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NON-THEMATIC REVIEW

Targeting the cytoskeleton against metastatic dissemination

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Abstract
Cancer is a pathology characterized by a loss or a perturbation of a number of typical features of normal cell behaviour. Indeed, the acquisition of an inappropriate migratory and invasive phenotype has been reported to be one of the hallmarks of cancer. The cytoskeleton is a complex dynamic network of highly ordered interlinking filaments playing a key role in the control of fundamental cellular processes, like cell shape maintenance, motility, division and intracellular transport. Moreover, deregulation of this complex machinery contributes to cancer progression and malignancy, enabling cells to acquire an invasive and metastatic phenotype. Metastasis accounts for 90% of death from patients affected by solid tumours, while an efficient prevention and suppression of metastatic disease still remains elusive. This results in the lack of effective therapeutic options currently available for patients with advanced disease. In this context, the cytoskeleton with its regulatory and structural proteins emerges as a novel and highly effective target to be exploited for a substantial therapeutic effort toward the development of specific anti-metastatic drugs. Here we provide an overview of the role of cytoskeleton components and interacting proteins in cancer metastasis with a special focus on small molecule compounds interfering with the actin cytoskeleton organization and function. The emerging involvement of microtubules and intermediate filaments in cancer metastasis is also reviewed.

Keywords Cytoskeleton · Migration · Invasion · Cancer metastasis · Small molecule compounds · Anti-metastatic drugs

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1 Introduction

Metastasis, which is defined as the dissemination of cancer cells from a primary tumour to a distant organ, represents the most difficult problem in cancer treatment and is the main cause of death of cancer patients [1]. The essential prerequisite for cancer cells to metastasize is the dramatic reorganization of their cytoskeleton. Under physiological conditions the three components of the cytoskeleton, microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs) collectively provide and maintain cell structure and shape and orchestrate key cellular events like cellular movement, cell division and intracellular transport. Several proteins belonging to/interacting with the cell cytoskeleton are mutated or abnormally expressed under pathological conditions, significantly affecting the invasive and metastatic phenotype of tumour cells [2, 3]. These findings pinpoint the cytoskeleton as an important provider of potential therapeutic targets against metastatic dissemination. We review here the actin cytoskeleton proteins implicated in cancer metastasis and the drugs developed so far to interfere with their signalling/function or with the polymerization and contractility of the actin cytoskeleton. The emerging role of MTs and IFs in cancer metastasis is also discussed.

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2 Cytoskeleton components and function

The cytoskeleton is a complex, dynamic network of highly ordered interlinking filaments forming the "infrastructure" of eukaryotic and prokaryotic cells [4]. In eukaryotic cells the cytoskeleton consists of a complex mesh of protein filaments and motor proteins, which regulate a variety of important cellular functions. Among them, the cytoskeleton supports cell

shape maintenance, it holds organelles in place and it provides the cell with the mechanical support to carry out essential processes like division, cytokinesis, motility, intracellular transport and organization of the organelles within the cell [5–8]. Three different types of protein filaments compose the cytoskeleton: MFs, MTs and Ifs (Table 1). Those fibres differ in their size, with MTs being the thickest and MFs being the finest. A description of each is reported below.

Concerning the cytoskeletal motor proteins, three superfamilies are known: myosins, kinesins and dyneins [36]. Myosin motors act upon actin filaments to generate cell surface contractions as well as vesicle motility, cytoplasmic streaming and muscle cell contraction [37]. The kinesin and dynein MT–based motors move vesicles and organelles within the cells, cause the beating of cilia and flagella and participate within the mitotic and meiotic spindles to the segregation of replicated chromosomes [25, 38].

2.1 Microfilaments

MFs are made of actin polymers and a variety of actin-binding proteins (ABPs). Indeed, in cells actin exists either as a monomer (globular, G-actin) or as a polymer (filamentous, F-actin). Monomeric actin that has a molecular weight of approximately 42 kDa is expressed in all eukaryotic cells and is highly conserved throughout evolution. Indeed, six isoforms of actin have been described in mammals: the cytoskeletal and cytoplasmic β- and γ-actins, cardiac muscle α-actin, skeletal muscle α-actin, and the smooth muscle α- and γ-actins [39–41]. MFs are assembled through the polymerization of G-actin monomers into F-actin polymers. This process is highly dependent upon the concentration of free actin monomers, which subsequently drives the equilibrium toward polymerization that is regulated also by a series of actin-associated proteins [42]. It is the case, for example, of profilin [43, 44] that binds to monomeric actin thereby occupying an actin-actin contact site. Once bound, profilin sequesters actin from the pool of polymerizable "free" monomers, thus impairing polymerization. G-actin binds ATP, which is hydrolyzed to ADP shortly after incorporation in the growing filament [45, 46]. Actin filaments display an inert minus end and a growing plus end to which new monomers are added. Generally, the plus terminus is oriented toward the cell surface. The rapid addition of monomers onto that end promotes the formation of actin surface protrusions for cell migration [17]. Various factors like calcium, ATP, cAMP and actin-binding proteins have been described to regulate the rate of actin polymerization. MFs are often subjected to "treadmilling", such that monomers are continuously added to the plus end and removed from the minus end while the filament maintains the same overall length [9–11]. Actin filaments are present in all cells; however, their proportion is different depending on the cell type. The most abundant and organized system of MFs are striated muscle cells. MFs are ordered and organized by a number of ABPs within the cytoplasm and on the surface of cell membranes [12]. Those proteins have different functions

Table 1 Cytoskeleton components

	Structure/composition	Diameter	Main functions
Microfilaments	Twisted double strands of actin, each a polymer of actin monomers, associated to a series of actin-binding proteins [9–12]	7 nm	Cell division (cleavage furrow formation) [13] Maintenance of (tension-bearing elements) and changes in cell shape [5, 6] Muscle contraction [14–16] Cell movement/migration [5, 17–24] Intracellular transport of organelles [5, 25]
Microtubules	13 linear protofilaments assembled around a hollow core and arranged in parallel, composed of a single type of the globular protein tubulin, which is a dimer of 2 closely related polypeptides, α- and β-tubulin [26]	25 nm	Maintenance of cell shape by resisting tension (compression-resisting "girders") [6] Separation of chromosomes during mitosis [8, 27–30] Intracellular transport of organelles [25, 31] Establishment of cell polarity [32] Cell motility (as in cilia et flagella) [22]
Intermediate filaments	Helical subunits of fibrous proteins (varying with function and cell type) supercoiled into thicker cables [33]	8–10 nm	Supply of mechanical strength and support necessary to reinforce cells and organize them into tissues [34] Anchoring of the nucleus and some organelles within the cytoplasm and nuclear lamina formation [35] Maintenance of cell shape (tension-bearing elements) [6]

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and act on MFs in various ways [12] (see next sections). The most abundant motor protein associated to MFs is myosin II, which moves toward the plus end of MFs, this process being driven by ATP hydrolysis. MFs are implicated in the regulation of important functions. A crucial one is to ensure mechanical stability to cells. An example is provided by microvilli on the surface of intestinal epithelial cells or epithelial cells of the kidney tubules. They are stabilized by MF wound around one another to form bundles of filaments [47–49]. MFs also play a crucial role in cell shape changes. For example, during cell division an actin ring that works in concert with myosin constricts the cell under division so that it forms a narrow waist, which finally breaks the connections between the two daughter cells [13]. Moreover, actin, in concert with myosin proteins, promotes cell contraction, as in the contractile apparatus of striated muscle cells [14–16]. Actin MFs are then implicated in several aspects of cell motility, from whole cell migration to the intracellular motility of the different organelles. Even if myosin motor proteins are responsible to generate the force needed for muscle contraction, cytokinesis and vesicle transport, actin MFs are able to generate force by themselves, assembling G-actin monomers into F-actin polymers producing force to deform the cell plasma membrane [50]. This phenomenon occurs for example at lamellipodia at the leading edge of a migrating cell [18, 19]. Other important cellular movements in which actin MFs participate are the rocket-like propulsions of pathogens, like the *Listeria* bacteria, which is responsible of pathogen spread among host cells [20, 21], the amoeboid movements observed in some protozoa [22] and the creeping of vertebrate white blood cells [23]. Finally, MFs are arranged in different ways according to the nature of the accessory proteins with which they associate. In contractile assemblies MFs associate with myosin and are antiparallel-oriented, as in the contractile ring responsible for cell division [13] and at the level of stress fibres, through which fibroblasts exert traction on their substratum. In actin-rich finger-like protrusions, like filopodia and other cell projections at the leading edge, MFs arrange as parallel bundles [24], whereas short randomly oriented filaments constitute the gels that are localized in the cortical region of the cell [51].

2.2 Microtubules

MTs are highly dynamic structures playing a crucial role both in cell shape determination and in a variety of cell movements, including some forms of cell locomotion, the intracellular transport of organelles, and the separation of chromosomes during mitosis [27, 31]. MTs are composed of a single type of globular protein, named tubulin, which is a dimer of two closely related 55 kDa polypeptides, α-tubulin and β-tubulin. A third type of tubulin, γ-tubulin, has been described which specifically localizes to the centrosome, where it is critically implicated in the initiation of MT assembly [52]. The polymerization of head-to-tail arrays of tubulin dimers originate MTs [26], which are generally composed by 13 linear protofilaments assembled around a hollow core and arranged in parallel. Like actin filaments, MTs are polar structures and exhibit two distinct ends: a fast-growing plus end and a slow-growing minus end [53]. This polarity is essential to establish movement direction. MTs are subjected to rapid cycles of assembly and disassembly thanks to continuous cycles of polymerization/depolymerization of tubulin dimers. Both α- and β-tubulin bind to GTP, whose hydrolysis (only when bound to β-tubulin) weakens tubulin-binding affinity for adjacent molecules, thus promoting their depolymerization. GTP hydrolysis also determines MT dynamic instability that consists in alternating cycles of growth and shrinkage [54]. The rate of tubulin addition relative to the rate of GTP hydrolysis determines the former or the latter. As long as new GTP-bound tubulin molecules addition is more rapid than GTP hydrolysis, a GTP cap is retained at the plus end and microtubules continue to growth. In contrast, by slowing of the polymerization rate, the GTP at the plus end of the MT will be hydrolyzed to GDP, with subsequent depolymerization and shrinkage of the MT. Like MFs, also MTs undergo "treadmilling" whereby tubulin molecules bound to GDP are lost from the minus end and replaced by GTP-bound molecules at the plus end of the same MT [55]. The rapid turnover of MTs is crucial for the remodelling of the cytoskeleton, for example during cell division, when MTs form the mitotic spindle. As the cell commits to divide, MTs nucleated by the centrosomes become shorter and more dynamic. This causes the disassembly of the interphase MT network [28]. After the breakdown of the nuclear envelope, MTs reorganize to form the mitotic spindle. Kinetochore MTs are attached to the condensed chromosomes, polar MTs overlap with each other in the centre of the cell, and astral MTs extend outward to the cell periphery [29]. The local stabilization and organization of those centrosomal and non-centrosomal MTs lead to the assembly of a bipolar spindle that is responsible of chromosome alignment on the metaphase plate and their segregation into two daughter cells [30]. Each daughter cell then contains one centrosome, which nucleates the formation of a new network of interphase MTs. The key protein in the centrosome nucleating MTs assembly is γ-tubulin. Complexes of γ-tubulin originate ring structures that contain from 10 to 13 γ-tubulin molecules. These γ-tubulin rings function as nucleation sites for the assembly of MTs and may remain bound to their minus ends [52, 56]. A large number of proteins favour MT stabilization or destabilization [57, 58]. Some proteins, for example, are implicated in MT disassembling, either by severing MTs or by increasing tubulin depolymerization rate from MT ends. Other proteins, known as MT-associated proteins (MAPs) bind to MT and increase their stability [59]. Those interactions

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are crucial to stabilize MTs at the level of specific cell locations and determine cell shape and polarity. Various MAPs have been described, which vary depending on the cell type. The well-characterized ones are MAP-1, MAP-2 and tau, which have been isolated from neuronal cells, and MAP-4, which is found in all non-neuronal vertebrate cell types. The tau protein has been object of great interest because it is the main component of the typical lesions identified in the brains of patients affected by Alzheimer disease [60]. MAP activity is mainly regulated by phosphorylation, which controls MT stability. Finally, MTs are critically involved in establishing cell polarity. It is widely accepted that one common role for MTs in generating polarity is their requirement for delivering positional information necessary to determine the proper site of cortical polarity [32].

2.3 Intermediate filaments

The name IFs derive by their diameter ranging between 8 and 10 nm, which is intermediate in size between the MTs (25 nm) and the MFs (7 nm). IFs are composed of a number of proteins which are expressed in different cell types. Indeed, more than 50 IF proteins have been described and classified into six groups on the basis of their amino acid sequence similarity [33]. Types I and II are two groups of keratins, each including about 15 different proteins expressed in epithelial cells [61]. Vimentin, a protein found in fibroblasts, smooth muscle cells and white blood cells is one of the best-known type III IF proteins [62]. Another type III protein is desmin [63], which is specifically expressed in muscle cells, with the function to connect the Z discs of individual contractile elements. The type IV group of IF proteins include the three neurofilament (NF) proteins NF-L (light), NF-M (medium) and NF-H (heavy), which constitute the major IFs of different types of mature neurons, being particularly abundant in the axons of motor neurons [64]. Type V IF proteins include the nuclear lamins, which are found in most eukaryotic cells as components of the nuclear envelope and differ from the other IF proteins by their capacity to assemble to form an orthogonal meshwork underlying the nuclear membrane [35]. Finally, the type VI IF group includes nestin, a protein expressed in several cell types during development, although its expression is usually transient and does not persist into adulthood. The expression of nestin has been well described at the level of neuronal precursor cells of the brain subgranular zone [65, 66].

The different IF proteins display a similar structural organization. They present a central α-helical rod domain (around 310 amino acids) flanked by amino- and carboxy-terminal domains (head and tail respectively), varying among the different IF proteins in size, sequence and secondary structure [67]. Differently from MFs and MTs, IFs are more stable and are not characterized by the dynamic behaviour typical of those other cytoskeletal components [68]. However, phosphorylation can regulate their assembly and disassembly within the cells, as in the case of nuclear lamins. Their phosphorylation results in disassembly of the nuclear lamina and breakdown of the nuclear envelope during mitosis [69, 70]. Furthermore, differently from MTs and MFs, assembled IFs have no polarity and association and dissociation of their dimers can occur all along the filament length.

Finally, IF organization and association with plasma membranes suggest that they essentially exert a structural role by providing the mechanical strength necessary to reinforce cells and organize them into tissues [34]. The best example is represented by the mechanical support provided by IFs to the plasma membrane, where it comes in contact with the extracellular matrix (ECM) or with other cells.

Importantly, major degenerative diseases of skin (epidermolysis bullosa simplex, EBS), muscle (desminopathy), and neurons (amyotrophic lateral sclerosis, ALS) depend on the disruption of the IF cytoskeleton or its connections to other cell structures [71–73]. However, this topic goes beyond the purpose of the present review.

3 The metastasization process

Metastasis is the leading cause of cancer-related deaths. Functionally, metastasis occurs *via* a series of discrete steps (Fig. 1), often termed the "invasion-metastasis cascade" [74]. They include: detachment of cells within the primary tumour; local migration and invasion of stromal tissue; intravasation into and transit through pre-existing and newly formed blood and lymph vessels; extravasation; establishment of disseminated cells (which can stay dormant for a prolonged period of time) at a secondary anatomical site; and outgrowth of micrometastases and macrometastases/secondary tumours, this last step being called "colonization" (Fig. 1) [75, 76]. Interestingly, recent data have suggested the existence of an additional step at the beginning of the "metastatic cascade": the creation of a "premetastatic niche" at the target site, before the arrival of the first tumour cells to this distant location [77]. Indeed, Lyden and colleagues have shown that bone marrow-derived cells are mobilized owing to the presence of a distant, intradermal tumour. As a consequence, these cells accumulate as clusters in the lungs, where they change the local microenvironment into a niche suitable for the establishment of secondary tumours. At a later stage, tumour cells arrive at these sites and co-localize with the bone marrow-derived cell clusters [77]. In line with these results, it has been reported that distant tumours induce elevated levels of pro-inflammatory chemokines in the lungs of tumour-bearing mice [78, 79] that attract tumour cells to the lungs *via* a positive feedback loop inducing the expression of nuclear factor-kB (NF-kB) [80]. These findings open the way to new possible therapeutical strategies, as the perturbation of the endocrine and paracrine

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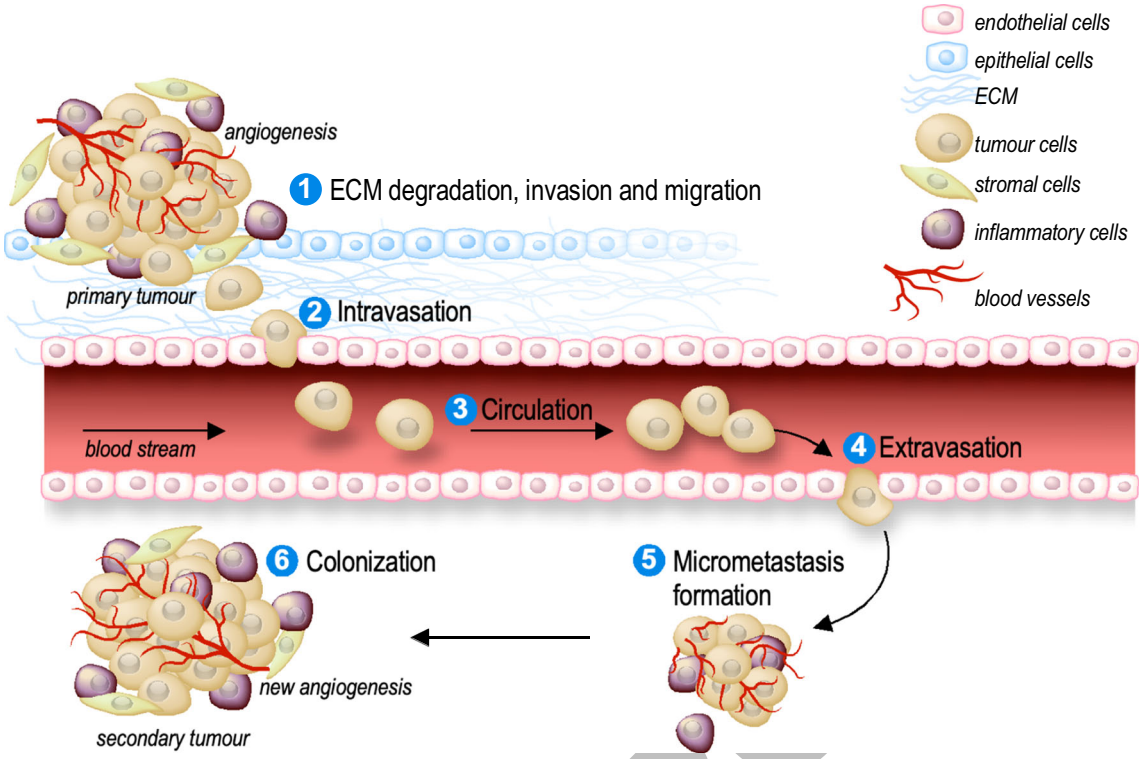

Fig. 1 Schematic overview of the essential steps of the metastatic process. Cancer cells detach from the primary tumour, degrade the extracellular matrix (ECM), invade through the basement membrane and migrate through the tumour stroma (1). They then intravasate into vasculature (2) and transit through pre-existing and newly formed blood vessels (3). After extravasation through the endothelial barrier (4), cancer cells form micrometastasis (5) to finally colonize target organ/s to originate secondary tumour/s (6)

signalling networks required for the establishment of a premetastatic niche could prevent the establishment and outgrowth of distant metastases.

According to the traditional metastasis models, metastatic cells are rare and appear during the late stages of tumour progression. However, expression profiling studies on human cancers, such as breast carcinomas, show that most cells in a primary tumour express the molecular signature that is associated with metastatic tumours [81]. Indeed, a specific gene-expression profile of primary breast cancers is associated with the development of metastasis and with a poor clinical outcome [82]. Those results suggest that the potential for metastasis is determined early in tumorigenesis, challenging the classical models.

To disseminate from a primary tumour to a distant organ, invasive cancer cells must subvert the molecular machinery that under normal conditions enable important physiological functions and facilitate the development and homeostasis of an organism. For example, signalling cascades are required for normal processes such as morphogenesis [83], neurogenesis [84] and angiogenesis [85]. Each of the stages involved in tumour metastasis requires numerous specific molecular interactions provided by the tumour cell, the surrounding ECM and the stromal cells (see below) [86]. These interactions are mediated by contact between the cell and the ECM, by direct cell-cell contact, and by secreted factors. Finally, each step of the metastatic process has one or more physiological barriers that act to inhibit the spread of malignant cells. Thus, to successfully metastasize, tumour cells need to overcome natural barriers such as the basement membrane and the interstitial matrix of connective tissue.

One important class of molecules that play a crucial role in cell invasion and metastasis are proteases, enzymes that are able to degrade the ECM. Although they are key mediators of many physiological processes, such as embryogenesis, tissue repair and immune response, they are highly expressed during tumour progression and metastasis [87]. Classically, their activity is associated with the late invasive stage of cancerogenesis, providing cells with the ability to breach barriers opposing their movement (such as the basal membrane), to facilitate intra- and extravasation of tumour cells and to promote growth in ectopic environments. However, multiple lines of evidence suggest they have important functional roles at additional stages of neoplastic progression. In pre-malignant cancer cells, protease activation favours the onset of angiogenesis and malignant conversion. In addition, their proteolytic activity is required for other functions apart from the local dissolution of basement membrane. Those functions include: the activation of latent proteases, growth factors, and chemotactic agents (and related receptors); the exposure of

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integrin-binding sites on the ECM with subsequent transmission from these sites of migratory and survival signals [88, 89]; the release of bioactive fragments of the ECM itself. These in turn are able to enhance protease gene expression or alternatively activate integrins.

During the past decade an important acceleration of the research on cancer cell ability to invade and metastasize has been registered thanks to emerging powerful research tools, novel experimental animal models and the identification by genomic studies of critical regulatory genes implicated in the metastatic cascade. However, major questions still remain unanswered.

An important developmental regulatory program called "epithelial-mesenchymal transition" has emerged as an essential mechanism by which transformed epithelial cells acquire the abilities to invade, to resist apoptosis and to disseminate [90, 91]. Carcinoma cells thus co-opt processes implicated in different steps of embryonic morphogenesis and wound healing and in parallel acquire multiple characteristics enabling them to invade and metastasize. Indeed, transcription factors like Snail, Slug, Twist and Zeb 1–2 orchestrating EMT and related migratory events during embryogenesis are also expressed at various levels and in different combinations in several malignant tumours. They have been demonstrated to be important to direct invasion in experimental models of carcinoma formation and some of them induce metastasis once ectopically overexpressed [92, 93]. The biological events triggered by such transcription factors are multiple: loss of adherens junctions with a parallel conversion from a polygonal/epithelial to a spindly/fibroblastic morphology, expression of matrix-degrading enzymes, increased migration and augmented resistance to apoptosis. Up to date it seems that EMT-inducing transcription factors have the ability to orchestrate most steps of the invasion-metastatic cascade excluding the final step of colonization. Although certain non-epithelial tumours, like sarcomas and neuroectodermal tumours, express EMT-inducing transcription factors, their role in triggering the initiation and progression of the metastatic cascade in those tumours is still unclear. Moreover, it remains to be established whether the EMT program is the only one through which carcinoma cells acquire invasive traits or whether other so far unknown regulatory programs cooperate to induce those malignant characteristics. Furthermore, two other distinct modes of invasion have been reported and implicated in cancer cell invasion [94]. "Collective invasion" has been described as the movement of groups of cancer cells advancing together into adjacent tissues and characterize, for example, squamous cell carcinomas, which are rarely metastatic. This suggests that this type of invasion lacks some characteristics that promote metastasis. The other one is the "amoeboid" invasion [95, 96], in which individual cancer cells display a morphological plasticity that allows them to slither through existing interstices in the extracellular matrix, as observed for both the mesenchymal and the collective forms of invasion.

Finally, it has been demonstrated that a crosstalk between cancer cells and cells of the neoplastic stroma contributes to invasive growth and metastasis [97, 98]. An example is provided by mesenchymal stem cells present in the tumour stroma which are shown to secrete CCL5/RANTES in response to signals released by cancer cells. CCL5 in turn acts on cancer cells to promote their invasive behaviour [99]. Very interesting is also the case of macrophages at the tumour periphery, which stimulate local invasion providing matrix-degrading enzymes [98, 100]. In an experimental model of metastatic breast cancer, tumour-associated macrophages provide breast cancer cells with epidermal growth factor (EGF), whereas cancer cells stimulate macrophages *via* CSF-1 secretion. This concerted action facilitates cancer cells intravasation into the circulatory system and metastatic dissemination [97, 101].

3.1 Actin cytoskeleton proteins implicated in cancer metastasis

3.1.1 Regulatory proteins: Rho small GTPases

Rho small GTPases belong to the Ras superfamily of GTPases. They have emerged as essential players in the regulation of key signalling pathways underlying cell migration, like cytoskeleton dynamics, assembly and disassembly of cell-cell junction, directional sensing and integrin-matrix adhesion. Since their discovery as homologous of Ras small GTPases, over 20 mammalian members have been reported and divided into 8 subfamilies [102]. Rac1, Cdc42 and RhoA are the best characterized members of the family. Their crucial role in regulating actin cytoskeleton organization and dynamics has been well documented [103]. Rac1, Cdc42 and RhoA were first reported to promote the formation of the following actin-rich structures in fibroblasts, respectively: lamellipodia/ruffles, filopodia and stress fibres [104]. The formation of lamellipodia/ruffles and filopodia by Rac1 and Cdc42 is a critical prerequisite for actin-driven protrusion at the leading edge of the cell, whereas RhoA-dependent stress fibres formation regulates actomyosin contraction of the cell body and retraction of the trailing edge. Subsequently, Rho small GTPases have been shown to have a role also in the regulation of key cellular functions, like polarity, survival, cell cycle progression and gene expression [105, 106]. Similarly to Ras small GTPases, Rho small GTPases are molecular switches that cycle between a GTP-bound active form and a GDP-bound inactive one [106]. Rho GTPase activity is regulated by 3 main families of regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEFs activate Rho GTPases catalysing the exchange of GDP for GTP [107], whereas GAPs inactivate Rho

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GTPases accelerating their intrinsic GTPase activity [108]. Eighty-two GEFs and 67 GAPs have been described to regulate GTPase activity downstream of several cell surface receptors such as growth factor receptors, integrins, cytokine receptors, and cadherins [107]. The role of GDIs is to bind to Rho GTPases in their inactive GDP-bound form and sequester them in the cytosol, thus acting as inhibitors of the Rho GTPase signalling [109]. Indeed, once translated, Rho GTPases are subjected to post-translational modifications like geranylgeranylation, or less commonly farnesylation, on a C-terminal CAAX motif, which promotes anchorage to intracellular membranes where they are activated [110]. Rho GTPases once bound to GTP undergo conformational changes and associate to a spectrum of effector proteins to stimulate downstream signalling pathways [111, 112]. The total number of effectors is too large to be discussed here. Excellent reviews containing a detailed description are available [111–113]. Some examples will be briefly reported here. Rac binds to the Wave complex to release active Wave, which induces actin polymerization in lamellipodia through Arp2/3 complex activation. Both Rac and Cdc42 bind and activate the p21-activated (PAK) kinases, which have multiple substrates including LIM kinase (LIMK) that promotes actin polymerization. PAK activity controls also myosin phosphorylation and cell contractility through a number of signalling cascades, including myosin light chain kinase (MLCK), myosin regulatory light chain, myosin heavy chain and caldesmon. Rac and Cdc42 also bind to the actin-binding protein IQGAP, which participates to the regulation of cell-cell adhesion. Moreover, both Rac and Cdc42 bind to and stimulate PI3 kinase (PI3K). The lipids phosphorylated by PI3K bind to and stimulate Rac GEFs in a positive feedback loop that promotes cell motility. Cdc42 mediates the formation of filopodia through WASP, which interacts directly with Arp2/3 complex to promote actin polymerization. Cdc42 is also able to activate the Myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKs), which are linked to Rho kinases and can stimulate myosin phosphorylation. Rho kinases 1 and 2 (ROCK1 and ROCK2) are key Rho effectors. Among the effectors of ROCKs, we can list (a) the myosin-binding subunit of myosin phosphatase (MBS), which causes the inhibition of its phosphatase activity, the increase of MLCK phosphorylation and a subsequent increase in general tension; (b) LIMK, which is responsible of cofilin phosphorylation and hence actin polymerization, and (c) myosin regulatory light chain itself that in turn promotes contractility. Finally, phosphatidylinositol 4,5 bisphosphate activates ezrin-radixin-moesin (ERM) proteins and promotes actin polymerization through WASP, profilin and multiple actin-capping proteins.

Rho small GTPases and their regulators and effectors participate in multiple aspects of cancer progression. Differently from Ras, which has been found to be mutated in around 20–30% of human cancers, Rho GTPases are rarely mutated. Recently, it has been reported that proline 29 in Rac1 is mutated in a subset of melanomas, breast tumours and head and neck tumours [114] and that RhoA is mutated in some tumours, like diffuse gastric cancer and angioimmunoblastic T cell lymphoma [115–118]. However, in the majority of the cases Rho GTPases are upregulated or display an increased activity as a result of altered expression levels of GEF, GAPs and/or GDIs. This is supported by abundant evidence based on *in vitro* experiments involving manipulation of the expression levels or the activity of Rho GTPases in cancer cell lines by overexpressing constitutive active or dominant-negative forms of Rho GTPases, their regulators or effectors, or exploiting small interfering RNAs (siRNAs) or small molecule inhibitors [119]. A series of tissue-specific conditional knock-out mouse models (as direct targeting of Rac1, Cdc42 and RhoA is embryonic lethal) has definitely provided strong evidence for the physiopathological role of Rho small GTPases [120, 121]. Rac1, Cdc42 and RhoA are overexpressed in a variety of malignancies [122–127]. Interfering with their expression and/or activity by knockdown or by expressing dominant-negative mutants or by using small molecule inhibitors (when available, see next sections) has been reported to inhibit lamellipodia/ruffles and filopodia formation, reduce the number of focal contacts, impair migration and invasion of a number of human cancer cell lines, inhibit colony formation in soft agar and decrease tumour metastasis *in vivo* [128–136].

The evidence that Rho GTPases are frequently overexpressed or hyper-activated but rarely mutated in several malignancies indicates that regulatory proteins like GEFs, GAPs and GDIs play a key role in the dysregulation of signalling pathways implicated in cancer initiation and progression. Indeed, Rho GTPase hyperactivation may occur through (a) their overexpression, (b) loss of GAP- or GDI-mediated inactivation, (c) upstream activation or overexpression of RhoGEFs. Altered expression or mutations of RhoGEFs, RhoGAPs and RhoGDIs have been reported in a variety of human cancers [137, 138]. Since GEF activation is the most common mechanism for signal-mediated GTPase activation, the theme that has emerged is that aberrant GEF regulation contributes to Rho GTPase activation in cancer, making Rho GEFs attractive therapeutic targets for cancer [138] (see next section).

Rho GAPs represent the flip-side of the coin to Rho GEFs. Surprisingly, differently from the many Rho GEFs that are altered in human malignancies, except for the deleted in liver cancer (DLC) family [139, 140], limited evidence exists for the role of Rho GAPs in cancer. It has been hypothesized that in many cancers GAP activity is normal, but Rho GTPase hyperactivation through GEFs or GTPase overexpression itself can override normal GAP-mediated inactivation.

Concerning RhoGDIs, even if changes in their expression levels have marked effects on the overall level of activities of

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Rho GTPases and have been linked to a number of cancers [141–144], little is known about how Rho GDI expression is regulated. Despite the wide variety in the Rho GTPase family, only three genes have been reported to encode RhoGDIs in mammals, implying that the consequences of changes in their expression are manifested through the action on more than 20 Rho GTPases. The effects of the Rho GDIs on malignancies are thus complex and do not fit a simple explanatory model.

3.1.2 Structural proteins: actin-binding proteins

As reported above, actin can exist in 2 forms: G-actin and F-actin. They both interact with a large variety of proteins in the cell. Up to date more than 100 ABPs have been described. They are critically implicated in regulating polymerization and depolymerization of actin filaments, organizing actin filaments in higher magnitude superstructures and controlling complex MFs dynamic properties [12]. Through those activities ABPs allow the actin cytoskeleton to rapidly respond to cellular and extracellular signals, being essential for the participation of the cytoskeleton to several cellular processes, like cell shape and motility, muscle contraction, intracellular trafficking, cell pathogenesis. ABPs can be divided into 7 groups according to their specific functions on actin polymers and/or monomers: (1) actin nucleators; (2) regulators of G-actin polymerization/F-actin depolymerization; (3) capping and actin severing proteins; (4) bundling and cross-linking proteins; (5) motor proteins; (6) anchoring actin filaments to the plasma membrane; (7) F-actin stabilizing and regulatory proteins (Fig. 2).

1- An important set of actin regulators initiates the formation of new actin filaments through a process called nucleation. Up to date 3 classes of proteins have been described that initiate new filament polymerization: the actin-related protein 2/3 (Arp2/3) complex, the formins and spire. Each promotes actin nucleation by a distinct mechanism. We will focus here on the Arp2/3 complex which has been the first to be identified and to critically participate in the formation of branched-actin-filament networks during different cellular processes ranging from cell migration to endocytosis. The Arp2/3 complex is a seven-subunit protein complex. Two of its subunits, the actin-related protein 2 and 3 (ARP2 and ARP3) closely resemble the structure of monomeric actin. Indeed, the complex is thought to mimic an actin dimer or trimer and to serve as a template for the initiation of a new (daughter) filament that branches off of an existing (mother) filament and generates y-branched (with a regular 70° branch angle) actin networks [145, 146]. This nucleating/branching action of the Arp2/3 complex is known as autocatalytic branching or dendritic nucleation and is crucial for its *in vivo* activity. The Arp2/3 complex displays little biochemical activity *per se*. However, when engaged by a nucleation promoting factor (NPF), it is activated to initiate actin nucleation. NPFs are divided into 2 main groups and are controlled by signalling cascades that coordinate actin polymerization in space and time. Class I NPFs include among others the Wiskott-Aldrich syndrome protein (WASP) and the suppressor of cyclic AMP repressor (SCAR, also known as WASP-family verprolin homologous protein WAVE) [147–149]. They display a common WCA domain, consisting of WASP-homology-2 (WH2) domain, a central (C; also called cofilin-homology or connector) and an acidic (A) region (both regions together are known as CA region). It has been proposed that for class I NPFs the A region mediates the binding to Arp2/3, the central region initiates an activating conformational change in the complex and the WH2 and central regions

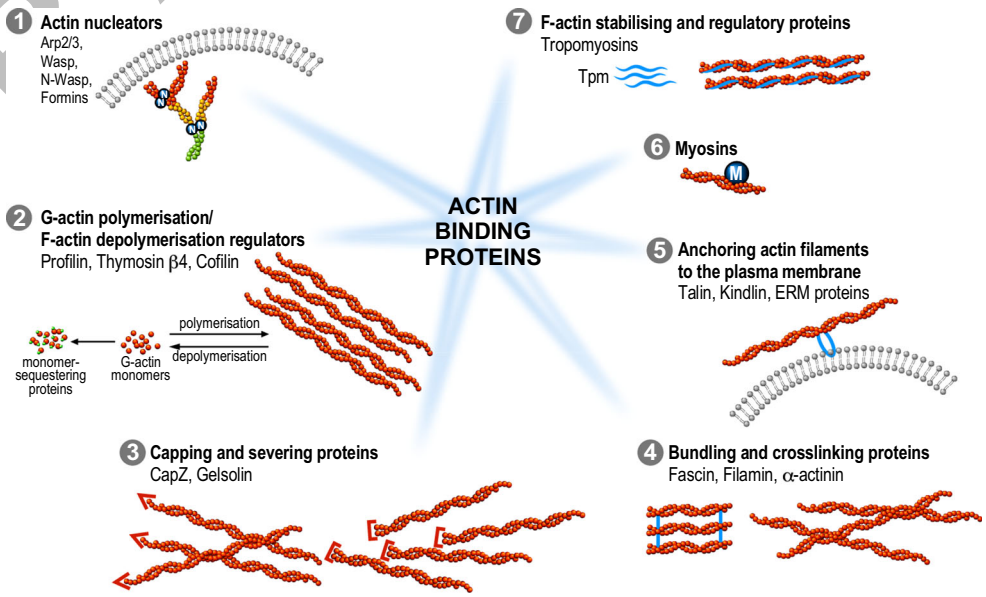

Fig. 2 Overview of the main families of actin-binding proteins. Actin-binding proteins are divided into seven groups according to their specific functions on actin polymers and/or monomers. They can (1) promote actin nucleation; (2) regulate G-actin polymerization/F-actin depolymerization; (3) cap or sever F-actin filaments; (4) bundle or crosslink F-actin filaments; (5) anchor F-actin filaments to the plasma membrane; (6) generate force; (7) stabilize F-actin filaments. Representative proteins are indicated for each group

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present an actin monomer to the complex, facilitating the formation of a nucleus for the polymerization of the daughter filament. The class II NPFs include, among others, cortactin. Like class I NPFs, they display an Arp2/3 binding to the acidic region, but they lack a G-actin – binding region and their mechanism of Arp2/3 activation is still unclear. The altered function of the Arp2/3 complex is linked to cancer metastasis. Indeed, the levels of Arp 2/3 mRNA as well as those of N-WASP (the closely related WASP neural isoform) are upregulated in a variety of tumours and invasive cells, being functionally associated with cell invasion and metastasis [150, 151]. The critical prerequisite for cancer cell invasion is the formation of actin-rich structures, like podosomes and invadopodia that possess adhesive and protrusive activities with the ability to degrade the ECM. The activation of the Arp2/3 complex is needed for podosome formation [152]. At their actin-reach core the Arp 2/3 complex colocalizes with WASP, N-WASP and cortactin [153, 154]. Similarly, the Arp 2/3 complex and N-WASP localize to invadopodia and are critically required for their formation [155]. The Arp2/3 complex and their activators are thus crucial for tumour cell invasion, being potential interesting target for therapeutic intervention (see next section).

Finally, just a few lines on formins. Formins are multidomain proteins containing a highly conserved actin assembly formin homology 2 FH2 domain, and the associated profilin-binding FH1 domain. FH1 and FH2 domains are flanked on either side by regulatory domains, the function of which is to regulate localization and activation [156, 157]. Some formins are activated by Rho GTPases and autoinhibited by association of their N- and C-terminal regulatory domains [156]. Once activated, the FH2 domain either nucleates actin filament assembly or associates with pre-existing filaments, remaining continuously linked to the elongating actin filament barbed end [158]. Through processive association formins protect barbed ends from capping proteins [159, 160] and promote the addition of profilin-actin bound to the FH1 domain [161, 162]. As a result, long-straight filaments often bundled in cells and pulled by myosin motors to generate contraction are produced.

2- The regulators of G-actin polymerization include proteins like profilin (see above) and thymosin β 4, a protein that sequesters actin monomers by forming complexes with G-actin, thus maintaining the pool of unpolymerized actin. Among F-actin depolymerization factors we would like to focus our attention on cofilin. It belongs to a family of related proteins sharing similar biochemical activities, the actin depolymerizing factor (ADF)/cofilin family. Cofilin and its regulatory proteins participate in the early steps of the motility cycle and evidence shows that the expression of a number of genes of the cofilin pathway is altered in invasive tumour cells. Cofilin is a small ubiquitous protein that binds to both G-and F-actin. Studies carried out using light microscopy to observe interaction between cofilin and actin filaments that are immobilized by cross-linking to a substratum have shown how cofilin can both increase the number of free barbed ends for polymerization and increase the rate of actin depolymerization (thus refilling the cell with new G-actin monomers) [163–166]. Those sets of experiments have also demonstrated that cofilin is a much more powerful severing protein than expected [165, 166]. Indeed, the appearance of free barbed filament ends together with further remodelling of the actin cytoskeleton can lead to the formation and retraction of path-finding structures like lamellipodia, invadopodia and filopodia used in chemotaxis, cell migration and invasion of tumour cells. The cofilin pathway has recently emerged as a central player in the generation of free barbed ends and actin filament-turnover in a large number of motile cells, including fibroblasts [163], mammary carcinoma cells [167, 168] and glioblastoma cells [169] during the formation of those structures. Furthermore, cofilin can greatly potentiate the dendritic nucleation activity of the Arp2/3 complex (see above), because through the severing of the mother filament it originates polymerized actin filaments that are preferred for the actin nucleation by the Arp 2/3 complex [170]. The cofilin pathway includes a group of kinases and phosphatases that control cofilin activation state in response to different stimuli, like EGF, transforming growth factor β (TGFβ), stromal cell-derived factor 1 (SDF1) and heregulin. The regulation of cofilin is very complex as it involves 4 independent mechanisms: (1) the phosphorylation on Ser 3 by LIMK1, LIMK2 and testicular protein kinase 1 and 2 (TESK1 and 2), which causes inhibition of cofilin actin-binding activity [171–173]; (2) the dephosphorylation of Ser 3 by phosphatase types 1, 2A and 2B, slingshot (SSH) and chronophin, resulting in the activation of actin-binding by cofilin [174–176]; (3) the binding to phosphatidylinositol 4,5 bisphosphate (PIP2, whose hydrolysis depends on phospholipase C γ, PLC γ) that inhibits actin binding by cofilin [177]; (4) the changes in pH over the physiological range (6.8–7.4) by the Na-H exchanger protein, which promote the severing activity of cofilin when it is dephosphorylated [178, 179]. Indeed, it is now clear that a balance of the stimulatory and inhibitory branches of the cofilin pathway is needed to ensure that protrusion, cell migration and chemotaxis occur in an optimal way. Expression profiling data have shown that the cofilin pathway is a major determinant of metastasis, several genes whose expression status is upregulated during metastasis being clustered in the cofilin pathway and its downstream effectors [180, 181]. This makes the cofilin pathway a potential druggable target for anti-metastatic therapy (see next section). Although studies of the effect of cofilin activity on metastasis have been carried out only in mammary tumours, it is likely that the cofilin cascade also plays a crucial role in the invasion and metastasis of other types of cancer, as demonstrated by the critical requirement of cofilin for the motility of different cell types and the

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correlation of cofilin pathway gene expression with the metastatic phenotype in various malignancies [182–186]. In particular, in rat and mouse mammary tumour models, it has been shown that 9 genes of the cofilin pathway are overexpressed, showing that many combinations are possible to increase the output of the cofilin pathway. Only cofilin and LIMK1 have been tested for their effects on the activity of the cofilin pathway, the results of those studies showing that cofilin signalling output is predictive for metastatic potential [187].

3- Capping and actin severing proteins regulate actin filament dynamics by blocking the addition and loss of G-actin monomers to F-actin filaments. Two of those proteins are CapZ, which caps the barbed end of actin filaments in muscle cells, and gelsolin, which controls actin filament length, being one of the most potent members of the actin-severing gelsolin/villin superfamily.

4- The actin-bundling and cross-linking proteins include among others filamin, a crucial actin crosslinker; α-actinin, an actin-bundling and cross-linking protein thought to anchor actin to a variety of intracellular structures, and the actin-bundling protein fascin (FSCN). We will focus our attention on this last protein. Three FSCN isoforms are known in mammals: FSCN1, which is widely expressed; FSCN2, which is expressed in retina and ear hair cells; FSCN3, which is specifically expressed in testis. FSCN1 has received considerable attention in recent years as its expression is very low or absent in normal adult epithelia, but it is dramatically upregulated at both the transcript and protein levels in all forms of human carcinomas studied to date [188–190]. FSCN1 crosslinks actin filaments into tightly packed parallel bundles, oriented with their growing ends toward the plasma membrane. It has been reported that its association with actin filaments can be increased by interaction with specific matrix [191] or upon cell stimulation by growth factors or cytokines [192]. FSCN1 localizes to actin-rich cytoskeletal structures, like stress fibres [193, 194], protrusive structures like microspikes and filopodia [195] and ECM-degrading structures, like podosomes and invadopodia [196, 197]. Cell biology studies have shown that FSCN1 is critically required for the activity of those structures and its expression levels correlate with higher cell motility and invasiveness [190, 198]. FSCN1 is mainly regulated by different factors like TGFβ [199], Wnt/β catenin [200], STAT3/NF-kB [201], microRNAs [202–204] at the transcriptional level, where it has been observed to be induced when epithelial tumours start to acquire invasive and migratory properties. FSCN1 can be post-translationally modified by PKC and, once phosphorylated on Ser 39, it shows a reduced actin binding and bundling activity [205, 206]. FSCN1 has been demonstrated to be a valuable prognostic biomarker in some epithelial cancers. Indeed, immunohistochemistry detection on conventional sections or tissue microarray (TMA) has revealed that higher FSCN1 expression levels positively correlate with tumour aggressiveness/malignancy and higher tumour grade/stage in carcinomas [207]. Elevated FSCN1 levels have been detected in advanced stage and lymph node metastasis of head and neck squamous cell and colorectal cancers with a bad prognosis [208–211]. In breast cancer, FSCN1 expression has been associated with oestrogen receptor-negative (ER2), progesterone receptor-negative (PR2) and basal-like phenotype, being correlated with an increased risk of mortality [212, 213]. Higher FSCN1 expression levels have also been detected in advanced tumour stage pancreatic cancers and lymph node metastasis of lung cancers [214–217]. Remarkably, we have recently shown that FSCN1 immunohistochemical detection is an independent prognostic factor in adrenocortical carcinoma (ACC), also refining results obtained with staging and Ki67 labelling index [>]]. The robust prognostic power of FSCN1 levels has been further confirmed in two independent ACC cohorts [218]. Moreover, we have provided evidence for a role of FSCN1 in promoting tumour cell invasion in a human ACC cell line that overexpresses the transcription factor steroidogenic factor-1 (SF-1) resulting in a more aggressive and invasive phenotype [219, 220]. Indeed, interfering with FSCN1 by gene silencing or chemical inhibition substantially impaired actin cytoskeleton reorganization affecting the formation of filopodia and reduced the increased tumour migration and invasiveness [218] observed in that cell line upon increased SF-1 dosage conditions that we had previously demonstrated to affect cytoskeleton dynamic and invasive properties through the upregulation of the Rho GEF VAV2 [220] (see next section). Overall, the above evidence indicates that besides being an excellent prognostic marker for a number of malignancies, FSCN1 is an attractive drug target. This is particularly interesting for a type of cancer like ACC, especially in the advanced stages, where therapeutic options are rather disappointing and the need for new and more effective drugs is urgent. Specific small molecule inhibitors for FSCN1 have been developed and shown to antagonize metastatic dissemination in pre-clinical cancer models (see next section).

5- Myosins are a superfamily of actin-dependent molecular motors which upon interaction with MFs are able to convert energy deriving from ATP hydrolysis to mechanical stress [221]. They have a crucial function in several aspects of eukaryotic motility, like cell movement, cytokinesis, organelle/particles trafficking, signal transduction and in maintaining cellular morphology. In the human genome around 40 different myosin-related genes have been identified. They encode 12 classes of myosins [221]. Myosins are composed of three functional subdomains: the NH2-terminal domain, which is required for actin binding and ATP hydrolysis; the neck domain, which binds calmodulin and light chains; the COOH-terminal domain, which is class-specific and is implicated in the transport of cargo along MFs. This tail region participates also in signal transduction and membrane interaction. Increasing evidence has pointed on myosins as critical actors

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in the process of tumorigenesis and thus potential druggable target for cancer therapy. Myosins are overexpressed in a variety of cancer types, including breast [222, 223], ovarian [224, 225], prostate [226, 227] and colorectal cancer [228, 229], intestinal [230, 231], gastric [232, 233] and pancreatic cancer [234, 235], melanoma [236, 237], anaplastic gliomas [238] and acute myeloid leukaemia [239]. In each type of malignancy myosins play different but essential roles in tumour progression. Myosin1e, for example, has been implicated in recruiting invadosome components to the plasma membrane and transport vesicles to the sites of new invadosome assembly [240]. Myosin II has been reported to drive cell invasion in pancreatic, breast and prostate cancer cells and in anaplastic glioma cells [222, 234, 238, 241, 242]. In breast cancer it also maintains cell polarization, stabilizes nascent focal adhesion complexes and mediates efficient integrin-based cell migration [243]. Moreover, it regulates tumour cell migration by interacting with P-cadherin [236]. Blocking of myosin II activity by specific MLCK inhibitors could prevent pancreatic and breast cancer cell adhesion and invasion [222, 234] (see next section). Myosin Va, positively regulated by Snail, seems to be a key factor in cytoskeletal organization and migration of metastatic colorectal cancer cells [244]. Its overexpression promotes actin assembly and cell motility, whereas its depletion impairs cell spreading, cytoskeletal remodelling and cell migration [245]. Myosin Vb has been reported to be a strong prognostic factor for disease recurrence [246]. Myosin VI is an early marker in prostate cancer development [247] and has a role in the dissemination of ovarian cancer cells [224]. Myosin IX has been shown to downregulate Rho activity [248] and to prevent actin bundle assembly during the nascent formation of cell adhesion, being critically required to sustain the collective migration of epithelial cells [249]. Finally, myosin X localizes at filopodia tips, being actively implicated in their formation [250, 251], and promotes metastasis development in primary glioblastoma and acute lymphoblastic leukaemia [252, 253]. It also appears to play a role in cancer cell protrusion and metastasis development by transporting β1 integrins to the filopodia tips [254]. Moreover, mutant p53-associated myosin X upregulation has been reported to promote breast cancer invasion and metastasis [255].

6- The actin-binding proteins which anchor actin filaments to the plasma membrane include among others talin, kindlin, and the three closely related proteins ezrin, radixin and moesin. Here we focus on talin, one of the major components of the focal adhesion complex, mainly acting as an interlink between transmembrane integrin receptors and cytosolic F-actin. Talin, besides actin, is also able to interact with a number of other proteins in the adhesion complex to regulate their functional dynamics. Indeed, 2 talin isoforms have been described in mammals (talin 1 and talin 2). They are about 270 kDa proteins in size and consist of an N-terminal head domain and a C-terminal flexible rod domain [256]. The talin head domain is composed of a FERM (4.1, ezrin, radixin, moesin) domain consisting of 3 subdomains (F1, F2 and F3) and an F0 subdomain that has no homology with other already known domains. The F3 subdomain is implicated in the binding to the cytoplasmic tails of integrins [256, 257]. The C-terminal rod domain is composed of a series of helical bundles that contain multiple binding sites for the F-actin-binding protein vinculin and a second integrin-binding site [258]. The C-terminal domain includes also a THATCH (talin/HIP1R/Sla2p actin tethering C-terminal homology) domain mediating the dimerization and providing a direct linkage between talin and F-actin [259, 260]. Talin is a recruited component of the focal adhesion complex where it functionally interacts with the cytoplasmic tails of integrins. Talin interconnects β-integrin cytoplasmic tails and actin filaments by directly binding both. It has been demonstrated that a single point mutation selectively abrogates the binding of talin head domain to integrin β tails, leading to the disruption of integrin localization to talin-rich focal adhesions [261]. The role of talin as a crucial regulator of β-integrin affinity for its ligands has been reported and the molecular mechanisms through which talin accomplishes this role have been described [256]. Talin function of regulating cell adhesion to the ECM is essential for both physiological processes during embryonic development, immune response, angiogenesis and pathological processes, like cell invasion and metastasis [262]. Talin1 has been shown to be implicated in focal adhesion dynamics, cell migration and cell invasion [263–265]. It enhances prostate cancer cell adhesion, migration, and invasion through the activation, in response to anoikis, of a FAK–Src complex and an AKT survival signalling leading to enhanced metastasis [265]. Further, talin-1 has been reported to be highly expressed in hepatocellular carcinoma cells relative to non-cancer liver epithelial cells and to promote tumour growth and metastasis [266]. It has also been proposed to be a potential marker for colon cancer and hepatocellular carcinoma diagnosis because of its high expression levels in serum specimens from cancer patients [267, 268]. In particular, in hepatocellular carcinoma Talin1 has been reported to have a higher sensitivity and specificity compared to the traditional biomarker alpha-fetoprotein (AFP). Indeed, both talin1 and talin2 expression levels correlate with the malignancy potential of human hepatocellular carcinoma cells [269], whereas talin2 has been implicated in the regulation of breast cancer cell migration and invasion [270]. Recent findings point to the clinical significance of talin's contribution to the metastasization process, talin1 being significantly upregulated in primary tumours and metastatic prostate cancer respect to the normal prostate gland [265]. Talin1 expression has been shown to be significantly higher in poorly differentiated prostate tumours (Gleason > 8) compared to moderately differentiated ones (Gleason 6 and 7). The same authors reported a

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strong inverse correlation between talin1 and E-cadherin expression in human prostate tumours and metastatic lesions. A proteomic analysis-based study showed that high levels of talin1 (> 16-fold) were expressed by highly metastatic cells respect to cells with low metastatic potential [271]. Interestingly, in a transgenic adenocarcinoma mouse prostate (TRAMP) model, talin1 is more highly expressed (more than 2-fold) in advanced disease than in early stage tumours [265].

7- The F-actin stabilizing and regulatory proteins include among others the tropomyosins (TPMs). They are key actin-binding proteins mainly implicated within the troponin complex in the calcium-dependent contraction of skeletal and smooth muscle cells or displaying a functional complexity in non-muscle cells, where they primarily maintain cytoskeleton stability [272]. Four TPM genes have been described in mammals: TPM1, TPM2, TPM3 and TPM4. Several isoforms (around 40) arise from those genes through exon splicing and alternate promoter usage [273]. All those isoforms fold into 2 parallel, linear chains of spiral α-helices [274]. It has been reported that the N- and C-termini of adjacent Tpm molecules originate a sort of "overlap complex" through which Tpms form cables along both sides of the helical actin filament. Among the identified isoforms, 3 are striated muscle-specific and constitute part of the actin thin filament of the contractile apparatus, where they are implicated in the control of actin-myosin interactions and in providing strength and stability to the contractile apparatus [275].The resting isoforms are the "non muscle" or "cytoskeletal" ones. Interestingly, it has been shown that distinct filament populations in various cell types are characterized by specific Tpm and actin isoforms [276–279], providing the opportunity to target well defined actin filament populations in cells based on their Tpm composition. Moreover, it emerges from the current knowledge that Tpms function as the "gatekeepers" of the actin cytoskeleton by virtue of their role as regulators of the interaction of other actin regulatory proteins with the MFs [280]. Functionally, distinct actin filament populations, characterized by their specific Tpm isoform composition, control a variety of physiological processes in yeast, insect and mammalian systems [273, 281, 282]. TPMs are implicated in the control of many benign myopathies, like myasthenia gravis and familial hypertrophic cardiomyopathy [283], their mutations being directly involved in cardiac and skeletal muscle diseases. Evidence has been shown that in malignancy the Tpm expression profile is dramatically altered, with a concomitant significant reorganization of the cytoskeleton [284], directly affecting cancer spread. TPM1 has been first reported to be downregulated in breast cancer, where it functions as a tumour suppressor gene (TSG) [285]. Later, it has been observed that TPM1 acts as a TSG in other types of cancer, like glioma, cholangiocarcinoma and oral squamous cell carcinoma [286–288]. Furthermore, downregulation of the TPM1 gene, in some cases caused by its deletion, has been shown to correlate with the development of colorectal cancer [289]. Interestingly, a number of studies have demonstrated that microRNA-21, a well-known oncogene overexpressed in several solid tumours, decreases TPM1 expression [290, 291]. In anti-miR21-treated MCF7 breast cancer cells, where TPM1 has been shown to be upregulated, identification of TPM1 as a miR-21 target gene has provided a possible explanation of the growth-inhibiting properties of this miR [290]. TPM1 has been shown to be significantly downregulated in both renal cell carcinoma tumours and cell lines [292]. *In vitro* assays demonstrated that TPM1 expression is associated with renal cell carcinoma apoptosis, invasion and migration [292]. Indeed, TPM1 overexpression has been shown to stabilize F-actin and increase the number of cell-cell junctions, thus reducing endothelial cell migration [293]. The low molecular weight isoform Tpm3.1 appears very interesting. Even if cell transformation is characterized by changes in the composition of cytoskeletal Tpm isoforms, this isoform together with Tpm4.2 have been reported to persist in all malignant cell types evaluated up to now, being required for melanoma and neuroblastoma cell survival [294–296]. This is the reason why Tpm3.1 has been considered a potential therapeutic target, small molecule compounds targeting Tpm3.1 being developed as anti-cancer agents [294, 297] (see next section).

4 Drugs targeting actin cytoskeleton organization and function

4.1 Interfering with Rho small GTPase signalling

Similarly to other signalling molecules which propagate the information by intracellular protein-protein interactions and are characterized by large contact surfaces, lacking the grooves and pockets for small molecule interactions, Rho GTPases together with their GEFs and GAPs have been considered for a long time as "undruggable" [138]. The complex picture of Rho small GTPases downstream signalling pathways (Fig. 3) also makes more complicated to achieve the goal of specifically targeting a given cellular process. However, a number of rational strategies have been studied to inhibit the activation of Rho GTPases. Among them, blocking interaction with GEFs or nucleotide binding emerged as the most promising ones to inhibit Rho GTPases in cancer cells and mouse models (Table 2 and Fig. 3). Other strategies to inhibit Rho GTPase activation will be briefly discussed and related small molecule compounds reported (Fig. 3 and Table 2).

- GEF interaction inhibitors

The multiple evidence of aberrant GEF activity in cancer has pointed on those proteins as attractive targets for anti-

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Table 2 Drugs interfering with Rho small GTPase signalling

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
NSC23766	Trio-Tiam1/Rac1	GEF interaction inhibitor	Prostate, breast, gastric cancer, chronic myelogenous leukaemia, anaplastic large cell lymphoma, glioblastoma cells	Impaired growth and invasion	[298–303]
ZINC69391	Tiam1/Rac1	GEF interaction inhibitor	Breast cancer cells	Reduced cell proliferation and migration	[304]
			Mouse model of breast cancer metastasis	Abrogation of *in vivo* metastatic dissemination	
1A-116	P-Rex1/Rac1	GEF interaction inhibitor	Breast cancer, glioma and leukaemia cells	Anti-proliferative and anti-invasive effects Tamoxifen-resistant cells chemosensitization	[304–307]
ITX-3	TrioN/Rac1, RhoG	GEF interaction inhibitor	Rat embryonic fibroblasts	Inhibition of dorsal membrane ruffling	[308]
EHop-016	VAV2/Rac1	GEF interaction inhibitor	Melanoma and breast metastatic cancer cells	Impairment of lamellipodia extension and cell migration	[309–311]
			Mouse model of breast cancer metastasis	Reduction of mammary tumour growth, angiogenesis and metastatic dissemination in nude mice	
MIBQ-167	Rac1/Cdc42	GEF interaction inhibitor	Metastatic breast cancer cells	Reduction of PAK signalling and STAT3 activation	[312]
			Mouse model of breast cancer metastasis	Impairment of migration, viability and mammosphere formation	
				Inhibition of cell surface actin–based protrusions	
				Inhibition of mammary tumour growth and metastasis *in vivo*	
AZA1	Rac1/Cdc42	GEF interaction inhibitor	Prostate cancer cells	Impairment of PAK signalling	[313]
			Prostate cancer mouse xenografts	Inhibition of cell migration	
				Decreased tumour growth *in vivo*	
AZA197	Cdc42	GEF interaction inhibitor	Colon cancer cells	Reduction of PAK and ERK activities and cyclin D expression	[314]
			Mouse xenograft model of colon cancer	Impaired cancer cell proliferation and tumour growth *in vivo*	
CASIN	ArhGEF4/Cdc42	GEF interaction inhibitor	Fast-cycling microadenoma-constructing colorectal cancer cells	Attenuated tumorigenicity	[315]
			Mouse and human intestinal organoids	Organoids abrogation	[316]
ZCL278	ArhGEF4/Cdc42	GEF interaction inhibitor	Metastatic prostate cancer cells	Suppression of actin-based motility and migration	[317]
EHT1864	Rac1	Nucleotide binding inhibitor	Fibroblasts	Inhibition of Rac1-dependent PDGF-induced lamellipodia formation	[318–323]
			Breast, fibrosarcoma and melanoma cells	Blocking of cell transformation caused by constitutively active Rac1 as well as Rac-dependent transformation caused by Tiam1 or Ras	
				Oestrogen receptor levels downregulation	
				Impairment of migration, invasion and tumour growth	
Compounds 1 and 6	Rac1	Nucleotide binding inhibitor	Pancreatic cancer cells	Interfering with the binding between Rac1 and its effector PAK1	[324]

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Table 2 (continued)

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
				Impairment of cell proliferation and migration	
MLS0005322233	Rho, Rac and Cdc42	Nucleotide binding inhibitor	Fibroblasts, rat basophilic leukaemia cells	Inhibition of actin rearrangements and changes in cell morphology typical of Rho GTPase activation	[325]
CID2950007 (ML141)	Cdc42	Nucleotide binding inhibitor	Fibroblasts, ovarian carcinoma cells	Impairment of filopodia formation upon bradykinin treatment Migration inhibition	[326, 327]
R-ketorolac	Rac1/Cdc42	Nucleotide binding inhibitor	Immortalized and primary patient-derived ovarian cancer cells	Decrease in PAK1/PAK2 activation Inhibition of cell adhesion, migration and invasion	[328–331]
			Aggressive mouse model of breast cancer	Reduction of mammary epithelial proliferation *in vivo* Suppression of tumour development *in vivo*	
PF-3758309	PAK	Downstream effector inhibitor: ATP-competitive group I and II PAKs inhibitor	Skin, colon, colorectal, melanoma cancer cells	Growth impairment	[332–335]
			Xenografts and K-ras-driven transgenic mouse model of skin cancer	Strong anti-cancer effects *in vivo*	
FRAX-597	PAK	Downstream effector inhibitor: ATP-competitive group I PAK inhibitor	K-ras-driven transgenic mouse model of skin cancer	Impairment of the initiation and progression of K-ras-driven tumours	[333, 336]
			Merlin-deficient schwannoma xenografts	Growth inhibition of primary tumours	
Secramine A	RhoGDI1/Cdc42	Rho GDI modulator	Monkey kidney epithelial cells	Inhibition of Cdc42 binding to membranes, GTP and effectors (*in vitro*)	[337]
			Astrocytoma cells	Inhibition of anterograde transport from the Golgi complex Disruption of Golgi polarization in migrating cells	
P61A6	Geranylgeranyl transferase type I/RhoA	Spatial regulation inhibitor	Pancreatic and non-small cell lung cancer cells	Inhibition of proliferation and cell cycle progression	[338, 339]
			Xenograft models of pancreatic and non-small cell lung cancer	Reduced growth of cancer cell lines xenograft tumour in mice Decreased RhoA association to the membranes within the tumours	
GGTI2418 (PTX100)	Geranylgeranyl transferase type I	Spatial regulation inhibitor	Xenograft model of breast cancer ErbB2 transgenic mouse model	Inhibition of tumour growth	[340]
Cerivastatin	Hydroxymethylglutaryl-coenzyme A (HMG-CoA)	Spatial regulation inhibitor	Breast cancer cells	Decrease in RhoA activity Impairment of cell migration and invasion	[341]
Atorvastatin	Hydroxymethylglutaryl-coenzyme A (HMG-CoA)	Spatial regulation inhibitor	Melanoma cells Mouse melanoma model	Impairment of *in vitro* and *in vivo* invasion	[342]
Lovastatin	Hydroxymethylglutaryl-coenzyme A (HMG-CoA)	Spatial regulation inhibitor	Umbilical vein endothelial cells Colon carcinoma cells	Inhibition of Rho-mediated expression of E-selectin Reduction of cell adhesion	[343, 344]
Simvastatin	Hydroxymethylglutaryl-coenzyme A (HMG-CoA)	Spatial regulation inhibitor	Colon cancer cells	Inhibition of RhoA/ROCK activation Increased cells sensitivity to doxorubicin treatment	[345]

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cancer therapy. An interesting point is that GEF activation defines spatially and temporally the activation of a given GTPase and likely of the deriving downstream events, with the advantage to convey high signalling specificity reducing off-target effects. Different studies allowed to define the structure of representative GTPase-GEF complexes [346, 347], which showed a very large protein-protein interface originating from the structural remodelling of the small GTPase upon GEF binding. Actually the shape, structural dynamics and chemistry of GEF-GTPase interaction surfaces largely differ from those of catalytic sites of enzymes, such as the ATP-binding site of signalling kinases, likely appearing inappropriate for small molecule binding. However, the mechanism of action of Brefeldin A (BFA), which targets the complex between Arf-GDP and the catalytic domain of the ArfGEF (the Sec7 domain) at the beginning of the exchange reaction, freezing the complex in a conformation that cannot proceed to nucleotide exchange [348], led to the general concept of "interfacial inhibition" [138]. This concept refers to inhibitors that act by stabilization of protein complexes and target regions in or near interfaces [138].

The computational screening of the Rac1 surface known to interact with GEFs has led to the discovery of the small molecule NSC23766. It inhibits Rac1 activation mediated by the Rac-specific GEFs Trio and Tiam1, but not GEF-mediated activation of Cdc42 and RhoA *in vitro* and in cells [298]. Indeed, NSC23766 impaired growth and invasion of prostate, breast and gastric cancer, chronic myelogenous leukaemia, anaplastic large cell lymphoma, and glioblastoma cancer cells [299–303], confirming the oncogenic role of Tiam1 and Trio in cancer. However, the presence of off-target effects in mouse platelets [349] and the high IC50 of around 50 mM make NSC23766 unsuitable for pharmacological applications. On the basis of the structural information derived from NSC23766 in complex with Rac1, *via* a similar approach followed by molecular docking calculations, other five small molecules inhibiting Rac1 have been identified. They were shown to be more effective than the reference compound NSC23766 in reducing the intracellular levels of Rac1-GTP [350]. However, those compounds do not directly target GEFs, rather they likely lack GEF specificity since they function by blocking the surface of GTPases and hence their activation by a variety of GEFs. Through another docking-based screening approach it has been possible to identify ZINC69391 as a molecule structurally distinct from NSC23766, which also affects Rac1-Tiam1 interaction, inhibits breast cancer cell proliferation and migration and abrogates metastatic dissemination *in vivo* [304]. A more potent analogue of ZINC69391 was identified by the same team and reported to affect the interaction of Rac1 with the GEF P-Rex1. This compound, named 1A-116, exerted, similarly to ZINC69391, anti-proliferative and anti-invasive effects on breast cancer, glioma and leukaemia cells [304–306], also chemosensitizing tamoxifen-resistant breast cancer cells [307]. High-throughput screens have largely helped in the discovery of inhibitors targeting specific Rho GEFs. The first was an aptamer screen carried out in yeast, in which peptides coupled to thioredoxin were selected for their binding to the catalytic domain of the GEF Trio [351]. This screening led to the identification of a potent Trio inhibitor, TRIPα, which was then optimized to inhibit the GEF activity of TGAT [352], an oncogenic isoform of Trio. The identified peptide, TRIP(E32G), significantly reduced Tgat-induced RhoA activation and foci formation. Furthermore, subcutaneous injection of cells expressing Tgat and TRIP(E32G) into nude mice reduced the formation of Tgat-induced tumours [352]. Another assay consisted in screening a small chemical compound library in order to monitor the interaction of the GTPase with an effector in the presence of a co-expressed GEF [353]. This approach allowed identifying a variety of inhibitors of Trio-mediated RhoG activation. One of the identified inhibitors, ITX3, was shown to block TrioN-mediated dorsal membrane ruffling and Rac1 activation in transfected mammalian cells with no effect on GEF337-mediated RhoA or Tiam1-/Vav2-mediated Rac1 activation [308]. However, the relatively high IC50 (ranging from 50 to 100 μM) of ITX3 still limits its clinical use. NSC23766 has served as a template for the design of other derivatives, which maintain the central pyrimidine core binding the critical Trp56 in the Rac protein. One of its derivatives is EHop-016 [309]. This compound inhibits the interaction of the VAV2 GEF with Rac. Its development has provided the proof of principle that this interaction represents a druggable target in cancer. By its GEF activity, VAV2 regulates cytoskeleton remodelling in response to stimuli such as growth factor receptor activation, leading to modulation of adhesion, motility and proliferation. In several cell types, VAV proteins function as potent proto-oncogenes that can induce cell transformation when mutated or overexpressed by activating Rho/Rac/Cdc42 GTPase function. We have been the first to describe a transcriptional regulatory loop involving upregulation of VAV2 driven by the overexpression of the transcription factor steroidogenic factor 1 (SF-1) in human ACC [220, 354]. VAV2 has a critical role in the processes of Cdc42 and Rac1 activation and cytoskeleton remodelling induced by SF-1 overexpression in ACC cells and in the regulation of their invasive capacities [220, 354]. Importantly, its expression is robustly correlated with prognosis in three different ACC patient cohorts [220, 354] and its assessment refines prognostic prediction in ACC [355]. Those findings open the way to the development of VAV2 inhibitors as novel targeted pharmacological tools against advanced ACC, which is of seminal importance as the lack of effective therapeutic options for this type of cancer makes the need for more selective and specific treatments urgent. EHop-016 has been reported to inhibit Rac activity with an IC50 of 1 μM and Cdc42 at ≥ 10 μM without affecting Rho [309, 310].

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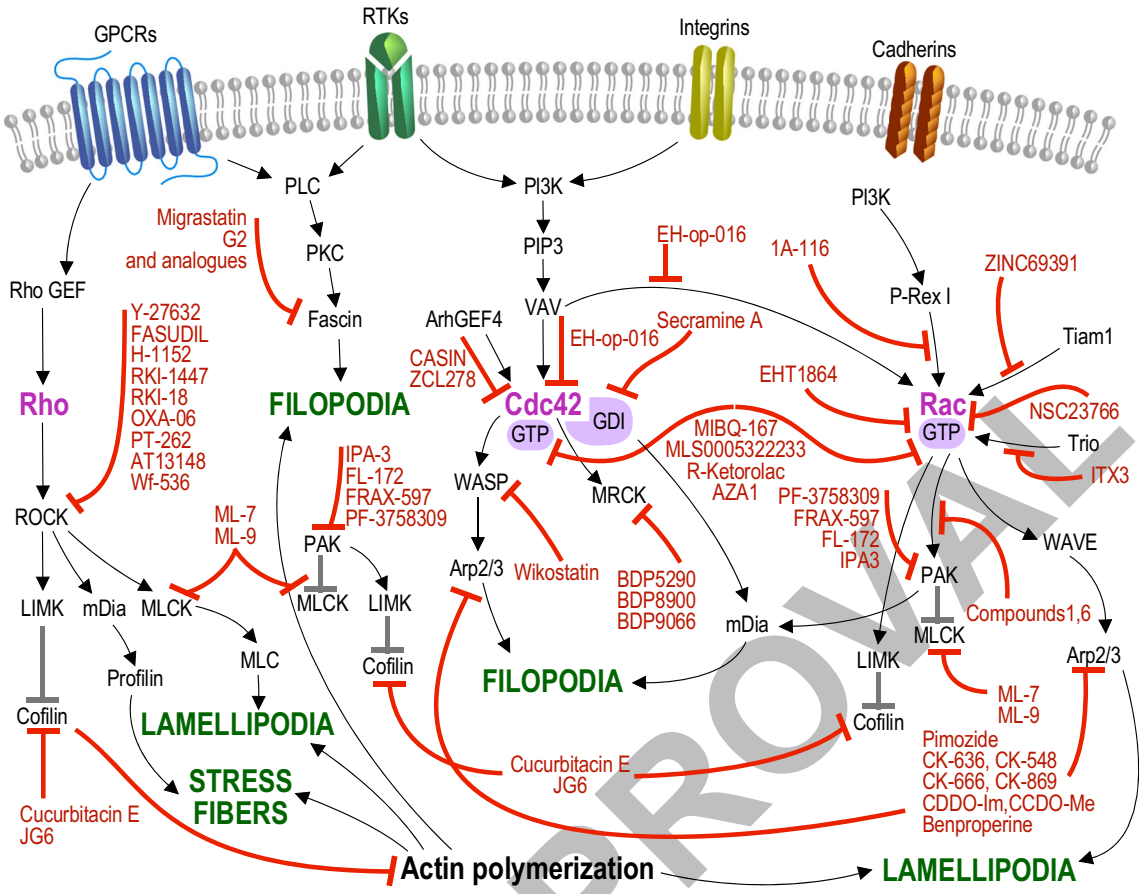

Fig. 3 Schematic representation of signalling pathways controlling actin cytoskeleton dynamics and currently available inhibitors. G protein–coupled receptor (GPCR), receptor tyrosine kinase (RTK), integrin and cadherin signalling pathways converge on the Rho family of small GTPases (Rho, Rac, and Cdc42, *fuchsia*), which are key regulators of actin cytoskeleton dynamics and organization. Some of their activators, like VAV, P-Rex1, Tiam1, Trio and downstream protein kinase effectors, including Rho kinase/ROCK and p21 activated kinase (PAK) are reported in the scheme. The cascades depicted here converge on proteins like cofilin, Wasp, Arp2/3, and profilin that directly regulate the remodelling of the actin cytoskeleton, through which the formation of distinct actin structures, such as lamellipodia, filopodia and stress fibres (*green*), occurs. The coordinated assembly/disassembly of those structures is the critical prerequisite for cells to migrate and invade into surrounding tissues. Small molecule compounds specifically inhibiting key signalling components of the pathways described and/or interfering with Rho small GTPases upstream regulators or downstream effectors are reported in the figure (*red*)

Moreover, it impaired lamellipodia extension and cell migration of melanoma and breast metastatic cancer cells [309] and reduced mammary tumour growth by around 80% in nude mice, inhibiting angiogenesis and metastasis [311]. Unfortunately, EHop-016 exhibited relatively high effective concentrations and a moderate bioavailability of around 30% with a half-life of 4.5 h [356]. In an attempt to find an optimized derivative of EHop-016, the MIBQ-167 compound has been synthesized [312]. This compound has been reported to inhibit Rac activity with an IC50 of 103 nM and Cdc42 activity with an IC50 of 78 nM [312] in metastatic breast cancer cells, which makes MIBQ-167 one of the most potent Rac and Cdc42 inhibitors so far described. MBQ-167 significantly decreased downstream effector p21-activated kinase (PAK) signalling and the activity of STAT3, without impacting on Rho, MAPK, or Akt signalling. Moreover, it impaired breast cancer cell migration, viability, and mammosphere formation and targeted cancer cells that have undergone EMT by a loss of cell polarity and inhibition of cell surface actin–based extensions with subsequent detachment from the substratum. *In vivo*, MBQ-167 inhibited mammary tumour growth and metastasis in immunocompromised mice by approximately 90% [312]. Other derivatives, retaining the central pyrimidine core of the NSC23766 molecule, have been developed; however, they exhibit IC50 values higher than those of EHop-016 or MBQ-167. AZA1 inhibited the activation of both Cdc42 and Rac1 at concentrations ranging from 5 to 20 μM, impairing PAK signalling and prostate cancer cell migration and tumour growth in mice [313]. AZA197, a Cdc42-delective small molecule inhibitor has been shown to be active at an IC50 ranging from 1 to 10 μM, with a reduction of PAK and ERK activities, cyclin D expression, and impaired colon cancer cell proliferation as well as tumour growth in mice [314]. Finally, the screening for Cdc42 specific inhibitors exploiting its unique

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Phe56 in its GEF-binding groove has led to the discovery of CASIN, a compound acting on the interaction between Cdc42 and ArhGEF4 (intersectin) with an IC50 of 2 μM [315]. CASIN-mediated inhibition of Cdc42-ArhGEF4 interaction led to an attenuated tumorigenicity of the fast-cycling microadenoma-constructing colorectal cancer cells and abrogated both mouse and human tumour organoids [316]. ZCL278 is another molecule interfering with the interaction between Cdc42 and ArhGEF4. It has been shown to suppress actin-based motility and migration in the metastatic prostate cancer cell line PC-3 without disrupting cell viability [317].

- Nucleotide binding inhibitors

An alternative to GEF inhibitors, which block GEFs from the binding to the GEF-interacting domains of Rho GTPases (see above), is represented by compounds that prevent nucleotide binding to GTPases. One of those molecules is EHT 1864, which possesses high affinity binding to Rac1 as well as to the related Rac1b, Rac2 and Rac3 isoforms. This association promotes the loss of the bound nucleotide and the inhibition of both guanine nucleotide association and Tiam1 GEF activity [318]. EHT1864 thus functions by freezing Rac in an inert and inactive state, preventing its engagement with downstream effectors. Indeed, EHT 1864 inhibited Rac1-dependent platelet-derived growth factor (PDGF)–induced lamellipodia formation and blocked NIH3T3 fibroblast transformation caused by constitutively active Rac1 as well as Rac-dependent transformation caused by Tiam1 or Ras [318]. Altogether, those data show that EHT 1864 inhibits Rac downstream signalling and transformation *via* a mechanism based on guanine nucleotide displacement. EHT 1864 also highlighted the key role of Rac in downregulating oestrogen receptor expression in breast tumours, migration in fibrosarcoma and melanoma cells, breast cancer tumour growth and invasion [319–323]. Critical off-target effects of this compound have been detected in mouse platelets [349], raising some doubts about its safety. To this class also belong compounds 1 and 6, which interfere with the binding of Rac1 to its effector PAK1, impairing cell proliferation and migration in a number of pancreatic cancer cell lines [324]. A flow cytometry bead–based multiplex assay for molecules inhibiting Rho GTPase activation by non-competitive inhibition of GTP binding led to the identification of MLS0005322233, which blocks Rho, Rac and Cdc42, inducing actin reorganization and cell morphology changes characteristic of Rho GTPases inhibition [325]. By the same flow cytometry type of assay, the CID2950007 (MIL141) compound, specifically inhibiting Cdc42, has been identified. CID2950007 is a potent, selective and reversible non-competitive inhibitor of Cdc42 suitable for *in vitro* assays, with low micromolar potency and selectivity against other members of the Rho family of GTPases, inhibiting filopodia formation in 3T3 cells upon bradykinin treatment [326, 327]. Moreover, CID2950007, without being cytotoxic in a panel of cell lines, inhibited the migration of human ovarian carcinoma cell lines OVCA429 and SKOV3ip in a dose-dependent manner [326]. Finally, through the high-throughput screening of the Prestwick Chemical Library and chemoinformatic studies, Oprea and colleagues [328] identified the R-enantiomers of two approved drugs (naproxen and ketorolac) as Rac and Cdc42 inhibitors, the corresponding S-enantiomers being considered the active components in racemic drug formulations for their activity as non-steroidal anti-inflammatory drugs (NSAIDs) selectively targeting cyclooxygenases [329]. The S-enantiomers of naproxen and ketorolac were reported to be inactive against the Rho GTPases as well as more than twenty other NSAIDs [328]. R-naproxen was first identified as a lead compound. Chemoinformatic-based substructure analysis on R-naproxen led to identification of racemic [R/S] ketorolac as a suitable FDA-approved candidate. Studies carried out on immortalized human ovarian adenocarcinoma cells and primary patient-derived ovarian cancer cells showed that R-ketorolac is a robust inhibitor of growth factor or serum-dependent Cdc42 and Rac1 activation with a potency and cellular efficacy similar to CID2950007/ML141 and NSC23766 [330]. Furthermore, GTPase inhibition by R-ketorolac was reported to decrease p21-activated kinases PAK1/PAK2 activation by more than 80%. R-ketorolac was also shown to significantly inhibit cell adhesion, migration, and invasion of SKOV3ip and primary patient-derived ovarian cancer cells [330]. R-ketorolac treatment caused a significant reduction of mammary epithelial proliferation and suppressed tumour development. The analysis of the proliferative mammary epithelium from R-ketorolac-treated mice showed a greater differentiation, based on significantly higher total E-cadherin and decreased keratin 5 staining than the epithelium of control-treated mice. Those findings pointed on R-ketorolac treatment as a novel therapeutic strategy to slow down tumour progression in an aggressive model of breast cancer, on the basis of its activity as a Rac1 and Cdc42 inhibitor [331]. R-ketorolac has been the first Rac and Cdc42 inhibitor to be used in a P0 clinical trial aimed at investigating whether administration of racemic (R,S) ketorolac after ovarian cancer surgery may lead to peritoneal distribution of R-ketorolac [357]. This study showed that R-ketorolac achieves sufficient levels in the peritoneal cavity of ovarian cancer patients to inhibit Rac1 and Cdc42 activity, potentially contributing to the observed survival benefit in women who received ketorolac [357].

- Downstream effector inhibitors

Another strategy to inhibit Rho GTPase activation is based on the prevention of their downstream signalling through the inhibition of key effector proteins. An example is provided by drugs targeting PAKs, which are key proteins at the nexus of

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several oncogenic signalling pathways. When activated by mutation, overexpression or upstream signalling molecules such as Rho GTPases, PAKs exert oncogenic effects, including promotion of invasion and metastasis, evasion of apoptosis and acquisition of deregulated growth signal. Indeed, a number of broad-range kinase inhibitors exhibited potent PAK inhibition; however, those compounds are not particularly useful due to their lack of selectivity. By sequence and structure, the six mammalian PAKs have been divided into two subgroups: group I (PAK1–3) and group II (PAK 4–6), displaying both overlapping and distinct function and being regulated by different autoinhibitory mechanisms exploitable for molecule design. PF-3758309 is a potent ATP-competitive PAK inhibitor, originally designed as a PAK4 inhibitor. It has been reported to efficiently target group I and II PAKs, including a series of off-target kinases [332]. PF-3758309 impaired the growth of a number of tumour cell lines and exhibited strong anti-cancer effects in xenografts and in a K-ras-driven transgenic mouse model of skin cancer [333–335]. Even if the biological effects of this compound *in vivo* have been supposed not to depend on PAK inhibition alone, its potency (around 4.7 nM) together with its oral availability allowed its enter into phase I clinical trials. Unfortunately, PF-3758309 failed clinical trials due to its undesirable pharmacokinetic characteristics and the lack of an observed dose-response relationship (https://clinicaltrials.gov/ct2/show/NCT00932126) [358]. FRAX-597 is a group I specific ATP-competitive PAK inhibitor which impairs the initiation and progression of K-ras-driven tumours in a mouse model of skin cancer and also inhibits the growth of Merlin-deficient schwannoma xenografts [333, 336]. Also, this compound has substantial off-target activity against receptor tyrosine kinases. To exploit the large ATP-binding pocket present in all PAK family members, a metallopyridocarbazole scaffold has been exploited to insert a rigid, bulky, octahedral ruthenium complex within the ribose binding site [359]. The resulting compound has been called FL172 and showed a high inhibitor efficacy toward PAK1 with an IC50 of around 1 μM as well as a good selectivity over other related kinases tested. However, organometal conjugates like FL172 are characterized by a poor solubility and a relatively high toxicity, which limits their application. Finally, in the attempt to develop allosteric PAK inhibitors, IPA-3 (inhibitor-p21-activated kinase-3), a sulphydryl-containing molecule targeting the N-terminal regulatory domain of group I PAKs, has been isolated [360]. IPA-3 acts by covalently binding in a reversible fashion to the PAK1 regulatory domain, thus preventing GTPase docking and its subsequent activation [361]. This mechanism of action confers to IPA3 exceptional target specificity. However, its pharmacokinetics properties as well as the continuous reduction of the sulphydryl moiety producing in the cell important redox effects, makes IPA3 unsuitable for clinical development. Another important downstream effector protein of Rho GTPase against which inhibitors have been developed is Rho kinase (together with its downstream effector kinase MLCK), also referred to as ROCK, which is the major effector of RhoA. Efforts were also made to target other Rho GTPase downstream effector proteins belonging for instance to the actin nucleation machinery like N-Wasp and Arp2/3 complex. ROCK, MLCK and N-Wasp-Arp2/3 small molecule inhibitors are described in the next sections.

- Rho GDI modulators

An additional strategy to inhibit Rho GTPase activation consists in the targeting of RhoGDIs, which, as reported above, sequester GDP-bound Rho GTPases in the cytosol. This approach is based on an interfacial interactor that prevents the release of a RhoGDI from a given Rho GTPase, with the ultimate effect to maintain the GTPase in its inactive state. Secramine A is a molecule that has been discovered through high-throughput synthesis and phenotypic screening as a compound able to inhibit membrane traffic out of the Golgi complex [337]. The mechanism underlying secramine A–dependent Cdc42 inhibition depends upon the Rho guanine dissociation inhibitor 1 (RhoGDI1). *In vitro* assays revealed that secramine A prevents Cdc42 activation (GTP loading) through the reduction of the binding of prenylated Cdc42 to membranes [337]. Those events are strictly dependent on the presence of RhoGDI1, which shuttles Cdc42 between the cytosol and the membranes. In cells, secramine A mimics the effects of dominant-negative Cdc42 expression on protein export from the Golgi and on Golgi polarization in migrating cells. RhoGDI-dependent Cdc42 inhibition by secramine A is representative of a novel approach to inhibit Rho GTPases by small molecules, providing new means to study Rho GTPases, RhoGDIs and the cellular processes they regulate.

- Spatial regulation inhibitors

The essential prerequisite for Rho GTPase activation by GEFs is localization of the GTPases at the plasma membrane. Hence the rationale of impairing Rho GTPase function by inhibiting the enzymes responsible for its post-translational modifications, such as the addition of isoprenoid moieties (prenylation) at the C-terminal "CAAX box", which are required for GTPase targeting to the plasma membrane. Similarly, Ras activation has been inhibited using farnesyltransferase inhibitors. A variety of small molecule candidate inhibitors of geranylgeranyltransferase type I (GGTIs) aimed at blocking membrane association of signalling proteins including Rho GTPases have been developed over the years. One of those compounds is P61A6, which showed inhibition of geranylgeranylation without affecting farnesylation and inhibited proliferation and cell cycle progression in a variety

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of human cancer cells lines, exhibiting tumour-suppressing effects on human pancreatic cancer xenografts [338]. P61A6 has been also reported to inhibit proliferation of various non-small cell lung cancer cell lines, interfering with geranylgeranylation and membrane association of RhoA [339]. The effects of P61A6 on cell proliferation has been mainly ascribed to RhoA, as the expression of the RhoA mutant which bypasses geranylgeranylation makes the cells resistant to P61A6 inhibition [339]. Further, P61A6 treatment reduced the growth of non-small cell lung cancer cell lines xenograft tumour in nude mice as well as the RhoA association to the membranes within the tumours [339]. Even if the GGTIs are not specific for the Rho GTPases, their effects being often difficult to interpret mechanistically, prenylation inhibition has emerged as an efficient strategy for anti-cancer therapy. An example is provided by the potent and selective peptidomimetic inhibitor of geranylgeranyltransferase type 1, GGTI2418 (also known as PTX100), which significantly inhibited the growth of breast tumours in mice [340]. This compound has been the first GGTase I inhibitor to enter clinical development in early 2009. Phase I clinical trials early results demonstrated that ~ 30% of patients with advanced solid tumours had stable disease following GGTI-2418 therapy, the drug being well tolerated and exhibiting minimal toxicity (https://drugs.ncats.io/drug/M67G28K74K). However, the Phase I trial of GGTI-2418 has been stopped due to its lack of efficacy in patients. Other molecules exploitable for their indirect action on Rho GTPases prenylation are statins. Indeed, those drugs inhibit hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the essential enzyme for cholesterol production, with the effect to lower farnesyl pyrophosphate and geranylgeranyl pyrophosphate synthesis and thus Rho GTPase prenylation. Cerivastatin (Baycol/Lipobay) decreases RhoA activity in MDA-MB-231 breast cancer cells, impairing cell migration and invasion [341]. Atorvastatin (Lipitor) inhibited *in vitro* and *in vivo* invasion of human melanoma cells [342]. Lovastatin (Mevacor) inhibited Rho-mediated expression of E-selectin, with subsequent reduction of tumour cell adhesion [343, 344]. Although this compound was also able to increase the levels of RhoA, RhoB and RhoC in human erythroleukaemia cells, those proteins accumulated in their unprenylated form, thus having impaired membrane localization and activation [344]. Simvastatin (Zocor) inhibited RhoA/ROCK activation and increased the sensitivity of human HT29 colon cancer cells to doxorubicin treatment [345]. Even if those examples show that statins display anti-cancer properties, their use is limited by their lack of selectivity. Further studies are needed to shed light on the specificity of their inhibition.

4.2 Interfering with actin-binding protein function

Numerous ABPs directly or indirectly have been shown to significantly affect the migratory and metastatic phenotype of tumour cells (see above). This is the reason why many efforts have been made in developing drugs which can interfere with the functions exerted by a number of ABPs (Fig. 3). Here we provide an overview of the compounds targeting the actin-bundling protein FSCN; the activator of actin nucleation N-Wasp and the actin nucleators Arp2/3 complex and formins; the focal adhesion complex protein talin and the F-actin depolymerization factor cofilin (Table 3).

- Fascin inhibitors

The first to suggest that the actin-bundling protein FSCN can be explored as a new molecular target for cancer treatment has been the group of Dr. Huang. Indeed, to understand the molecular basis by which the analogues of the natural product migrastatin inhibit tumour cell migration and metastatic dissemination [362–364], this team pursued the biochemical identification of the protein targets of those compounds [365] by an affinity protein purification approach using a synthesized biotin-labelled macroketone [363]. They were thus able to show that the migrastatin analogues target the actin-bundling protein FSCN to inhibit its activity [365]. Further, they solved X-ray crystal structures of the wild-type FSCN and four FSCN mutants to identify the active and inactive configurations of FSCN and showed the structural basis for the conformational changes of FSCN during the actin-binding process [365, 382]. They also carried out a systematic mutagenesis study employing 100 FSCN mutants which provided evidence for the existence of at least two major actin-binding sites on FSCN, each of those sites being essential for filopodia formation in cells [382]. With the unpredicted nature of potential toxicity of small molecule drugs in mind, the same group screened chemical libraries in order to identify novel molecules still inhibiting FSCN actin-bundling activity but being structurally different from migrastatin analogues, which share the similar core chemical scaffold [366]. By high-throughput screening, they identified and characterized novel small molecules able to impair FSCN actin-bundling activity. In particular, they focused on one such inhibitor, the G2 compound [366], and they demonstrated that it specifically blocks filopodial formation in two-dimensional and 3D environment, breast tumour cell migration and invasion *in vitro*, and metastasis *in vivo* [366]. Altogether those findings indicated that target-specific anti-FSCN agents exhibit a therapeutic potential for the treatment of cancer. To optimize the structure-activity-relationship of the G2 compound, which inhibits the actin-bundling activity of FSCN with an IC50 of 5–8 μM, they then designed, synthesized and biologically evaluated a number of new G2 analogues [367]. Those molecules resulted to be more potent than G2, one of them, the NP-G2–044 compound, exhibiting an IC50 value of about 0.2 μM [367]. They focused on this analogue and on another one, the NP-

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G2–011 compound, and they demonstrated that the two compounds inhibited *in vitro* actin-binding and bundling activities of FSCN, actin cytoskeleton reorganization with a decrease in filopodia and lamellipodia formation, breast tumour cell migration and metastasis in mouse models [367]. Those data provided evidence for a great potential of NP-G2–044 and NP-G2–011 as novel potential pharmacological tools to exploit for the treatment of advanced metastatic tumours. Remarkably, NP-G2–044 is under investigation since 2017 in a phase 1 clinical trial in patients with advanced or metastatic treatment-refractory solid tumour malignancies (https://clinicaltrials.gov/ct2/show/NCT03199586).

- N-Wasp and Arp2/3 complex and formin inhibitors

Almost 20 years ago, Peterson and colleagues used a chemical screen in *Xenopus* cell-free extracts to identify compounds that affect signalling pathways controlling actin polymerization. As a result of high-throughput screens for compounds inhibiting phosphatidylinositol 4,5-bisphosphate (PIP2)-induced actin assembly, they identified the first molecule known to block actin assembly through the inhibition of an upstream signalling component. They indicated N-Wasp as the target of this molecule and showed that this compound prevented Arp2/3 complex activation through the allosteric stabilization of the autoinhibited conformation of N-WASP [383]. The same group some years later reported the identification of a small molecule inhibitor of N-WASP, called wikostatin [368]. They showed that this compound interacts with a cleft in the regulatory GTPase-binding domain (GBD) of WASP in the solution structure of the complex inducing folding of the isolated, unstructured GBD into its inactive autoinhibited conformation. Those findings indicate that wikostatin functions by stabilizing N-WASP in this native conformation, thus causing N-Wasp inhibition [21]. Therefore, wikostatin has been considered to function by biasing an allosteric equilibrium toward an inactive state.

More recently, Nolen and colleagues described two compounds that bind to different sites on Arp2/3 complex inhibiting its actin nucleating filament activity [369]. CK-0944636 (abbreviated CK-636) has been described to bind between Arp2 and Arp3, where it seems to inhibit the shift of Arp2 and Arp3 to their active conformation. CK-0993548 (abbreviated CK-548) likely inserts into the hydrophobic core of Arp3 altering its conformation. Further, treatment of cultured cells with those compounds inhibited the formation of actin structures known to require Arp2/3 complex, like *Listeria* actin comet tails and podosomes [369]. The same group then used a combination of biochemical and biophysical methods to define the mechanisms of action of CK-666 and CK-869, which are more potent versions of the parent compounds CK-636 and CK-548 [370] providing evidence that those molecules inhibit Arp2/3 complex by blocking an activating conformational change. Different mechanisms underly the conformational trapping exerted by each inhibitor. CK-666 works as an allosteric effector, stabilizing the inactive state of the complex, whereas CK-869 likely disrupts crucial protein-protein interfaces in the short pitch Arp2-Arp3 dimer to destabilize the active state [370].

The synthetic triterpenoids 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO)-Im and CDDO-Me have been shown to associate with actin cytoskeleton proteins, including the Arp3 subunit of the Arp2/3 complex [371]. They are able to inhibit the localization of Arp3 and actin at the leading edge of the cell, perturb cell polarity and impair Arp2/3-dependent branched actin polymerization, ultimately inhibiting cell migration [371].

Through a Connectivity Map (CMap)–based drug discovery strategy as a new screening method, Choi and co-workers identified pimozide, an antipsychotic drug used in the clinic, as an ARPC2 inhibitor [372]. This compound has been shown to strongly impair migrating and invasive abilities of different cancer cells. Direct interactions between ARPC2 and pimozide have been demonstrated by computational docking studies and label-free biochemical assays. The compound has also been shown to increase the lag phase of Arp2/3 complex–dependent actin polymerization and to inhibit vinculin-ARPC2 interaction crucial for focal adhesion turnover. Pimozide has been thus proposed as a lead molecule for the development of anti-metastatic drugs. The same group by a phenotype-based screening of a library of 719 FDA-approved drugs or clinically tested compounds identified Benproperine (Benp), an antitussive drug used in the clinic, as a potent anti-migratory drug [373]. Its anti-metastatic activity was also shown *in vivo*. ARPC2 was identified as a direct target of Benp and further validated by computational docking study and label-free biochemical and biophysical assays. Benp inhibited Arp2/3 function, disrupting lamellipodial structure and inhibiting actin polymerization. Moreover, Benp showed anti-migratory activity at a lower dose (2 μM) respect to others Arp2/3 inhibitors, like CK666 (100 μM) and CK869 (20 μM). Remarkably, differently from Arp2/3 inhibitors, Benp selectively inhibited migration and invasion of cancer cells but not normal cells [373], suggesting that this drug has the clinical potential to block metastasis.

As reported above, another class of actin nucleators is represented by formins. By screening compounds for the ability to prevent formin-mediated actin assembly *in vitro* Rizvi and co-workers identified a general small molecule inhibitor of formin homology 2 domains (SMIFH2) [374]. SMIFH2 has been shown to prevent both formin nucleation and processive barbed-end elongation, also decreasing formin's affinity for the barbed end. Moreover, it has been demonstrated that low micromolar concentrations of SMIFH2 affected only formin-dependent, but not Arp2/3 complex–dependent actin cytoskeletal structures in both fission yeast and mammalian NIH3T3 fibroblast cells [374].

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Table 3 Drugs interfering with actin-binding protein function

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
Migrastatin and analogues	Fascin	Inhibition of Fascin actin-bundling activity	Ovarian, colon, mammary carcinoma cells, B lymphoblastoid cells Breast cancer metastasis mouse model	Inhibition of tumour cell migration Inhibition of metastatic dissemination	[362–365]
G2	Fascin	Inhibition of Fascin actin-bundling activity	Breast cancer cells Orthotopic spontaneous breast tumour metastasis	Blocking of filopodia formation in 2D and 3D environment	[366]
			Mouse model	Reduction of tumour cell migration and invasion	
			Xenograft model of breast tumour metastasis	Reduction of metastasis	
NP-G2-044/NP-G2-011	Fascin	Inhibition of Fascin actin-binding and bundling activity	Mouse and human breast cancer cells Orthotopic spontaneous breast tumour metastasis mouse model	Inhibition of actin cytoskeleton reorganization Reduced number of filopodia and lamellipodia Impairment of cell migration Impairment of metastasis *in vivo*	[367]
Wikostatin	N-Wasp	Allosteric stabilization of the autoinhibited conformation of N-WASP	Xenopus laevis egg extract	Impaired Arp2/3 activation	[368]
CK-636/CK-548/CK666/CK-869	Arp2/3 complex	Blocking of an activating conformational change *via* the stabilization of the inactive state or the destabilization of the active state of the complex	Monocytic leukaemia cells, ovarian cancer cells, *Listeria monocytogenes* cells	Inhibition of actin nucleation by Arp2/3 Inhibition of actin-rich structures (podosomes and Listeria comet tails) assembly	[369, 370]
CDDO-Im/CDDO-Me	Arp2/3 complex	Investigated but not yet defined	Fibroblasts	Altered localization of Arp3 and N-Wasp at the Leading edge of polarized cells Reduction of branched actin at the leading edge of migrating cells Impairment of cell migration	[371]
Pimozide	Arp2/3 complex (ARPC2)	Direct interaction with ARPC2 with impairment of its function	Colon, pancreas, lung, melanoma cancer cells Xenograft mouse model of lung metastasis	Increase of the lag phase of Arp2/3 complex–dependent actin polymerization Inhibition of vinculin-ARPC2 interaction Inhibition of growth, migration and invasion *In vivo metastasis suppression*	[372]
Benproperine					[373]

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Table 3 (continued)

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
	Arp2/3 complex (ARPC2)	Disruption of ARP2C function without affecting the expression levels of Arp2/3 complex subunits	Colon, breast, melanoma, pancreas and prostate cancer cells	Disruption of lamellipodial structure Inhibition of actin polymerization	
			Xenograft orthotopic and metastatic models of pancreatic cancer	Impaired migration Reduced *in vivo* metastatic dissemination	
SMIFH2	Formins (homology 2 domain)	Prevention of formin nucleation and barbed-end elongation	Fission yeast Fibroblasts Lung cancer cells	Inhibition of formin-mediated actin assembly Disruption of formin-dependent actin cytoskeletal structures Inhibition of death, growth and migration	[374]
Indothiazinone	talin/αIIbβ3	Binding to talin and interfering with integrin/talin interaction	Chinese hamster ovary cells expressing integrin αIIbβ3	Antiplatelet activity Inhibition of integrin αIIbβ3–dependent cell adhesion Impairment of platelet spreading Inhibition of talin-induced integrin activation	[375]
Trastuzumab	miR-194/talin2	miR-194 upregulation	HER2-overexpressing human breast cancer cells Human breast cancer xenografts in nude mice	Talin2 downregulation disruption of cytoskeletal organization Disruption of normal vinculin staining pattern Inhibition of cell migration and invasion	[376]
Resveratrol	Talin/pFAK	Investigated, but not yet defined	Colon cancer cells	Suppression of cell proliferation Induction of apoptosis Suppression of talin and phosphorylated FAK protein levels Abrogation of cell-ECM interaction	[377]
DZ-50	Talin/fibronectin/integrin α6	Downregulation of critical effectors of focal adhesion integrity and tight junction formation	Prostate cancer cells	Structural destruction of both focal adhesions and tight junctions	[378]

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Table 3 (continued)

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
				Decreased cell survival, migration and adhesion to ECM components	
Cucurbitacin E	Cofilin	Cofilin phosphorylation inhibition	Breast cancer cells	Inhibition of cell migration and invasion	[379, 380]
		Abrogation of Arp2/3-dependent actin polymerization Inhibition of the Src/FAK/Rac1/MMP cascade	Spontaneous and experimental breast tumour metastasis models	Reduction of tumour metastasis *in vivo*	
JG6	Cofilin	Occupation of the actin-binding sites of cofilin with subsequent suppression of depolymerization/severing activities	Breast cancer cells	Inhibition of migration and invasion	[381]

- Talin and cofilin inhibitors

An in-house chemical library screening aimed at finding natural products capable of inhibiting the interaction between platelet integrin aIIbβ3 and fibrinogen, which is a crucial step in platelet aggregation, led to the identification of indothiazinone, an alkaloid found in microbial cultures, as a potential antiplatelet agent [375]. Surface plasmon resonance and molecular dynamics studies revealed that this compound exhibited antiplatelet activity, inhibiting integrin aIIbβ3–dependent cell adhesion, platelet spreading, and talin-induced integrin activation [375]. However, the potential of indothiazinone of impairing cancer cell migration and invasion by interfering with talin-induced activation of other β-integrin isoforms has not yet been tested, the molecule being proposed as a lead compound for the development of antiplatelet drugs with a new mechanism of action.

Some established and putative cancer therapies have been reported to affect directly talin levels in the cell. Le and coworkers, in the attempt to measure the effect of the humanized monoclonal antibody trastuzumab on miRNAs and their role in trastuzumab-mediated regulation of human breast cancer cells overexpressing HER2, showed that trastuzumab treatment impacted the expression of several miRNAs [376]. In particular, miR-194 was shown to be upregulated and to downregulate talin2, with a subsequent inhibition of cell migration and invasion. The authors proposed that trastuzumab treatment may exert its cell migration-inhibitory effect through miR-194-mediated downregulation of cytoskeleton protein talin2 in HER2-overexpressing human breast cancer cells [376].

Vanamala and colleagues [377] unravelled key components of the cancer chemoprevention ability of Resveratrol (RSV, 3,5,4′-trihydroxy-trans-stilbene) by functional proteomic studies carried out in HT29 human advanced-stage colon cancer cells. RSV is a stilbenoid and a potent chemopreventive bioactive compound, which exerts anti-cancer properties by the inhibition of tumour initiation, promotion and progression [384]. They showed that RSV suppressed talin and phosphorylated focal adhesion kinase (FAK) protein levels even in the presence of the potent mitogen insulin growth factor 1 (IGF-1), suggesting that RSV anti-cancer effects against HT29 cells might be partly due to the abrogation of cell-ECM interaction, the talin-FAK signalling being a critical target of RSV [377].

DZ-50 is a quinazoline-based compound of new generation derived from the adrenoreceptor antagonist Doxazosin® and exerting strong anoikis-inducing effects on cancer cells [385]. DZ-50 has been shown to inhibit the growth of human prostate cancer xenografts and metastatic dissemination in animal models affecting angiogenesis, migration and invasion [386] by targeting the focal adhesion signalling cascade [387]. Hensley and co-workers carried out a genome-wide analysis in

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the human prostate cancer line DU-145 and identified as primary downregulated targets of DZ-50 critical effectors of focal adhesion integrity and tight junction formation [378]. Talin, together with fibronectin and integrin-α6, were among the identified genes. Confocal microscopy studies in two human prostate cancer cells showed as a consequence of the downregulation of those targets the structural disruption of both focal adhesions and tight junctions accompanied by decreased cell survival, migration and adhesion to ECM components [378]. Remarkably, overexpression of talin1 stabilized cell-ECM interactions mitigating DZ-50 effects, while talin-1 loss of expression sensitized human prostate cancer cells to anoikis [378].

Nakashima and colleagues synthesized a biotin-linked cucurbitacin E, a cucurbitane-type triterpene molecule reported to exert cytotoxic effects on a number of cancer cell lines, to isolate target proteins based on their affinity for this molecule. As a result, cofilin was isolated and proposed to be a target of cucurbitacin E [379]. Indeed, cucurbitacin E inhibited cofilin phosphorylation in a concentration-dependent manner, effective concentrations being in the same range as those at which the compound had cytotoxic effects on human leukaemia U937 cells [379]. Moreover, the treatment of human fibrosarcoma cells HT1080 with low doses of cucurbitacin E decreased the F-actin/G-actin ratio. Collectively, those results suggested that the inhibition of cofilin's phosphorylation *via* cucurbitacin E promoted cofilin severing activity, low doses of the drug increasing actin depolymerization. In contrast to those results, Cucurbitacin E has been reported by Sorensen and co-workers to inhibit depolymerization of actin filaments by specifically binding to F-actin (but not to monomeric G-actin) forming a covalent bond at residue Cys257 [388]. Interestingly, in *in vivo* experiments the intraperitoneal administrations of cucurbitacin E significantly reduced breast tumour metastasis to the lung with no effects on apoptosis or proliferation of inoculated breast cancer cells [380]. *In vitro* cucurbitacin E strongly inhibited migration and invasion of metastatic breast cancer cells. The abrogation of Arp2/3-dependent actin polymerization and the inhibition of the Src/FAK/Rac1/MMP cascade have been identified as the molecular events triggered by cucurbitacin E and underlying the reduction in the cell migratory and invasive abilities [380]. Those data point on cucurbitacin E as a potential candidate for treating breast cancer metastasis.

Finally, it has been shown that JG6, a marine-derived oligosaccharide, binds to cofilin and inhibits the migration of human breast cancer cells *in vitro* and cancer metastasis *in vivo* [381]. From a mechanistic point of view, JG6 occupies the actin-binding sites of cofilin, thus suppressing its depolymerization/severing activities of cofilin, the ultimate result being an inhibition of breast cancer cell migratory and invasive potential [381].

Overall, the presented evidence supports cofilin as an emerging target in cancer therapy. However, cofilin activity is more frequently modulated *via* the targeting of the upstream LIM kinase, which, as reported above, phosphorylating cofilin on Ser 3 controls its actin severing activity. An example is provided by diallyl disulphide (DADS), which has been reported to inhibit the phosphorylation of ADF/cofilin *via* downregulation of LIMK1, those events leading to a significant impairment of colon cancer cell migration and invasion [389].

4.3 Interfering with actin cytoskeleton polymerization

Compounds which affect actin cytoskeleton polymerization produce dramatic effects on crucial cell processes, like cell migration, cell division, maintaining of cell shape, endocytosis, exocytosis and the ensemble of the events depending on the remodelling of the actin cytoskeleton [390, 391]. Those drugs have also had a key role in the definition of the actin biochemical properties and dynamics in certain cellular functions, like cell motility. Two main categories can be identified: (a) molecules that stabilize filaments, thus inducing actin polymerization and (b) molecules that abrogate the assembly of actin filaments and produce filament destabilization (Fig. 4 and Table 4). Multiple are the compounds that belong to the latter group. Some of them destabilize F-actin through filament severing, while others simply bind monomers, thus preventing polymerization or disassembly existing filaments. In general, those compounds cause a sort of shift in the dynamic equilibrium between monomers and filaments toward monomers, the final result being a loss of the filamentous fraction since depolymerization is not counterbalanced by polymerization in the presence of those molecules. The opposite occurs to compounds that stabilize the assembled polymer by binding to F-actin which promote polymerization through a shifting of the equilibrium toward filaments.

- Actin cytoskeleton stabilizing drugs

The best-known compound stabilizing actin filaments and promoting actin polymerization is phalloidin, a phallotoxin from the deadly mushroom *Amanita phalloides*. It is a bicyclic heptapeptide that shifts the equilibrium between G- and F-actin toward F-actin lowering the critical concentration for polymerization by an order of magnitude [390]. Phalloidin is not cell permeant, which makes it not useful as an inhibitor. However, fluorophore-conjugates of phalloidin are used since a long time for the staining and visualization of F-actin in permeabilized cells [422].

Jasplakinolide is a cyclodepsipeptide derived from marine sponges [423, 424]. It promotes actin polymerization, binds F-actin competitively with phalloidin and induces actin filament stabilization [425]. Differently from phalloidin, jasplakinolide crosses the cell membranes even in the absence of cell

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Table 4 Drugs interfering with actin cytoskeleton polymerization

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
Jasplakinolide	Actin	Actin stabilizer (binding to F-actin competitively with phalloidin and filament stabilization induction)	Endothelial cells	Impairment of lamellipodia protrusion	[]
			Primary fibroblasts	Decreased migration	
			Prostate and lung carcinoma cells	Inhibition of Listeria monocytogenes	
			Lung carcinoma and prostate carcinoma mouse xenografts	Actin-based motility	
				Decreased metastasis *in vivo*	
				Cell sensitization to radiation	
Chondramides	Actin	Actin stabilizers (binding to F-actin competitively with phalloidin and filament stabilization induction)	Breast cancer cells Breast carcinoma mouse xenografts	Inhibition of invasion and metastasis	[392, 393]
Cytochalasin B	Actin	Actin destabilizer (binding and blocking of the barbed end of actin filaments, shortening/severing of actin filaments)	Mouse lung carcinoma and melanoma cells	Delayed appearance of tumour nodules	[390, 394–400]
			Mouse mammary ascites carcinoma cells	Delayed tumour growth	
			Lung carcinoma and melanoma xenograft models	Inhibition of spontaneous lung metastasis	
Immunosuppression induction of murine allogeneic anti-tumour response					
Cytochalasin D	Actin	Actin destabilizer (binding and blocking of the barbed end of actin filaments, binding to and sequestering of actin monomers, actin ATPase activity acceleration)	Gastric carcinoma cells Breast carcinoma cells Experimental model of melanoma metastasis to lungs	Invasion inhibition Induction of pulmonary metastasis	[401–408]
Latrunculin A and derivatives	Actin	Actin destabilizer (inhibition of actin polymerization by sequestering of G-actin monomers, inhibition of nucleotide exchange in the monomer)	Tumorigenic S-adenosylmethionine decarboxylase--transfected fibroblasts	Actin cytoskeleton disruption in yeast Induction of changes in cell shape and actin	[409–421]
			Breast, cervical and prostate cancer cells	Filament organization in cells	
			Mouse model of human gastric cancer	Impairment of invasion	
				Impairment of metastatic dissemination	

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permeabilization, being a useful tool for cellular studies aimed at stabilizing F-actin and increasing actin polymerization. Even if the F-actin stabilization activity of jasplakinolide has been directly detected in cells [426, 427], when treated with this compound certain cells exhibit a disruption of the actin cytoskeleton. This effect can appear paradoxical, but it has been explained as the result of an increased *de novo* actin nucleation which produces an important amount of amorphous F-actin filaments in spite of insufficient remaining G-actin monomers for the remodelling of organized structures like stress fibres [428]. Jasplakinolide has been reported to impair lamellipodia protrusion and migration of fibroblasts, where the inhibition of F-actin disassembly is observed upon short-term treatment whereas only upon longer treatment a net increase in actin polymer amount is observed [427]. The drug also inhibits the actin-based motility of the intracellular pathogen *Listeria monocytogenes* [427]. Finally, jasplakinolide was an active antitumor agent against the Lewis lung carcinoma and the DU-145 prostate carcinoma xenograft [429]. Moreover, it was shown to be a radiation sensitizer in the

Lewis lung carcinoma and to decrease lung metastases in systemic Lewis lung carcinoma [429]. Lung metastases were further decreased upon jasplakinolide administration along with radiation to the subcutaneous primary tumour [429].

Other compounds the binding of which to F-actin is competitive with phalloidin are chondramides, cyclodepsipeptides isolated from the mycobacterium *Chondromycescrocatus crocatus* [392]. They have been reported to inhibit the invasion of human MDA-MB-231 breast carcinoma and to inhibit metastatic dissemination of 4 T1 breast carcinoma cells to the lung [393]. Among F-actin assembly inducing-agents are also included dolastatin 11 [430], hectochlorin [431], and doliculide [432]. Dolastatin 11 and hectochlorin, differently from jasplakinolide, are not competitive with phalloidin for F-actin binding.

- Actin destabilizing drugs

The fungal metabolite cytochalasins are among the best-known drugs interfering with actin polymerization [390]. Those molecules act by binding and blocking the barbed end of actin filaments, behaving like barbed-end capping proteins, thus blocking further polymerization. More than 60 different cytochalasins have been identified; however, only cytochalasins B and D have been extensively studied for their chemotherapeutic potential and are the most largely used members of this group of compounds.

Cytochalasin D has been reported to have a greater selectivity for actin than cytochalasin B, and a lower Ki for inhibition of dynamics at the barbed end. Further, cytochalasin D also binds to and sequesters actin monomers and perhaps dimers [401–403]. Cytochalasin D and B, even if the latter to a lesser extent, have also been shown to accelerate the ATPase activity of actin [404, 405]. Evidence indicates that cytochalasin B has the ability to shorten or sever actin filaments, thus resembling to actin filament-severing proteins [394, 395]. Cytochalasin D has been shown to inhibit invasion of AGS gastric cells [406] and MDA-MB-231 breast carcinoma cells [407], and to induce pulmonary metastasis of B16 melanoma cells [408]. Indeed, several studies on the anti-cancer activity of those class of compounds focused on cytochalasin B because of its safer and less toxic profile respect to the more potent analogue cytochalasin D [396]. Cytochalasin B has been known for its anti-metastatic properties since the late 1970s [397]. It was reported to inhibit the metastatic dissemination of Madison 109 mouse lung carcinoma cells [398] and mouse B16-F10 mouse melanoma cells [399]. In mouse lung carcinoma cells, it exhibited an immunosuppressive effect that was however completely abrogated by human recombinant interleukin-2 [400].

Latrunculins are compounds derived from marine sponges that inhibit actin polymerization by sequestering G-actin monomers [409]. Compared to cytochalasins they are generally more potent and are characterized by a simpler and more definable mode of action. Latrunculin A is the most potent member of this class of compound. It impairs actin polymerization through the binding to G-actin in a 1:1 complex [410]. Moreover, it inhibits nucleotide exchange in the monomer [411]. Thus, differently from cytochalasins, which bind to the barbed end of filaments, latrunculin A seems to associate only with the actin monomer [411], this mode of action resembling that of the G-actin-sequestering protein thymosin 4. Latrunculins have been shown to induce changes in cell shape and actin filament organization in mammalian cells in culture [412–414] and to disrupt the actin cytoskeleton in yeast [411]. The high-resolution structure of the actin/latrunculin A complex indicates that latrunculin alters the actin-monomer subunit interface interfering with the conformational changes that are necessary for actin polymerization [415]. Latrunculin A has been reported to impair the invasion of tumorigenic S-adenosylmethionine decarboxylase-transfected fibroblasts [416], G3S1 human breast cancer cells [417] and HeLa-O3 cells, a variant of HeLa adenocarcinoma cells formerly mistaken for an oral cancer cell line [418]. It has also been shown to impair the peritoneal dissemination of human gastric carcinoma MKN45 and NUGC-4 cells [419]. A number of semisynthetic derivatives of Latrunculin A (acetylated, esterified and N-alkylated) affected the invasive properties of MDA-MB-231 cells [420], whereas Latrunculin A-17-O-carbamates inhibited PC3 human prostate cancer cells and T47D breast carcinoma cells [421].

Another compound, first isolated form a Red Sea sponge, is swinholide A [433]. It binds dimers of G-actin with high affinity, displaying F-actin severing activity [434, 435]. With a chemical structure very similar to swinholide A, misakinolide A (also called bistheonillide A) a compound isolated from an Okinawan marine sponge [436], exhibits barbed ends capping activity rather than filament-severing activity as swinholide A [435, 437]. Finally, scytophycins, like tolytoxin, which resembles a monomeric unit of swinholide A, have been shown to impair *in vitro* actin polymerization even if their mechanisms of action remain to be elucidated [438].

Mycalolide B is another compound isolated from a marine sponge [439] which has been shown to inhibit G-actin polymerization and to promote rapid F-actin depolymerization *in vitro* likely through F-actin severing activity and binding to G-actin in a 1:1 complex [440–442]. It appears to covalently modify actin, thus acting in an irreversible-fashion [435, 442]. Aplyronine A, a compound exhibiting a similar side-chain structure of that of mycalolide B, seems to act very similarly to mycalolide B. Finally, other two compounds structurally related to mycalolide B are halichondramide and dihydrohalichondramide. They exhibit both barbed-end capping and F-actin severing activity [391].

4.4 Interfering with actin cytoskeleton contractility

The following section is dedicated to compounds that interfere with the contractility of the actin cytoskeleton (Fig. 3 and

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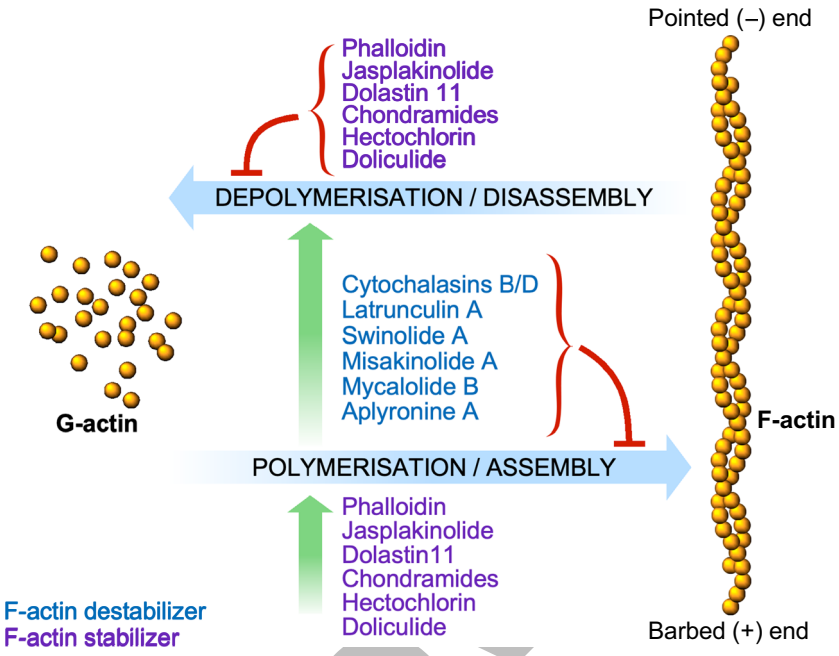

Fig. 4 Drugs interfering with actin cytoskeleton polymerization. F-actin destabilizer and stabilizer compounds are reported in the figure (blue and purple respectively). F-actin destabilizer compounds exert their action either promoting F-actin depolymerization disassembly (green arrow) or inhibiting G-actin polymerization/assembly (red lines), whereas F-actin stabilizer compounds act either promoting actin polymerization/assembly (green arrow) or inhibiting actin depolymerization/disassembly (red lines)

Table 5). They include: myosin and tropomyosin inhibitors, ROCK, MRCK and MLCK inhibitors.

- Myosin inhibitors

Blebbistatin is the most well-known and largely used small molecule inhibitor of myosins. It has been identified through a high-throughput screening assay as a compound inhibiting the ATPase activity of non-muscle myosin IIa [443]. Blebbistatin functions as a non-competitive inhibitor of myosin II. It acts by hindering a crucial step in myosin II ATPase cycle [444–446]. It has been reported to suppress myosin II activity in an actin-detached state, inhibiting the ATPase activity and *in vitro* motility of vertebrate non-muscle myosin IIA and IIB, porcine, rabbit and scallop striated muscle myosin II proteins, and *Dictyostelium* myosin II (half-maximal IC50 0.5–5 μM) [443–446, 496]. In contrast, the ATPase activity of myosins from other classes, like myosins I, V and X are not inhibited by blebbistatin at concentrations up to 50 μM [496]. Blebbistatin displays high cell permeability and its effects are reversible. Those characteristics have made this molecule a powerful tool to study myosin II–dependent cellular events [443]. However, blebbistatin displays two limitations for its use: the low solubility in aqueous solution and the light sensitivity. This second feature limits its application for live cell imaging, as blebbistatin photoinactivation reaction has been reported to be cytotoxic [497]. The use of blebbistatin has allowed demonstrating that the position of the cytokinetic cleavage furrow is maintained by signals coming from MTs and regulating non-muscle myosin II localization [443]. Moreover, studies employing blebbistatin has unveiled that non-muscle myosin II is implicated in fear memory consolidation in the lateral amygdale [498]; non-muscle myosin IIA and IIB control rat hepatic stellate cell motility [499] and non-muscle myosin II is essential for ligand-induced internalization of the EGFR [500]. Furthermore, blebbistatin treatment of muscle fibres has been crucial to highlight key aspects of muscle contraction, like the dynamic and variable range of mitochondrial ADP-stimulated respiratory kinetics in skeletal muscle [501]. Blebbistatin has been also successfully used to improve some cell biology techniques. An example is represented by its use as a tool to synchronize mammalian culture cells during various steps of mitotic division [502]. Interestingly, blebbistatin appears as a lead for anti-cancer agent development. Indeed, it has been shown to impair the invasive properties of a number of cancer cell lines, like pancreatic adenocarcinoma cells [447], BE human colon carcinoma cells [448], MDA-MB-231 [448] and MCF7 human [449] and 4 T1 mouse [450] breast cancer cells, 501Mel melanoma cells [451], A337/311RP rat and PR9692 avian sarcoma cells [452], and D54 glioblastoma cells [453]. However, no *in vivo* data are yet available for this molecule.

Aryl sulphonamide N-benzyl-p-toluene sulphonamide (BTS) [503] and 2,3-Butanedione monoxime (BDM) [504] have been also reported as non-competitive inhibitors of myosin II. The use of BTS has allowed unveiling the structural orientation and contractile properties of skeletal muscle in zebrafish [505]. Concerning BDM, its low binding affinity [506] together with its action on a number of proteins and cellular events which are independent of the ATPase activity [507] has obliged to carefully considerate its use to determine myosin II function *in vivo*. However, BDM treatment has been employed to provide evidence that fibronectin integrin

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Table 5 Drugs interfering with actin cytoskeleton contractility

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
Blebbistatin	Myosin II	Hindering of a crucial step in myosin II ATPase cycle	Pancreatic adenocarcinoma, colon carcinoma, melanoma and glioblastoma cells Human and mouse breast cancer cells Rat and avian sarcoma cells	Impairment of invasive properties	[443–446], [447–453]
TR100	Tpm3.1	Disruption of tpm3.1-regulated actin filament dynamics by increasing the filament depolymerization rate	Neural crest-derived tumour cell lines	Impairment of cell viability and motility in 2D and 3D Reduction of tumour cell growth *in vivo*	[294, 297, 454]
			Melanoma and neuroblastoma xenograft models Tpm3.1 knockout mouse line Pancreatic beta cells	In combination with anti-microtubule agents: induction of synergistic cancer cytotoxicity; G2-M phase arrest; mitotic spindle formation and cellular apoptosis abrogation Inhibition of tumour growth and improved survival *in vivo* On-target impact on glucose clearance in mice Inhibition of insulin-stimulated glucose uptake in skeletal muscle (*ex vivo*) Inhibition of insulin secretion in cells (by disruption of cortical actin filaments in β cells)	
ATM-3507	Tpm3.1	Disruption of tpm3.1-regulated actin filament dynamics by increasing the filament depolymerization rate	Neuroblastoma cell lines Neuroblastoma xenograft model	In combination with anti-microtubule agents: induction of synergistic cancer cytotoxicity; G2-M phase arrest; mitotic spindle formation and cellular apoptosis abrogation Inhibition of tumour growth and improved survival *in vivo*	[297]
ATM-1001	Tpm3.1	Disruption of tpm3.1-regulated actin filament dynamics by increasing the filament depolymerization rate	Tpm3.1 knockout mouse line Pancreatic beta cells	On-target impact on glucose clearance in mice Inhibition of insulin-stimulated glucose uptake in skeletal muscle (*ex vivo*) Inhibition of insulin secretion in cells (by disruption of cortical actin filaments in β cells)	[454]
Y-27632	ROCK	ATP-competitive inhibitor	Rat and human hepatoma cells, human breast, melanoma, colon, ovary, oesophageal, non-small cell lung cancer cells, mouse melanoma and human uveal melanoma cells, human anaplastic thyroid, glioma, retinoblastoma and tongue squamous cell carcinoma cells, human gastric, colorectal, hepatocellular and prostate cancer cells	Impairment of invasion under basal or stimulated conditions	[184, 455–475]

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Table 5 (continued)

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
			Reduction of metastatic growth and dissemination *in vivo*	Orthotopic mouse model of liver metastasis Xenograft mouse models of prostate cancer	
Experimental mouse model of hepatocellular carcinoma					
Fasudil	ROCK	ATP-competitive inhibitor	Lung cancer, adenocarcinoma and small cell lung cancer cells, highly metastatic liver cancer cells, oral squamous carcinoma cells, sarcoma, glioblastoma and ovarian cancer cells	Inhibition of proliferation, adhesion, migration and invasiveness Decrease of invasion-related gene expression levels	[476–483]
			Glioblastoma intracranial xenograft models	Metastasis suppression *in vivo*	
H-1152	ROCK	ATP-competitive inhibitor	Triple-negative breast cancer cells	Inhibition of invasive behaviour	[484]
RKI-1447/RKI-18	ROCK I and II	ATP-competitive inhibitors	Breast cancer cells	Inhibition of migration and invasion	[485, 486]
OXA-06/PT262	ROCK	ATP-competitive inhibitors	Non-small cell lung carcinoma cells	Inhibition of cytoskeleton remodelling, migration and invasion Inhibition of anchorage-independent growth	[487, 488]
AT13148	ROCK	ATP-competitive inhibitor	Melanoma cells Pre-clinical models of pancreatic, breast, prostate and uterine cancer	Inhibition of tumour growth Inhibition of cell migration and invasion Inhibition of metastatic dissemination *in vivo*	[488–492]
Wf-536/Y-27632	ROCK	ATP-competitive inhibitor	Mouse melanoma and lung carcinoma cells Mouse model of experimental melanoma metastasis	Anti-invasive effects *in vitro* Decrease of lung tumour colony formation *in vivo*	[493]
BDP5290	MRCK α and β ROCK I and II	ATP-competitive inhibitor	Breast cancer cell Squamous cell carcinoma cells	Reduction of myosin light chain phosphorylation Inhibition of invasion through Matrigel Inhibition of invasion through 3D collagen matrix	[494]
BDP8900/BDP9066	MRCK	ATP-competitive inhibitors	Breast cancer cells, cancer-associated fibroblasts, squamous cell carcinoma cells Mouse model of cutaneous squamous cell carcinoma	Reduction of substrate phosphorylation Induction of morphological changes along with inhibition of migratory and invasive abilities Anti-proliferative effects Reduction of skin papilloma outgrowth	[495]
ML-7/ML-9	MLCK	ATP-competitive inhibitors	Pancreatic cancer cells, rat prostatic adenocarcinoma cells, mouse mammary adenocarcinoma cells Mouse mammary organ culture model	Inhibition of migration and adhesion Induction of cell rounding up and decrease in stress fibre number	[234, 241, 436]

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Table 5 (continued)

Inhibitor	Target	Mechanism of action	Models	Outcomes	References
			Xenograft models of mammary and prostate tumours (mouse and rat respectively)	Impairment of invasive abilities Stimulation of etoposide-induced apoptosis Chemopreventive effect on organ culture models Decrease of tumour growth *in vivo* Enhancement of etoposide ability to prevent tumour growth *in vivo*	

interactions are controlled by cardiomyocyte contractile status [508]. Further, BDM as well as blebbistatin treatment has been used to lengthen culture lifetime of mouse cardiac myocytes [509, 510]. Therapeutically, treatment with BDM turned out to be useful in increasing the storage lifetime and efficiency of heart transplantation [511, 512]. Pentachloropseudilin (PCIP) is a natural antibiotic which has been shown to reversibly inhibit myosins 1 function [513], while Pentabromopseudilin (PBP), an antibiotic originally isolated from *Pseudomonas bromoutilis* [514, 515], has been described as a potent and reversible inhibitor of the ATPase activity and *in vitro* motility of myosin Va. Another inhibitor of myosin V ATPase activity is represented by the chemically synthesized compound MyoVin-I, whereas through a series of computational and binding studies the halogenated phenol 2,4,6-triiodophenol (TIP) has been identified as an inhibitor of the ATPase activity of myosin VI [516]. To summarize, although small molecule inhibitors of myosins are well-established as a tool for understanding myosin activity and myosin-based biological processes, also being useful for the optimization of tissue culture and imaging techniques (see above), blebbistatin is the only one with a proven anti-invasive activity on a panel of cancer cells (see above), potentially serving as a lead for the development of anti-cancer drugs.

- Tropomyosin inhibitors

As reported above, the tropomyosin isoform Tpm3.1 is highly and specifically upregulated in each cancer cell line that has been tested so far, thus representing a potentially selective target to exploit for actin-oriented cancer therapy [454, 517]. Remarkably, therapies directed against actin cytoskeleton failed to progress to the clinic because of their cardiac (and other muscle) toxicity. The targeting of Tpm3.1 potentially circumvents those side effects. Three compounds with selective anti-Tpm3.1 activity, TR100, ATM1001 and ATM3507 [294, 297, 454] have been identified by the same group. Interestingly, although those molecules are structurally distinct, lead to comparable biological effects. Stehn and co-workers developed this new class of anti-tropomyosin compounds, which preferentially target cytoskeletal tropomyosin-containing filaments in malignant cells. TR100, the lead compound of this class of molecules, has been reported to impact tumour cell viability and motility in two-dimensional (2D) and three-dimensional (3D) cultures of a panel of neural crest-derived tumour cell lines, minimally affecting the contractile properties of isolated rat adult cardiomyocytes (ACM). Moreover, using melanoma and neuroblastoma mouse xenograft models, the authors provided evidence that TR100 reduces tumour cell growth *in vivo* displaying minimal cardiotoxicity [294]. They then screened over 200 other anti-tropomyosin analogues for their anti-cancer and on-target activity through a number of *in vitro* cell-based and biochemical

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assays. This screening allowed identifying ATM-3507 as the new lead for its ability to impair Tpm3.1-containing filaments, its cytotoxicity potency, and its better drug-like features [297]. *In vitro* experiments based on the use of purified proteins have reported that those molecules disrupt Tpm3.1-regulated actin filament dynamics by increasing the rate of filament depolymerization [294, 297, 518]. ATM-3507 and TR100 have been shown to exhibit a high degree of synergy with *Vinca* alkaloid and taxane anti-microtubule compounds *in vitro*. Moreover, the combination of those anti-tropomyosin agents with vincristine produced in animals bearing human neuroblastoma xenografts a strong inhibition of tumour growth and an improved survival, together with a minimal loss of weight respect to control and either drug alone. Moreover, in combination with vincristine they led to G_2-M phase arrest, abrogation of the mitotic spindle formation and cellular apoptosis. Overall those results showed for the first time that the targeting of the actin cytoskeleton through tropomyosin small molecule inhibitors sensitize cancer cells to anti-microtubule drugs, being also well tolerated *in vivo* [297]. Recently, it has been shown that TR100 and ATM-1001, another molecule identified through the same screening that led to the discovery of ATM-3507 [297], impacted two processes known to be controlled by the actin cytoskeleton, insulin-stimulated uptake of glucose and insulin secretion [519, 520]. Comparing the effect of those drugs in wild-type (WT) *versus* Tpm3.1 knock-out (KO) mice demonstrated that those molecules act through the specific inhibition of the Tpm3.1 function [454].

- Kinase inhibitors (ROCK, MRCK and MLCK inhibitors)

Important effort has been made to develop ROCK inhibitors for cancer therapy; however, the most part of compounds has failed to progress to clinical trials. One extensively employed ROCK inhibitor, Y-27632, is a pyridine-analogue that competes with ATP for binding to ROCKs [455]. It has been the first-generation ROCK inhibitor to be identified [455]. Y-27632 has been reported to impair the invasive ability of rat hepatoma MM1 cells and their dissemination in the peritoneal cavity [456]; to reduce the intrahepatic metastasis of primary human hepatoma LI7 cells [457]; to decrease the invasion of human MDA-MB-231 breast carcinoma cells [184, 458], A375m2 and WM266.4 human melanoma and LS174T human colon carcinoma cells [459]. Moreover, Y-27632 has been shown to reduce bombesin-stimulated invasiveness of Isreco 1 human colon carcinoma cells [460], LPA-induced invasion of human hepatoma SMMC-7721 cells [461] and human ovarian cancer CAOV-3 and PA-1 cells [462]; shear stress-induced invasion of human oesophageal cancer OC-1 cells [463] and VMRC-LCD human non-small cell lung cancer cells [464]. Further, it impaired the invasive abilities of B16F1 mouse melanoma cells; UvMel 1.3, UvMel 1.5, and UvMel 270 human uveal melanoma cells [465]; human anaplastic thyroid cancer ARO cells [466], U87MG human glioma cells [467]; Y79 human retinoblastoma cells [468]; and Tca8113 and CAL-27 human tongue squamous cell carcinoma cells [469]. Y-27632 anti-invasive effects were also visible on SGC-7901 human gastric carcinoma cells [470]; human colorectal carcinoma SW620 cells [471] and human hepatocellular carcinoma cells [472]. Finally, Y-27632 has been shown to reduce the metastatic growth of human prostate cancer PC3 cells in immune-compromised mice [473]; to significantly decrease intrahepatic metastasis orthotropic implantation of CBO140C12 HCC tumour fragments into mice liver [474], and to impair the metastatic dissemination of HT29 human colorectal carcinoma cells in an orthotropic mouse model of liver metastasis [475]. However, it is important to underline that ROCK inhibition by Y-27632 has been also shown to increase the proliferation and migration of a series of *in vitro* and *in vivo* cancer models [521–524], those effects being explained by the observation that ROCK activation can be implicated in the negative feedback mechanisms controlling pro-proliferative pathways [525].

Fasudil (also known as HA-1077) is another widely studied ROCK inhibitor. As Y-27632 is an ATP-competitive inhibitor, which exerts its function on a wide spectrum of kinases other than ROCKs [526]. Among ROCK inhibitors, Fasudil has been the only clinically approved for the treatment of cerebral vasospasm and pulmonary hypertension in Japan since 1995 [527]. It has been reported to impair invasive abilities of human lung cancer A549 cells [476], human lung 95D adenocarcinoma cells [477] and human small cell lung cancer NCI-H446 cells [478]. Moreover, inhibitory effects on the invasive properties of human highly metastatic HCCLM3 liver cancer cells [479] and human oral squamous SCC-4 carcinoma cells [480] have been shown. Finally, Fasudil inhibits metastatic dissemination of HT1080 sarcoma cells to the lungs [481]; *in vitro* and *in vivo* invasive abilities of T98 and U251 human glioblastoma cells [482] and LPA-induced invasiveness of human ovarian cancer cells [483]. Although this compound is clinically approved to treat cerebral vasospasm in Japan and China, it displays a pharmacokinetic profile, which makes it unsuitable for chemotherapy application [30].

A number of recently developed ROCK inhibitors have been reported to be more potent and/or selective respect to Y-27632 or Fasudil. Some of them are effective in pre-clinical models. H-1152 is a membrane-permeable compound displaying high specificity for ROCK over other kinases of the AGC family [528]. It has been reported to impair invasive abilities of human breast TMX2-28 carcinoma cells [484].

RKI-1447 and RKI-18 have been both shown to decrease human breast carcinoma MDA-MB-231 cell invasion [485, 486]. OXA-06 and PT262 reduce both migration and invasion of non-small cell lung carcinoma (NSCLC cells) [487, 488], whereas CCT129253 and AT13148 negatively regulate

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melanoma cell invasion and *in vivo* metastatic dissemination [490]. AT13148 deserves special consideration as it is the only ROCK inhibitor that has progressed into clinical trials for cancer. It is an orally available multi-AGC kinase inhibitor that has been identified by a fragment-based screen [491]. It has been shown to also potently inhibit ROCK1 and ROCK2 [491] and to exert anti-tumour effects in pre-clinical models of pancreatic [489], breast, prostate and uterine cancer [488]. It has proven to be well tolerated in a phase I clinical trial initiated in 2012 (https://clinicaltrials.gov/ct2/show/NCT01585701) in patients with advanced solid tumours [492]. Finally, Wf-536 is a Y-27632 derivative reported to impair invasion and metastasis of B16 mouse melanoma cells and LLC mouse Lewis lung carcinoma cells [493]. A series of molecules displaying selectivity for ROCK2 over ROCK1 have also been described. However, their efficacy has been essentially evaluated in models of pathologies other than cancer, like glaucoma, hypertension and chronic kidney disease [529]. It is also important to underline that ROCK targeting implies some consequences on blood vessels contraction, ROCK inhibitors likely producing hypotension [530]. Finally, ROCK inhibition has been also reported to increase survival of cancer stem cells [531, 532].

MRCKs are three serine/threonine protein kinases (α, β and γ isoforms) evolutionarily related to ROCKs, which are critically implicated in the regulation of actomyosin contractility [533, 534]. MRCKα has been reported to be upregulated in a variety of human cancers [494]. Remarkably, the dual inhibition of ROCK and MRCK led to a stronger impairment of cancer cell migration and invasion than ROCK inhibition alone [535, 536], the dual ROCK/MRCK inhibition representing an efficient therapeutic strategy. It has to be noted that selective MRCK inhibitors might be employed to counteract the hypotension caused by long-term ROCK inhibitor-base therapies [530]. Unbekandt and colleagues by screening a kinase-focused small molecule chemical library identified a series of compounds with inhibitory activity toward MRCK. Medicinal chemistry combined with *in vitro* enzyme profiling led to the identification of BDP00005290 (abbreviated as BDP5290) as a potent MRCK inhibitor. BDP5290's activity resulted in reduced myosin light chain (MLC) phosphorylation, more efficient reduction of MDA-MB-231 human breast cancer cell invasion through Matrigel compared to Y27632 and stronger inhibition of human SCC12 squamous cell carcinoma cell invasion through 3D collagen matrix compared to the same concentration of Y27632 [494]. More recently, the same group discovered the azaindole compounds BDP8900 and BDP9066 as potent and selective MRCK inhibitors that reduce substrate phosphorylation, producing morphological changes in cancer cells along with inhibition of their migratory and invasive abilities [495]. Moreover, they provided evidence that in over 750 human cancer cell lines tested form 40 different cancer types, BDP8900 and BDP9066 exerted significant anti-proliferative effects, the highest activity being observed in hematologic cancer cells [495]. Further, they showed that in a two-stage chemical carcinogenesis model of murine squamous cell carcinoma (SCC), BDP9066 topical treatment reduced MRCKα activation skin papilloma outgrowth. Altogether, those findings provided an initial pre-clinical proof of concept for MRCK inhibition as a valid therapeutic strategy. However, the toxicity of systemic BDP8900 and BDP9066 administration has not been evaluated and nothing is known about the bioavailability of those drugs.

Two are the compounds that have been reported to specifically inhibit MLCK, ML-7 and ML-9. They both strongly inhibited migration and adhesion of pancreatic cancer cells in a dose-dependent manner, those data supporting a specific and competitive inhibition of MLCK by the two molecules [237]. In particular, ML-7 induced pancreatic cancer cell rounding up and a marked decrease of stress fibre numbers [234]. ML-7 and ML-9 also have been reported to inhibit Mat-Ly-Lu (MLL) rat prostatic adenocarcinoma cell invasiveness [241]. Moreover, ML-7 has been found to stimulate the ability of etoposide to induce apoptosis in Mm5MT mouse mammary adenocarcinoma cells and MLL cells, also exerting a chemopreventive effect in an *in vitro* mouse mammary organ culture model [537]. Finally, *in vivo* experiments showed that ML-7 is able to reduce the growth of mammary tumours in mice and prostate tumours in rats, significantly promoting etoposide ability to prevent the growth of those tumours [537]. Those findings have unveiled a role of ML-7 as an etoposide adjuvant, opening the way to further development.

5 The emerging role of microtubules and intermediate filaments in cancer metastasis

Although most of the literature among the three different types of protein filaments composing the cytoskeleton focuses on the implication of MFs and their binding and regulatory proteins in tumour cell motility, invasion and metastasis, the MT system is emerging as a novel crucial player of those complex cellular processes. A first point to be underlined is that MTs and MFs share the vast majority of the same signalling machinery, including, for example, the Rho GTPases and their upstream regulators and downstream effectors, being often coordinately regulated. ß-Tubulin, one of the components of MTs (see above) is well known to be the cellular target of the vinca alkaloids and taxanes, ones of the most powerful classes of anti-cancer drugs [538]. It has been reported that the alterations in specific β-tubulin isotypes, as the neuron-specific βIII-tubulin are linked to resistance development to chemotherapy based on tubulin-binding agents in epithelial cancers [539] Indeed, specific β-tubulin isotypes have been reported to be implicated in mediating sensitivity to

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chemotherapeutic drugs [540, 541], βIII-tubulin depletion reducing tumour incidence [541]. An interesting clinical study has reported that the mRNA expression levels of β-tubulin-III in the tumour tissue of patients with stage II NSCLC might be considered as an index of prognosis and chemosensitivity, as well as a reference for personalized chemotherapeutic applications in patients [542].

MTs are also emerging to influence the activity of Rho GTPases, which in turn control MTs dynamics [543]. An example is represented by the relationship between Cdc42 and MTOC. Cdc42 is critically implicated in the regulation of directional cell migration [544], which is fundamental for metastatic dissemination, and results in a high polarized cell in which signalling molecules and the cytoskeleton are asymmetrically distributed. It is well known that in the migrating cell Cdc42 controls both MTOC and Golgi apparatus reorientation toward the direction of migration where MT plus ends stabilization occurs [144, 544–547]. Moreover, MTs are crucial tracks along which cargo molecules are transported within the cell to various subcellular locations, including the leading edge of a migrating cell [548]. MTs stability has thus an important impact on the modulation of the vesicular trafficking of proteins mediating the transport of molecules required for the invasion machinery. It is the case of Rab11, an important molecule for vesicular trafficking, particularly membrane protein recycling and translocation of proteins from the trans-Golgi network to the plasma membrane. Yoon and colleagues have shown that hypoxia increases MDA-MB-231 breast cancer cell invasion by Rab1 modulation. Indeed, they showed that hypoxia-induced Rab11 trafficking is regulated by MT stability, as evidenced by the fact that hypoxia increases glutamylated (Glu) tubulin and that colchicine (microtubule polymerization inhibitor) blocks Rab11 trafficking and invasion. A target of Rab11-mediated trafficking that contributes to invasion is the integrin α6β4. Hypoxia increased α6β4 in a Rab11- and stable microtubule-dependent fashion [549].

Finally, we wish to mention just two examples of MT-interacting proteins which participate in MT dynamics that have been shown to be implicated in cancer metastasis. End binding 1 (EB1) is a plus-end-tracking protein (+TIP) that localizes to MT plus ends where it modulates their dynamics and interactions with intracellular organelles [550, 551]. EB1 expression has been shown to be upregulated in human breast cancer specimens and cell lines. Indeed, EB1 levels correlate with clinicopathological features indicative of breast cancer malignancy. EB1 expression knock-down significantly reduced cancer cell proliferation, whereas its overexpression stimulated cell proliferation, colony formation and tumour growth in nude mice [552]. Moreover, EB1 together with adenomatous polyposis coli (APC) has been reported to bind to the formin mDia to stabilize MTs downstream of Rho and stimulate cell migration [553]. ATIP3 is a potent MT-stabilizing protein, the depletion of which has been shown to increase MT dynamics. Molina and co-workers showed that ATIP3 expression significantly inhibits cell motility, its knock-down increasing breast cancer cell migration. That evidence was confirmed *in vivo*, where ATIP3 expression was shown to slow metastatic progression limiting the number and size of metastases [554]. The mechanism accounting for ATIP3-dependent impairment of migration and metastasis has been identified in its ability to control the capacity of MT tips to reach the cell cortex during migration through the reduction of MT dynamics [554]. Furthermore, ATIP3 has been reported to be a prognostic marker for breast cancer patients' overall survival, its low levels associating with decreased survival of patients with highly proliferative breast carcinomas [555]. Very recently, the same group has shown that the combinatorial expression of EB1 and ATIP3 improves breast cancer prognosis, a population of highly aggressive breast tumours which express high-EB1 and low-ATIP3 levels being potentially considered for the development of novel molecular therapies [556].

Concerning IFs, although most of IF research has focused on diseases other than cancer [557], increasing evidence indicates that oncogenic transformation modifies the specific IF signature of the cell causing an alteration in the expression of a subset of IF proteins. They include vimentin, which is upregulated in cancer cells that have undergone EMT [558, 559], and various keratins, which display an altered expression in a number of tumours compared to the normal tissues [560]. Modifications in the expression patterns of IFs are thus associated with cancer development and progression, specifically with an increase of cell migration and invasiveness into the surrounding tissues. Those findings unveil a novel role for IF proteins, like keratins, in cancer, which goes beyond their traditional ones as diagnostic and prognostic markers [560]. This opens the way to the development of IF-targeted therapies, no compounds specifically targeting IFs being available up to date. Regarding IF proteins implication in cancer metastasis, firstly they provide mechanical elements that are needed to the cell to migrate through and invade into neighbouring tissues during initial steps of the metastatic cascade. This task is accomplished in tight relationship with MF and MT proteins [561–564]. Secondly, they regulate migration and invasion participating to signalling events that are crucial for cells to invade. It is the case of vimentin, which promotes migration and invasion partly through its involvement in the signalling cascades underlying EMT [563, 565, 566]. Vimentin has been reported to be implicated in invasion and metastasis in a number of studies. It was found to be significantly associated with a poor prognosis in NSCL cancer, being lower expressed in squamous cell carcinoma than in adenocarcinoma [567]. Furthermore, highly invasive subpopulations of A549 cells displayed higher expression levels of vimentin than did parental cells, also being more invasive. Vimentin knock-down significantly decreased invasion through Matrigel of those

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aggressive cell populations [567]. Similar effects have been observed in oral squamous cell carcinoma, which formed fewer colonies in soft agar and were less invasive through Matrigel upon vimentin depletion [568]. Moreover, vimentin has been reported to be critically implicated in the ERK2-Slug-Axl-dependent EMT induction, migration and invasion through 3D matrix, this signalling cascade also inducing cell extravasation and metastases formation in mice [569, 570]. Clusters of β4 integrin have been shown to organize into distinct puncta and to localize along vimentin filaments within lamellipodia at the cell edge of A549 lung epithelial carcinoma cells. The knock-down of β4 leads to a loss of vimentin filaments from lamellipodia and an impaired motility, whereas vimentin-depleted A549 cells, although they do not exhibit an alteration in β4 integrin organization, migrate in a "less-directed" manner and display a reduction in Rac1 activity [571]. The aberrant motility phenotype produced by both β4 integrin and vimentin-depleted cells is rescued by active Rac1 expression, suggesting that complexes of β4 integrin and vimentin act as signalling hubs, thus regulating cell motility behaviour [571]. Vimentin is also the target of some kinases, which phosphorylate key Ser or Tyr residues and regulate its dynamics and migrating/invasive abilities. An example is provided by phosphorylation on S38 by Akt, which has been reported to increase migration and invasion of soft tissue sarcoma and metastatic dissemination in xenograft model [572]. Moreover, phosphorylation of Tyr 117 by Src led to vimentin IF disassembly, VAV2 recruitment to the cell membrane and induction of lamellipodia formation [573]. Liu and co-workers showed that vimentin was the protein with the most increased expression in metastatic oral squamous carcinoma cell lines respect to the parental ones [574]. Moreover, they showed that high vimentin expression levels correlated with a high amount of lymph node metastases [574]. Interestingly, it seems that vimentin facilitates metastatic dissemination through the tumour microenvironment. Studies carried out on a full-body vimentin knock-out (VIM−/−) mouse model of lung adenocarcinoma which carries *LSL-KRas*^{G12D} and *Lkb1*$^{fl/fl}$ driver mutations showed that vimentin depletion does not have an impact on primary tumours development, whereas lymph node metastases were reduced by 50%. The authors reported that in VIM+/+ mice vimentin, although absent from tumour cells, was present at the level of cancer-associated fibroblasts (CAFs) that surrounded the cells that became detached from the primary tumour. The number of those groups of cells, which are known as collective invasion packs, decreased in VIM−/− mice, even if the number of cells per pack was unaltered. The presence of CAFs, which were absent in VIM−/− mice, was the main difference in the microenvironments of the two mouse models. Remarkably, when CAFs were added to spheroids of lung adenocarcinoma cells in a 3D invasion assay *in vitro*, spheroids increased their branching, an opposite effect being observed upon vimentin knock-down [575]. Overall, those results show the key role played by vimentin with other cell types associated with metastasis formation.

Concerning keratins, the studies carried out showed that they affect cell motility and invasion in a "context-dependent" fashion. For example, upon genetic ablation of all keratins, keratinocytes display decreased stiffness and increased (confirm here) invasiveness. This phenotype was reversed by re-expression of basal keratins K5 and K14, suggesting that those two keratins exert an inhibitory role on cell invasion [576]. In contrast, K14 expression has been reported to be required by breast cancer cells for collective invasion [577]. Similarly, K14 contributes to collective invasion of salivary adenoid cystic carcinoma [578]. Opposing roles for K19 in migration and invasion have been also shown [579, 580]. In spite of the positive correlation observed between increased K19 expression and metastatic dissemination in cancer patients [581–586], K19 knock-down in hepatocellular carcinoma Huh7 cells decreased invasiveness [581]. On the other hand, it has been reported that K19 overexpression in K19-negative BT549 breast cancer cells impaired cell migration [587]. Similar effects were observed in oral squamous cell carcinoma cells [580]. Those effects of K19 on cell migrating and invasive abilities were also detected upon K19 knock-down in BT474 and SKBR3 breast cancer cells, which led to increased cell proliferation, migration, invasion, and survival [579]. Also, K8 and K18 have been shown to negatively regulate invasion and migration of multiple cancer cell lines [588–591]. However, in a renal cancer cell line K8 knock-down provided evidence that K8 is required for cancer metastasis [592].

6 Concluding remarks

It has been widely shown that the cytoskeleton is deregulated in cancer and several cytoskeletal-interacting proteins strongly affect the migratory and metastatic phenotype of cancer cells. Moreover, the tight relationship between cytoskeletal alterations and metastasis in the clinic has unveiled novel potential therapeutic targets for anti-cancer therapy. However, so far the cytoskeleton has not translated as a druggable clinical target as its pharmacological targeting has been considered for a long time to cause a number of toxic side effects. Actually, there is no doubt that targeting actin cytoskeleton dynamics and/or contractility produces multiple effects on a variety of crucial biological processes, like cell migration, division, exocytosis in normal as well as cancer cells. This represents a key challenge in the development of drugs interfering with cytoskeleton organization and function. In addition, several actin regulators have been so far considered undruggable, because of their structures which lack the classical functional sites suitable for targeted drug design. However, the increasing evidence pointing on the critical implication of the cytoskeleton

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in tumour cell migration, invasion and metastasis has pushed researchers to focus their studies on the possibility to exploit the cytoskeleton as a target to treat metastatic disease. The studies reviewed here demonstrate the feasibility of targeting different cytoskeletal components and cytoskeleton-binding proteins through compounds specifically interfering with cytoskeleton organization and function. However, the example of Rho GTPases shows how, although those signalling molecules and their modulators and effectors have been recognized for decades as key regulators of cell migration and invasion, yet only a few compounds targeting this signalling network have been developed beyond an early pre-clinical stage. Actually, despite the evidence that the ability of cells to metastasize accounts for 90% of death from solid tumours, the acquisition of an invasive phenotype being the feature that mainly distinguishes malignant from benign tumours, a specific class of anti-invasion and anti-metastatic drugs is still lacking. This limits the effective therapeutic options currently available for patients with advanced disease. Among others, the factors that have led to the paucity of therapeutic options specifically targeting metastasis are the complexity of the metastatic process, the relatively limited number of the available metastatic pre-clinical *in vivo* models and the limitations in the current clinical settings. The ever-growing understanding of the cytoskeletal machinery with several well-characterized molecular targets largely shared across multiple malignancies opens the way to the development of novel anti-metastatic drugs as efficient potential therapeutic tools to treat patients with advanced-stage cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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