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Abstract

Cell migration is a fundamental process responsible for numerous physiological and physiopathological conditions such as inflammation, embryogenesis and cancer. This central aspect of cell biology has seen quantum leaps in our understanding of the coordinated regulations, both spatial and temporal of numerous cytoskeletal proteins and their orchestrations. At the molecular level, this dynamic cellular process can be naively summarised as an engineered cycle composed of three distinct phases of (1) formation of cellular protrusion to initiate contact followed by (2) the adhesion with the external environment/cell-extracellular established connection and (3) the actomyosin force generation to consequently remodel the cytoskeleton. A prominent factor that regulates cellular motility is S100A4, a protein that has received constant attention for its significant role in cellular migration. Consequently, and in order to focus further the impact of this work, the present chapter aims to review some of the actomyosin proteins/complexes that have been demonstrated to be crucial players of the cyclic migration process but are also S100A4 interactors. In doing so, this chapter aims to capture a picture of how expression of this small, calcium-binding protein may, in essence, remodel at different levels the actin organisation and fulfil the motility engineered cycle of protrusion, attachments and contractions.

Keywords: S100A4, Actin, Arp2/3, formin, tropomyosin, myosin, Rho-GTPases, Rhotekin

1. Introduction

Cellular motility has been an essential cellular phenomenon throughout phylogeny that has allowed organisms to survive, adapt and prosper in different environments. It is engrained in the chemoattraction and nutrient-seeking mechanisms in protozoa such as Dictyostelium.
discoideum [1]; whilst in metazoan, it is found to be a key concept for physiological regulations during all aspects of life. For instance, cellular migration in the early stages of gastrulation allows the coordinated movements of progenitor cells for the subsequent development of the different layers of precursor tissues and organs [2, 3]. Equally important is the profound effects cellular migration occupies in the process of healing during wound closure and/or tissue regeneration undertaken by tissues of the mesenchyme or epithelium [4]. Cell motility also plays essential functions during all stages of the immune response, from the development of mature effector cells, to endothelium trans-crossing and phagocytosis [5–7]. Given the indispensable roles of cellular migration in these events, and others, it is therefore not surprising to learn that loss of functions of many actin-regulating genes result in embryonic lethality or severe immunodeficiency syndromes [8].

Other than these physiological conditions, cellular motility is essential in regulating some of the physiopathological steps seen in disease. As example, it is well-documented that cellular migration is one of the prominent factors involved in the later stages of carcinogenesis and the subsequent phases of metastasis [9–11]. Cancer cell dissemination is clearly dependent upon the ability of migratory tumour cells to evade away from their initial niche, leading to the colonisation and formation of distant secondary lesions in the body [12].

At the molecular level, cell migration requires the coordinated regulations, both spatial and temporal of numerous cytoskeletal proteins, to orchestrate the dynamic cellular processes needed for cells to acquire movement. In this context, the actin cytoskeleton and the closely linked myosin network play essential functions [13, 14]. The process of cellular motility can be summarised as an engineered cycle composed of three distinct phases which are, (1) formation of cellular protrusion in the forms of lamellipodia and fillopodia to initiate contact and adhesion with the external environment, (2) regulation of cell-extracellular matrix established connections, usually integrin-dependent, and (3) force generation by the actomyosin network which will control both the structure and organisation of the motile architecture [15]. I provide here a brief overview of some of the different elements and protein complexes that are regulated during this migratory cycle, focusing primarily on specific components of the actomyosin complexes.

A group of low-molecular weight polypeptides that has been demonstrated to have key functions in remodelling the overall actin cytoskeletal network is the S100 protein family [16]. Composed of approximately 25 members, the presence of the majority of these in different cellular systems, both in vivo and in vitro has been associated with significant changes in cellular migration. One of the most prominent members of this family to have been linked to regulate cellular motility is S100A4, a protein that has received constant attention for its significant role in promoting cancer metastasis [16–18]. Consequently, and in order to emphasise the impact of this work and strengthened its delivery, I have concentrated our attentions on actomyosin proteins/complexes that have both been demonstrated to be crucial players of the migration process but also S100A4 interactors. In doing so, this chapter aims to capture a picture of how expression of this small, calcium-binding protein may in essence remodel at different levels the actin organisation and fulfil the motility engineered cycle of protrusion, attachments and contractions.
2. The actomyosin machinery in cellular migration

Motility can be seen as a lone activity where a single cell may migrate (also known as amoeboid or mesenchymal migration [19, 20]) or is referred to as collective, if this effort is the result of concerted effort undertaken by numerous cells, either in sheet or clusters [9]. Equally important is the cell physiognomy that will be regulated in the process. Mesenchymal motility as seen during fibroblast migration leads to cellular characteristic of a more elongated spindle-like shape. In this type of migration, an actin-rich leading edge can be observed, where extension of the front leading edge is driven by actin polymerisation [21]. In amoeboid migration, cells adopt a more rounded morphology and rely on the contraction-based membrane blebbing and enriched levels of myosin II at the cell rear [22]. Both of these migratory processes have been shown to play important roles in both physiological and pathological events.

The complexity of the different types of cell migration that can be used is mirrored by the number of different molecular pathways that are available to orchestrate these processes. Among them, however, the remodelling of the actin cytoskeleton and its organisation stands as an irreplaceable circuitry which is undisputably common to all. At the molecular level, this network is considered to provide the platform where physical forces will be exerted. Pushing forces generated by the assembly of filamentous actin (F-actin) will encourage the formation of cellular protrusions, such as filopodia, lamellipodia, blebbing and the most recently characterised invadopodia [23–25]. These changes in actin polymerisation and their dynamics will be directly responsible for reshaping and remodelling the underlying plasma membranes.

2.1. Cellular protrusions and regulators

Actin polymerisation. The actin filaments are considered to be the backbone of cellular protrusion, providing the physically necessary special platform that will provide sufficient force to deform the plasma membrane. Their overall organisation relies primarily on their polymerisation from monomeric globular actin (G-actin) into long arrays. This process is regulated by numerous partners but the core regulator lies in ATP hydrolysis to promote actin molecule recognition and bonding between two monomers. When ATP bound G-actin is hydrolysed, the newly created ADP+Pi G-actin structure can form stable filaments. Binding of the nucleotide takes place in the high-affinity binding site located in the deep upper inter-domain cleft of actin (Figure 1). The presence of a cleft around exposed subdomains II and IV results in the polarisation of the monomeric structure and is referred to as the pointed end (Figure 1). The other exposed side, composed of subdomains I and III is known as the barbed end [26] and constitute the major binding site for most actin binding proteins ([27], Figure 1). This is a very important distinction which will result in sticking difference in behavioural characteristics in both G-actin and F-actin, of which polarised polymerisation is only one aspect.

In the early stages of assembly, also known as nucleation, actin protomers aggregate in an energetically unfavourable process to form a dimer that is more likely to dissociate. Addition of another subunit stabilises the complex and represents the nucleus, a state where actin polymerisation is now more favourable than dissociation (Figure 1). The association of monomers
Figure 1. Actin structure and cartoon of F-actin polymerisation. (A) G-actin monomer at 1.54-Å resolution bound to ADP (PDB code 1J6Z) by Otterbein et al. [165] obtained from striated rabbit muscle tissue. Subdomain I (red, residues 1–32, 70–144 and 338–374), subdomain II (yellow, residues 3369), subdomain III (green, residues 145–180 and 270–337) and subdomain IV (grey, residues 181–269) are highlighted, resulting in the orientation of the actin molecule with the pointed end (− end) and the nucleotide cleft in the upper part, and the barbed end (+ end) in the lower part. (B) Process of actin polymerisation highlighting the steps of nucleus formation and filament formation. Please note this is a schematic representation which does not illustrate the current model of actin polymerisation initially proposed by Holmes et al. [166] suggesting that actin filaments are structured as a two right handed long pitch helices of head to tail bound actin subunits or a single left handed short pitch helix with consecutive lateral subunits staggered with respect to another by half a monomer length.
into a trimeric structure is seen as the rate limiting step of the whole polymerisation process as it is reversible where monomers can easily dissociate [28–30]. It is during the stage of nucleation that addition of further actin subunits is supported at both ends. Once the nucleus and newly added monomers have been locked into position by conformational changes, the process of elongation begins and the addition of actin molecules at the barbed end of the filament can be seen, resulting in the formation of structural polarised complexes (Figure 1). Whilst G-actin subunits can self-assemble, this process only occurs if the concentration of actin exceeds a critical concentration.

Within cells, a growing number of binding partners, or actin-binding proteins, will act both antagonistically and agonistically to regulate the polymerisation process. Some factors will act as nucleators, such as formins and Arp2/3, facilitating the process through providing a scaffold structure which encourages de novo assembly. Others will regulate the overall structure of filaments through their remodelling in larger structures. Examples provided here will control the cross-linked state of actin filaments through the involvement of bundling regulators such as the tropomyosins and to an extent myosins. Involvements of all these factors, as well as many others that are too numerous to be listed, here, will be responsible for the remodelling of the actin cytoskeleton into different substructures seen during cell migration (Figure 2).

When grown in a 2D environment, cells will encourage the formation of differential planar filamentous actin, in the form of filipodia/microvilli or sheet-like structures referred to as lamellipodia (Figure 2, [31, 32]). Whilst the former act as sensory organelles that enable cells to probe their local environment, through the formation of thin extensions that are mainly made of long, unbranched bundles, the latter is viewed as the main driving force for locomotion, through the organisation of short branched actin networks (Figure 2). In both instances, however, regulation of F-actin polymerisation, especially at their barbed end is essential, in order to control their elongation in the direction of the plasma membrane and is thought to require nucleation-promoting factors where both formins and Arp2/3 have been shown to play key functions (Figure 2, [31, 33]).

**Formins.** The family of formins, encoded by 15 different genes in mammals represent a cluster of large multi-domain proteins, grouped in eight different subfamilies, that regulate actin nucleation and polymerisation, primarily at the barbed end [34, 35]. Their nucleation abilities are regulated by signature regions of the proteins, the formin homology domains 1 and 2 (FH1/FH2), located at the C-terminus (Figure 3). Although a clear picture as to how formins nucleate the assembly of actin filament is still under investigation, the C-terminal region has been demonstrated to be a key regulator as it recruits actin monomers in the presence of profilin. The FH2 domain also plays key function during the polymerisation of F-actin as it allows addition of large amounts of actin subunits at the barbed end [36]. This continuous tracking results from alternate contact of the two halves of the FH2 domain with the two most terminal actin subunits in the filament, allowing the sliding of the whole formin molecule through an open/closed conformation as the subunits, remaining bound as subunits are added [37–39]. For some formins, activation is also controlled through the release of the head to tail auto-inhibition as well as through the movement of proteins away from the leading edge [40]. For such formins, classified as diaphanous-related formins (DRF) [41], comprised
of 4 families; diaphanous (Dia including mDia), Dishevelled associated activators of morphogenesis (Daam), formin-like proteins (FMNL) and FH1/FH2 domain-containing proteins (FHOD) in mammals, the auto-inhibitory mechanism relies on the folding of the N-terminal portion, containing domains FH3, which physically obstructs the diaphanous autoregulatory domain (DAD) at the C-terminus and prevents it to interact with actin molecules ([42]).

Binding of the Rho-GTP to the formin polypeptide in the GBD (GTPase binding domain) region is thought to result, at least in part, to the displacement of the masking DAD region away from the FH3 domain [43]. Molecular mechanisms to explain this process are currently being investigated. The relocalisation of formin to the leading edge is also a key concept to control their activities. Membrane relocalisation has been reported to be performed primarily by Rho-GTPases through their binding to the GBD [44]. Other studies have also revealed that

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**Figure 2.** S100A4 interacting actomyosin complexes and their simplified organisation in the different protrusions of a migrating cell. (A) Actin and focal adhesion organization in a HeLa migrating cell. Staining for F-actin using Phalloidin-rhodamin (red) and paxillin with antibodies coupled to FITC (green) in a migrating HeLa cell. In this image, the actin mediated structures of the filopodium and lamellipodium/lamellum are distinctly visible at the leading edge of the cell. B and C present models for the lamellipodium/lamellum and filopodium and the respective molecular organisation within, focusing on the proteins presented in this chapter. (B) A simplified model for lamellipodium/lamellum formation. In the lamellipodium, the Arp2/3 complex via activation by WASP/WAVE complex interacts with actin filaments resulting in the nucleation of new actin filaments from the side of existing filaments. Formin proteins are also found at the barbed end of filaments. Limprin and the Rho-GTPase-Rhotekin complexes could get regulated by S100A4 to promote lamellipodia protrusions. In the lamellum, tropomyosin wrapping around the actin filaments prevents interactions with other actin binding proteins. NMMIIA regulates retrograde flow in the lamellum. At the interface of the lamellipodium–lamellum, actin is depolymerised. Interactions of S100A4 with tropomyosins and the NMMII complexes have been reported and could result in significant changes in their overall organisation. (C) A simplified model for filopodia formation. In this diagram, actin polymerisation promoted by the Arp2/3 complex leads to the branching and extension of nascent individual actin filaments in the filopodium. Recruitment of the formins to this location promotes the elongation of the filaments through the addition of actin monomers at the barbed end. Other actin bundling proteins such as fascin regulates filopodia stability through the clustering of actin filaments. Both the Limprin and the Rho-GTPase-Rhotekin complexes could be regulated by S100A4 to control filopodial protrusions.
the FH3 and DD (dimerization domain) regions on mDia also mediate its membrane localisation [45, 46], indicating that other proteins capable of interaction with such domains could be efficient regulators. The liprin family have been suggested to possess such properties and have been put forward as another series of proteins which may affect formin cellular functions [47].

**Arp2/3 complex.** Another regulator of actin nucleation and polymerisation that plays a critical role in the process of formation of lamellipodia and filopodia structures is the 220kDa Arp2/3 factor [48]. Composed of seven different subunits (ARPC1-5, Arp2 and Arp3), this complex promotes the formation of newly formed actin filament from the sides of existing filaments, forming a 70° side-branched network from pre-existing filaments [49, 50] (Figure 3). This property is predominantly the result of a striking similarity between the Arp2 and Arp3 proteins and that of monomeric actin molecules [51], providing a mimicking dimer that serves as a cooperative docking for other actin subunits and in doing so, accelerates the nucleation

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**Figure 3.** Cartoon showing some of the regulation steps for different actin nucleating proteins. (A) Activation of the Diaphanous-related formin. Autoinhibition of the actin nucleating ability is due to the interaction of the C-terminal Diaphanous auto-regulation domain (DAD) with the N-terminal FH3 (Formin homology) domain. Rho-GTP Binding of the GTP bound Rho to the GTPase binding domain (GBD) region is thought to lead to a partial displacement of the DAD as well as relocalisation of the complex, resulting in the unfolding of the protein and the relieve of the autoinhibition (DD dimerization domain). (B) Cartoon representation of the Arp2/3 complex and nucleation of a branched filament. The Arp2/3 complex initially binds to the pointed end of the mother F-actin. Binding of the WCA domain of a nucleation promoting factor (NPF) to exposed regions of Arp2 and Arp3 allows the delivery of actin monomer and initiate the polymerisation of a nascent branched filament as elegantly demonstrated [52].
process and thereby reduces the rate of the limiting step at this stage [52]. Whilst all components of this hetero-heptamer are critical for the generation of newly formed actin arrays from the pointed end, albeit with distinct functions, the Arp2 and Arp3 proteins are seen as the principal components responsible for establishing the initial base of the newly assembled filament [52]. The other components, especially ARPC1, are mainly involved in the binding to the mother filament [53, 54] (Figure 3). Interestingly, weak basal activity of the purified Arp2/3 complex in promoting actin nucleation and branch formation [55, 56] highlights its intrinsic association with other regulators [57]. Activation of the Arp2/3 complex is regulated by different complexes at distinct cellular locations. Whilst Arp2/3 is controlled by the WAVE regulatory complex in a Rac-GTPase pathway in lamellipodia, the Wiskott-Aldrich syndrome protein family (WASP), downstream of Cdc42, is predominantly implicated with the regulation of Arp2/3 in filopodia [58]. By all accounts, these nucleation-promoting factors (NPF) stimulate Arp2/3 mediated-nucleation through a WCA domain found at the C-terminus (Figure 3). It is thought that the WH2 region within the WCA domain is responsible for binding and therefore delivering the actin monomer, whilst the CA sequence promotes binding to the exposed regions of both Arp2 and Arp3 [59]. It is the clustering of the different subunits, along with the newly added actin molecule that encourages formation of a new nucleus and further actin polymerisation, resulting in the elongation of 70° side-branched network. Since the NPF family has been continuously expanding, it is now subcategorised into five groups including WASp and neural Wiskott-Aldrich syndrome protein (N-WASP), three SCAR/WAVE proteins and the recently identified factors WASH, WHAMM and JMY [60].

Taken all together, actin polymerization at the leading edge is a vital process for cellular migration, through the orchestrations of events that will ultimately lead to different cellular protrusion events. In this section, different actin polymerization factors and their functions (Arp2/3 and Formin) were briefly explored. One should remember that this is only a preferential view in regards to their potential involvements through a S100A4-dependent process and that numerous other regulators not mentioned here play equally vital roles in the process of actin remodelling and cellular migration.

Away from the leading edge and the protrusions of the lamellipodia and filopodia, the array of filamentous actin is seen to exist as more bundling rather than the branched sheets reported previously, mainly due to the interaction of different actin-binding proteins. This contractile network is seen as a unique structural complex, spatially posterior to the lamellipodium, and is referred to as the lamellum [61].

2.2. Lamellum and cellular contractions

In the spatial arrangement of the lamellum, filaments are organised in different structures, known as stress, dorsal and ventral fibres; they are the result of interaction of the actin filaments with different partners (Figure 2). It is in this context and primarily through the control of the tropomyosin and myosins that contractile forces are exerted to manipulate their overall organisation. Generation of such tensile forces is provided by the myosin network, mainly non-muscle myosin II (NMMII) which is responsible for the majority of the morphological and architectural reorganisations that promote cell movement.
Tropomyosin. The tropomyosin (tpm) family is composed of four separate genes, TPM1-4 which can be further subdivided, due to different alternative splicing and post-translational modifications, resulting in the presence of more than 40 tpm products [62, 63]. Interestingly, these isoforms have been shown to interact differentially with actin filaments, ensuing biophysical and dynamic property changes, as well as different subpopulations occurring in different locations and in abundance [64]. It is unclear today, how these association-promoting mechanisms are regulated over time and space, to result in such highly selective and discriminatory organisation [65, 66], but all interactions necessitate dimerization as well as head to tail contact between individual complexes to form continuous actin/tropomyosin filaments [67]. The formation of these highly selective complexes is thought to seclude, or at least regulate the interactions of other actin-binding proteins with these actin filaments, therefore playing a major role in determining the functions of different filaments [68, 69]. For instance, the absence of tropomyosin in the leading edge is thought to be a predominant factor that allows specific branching of the actin network, since different isoforms have been shown to compete and inhibit the actin polymerisation of Arp2/3, at least in vitro [52, 70]. Equally important is the fact that tropomyosin has been implicated in the regulation and recruitments of NMMII in stress fibre formation [68], regulating both elasticity and stiffness [71].

The overall organisation of the actin cytoskeleton can also be dictated by actin bundling and contractile motor proteins. Binding of individual filaments, actin cross-linking and motor proteins allow the formation of thicker, linear and either paralleled or antiparallel filamentous F-actin networks that can be found in all subcellular localisations. In the lamellum, the class II non-muscle myosin family has been shown to be a key regulator, participating in the bundling of actin filaments and generating mechanical forces, which result in filaments sliding and/or contractions [72, 73].

Non-muscle Myosin II family. The myosin II family, which encompasses a group of 34 different isoforms, are expressed in all eukaryotes, except plants with 15 genes corresponding to the myosin II cluster. These myosin II motor proteins are exclusively expressed in non-muscles cells and can therefore be referred to as non-muscle myosin II (NMMII). In its fully formed state, the NMMII complex corresponds to a 525 kDa structure composed of six non-covalently associated polypeptides. The backbone of this is a homodimeric myosin heavy chain containing a head domain and a long coiled-coil rod domain, separated by a neck area. Two essential light chains and two regulatory light chains bind to this backbone [74]. The N-terminal head portion of the heavy chain is globular in structure and possesses the actin-binding domains as well as the ATPase activity which is required for movement towards the plus end of the actin filament, thereby inducing sliding between filaments and force generation. In contrast, the long coiled-coil C-terminal part of this protein is essential for dimerization and further assembly of one hexamer to another thereby forming a multimeric network of bipolar NMMII with motor domains positioned at both ends of the filaments. Bipolar filaments of NMMII formation are the result of electrostatic interactions between these C-terminal helical tails [75] and are essential for its cellular functions. Stability of these NMMII filaments is controlled by phosphorylation of the myosin heavy chain [76, 77] or by interaction with proteins that recognize the C-terminal helical tail region.
NMMII isoforms in the form of NMMIIA, NMMIIB and NMMIIC are found in most, but not all human cells and most mammals. NMMIIA and NMMIIB are expressed at similar levels in endothelial and epithelial cells. There is, however, little or no NMMIIC present in these cell subsets although it is known to be much more prominent in cells of lung and nervous tissues. Their location appears to be cell-specific and will be regulated differentially depending on the type of cell migration. Perhaps their expression patterns further reflect their function, since NMMIIA and NMMIIB play fundamental roles in mediating cell shape and matrix interactions during migration, whilst a clear role of NMMIIC in this process is still missing. As a consequence, during mesenchymal motility, NMMIIA is localised throughout the cells, in cellular protrusions and in both the lamellipodia and lamella of migrating cells [78] where, with Arp2/3-dependent actin polymerisation in the former, it results in actomyosin contraction and the retrograde flow of F-actin towards the cell body [61]. NMMIIB is predominantly found in the central and rear regions of the cell but not at the cell front [79].

Whilst formation of protrusions is a key element of cellular motility, it is equally critical that these newly created extensions also adhere and attach to the substratum, as their inability to do so result in their rearward movements in waves due to cellular tension [80]. Nascent adhesions are the first observable adhesive structures established in the lamellipodium [72, 81]. Their transient nature will force them to either disassemble quickly or mature into larger complexes known as focal adhesions which reside at the boundary of the lamellipodium and lamellum [82]. Actomyosin contraction is the main regulator that controls nascent adhesion enlargement [72]. This initial interaction with the extracellular environment will in turn induces activation of downstream effectors transmitted to the plasma membrane to encourage further integrin adhesion and clustering through their activation as well as changes in confirmation of extracellular matrix proteins [83]. Following on from this integrin activation, other downstream cellular pathways are also instigated; these lead to the recruitment, in a force dependent manner, of numerous framework and adaptor proteins which associate to form an adhesome [84]. Indeed mechanical stretch generated during this process induces changes in structural protein configurations, thereby encouraging binding of certain factors to other partners, as is the case for paxillin, vinculin, talin and actin, three essential component of the adhesome/adhesion complex [85, 86] (Figure 4).

NMMII cross-linking and contractile functions to remodel the actin cytoskeleton are regulated by phosphorylation of (1) regulatory light chains (RLC), (2) heavy chains (HC) and (3) myosin’s ability to assemble into filaments. The RLC polypeptide can be specifically phosphorylated at different regulatory sites. The most prominent site is Ser19, leading, when phosphorylated, to a significant increase in ATPase activity of the head domain in the presence of actin [87]. Phosphorylation sites in the C-terminus of the myosin heavy chain have also been identified, although the true implications of such changes in ATPase activity remain unclear. Regulation of the reversible phosphorylation of specific residues is obtained through the activation of different kinases and phosphatases, depending on the residues considered. Whilst phosphorylation at residue Ser19 will be regulated by MLCK (myosin light chain kinase) and MYPT1 (myosin phosphatase target subunit 1), the most prominent examples, respectively [88, 89], the C-terminal region of the myosin heavy chain is directly targeted by kinases such as TRPM7 (transient receptor potential cation channel subfamily M member 7) and PKC (protein kinase C) [77, 90].
Figure 4. Myosin IIA expression in Cos7 cells regulates actin fiber formation and focal adhesion maturation. (A) Regulation of the actin organisation in Cos7 cells in the presence of NMMIIA. Cos7 cells were seeded into 24 well plates and left to incubate for 24 h. Cells were washed and fresh medium without antibiotics were added into each well 4 h prior to adding transfection mixture containing either the empty PeGFP-C3 plasmid (green cells labelled as A’) or the PeGFP plasmid expressing NMMIIA (green cells labelled B’). Following a 48-h incubation, cells were fixed, permeabilised and stained either for actin (Phalloidin-rhodamine (red)) prior to mounting and viewing by epifluorescence microscopy. Note that the expression of NMMIIA leads to significant increases in stress fibres formation (B’ where both NMMIIA and actin colocalise). (B) Focal adhesion organisation in Cos7 cells in the presence of NMMIIA. Cells grown as above were transfected with an empty PeGFP-C3 plasmid (green cells labelled as C’) or the PeGFP plasmid expressing NMMIIA (green cells labelled D’) prior to fixing, permeabilisation and immunostaining for paxillin using a mouse anti-paxillin primary antibody and a secondary anti-mouse rabbit Alexa-568 secondary antibody (red), prior to mounting and viewing by epifluorescence microscopy. Note that the expression of NMMIIA leads to significant increases in formation of paxillin cluster at the end of the myosin fibres (D’ where paxillin foci are seen).
Compelling evidence also demonstrates that phosphorylation and interactions with the C-terminal region of NMMII regulate assembly of the complex into filaments. Such post-translational modifications also result in a change in overall structure. In its fully dephosphorylated form, the compact NMMII complex adopts an asymmetric state, unable to polymerise which is relaxed into an extended conformation following phosphorylation of RLC. Phosphorylation of the C-terminal region of NMMII by kinases such as TRPM7 and PKC has also been shown to interfere with its abilities to form filaments. Finally, it is predominantly through changes at the C-terminus, via the coiled-coil domain that NMMII assembly into filaments is controlled. Truncation experiments have demonstrated that the C-terminal region containing an ACD (assembly competence domain), as well as the C-terminal tail piece, are both important to promote correct assembly of NMMII into parallel and anti-parallel filaments.

Another key regulator of motility is the cell’s ability to orchestrate the different contraction and tension forces that are necessary to promote movement. In this section, lamellum components such as those of the NMMII and tropomyosin networks have been briefly reviewed. The overarching purpose here being once again to focalise the reader’s mind on certain complexes, which are known interactors for the S100A4 proteins and could be key players in the process of motility observed when this protein is aberrantly expressed. Other pivotal factors that regulate motility via contractions have not been mentioned here but have been addressed in numerous other reviews and chapters.

3. S100A4 protein regulation of the actomyosin cytoskeleton

Initially, referred to as mts1, FSP1, metastatin, p9Ka, PEL98, calvasculin, 42A and placental calcium-binding protein, S100A4 is a low-molecular weight acidic protein belonging to the S100 family. This small polypeptide is characterised, as all other members of this family, by a pair of calcium-binding helix-loop-helix regions referred to as EF-hand calcium-binding domains located at either side of the protein and separated by a hinge region. Over the years, S100A4 has received a large degree of interest in the field of cancer cell biology, since its expression is linked to increased motility and invasion directly promoting metastasis in animals, and it is now considered a potent marker for human metastasis and predictor for poor patient outcomes. Its expression is also observed in non-physiopathological states in different motile cell types in vivo, including those of the immune system (lymphocytes, macrophages and neutrophils) as well as mesenchymal fibroblastic cells. The biological implications of S100A4 expression on cellular migration are well-known, but the mechanisms required to attain such phenotypic changes are not fully characterised. In this part of the chapter, I will review its interactions with the different actomyosin components that have been discussed, whether actin nucleating activators, actin binding proteins or myosin regulators, highlighting how their functions are being affected in the presence of the S100A4 protein.

3.1. S100A4 regulates cellular protrusion

Expression of S100A4 has been correlated to significant changes in overall actin organisation and cellular extensions, with extensive increases in lamellipodia and forward protrusions.
[104, 105] which, in turn, are thought to result in greatly enhanced cellular motility. Similarly, S100A4 has been found to be enriched at both the leading edge and in pseudopodia of migrating cells [104, 106]. A clear molecular explanation as to how S100A4 can regulate such processes is currently lacking, but different hypotheses have been formulated in view of what is known about its different binding partners and regulatory functions.

**Actin interaction.** Direct interaction and binding of S100A4 with actin has been demonstrated. This process is obtained, in the presence of calcium ions, at a ratio of 3 to 5:1. This high ratio of S100A4 to actin is intriguing, especially in view of that of other actin-binding proteins, suggesting possible oligomerisation of S100A4 with itself as well as its ability to cross-link actin filaments [107, 108]. Further association of S100A4 with actin stress fibres have been reported both in vitro and in vivo [108, 109], although it is important to highlight that it is unsure whether this interaction is the result of direct protein interactions or is the result of an association of S100A4 with other actin-associated factors. To this date, the biological consequences of such a partnership have not been deciphered and the true role of their interaction remains to be elucidated.

**Liprin interaction.** S100A4 has been shown to interact with the liprin family of proteins and both can be seen to colocalise at the plasma membrane [110]. Interaction of these two proteins leads to reduction in phosphorylation of the liprin β1 molecule. Current thinking indicates that the liprin family of proteins are key promoters of cellular migration [47, 111]. Among possible functions to regulate cytoskeletal remodelling at the leading edge, liprins have been shown to interact with actin stress fibres and affect the appropriate localisation of the formin mDia to the plasma membrane. Their interaction is modulated by liprin binding to the FH3-DD domains on mDia, preferably when in the open configuration after Rho-GTPase activation (Figure 3, [47]). In cells, Liprin α1 has been shown to form a pentameric complex with another isoform, liprin –β1 as well as LL5 and ERC1/ELKS proteins. This complex is located primarily at the leading edge of migrating cells and stimulates lamellipodia formation [112] as well as integrin-mediated focal adhesion stability [113]. Other reports further demonstrate the essential functions of liprin on stabilising lamellipodia and invadopodia [114]. The true involvement of S100A4 in the regulation of liprin and/or formin activities is currently missing, but one could hypothesise that the three protein functions may be intertwined. Given that S100A4 expression has been linked to filopodia instability [105], and the critical known role of formins in the process [58], one could for instance speculate and put forward a new formin-liprin-dependent function for this small calcium-binding protein.

**Rhotekin interaction.** S100A4 has recently been linked to into the Rho-GTPase pathway through its association with the scaffold protein Rhotekin. The Rho-GTPase family has been demonstrated to be a key regulator of the actin cytoskeleton structure and organisation. Initially, their roles appeared to remodel the formation of stress fibres, myosin activities and focal adhesion formation through modulations of its two main effectors ROCK and mDia [115, 116]. Activation of ROCK, a Rho-associated coiled-coil serine/threonine kinase, would result in phosphorylation of myosin phosphatases, which, in turn, would lead to increase phosphorylation of RLC and subsequent contractibility of NMMIIA (as discussed above). Actin polymerisation through the mDia axis would also be stimulated by ROCK and Rho-GTPase [116, 117], resulting in actin filament and ultimately fibre formation. More recent analysis
clearly demonstrates that Rho activity is a key in the process of restructuring the leading edge in migrating cells, particularly RhoA [118–121]. Activation of RhoA has further been demonstrated to take place within a short distance of the leading edge, regulating cellular protrusion in the process, preceding the activation of other GTPases such as Rac1 and Cdc42 [120]. A ternary complex consisting of RhoA, Rhotekin and S100A4 has been shown to play a key role in hijacking this complex towards the leading edge to stimulate membrane ruffling in lieu of stress fibres [122]. S100A4 binding specifically to the Rho-binding domain of Rhotekin does not interfere with the association of the scaffolding protein with RhoA, and instead, leads to further activation of the complex, although the direct effectors of this process to fully explain how this enhances lamellae formation remain unknown [122].

**NMMIIA interactions.** The best studied S100A4 interacting partner is undoubtedly the NMMIIA heavy chain [92, 123–126]. Structural models have revealed that the S100A4 dimer binds both the C-terminal region of the coiled-coil ACD1 domain as well as the N-terminal part of the disordered tailpiece [127, 128]. Such interaction is thought to induce unwinding and destabilisation of the coiled-coil region, potentially disrupting intermolecular interaction between myosin molecules and subsequent disassembly and losses in contractility [123, 128, 129].

Recent evidence indicates that the retrograde flow exerted by NMMII impedes extension of the leading edge and subsequent rates of protrusions because of reduced actin polymerisation [130, 131]. Therefore, a reduction in contractile forces, as observed when NMMII activity in cells is inhibited by blebbisstatin, leads to significant decreases in actin retrograde flow in the lamellum. This reduced flow leads, in turn, to subsequent increases in actin clustering into bundles at the lamellipodium–lamellum interface, and increased leading edge extension [132, 133]. The opposite experiment where activation of the NMMII complex is achieved through a Eph receptor/RhoA/ROCK signalling pathway was found to inhibit lamellipodial extension [134].

It is therefore rational to suggest that one of the protrusion-promoting abilities of S100A4 may be directly linked to its ability to interfere with formation of myosin fibres and their role in protrusion formation. Experimental support for a S100A4-NMIIA role in cellular protrusion was obtained when S100A4 overexpression in tumour cells led to significant increase in leading edge protrusive activity [104]. This change in phenotype was shown indirectly to be NMIIA dependent since the exogenous addition of antibody targeted towards the NMIIA-binding site mimicked the cellular behaviour [104]. Interestingly, a reverse experiment also confirmed a clear role for S100A4 in this process, since S100A4 (−/−) macrophages demonstrated large amounts of over assembled NMIIA filaments, leading to significant lamellipodia instability and reduced persistence [135]. Because NMIIA has been clearly demonstrated to be the preferential cytoskeletal target for S100A4 to date, it is expected that their partnership may also influence the overall organisation of the actomyosin network in the lamellipodium and lamellum where the majority of the NMIIA pools are located.

Whilst S100A4 expression in cells has undoubtedly been linked to regulation of cellular protrusions, mainly increases in lamellipodia and forward protrusions, and arguably being responsible at least in part for some of the enhanced cellular motility observed, the true molecular processes responsible are yet to be fully characterized. In this section, S100A4 partners
such as Rhotekin, actin or liprin possibly found at the leading edge are presented in order to provide possible explanations as to how such biological properties may be regulated and it is expected that future researches will shed new lights into the true mechanisms that are involved in this process.

3.2. S100A4 functions in the lamellum and cellular contractions

Behind the cellular protrusion of the highly dynamic lamellipodium, the actomyosin network contributes to cell migration through contractility and substrate adhesion [61, 72, 81]. Although direct evidence of S100A4 being localised in the lamella of migrating cells is still lacking; numerous reports have highlighted potential regulatory functions of the S100A4 within this subcellular fraction since its expression has been shown to lead to dramatic changes in numbers and organisation of focal adhesions and actin stress fibres [104, 105, 109, 122, 128, 136]. I will briefly discuss here the different properties that S100A4 encompasses towards a remodelling of this cellular architecture.

**Tropomyosin interactions.** The ability of S100A4 to bind to tropomyosin has been put forward both in vitro and in vivo [137], but with relatively low affinity if at all [138]. The true consequences of this interaction remain to be fully elucidated in regards to biological cellular consequences.

**NMMIIA interactions.** As discussed previously, binding of S100A4 leads to significant disassembly of NMMIIA filaments. Mechanical forces exerted by the myosin network have been shown to be key regulators for the growth and maturation of focal adhesions since altering contractility using either inhibitors [139] or knockout studies [79] results in impeded maturation and stabilisation. Although myosin is not physically present in the adhesion, it influences the process through its attachment to actin bundles with which adhesion is associated [79]. Whilst no direct evidence has been presented to demonstrate that S100A4 interacting with the myosin network results in the loss of adhesion of cells with their substratum, there is a compelling number of reports which have linked S10A4 expression to either stress fibre losses or reduction of focal adhesion stability or maturation [104, 105, 109, 122, 128, 136]. Similarly, regulating the assembly status of myosin via phosphorylation of sites in the C-terminal coiled coil and tailpiece regions have also been associated with reorganisation of the cytoskeleton and focal adhesions. Phosphorylation of serine residue S1943 results in disassembly of NMMIIA filaments [76] and leads in vivo to cytoskeletal reorganisation, whilst conversely inhibiting phosphorylation of the C-terminal tail pieces was shown to induce stabilisation of focal adhesion [140]. Other myosin-disassembling factors which bind to the C-terminal coiled coil and tailpieces regions such as S100P or lethal giant larvae have also been shown to regulate focal adhesions [141–143].

This final section briefly summarizes the best characterized and known S100A4 interactor, namely NMMII, in view of their high binding affinity, as well as by the number of reports highlighting their association. Yet again, whilst we are gathering further understanding related to their association and the different regions of the proteins that are responsible, a true model as to how their interactions regulate cellular motility remains elusive both theoretically and more importantly experimentally.
4. Conclusion

Controlling the actin cytoskeleton and the motility process is a key function, that when going awry, leads to significant pathological conditions. Not surprisingly, mutations or aberrant expression of all actin interacting proteins listed in this section have been linked to diseases, thought to be the result of significantly reduced cellular motility. Mutations in cytoplasmic actin have been related to autosomal dominant hearing loss [144]. Mutation or molecular mechanisms that result in changes of activity of actin-binding proteins, such as the nucleating facilitating protein complex Arp2/3 have been linked, albeit indirectly, to bacillary dysentery, as their functions are high jacked by the *Shigella* bacterial strains to disseminate in the colonic epithelium [145]. Involvement of the Arp2/3 complex and their regulator WASp have also been shown to lead to immunodeficiency and reduced platelet numbers, as the loss of WASp expression leads to the Wiskott-Aldrich syndrome [146]. Formin has also been linked to pathological conditions such as deafness [147] and immunity deficiencies, at different levels, presumably because of the differential expression of formins isoforms in different cell systems [148]. Mutation in NMMII has also been associated with a multitude of defects [89], with for instance, loss in NMMIIA affecting platelet and leukocyte dysfunction, renal diseases, loss to cataracts formation and neuronal disorders [14]. Equally important is the fact that differential expression of these factors and/or their activations are also key regulator of changes in cell division and migration/invasion, playing a predominant role in tumorigenesis and metastasis [14, 15, 149]. In this context, the high levels of S100A4 expression have also been shown to be significant determinant allowing cellular spreading and distant tumour formation. Beside its role in cellular motility through its interactions with actomyosin components which have been discussed throughout, S100A4 has also been demonstrated to play key roles in both cellular motility through the interactions with other partners. Both the Wnt/β-catenin [150] and the AKT/slug [151] pathways have been shown to be capable of regulation by S100A4, leading to changes in cytoskeletal architecture and overall cellular migration. Other signalling pathways such as the PI3K/AKT/mTOR [152] or the NF-κB [153] route have also been shown to be capable of activation by S100A4 leading to significant changes in migration but without directly linking this process to cytoskeletal reorganisation.

Another important aspect of S100A4 ability to encourage cellular migration and potential chemotaxis relates to its presence in the extracellular matrix. In the context of this chapter, I have concentrated our efforts to consider cytoplasmic S100A4, where it is found at concentration as high as 10 μM [154]. Traces of the protein have, however, also been reported both in medium of cultured cells [155, 156] and in biological fluids [157, 158], whether physiological or pathological, where it is thought to regulate the activities of metalloproteinases [159] or cellular receptors such as annexin2/plasmin and RAGE [156, 160]. Perhaps not surprisingly, different approaches have been utilised to combat the S100A4 motility and invasion inducing capability through the isolation of inhibitors. The result of this early work has demonstrated that some of these inhibitors regulate the interactions of S100A4 with the actomyosin network. For instance, trifluoperazine, a phenothiazine-based compound, has been reported to block S100A4 ability to depolymerise NMMIIA filaments [161, 162]. The true potential of this compound in cells and possibly in vivo, along with deciphering the mode of actions of other molecules that have been isolated as specific inhibitors of the S100A4 associated cell motility/
invasion/metastasis [163, 164] will pave the way for the development of further drugs that can regulate S100A4 interaction with the actomyosin architecture.

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I would like to apologise for the numerous studies, which have significantly improved our understanding of actin dependent processes regulating cellular motility and invasion, but could not be included in this work owing to limits on the number of references. Special thanks are given to Prof. Philip S. Rudland (the University of Liverpool) for his critical comments on the chapter and for Prof. David Poyner (Aston University) for his invaluable help with the preparation of the different actin structures used.

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