC tagged TNKS1 alone, or mixed with purified Arpin in a 1:10 ratio, was applied to carbon-coated glow-discharged copper grids for 30 sec, the excess of liquid was blotted away, and grids were negatively stained with 1% Uranyl acetate for 30 sec at room temperature, air-dried, and analyzed in a JEOL 2100 electron microscope at 200 kV at low-dose conditions. When indicated, 5 nm Ni-NTA-Nanogold (Nanoprobes Inc.) were incubated for 30 min at room

temperature with the fraction containing His tagged TNKS1. Grids were then washed with water and negatively stained as described above. Images of TNKS1 alone, of TNKS1 labeled with Ni-NTA-Nanogold and TNKS1 bound to Arpin were manually collected from electron micrographs using the graphical program BOXER. Three-dimensional reconstructions were first built using EMAN 2.1 (Ludtke et al., 1999; Ludtke, 2016) and refined with RELION 5.0 (Scheres, 2012).

### **Author contributions**

AC isolated most stable cell lines, performed migration assays, fractionation experiments and single particle analysis. ID and KO performed most co-immunoprecipitations and pull-down experiments. SF and VC produced expression constructs and purified proteins. MAN performed the ITC experiment. JC supervised the work of SF and VC. OSS supervised EM structural analysis. AG supervised biochemistry and cell biology experiments and the overall project. AC and OSS drafted the manuscript and AG wrote it in its final form.

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## References

- Bae, J., J.R. Donigian, and A.J.W. Hsueh. 2003. Tankyrase 1 interacts with Mcl-1 proteins and inhibits their regulation of apoptosis. *J Biol Chem.* 278:5195–5204. doi:10.1074/jbc.M201988200.
- Bao, R., T. Christova, S. Song, S. Angers, X. Yan, and L. Attisano. 2012. Inhibition of tankyrases induces Axin stabilization and blocks Wnt signalling in breast cancer cells. *PLoS ONE.* 7:e48670. doi:10.1371/journal.pone.0048670.
- Bisht, K.K., C. Dudognon, W.G. Chang, E.S. Sokol, A. Ramirez, and S. Smith. 2012. GDP-mannose-4,6-dehydratase is a cytosolic partner of tankyrase 1 that inhibits its poly(ADP-ribose) polymerase activity. *Mol Cell Biol.* 32:3044–3053. doi:10.1128/MCB.00258-12.
- Dang, I., R. Gorelik, C. Sousa-Blin, E. Derivery, C. Guérin, J. Linkner, M. Nemethova, J.G. Dumortier, F.A. Giger, T.A. Chipysheva, V.D. Ermilova, S. Vacher, V. Campanacci, I. Herrada, A.-G. Planson, S. Fetics, V. Henriot, V. David, K. Oguievetskaia, G. Lakisic, F. Pierre, A. Steffen, A. Boyreau, N. Peyriéras, K. Rottner, S. Zinn-Justin, J. Cherfils, I. Bièche, A.Y. Alexandrova, N.B. David, J.V. Small, J. Faix, L. Blanchoin, and A. Gautreau. 2013. Inhibitory signalling to the Arp2/3 complex steers cell migration. *Nature.* 503:281–284. doi:10.1038/nature12611.
- Derivery, E., and A. Gautreau. 2010. Assaying WAVE and WASH complex constitutive activities toward the Arp2/3 complex. *Methods Enzymol.* 484:677–695. doi:10.1016/S0076-6879(10)84033-9.
- Eisemann, T., M. McCauley, M.-F. Langelier, K. Gupta, S. Roy, G.D. Van Duyne, and J.M. Pascal. 2016. Tankyrase-1 Ankyrin Repeats Form an Adaptable Binding Platform for Targets of ADP-Ribose Modification. *Structure.* 24:1679–1692. doi:10.1016/j.str.2016.07.014.
- Fetics, S., A. Thureau, V. Campanacci, M. Aumont-Nicaise, I. Dang, A. Gautreau, J. Pérez, and J. Cherfils. 2016. Hybrid Structural Analysis of the Arp2/3 Regulator Arpin Identifies Its Acidic Tail as a Primary Binding Epitope. *Structure.* 24:252–260. doi:10.1016/j.str.2015.12.001.
- González, F., Z. Zhu, Z.-D. Shi, K. Lelli, N. Verma, Q.V. Li, and D. Huangfu. 2014. An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. *Cell Stem Cell.* 15:215–226. doi:10.1016/j.stem.2014.05.018.
- Gorelik, R., and A. Gautreau. 2014. Quantitative and unbiased analysis of directional persistence in cell migration. *Nature Protocols.* 9:1931–1943. doi:10.1038/nprot.2014.131.
- Gorelik, R., and A. Gautreau. 2015. The Arp2/3 inhibitory protein arpin induces cell turning by pausing cell migration. *Cytoskeleton (Hoboken).* 72:362–371. doi:10.1002/cm.21233.
- Guettler, S., J. LaRose, E. Petsalaki, G. Gish, A. Scotter, T. Pawson, R. Rottapel, and F. Sicheri. 2011. Structural basis and sequence rules for substrate recognition by Tankyrase explain the basis for cherubism disease. *Cell.* 147:1340–1354. doi:10.1016/j.cell.2011.10.046.



- Krause, M., and A. Gautreau. 2014. Steering cell migration: lamellipodium dynamics and the regulation of directional persistence. *Nat Rev Mol Cell Biol.* 15:577–590. doi:10.1038/nrm3861.
- Li, C., X. Zheng, Y. Han, Y. Lv, F. Lan, and J. Zhao. 2018. XAV939 inhibits the proliferation and migration of lung adenocarcinoma A549 cells through the WNT pathway. *Oncol Lett.* 15:8973–8982. doi:10.3892/ol.2018.8491.
- Li, T., H.-M. Zheng, N.-M. Deng, Y.-J. Jiang, J. Wang, and D.-L. Zhang. 2017. Clinicopathological and prognostic significance of aberrant Arpin expression in gastric cancer. *World J. Gastroenterol.* 23:1450–1457. doi:10.3748/wjg.v23.i8.1450.
- Liu, X., B. Zhao, H. Wang, Y. Wang, M. Niu, M. Sun, Y. Zhao, R. Yao, and Z. Qu. 2016. Aberrant expression of Arpin in human breast cancer and its clinical significance. *J. Cell. Mol. Med.* 20:450–458. doi:10.1111/jcmm.12740.
- Lomakina, M.E., F. Lallemand, S. Vacher, N. Molinie, I. Dang, W. Cacheux, T.A. Chipysheva, V.D. Ermilova, L. De Koning, T. Dubois, I. Bièche, A.Y. Alexandrova, and A. Gautreau. 2016. Arpin downregulation in breast cancer is associated with poor prognosis. *Br. J. Cancer.* 114:545–553. doi:10.1038/bjc.2016.18.
- Ludtke, S.J. 2016. Single-Particle Refinement and Variability Analysis in EMAN2.1. *Methods Enzymol.* 579:159–189. doi:10.1016/bs.mie.2016.05.001.
- Ludtke, S.J., P.R. Baldwin, and W. Chiu. 1999. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* 128:82–97. doi:10.1006/jsbi.1999.4174.
- Lupo, B., J. Vialard, F. Sassi, P. Angibaud, A. Puliafito, E. Pupo, L. Lanzetti, P.M. Comoglio, A. Bertotti, and L. Trusolino. 2016. Tankyrase inhibition impairs directional migration and invasion of lung cancer cells by affecting microtubule dynamics and polarity signals. *BMC Biol.* 14:5. doi:10.1186/s12915-016-0226-9.
- Mariotti, L., C.M. Templeton, M. Ranes, P. Paracuellos, N. Cronin, F. Beuron, E. Morris, and S. Guettler. 2016. Tankyrase Requires SAM Domain-Dependent Polymerization to Support Wnt- $\beta$ -Catenin Signaling. *Mol Cell.* 63:498–513. doi:10.1016/j.molcel.2016.06.019.
- Mariotti, L., K. Pollock, and S. Guettler. 2017. Regulation of Wnt/ $\beta$ -catenin signalling by tankyrase-dependent poly(ADP-ribosyl)ation and scaffolding. *Br. J. Pharmacol.* 174:4611–4636. doi:10.1111/bph.14038.
- Molinie, N., and A. Gautreau. 2018. The Arp2/3 Regulatory System and Its Deregulation in Cancer. *Physiol Rev.* 98:215–238. doi:10.1152/physrev.00006.2017.
- Morrone, S., Morrone, S., Z. Cheng, Z. Cheng, R.T. Moon, R.T. Moon, F. Cong, F. Cong, W. Xu, and W. Xu. 2012. Crystal structure of a Tankyrase-Axin complex and its implications for Axin turnover and Tankyrase substrate recruitment. *Proc Natl Acad Sci U S A.* 109:1500–1505. doi:10.1073/pnas.1116618109.

- Popinako, A.V., M.Y. Antonov, A.S. Chemeris, K.V. Shaitan, and O.S. Sokolova. 2017. Analysis of the Interactions between Arp2/3 Complex and an Inhibitor Arpin by Molecular Dynamics Simulation. *Biophysics*. 62:885–891. doi:10.1134/S0006350917060203.
- Qiu, W., R. Lam, O. Voytyuk, V. Romanov, R. Gordon, S. Gebremeskel, J. Vodsedalek, C. Thompson, I. Beletskaya, K.P. Battaile, E.F. Pai, R. Rottapel, and N.Y. Chirgadze. 2014. Insights into the binding of PARP inhibitors to the catalytic domain of human tankyrase-2. *Acta Crystallogr. D Biol. Crystallogr.* 70:2740–2753. doi:10.1107/S1399004714017660.
- Riccio, A.A., M. McCauley, M.-F. Langelier, and J.M. Pascal. 2016. Tankyrase Sterile  $\alpha$  Motif Domain Polymerization Is Required for Its Role in Wnt Signaling. *Structure*. 24:1573–1581. doi:10.1016/j.str.2016.06.022.
- Ridley, A.J. 2011. Life at the leading edge. *Cell*. 145:1012–1022. doi:10.1016/j.cell.2011.06.010.
- Riffell, J.L., C.J. Lord, and A. Ashworth. 2012. Tankyrase-targeted therapeutics: expanding opportunities in the PARP family. *Nat Rev Drug Discov*. 11:923–936. doi:10.1038/nrd3868.
- Scheres, S.H.W. 2012. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* 180:519–530. doi:10.1016/j.jsb.2012.09.006.
- Sokolova, O.S., A. Chemeris, S. Guo, S.L. Alioto, M. Gandhi, S. Padrick, E. Pechnikova, V. David, A. Gautreau, and B.L. Goode. 2017. Structural Basis of Arp2/3 Complex Inhibition by GMF, Coronin, and Arpin. *J Mol Biol*. 429:237–248. doi:10.1016/j.jmb.2016.11.030.
- Steffen, A., M. Ladwein, G.A. Dimchev, A. Hein, L. Schwenkmezger, S. Arens, K.I. Ladwein, J. Margit Holleboom, F. Schur, J. Victor Small, J. Schwarz, R. Gerhard, J. Faix, T.E.B. Stradal, C. Brakebusch, and K. Rottner. 2013. Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. *J Cell Sci*. 126:4572–4588. doi:10.1242/jcs.118232.
- Steffen, A., S.A. Koestler, and K. Rottner. 2014. Requirements for and consequences of Rac-dependent protrusion. *Eur J Cell Biol*. 93:184–193. doi:10.1016/j.ejcb.2014.01.008.
- Tian, X., W. Hou, S. Bai, J. Fan, H. Tong, and H. Xu. 2014. XAV939 inhibits the stemness and migration of neuroblastoma cancer stem cells via repression of tankyrase 1. *Int. J. Oncol.* 45:121–128. doi:10.3892/ijo.2014.2406.
- Zhang, Y., S. Liu, C. Mickanin, Y. Feng, O. Charlat, G.A. Michaud, M. Schirle, X. Shi, M. Hild, A. Bauer, V.E. Myer, P.M. Finan, J.A. Porter, S.-M.A. Huang, and F. Cong. 2011. RNF146 is a poly(ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. *Nat Cell Biol*. 13:623–629. doi:10.1038/ncb2222.

# FIGURE LEGENDS

Figure 1

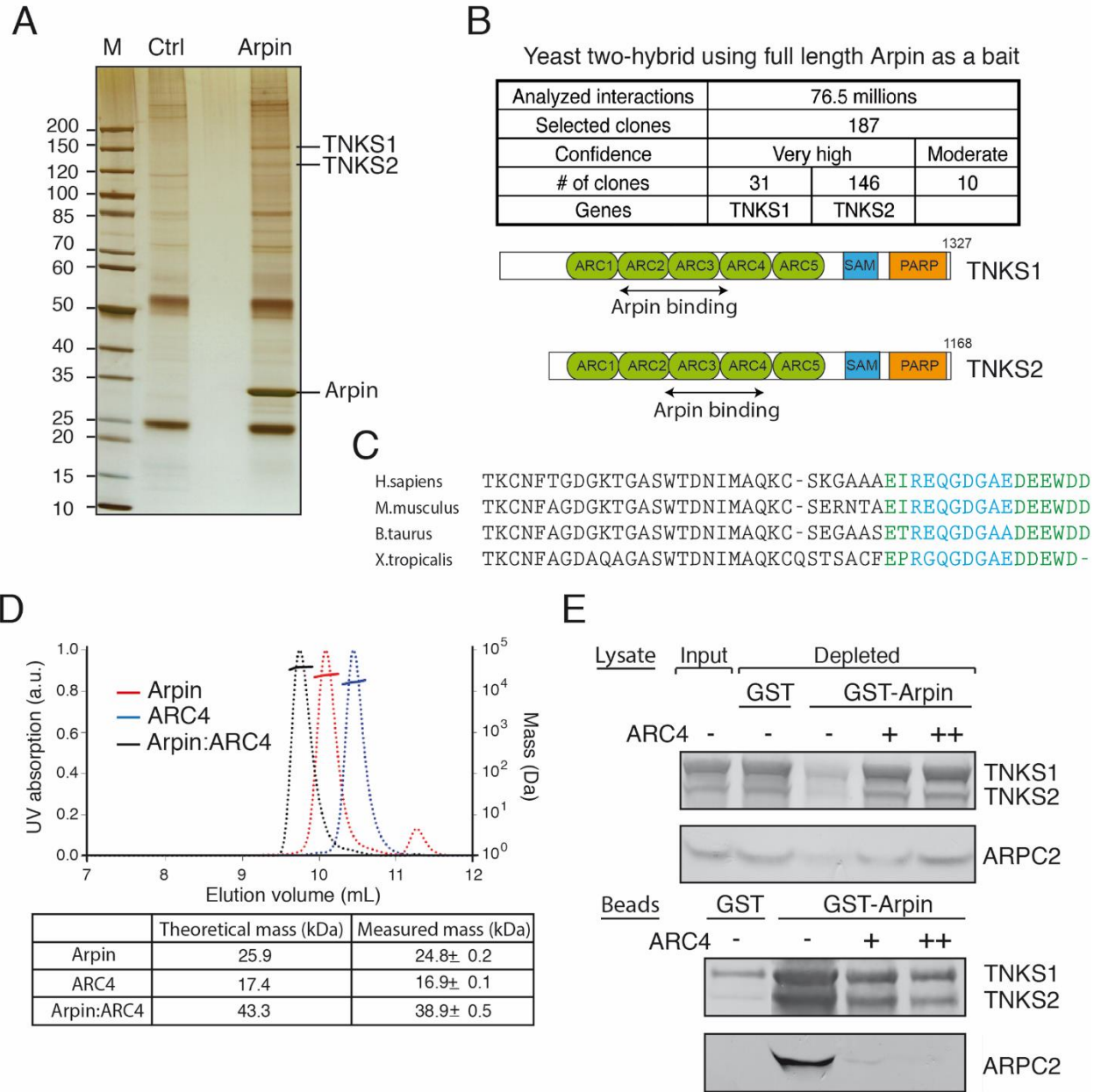


Figure 1. **Identification of Tankyrases as partners of Arpin.** (A) A 293 stable cell line expressing tagged Arpin was used to immunoprecipitate Arpin and associated proteins (silver staining). Tankyrase 1 (TNKS1) and Tankyrase 2 (TNKS2) were identified as specific Arpin partners by mass spectrometry. (B) The two Tankyrases were also the major hits of a yeast two hybrid screen. The retrieved clones of Tankyrases contained the indicated Ankyrin Repeat Clusters (ARCs), but not the Sterile Alpha Motif (SAM) nor the Poly ADP Ribose Polymerase (PARP) catalytic domain. (C) A consensus motif reported to bind tankyrases (blue) was identified within the Arp2/3 binding site (green) at the C-terminus acidic tail of Arpin. (D) Purified Arpin and purified ARC4 form a 1:1 complex as revealed by SEC-MALS. (E) MEF lysates were incubated with purified GST- or GST-Arpin immobilized on glutathione beads. Increasing concentrations of purified ARC4 was added to the lysate when indicated. Western blots indicate that Arpin beads bind to the Arp2/3 complex and the two Tankyrases. Both interactions are competed by soluble ARC4. Depleted lysates show the extent of depletion of Arp2/3 and Tankyrases by Arpin beads.

Figure 2

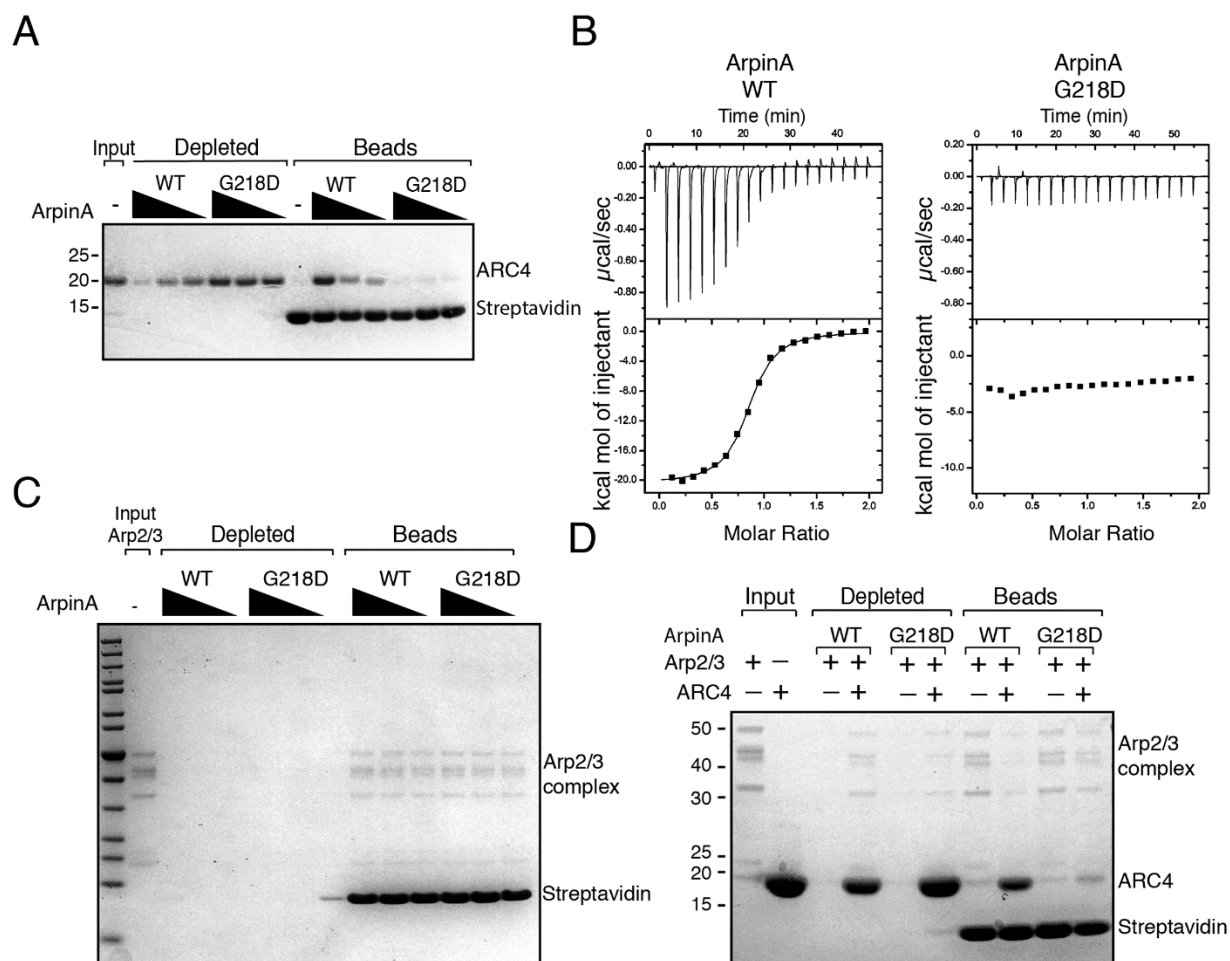
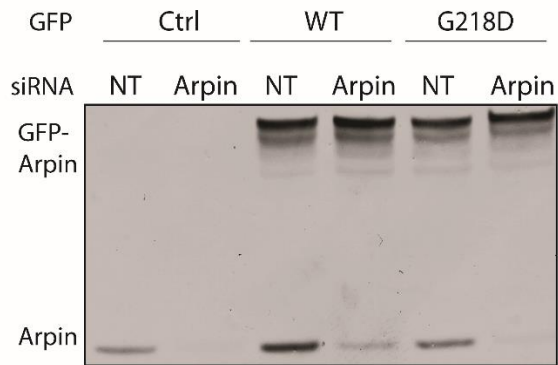


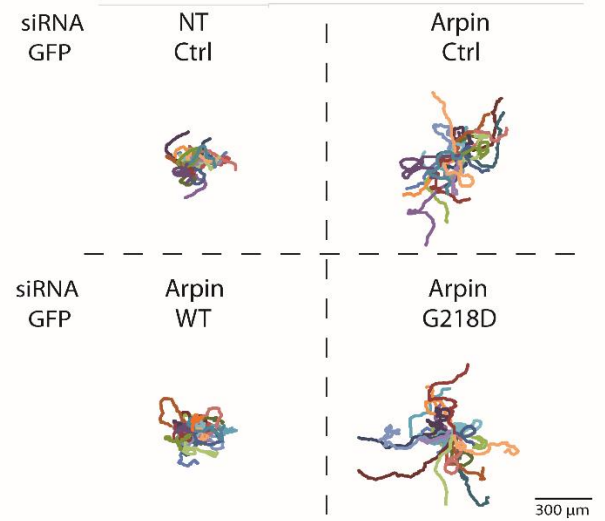
Figure 2. **In vitro the G218D mutation of Arpin abolishes binding to Tankyrases but not to the Arp2/3 complex.** (A) Biotinylated peptides corresponding to the acidic tail of Arpin (ArpinA, the last 20 amino-acids) in either its wild type (WT) form or containing the G218D mutation were incubated with streptavidin beads and purified ARC4. Input, depleted lysates and washed beads were analyzed by SDS-PAGE and Coomassie staining. (B) Isothermal Titration Calorimetry (ITC) analysis of the ARC4 interaction with Arpin peptides. The WT peptide interacts with a  $K_d$  of 0.4  $\mu$ M, whereas the G218D peptide does not interact. (C) Interaction of Arpin peptides with purified Arp2/3 was analyzed as in panel A. The G218D Arpin peptide interacts with the Arp2/3 complex as the WT. (D) Purified ARC4 competes the Arp2/3 interaction of the WT peptide, but much less so in the case of the G218D peptide.

Figure 3

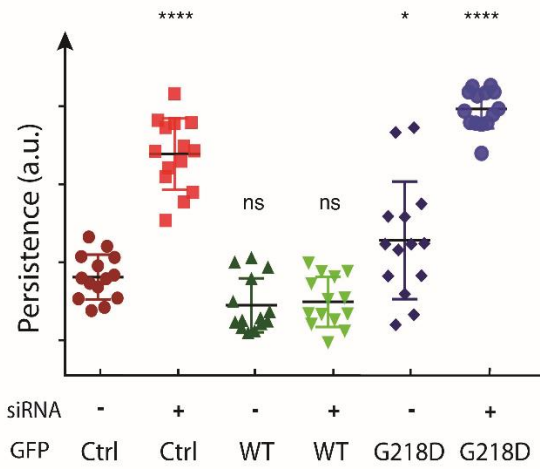
A



B



C



D

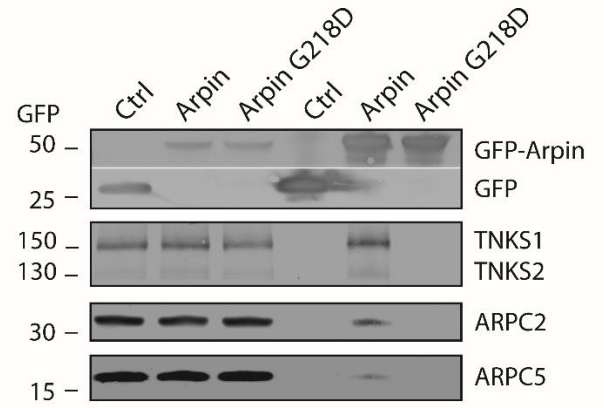
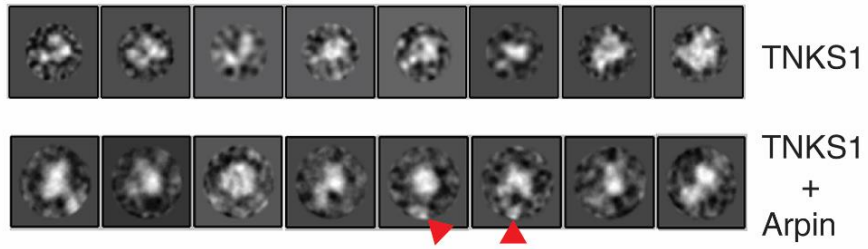


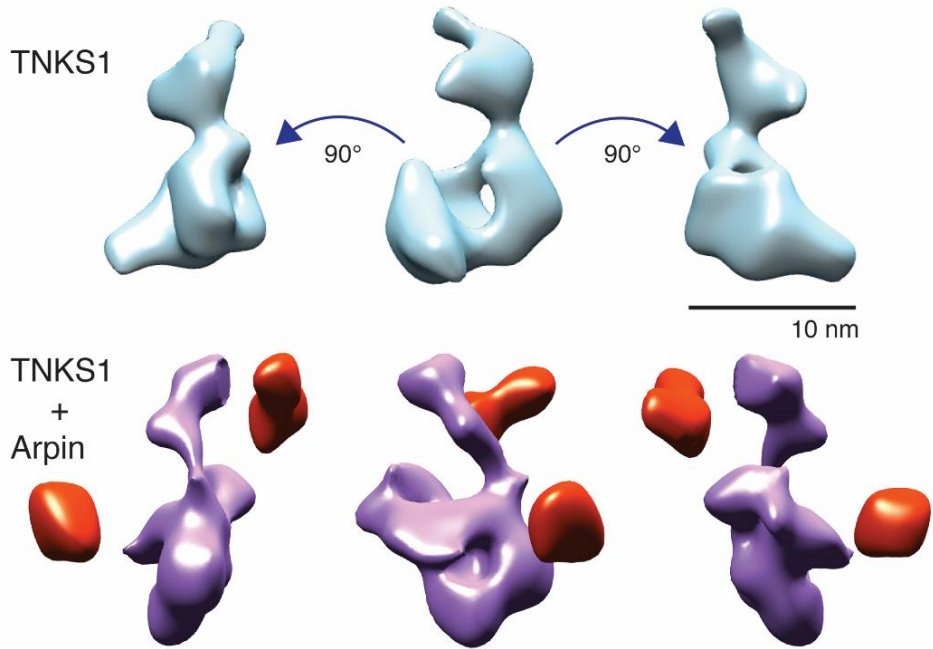
Figure 3. **Arpin binding to Tankyrases is required for its activity.** (A) Stable MCF10A cell lines expressing GFP, GFP-Arpin WT or GFP-Arpin G218D were transfected with siRNA specifically targeting endogenous Arpin or with a non-targeting siRNA (NT). Endogenous and exogenous Arpin were revealed by a Western blot with Arpin antibodies. (B) Single cell trajectories in a random migration assay show that Arpin depleted cells explore a larger territory than control cells (n = 15 cells). This effect is rescued by WT Arpin. In contrast, G218D Arpin is inactive, since it does not rescue the effect of Arpin depletion. (C) Arpin decreases migration persistence (two way ANOVA; \* P<0.05, \*\*\*\* P<0.0001, n = 15 cells). G218D Arpin is unable to rescue the depletion of endogenous Arpin. G218D Arpin is even slightly dominant negative on endogenous Arpin, since its expression increases migration persistence in presence of endogenous Arpin. (D) GFP trap beads coprecipitate Tankyrases and the Arp2/3 complex with wild type GFP-Arpin. With the G218D mutation, GFP-Arpin does not associate with the Arp2/3 complex in line with its lack of activity.

Figure 4

A



B



C

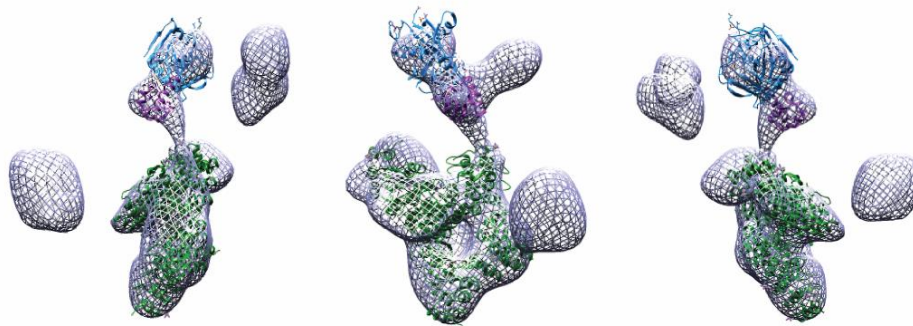
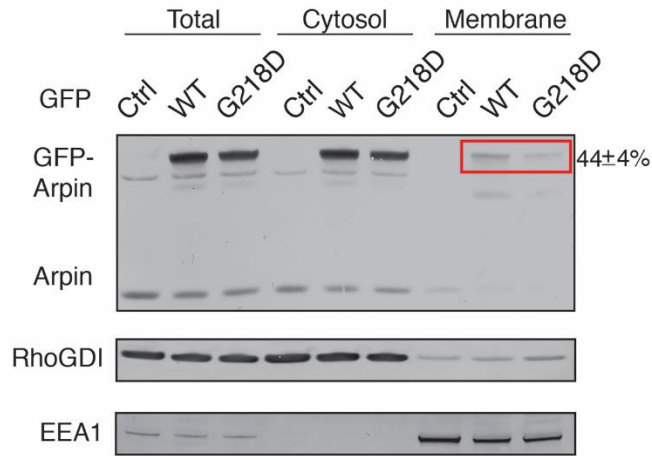




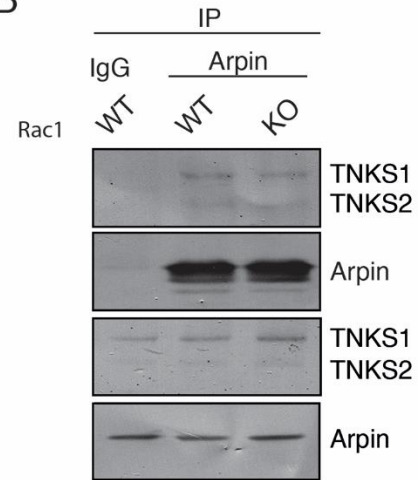
Figure 4. **Tankyrase changes conformation upon Arpin binding.** (A) TNKS1 was purified by affinity from a stable 293 cell line and visualized by negative staining in transmission electron microscopy with or without an excess of purified Arpin. Several classes of 2D projections of single particles are shown. Extra densities attributed to Arpin are indicated with a red arrowhead (B) 3D reconstructions of both forms are displayed. The extra-densities attributed to Arpin molecules are in red. (C) Docking of the crystal structures of PARP domain (blue), SAM domain (fuchsia) and the 5 Ankyrin Repeats Clusters (green) into the electron density model of TNKS1 bound to Arpin.

Figure 5

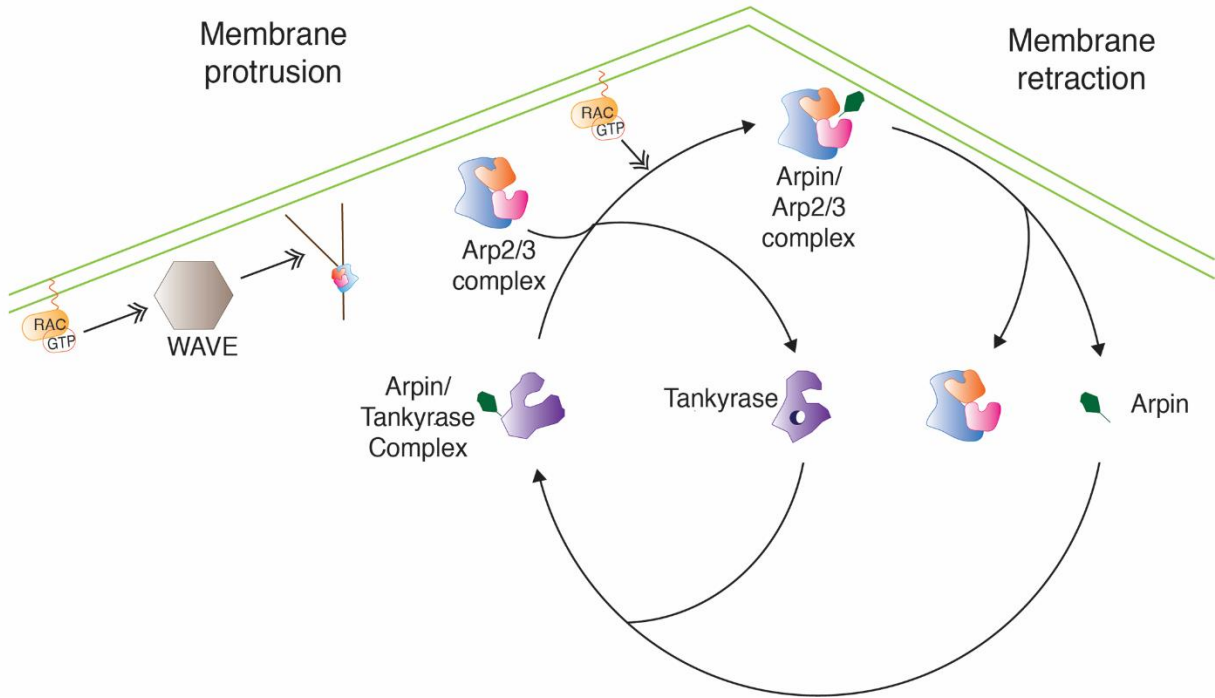
A



B



C



**Figure 5. Tankyrase binding promotes membrane association of Arpin.** **(A)** Biochemical fractionation of stable MCF10A cells expressing GFP, GFP-Arpin WT or GFP-Arpin G218D into cytosol and membrane (10 x concentrated compared to total or cytosol). Soluble RhoGDI and EEA1 were markers of cytosol and membrane fractions, respectively. Arpin G218D is less associated with membranes than WT ( $44 \pm 4\%$  based on the densitometry of 3 experiments). **(B)** Endogenous co-immunoprecipitations reveal that Arpin similarly binds to tankyrases in WT and Rac1 knock-out (KO) mouse embryonic fibroblasts. **(C)** Model of the Arpin activation cycle. The interaction of Arpin with Tankyrase is a prerequisite for Arp2/3 binding. For clarity, a single Arpin molecule is represented bound to Tankyrase. Arpin binding to the Arp2/3 complex takes place in membrane protrusions and depends on Rac1 activity (Dang et al., 2013). Arpin induces a change of Tankyrase conformation in the cytosol, which promotes its membrane translocation. Arpin then maintains the Arp2/3 complex in an inactive conformation (Sokolova et al., 2017), thereby inducing the retraction of membrane protrusions.

## **Discussion**

## **1. Tankyrase1/2 is necessary for inhibitory activity of Arpin**

Arp2/3 complex is the key machinery that drives the growth of branched actin network and thus provides the energy for membrane protrusion at the leading edge of the motile cell. Formation of membrane protrusions called lamellipodia plays a crucially important role for the process of cell migration. Arp2/3 complex requires activation by Nucleating Promoting Factors, that are the proteins from the WASP family proteins. In lamellipodial tip Arp2/3 complex is known to be activated by WAVE complex that itself should be activated by small GTPase Rac in response to extracellular signals.

Arpin is a recently found inhibitor of Arp2/3 complex that counteracts WAVE complex activity at the lamellipodial tip of the motile cells. Arpin was found to be activated by small GTPase Rac as well as WAVE complex, activator of Arp2/3 complex at the lamellipodial tip. Thus, Arpin is involved in so called «incoherent feed-forward loop» that is formed by two antagonistic pathways: activating Rac-WAVE complex-Arp2/3 complex and inactivating Rac-Arpin-Arp2/3 complex (Irene Dang et al. 2013).

In my thesis we showed that Arpin has two binding partners - Tankyrase-1 and Tankyrase-2. Tankyrases are the poly(ADP-ribose)polymerase (PARP) family proteins that are localized to the telomeres and bind telomeric DNA-binding proteins TRF1 and TRF2, while TRF2 is known to protect telomere ends, and TRF1 negatively regulates the length of telomeres (S. Smith et al. 1998). Overexpression of Tankyrases causes a telomere elongation in cancer cells due to the release of TRF1 from telomeres.

Tankyrase has two homologs: 142 kDa Tankyrase-1 (TNKS1) and 130 kDa Tankyrase-2 (TNKS2) that are ubiquitously expressed in mammalian cell lines (S. Smith et al. 1998). TNKS1 and TNKS2 share 85% amino acid identity and they are supposed to have the same functions. However, unlike Tankyrase-1, overexpression of Tankyrase-2 caused a rapid cell death.

Tankyrases interact with a wide spectrum of proteins and mediate poly(ADP-ribosyl)ation of these proteins thus regulating essential cellular processes. Tankyrases bind telomeres, centrosomes, Golgi apparatus, NuMA (nuclear mitotic apparatus protein), SH3BP2 and others

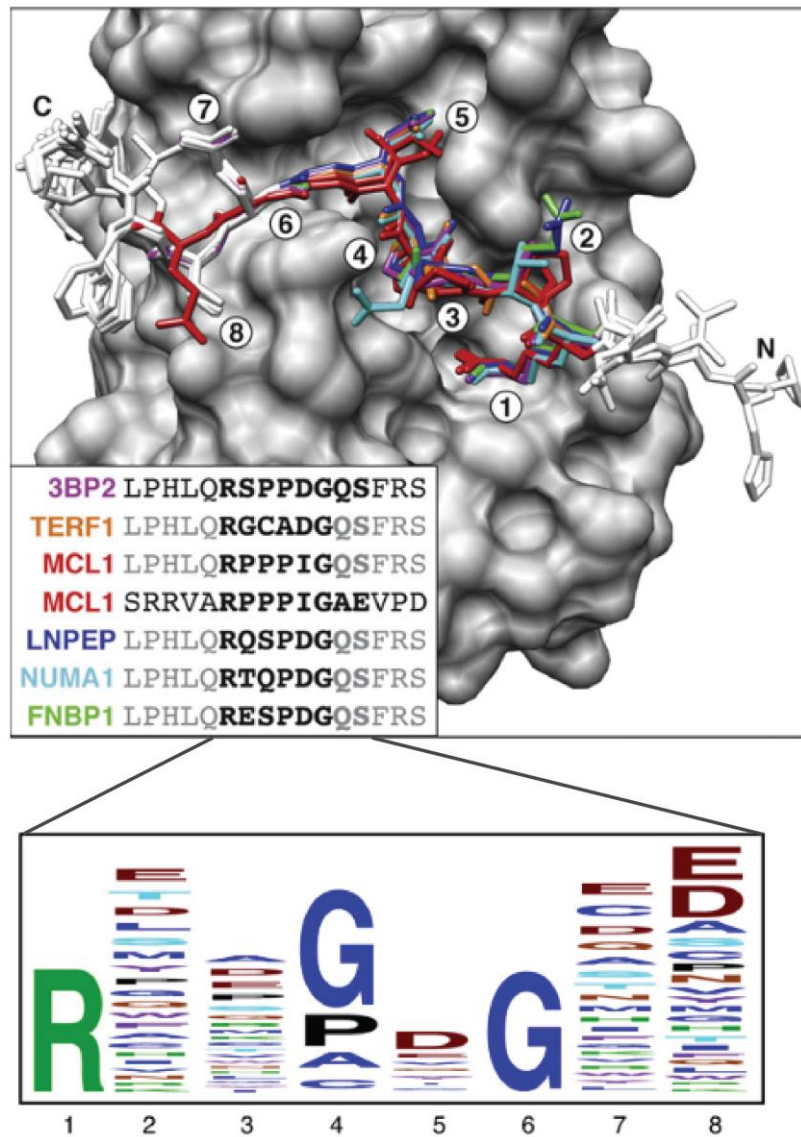
proteins. Mutation that abolishes binding of SH3BP2 to Tankyrases causes human disease cherubism. Tankyrases are also involved in the regulation of telomere length regulation, sister telomere separation, mitosis and glucose metabolism. Tankyrases were found to be the signalling targets of mitogen-activated protein kinase MAPK in Golgi (Chi and Lodish 2000).

It was recently found that Tankyrase-mediated PARylation regulates Wnt/ $\beta$ -catenin signalling pathway. Tankyrases disrupt  $\beta$ -catenin destruction complex via PARylation of AXIN1/2 (Mariotti, Pollock, and Guettler 2017). Wnt/ $\beta$ -catenin signalling pathway can be modified by inhibition of Tankyrases: inhibitor of Tankyrases XAV939 causes stabilization of axin and facilitates degradation of  $\beta$ -catenin (Huang et al. 2009).

Taking into consideration that PARPs are involved in the DNA single-strand breaks repair, inhibition of PARP catalytic activity leads to the accumulation of DNA breaks. PARP inhibitors are suggested to be applied in therapy of cancer with defects in homologous-recombination DNA repair. For example, PARP inhibitor AZD2281 (olaparib) showed an activity against BRCA1- , BRCA2-mutant tumours.

Tankyrase inhibition has also shown a therapeutic effect in lung fibrosis and neurodegenerative diseases. Breast cancer, the most common cancer type in women, includes several subtypes based on its molecular characteristics. The most aggressive subtype is a triple-negative breast cancer (TNBC) which is characterised by the lack of estrogen/progesterone/Her2 receptors. TNBC is associated with poor prognosis and barely responds to the therapies. It was shown that TNBC metastasis and poor prognosis were associated with up-regulation of Wnt/ $\beta$ -catenin signalling pathway. Recently, the combination of Tankyrase inhibitor and polo-like kinase inhibitor decreased both cell migration and invasion and increased the apoptosis of triple-negative breast cancer cells (Ha et al. 2018).

Tankyrase binding proteins share «hexapeptide» consensus motif RXXGXGXX that facilitates binding with ARC (Guettler et al. 2011) (Fig.10). We found that this motif is located in acidic C-terminus of Arpin and it overlaps Arp2/3 complex binding region. We showed that Tankyrase and Arpin compete for interaction with Arp2/3 complex *in vitro*.



**Figure 10. Tankyrase binding motif.** Modified from (Guettler et al. 2011)

Diverse TNKS-binding motifs were identified by the analysis of peptide library. The sequences of the peptides and peptide chimeras are shown.

In my thesis I did an analysis of Tankyrase binding importance for Arpin activity *in vivo* via point mutation of one of the conserved residues in «hexapeptide» (G218D). Thus we showed that Arpin mutation impairing Tankyrase-Arpin interaction abrogates interaction of Arpin with Arp2/3 complex *in vivo*.

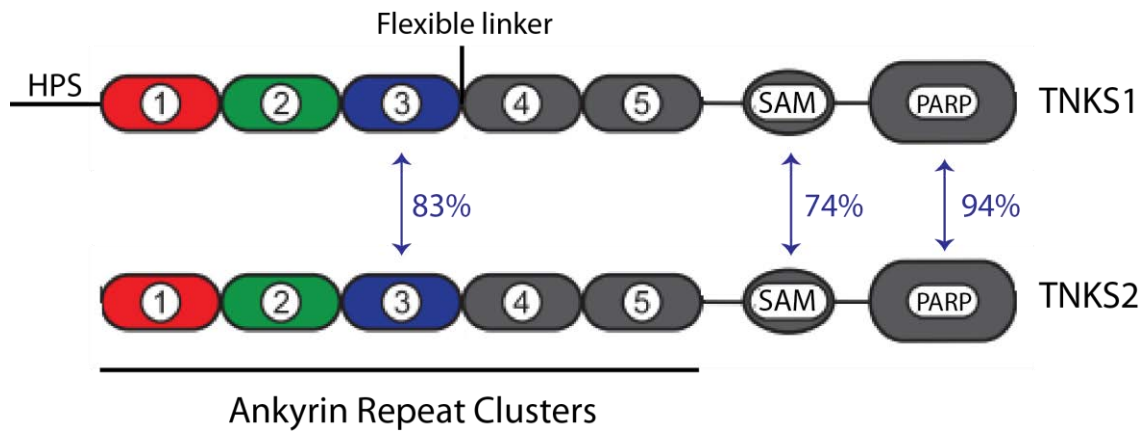
It was known before that the efficiency of cell migration depends on the cell speed and directionality of persistence. Arpin was identified as a turning factor in motile cells: microinjections of Arpin in highly directional persistent fish keratocytes forced these cells to turn (Irene Dang et al. 2013; Gorelik and Gautreau 2015). Thus, decrease of cell directional persistence was identified as a key feature of Arpin. Our analysis of directional persistence of cells expressing Arpin with lack of Tankyrase binding showed that these cells were acting similar to the cells with endogenous Arpin knockdown. Cell with overexpression of mutated Arpin had higher directional persistence comparing to the cells with overexpression of wild-type Arpin. We suggested that mutated Arpin (Arpin G218D) has no inhibitory activity *in vivo* consistent with our biochemical analysis of Arpin G218D - Arp2/3 complex missing interaction *in vivo*.

We made an assumption that if Tankyrase1/2 is mostly located in the cytoplasm and the nucleus and Tankyrase1/2 binding is necessary for Arpin activity, then Tankyrase1/2 association could be important for the delivery of Arpin to the lamellipodial tip of motile cell and the subsequent recognition of Arp2/3 complex by Arpin. We analyzed the protein amount of both Arpin WT and Arpin G218D at the membrane fraction of the moving cells. We observed that Arpin G218D was 66% in average less in the membrane fraction comparing to the amount of Arpin WT. Therefore, we suggest, that Tankyrase1/2 is important for the localization of Arpin at the lamellipodium tip where it can consequently bind and inactivate Arp2/3 complex. Thus, Tankyrase1/2 may act as a platform for Arpin delivery to the membrane.



## **2. First full-length three-dimensional structure of Tankyrase-1 and Tankyrase-2 bound to Arpin**

Tankyrase-1 consists of N-terminal histidine-, proline-, serine-rich (HPS) domain, five ankyrin repeat clusters (ARCs domain), sterile alpha-motif SAM and catalytic domain PARP. Tankyrase-2 has 74-94% homology to Tankyrase 1 with lack of HPS domain (Fig.11).



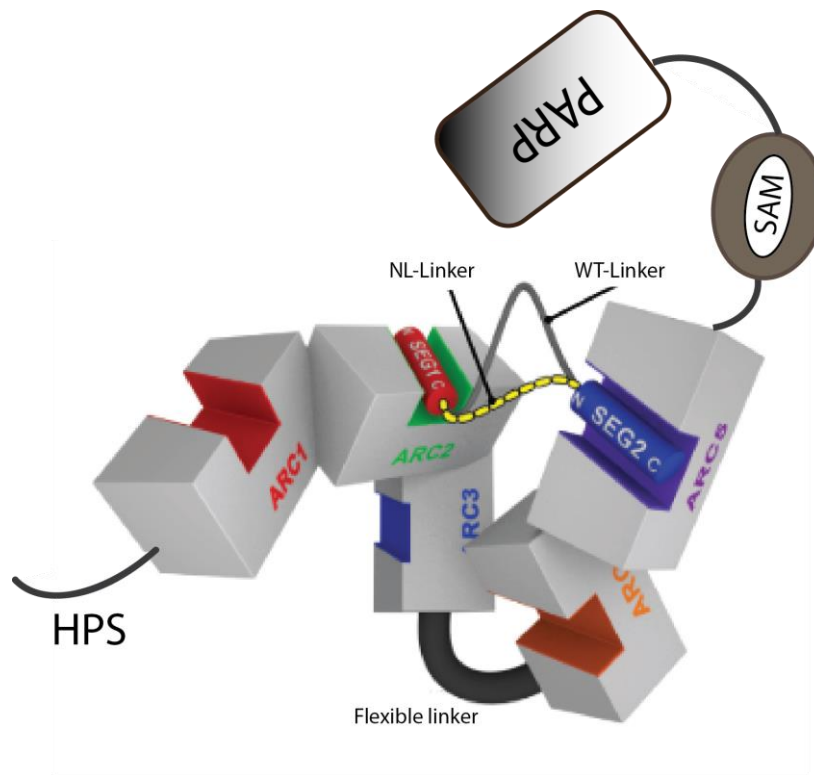
**Figure 11. Homology of TNKS1 and TNKS2.** Inspired by (Eisemann et al. 2016)

As was mentioned before, PARP domain is responsible for the PARylation of substrate proteins (PARylation) of substrate proteins. PARylation of proteins is a post-translational modification that regulate processes like DNA damage response, apoptosis and mitosis. Poly(ADP-ribose)polymerase acts as a DNA damage sensor, that covalently binds poly(ADP-ribose)-chain using  $\text{NAD}^+$  as a substrate to the proteins and thereby this chain recruits DNA repair factors. Moreover, PARylation can be involved in the process of ubiquitination and subsequent degradation of PARylated proteins. Most of the binding proteins were PARylated by Tankyrase1/2. Aberrations in PARP activity are coupled with tumorigenesis (Wei and Yu 2016).

SAM domain mediates oligomerization of Tankyrase 1 and is crucial for its catalytic activity (Riccio et al. 2016).

ARCs domain consists of 24 ankyrin (ANK) repeats that are organized into five ARCs. One ankyrin repeat consists of 33 amino acids and it is responsible for protein-protein interactions (Sedgwick and Smerdon 1999). It was found that all clusters (except ARC3) may bind proteins. ARC3 showed no activity in protein binding suggesting that ARC3 should play structural role in Tankyrase1/2. Thus Tankyrase1/2 can bind four target proteins simultaneously (Guettler et al. 2011).

It was previously shown that Tankyrase may fold into compact structure thus enables the interaction between catalytic PARP domain and proteins, bound to ARCs region. Moreover, it was shown that ARC3 and ARC4 clusters are separated by a flexible linker (Eisemann et al. 2016) (Fig.12). Due to the flexibility of Tankyrase, obtaining of its full-length crystal structure was not successful.



**Figure 12. Model of TNKS1.** Modified from (Eisemann et al. 2016)

Orientation of ARCs is represented based on the crystallization of ARC1-3, Ab initio protein modelling and bivalent binding of Axin-WT and Axin-NL (“no linker”, short version of Axin peptide) to ARC1-2 and ARC4-5.

Transmission Electron Microscopy and negative staining of biological samples are able to detect and display distinct transformational states of flexible proteins. TEM and Single Particle Analysis of images allowed us to determine the first full-length three-dimensional structure of Tankyrase-1 in a free state and structure of Tankyrase bound to Arpin. Moreover, we could explain the role of Tankyrase-1 in Arpin function.

In this work we perform for the first time the three-dimensional reconstruction of full-length Tankyrase-1 and Tankyrase-1 bound to Arpin. Reconstruction was obtained using 200 kV TEM and Single Particle Analysis. The same strategy was used by us before to characterize interaction between Arpin and Arp2/3 complex. It was shown that Arpin binds to Arp2/3 complex via its 50 nm acidic C-terminal linear tail and this tail was not visible at 3D reconstruction (Sokolova et al. 2017).

Using SEC-MALS analysis we showed that the stoichiometry of ARC4:Arpin interaction is 1:1 and consistent with the previous data we expected maximum four Arpins bound to Tankyrase. Our EM 3D reconstruction of Tankyrase-1 with Arpin showed 3 additional densities at the distance of 45-50 Å from the Tankyrase-1. Two additional densities had the diameter of 25 Å that was consistent with our reconstruction of Arpin bound to the Arp2/3 complex (Sokolova et al. 2017). The third density was bigger than the others and was about the diameter of 30 Å. We suggest that this distinct density could be the 2 overlapping Arpins since we expected that ARCs domain of Tankyrase1/2 is able to bind four Arpins. However, we cannot exclude the possibility that the biggest additional density was the result of high flexibility of Arpin bound to Tankyrase-1. Moreover, it was recently found that ankyrin repeat clusters may cooperate pairwise to enhance the affinity to target proteins (Eisemann et al. 2016). It is like enough that due to this cooperation of ARCs there are three Tankyrase-bound Arpins.

Moreover, comparing reconstructions of free Tankyrase-1 and Tankyrase-1 bound to Arpins we observed dramatical conformational changes in the part of Tankyrase reconstruction that corresponds to the ARCs domain according to the computational docking of known PDB crystal structures into the derived reconstructions.

### **3. Model of Arpin-Tankyrase interaction**

During my thesis we showed that Tankyrase1/2 and Arp2/3 complex compete for Arpin binding and these proteins are not able to bind Arpin at the same time. We also observed that mutated ArpinG218D was not able to bind both Arp2/3 and Tankyrase1/2 *in vivo*, although G218D mutation impaired Tankyrase1/2 binding *in vitro* only. Moreover, we showed that Arpin activity did not require activation by small GTPase Rac1. Based on this data we proposed a model of Arpin-Tankyrase interaction at the lamellipodium tip. According to our model Tankyrase1/2 binding is required for the further Rac1-dependent Arpin-Arp2/3 complex interaction.

We suggest that Tankyrase1/2 attaches several Arpins in the cytoplasm and undergoes significant conformational changes. Modification of Tankyrase1 conformational state is possibly responsible for the interaction of Arpin with Arp2/3 complex and plasma membrane.

Consequently, we suggest that Tankyrase delivers Arpin towards the lamellipodial tip where Tankyrase1/2 facilitates association of Arpin with Arp2/3 complex and its consequent inactivation due to the small GTPase Rac signalling. How Arpin attaches the plasma membrane and the role of Tankyrase1/2 in this association are open questions that should be answered in the future studies.

## References

- Anand-apte, Bela et al. 1997. "Platelet-Derived Growth Factor and Fibronectin-Stimulated Migration Are Differentially Regulated by the Rac and Extracellular Signal-Regulated Kinase Pathways \*." *Journal of Cell Biology* 272(49): 30688–92.
- Bear, James E. et al. 2002. "Antagonism between Ena/VASP Proteins and Actin Filament Capping Regulates Fibroblast Motility." *Cell* 109(4): 509–21.
- Bhatt, Amit, Irina Kaverina, Carol Otey, and Anna Huttenlocher. 2002. "Regulation of Focal Complex Composition and Disassembly by the Calcium-Dependent Protease Calpain." *Journal of cell science* 115(Pt 17): 3415–25. <http://www.ncbi.nlm.nih.gov/pubmed/12154072>.
- Bieling, Peter et al. 2017. "WH2 and Proline-rich Domains of WASP-family Proteins Collaborate to Accelerate Actin Filament Elongation." *The EMBO Journal* 37(1): e201797039. <http://emboj.embopress.org/lookup/doi/10.15252/emboj.201797039>.
- Blanchoin, Laurent, and Thomas D Pollard. 1999. "Mechanism of Interaction of Acanthamoeba Actophorin ( ADF / Cofilin ) with Actin Filaments \*." *The Journal of Biological Chemistry* 274(22): 15538–46.
- Boczkowska, Malgorzata, Grzegorz Rebowski, and Roberto Dominguez. 2013. "Glia Maturation Factor (GMF) Interacts with Arp2/3 Complex in a Nucleotide State-Dependent Manner." *Journal of Biological Chemistry* 288(36): 25683–88.
- Buck, C A, and A F Horwitz. 1987. "Cell Surface Receptors for Extracellular Matrix Molecules." *Annu.Rev.Cell Biol.* 3: 179–205.
- Burianek, Lauren E., and Scott H. Soderling. 2013. "Under Lock and Key: Spatiotemporal Regulation of WASP Family Proteins Coordinates Separate Dynamic Cellular Processes." *Seminars in Cell and Developmental Biology* 24(4): 258–66. <http://dx.doi.org/10.1016/j.semcd.2012.12.005>.
- Burridge, Keith, and Krister Wennerberg. 2004. "Rho and Rac Take Center Stage." *Cell* 116(2): 167–79.
- Bustelo, Xosé R. 2018. "RHO GTPases in Cancer: Known Facts, Open Questions, and Therapeutic Challenges." : 1–20. <http://www.biochemsoctrans.org/content/ppbiost/46/3/741.full.pdf>.
- Calderwood, David A. et al. 1999. "The Talin Head Domain Binds to Integrin  $\beta$  Subunit Cytoplasmic Tails and Regulates Integrin Activation." *Journal of Biological Chemistry* 274(40): 28071–74.
- Campellone, Kenneth G, Neil J Webb, Elizabeth A Znameroski, and Matthew D Welch. 2009. "WHAMM Is an Arp2/3 Complex Activator That Binds Microtubules and Functions in ER to Golgi Transport." *Cell* 134(1): 148–61.
- Carragher, Neil O., Bodo Levkau, Russell Ross, and Elaine W. Raines. 1999. "Degraded Collagen



- Fragments Promote Rapid Disassembly of Smooth Muscle Focal Adhesions That Correlates with Cleavage of Pp125(FAK), Paxillin, and Talin." *Journal of Cell Biology* 147(3): 619–29.
- Charras, Guillaume, and Ewa Paluch. 2008. "Blebs Lead the Way: How to Migrate without Lamellipodia." *Nature Reviews Molecular Cell Biology* 9(9): 730–36.
- Chen, W.-T. 1981. "Mechanism of the Trailing Edge during Fibroblast Movement." *J. Cell Biol.* 90: 187–200.
- Chen, Zhucheng et al. 2010. "Structure and Control of the Actin Regulatory WAVE Complex." *Nature* 468(7323): 533–38. <http://dx.doi.org/10.1038/nature09623>.
- Chesarone, Melissa A, and Bruce L Goode. 2010. "NIH Public Access." *Cell* 21(1): 28–37.
- Chi, Nai Wen, and Harvey F. Lodish. 2000. "Tankyrase Is a Golgi-Associated Mitogen-Activated Protein Kinase Substrate That Interacts with IRAP in GLUT4 Vesicles." *Journal of Biological Chemistry* 275(49): 38437–44.
- Clainche, Christophe L E, and Marie-france Carlier. 2008. "Regulation of Actin Assembly Associated With Protrusion and Adhesion in Cell Migration." *the American Physiological society*: 489–513.
- Condeelis, John. 1993. "Life at the Leading Edge: The Formation of Cell Protrusions." *Annual Review of Cell Biology* 9(1): 411–44.  
<http://www.annualreviews.org/doi/10.1146/annurev.cb.09.110193.002211>.
- Cooper, John A., and David Sept. 2008. "New Insights into Mechanism and Regulation of Actin Capping Protein." *Int Rev Cell Mol Biol* 267(08): 183–206.  
[http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=18544499](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18544499).
- Dang, Irene et al. 2013. "Inhibitory Signalling to the Arp2/3 Complex Steers Cell Migration." *Nature* 503(7475): 281–84.
- Dang, Irène et al. 2017. "The Arp2/3 Inhibitory Protein Arpin Is Dispensable for Chemotaxis." *Biology of the Cell* 109(4): 162–66.
- Dayel, Mark J., and R. Dyche Mullins. 2004. "Activation of Arp2/3 Complex: Addition of the First Subunit of the New Filament by a WASP Protein Triggers Rapid ATP Hydrolysis on Arp2." *PLoS Biology* 2(4): 476–85.
- Derivery, Emmanuel et al. 2008. "Free Brick1 Is a Trimeric Precursor in the Assembly of a Functional Wave Complex." *PLoS ONE* 3(6).
- Derivery, Emmanuel, and Alexis Gautreau. 2010a. "Evolutionary Conservation of the WASH Complex, an Actin Polymerization Machine Involved in Endosomal Fission." *Communicative and Integrative*

- Biology* 3(3): 227–30.
- . 2010b. "Generation of Branched Actin Networks : Assembly and Regulation of the N-WASP and WAVE Molecular Machines." *BioEssays* (2): 119–31.
- Eisemann, Travis et al. 2016. "Tankyrase-1 Ankyrin Repeats Form an Adaptable Binding Platform for Targets of ADP-Ribose Modification." *Structure* 24(10): 1679–92.
- Etienne-Manneville, S. 2004. "Cdc42 - the Centre of Polarity." *Journal of Cell Science* 117(8): 1291–1300. <http://jcs.biologists.org/cgi/doi/10.1242/jcs.01115>.
- Fetics, Susan et al. 2016. "Hybrid Structural Analysis of the Arp2/3 Regulator Arpin Identifies Its Acidic Tail as a Primary Binding Epitope." *Structure* 24(2): 252–60.
- Franco, S. J. 2005. "Regulating Cell Migration: Calpains Make the Cut." *Journal of Cell Science* 118(17): 3829–38. <http://jcs.biologists.org/cgi/doi/10.1242/jcs.02562>.
- Friedl, Peter, and Darren Gilmour. 2004. "Collective Cell Migration in and Cancer." *Nature Publishing Group* 10(7): 445–57. <http://dx.doi.org/10.1038/nrm2720>.
- Friedl, Peter, and Katarina Wolf. 2003. "Tumour-Cell Invasion and Migration: Diversity and Escape Mechanisms." *Nature Reviews Cancer* 3(5): 362–74.
- Galbraith, Catherine G., Kenneth M. Yamada, and Michael P. Sheetz. 2002. "The Relationship between Force and Focal Complex Development." *Journal of Cell Biology* 159(4): 695–705.
- Goley, E. D. et al. 2010. "An Actin-Filament-Binding Interface on the Arp2/3 Complex Is Critical for Nucleation and Branch Stability." *Proceedings of the National Academy of Sciences* 107(18): 8159–64. <http://www.pnas.org/cgi/doi/10.1073/pnas.0911668107>.
- Goley, Erin D, and Matthew D Welch. 2006. "The ARP2/3 Complex: An Actin Nucleator Comes of Age." *Nature Reviews Molecular Cell Biology* 7: 713–26.
- Gorelik, Roman, and Alexis Gautreau. 2015. "The Arp2/3 Inhibitory Protein Arpin Induces Cell Turning by Pausing Cell Migration." *Cytoskeleton* 72(7): 362–71.
- Goroncy, Alexander K. et al. 2009. "NMR Solution Structures of Actin Depolymerizing Factor Homology Domains." *Protein Science* 18(11): 2384–92.
- Guettler, Sebastian et al. 2011. "Structural Basis and Sequence Rules for Substrate Recognition by Tankyrase Explain the Basis for Cherubism Disease." *Cell* 147(6): 1340–54.
- Ha, Geun-Hyoung, Dong-Young Kim, Eun-Kyoung Breuer, and Chung Kwon Kim. 2018. "Combination Treatment of Polo-Like Kinase 1 and Tankyrase-1 Inhibitors Enhances Anticancer Effect in Triple-Negative Breast Cancer Cells." *Anticancer Research* 38(3): 1303–10.
- Hadler-Olsen, Elin et al. 2011. "Regulation of Matrix Metalloproteinase Activity in Health and Disease."

- FEBS Journal* 278(1): 28–45.
- Hansen, Scott D., and R. Dyche Mullins. 2010. "VASP Is a Processive Actin Polymerase That Requires Monomeric Actin for Barbed End Association." *Journal of Cell Biology* 191(3): 571–84.
- Haug, Jason M., Franca Codazzi, Mary Teruel, and Tobias Meyer. 2000. "Spatial Sensing in Fibroblasts Mediated by 3' Phosphoinositides." *Journal of Cell Biology* 151(6): 1269–79.
- Hetrick, Byron, Min Suk Han, Luke A Helgeson, and Brad J Nolen. 2014. "NIH Public Access." 20(5): 701–12.
- Hotulainen, Pirta, and Pekka Lappalainen. 2006. "Stress Fibers Are Generated by Two Distinct Actin Assembly Mechanisms in Motile Cells." *Journal of Cell Biology* 173(3): 383–94.
- Huang, Shih-min A et al. 2009. "Tankyrase Inhibition Stabilizes Axin and Antagonizes Wnt Signalling." *Nature* 461(7264): 614–20. <http://dx.doi.org/10.1038/nature08356>.
- Insall, Robert H, Orion D Weiner, Longwood Ave, and Cell Biology Kirschner C-. 2001. "Similar Messages , Different Meanings?" *Oncogene* 1: 743–47.
- Jean, S., and A. A. Kiger. 2014. "Classes of Phosphoinositide 3-Kinases at a Glance." *Journal of Cell Science* 127(5): 923–28. <http://jcs.biologists.org/cgi/doi/10.1242/jcs.093773>.
- Jégou, Antoine et al. 2011. "Individual Actin Filaments in a Microfluidic Flow Reveal the Mechanism of ATP Hydrolysis and Give Insight into the Properties of Profilin." *PLoS Biology* 9(9).
- Jia, Da et al. 2010. "WASH and WAVE Actin Regulators of the Wiskott – Aldrich Syndrome Protein ( WASP ) Family Are Controlled by Analogous Structurally Related Complexes." *PNAS* 107(23): 10442–47.
- Kaverina, Irina, Olga Krylyshkina, and J. Victor Small. 2002. "Regulation of Substrate Adhesion Dynamics during Cell Motility." *International Journal of Biochemistry and Cell Biology* 34(7): 746–61.
- Kelly, Alexander E., Heather Kranitz, Volker Dötsch, and R. Dyche Mullins. 2006. "Actin Binding to the Central Domain of WASP/Scar Proteins Plays a Critical Role in the Activation of the Arp2/3 Complex." *Journal of Biological Chemistry* 281(15): 10589–97.
- Kim, Annette S et al. 2000. "Autoinhibition and Activation Mechanisms of the Wiskott ± Aldrich Syndrome Protein." 3: 151–58.
- Korn, E D, M F Carlier, and D Pantaloni. 1987. "Actin Polymerization and ATP Hydrolysis." *Science (New York, N.Y.)* 238(4827): 638–44.
- Krause, Matthias, and Alexis Gautreau. 2014. "Steering Cell Migration: Lamellipodium Dynamics and the Regulation of Directional Persistence." *Nature Reviews Molecular Cell Biology* 15(9): 577–90.

<http://dx.doi.org/10.1038/nrm3861>.

- Lan, Tian et al. 2016. "Displacement Correlations between a Single Mesenchymal-like Cell and Its Nucleus Effectively Link Subcellular Activities and Motility in Cell Migration Analysis." *Scientific Reports* 6(March): 1–12. <http://dx.doi.org/10.1038/srep34047>.
- Lauffenburger, Douglas A., and Alan F. Horwitz. 1996. "Cell Migration: A Physically Integrated Molecular Process." *Cell* 84(3): 359–69.
- Lebensohn, Andres M., and Marc W. Kirschner. 2009. "Activation of the WAVE Complex by Coincident Signals Controls Actin Assembly." *Molecular Cell* 36(3): 512–24. <http://dx.doi.org/10.1016/j.molcel.2009.10.024>.
- Linder, Stefan, Christiane Wiesner, and Mirko Himmel. 2011. "Degrading Devices: Invadosomes in Proteolytic Cell Invasion." *Annual Review of Cell and Developmental Biology* 27(1): 185–211. <http://www.annualreviews.org/doi/10.1146/annurev-cellbio-092910-154216>.
- Lomakina, Maria E. et al. 2016. "Arpin Downregulation in Breast Cancer Is Associated with Poor Prognosis." *British Journal of Cancer* 114(5): 545–53.
- Luan, Qing et al. 2018. "Identification of Wiskott-Aldrich Syndrome Protein (WASP) Binding Sites on the Branched Actin Filament Nucleator Arp2/3 Complex." *Proceedings of the National Academy of Sciences*: 201716622. <http://www.pnas.org/lookup/doi/10.1073/pnas.1716622115>.
- Machesky, Laura M. et al. 1994. "Purification of a Cortical Complex Containing Two Unconventional Actins from *Acanthamoeba* by Affinity Chromatography on Profilin-Agarose." *Journal of Cell Biology* 127(1): 107–15.
- Mariotti, Laura, Katie Pollock, and Sebastian Guettler. 2017. "Regulation of Wnt/ $\beta$ -Catenin Signalling by Tankyrase-Dependent Poly(ADP-Ribosyl)ation and Scaffolding." *British Journal of Pharmacology* 174(24): 4611–36.
- Maritzen, T. et al. 2012. "Gadkin Negatively Regulates Cell Spreading and Motility via Sequestration of the Actin-Nucleating ARP2/3 Complex." *Proceedings of the National Academy of Sciences* 109(26): 10382–87. <http://www.pnas.org/cgi/doi/10.1073/pnas.1206468109>.
- Molinie, Nicolas, and X Alexis Gautreau. 2018. "The Arp2/3 Regulatory System and Its Deregulation in Cancer." *American Physiological Society*: 215–38.
- Murphy, Da, and Sa Courtneidge. 2011. "The 'ins' and 'outs' of Podosomes and Invadopodia: Characteristics, Formation and Function." *Nature reviews Molecular cell biology* 12(7): 413–26. <http://www.nature.com/nrm/journal/v12/n7/abs/nrm3141.html>.
- Nobes, C D, P Hawkins, L Stephens, and a Hall. 1995. "Activation of the Small GTP-Binding Proteins Rho

- and Rac by Growth Factor Receptors." *Journal of cell science* 108 ( Pt 1: 225–33.
- Nürnberg, Alexander, Thomas Kitzing, and Robert Grosse. 2011. "Nucleating Actin for Invasion." *Nature Reviews Cancer* 11(3): 117–87. <http://dx.doi.org/10.1038/nrc3003>.
- Oikawa, Tsukasa et al. 2004. "PtdIns(3,4,5)P3 Binding Is Necessary for WAVE2-Induced Formation of Lamellipodia." *Nature Cell Biology* 6(5): 420–26.
- Okreglak, Voytek, and David G. Drubin. 2010. "Loss of Aip1 Reveals a Role in Maintaining the Actin Monomer Pool and an in Vivo Oligomer Assembly Pathway." *Journal of Cell Biology* 188(6): 769–77.
- Padrick, S. B. et al. 2011. "Arp2/3 Complex Is Bound and Activated by Two WASP Proteins." *Proceedings of the National Academy of Sciences* 108(33): E472–79. <http://www.pnas.org/cgi/doi/10.1073/pnas.1100236108>.
- Pantaloni, Dominique. 1993. "How Profilin Promotes Actin Filament Assembly in the Presence of Thymosin P4." 75: 1007–14.
- Parent, Carole a, and Peter N Devreotes. 1999. "A Cell ' s Sense of Direction." *Science* 284(April): 765–70.
- Perrin, Benjamin J., and James M. Ervasti. 2010. "The Actin Gene Family: Function Follows Isoform." *Cytoskeleton* 67(10): 630–34.
- Pfaendtner, Jim, Edward Lyman, Thomas D Pollard, and Gregory a Voth. 2011. "NIH Public Access." 396(2).
- Pocha, Shirin M., and Giles O. Cory. 2009. "WAVE2 Is Regulated by Multiple Phosphorylation Events within Its VCA Domain." *Cell Motility and the Cytoskeleton* 66(1): 36–47.
- Pollard, Jeffrey W. 2009. "NIH Public Access." *Nature reviews. Immunology* 9(4): 259–70.
- Pollard, Thomas D. 2007. "Regulation of Actin Filament Assembly by Arp2/3 Complex and Formins." *Annual Review of Biophysics and Biomolecular Structure* 36(1): 451–77. <http://www.annualreviews.org/doi/10.1146/annurev.biophys.35.040405.101936>.
- Pollard, Thomas D., and Gary G. Borisy. 2003. "Cellular Motility Driven by Assembly and Disassembly of Actin Filaments." *Cell* 112(4): 453–65.
- Pollard, Thomas D. 2016. "Actin and Actin-Binding Proteins." : 1–18.
- Ren, X. D. et al. 1996. "Physical Association of the Small GTPase Rho with a 68-KDa Phosphatidylinositol 4-Phosphate 5-Kinase in Swiss 3T3 Cells." *Molecular Biology of the Cell* 7(3): 435–42. <http://www.molbiolcell.org/cgi/doi/10.1091/mbc.7.3.435>.
- Riccio, Amanda A., Michael McCauley, Marie-France Langelier, and John M. Pascal. 2016. "Tankyrase SAM Domain Polymerization Is Required for Its Role in Wnt Signaling." 24(9): 1573–81.

- Ridley, Anne J. et al. 1992. "The Small GTP-Binding Protein Rac Regulates Growth Factor-Induced Membrane Ruffling." *Cell* 70(3): 401–10.
- Rocca, Daniel L., Stéphane Martin, Emma L. Jenkins, and Jonathan G. Hanley. 2008. "Inhibition of Arp2/3-Mediated Actin Polymerization by PICK1 Regulates Neuronal Morphology and AMPA Receptor Endocytosis." *Nature Cell Biology* 10(3): 259–71.
- Rodal, Avital A. et al. 2005. "Conformational Changes in the Arp2/3 Complex Leading to Actin Nucleation." *Nature Structural and Molecular Biology* 12(1): 26–31.
- Rouiller, Isabelle et al. 2008. "The Structural Basis of Actin Filament Branching by the Arp2/3 Complex." *Journal of Cell Biology* 180(5): 887–95.
- Sander, Eva E. et al. 1999. "Rac Downregulates Rho Activity: Reciprocal Balance between Both GTPases Determines Cellular Morphology and Migratory Behavior." *Journal of Cell Biology* 147(5): 1009–21.
- Sanz-Moreno, Victoria et al. 2008. "Rac Activation and Inactivation Control Plasticity of Tumor Cell Movement." *Cell* 135: 510–23.
- Sedgwick, S. G., and S. J. Smerdon. 1999. "The Ankyrin Repeat: A Diversity of Interactions on a Common Structural Framework." *Trends in Biochemical Sciences* 24(8): 311–16.
- Silva, Jose M. et al. 2009. "NIH Public Access." *Cell* 137(6): 1047–61.
- Smith, Benjamin A, Karen Daugherty-clarke, Bruce L Goode, and Jeff Gelles. 2013. "Pathway of Actin Filament Branch Formation by Arp2 / 3 Complex Revealed by Single-Molecule Imaging." *PNAS* 110(4): 1285–90.
- Smith, Susan, Izabela Gariat, Anja Schmitt, and Titia de Lange. 1998. "Tankyrase, a Poly (ADP-Ribose) Polymerase at Human Telomeres." *Science* 282(5393): 1484–87.
- Sokolova, O.S. et al. 2017. "Structural Basis of Arp2/3 Complex Inhibition by GMF, Coronin, and Arpin." *Journal of Molecular Biology* 429(2).
- Suetsugu, Shiro et al. 2002. "Sustained Activation of N-WASP through Phosphorylation Is Essential for Neurite Extension." *Developmental Cell* 3(5): 645–58.
- . 2006. "Optimization of WAVE2 Complex-Induced Actin Polymerization by Membrane-Bound IRSp53, PIP3, and Rac." *Journal of Cell Biology* 173(4): 571–85.
- Svitkina, Tatyana M., and Gary G. Borisy. 1999. "Arp2/3 Complex and Actin Depolymerizing Factor/Cofilin in Dendritic Organization and Treadmilling of Actin Filament Array in Lamellipodia." *Journal of Cell Biology* 145(5): 1009–26.
- Svitkina, Tatyana M et al. 2002. "Mechanism of Filopodia Initiation by Reorganization of a Dendritic Network." *Journal of Cell Biology*: 409–21.

- Takenawa, Tadaomi, and Shiro Suetsugu. 2007. "The WASP-WAVE Protein Network: Connecting the Membrane to the Cytoskeleton." *Nature Reviews Molecular Cell Biology* 8(1): 37–48.
- Tanaka, Masahito et al. 2017. "Turnover and Flow of the Cell Membrane for Cell Migration." *Scientific Reports* 7(1): 1–13. <http://dx.doi.org/10.1038/s41598-017-13438-5>.
- Tang, Haoran et al. 2013. "Loss of Scar/WAVE Complex Promotes N-WASP- and FAK-Dependent Invasion." *Current Biology* 23(2): 107–17. <http://dx.doi.org/10.1016/j.cub.2012.11.059>.
- Tojkander, Sari, Gergana Gateva, and Pekka Lappalainen. 2012. "Actin Stress Fibers - Assembly, Dynamics and Biological Roles." *Journal of cell science* 125(Pt 8): 1855–64. <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22544950&retmode=ref&cmd=prlinks%5Cnpapers3://publication/doi/10.1242/jcs.098087>.
- Visweshwaran, Sai P et al. 2018. "The Trimeric Coiled-coil HSBP1 Protein Promotes WASH Complex Assembly at Centrosomes." *The EMBO Journal*: e97706. <http://www.ncbi.nlm.nih.gov/pubmed/29844016%0Ahttp://emboj.embopress.org/lookup/doi/10.15252/emj.201797706>.
- Wagner, Johanna et al. 2014. "It's How You Get There: Walking down a Virtual Alley Activates Premotor and Parietal Areas." *Frontiers in Human Neuroscience* 8(February): 1–11. <http://journal.frontiersin.org/article/10.3389/fnhum.2014.00093/abstract>.
- Wang, Fei. 2009. "The Signaling Mechanisms Underlying Cell Polarity and Chemotaxis." *Cold Spring Harbor perspectives in biology* 1(4): 1–16.
- Weaver, Alissa M. et al. 2001. "Cortactin Promotes and Stabilizes Arp2/3-Induced Actin Filament Network Formation." *Current Biology* 11(5): 370–74.
- Wei, Huiting, and Xiaochun Yu. 2016. "Functions of PARylation in DNA Damage Repair Pathways." *Genomics, Proteomics and Bioinformatics* 14(3): 131–39.
- Winder, S. J. 2005. "Actin-Binding Proteins." *Journal of Cell Science* 118(4): 651–54. <http://jcs.biologists.org/cgi/doi/10.1242/jcs.01670>.
- Wu, Shuang et al. 2012. "Nudel Is Crucial for the WAVE Complex Assembly in Vivo by Selectively Promoting Subcomplex Stability and Formation through Direct Interactions." *Cell Research* 22(8): 1270–84.
- Yang, Xuejun. 2013. "Expression of the Arp2/3 Complex in Human Gliomas and Its Role in the Migration and Invasion of Glioma Cells." *Oncology Reports* (9): 2127–36. <http://www.spandidos-publications.com/10.3892/or.2013.2669>.
- Zuchero, J Bradley et al. 2009. "P53-Cofactor JMY Is a Multifunctional Actin Nucleation Factor." 11(4):

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**Titre :** Régulation du suppresseur d'invasion Arpin par les Tankyrases

**Mots clés :** Arp2/3, complexe multiprotéique, motilité

**Résumé :** Le complexe Arp2/3, conservé sur le plan évolutif, joue un rôle central dans la nucléation d'actine branchée, qui entraîne la migration cellulaire, l'endocytose et d'autres processus cellulaires. Récemment, une petite protéine, Arpin, qui inhibe le complexe Arp2/3 au front du lamellipode a été découverte et caractérisée. Ici, nous utilisons la microscopie électronique de particules uniques pour obtenir une reconstruction 3D du complexe Arp2/3 lié à Arpin, à une résolution de 25 Å.

Nous avons, ensuite, identifié Tankyrases1/2, comme un nouveau partenaire qui se lie à Arpin. Nous avons démontré qu'il y a une compétition dose-dépendante entre le domaine ARC4 de Tankyrase1 et le complexe Arp2/3.

Pour comprendre les principes de l'interaction entre Arpin et Tankyrases, nous avons créé un mutant d'Arpin (ArpinG218D) qui, *in vitro*, se lie toujours au complexe Arp2/3, mais plus aux Tankyrases. *In vivo*, ArpinG218D n'est pas capable d'inhiber le complexe Arp2/3, ce qui suggère que Tankyrase pourrait être nécessaire pour l'interaction entre Arpin et le complexe Arp2/3. Arpin est le facteur responsable du changement de direction des cellules migrantes.

Arpin is the turning factor of migrating cells, so we performed a migration analysis of MCF10-A cells expressing either wild type Arpin (ArpinWT) or mutant ArpinG218D in parallel with the depletion of endogenous Arpin. Cells expressing ArpinG218D had higher directional persistence, similar to the cells where the endogenous Arpin was knocked down. Thus, we suggested that mutant ArpinG218D cannot inactivate the Arp2/3 complex since it is not present at the lamellipodial tip. We compared the amount of protein for both ArpinWT and ArpinG218D in the membrane fraction of the migrating cells. A significant difference (44%) in the amount of ArpinWT and ArpinG218D was consistent with our hypothesis. According to our model, Tankyrases bind Arpin in the cytoplasm, change their conformational state and bring Arpin closer to the membrane in the lamellipodia. Deciphering the extracellular signals, Rac GTPase activates Arpin, which sequentially inactivates the Arp2/3 complex, while Tankyrases are released.

**Title :** Regulation of the invasion suppressor Arpin by Tankyrases

**Keywords :** Arp2/3, multiprotein complex, motility

**Abstract :** The evolutionarily conserved Arp2/3 complex plays a central role in nucleating the branched actin filament arrays that drive cell migration, endocytosis, and other processes. Recently, an inactivator of the Arp2/3 complex at the lamellipodium tip, a small protein, Arpin, was discovered and characterized. Here, we used single particle electron microscopy to obtain a 3D reconstruction of the Arp2/3 complex bound to Arpin at a 25Å resolution. Next, we identified the new Arpin binding partners, Tankyrases1/2. We demonstrated a competition between the ARC4 domain of Tankyrase1 and the Arp2/3 complex in a dose-dependent manner. To understand the principles of Tankyrases-Arpin interaction, we created a mutant Arpin (ArpinG218D) that lacks its ability to interact with Tankyrases, but not with the Arp2/3 complex *in vitro*. Interestingly, ArpinG218D was not able to inhibit the Arp2/3 complex *in vivo*, suggesting that Tankyrase may be necessary for Arpin-Arp2/3 complex interaction.

Arpin is the turning factor of migrating cells, so we performed a migration analysis of MCF10-A cells expressing either wild type Arpin (ArpinWT) or mutant ArpinG218D in parallel with the depletion of endogenous Arpin. Cells expressing ArpinG218D had higher directional persistence, similar to the cells where the endogenous Arpin was knocked down. Thus, we suggested that mutant ArpinG218D cannot inactivate the Arp2/3 complex since it is not present at the lamellipodial tip. We compared the amount of protein for both ArpinWT and ArpinG218D in the membrane fraction of the migrating cells. A significant difference (44%) in the amount of ArpinWT and ArpinG218D was consistent with our hypothesis. According to our model, Tankyrases bind Arpin in the cytoplasm, change their conformational state and bring Arpin closer to the membrane in the lamellipodia. Deciphering the extracellular signals, Rac GTPase activates Arpin, which sequentially inactivates the Arp2/3 complex, while Tankyrases are released.

