

Dynamics of nuclear actin

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I LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. In the text, they are referred to with their roman numerals.

- I** Skarp KP, Vartiainen MK. 2013. Actin as a model for the study of nucleocytoplasmic shuttling and nuclear dynamics. *Methods Mol Biol.* 1042: p. 245-55.
- II** Dopie J*, Skarp KP*, Rajakylä EK, Tanhuanpää K, Vartiainen MK. 2012. Active maintenance of nuclear actin by importin 9 supports transcription. *Proc Natl Acad Sci U S A.* 109(9): p. E544-52.
- III** Skarp KP, Huet G, Vartiainen MK. 2013. Steady-state nuclear actin levels are determined by export competent actin pool. *Cytoskeleton (Hoboken).* 70(10): p. 623-34.

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Contributions:

- I** Skarp KP carried out the experiments, analyzed the data and prepared the figures. Skarp KP together with Vartiainen MK designed the experiments and wrote the paper.
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II ABBREVIATIONS

ABP	actin-binding protein
ADP	adenosine diphosphate
Arp	actin related protein
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Å	ångström
CRM1	chromosome region maintenance 1
DNA	deoxyribonucleic acid
dsRNA	doublestranded RNA
eGFP	enhanced GFP
F-actin	filamentous actin
FCS	fluorescence correlation spectroscopy
FG	phenylalanine-glycine
FH	formin homology
Fli1	flightless 1
FLIP	fluorescence loss in photobleaching
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
G-actin	globular actin
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	GDP/GTP exchange factor
GFP	green fluorescent protein
GTF	general transcription factor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
hnRNP	heterogeneous nuclear RNP
INM	inner nuclear membrane
kDa	kilodalton
latB	latrunculin B
LMB	leptomycin B
mDa	megadalton
miRNA	micro RNA
mRNA	messenger RNA
mDia	mammalian homolog of Diaphanous
MRTF-A	myocardin-related transcription factor A
NE	nuclear envelope
NES	nuclear export signal
NLS	nuclear localization signal
NPC	nuclear pore complex
NR	nuclear receptor
NTR	nuclear transport receptor

NUP	nucleoporin (nuclear pore protein)
ONM	outer nuclear membrane
phactr	phosphatase and actin regulator
P _i	inorganic phosphate (PO ₄ ⁻³)
PNS	perinuclear space (between INM and ONM)
PP	protein phosphatase
Ran	ras related nuclear protein
rDNA	ribosomal DNA
RCC1	regulator of chromosome condensation 1
RNA	ribonucleic acid
RNAi	RNA interference
RNAP	RNA polymerase
RNP	ribonucleoprotein
ROCK	Rho-associated protein kinase
siRNA	small interfering RNA
snRNP	small nuclear RNP
SRF	serum response factor
tβ4	thymosin β4
tDNA	transfer DNA
UTR	untranslated region
WASp	Wiscott-Aldrich syndrome protein
WH2	WASp homology domain 2
wt	wild type

III SUMMARY

The notion of actin in the nucleus has slowly garnered popularity over the decades transforming the protein from an obscure artefact into a target of extreme curiosity in that compartment. Actin has been associated with a whole spectrum of nuclear functions, which directly or indirectly connect the protein with the most important nuclear function, transcription. However, it is currently not known how actin enters the nucleus in the first place and whether the protein is subjected to constitutive transport between the two eukaryotic compartments. The question regarding nucleocytoplasmic shuttling of actin is further complicated by the fact that actin is near the nuclear pore complex size exclusion limit, which sets a physical barrier for passive diffusion but not active transport.

To investigate the nature of actin transit between the nucleus and the cytoplasm we developed microscope assays to monitor the shuttling of fluorescently labeled particles in real time in living cells. Using our assays it was possible to quantitate nuclear actin import and export rates. This immediately revealed that actin constantly shuttles in steady state cells. We used fluorescent probes of various sizes to explore the limits of passive vs. active import and found that even if the size of the fluorescent actin construct is increased, it retains the import rate. This suggests an active mechanism is behind the nuclear import of actin, because passively traveling constructs slow down when size increases as shown by controls. Longer observations of nuclear import revealed that nuclear actin exists in at least three pools of different motility and the largest one exhibits retarded exchange rates with the environment. This is consistent with the reports of actin bound to various nuclear complexes, which may render the actins in question less mobile. We were also able to clarify the status of actin export, where two export receptors have been reported. By using a small molecule inhibitor, we showed that CRM1 does not participate in the export of actin, which at least during steady state seems to fall exclusively in the domain of exportin 6.

Next we identified the components of active import machinery by using RNAi vs. exportin 6 and suspected import factors. We found that actin is imported in complex with importin 9 and unphosphorylated cofilin and the process is dependent on Ran. We then showed that RNA polymerase II dependent transcription requires a suitable amount of actin in the nucleus or it is disrupted.

Finally, we wanted to investigate which cellular features might correlate with the transport rates of actin and how actin shuttling is conducted in cells of varying amount of actin in the

nucleus in relation to cytoplasm (N/C ratio). We found that factors such as the size or shape of the nucleus or cytoplasm or the ratio of their sizes does not affect the import or export rates of actin. Instead, we made the surprising discovery the the N/C ratio of actin inversely correlates with both export and import. This means that the fastest shuttling rates can be found in cells with least actin in the nucleus while in cells of high amount of actin in the nucleus, the transport is slow. The latter situation suggests the nucleus is filled actin, which is somehow made export incompetent, perhaps by associating with nuclear complexes. To explore the matter further, we performed studies on the motility of nuclear and cytoplasmic actin pools. However, we found no relation with high N/C ratio of actin and the size of the G-actin pool. This suggests that the primary mode of regulation of nucleocytoplasmic shuttling does not take place through manipulating the size of the global G-actin pool.

IV INTRODUCTION

1. Actin

1.1 Introduction to actin

Actin was first purified from muscle tissue in the 1940s as an “activator” of myosin (Szent-Gyorgyi, 1945). Decades of research ever since have elucidated the cell biological nature and various roles of actin also outside the muscle tissue. Actin is a ubiquitous protein across domains of life and is in all instances involved in the movement of macromolecules. To best understand the function of a protein, it is appropriate to start with the structure which typically translates into function.

The structure of actin was reported in 1990 with 2.8 Å accuracy (Kabsch et al., 1990) in complex with DNase I. The amino acid sequence is 375 residues long (GenBank: HS-β: AAA51567.1) and folds into a box of approximately 55 x 55 x 35 Å. The tertiary structure of the globular polypeptide consists of “large” and “small” domains, which are thus called for historical reasons despite being of similar size. They are further divided into subdomains so that subdomains 1 and 2 comprise the small domain and subdomains 3 and 4 the large domain (Figure 1). Both the N-terminus and C-terminus of the protein are found in subdomain 1 of the small domain. In the center of this globular structure in a cleft between the domains lies the nucleotide binding site. Nearby in a hydrophobic pocket formed by Asp11 - Gln137 - Asp154 is a binding site for a divalent cation, which also forms bonds with the phosphate groups of the bound nucleotide. The structures of ATP-actin and ADP-actin are quite similar, which suggests the protein does not undergo major structural changes upon ATP hydrolyzation. A later study on uncomplexed ADP-actin structure refined to 1.54 Å resolution showed that subdomains 2 and 4 only undergo minor rotation of 10° and 5°, respectively, when compared to models of ATP-actin (Otterbein et al., 2001). Also the DNase I binding site in this study consisting of residues 40-48 in subdomain 2 is slightly different from actin structures described earlier. Here it folds as an α -helix while Kabsch *et al.* found it to be a β -turn.

The most striking aspect of actin is the capability to polymerize monomeric G-actin (globular) subunits into polymeric F-actin (filamentous). The actin turnover from monomers to filaments and back to monomers is called treadmilling and during the process the net

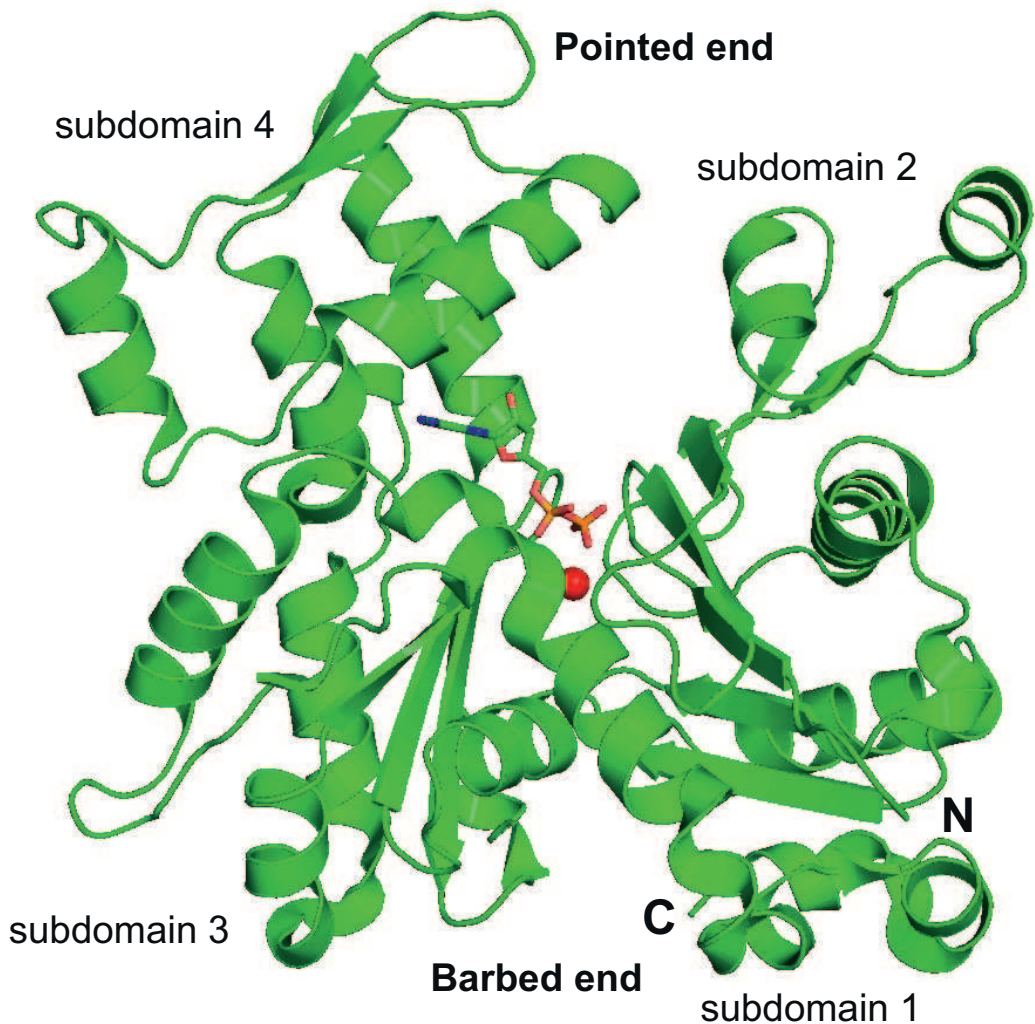


Figure 1. A ribbon diagram showing the structure of ADP-actin. Amino and carboxy termini are labeled with N and C. Due to the orientation of the molecule, N-terminus is in the front and C-terminus in the back of the picture. Bound ADP is shown in the central cleft between the small domain (subdomains 1-2) and large domain (subdomains 3-4). Me²⁺ is shown in red. Barbed end is formed by subdomains 1 and 3 and pointed end by subdomains 2 and 4. Image was rendered with pymol 1.7.1.7 (PDB: 1j6z).

consumption of actin monomers is zero (Wegner, 1976). An actin filament consists of two intertwined right-hand helices with a width of approximately 90-95 Å, which roughly corresponds to the width of two actin monomers (Holmes et al., 1990). The nucleation of a filament starts with the formation of an actin dimer, which forms a biochemical bottleneck

before elongation, which is a faster process (Wegner and Engel, 1975). Actin filaments are asymmetrical macromolecules and this polarity is based on the orientation of individual actin subunits within the polymer. Subdomains 1 and 3 form the barbed end of actin and subdomains 2 and 4 the pointed end. Actin filaments can elongate by the addition of actin monomers to either end. However, whether actual net polymerization takes place is dictated by the critical concentration, which is the concentration of G-actin where association and dissociation rates with the filament are equal. Critical concentrations vary between ATP-actin and ADP-actin and between pointed and barbed ends (Wegner and Isenberg, 1983). Consequently, the barbed end shows a strong preference for ATP-actin when compared to the pointed end and thus predisposes the barbed end for elongation. Conversely, the probability of ADP-actin dissociation from the pointed end is significantly higher than from the barbed end making the pointed end the preferable site of disassembly. The barbed end part of the filament contains most ATP-actin, which becomes increasingly hydrolyzed towards the pointed end. The hydrolysis of bound ATP therefore is not directly coupled to the process of filament elongation but rather appears signify the age of the filament (Ohm and Wegner, 1994).

The process of actin polymerization is spontaneous in physiological salt concentration (Nishida and Sakai, 1983). However, to expedite the process and to prevent unnecessary polymerization cycles, the polymerization of actin is extensively regulated by actin binding proteins (ABPs), which play a role in several key steps of the polymerization process (Figure 2). The eukaryotic actin interactome contains hundreds of proteins but the foremost regulators of actin are usually proteins, which directly engage actin filaments using their various actin binding domains. Their equal counterparts are G-actin binding proteins, which constitute a critical link in the chain of actin regulation by e.g. recycling actin between monomeric and polymeric forms. Together the various ABPs mold the cytoskeleton into a dynamic machine (Figure 2) which under continuous regulation can, for example, move the cell.

Cellular motility has been extensively studied using the standard 2D cell culture model. In those conditions the cell moves by the virtue of a protrusion in the leading edge of the cell called lamellipodium. This structure typically exhibits rapid actin turnover, which is accomplished with the help of Arp2/3 complex, which nucleates new branches from existing filaments (Pantaloni et al., 2000). Arp2/3 activation is typically carried out by proteins such as WASp (Wiscott-Aldrich syndrome protein) (Yarar et al., 1999), which are carefully

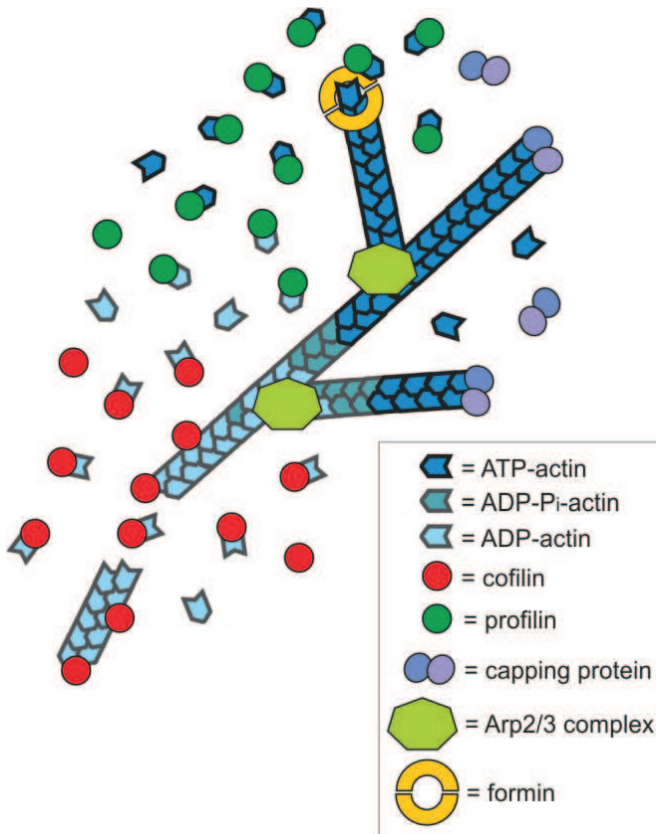


Figure 2. The classical model for actin turnover. Profilin feeds actin monomers to the barbed ends and as filaments age, they become increasingly susceptible to severing and depolymerizing action from cofilin. Arp2/3 complex nucleates the formation of branched filaments and formins nucleate and elongate straight filaments.

regulated by other proteins (Rohatgi et al., 2000). Formins, which nucleate straight filaments have a distinct mechanism for filament elongation compared to Arp2/3 complex. Formins

can nucleate filaments by stabilizing an actin dimer used to nucleate the growing filament (Li and Higgs, 2003; Sagot et al., 2002). This functions as a seed for formin dependent elongation. Formins then processively add actin monomers to the barbed end and simultaneously protect it from capping (Kovar and Pollard, 2004). As the filaments incorporate ATP-actin provided by profilin (Pollard and Cooper, 1984; Pring et al., 2003), a small ABP, the nucleotide carried by actin is slowly hydrolyzed indicating the age of the filament. Filaments may become capped by capping proteins (Schafer et al., 1996), which prevent further elongation.

When the actin filament ages, it becomes increasingly available for attack by ADF (actin depolymerizing factor)/cofilins, which sever and depolymerize filaments (Lappalainen and Drubin, 1997). Cofilin together with thymosin- β 4 sequesters G-actin making them unavailable for polymerization. However, the role of cofilin is not to downregulate actin filaments *per se* but rather to ensure older ADP-actin filaments are recycled and can be reused, thus making it critical in sustaining continuous actin treadmilling. Once ADP-actin is

available, profilin, which performs almost the opposite function of cofilin, catalyzes the exchange of ADP to ATP and prepares to feed the ATP-actin to the barbed end of growing filaments (Courtemanche and Pollard, 2013; Selden et al., 1999), restarting the cycle of actin polymerization. The small G-actin binding proteins are important for the maintenance of G-actin pool and their levels are changed according to needs during cellular life and development. For example, thymosin- β 4 is still expressed in embryonic skeletal muscle cells but not in adult cells (Nagaoka et al., 1996) and the form of cofilin is switched to a muscle specific form when muscle cells mature (Obinata et al., 1997). It is important to acknowledge the role of these small mobile ABPs as the dynamic architects of the cytoskeleton whereas the roles of larger ABPs are many times more structural. They are the proteins ensuring critical concentration is exceeded in barbed ends, depolymerization takes place near pointed ends and a pool of sequestered G-actin exists in balance with F-actin (Nishida et al., 1984; Ozaki and Hatano, 1984; Pantaloni and Carlier, 1993). In this way the small ABPs form an important bridge between the monomeric and polymeric actin pools.

Such a process where chemical energy is converted into mechanical energy is of extreme importance to fundamental cellular functions such as motility, intracellular vesicle transport and cytokinesis (reviewed in (Pollard, 2008; Pollard and Borisy, 2003; Pollard and Cooper, 2009)). In each of these actin-based mechanisms, different actin structures are molded by the ABPs, which thus contribute to higher order regulation of the cytoskeleton. For example, α -actinin bundles and crosslinks actin filaments. Interestingly, the architecture of these structures are determined by nucleation and elongation kinetics until dynamic arrest occurs due to steric hindrance from filament entanglement or crosslinking (Falzone et al., 2012). The cytoskeleton is anchored to the plasma membrane via proteins like α 1(E)-catenin (Rimm et al., 1995) and BAR domain proteins (Aspenstrom, 2014; Fricke et al., 2010; Mattila et al., 2007; Pykalainen et al., 2011; Zhao et al., 2011). Consequently, the shape of the cytoskeleton usually corresponds quite well to the morphology of the plasma membrane.

As mentioned above, cellular motility is influenced by the dense actin meshwork in the lamellipodium of the leading edge, which is mainly thought to be orchestrated by Arp2/3 complex (Welch et al., 1997). Arp2/3 complex also plays a role in smaller plasma membrane extensions called filopodia, which are important for cell migration (Mattila and Lappalainen, 2008). Filopodia help forming focal adhesions which are anchored to the substratum as well as to the cytoskeleton (Partridge and Marcantonio, 2006). The connection to cytoskeleton is made by large bundles of actin and myosin called stress fibers, which are contractile

structures with a prominent role in cell morphology (Tojkander et al., 2012). Myosins belong to a superfamily of ABPs, which have almost 40 copies in the human genome (Krendel and Mooseker, 2005). Their hallmark is the ability to convert ATP into mechanical energy to move them along actin filaments. In addition to their contribution in muscles, a group of myosins called unconventional myosins have important cell biological roles also in non-muscle cells (Pollard and Korn, 1973). They are literally the driving force behind intracellular transport, because they have distinct general roles as transporters of cargo along actin filaments (DePina and Langford, 1999; Vale, 2003). Actin and myosin are also involved in cytokinesis, where a contractile ring made of actin and myosin appears to constrict the plasma membrane and eventually isolates the two new cytoplasms from each other. This process is guided by formins, which nucleate straight actin filaments (Coffman et al., 2013).

1.2 Types of actin

Several different actin genes are encoded by the vertebrate genome. They are very well conserved from species to species (Chang et al., 1985) but certain differences exist between the different isoforms (Alonso et al., 1986). In mammals and birds, 6 different gene loci are known to encode actin (Vandekerckhove and Weber, 1978, 1981). Such divergence supports the more specific needs of larger organisms with more complex body plans. For example, α -actin, which is the isoform found in muscles is further divided into cardiac, skeletal and smooth isoforms, depending on type of the muscle tissue. Smooth muscle isoform also exists for γ -actin but perhaps the most relevant actins for the cell biologist are non-muscle γ - and β -actins, which are ubiquitously expressed. While the function of α -actins in muscles is somewhat self-explanatory on an organ level, confusion reigns over the isoform specific contributions of actins β and γ . The coding sequence of these two non-muscle actins only differ in 4 N-terminal residues (GenBank: HS- β : AAA51567.1, HS- γ :AAH53572.1), which results in a minor difference in their isoelectric points. This is because three aspartic acid residues of β -actin in positions 2-4 of the coding sequence are glutamic acid in γ -actin making it slightly more acidic. However, the β -actin mRNA contains signals in the 3' untranslated region (UTR), which are used as zipcodes for the intracellular delivery of mRNA (Sharpless et al., 1993). Currently, such β -actin mRNA polarization is known to be employed during injury (Hooek et al., 1991), cell movement (Shestakova et al., 2001) and neuronal growth cones of *Xenopus laevis* (Yao et al., 2006). As a testament to at least some

degree of interchangeability among actins, even the severe phenotypes of some α -actin knockout mice can be reversed or alleviated with the expression of another actin isoform (Kumar et al., 1997; Nowak et al., 2009). However, the non-muscle γ -actin was not able to compensate for the loss of skeletal α -actin despite being readily incorporated into filaments and total actin levels being restored (Jaeger et al., 2009). In *Drosophila*, all cells express two non-muscle actin isoforms, actin5C and actin42A. Also here, the lethal phenotype of actin5C knockout can be rescued with the expression of a hybrid transgene retaining the regulatory sequences of actin5C but containing the encoding sequence of actin42A (Wagner et al., 2002).

Despite the highly overlapping roles of the various actins in different organisms, some reports may offer clues to the reasons behind the existence of different isoforms. For instance, *C. elegans*, which has 5 actins expresses one of them specifically in the apical gut layer. If a mutant actin is used, microvilli is lost resulting in smaller worms, which grow slowly (heterozygous) or die during the first larval stage (homozygous) (MacQueen et al., 2005). The latter somehow evade a starvation checkpoint, because they die in 72 hours while normal larvae can survive at least 10 days without food. Another organ level failure due to a mutated actin isoform can be found in the mammalian inner-ear, where a mutation in the non-muscle γ -actin have been linked to loss of stereocilia and thus hearing loss (Zhu et al., 2003). Curiously, both tissues contain cells, where small protrusions on the cell surface, stereocilia and microvilli, are compromised if specific actin isoform is not available. Thus, it appears that higher organisms may have evolved actin isoforms to function in specific niche environments. Other studies on higher organisms suggest that different actin isoforms may be differentially involved in the regulation of cell size (Schevzov et al., 1992), cell-to-cell junctions (Baranwal et al., 2012) and meiosis (Brockmann et al., 2011). In agreement with the mice knockout studies, the largest differences among actin isoforms are typically discovered in experiments, where α -actins are compensated with non-muscle β/γ -actins (Fyrberg et al., 1998; Kaeck et al., 1997; Mounier et al., 1997; Schevzov et al., 1992; von Arx et al., 1995).

2. Nucleus

2.1 The structure of the nucleus

The nucleus contains the DNA, which together with proteinaceous components forms chromatin, the medium for genetic information. The nucleus is encased in a double lipid membrane called the nuclear envelope (NE), which is continuous with the endoplasmic reticulum (Watson, 1955). The inner nuclear membrane (INM) is connected to the outer nuclear membrane (ONM) at nuclear pore complexes (NPC), which serve as highways for molecules in and out of the nucleus (reviewed in (Grossman et al., 2012)). Outside NPCs, INM and ONM are separated by a ~50 nm perinuclear space (PNS) (Watson, 1955). Several proteins on both membranes span the gap, bind each other (Sosa et al., 2012) and thus connect nuclear architecture to the cytoplasm and there to the cytoskeleton (Maniotis et al., 1997). Most protein domains present in the NE proteome are deeply conserved (Mans et al., 2004). One well conserved domain among the proteins occupying the INM is the SUN (Sad1 and UNC-84) domain, which links the INM to the ONM. SUN proteins are anchored to the INM by a transmembrane domain followed by a coiled coil region and the SUN domain. The proteins reach through the PNS and use the SUN domain to bind KASH (Klarsicht/ANC-1/Syne Homology) domain proteins on the ONM (Sosa et al., 2012). Together with nuclear intermediate filaments known as lamins, SUN and KASH proteins form a LINC (LInker of Nucleoskeleton and Cytoskeleton) complex. These complexes link nucleus to the cytoskeleton with the help of a cytoplasmic structure called actin cap. This actin apparatus seems to play an important role in defining the shape of the nucleus (Khatau et al., 2010) and it can also be used to transfer mechanical information from the extracellular environment to the nucleus (Chambliss et al., 2013). Several KASH domain proteins are part of the spectrin superfamily due to containing several spectrin repeats and their actin binding domains tend to be closely related to α -actinins and spectrins (Autore et al., 2013; Mellad et al., 2011; Simpson and Roberts, 2008). The lamins at the INM together with other structural proteins create an elastic layer on the nuclear side of the membrane (Dahl et al., 2004). This layer on the INM is critical for nuclear assembly (Krauss et al., 2003; Krauss et al., 2002). Lamins and other key components occupy the inner surface of the INM and facilitate the attachment of other proteins by acting as a scaffold. Diseases associated with the NE are called laminopathies and range from muscular dystrophy to premature aging (Burke and Stewart, 2002). The molecular scope of the disease is reflected in the behaviour of lamin binding partners including actin, which is unable to operate normally (Nikolova-Krstevski et al.,

2011). Interestingly, interaction between lamin A/C and emerin, another ABP, is crucial for the proper nucleocytoplasmic shuttling of MRTF-A, which connects them to the regulation of cytoplasmic and nuclear actin pools (Ho et al., 2013; Talwar et al., 2014).

Inside the boundary of NE is the nucleoplasm, which was already early (Merriam and Koch, 1960) noted to be less concentrated than the cytoplasm. Studies using FCS (fluorescence correlation spectroscopy) show that the level of molecular crowding is conserved in mammalian cells throughout cell types (Guigas et al., 2007). The nucleoplasm also contains several types of subnuclear bodies including nucleoli, Cajal bodies, promyelocytic leukemia (PML) bodies, speckles (Handwerger and Gall, 2006) and paraspeckles (Fox and Lamond, 2010). They are punctate collections of proteins and RNA usually tasked with a specific function regarding some phase of gene expression. Some of these nuclear bodies such as the nucleolus exhibit differences in accessibility to macromolecules although the viscosity remains the same as compared to the nucleoplasm (Speil and Kubitscheck, 2010). Because subnuclear bodies are not restricted by membranes, their contents can exchange relatively freely with the nucleoplasm (Lang et al., 2010). For example, U1 snRNP (small nuclear ribonucleoprotein) is enriched in nuclear speckles, which are locations rich in splicing factors (Spector and Lamond, 2011). The protein nevertheless exhibits similar dwelling times in the speckles as in the nucleoplasm (Grunwald et al., 2006). The lack of membranes enables nuclear organelles to be extremely dynamic structures, which can be constructed and disassembled based on demand (Dundr et al., 2004; Kaiser et al., 2008; Mao et al., 2011). When micron sized paramagnetic beads are moved within the nucleoplasm with an electromagnet their trajectories appear as a series of hops, which are ATP-dependent. This may reflect the opening of chromatin in that area, because they coincide with regions of low chromatin compaction and the authors suggest it may be the general type of movement for nuclear foci such as nuclear bodies (Hameed et al., 2012).

If a eukaryotic genome is linearized, the length is approximately 2 meters (Ross, 1999). In order to pack this polymer into a volume of a single nucleus, extensive compaction must take place. First, DNA is wrapped around histones forming nucleosomes (Bednar et al., 1998). They look like beads on a string and assemble further into higher order structures (Woodcock, 2006). On a higher level, chromatin can be roughly divided into euchromatin and heterochromatin, which vary in how accessible the DNA is to molecular machines. Euchromatin is more lightly packed and thus more transcription compatible than heterochromatin, which is typically enriched in the nuclear periphery.

2.2 Nucleocytoplasmic transport

The genetic material and other nuclear contents are encapsulated in a membrane and well isolated from the rest of the cell but a mechanism must exist that allows the exchange of molecules between the nucleus and the cytoplasm. Such mechanism is provided by nuclear pore complexes (NPC), which were first discovered in 1950 (Callan and Tomlin, 1950). They are large protein complexes embedded in the NE functioning as selective gateways. All transport through the NE, active and passive, goes through the NPCs (Figure 3). Consequently, the nucleus and the cytoplasm are not isolated from each other but exhibit a steady exchange of molecules of various sizes. This is critical for the cell, because for example, genetic information must flow to the cytoplasm for translation and regulatory cues must reach the nucleus to deliver instructions for the gene expression machinery. It is therefore no surprise that NPCs and nucleocytoplasmic transport play a pivotal role in many diseases (reviewed in (Hill et al., 2014; Jamali et al., 2011)). NPCs are the largest molecular machineries known to exist and are readily visible with an electron microscope. The basic blueprint of the massive structure is conserved from yeast to mammals and is larger and more complex in more developed organisms. In *Saccharomyces cerevisiae* the complex mass is 66 MDa (Rout and Blobel, 1993) whereas the NPC in vertebrates contains additional components, which can increase the mass of the complex to over 100 MDa (Reichelt et al., 1990). The NPC structure penetrates both membranes of the NE and forms a ring around a central channel where cargo can pass through. The NPC ring exhibits eight-fold symmetry and is composed of ~30 nucleoporins (NUPs, nuclear pore proteins) all of which are expressed in single or multiple copies of eight (Cronshaw et al., 2002; Rout et al., 2000). In the structure, hydrophobic spokes penetrate the NE and are attached to rings on both sides. In the nuclear side, the ring is attached to a basket, which ends in a terminal ring while on the cytoplasmic side the ring acts as a scaffold for filaments. The central pore contains unstructured polypeptide chains containing repeats of phenylalanine and glycine (FG), which are the molecular basis for the sieve function in nucleocytoplasmic shuttling (Maimon et al., 2012). The FG-sieve is thought to accept items for transport based on their hydrophobicity and the addition of as few as four hydrophobic amino acid analogues is enough to confer transport capabilities to a protein intrinsically lacking them (Jovanovic-Talisman et al., 2009; Naim et al., 2009). Some of the FG-nups have distinct functions either in import or export (Sabri et al., 2007) and phosphorylation affects interactions with their cargo at least in some cases (Kosako and Imamoto, 2010). Despite the general purpose of the NPC for all cells,

there are developmental and tissue specific modifications to the complex (Raices and D'Angelo, 2012) and some nucleoporins are physically uncoupled from the NPC (Grunwald and Singer, 2012) and assist in shuttling as mobile factors (Oka et al., 2010).

The NPC molecular filter allows the passive translocation of smaller molecules of ~ 45 Å in diameter or ~ 40 kDa in mass across the NE but blocks larger macromolecules (Keminer and Peters, 1999; Paine et al., 1975). NPCs require the assistance of the active transport machinery, which enables rapid transport of even larger macromolecules such as ribosomal subunits or mRNP complexes at significant rates (Grunwald and Singer, 2010). The machinery for active transport is composed of karyopherins collectively referred to as nuclear transport receptors (NTRs), which are importin and exportin proteins acting as adaptors for the shuttling of proteins containing an NLS (nuclear localization signal) or NES (nuclear export signal) (Figure 4). NLSs are usually short, basic polypeptide sequences. The first characterized NLS was a monopartite signal from the Simian Vacuolating Virus 40, where the large T antigen contains a PKKKRKV sequence (Kalderon et al., 1984). Bipartite NLSs, which are discontinuous in the sequence, are also known to exist and they are constructed of two such basic sequences separated by a gap of usually ~ 10 amino acids (Dingwall et al., 1988). NES sequences are of similar length as monopartite NLS signals and typically contain

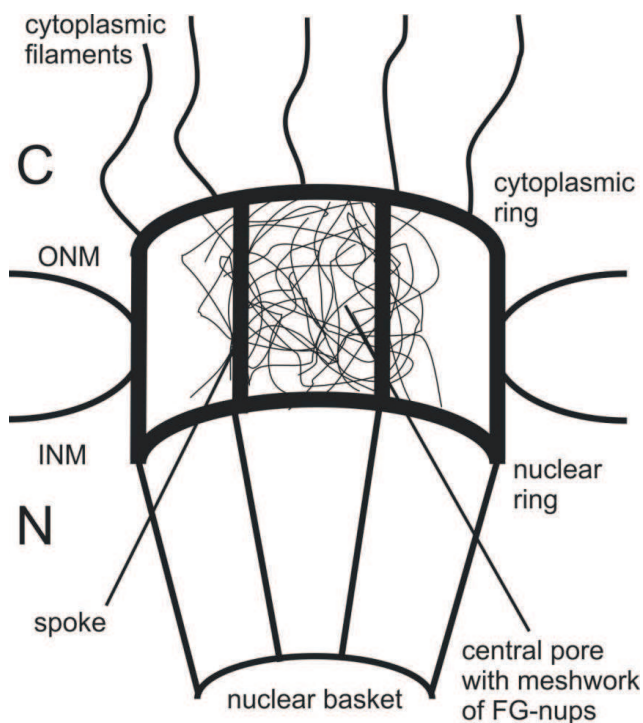


Figure 3. Schematics of the nuclear pore complex shown from the side. NPCs are the huge portals required for nucleocytoplasmic shuttling of molecules. The structure is embedded in the NE and forms a pore, which contains a selective barrier formed by non-structured phenylalanine-glycine repeat containing nucleoporins.

4 hydrophobic residues (usually leucines) flanked by other amino acids (reviewed in (Kutay and Guttinger, 2005)). Eukaryotic

genomes typically contain a set of approximately dozen karyopherins which act as importins and exportins. Their expression levels greatly dictate the contents of the nucleus and the cargo sent to the cytoplasm and vary according to the cell fate (reviewed in (Okada et al., 2008)). Importins and exportins are guided by a RanGTP/RanGDP gradient, which enables the proteins to dump their cargo in the correct cellular compartment. Ran is a small GTPase and its guanine nucleotide exchange factor (GEF), RCC1 (Regulator of Chromosome Condensation 1), binds chromatin (Bischoff and Ponstingl, 1991). Therefore, RanGTP is high in the nucleus and low in the cytoplasm (and vice versa for RanGDP). Consistently, RanGAP (GTPase activating protein) lies on the cytoplasmic side of the NE either free or tethered to the nucleus ready to assist in hydrolysing the GTP of exported cargo (Bischoff et al., 1995a).

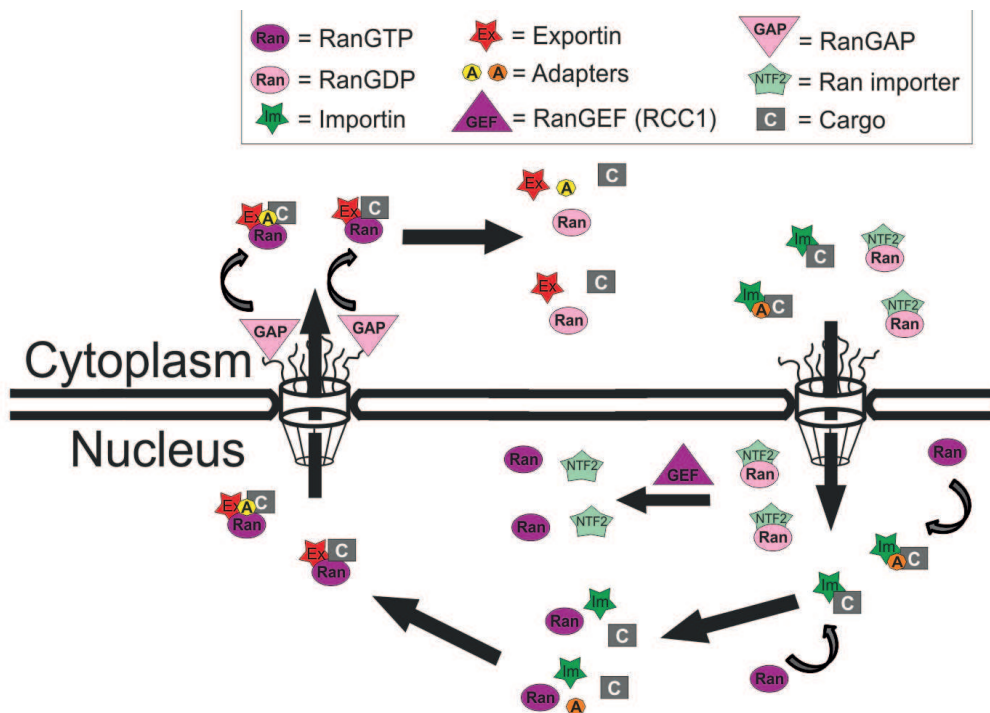


Figure 4. General schematics of nucleocytoplasmic shuttling. Cargo is recognized in the cytoplasm by importins, which may use adapters in the process. Upon encountering RanGTP in the cytoplasm, cargo is released. Export mirrors this process with the exception that RanGTP is now part of the cargo complex. When cytoplasm is reached, RanGAP hydrolyzes the GTP of Ran, which destabilizes the complex and cargo is released to the cytoplasm. Ran is imported by nuclear transport factor 2 (NTF2), which recycles the protein back to the nucleus, where RCC1 acts as RanGEF and catalyzes the reaction RanGDP \rightarrow RanGTP.

The active shuttling along the Ran gradient between the nucleus and the cytoplasm consists of the following steps: cargo with a NLS docks with an importin monomer or heterodimer depending whether additional adapters are needed to recognize the cargo. This complex can now access the FG-mesh within the NPC pore and can traverse the NE into the nucleus. Crystallization studies with N-terminal half of importin β suggested overlapping binding sites in a hydrophobic groove for FxFG and GLFG amino acid repeats of nucleoporins (Bayliss et al., 2002), while another study reveals another, weaker binding site in the C-terminal side (Bednenko et al., 2003). Structurally, these sites are on opposing sides of importin β and molecular dynamics simulations suggest several more binding sites for FG-repeats (Isgro and Schulten, 2005). *In vitro* experiments with hydrogels (Frey and Gorlich, 2007) and using surface plasmon resonance suggest FG-barrier has self-healing properties, which immediately reseal the barrier behind the cargo complex, thus facilitating the procedure (Frey and Gorlich, 2009; Schoch et al., 2012). Once inside the nucleus, interaction with RanGTP dissociates the importin(s) from the cargo (Percipalle et al., 1997). For example, cargo release by importin α/β heterodimer is directly coupled to the importin- β release from the NPC. Obstructing this step prevents importin- β /RanGTP interaction and arrests imported cargo to the nuclear side of the NPC (Gorlich et al., 1996; Lowe et al., 2010).

A similar chain of events takes place upon exit from the nucleus with the slight exception that Ran actually shuttles together with the complex: cargo with a NES docks with an exportin and forms a ternary complex with RanGTP. Again, the karyopherin enables passage as the complex in transit is engulfed in the web of tentacles within the NPC pore and reaches the cytoplasm through hydrophobic interactions. Here, Ran encounters its RanGAP, which with the help of RanBP1 (Bischoff et al., 1995b) and RanBP2 (Melchior et al., 1995) facilitates the GTPase activation of Ran resulting in $GTP \rightarrow GDP + P_i$ reaction. The presence of RanGDP destabilizes the export complex, which dissociates in the cytoplasm, freeing the cargo.

Since Ran only traverses the NE during export, it has its own importer, NTF2, which is required to shuttle Ran into the nucleus (Moore and Blobel, 1994) and can itself affect global nucleocytoplasmic shuttling through Ran (Chafe et al., 2012). Now inside the nucleus, RCC1 can catalyze the exchange of GDP to GTP and RanGTP is then free to participate in the export process described above and subsequently another round of import by NTF2.

As mentioned, importins and exportins are the mobile receptors, which are acting as the workhorse of energy dependent active transport through NPCs. They are very conserved and

both unicellular and multicellular eukaryotes contain a highly similar set of proteins functioning in nucleocytoplasmic shuttling in this fashion (Pemberton and Paschal, 2005). Importins and exportins diverge among themselves to specialize in the transport of specific cargo while also a more generic transporter is required for both directions. For example, importins 4, 5 and 9 carry histones and ribosomal proteins while the more variable cargo is recognized by importin β sometimes with the help of importin α or snurportin as an adapter (Lott and Cingolani, 2011; Pemberton and Paschal, 2005). Both of these adapters, once nuclear, are actively recycled back to the cytoplasm (Kutay et al., 1997; Paraskeva et al., 1999). In export, the scenario is not very dissimilar. For example, exportin-t transports tRNA out of the nucleus, exportin 5 transports miRNA (Leisegang et al., 2012) while a family of homo- and heteromeric export factors known as NXF and NXT are needed for mRNA export (Matzat et al., 2008; Tretyakova et al., 2005). The latter process is highly manipulated by viruses trying to take advantage the cellular gene expression machinery (reviewed in (Kuss et al., 2013)). The master exporter is CRM1 (exportin 1), which recognizes hydrophobic NES sequences (Pemberton and Paschal, 2005) and similar to importin beta, has the ability to use adapters to expand the range of cargo recognized by the molecule (Ohno et al., 2000). Curiously, the role of CRM1 is not entirely restricted to guiding cargo through the NPCs but is also implicated in bringing proteins to the nucleoli (Verheggen and Bertrand, 2012).

It has been suggested that the two modes of transport, passive diffusion and active transport facilitated by karyopherins along the Ran gradient may occur in spatially different regions within the pore (Kramer et al., 2007). This hypothesis was strengthened by a recent paper where human cells were imaged with 3D super-resolution fluorescence microscopy. Accordingly, it was discovered that a single central channel is solely responsible for passive transport while active transport takes distinct routes through the periphery of the pore among the FG-mesh (Ma et al., 2012). The active and passive pathways have distinct shuttling speeds and import and export processes are uncoupled from each other (Cardarelli et al., 2012).

2.3 Nuclear functions

The nucleus contains the molecular machinery, which maintains, reads and writes genetic information. Although all the molecular machinery needed for transcription is in theory freely available in the nucleoplasm, certain functionality tends to coalesce into intranuclear foci. For

example, rDNA is concentrated and transcribed at the nucleoli, Cajal body serves in the biogenesis of smaller RNA species which are incorporated into snRNPs and snoRNPs (small nucleolar) and speckles and paraspeckles assist in splicing and mRNA processing among other tasks (Morimoto and Boerkoel, 2013). The lack of membranes on the nuclear bodies and their relatively high exchange rate with the nucleoplasm makes it challenging to unambiguously pinpoint the exact duties of each type of nuclear body. However, they are all linked to transcription, which itself has been suggested to take place in concentrated foci called transcription factories (Sutherland and Bickmore, 2009). It is important to note that many transcription related functions such as splicing are considered to occur co-transcriptionally in the eukaryotic system. Their careful coordination is imperative, because for example too fast transcription may result in decreased splicing efficiency (Moehle et al., 2014). Eukaryotic transcription is divided among 3 RNA polymerases (RNAPs). RNAPs I and III transcribe rDNA (in the nucleoli) and tDNA genes while the bulk of gene expression is dependent on RNAP II. The molecular machinery which facilitates transcription among the different RNAPs is very conserved and they all need transcription factors, DNA topology manipulating factors, chromatin remodeling complexes and RNPs (ribonucleoproteins) to complete the task (Melnik et al., 2011).

Of the three metazoan RNAPs, RNAP II dependent transcription is best understood and for the same reason it is also the most relevant for the study of nuclear actin. The first stage in a cascade of events, which result in newly made mRNA, takes place at an enhancer region upstream of the gene (Figure 5). Here, transcription factors help recruit the Mediator complex (Kuras et al., 2003; Wang et al., 2014), which is a huge multisubunit complex of approximately 2 MDa in mass (components listed in (Poss et al., 2013)). Together with Mediator are summoned factors assisting in the physical manipulation of chromatin to make space for the transcription machinery. Mediator facilitates the assembly of chromatin remodelers (Huang et al., 2003; Khorosjutina et al., 2010; Lin et al., 2011), histone acetyl transferases (HATs) and general transcription factors (GTFs) (Black et al., 2006) to the gene promoter, which form the RNAP II pre-initiation complex (PIC) downstream of the enhancer region near the transcription initiation site (Ansari et al., 2014; Wang et al., 2005) (Figure 5). Although transcription competency can be achieved with less PIC components (Kotova et al., 2001; Myers et al., 1997; Parvin and Sharp, 1993), transcription initiation *in vivo* has been considered to include the polymerase, TATA-binding protein (TBP) and 6 GTFs; TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIF (reviewed in (Barrero and Malik, 2013; Liu et al.,

2013). The complex unwinds the DNA (promoter melting) to create a transcription bubble on the DNA, which is anchored to the polymerase (modeled in (He et al., 2013b)). The PIC then launches the polymerase while some GTFs and other components are retained at the promoter region, which may facilitate consecutive rounds of transcription (Figure 5).

During the first ~50 basepairs, the polymerase may pass regulatory checkpoints and pause (Margaritis and Holstege, 2008) but after this, elongation is more continuous although pauses may still occur (Maiuri et al., 2011; Palangat and Larson, 2012). RNAP II is accompanied during elongation by somewhat changed set of factors (Kwak and Lis, 2013) but the Mediator is considered to facilitate this part of transcription as well. Some newcomers include positive transcription elongation factor (pTEFb) (Price, 2000) and PSF-NonO complex (Ferrai et al., 2009). During this time the nascent mRNA begins to attract factors which help in the processing and transport of the molecule. These factors are usually hybrid macromolecules containing both protein and RNA called snRNPs and hnRNPs (heterogeneous nuclear ribonucleoprotein). They assist in splicing and prepare the complex, which has matured into a mRNP (messenger ribonucleoprotein) complex, for export out of the nucleus. During these processes RNAP II is further regulated by the phosphorylation of its C-terminal domain (CTD), which varies in different phases of transcription (Hsin and Manley, 2012). CTD is also known to be involved in the last stages of mRNA manufacturing by 3' end capping and pre-mRNA processing including polyadenylation (Hirose and Manley, 1998). As mentioned, the ready transcript is then spliced and prepared for transport out of the nucleus in complex with hnRNPs, which facilitate the passage through NPCs and deliver the cargo for ribosomes for translation.

Among RNAP II transcribed sequences, the pertinent components for the optimal transcription of a particular gene vary and for example the Mediator has been suggested to focus on heat shock activated genes (Auesukaree et al., 2009; Park et al., 2001). It is therefore possible that factors like Mediator, which appears to enhance basal transcription activity several fold (Kim et al., 1994) are recruited in strenuous conditions. The general mechanism described above is relevant for RNAP II but similar mechanisms are undoubtedly applied by other RNAPs. However, the differences in the polymerases themselves or in the choice of partners might also make them process the read DNA differently. One abundant obstacle in the one-dimensional course of an elongating polymerase are histones. They are octameric globular proteins typically consisting of two sets of H2A, H2B, H3 and H4. The octamers wrap DNA around them to form nucleosomes, which are the first layer of chromatin

compaction. Nucleosomes need to be somehow disassembled or displaced to allow the transcription machinery physical access to the DNA. Histones are targets for various post-translational modifications such as acetylation, which affect transcription efficiency (Workman, 2006). Interestingly, it has been proposed that in RNAP II dependent elongation, only 2 histones are displaced from the nucleosome leaving a hexamer of histones intact, which are elongation compatible. In contrast, in the same paper the authors suggest that RNAP III dependent elongation requires the disassembly of the whole nucleosome before elongation can proceed (Kulaeva et al., 2009).

While our understanding of the events resulting in the transcription of a gene has increased significantly over the years, it is still very much open to debate how the greater macromolecular neighborhood behaves in transcriptional context. Obviously, the 3D structure of chromatin must somehow be brought to accommodate transcription and rigorous studies are starting to reveal some of the dynamics of these events. For example, co-expressed genes tend to cluster and transcription itself may induce association with nuclear bodies (Rieder et al., 2014). Also promoters responding to the same transcription factors may result in co-transcription of genes in the same transcription factories (Larkin et al., 2013). Genes that are brought together for transcription do not usually even need to be adjacent to each other in the linear sequence of DNA (Li and Heermann, 2013), which significantly increases the combinations of genes the cell can co-express. Further studies are required for us to fully appreciate the spatiotemporal regulation of nuclear events, which all come together to drive gene expression.

3. The case for nuclear actin

Traditionally, the primary focus of actin studies has been the cytoplasm where light microscopical methods have shown the elegant dynamics of microfilaments. However, circumstantial evidence from various organisms has long suggested actin resides also in the nucleus (Jockusch et al., 1971) and most importantly, affects chromatin (Goldstein et al., 1977; Henney and Yee, 1979; Rungger et al., 1979) and transcription (Egly et al., 1984). These preliminary reports were met with much skepticism mainly because contamination from the massive cytoplasmic actin pool could not be ruled out. Also, fluorescently labeled phalloidin, which has been routinely used to visualize F-actin from early on (Wulf et al., 1979), does not show signal in the nucleus under normal exposure. Thus, technical

constraints stalled the early acknowledgement of nuclear actin. Nevertheless, evidence for nuclear actin has accumulated over the years and two nuclear export receptors have been described for actin.

The first report of energy dependent actin exclusion from the nucleus involves CRM1, a known export receptor for different cargos. It can be specifically inactivated by a small molecule, Leptomycin B (LMB). Treatment of rat fibroblast cells with this drug resulted in actin accumulation in the nucleus (Wada et al., 1998). However, increased nuclear actin levels ensued only after extensive incubation times with the drug, possibly resulting in secondary effects. This report was contradicted by another report proposing the actin nuclear export receptor to be exportin 6 (Stuven et al., 2003b). They found no evidence of CRM1 facilitated actin export in neither experimental system used, mammalian nor insect.

3.1 Forms of nuclear actin

Ever since the beginning of the actin-in-the-nucleus debate, one question above all else has dominated the discourse: Does it polymerize into filaments like in the cytoplasm? Attempts to detect nuclear actin filaments microscopically with a fluorescently labeled version of the classical actin binding reagent, the mucosal toxin phalloidin, have been primarily unsuccessful. The reason for this is not completely understood but because phalloidin requires at least 7 actin monomer long filament to bind, it has been speculated that nuclear actin would adopt a special conformation incompatible with phalloidin binding. To clarify the issue, various laboratories have tried to detect nuclear actin by raising antibodies against monomeric actin (Gonsior et al., 1999; Schoenenberger et al., 2005) but unfortunately with rather ambiguous results.

However, in 2006 the presence of polymeric actin in the cell nucleus was shown by using FRAP (fluorescence recovery after photobleaching) on GFP-actin expressing cells (McDonald et al., 2006). In FRAP experiments, fluorescent particles are photolytically destroyed in the area of interest and then the recovery of the fluorescence signal, which comes at the expense of surrounding unbleached area, is recorded. With actin, the recovery graph in the cytoplasm traditionally exhibits two phases, which represent monomeric and polymeric actin. The monomeric phase recovers fast, because it is limited by the diffusion of individual G-actin molecules while the polymeric phase recovers at a slower speed. This is

because bleached actin polymers have to be first disassembled in order to become exchanged with unbleached actin. In their experiments, McDonald *et al.* observed that the shape of the recovery graph in the nucleus contains the same two phases as in the cytoplasm. Very interestingly and also similar to cytoplasm, the slower phase was sensitive to actin drugs or mutants affecting polymerization, which demonstrates the presence of a polymeric actin pool also in the nucleus.

Despite the general lack of phalloidin stainable actin in the nucleus, some evidence exists of phalloidin-stainable nuclear actin. For example, nuclear DNA helicase II/RNA helicase A binds F-actin (Zhang *et al.*, 2002b) and localizes to nucleoli and the localization is dependent on rRNA synthesis (Zhang *et al.*, 2004). *Xenopus laevis* oocytes, which are cells with huge nuclei do not express exportin 6 and in fact seem to require larger amounts of actin in the nucleus than the average somatic cell. If exportin 6 is microinjected, the nuclei become extremely fragile after the first hour and burst if dissected (Bohnsack *et al.*, 2006). Interestingly, the actin in these nuclei is readily stainable with phalloidin and reveals a thick meshwork of F-actin, which apparently serves at least as a support structure. Indeed, it was recently shown that weak physical forces such as gravity start to play a role in cell nuclei once they become larger than $\sim 10 \mu\text{m}$ (Feric and Brangwynne, 2013). In *Drosophila* S2 cells, artificial increase in the amount of nuclear actin by depleting exportin 6 with RNAi results in the formation of large, several micrometer long nuclear actin bars or rods (Stuven *et al.*, 2003b). These take various shapes from linear to horseshoes and are easily stainable with phalloidin. In agreement with the notion of phalloidin stainable nuclear F-actin, a screen was recently performed using the actin binding reagent in question (Samwer *et al.*, 2013). Using a phalloidin based affinity matrix, the authors probed nuclear extracts from *Xenopus* oocytes for any F-actin binding partners. As mentioned, these cells use nuclear actin as a mechanical support for their enormous size. Consequently, the screen revealed many ABPs known to function in this context such as filamin A, supervillin and capping proteins. Interestingly, the most enriched F-actin binder in these nuclei was NabKin (Nuclear and meiotic actin binding Kinesin). Kinesins bind microtubules and can move along them towards cell periphery. The study found that NabKin can bundle actin and during meiotic cytokinesis colocalizes with microtubular and F-actin based cytoskeletal structures such as meiotic spindle and the contractile ring, respectively. The interaction of actin and NabKin is dependent on RanGTP supposedly due to RanGTP aiding NabKin to dissociate from a bound importin β . Interestingly, despite containing a wealth of F-actin regulators and DNA-binding proteins, the

Xenopus oocyte F-actin interactome does not include any myosins. Some co-localization studies with F-actin in HeLa cells suggests that the F-actin interactome may also contain histone deacetylases (He et al., 2013a) but further research is certainly needed to properly validate such ideas.

A very interesting recent finding are the transient phalloidin stainable nuclear actin filaments, which polymerize and depolymerize swiftly after serum stimulation of mouse fibroblasts (Baarlink et al., 2013). Baarlink *et al.* showed that this activity involves formins mDia1 and mDia2 and regulates the Serum Response Factor (SRF) coactivator MRTF-A. In conditions of lowered G-actin, this protein accumulates into the nucleus and activates transcription as a dimer via serum responsive elements (SREs). Another recent finding about the nature of nuclear actin came from the Mullins lab, where they tested fluorescent labeled actin probes containing various filamentous and monomeric actin binding domains from a number of ABPs (Belin et al., 2013). The monomeric actin probe based on the RPEL domain from MRTF-A was found in the nucleoplasm but also localized strongly to nucleoli and nuclear speckles. Interestingly, a probe for polymeric actin containing the tandem calponin homology (CH) domains from utrophin, apparently stained nuclear actin filaments enough to make them visible in the light microscope. Importantly, these filaments were also stainable with phalloidin although only after latrunculin A treatment. However, whether the structures have any connection to the physiologically important nuclear actin filaments described by Baarlink *et al.* remains to be shown.

3.2 Actin and myosin in transcription

Many of the reports of nuclear actin involve nuclear complexes or machines, which are directly or indirectly involved in gene expression. In this process, actin is involved in several steps (Figure 5). Right from the start of gene expression, actin can bind transcription factors and regulate their downstream activity. For example, MRTF-A is driven to activate serum response factor (SRF) dependent transcription when it fails to bind actin (Vartiainen et al., 2007) and coronin 2a together with oligomeric actin is implicated in relieving constitutively repressed genes important for immunity (Huang et al., 2011). Actin together with certain actin related proteins (Arps) has been purified as components of several chromatin remodeling complexes (Cairns et al., 1998; Fuchs et al., 2001; Shen et al., 2003; Zhao et al., 1998). These machines modify chromatin structure in an ATP-dependent manner, which

helps in providing physical access to DNA for larger molecular assemblies such as those involved in transcription (Oma and Harata, 2011). Actin also co-purifies with all 3 RNA polymerases and is required for their function (Hofmann et al., 2004; Hu et al., 2004; Philimonenko et al., 2004). Once transcription by RNAP II ensues, actin can be found in complexes with ABPs interacting with the transcription complex. Positive transcription elongation factor b P-TEFb is a heterodimer of cdk9 and cyclin T1. Actin binds the catalytic subunit in cdk9 and recruits P-TEFb to elongating complexes (Qi et al., 2011). Also N-WASp and Arp2/3 complex have been both implicated in RNAP II dependent transcription and copurify with the polymerase (Wu et al., 2006; Yoo et al., 2007).

The inseparable binding partner of actin in muscles, myosin, is also found in certain transcriptional contexts. Perhaps the most interesting myosin localizing to the nucleus is nuclear myosin I (NMI), which is expressed in three different isoforms: A, B and C (Schwab et al., 2013). Their differences lie in the N-terminus, which is 16 residues longer in the B isoform and 35 residues longer in the A isoform as in C isoform. The three isoforms contain a nucleolar localization signal in a region common to all and another in isoform B specific region. Consistently, it is the isoform B which most prominently localizes to the nucleus. They also show differences in colocalization with transcriptional machinery (Ihnatovych et al., 2012). However, because certain level of similarity in localization remains, functional overlap among the isoforms may explain the lack of abnormalities in NMI null mice (Venit et al., 2013). NMI has been linked to both RNAP I and II dependent transcription (Hofmann et al., 2006; Philimonenko et al., 2004; Ye et al., 2008). In RNAP II dependent transcription, the requirement for both actin and NMI is in the PIC formation, which is stimulated by actin (Hofmann et al., 2004) and NMI (Hofmann et al., 2006) although actin is also found in the coding region of active genes (Obrdlik et al., 2008). NMI can also be found in the coding region of rDNA genes and binds chromatin remodeling complex B-WICH (Percipalle et al., 2006; Vintermist et al., 2011), which is required for faithful cell cycle progression (Sarshad et al., 2013). NMI and actin seem to react and change localization according to the transcriptional status of the cell (Kysela et al., 2005). NMI and actin are also involved in intranuclear motility such as movement of chromosome sites (Chuang et al., 2006; Dundr et al., 2007) and accompanying mature rRNA transcripts to the NPC (Obrdlik et al., 2010). NMI and actin are also required to arrange the changes in chromatin structure, which facilitate nuclear receptor mediated transcription (Hu et al., 2008). Interestingly, also myosin II has been implicated in the first steps of RNAP II PIC assembly by associating with promoter

region of *ICAM-1* (Li and Sarna, 2009) while myosin VI associates with promoter and intragenic regions of active genes (Vreugde et al., 2006). In addition to NMI, myosin Vb is the only other myosin implicated in RNAP I dependent transcription (Lindsay and McCaffrey, 2009).

Actin continues to affect gene expression also in later stages by associating with nascent mRNA. This takes place through adaptors such as hnRNPs, which bind poly A tails of transcripts in addition to actin (Percipalle et al., 2002). Particularly interesting ribonucleoprotein is hnRNP U, which, just like actin, binds both RNAP II CTD and nascent transcripts (Kukalev et al., 2005). In *Chironomus tentans*, the hnRNP U counterpart is hrp65 and in both models they recruit histone acetyltransferases to the sites of transcription, perhaps to ensure the presence of transcription compatible chromatin (Obrdlik et al., 2008; Percipalle et al., 2003; Sjolinder et al., 2005), reviewed in (Skrp and Vartiainen, 2010). In the dipteran, also hnRNP A1 homolog hrp36 binds actin and accompanies transcripts all the way to ribosomes (Percipalle et al., 2001) although its detailed function in this process remains to be elucidated. During the lifespan of a hnRNP A1 binding transcript, the ribonucleoprotein participates in a number of tasks, which facilitate the mRNP progression from nucleus to cytoplasm (reviewed in (Jean-Philippe et al., 2013)).

3.3 Arps in the nucleus

Eukaryotic genomes encode several proteins related to actin, which are collectively called Arps (actin related proteins) and are well conserved from yeast to mammals. Arps, like actin have been suggested to assist in a number of tasks in the nucleus including chromatin remodeling (Figure 5). Sizewise, they range from approximately equal to actin up to $\sim 2x$ the mass of actin and they all have similar actin fold (Oma and Harata, 2011). Arguably the best known Arps are Arp2 and Arp3, which in complex with 5 other proteins nucleate branch points to existing F-actin in 70° angles (Mullins et al., 1998). They have well characterized functions in the cytoplasm, especially at the leading edge (Bisi et al., 2013) but have also been implicated in RNAP II regulation after activation by N-WASp (Yoo et al., 2007).

As members of the versatile actin family, the roles of Arps range from development (Oma et al., 2003) to the formation of long term memories (Vogel-Ciernia et al., 2013). Continuing studies made it soon obvious that the deployment of actin-Arp modules in eukaryotic cells is

a standard operating procedure to modulate the function of chromatin remodelers. Specifically, the heterodimer seems to maintain the ATPase activity of the complex by binding the HSA (helicase-SANT-associated) domain of the catalytic subunit (Szerlong et al., 2008). Arp4-actin is required for the proper function of e.g. Brg1 containing chromatin remodeling complexes (Nishimoto et al., 2012). It is interesting that Arp4 is also recognized by flightless 1, which is a nuclear receptor activator and a gelsolin ABP family member involved in hormone regulated transcription (Jeong et al., 2009). The authors suggest Fli1-Arp4 could recruit chromatin remodeling complex SWI/SNF to target genes of the estrogen receptor. In addition to actin-Arp4, also Arp5, Arp6 and Arp8 (also Arp7 and Arp9 in yeast) are known to associate with chromatin remodelers. In humans, the currently known actin and Arp containing chromatin remodeling complexes are INO80, SRCAP, BAF, p400 and Tip60 with yeast having orthologs for many of the mentioned (reviewed in (Oma and Harata, 2011)).

How exactly do the actins interact within the chromatin remodeler and with the rest of the nucleus remains unclear although the determination of the molecular structures of Arp4 and Arp8 (Fenn et al., 2011; Gerhold et al., 2012) shed some light to the issue. The insertions in both Arps ensure they prefer to stay monomeric and are not incorporated into F-actin. In fact, Arp8 exhibits an ability to depolymerize actin filaments *in vitro*, which improves in the presence of Arp4. Within the INO80 complex, Arp8 complexes together with actin and Arp4 and the latter interaction has been shown to take place via the barbed end of actin, which would prevent filament elongation (Kapoor et al., 2013). INO80 complex also contains actin and Arp5. Experiments with yeast mutants have shown that Arp5 or Arp8 defective strains have decreased INO80 ATPase activity and in the Arp8 defective strain, also Arp4 and actin was lost from the complex (Shen et al., 2003). This is because unlike Arp8, Arp5 is thought to associate with the complex differently than through the HSA domain and does not therefore affect actin and Arp4 binding. The importance of proper complex assembly through Arp8 seems to be conserved, because sites of DNA damage in mammalian cells fail to recruit INO80 in the absence of Arp8 (Kashiwaba et al., 2010). Recently, the 1.3 MDa INO80 complex from yeast was imaged with electron microscopy with the help of interaction mapping by crosslinking and mass spectrometry (Tosi et al., 2013). Their model takes shape with four distinct parts: globular head connected to a body and foot via a flexible linker. This places Arp5 at the linking region between the head and the body and leaves Arp4, Arp8 and actin to the end of the foot. The mode of action is suggested to be the movement of the leg

towards the head with the nucleosome between them. The insertions in Arp4 and Arp8 are acidic and can thus mediate interactions with basic histones. Each Arp prefers their own set of histones suggesting they could serve as a nucleosome recognition module for INO80 (Harata et al., 1999; Shen et al., 2003). It has been known already for 15 years that during lymphocyte activation, phosphatidylinositol 4,5-bisphosphate (PIP2) stimulus drives the mammalian BAF complex to chromatin (Zhao et al., 1998). The catalytic subunit of this complex, Brg1 binds actin and Arp4 but also PIP2. Remarkably, PIP2 enables the complex to associate with actin branch points and pointed ends by enhancing the actin binding capability (Rando et al., 2002). This is suggested to take place by PIP2 removing an intramolecular cap, which reveals a new F-actin binding site in the complex on the surfaces of actin and Arp4. In addition to chromatin remodelers and histones, Arps are also known to bind a spectrum of other nuclear proteins, such as transcription factors and other regulators of gene expression (listed in (Dion et al., 2010)).

4. Actin-binding proteins implicated in transcription

Many of the ABPs known to localize to the nucleus are proteins with relatively well characterized roles in the cytoplasm. Therefore on several occasions, the nuclear localization has become as a surprise, because no clear nuclear role might be immediately obvious for the protein. This set of dozens of proteins includes all the necessary machinery for classical actin behaviour including proteins capable of sequestering, nucleating, capping, depolymerizing, branching, bundling and crosslinking actin monomers/polymers. Their presence in the nucleus means that at least in theory similar actin turnover and regulation can take place in the nucleus as in the cytoplasm. Some nuclear ABPs are also present at the nuclear envelope, where many of them have a structural role (Lattanzi et al., 2003; Zhang et al., 2002a). For some of these ABPs, a NLS/NES has been characterized but for many the details of their nucleocytoplasmic shuttling remain obscure. Interestingly, most of the nuclear ABPs seem to be intimately tied to gene expression. Either they are components of the actual nuclear machines doing the job or they have an indirect regulatory role in the process.

4.1 Small actin binding proteins

The smallest ABP in cells is thymosin β 4. This 5 kDa protein consists of a single 43 amino acid polypeptide chain capable of binding actin (Low et al., 1981). The sequence is closely related to WH2 domain, which is common actin binding motif. Thymosin β 4 is considered the foremost ABP and actin sequestering agent in the cytoplasm. Thus, it comes as no surprise that it is connected to many organ/organism –level events orchestrated by actin, such as cancerogenesis, apoptosis, angiogenesis, blood coagulation and wound healing (Crockford et al., 2010). However, what was not immediately obvious is the fact that its nucleocytoplasmic shuttling appears to be actively regulated despite the small size. Evidence suggests that thymosin β 4 does not travel to the nucleus together with actin, since crosslinked t β 4-actin complex is cytoplasmic (Huff et al., 2004) and nuclear entry of thymosin β 4 is not actin dependent (Zoubek and Hannappel, 2007). Indeed, the expression as well as nuclear localization of t β 4 is under the control of hMLH1, which is involved in DNA mismatch repair and is mutated in cancers (Brieger et al., 2007). Interestingly, a recent paper describes a nuclear role for t β 4 in the regulation of localization and activity of MRTF-A, resulting in SRF activation (Morita and Hayashi, 2013). This result is expected, because t β 4 regulates the amount of available G-actin and MRTF-A specifically senses the amount of available G-actin (Vartiainen et al., 2007).

Profilin was the first G-actin binding protein characterized (Carlsson et al., 1977). Like t β 4, profilins are small ABPs, which bind actin monomers. However, unlike actin sequesters, profilin delivers G-actin for assembly into higher order actin structures and thus promotes polymerization. In the nucleus, profilin colocalizes with Cajal bodies and speckles and has thus been suggested to have a role in pre-mRNA splicing as both of these nuclear subcompartments are enriched in spliceosomal components such as snRNPs (Skare et al., 2003). Cajal bodies also contain Survival of motor neuron protein (SMN), which colocalizes with profilin and is required for snRNP assembly. It contains a profilin-binding polyproline motif and preferentially binds profilin isoform II (Giesemann et al., 1999). Such motif is also present in p42^{POP}, which is a Myb-related transcription factor. It is a multidomain protein with apparently working NLS and NES sequences and loses profilin affinity upon dimerization. Like many Myb-related transcription factors, p42^{POP} can modulate transcription with its C-terminal half in transcriptional assays: p42^{POP} acts as a suppressor of transcription and this effect is countered by profilin and dependent on the interaction between profilin and the polyproline stretch in p42^{POP} (Lederer et al., 2005). Recently, antibodies were raised

against the profilin of *Chironomus tentans*. The salivary glands of this dipteran contain polytene chromosomes, which are relatively easy to image because of their large size. Staining experiments with the antibody show profilin associated with multiple bands of the polytene chromosomes but not with dense chromatin. Interestingly, in the polytene chromosomes profilin localization seems to depend on on-going transcription. However, unlike the association of actin, the association of profilin with the actual gene loci was not sensitive to RNase (Soderberg et al., 2012). Finally, profilin has been shown to be an important part of the nuclear actin export pathway together with exportin 6. There, actin binding to exportin 6 is greatly enhanced in the presence of profilin (Stuven et al., 2003b).

Cofilin/ADF belong to a family of small single domain ABPs, which bind both actin monomers and filaments. However, regarding treadmilling, cofilin seems to perform almost the exact opposite function to profilin: cofilin can depolymerize and sever actin filaments (Lappalainen and Drubin, 1997). Cofilin has been known to localize to the nucleus from early on and the overexpression of cofilin has been linked to DNA damage response (Lee et al., 2005; Leu et al., 2013). Interestingly, excess cofilin in the nucleus tends to form intranuclear rods (Nishida et al., 1987), which have been suggested to play a role in Huntington's disease due to the presence on huntingtin, which is released from ER, imported to the nucleus and incorporated into such cofilin-actin rods upon stress (Munsie et al., 2011; Munsie and Truant, 2012). The function of nuclear cofilin-actin rods is not clear but a study in stressed neurons showed that cells with cofilin rods were able maintain their mitochondrial membrane potential better immediately after the insult. Also the amount of ATP declined slower in cells with nuclear rods (Bernstein et al., 2006). Such rods in stressed conditions may alleviate the effects of the insult by arresting actin turnover and saving energy. Under stressed conditions, actin seems to be imported into the nucleus together with cofilin (Munsie et al., 2012; Pendleton et al., 2003), which may facilitate the formation of nuclear cofilin-actin rods as new particles imported from the cytoplasm can be readily incorporated into nuclear structures. Perhaps the thermodynamic stability of this complex compared to profilin-actin complex (Kardos et al., 2013) is preferable during stress although FRET experiments suggest actin may prefer cofilin as a nuclear interaction partner also in steady state conditions (Chhabra and dos Remedios, 2005).

In stress induced nuclear rods the bond between the heterodimer becomes covalent but usually the interaction between actin and cofilin is regulated by the latter's phosphorylation on serine-3, which inhibits actin binding. Cofilin is phosphoregulated in this manner by

several proteins – some of which are specific to cofilin. For example, LIM kinase (Arber et al., 1998) and slingshot (Niwa et al., 2002), a cofilin phosphatase, are cofilin specific but cofilin is also regulated by TES kinases (Toshima et al., 2001a; Toshima et al., 2001b) and chronophin (Gohla et al., 2005), another phosphatase. In addition to these, cofilin is also regulated by general protein phosphatases PP1, PP2A and PP2B. Recent studies have shown that with PP1 this regulatory feedback loop employs phactr4, which activates cofilin via PP1 when actin monomer levels are low (Huet et al., 2013; Wiezlak et al., 2012).

Cofilin has been also described to function in RNA polymerase II mediated transcription, where it is found in the same complex with actin and phosphorylated RNAP II. Chromatin immunoprecipitation experiments placed cofilin on the transcribed regions of active genes. When cofilin was silenced with RNAi, general transcription levels decreased and in addition to cofilin, also actin and phosphorylated RNAP II was missing from active genes (Obrdlik and Percipalle, 2011). Interestingly, *in vitro* assays with RNAP I showed that excess cofilin decreased transcription in a dose-dependent manner (Ye et al., 2008), suggesting the role of cofilin may vary among RNA polymerases. There is also evidence that cofilin can repress glucocorticoid receptor, which belongs to class of mobile nuclear receptors capable modulating gene expression of specific genes by binding to promoter regions. This activity was dependent on phosphorylation status of cofilin (Ruegg et al., 2004).

4.2 WH2 domain containing ABPs

In addition to the small actin binders, many of the multidomain ABPs, which are able to induce more complex actin modulation also localize to the nucleus. Many of them contain WH2 domains, which is also present in thymosin β 4. They include N-WASp, WASp and WAVE1, which are part of the Wiscott-Aldrich syndrome (WAS) family proteins and have well defined cytoskeletal roles in cell motility. They all possess similar C-terminal domains, which can activate Arp2/3 dependent actin polymerization, which generates branches into actin filaments (Derivery and Gautreau, 2010).

N-WASp (Neural-) is found in steady state (Wu et al., 2006) as well as stimulated (Ferrai et al., 2009) conditions in complex with RNAP II via its interaction with PSF-p54Nrb (NonO) complex. This transcription factor complex is probably an obligate heterodimer (Passon et al., 2012) and is regulated by protein phosphatase 1 (Liu et al., 2011). In stimulated conditions,

inhibition of actin polymerization inhibits PSF-p45Nrb complex recruitment specifically to inducible *HoxB* genes but not on constitutively expressed genes (Ferrai et al., 2009). A recent study localizes this activity to nuclear speckles (Naum-Ongania et al., 2014). Experiments with truncated proteins narrowed the N-WASp NLS to the N-terminal region of the protein while the export is CRM1 dependent and is regulated by phosphorylation by Src kinases (Suetsugu and Takenawa, 2003).

WASp has been described in T-helper 1 cell differentiation defects underlying Wiscott-Aldrich syndrome where it was found on the proximal promoter region of the *TBX21*, which is a gene regulating TH₁ differentiation (Taylor et al., 2010). In cells depleted of WASp, chromatin modifications by WASp interacting histone modifiers were impaired, suggesting a role for WASp in the creation of transcription permissive chromatin.

WAVE1 knockdown in *Xenopus laevis* oocytes have been shown to result in abnormal development because of defective *HoxB* gene activation. The effect is dependent on the N-terminal WHD (WAVE homology domain) of WAVE1, which is needed for the facilitation of RNAP II mediated transcription (Miyamoto et al., 2013). Future studies are needed to elucidate whether N-WASp and WAVE1 act in parallel and/or in serial in the actin dependent expression of *HoxB* genes. The fact that N-WASp lacks a WHD, which was elemental to the function of WAVE1 in this context, suggests their mechanism may be at least partially different.

JMY is structurally similar to WAS family proteins in the sense that it contains similar C-terminal domain capable of activating Arp2/3 complex. However, it also has multiple WH2 domains, which can induce the polymerization of filaments on their own (Zuchero et al., 2009). Interestingly, actin binding WH2 domains overlap with a bipartite NLS, which is responsible for the nuclear infusion of JMY upon DNA damage, which causes actin polymerization in the cytoplasm. The decrease in available cytoplasmic G-actin pool reveals the NLS, which can then be bound by importin β (Zuchero et al., 2012). Once nuclear, JMY participates in DNA damage response by binding p300 and Strap, which facilitate the p53 dependent transcription (Adams et al., 2012; Shikama et al., 1999). This action may take place through the WH2 domains, because mutations that decrease Arp2/3 activation but do not affect the intrinsic ability of JMY to polymerize actin had no effect on the nuclear functions of JMY. This is in contrast to JMY's cytoplasmic role of enhancing cell motility, which is compromised by such mutations (Coutts et al., 2009).

4.3 RPEL domain containing ABPs

MRTF-A is one of the best characterized actin dependent transcription factors. It is a binding partner of SRF, which is a transcription factor regulating many immediate-early genes important for development (reviewed in (Posem and Treisman, 2006)). MRTF-A contains 3 N-terminal RPEL repeats, which can bind actin and it rapidly shuttles between the nucleus and cytoplasm in steady state cells (Vartiainen et al., 2007). This shuttling is facilitated by a bipartite NLS, which overlaps the RPEL repeats and is recognized by importin α/β complex (Pawlowski et al., 2010). MRTF-A is exported out of the nucleus by CRM1 (exportin 1) and this interaction is sensitive to actin binding (Vartiainen et al., 2007). Such behaviour makes MRTF-A to function in practice as a G-actin sensor: when G-actin is abundant, MRTF-A remains cytoplasmic. When the depletion of G-actin pool occurs by, for example, inducing actin polymerization by serum stimulation, the NLS is now free to direct actin into the nucleus (Pawlowski et al., 2010) and the lack of actin-MRTF-A interaction ensures export does not leak the protein back into the cytoplasm (Vartiainen et al., 2007). Once confined to the nucleus, MRTF-A is better positioned to activate SRF dependent transcription, which controls the expression of ~ 2600 genes (Esnault et al., 2014). The shift in MRTF-A localization to nuclear can be used e.g. in cell differentiation (Gupta et al., 2013) and even in maintaining the circadian rhythm of the whole organism (Gerber et al., 2013). Interestingly, a recent paper shows MRTF-A can be made nuclear by Mical-2, which contains a putative bipartite NLS and is a nuclear protein (Lundquist et al., 2014). Mical-family proteins are able to disassemble F-actin by catalyzing the oxidation of methionine-44, which severs filaments and decreases polymerization (Hung et al., 2011). Surprisingly, the disassembly of nuclear F-actin resulted also in decreased nuclear G-actin (Lundquist et al., 2014). The exact mechanism behind this G-actin depletion remains to be elucidated but nevertheless the mechanism of MRTF-A nuclear retention by Mical-2 functions through lowering G-actin availability (Vartiainen et al., 2007).

In addition to MRTF-A, the usage of RPEL as actin binding domain in cells is rather limited according to current knowledge. Another group of proteins known to contain RPEL domains is phactr1-4 (phosphatase and actin regulator) family of proteins, which are especially expressed in brain (Kim et al., 2012). Unlike in MRTF-A, their RPEL domains lie in the C-terminus although a single RPEL domain remains in the N-terminal end (Wiezlak et al., 2012). Interestingly, only with phactr1 but not phactr2-4, the actin-concentration sensing RPEL domains regulate the subcellular localization of the protein similar to MRTF-A

(Wiezlak et al., 2012). Thus, phactr1 reacts to serum stimulation in a similar manner to MRTF-A – by nuclear accumulation and also here the bipartite NLS is hidden among the RPEL domains. Phactr interaction with protein phosphatases has been studied in more detail with phactr1 and phactr4 (Huet et al., 2013; Wiezlak et al., 2012). Both contain a C-terminal protein phosphatase 1 (PP1) binding site. This is close enough to the actin binding sites that actin and PP1 compete for phactr interaction. Phactr1 is suggested to play a role in actomyosin assembly in an RPEL dependent manner (Wiezlak et al., 2012). Interestingly, the RPEL of phactr4 is utilized in a different context and also there PP1 plays a crucial role. The RPEL in phactr4 is used to monitor the cellular G-actin/F-actin balance and react by inducing cofilin if needed to replenish the G-actin pool (Huet et al., 2013). It is currently not known whether phactr4 plays a role in regulating G-actin also in the nucleus.

4.4 Formins

Formins are ABPs, which polymerize straight actin filaments using their FH1/FH2 (formin homology) domains. Some formin family members are also present in the nucleus. These include FHOD1, which plays a role in related (Thummel, 2001) mechanisms; apoptosis in human cells (Menard et al., 2006) and autophagic cell death in drosophila cells (Anhezini et al., 2012). Upon apoptotic signals in HeLa cells, FHOD1 is cleaved into 2 parts by Caspase-3. While the N-terminal fragment remains cytoplasmic, the C-terminal part is imported into the nucleus and nucleoli and reduces transcription by RNAP I if overexpressed (110). FHOD1 has also been implicated in regulating SRF mediated transcription (Westendorf, 2001). Like all formins related to Diaphanous, FHOD1 contains N-terminal Diaphanous inhibitory domain (DID) and C-terminal Diaphanous activation domain (DAD). These domains keep the protein in a state of autoinhibition. Cleaving the regulatory terminus from either end results in stimulated transcription of a serum response element (SRE).

Another formin which was very recently shown to be required for SRF mediated transcription is mDia (Baarlink et al., 2013). The nuclear transport receptors for mDia2 have been identified and it is imported via importin α/β pathway and exported with CRM1 (Miki et al., 2009). Although we understand more of the nucleocytoplasmic shuttling of mDia2 than mDia1, they both seem to play a part in SRF mediated transcription. Fusing NLS or NES sequences and using unpolymerizable mutant actin, constitutively active mDia1 and photoactivatable DAD domain from mDia2, it was shown that it is indeed nuclear and not

cytoplasmic mDia, which is responsible for the effect. This is important, because it suggests that the effect flows through MRTF-A reacting to the decreased G-actin pool in the nucleus. When SRF is activated by serum stimulation, it also appears to induce transient nuclear F-actin polymerization. It happens only ~15 seconds after stimulation of starved cells and dissipates rapidly within 2 minutes (Baarlink et al., 2013). The only way to visualize the effect in live cells was to use LifeAct-GFP-NLS. This is a short 17 amino acid F-actin binding element, which ignores G-actin (Riedl et al., 2008) and some stress induced twisted F-actin conformations (Munsie et al., 2009). However, using extreme exposure times, these small nuclear actin filaments could also be visualized with traditional phalloidin staining. Depletion of either mDia1 or mDia2 or using small molecule inhibitors abrogated the transient nuclear polymerization upon serum stimulation. Also nuclear extracts in these samples showed markedly decreased actin polymerization. Interestingly, depleting Arp2/3 or FHOD1 had no effect on nuclear actin polymerization rates (Baarlink et al., 2013). This suggests that the effect of FHOD1 on SRE described earlier probably works through cytoplasmic G-actin pool.

V AIMS OF THE STUDY

Various lines of evidence point to an important role for actin in the nucleus. However, important pieces of the nuclear actin puzzle remain unsolved. The manner in which actin enters the nucleus is not known and there are conflicting reports on actin export. To investigate these matters, our specific aims were:

1. Establish the methodology to study the nucleocytoplasmic shuttling of actin in living cells
2. Apply this methodology to quantitate actin transport rates, with specific aims to:
 - a. Determine if the nuclear import of actin is active or passive
 - b. Clarify the nuclear export pathway of actin (Exp6 vs Crm1)
3. Determine the cellular parameters that affect actin levels under steady-state conditions

VI MATERIALS AND METHODS

Table 1. Methods, which I have applied in these studies. Roman numerals indicate the publication in question.

Method	Publication
Cell culture	
NIH 3T3	I, II, III
MCF-7	III
Cell biology	
DNA transfection	I, II, III
Microinjection	I, II
Sample preparation for microscopy	
Immunofluorescence	I, II, III
Microscopy	
Laser scanning confocal microscopy (fixed cells)	II
Laser scanning confocal microscopy (live cells)	I, II, III
Method development	
FRAP import assay, short	I, II, III
FRAP import assay, long	I, II
FLIP export assay	I, II, III
intranuclear FRAP assay	III
cytoplasmic FRAP assay	III
Image acquisition, quantitation and statistical analysis	
Las-AF	I, II, III
Photoshop CS 5.1	I, II, III
ImageJ	I, II, III
Excel	I, II, III

VII RESULTS

5. Development of methodology for the study of nucleocytoplasmic shuttling (I)

To start our investigations into nuclear actin dynamics we first had to design the methodology, which would enable us to observe the movement of actin between the nucleus and the cytoplasm. To this end, a confocal laser scanning microscope (CLSM) would be used, which allows the imaging of optical sections. This technology excludes most of the signal coming from above and below of the chosen z-level enabling, for example, the proper imaging of the nucleus (Pawley, 2006). When living cells containing fluorescent particles are imaged under a microscope, the particles are in a steady-state situation regarding localization. This means they are in constant motion diffusing throughout the cell. The particles may appear almost immobile, because the net change in their concentration in different places in the cell is zero and because cellular movement typically takes minutes, not seconds. However, by destroying the fluorescence in one part of the cell, it is possible to directly observe these diffusion kinetics, because the fluorescence from the area that was left untouched rapidly exchanges with the area where fluorescence was diminished. Recording either the gain or loss in fluorescence after such perturbations therefore offers a possibility to quantitate the dynamics of fluorescent molecules in living cells. We therefore proceeded to develop microscope assays where we would destroy fluorescent chromophores with a high power laser (photobleach) within a region of interest (ROI) and then observe how the remaining fluorescence redistributes with time by exchanging particles between the ROI and the rest of the cell.

5.1 Nuclear import (I)

To study import into the nucleus we developed a FRAP (Fluorescence Recovery After Photobleaching) assay, where we bleach the nucleus once or twice and then observe the recovery of fluorescence in the nucleus [I, Fig 1A, Table 1]. Because the nuclear envelope isolates the nucleoplasm from the cytoplasm, the only source of fluorescence recovery comes from the cytoplasm, thus making the recovery representative of nuclear import rate. When the assay proceeds and nuclear fluorescence increases, nuclear export begins to undermine the import rate quantitation, because all the time more and more of the increasing nuclear signal is simultaneously exported back to the cytoplasm. It is therefore imperative that the quantitation is made from the very beginning of the fluorescence recovery curve [I, Fig 2]. In

principle, FRAP experiments should be performed until the recovering signal has reached the pre-bleach value. Because this is not achieved with our short import FRAP assay designed for the quantitation of the crucial beginning of the curve, we also designed a longer FRAP import assay, where we observe the recovery approximately 40 minutes [I, Table 1]. To ensure the size of the fluorescent tag does not interfere with the import dynamics of actin, it is important to use smaller labels such as Alexa Fluor 488 labeled actin. Also, to properly set the studied fluorescent construct in the context of passively transporting particles, fluorescent probes, which are smaller and larger than the NPC size exclusion limit should be employed. To this end, we wanted to use particles, which could be guaranteed to use passive transport mechanism to enter the nucleus. Therefore, FITC labeled dextrans, which are complex polysaccharides were used. The differential behaviour of dextrans of 40 and 70 kDa, which manifests as markedly different localization is a testament to the size exclusion of passively transporting particles. We also wanted controls, which would give us more hints of the active/passive nature of actin transport by increasing the construct size. This was most conveniently accomplished by the addition of more GFPs to the GFP-actin construct.

5.2 Nuclear export (I)

For the study of export, we initially we explored the possibility to use 2-photon photoactivation instead of photobleaching but since our ROIs tend to be large (e.g. the nucleus), this was deemed unfeasible with the hardware available to us. This approach has been applied to the export of Smad, MRTF-A and STAT (Cimica and Reich, 2013; Schmierer and Hill, 2005; Vartiainen et al., 2007). The advantage of this approach lies in the fact that using more than one photon to excite a chromophore critically decreases the probability of an excitation event taking place anywhere else except exactly at the focal z-level, which greatly increases signal to noise ratio. Instead of photoactivation, we opted a different approach, where we use FLIP (Fluorescence Loss in Photobleaching). In the assay, the whole cytoplasm is continuously bleached and the loss of nuclear fluorescence is taken as a measure of export rate [I, Fig 1B, Table 1]. Here, it is extremely important to draw the ROI very close to the NE without touching it in order to minimize the possibility of exported particles being re-imported into the nucleus before being bleached in the cytoplasm. To ensure this, constitutively nuclear control constructs should be utilized. For example, a shuttling protein made nuclear by NES deletion works well [I, Fig 3]. Again, to minimize the contamination

from re-import, it is important to make the quantitation from the beginning of the curve [I, Fig 3B].

6. Actin is exported out of the nucleus by exportin 6, not CRM1 (II)

Two export receptors for actin are described in the literature, *crm1* (Wada et al., 1998) and exportin 6 (Stuven et al., 2003b). Because the latter study found no evidence for *crm1* in actin export, we wanted to further clarify this by observing how actin export takes place in cells where *crm1* is blocked. Thus we began our exploration into understanding nuclear actin shuttling by addressing the issue of actin export. When we continuously bleach the cytoplasm excluding the nucleus, we see a steady drop in nuclear signal, which shows that actin is constantly exported out of the nucleus of steady state cells. The rate is relatively fast, because in the course of two and a half minutes, the nuclear signal has decreased to 2/3. During the runtime of our 5 minute export assay, actin was never completely exported out of the nucleus, suggesting some part of nuclear actin pool may not be readily available for export. We knew from localization studies with leptomycin B (LMB), which can specifically disable CRM1 that GFP-actin was not arrested into the nucleus in LMB treated NIH 3T3 fibroblasts [II, Fig 1C], which is one of our primary cell culture models in the study of nuclear actin. In contrast, a known cargo of CRM1, MRTF-A-GFP was clearly confined to the nucleus after a short period of incubation with the drug [II, Fig 1C], as expected (Vartiainen et al., 2007). We then proceeded to corroborate these findings in living cells by measuring the nuclear export rates with the FLIP assay described above [II, Fig 1A]. We recorded export rates in the presence and absence of LMB for GFP-actin and GFP-actin-R62D, which is an actin mutant incapable of polymerization [II, Fig S1A] (Posern et al., 2002). The results show that actin travels as a monomer, because actin mutant R62D is exported at a much greater rate than the wild type [II, Fig 1B]. In agreement with the data from fixed cells, we detected no statistically significant differences in export rates with neither GFP-actin nor GFP-actin-R62D when LMB had been applied. In contrast, the export of LMB sensitive MRTF-A-GFP was as slow as the export of RanBP1 Δ NES-GFP, which lacks an export signal [II, Fig 1B]. This shows that LMB had worked, because the usually fast export of MRTF-A (Vartiainen et al., 2007) had been reduced to background levels. These results suggest that CRM1 does not play a significant role in the export of actin in steady state cells. This was confirmed by exportin depletion studies in *drosophila* cells, where excess nuclear actin accumulates into large intranuclear bars [II, Fig S1C], which is an easy phenotype to score microscopically [II, Fig

1E, middle panel, arrow]. The bars were crystal clear in approximately half of the cells depleted of exportin 6 but were as absent in CRM1 depleted cells as in negative control [II, Fig 1E and F]. Also mammalian cells showed an increase in the levels of nuclear actin upon depletion of exportin 6 but not CRM1 [II, Fig 1D]. The fact that MRTF-A-GFP responded to CRM1 depletion in these cells shows that the depletion was successful [II, Fig S1B]. These results confirm that it is exportin 6 but not CRM1, which primarily exports actin out of the nucleus. If they were both involved, our negative result for LMB blocked CRM1 from fixed cells could in theory be explained by lesser participation of CRM1, which might require time (Wada et al., 1998) to show a detectable phenotype. However, because 1) measurements of the *in vivo* export rates did not drop upon LMB treatment, almost to the contrary and 2) CRM1 blockage taking days does not accumulate actin whereas other CRM1 responding cargo is arrested to the nucleus show together that it is extremely unlikely that CRM1 plays a role in actin steady state export. Because CRM1 exports also other cargo, secondary effects cannot be ruled out in the experiment by Wada *et al.* For example, merely the accumulation of actin binding proteins into the nucleus might appear to produce a phenotype of actin accumulation.

7. Actin constantly shuttles in steady state cells and the import is active (II)

We then shifted our focus on the nuclear import of actin and addressed the matter with the import FRAP assays described above [II, Fig 2A]. Our selection of fluorescent constructs in the assay was based on shedding light on the active/passive transport dilemma of actin. Actin has a size of 42 kDa, which might barely allow it to translocate passively through the NPCs into the nucleus. However, the existence of an export receptor tentatively suggests that also an active import machinery might be in use but no evidence exists in the literature one way or the other. When we started bleaching the nuclei of GFP-actin expressing cells, we found that the nuclear signal, which drops significantly upon bleaching, rapidly recovers the fluorescence intensity. Put together with our export data, our experiments show that monomeric actin undergoes constant and rapid nucleocytoplasmic shuttling in steady state cells. Our data shows that GFP-actin fusion protein, which is almost a 70 kDa in mass and well over the NPC size exclusion barrier for passive diffusion, nonetheless enters the nucleus at a speed comparable to smaller, passively traveling particles [II, Fig 2C]. With passive diffusion, the speed of NPC translocation decreases with size, because larger particles have increasing steric difficulties to rapidly navigate the geometry of the central pore. This is

evident with the decreased import rate of 2GFP compared to GFP, despite being still much smaller than GFP-actin [II, Fig 2C]. Size exclusion with passive diffusion is also demonstrated with FITC labeled dextrans, where the 40 kDa dextran exhibits similar import rates to GFP while the 70 kDa dextran was already incompatible with our assay due to lack of nuclear signal [II, Fig 2C]. Therefore, the capability of actin to maintain high import rates even when encumbered with 2 GFPs (2GFP-actin), which brings the size to almost 100 kDa, is indicative of an active import mechanism. It was also important to establish, that the relatively large GFP tag would not hinder the translocation properties of actin. To ensure this, we microinjected cells with Alexa Fluor 488 labeled α -actin, where the size of the chemical label is $\sim 100\times$ smaller than GFP and should minimize any steric hindrance. AF488-actin performed very similarly to GFP-actin, validating GFP-actin for the study of nucleocytoplasmic shuttling [II, Fig 2C]. These three actin constructs of very different sizes, AF488-actin, GFP-actin and 2GFP-actin nevertheless distributed themselves in a similar manner between the nucleus and the cytoplasm as expected based on their similar transport rates [II, Fig S1D]. It is also interesting to note that there was no apparent discrimination by the import machinery with actin α/β isoforms, because AF488 labeled actin was the α -isoform while the GFP-fusions encoded by transfected plasmids were β -actins. The results also show that similar to export, actin is imported as a monomer, because actin mutant R62D is imported at a greater rate than the wild type actin [II, Fig 2C]. Appropriately, the treatment of cells with jasplakinolide, which stabilizes F-actin and tips the balance of the actin pool towards the polymeric, decreases actin import rate, because less monomers are available for transport [II, Fig 2C].

8. Three pools of actin with different mobilities are present in the nucleus (II)

We then performed the longer import assay to better understand the long term recovery of the nuclear actin fluorescent profile [II, Fig 2B]. The traditional cytoplasmic actin FRAP experiment contains two phases of recovery, one for monomeric G-actin, which is fast and another for polymeric F-actin (Amato and Taylor, 1986). The latter recovers slower, because it is restricted by the rate of treadmilling, which is required to exchange the bleached particles within the filaments. Very interestingly, our longer GFP-actin FRAP curve contains 3 phases of recovery while GFP-actin-R62D contains only 2 [II, Fig 2B]. Since we know that R62D is not incorporated into filaments and thus lacks a phase, there is still an additional phase present in both recovery curves. The half lives of the 3 phases of GFP-actin were 6.8 s, 230 s

and 1800 s and the half lives of the 2 phases of GFP-actin-R62D were 4.5 s and 330 s [II, Materials and Methods]. The phases with the lowest half lives present with both constructs correspond to monomeric actin while polymeric form of actin is likely responsible for the middle phase of GFP-actin. The remaining GFP-actin phase with the slowest turnover shows the presence of a detained nuclear actin pool and suggests approximately 60 % of the nuclear actin pool is relatively tightly bound to nuclear complexes. This third phase may partially correspond to the second phase of GFP-actin-R62D although the actin mutant may be excluded from some nuclear complexes harboring actin.

9. Nuclear import of actin is dependent on importin 9, cofilin and the Ran gradient (II)

We then proceeded to identify factors involved in the import of actin using exportin 6 depleted *drosophila* S2R+ cells with their unambiguous nuclear actin bar phenotype. By coupling the depletion of exportin 6 with another factor, one can study whether that factor is involved in the nuclear import of actin by examining the abundance of nuclear actin bars. We wanted to examine whether Ran-gradient is required for the transport of actin into the nucleus. We also included a number of transport receptors and cofilin, which is known to localize to the nucleus upon stress like actin (Nishida et al., 1987) together with its known activator, a cofilin phosphatase slingshot. Indeed, the ablation of Ran by RNAi rescued the exportin 6 phenotype, indicating that the import of actin falls under the jurisdiction of Ran-gradient like all active nucleocytoplasmic shuttling [II, Fig 3A and B]. While this effect may be unspecific, the unaltered localization of GFP in Ran depleted cells shows passive transport remained intact [II, Fig S1E]. Also co-depletion with cofilin or slingshot rescued the nuclear actin phenotype, which shows cofilin plays a role in actin import also in steady state cells [II, Fig 3A and B]. Disappointingly, none of the tested *drosophila* import receptors were able to prevent the formation of nuclear actin bars [II, Fig S1F].

The involvement of cofilin was also clear in mammalian cells, where simultaneous depletion of exportin 6 and cofilin suppressed the nuclear accumulation of actin [II, Fig 4A and B]. However, the involvement of cofilin could also be interpreted differently; the lack of cofilin might result in impaired actin import, because in general cellular G-actin levels have decreased due to missing F-actin depolymerization and severing activity. To exclude the lack of actin monomers as a deciding factor in import during cofilin depletion, we also studied the localization of the R62D mutant, which was similarly dependent on cofilin [II, Fig 4C] but

independent of F-actin. We then performed a similar screen involving nuclear transport factors using mammalian cells, which showed a clearly quantifiable increase in nuclear actin under the microscope upon exportin 6 depletion although not actin bars like the fly hemocytes. Of the factors tested, only importin 9 abolished the nuclear accumulation of actin [II, Fig 4A and B, S2D and E]. Fractionation of importin 9 depleted cells showed a marked decrease in actin levels of the nuclear fraction [II, Fig 4D and E].

We then performed biochemical analysis on the interaction between actin, cofilin and importin 9. It turned out that the receptor co-immunoprecipitates with actin in a cofilin dependent manner [II, Fig 5A]. This interaction could be disrupted by Ran-Q69L mutant, which predominantly binds GTP and fools the import cargo complex it is already in the nucleus and ready to dissociate [II, Fig 5B]. Such usage of a small ABP as a binding partner is reminiscent of the interaction of profilin with actin during nuclear export (Stuven et al., 2003a). These experiments demonstrate that the shuttling of actin between the nucleus and the cytoplasm is not a passive process, it requires energy and involves a number of transport factors and can therefore be carefully regulated.

10. Transcription is dependent on a suitable amount of actin in the nucleus (II)

Finally, we wanted to measure how transcription is affected by the various levels of nuclear actin we can now artificially induce by manipulating the corresponding nuclear actin transport factors. To this end, we depleted cells of the indicted transport factors and labeled the nascent mRNA transcripts with 5-fluorouridine (5-FUrd). According to our findings, nuclear actin is required to maintain proper levels of transcription, because too low amount results in decreased RNAP II dependent transcription [II, Fig 6A and B]. Also too much actin in the nucleus may be unoptimal for the cell, because transcription decreased also in exportin 6 depleted cells but this result was not statistically significant. To ensure the effect was specific to actin, we prepared a cell line expressing Flag-NLS-actin, which delivers actin into the nucleus via an importin 9 independent way by using importin α/β pathway. In these cells, abrogation of importin 9 had no effect on transcription but very interestingly, the loss of cofilin still did [II, Fig 6C]. This is in agreement with previous studies pinpointing a specific role for cofilin in RNAP II mediated transcription (Obrdlik and Percipalle, 2011). Our studies show that the eukaryotic genome contains a conserved transport machinery, which constantly shuttles actin between the cytoplasm and the nucleus. The nuclear actin levels have a direct

impact on global transcription rates, which decrease dramatically when actin is absent and excess actin also seems to have a detrimental effect on transcription rates. This implies that nuclear actin levels must be under careful regulation.

11. Actin shuttling rates depend inversely on the amount of actin present in the nucleus (III)

The finding of transcription compatible nuclear actin levels is interesting because we have observed a lot of variance in the amount of nuclear actin in cultured cells [II, Fig 1A]. We therefore figured mapping of cellular parameters possibly involved in the transport of actin might give us clues about the nature of the phenomenon. To entertain this line of thinking, we performed our standard import and export assays extensively with two cell lines, NIH 3T3 mouse fibroblasts and MCF-7 breast carcinoma cells. Our goal was to gather enough statistical data to understand how various cellular features such as cytoplasm size, nucleus size and shape or the ratio of nuclear to cytoplasmic actin (N/C ratio) correlate with actin transport. First we wanted to ensure that the expression levels themselves would not be responsible for guiding GFP-actin or GFP-actin-R62D towards nuclear or cytoplasmic compartments. Neither cell line displayed correlations towards this scenario [III, Fig 1B-E]. We then sought to establish that the fluorescence levels of the cells would not be indicative of the actual transport rates. Accordingly, we found no correlation with the total fluorescence of the cells with the *in vivo* nucleocytoplasmic shuttling rates [III, Fig 2B-E], which legitimizes us to compare the rates of transport in cells of varying amounts of fluorescence.

We proceeded to compare a number of cell physiological features to the rates of actin import and export. We plotted the shape of the nucleus, nuclear area, cytoplasmic area or the ratio of nuclear area to cytoplasmic area vs. nucleocytoplasmic transport speeds [III, Fig 3A-H]. None of these parameters exhibited statistically significant dependence on the import or export rate of actin.

However, what we found when we started to compare the ratio of nuclear actin to cytoplasmic actin (fluorescence N/C ratio) to the transport rates was extremely intriguing and initially puzzling. Our data shows that in cells with a high N/C ratio i.e. high concentration of actin in the nucleus as compared to the cytoplasm, actin is in fact imported at a relatively slow speed [III, Fig 4A, and E]. Accordingly, when the N/C ratio of the cell is low, which means low amount of nuclear actin compared to cytoplasmic actin, actin is rapidly imported

into the nucleus. The same scenario is prevalent also in the export of actin, which is slow in cells of high actin N/C ratio and *vice versa* [III, Fig 4B and F]. Also the export of mutant actin-R62D correlates negatively with the N/C ratio in a statistically significant manner [III, Fig 4D, and H] but interestingly not the import of actin-R62D [III, Fig 4C, and G]. This may indicate that once G-actin is overly abundant, it no longer responds to regulatory cues regarding actin import as concisely. These results show that when the nuclei are relatively low on actin, an active transport mechanism is more extensively utilized in both directions to refresh the nuclear actin pool, perhaps to ensure the minimum needed for ongoing nuclear tasks is maintained.

12. Shuttling rates of nuclear actin are not dictated by the global G-actin pool (III)

Next we asked how does actin behave compartmentwise in these different scenarios of varying actin N/C ratios. To investigate this, we performed FRAP assays both in the nucleus and the cytoplasm [III, Fig 5A]. In the intranuclear FRAP assay, it would be very important to keep the ROI small enough to ensure the recovery comes from within the nucleus and is thus representative of the intranuclear motility of actin instead of actin import rate. We therefore bleached an oval shaped area in the center of the nucleus with x and y diameters of only a quarter of those of the nucleus. To further minimize the effect the constant shuttling of actin has on this assay, time per frame was lowered and the recovery was normalized to total nuclear fluorescence. The intranuclear and cytoplasmic FRAP assays did not show a statistically significant correlation between the motility of actin and actin N/C fluorescence ratio [III, Fig 4B-E]. This suggests that actin shuttling rates are not adjusted by direct manipulation of the global G-actin pool and the nuclear actin in high N/C ratio cells retains its motility but is somehow export incompetent.

VIII DISCUSSION

Our results have revealed fascinating novel aspects of nuclear actin. It is a protein undergoing constant shuttling between the nucleus and the cytoplasm in steady state cells and this activity is dependent on energy consuming transport machinery. Interestingly, when the amount of actin in the nucleus increases, the nuclear actin pool retains the intranuclear motility despite becoming relatively inert in terms of nucleocytoplasmic exchange. Because this implies

nuclear actin shuttling rates are not dictated by the global G-actin pool, we suggested that in cells of high actin N/C ratio, nuclear actin is bound to nuclear complexes or alternatively post-translationally modified to prevent its export (Skarp et al., 2013).

Curiously, the latter of those two scenarios is exactly what appears to take place in a recent paper describing MRTF-A activation by Mical-2 (Lundquist et al., 2014). In order to activate MRTF-A, it must first be confined to the nucleus. This is typically and also with Mical-2 achieved by lowering the amount of available G-actin. Mical-2 depolymerizes nuclear actin by oxidizing a methionine residue (M44) in the pointed end of actin. One would assume this results in an increase in available G-actin in the nucleus but counterintuitively the result is the exact opposite and the nucleus is depleted of actin. The finding is in agreement with our hypothesis of export competent and incompetent (or rather export competent and more export competent) G-actin pools in the nucleus. Perhaps in general the lowered nuclear actin in cells is achieved through Mical-2 type mechanism, which first disassembles nuclear F-actin and subsequently predisposes G-actin for export. The exact mechanism of nuclear actin depletion by Mical-2 remains to be elucidated but could, for example, be based on enhanced interaction with exportin 6. Because profilin binds actin in the barbed end (Schutt et al., 1993), it is unlikely that the enhanced export competence of M44 oxidized actin is due to increased interaction with profilin, which helps exportin 6 to carry actin into the cytoplasm.

What are then the steps where the migration of actin either from cytoplasm to the nucleus or *vice versa* can be regulated? As suggested (Author summary in (Dopie et al., 2012)), there are several situations, where this can be achieved (Figure 6).

First, the number of actin monomers constitute a limiting factor, because all actin shuttling happens with monomers. This makes the balance between G-actin and actin arrested in nuclear complexes or polymeric form important for shuttling. Although our results show continuous exchange between cytoplasmic and nuclear actin pools, Mical-2 is a good example that the G-actin pools in both compartments can also be regulated separately. Published data from other laboratories and our microscopy experiments make it evident that actin in the nucleus has a plethora of binding partners and is retained in the nucleus in terms of export. Currently, it is extremely challenging to distinguish between actin bound to nuclear complexes and a possible export incompetent G-actin pool but I consider it likely that both of these scenarios, which can in fact be two faces of the same phenomenon, contribute to the

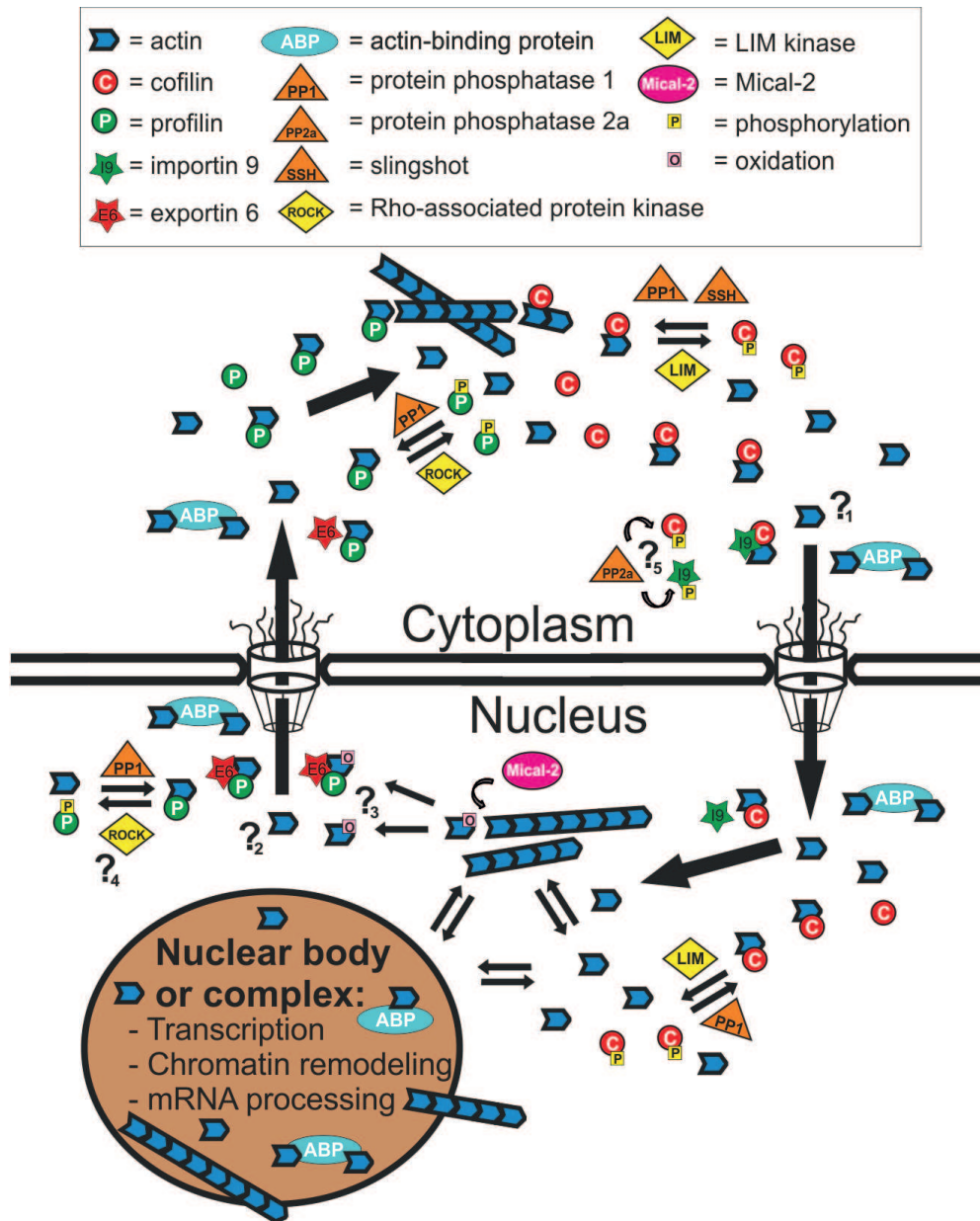


Figure 6. Current working model of the nucleocytoplasmic shuttling of actin. Actin is actively imported by importin 9 in complex with cofilin and exported by exportin 6 in complex with profilin. In addition, actin is carried over the NE by several ABPs and might also shuttle passively (?₁ and ?₂). Actin import is influenced by the phosphorylation of cofilin and export by oxidation of actin by Mical-2 although the exact details of Mical-2 dependent mechanism remain unknown (?₃). Additional layers of regulation may be provided by profilin phosphorylation (?₄) and the actions of PP2a in phosphorylating both cofilin and importin 9 (?₅). In the nucleus, actin and many ABPs can be found in the nucleoplasm as well as in several nuclear foci, which are connected, for example, to transcription, chromatin remodeling and DNA damage.

third phase of our longer import FRAP assay. Whether individual nuclear events such as the formation of nuclear F-actin and sudden increase in application of actin containing nuclear machinery such as polymerases (Baarlink et al., 2013) have an impact on actin shuttling remains to be studied.

The second level of nuclear actin transport regulation can take place at the level of small ABPs, cofilin and profilin, which facilitate import and export, respectively. Because they are universally required for numerous critical tasks all around the cell, I would imagine alterations in their expression levels *per se* are not easily used - at least to a great effect - to influence nucleocytoplasmic shuttling. Rather because these proteins are integral components of cytoskeletal dynamics and thus subject to various regulatory cues, it is probably more energetically favorable to modify their behaviour in transport by, for example, post-translational modifications. In addition to slingshot, which had a significant enabling impact on the nuclear import of actin via cofilin, cofilin is also dephosphorylated by chronophin and several protein phosphatases. These actions on cofilin are countered by LIM (Arber et al., 1998) and TES kinases (Toshima et al., 2001a; Toshima et al., 2001b). Like cofilin, profilin is also dephosphorylated by protein phosphatase 1 (Shao and Diamond, 2012). This action takes place at Ser-137 and is countered by the kinase ROCK (Shao et al., 2008). The presence of a phosphate group at Ser-137 hinders the interaction with actin. In contrast, phosphorylation of profilin at Tyr-129 enhances the interaction with actin (Fan et al., 2012). It is not known whether these modifications have an impact on the shuttling of profilin and thus actin but it should be investigated whether the protein is modified in such a manner also in the nucleus. Logic suggests that modifications enhancing actin interaction might have a positive effect on actin export although careful studies are required to determine such an impact on actin interaction is not mainly applied towards increased polymerization in the nucleus. Nevertheless, it appears phosphatase activity in general could have a positive effect in the shuttling of actin both in import and also tentatively in export. Many of the factors involved in the regulation of actin such as Rho GTPases are also found in the nucleus and could together create a regulatory circuit for the benefit of actin in this compartment as well (Rajakyla and Vartiainen, 2014). How such tinkering of general actin pathways in the name of nucleocytoplasmic shuttling affects general G-actin/F-actin balance and how that might undermine or increase the effect would be of interest to know.

The third aspect in the regulation of nuclear actin transport is provided by the transport machinery itself, namely exportin 6, importin 9 and Ran. While Ran serves to establish the

RanGTP/RanGDP gradient required for active transport in general, beyond this role it is an unlikely candidate for the regulation of nuclear actin in interphase cells. However, it is imported by NTF2 together with CapG (Van Impe et al., 2008), an actin capping protein, perhaps to ensure filamentous actin in the nucleus remains short in length.

Unlike postulated for cofilin and profilin, the two karyopherins transporting actin may well be regulated by their expression levels. This is already known to be true for exportin 6, which remains unexpressed in *Xenopus* oocytes to ensure enough actin remains in the nucleus to form a nucleoskeleton supporting their immense size (Bohnsack et al., 2006). Increase of nuclear actin has also been connected to macrophage differentiation induced by phorbol 12-myristate 13-acetate (Xu et al., 2010). Although they did not investigate the matter in detail, it is quite possible that also here, nuclear actin increase is caused by reduction of exportin 6 expression, because when macrophage differentiation is induced by another agent, all-trans-retinoic acid (ATRA), exportin 6 levels drop (Suzuki et al., 2008). Exportin 6 levels have also been noted to significantly drop in prostate cancer by miRNA-1 (Ambs et al., 2008). In the *drosophila* brain, excess nuclear actin resulting from mutant exportin 6 disrupts working memory and this effect appears to propagate through misregulated MRFT-A (Thran et al., 2013).

When is importin 9 dependent regulation of nuclear actin then used? The shortest and most honest answer currently is that it is not known. For example, when cells of high actin N/C ratio slow down the shuttling of actin, we do not know which system is affected first or whether the import and export processes are regulated in concert. It stands to reason that it may be energetically favorable to just reduce the expression of exportin 6 to induce accumulation of nuclear actin rather than increase the expression of importin 9, as the examples above show. According to this logic, it might a similarly economic solution to deplete nuclear actin by reducing the expression of importin 9. Something like this may very well take place in epithelial cells, where nuclear actin depletion has been linked to cellular senescence (Spencer et al., 2011). However, fibroblasts may assume a completely contrary strategy upon senescence (Kwak et al., 2004), which may give us hints regarding which pathway is initially regulated at least in this particular case. When young human diploid fibroblasts were exposed to stress-induced premature senescence, cofilin was activated while exportin 6 and Ran levels remained intact. This was in contrast to older cells in replicative senescence, where also exportin 6 and Ran levels had begun to decrease (Park et al., 2011). It is therefore possible that nuclear actin accumulation is triggered by increased import and as

process moves forward, augmented also by the collapse of Ran gradient and exportin 6 pathway. Unsurprisingly, the authors did not address the role of importin 9 in these studies but it would be interesting to know whether cofilin activation is coupled to some level of increase or activation of importin 9. While pondering the possible ways of importin 9 regulation, it is important to keep in mind that the ~115 kDa isoform used in our studies may not be the only isoform of the protein and some reports describe versions with different electrophoretic mobility, suggesting the presence of degradation products, splice variants or posttranslational modifications (Kortvely et al., 2005). Importin 9 can also bind protein phosphatase 2A (Lubert and Sarge, 2003), which can also target cofilin (Ambach et al., 2000), potentially subjugating these two important actin import factors under the same regulatory cues.

Our studies have unambiguously revealed the machinery behind the active translocation of actin into the nucleus and clarified the confusion that reigned over the export of actin. However, is it not possible that in addition to this, actin might also cross the NE passively, even if only in miniscule amounts? Our data does not exclude this possibility. In cells where both exportin 6 and importin 9 are depleted, still over half of the nuclear actin is present when compared to control cells [II, Fig 4D]. Of course, RNAi never completely negates the presence of the suppressed protein product but nonetheless, some actin may continuously leak in and out of the nucleus in small amounts. Such passive background actin shuttling could be harnessed, for example, to guarantee some actin is always retained in the nucleus for the critical functions described for it (Skarp and Vartiainen, 2010). For the actual regulation of nuclear actin levels though, the cell may need to resort to active measures. Actin has a negative net charge but also contains a hydrophobic loop, which has been suggested to play a role in filament stability (Scoville et al., 2006). In support of the scenario described for passive transport above, perhaps these features might make actin amphiphilic enough to take advantage of a spontaneous transport across NE described for certain ABPs (Kumeta et al., 2012). A negative charge itself has been shown to increase the probability of binding events with NPCs (Goryaynov and Yang, 2014). If actin is transported in such a manner, it is possible that posttranslational modifications, such as oxidizing M44 (Lundquist et al., 2014), might contribute to this pathway. The proposed hypothetical passive “tunneling” of actin in and out of the nucleus even if not constitutively used, might become more probable in certain cellular situations. For example, when cells are stressed, nuclear pore size tends to increase, which just might be enough to facilitate the passive translocation of actin (Feldherr et al.,

2001; Willison and Johnston, 1978). Finally it should be noted that a large number of ABPs localize to the nucleus and whether they reach the nucleus passively or actively, those that shuttle in steady state cells are likely to smuggle bound actin into the nucleus although their direct contribution to nuclear actin levels may not be physiologically relevant.

IX FUTURE PERSPECTIVES

In the light of our findings, it would be interesting to proceed further into the transcriptional regulation by actin dependent processes. For example, regarding the amount of actin in the nucleus, what is the concentration where RNAP II dependent transcription is most optimal and at what concentration it starts to decline? Or what is the nuclear actin concentration where MRTF-A export becomes less than its import, it certainly should be in the range of the concentration required for general RNAP II functionality? However, certain cell biological realities make questions like this and other inquiries into the function of nuclear actin extremely difficult and can be reduced to one word: dynamics. The contents of the cell are extremely mobile and it is non-trivial to understand at a molecular level e.g. chromatin organization (Bolzer et al., 2005; Kalhor et al., 2012). To the untrained eye, it may appear chaotic but it nonetheless accommodates all critical nuclear processes such as transcription, chromatin remodeling and DNA damage (Baltanas et al., 2011; Cheutin and Cavalli, 2012; Davidson et al., 2013; Gagniuc and Ionescu-Tirgoviste, 2013; Lee et al., 2011; Mehta et al., 2013) many of which coincide spatially and temporally. The fact that nuclear actin and sometimes also myosins have been deemed indispensable for these processes prompts the question how exactly do they do it? Two alternatives come to mind.

1) Higher order actin structures are basically non-existent in the nucleus and are only formed upon specific action such as transcription or chromatin remodeling, which requires changes in the 3D architecture.

2) Higher order actin structures are continuously present in the nucleus forming a mesh, which together with other components can be described as the nucleoskeleton. Upon actions like transcription or chromatin remodeling, this mesh needs to be manipulated to permit these events.

Some hints regarding the feasibility of the mentioned scenarios can be derived from existing literature. For instance, in the huge oocytes of the frog, nuclear actin forms an apparently

homogenous network throughout the whole nucleus and due to the size of the nucleus, DNA concentration is only a fraction of somatic cells (Bohnsack et al., 2006). Due to ample space in these nuclei, there might not be any need to clear space when nuclear architecture requires modification. The *Xenopus* actin mesh supports the nucleus structurally but what if such a network is in fact present also in somatic cells but due to tight packaging of chromatin, can only manifest in a limited fashion and therefore does not provide similar structural support? Such actin network would then have to be remodeled during transcription events, of which the nuclear actin seen by Baarlink *et al.*, could be an example (Baarlink et al., 2013). In that paper, the transient F-actin is dependent on formins, which create straight actin cables and the network was present throughout the nucleus. The transcription facilitated by them is dependent on SRF, which has thousands of binding sites in the chromatin and regulates almost an equal number of genes (Esnault et al., 2014). These studies therefore show that actin-based nucleoskeleton is a substantial structure, which may literally support transcription.

But how, exactly, could actin polymers support transcription? Although Arp2/3 complex associates with RNAP II (Yoo et al., 2007), it was not required for the SRF dependent transcription based on formins (Baarlink et al., 2013). A hypothetical Arp2/3 based actin structure in the nuclei of somatic cells would then have to be similarly transient as the formin network and thus remain dispensable for the described SRF activation. Another possibility although quite improbable is that Arp2/3 based nuclear actin structures are not transient but permanent or at least extremely resilient and crowded, because it would have to escape depletion by RNAi and staining by phalloidin but still remain dispensable for SRF activation. Going through so much trouble sounds energetically inefficient and because formins already function in this context, maybe Arp2/3 complex is indeed dispensable for general gene expression. Or perhaps constitutive Arp2/3 dependent F-actin remodeling does not take place in the nucleus, because the formin based F-actin which happens to exist, is itself so crowded in the nuclear environment that branching is severely restricted? Circumstantial support for this idea is given by a study, which shows that a chromatin remodeling complex binds F-actin (Rando et al., 2002), possibly to increase its spatial stability in the changing microenvironment of the nucleoplasm. However, the fact that formin based nuclear actin filaments retain their phalloidin stainability – albeit at a weak level – shows, that at least the formin based filaments are not so crowded to exclude this small molecule with F-actin affinity.

I favor a model (Figure 5), where the actin based nucleoskeleton is run by formins but should nevertheless require Arp2/3 or some other factors to provide some degree of branching. With or without Arp2/3, nuclear actin filaments under the guidance of formins physically exert force on chromatin. This action together with chromatin remodelers and histone modifying enzymes results in changes in the 3D structure of chromatin, which facilitates e.g. transcription. Perhaps different actin polymerizing machinery is required for different subtasks in this process. For example, formins may be required to rapidly conquer larger nuclear areas and make them transcription compatible, which is greatly needed during sudden situations like stimulation by serum. Arp2/3 polymerization may be required for specific things like different polymerases or perhaps just to move the histones a bit differently (Kulaeva et al., 2009). It would also be interesting to investigate whether the Mediator complex binds actin. In the literature, the links between the Mediator and actin are few. Some early reports implicate TFG3/TAF30/ANC1 as a potential Mediator interacting ABP, because it is a component of the yeast Mediator and SWI/SNF, binds RNAP II and has been implicated in cytoskeletal function (Cairns et al., 1996; Henry et al., 1994; Kim et al., 1994; Welch and Drubin, 1994). Also the F-actin screen from Görlich laboratory revealed a couple of components, TRRAP and BAF155, which have been placed in close proximity with the Mediator either physically or functionally (D'Alessio et al., 2009; Meyer et al., 2008).

Also the actin probes developed by the Mullins lab, which show enrichment of actin filaments in the interchromatin space may be reflection of either of the two presented scenarios (Belin et al., 2013). Perhaps the probes betray the dynamics of the actin based phenomena in question and stabilize the actin structures so that they can be more easily visualized. The first scenario is also supported by the general observation that the nucleus is continuously flooded by at least two factors limiting filament elongation, CapG by NTF2 and cofilin by importin 9 while a filament promoting factor, profilin, is efficiently exported from the nucleus by exportin 6.

Such basal actin network as suggested in the second scenario would have to be microscopic enough to escape staining by phalloidin, and in general, detection by light microscopy. Or perhaps not; Baarlink *et al.* already showed some phalloidin stainable nuclear actin becomes visible when the exposure is increased enough – they are just usually obfuscated by the extreme glow of cytoplasmic actin signal (Baarlink et al., 2013). If they were able to display such formin dependent actin mesh when cells were stimulated, perhaps the basal actin network in non-stimulated cells could be detected by yanking up the exposure still a notch or

two? If not, super resolution light microscopy methods could be applied to investigate this issue. Certainly, super resolution approaches are not easy to implement in practice and some of them are unfeasible for the imaging of nuclear dynamics due to their high sample acquisition times (Deng et al., 2014). However, the super resolution field is progressing (Han et al., 2013; Jungmann et al., 2014; Small and Stahlheber, 2014; Uphoff and Kapanidis, 2014; Zhang et al., 2014) and even conventional diffraction limited microscopes can be converted to acquire super resolution images (Yuan et al., 2014). The scientist is ultimately limited and imprisoned by the methodology of choice and sometimes it feels we are unable to see the forest because of the trees. Application of such strategies would allow us to literally see more clearly.

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