REVIEW

Cytoskeleton actin-binding proteins in clinical behavior of pituitary tumors

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Abstract

Although generally benign, pituitary tumors are frequently locally invasive, with reduced success of neurosurgery and unresponsive to pharmacological treatment with somatostatin or dopamine analogues. The molecular basis of the different biological behavior of pituitary tumors are still poorly identified, but a body of work now suggests that the activity of specific cytoskeleton proteins is a key factor regulating both the invasiveness and drug resistance of these tumors. This review recapitulates the experimental evidence supporting a role for the actin-binding protein filamin A (FLNA) in the regulation of somatostatin and dopamine receptors expression and signaling in pituitary tumors, thus in determining the responsiveness to currently used drugs, somatostatin analogues and dopamine receptor type 2 agonists. Regarding the regulation of invasive behavior of pituitary tumoral cells, we bring evidence to the role of the actin-severing protein cofilin, whose activation status may be modulated by dopaminergic and somatostatinergic drugs, through FLNA involvement. Molecular mechanisms involved in the regulation of FLNA expression and function in pituitary tumors will also be discussed.

Key Words

- filamin A
- cofilin
- somatostatin receptor 2
- dopamine receptor type 2
- pituitary tumors

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Introduction

Pituitary tumors, accounting for 16.2% of all primary brain and other central nervous system tumors (Ostrom *et al.* 2017), are classified based on their secretory activity in non-functioning pituitary tumors (NFPTs) or hormone-secreting tumors, including prolactin (PRL)-, growth hormone (GH)- and adrenocorticotrophic hormone (ACTH)-secreting tumors. The 4th edition of the World Health Organization (WHO) classification of endocrine tumors (Lloyd *et al.* 2017) has adopted an adenohypophyseal cell lineage designation, with subsequent categorization of histological variants according to hormone content and the expression of pituitary-specific transcription factors.

Therapy is aimed to remove or reduce tumor mass and to normalize hormone secretion. Transsphenoidal surgery is the first choice of treatment in many cases. However, incomplete tumor resection and high rate of recurrence are associated with pituitary tumors invasion into surrounding tissues, a feature found in 30–50% of the lesions (Meiji *et al.* 2002). For PRL-secreting tumors, dopamine receptor type 2 (DRD2) agonists represent the first-line therapy, whereas somatostatin analogs (SSAs) are used for the pharmacological treatment of GH- and ACTH-secreting tumors. However, a variable percentage of patients (about 10% of PRL-, 30% of GH-, 50–70% of ACTH-secreting tumors) is resistant to these drugs

(Colao *et al.* 2011, Cuevas-Ramos & Fleseriu 2014, Guelho & Grossman 2015, Tirosh & Shimon 2015).

Little is known about the molecular determinants underlying the local invasiveness and the pharmacological resistance of pituitary tumors. However, cytoskeleton involvement in both these critical issues has been recently demonstrated by several studies.

Cell cytoskeleton and pituitary tumors: old and new players

Cell cytoskeleton is a complex, dynamic and multifunctional network of protein filaments that can be classified in three main types: microfilaments, intermediate filaments and microtubules, originating by polymerization of different protein subunits. Microfilaments are composed by actin, intermediate filaments mainly by vimentin and keratin and microtubules by tubulin. They are characterized by different structural and physical properties, enabling specific cellular functions. In addition to provide and maintain cell shape and structure, they participate in a variety of cellular processes. In particular, microfilaments are involved in cell movement, shape, differentiation, division and intracellular transport, these activities being regulated by specific interactions with a variety of actin-binding proteins. Intermediate filaments participate in cell–cell and cell–matrix junctions. Microtubules are involved in mitosis, organelles transport and cell shape. In addition, these three types of filaments interact both directly, by physical contact, and indirectly, via biochemical signaling and gene transcription, allowing reciprocal regulation.

A cytoskeleton involvement in secretory granules' transport and exocytosis in anterior pituitary cells has been demonstrated by electron microscopy techniques. Both actin filaments (Ostlund *et al.* 1977, Senda *et al.* 1989) and microtubules (Labrie *et al.* 1973, Sherline *et al.* 1977) bind anterior pituitary secretory granules *in vitro*, playing a role in their intracellular transport, approach to the plasma membrane and release. Moreover, agents that disrupt microtubules, such as colchicine or vinblastine, inhibited the transport of GH storage granules from the Golgi complex to the cytoplasmic pool (Howell & Tyhurst 1978).

Specific cytoskeleton features have been associated to pituitary tumors clinical behavior. Cytokeratins, components of the intermediate filaments, that are expressed in both normal and tumoral anterior pituitary (Halliday *et al.* 1990) are specific histological markers that designate different subtypes of GH-secreting pituitary

tumors. In particular, densely granulated tumors are defined by perinuclear cytokeratin distribution, whereas sparsely granulated tumors are characterized by dot-like keratin immunoreactivity, commonly referred to as fibrous bodies. Histological subtype of the tumor correlates with the expression of SS receptor type 2 (SSTR2) and response to SSA (Brzana *et al.* 2013, Chinezu *et al.* 2014, Kiseljak-Vassiliades *et al.* 2015), but to date, the molecular mechanisms involved are unknown.

Regarding actin-binding proteins, a role in regulating migration and invasion of pituitary adenomas has been attributed to fascin, which organizes actin filaments in parallel bundles (Liu *et al.* 2016). The authors demonstrated that silencing of fascin in GH3 cells reduced cell invasion, with a mechanism involving NOTCH1/DLL pathway. They also found an association between fascin expression and invasion and increased risk of recurrence in NFPT and GH-secreting tumors.

In the last years, two major actin-binding proteins, filamin A (FLNA) and cofilin, emerged as important players in the regulation of the complex intracellular processes that dictate pituitary tumors drug responsiveness and invasiveness. They participate in actin filaments crosslinking and remodeling, respectively, and in mediating SS and DA receptors intracellular effects.

FLNA: structure and function

Filamins (FLNs) are high-molecular-weight homodimeric actin-binding proteins which cross-link actin cytoskeleton filaments. FLNs family includes three homologous proteins, FLNA, B and C, encoded by different genes located on chromosome X, 3 and 7, respectively (van der Flier & Sonnenberg 2001). While FLNC is primarily expressed in cardiac, smooth and striated muscle, FLNA and B are both ubiquitously expressed, but FLNA is the most abundant isoform (Feng & Walsh 2004).

A complete loss of FLNA expression in mice causes embryonic lethality, cardiac malformations and skeletal defects (Hart *et al.* 2006). In humans, FLNA mutations lead to a broad spectrum of clinical disorders, called filaminopathies, which can be classified in loss of function or gain of function. FLNA loss-of-function mutations, leading to reduced or absent FLNA expression, are embryonic lethal in males but are manifest in females as periventricular nodular heterotopia (PVNH), a localized neuronal migration disorder during late embryonic and early fetal development, Ehlers–Danlos syndrome-like collagenopathy, macrothrombocytopenia and X-linked cardiac valvular dystrophy (XCVD) (Fox *et al.* 1998, Sheen

et al. 2005, Bernstein *et al.* 2011, Ieda *et al.* 2018). FLNA variants can also present with seizures, cardiovascular and pulmonary findings (Robertson 2005).

In contrast, gain-of-function mutations of FLNA cause various skeletal dysplasias and congenital malformations affecting brain, viscera and urogenital tract (otopalatodigital syndromes, frontometaphyseal dysplasia and Melnick–Needles syndrome) (Robertson *et al.* 2003, Robertson 2005), strongly suggesting a FLNA role in modulating signaling during organogenesis in multiple tissues.

The structure of a FLNA monomer is represented in Fig. 1. FLNA was originally discovered as an actin-crosslinking protein (Hartwig & Stossel 1975), a function derived from its ability to homodimerize in V-shaped flexible structures that cross-link perpendicular actin filaments, conferring membrane integrity and defending cells against mechanical stress. Moreover, the binding of FLNA with several transmembrane proteins, including channels and receptors, anchors actin cytoskeleton to the cell membrane. Besides these structural functions, mounting evidence suggests a major role of FLNA in signal transduction, due to its ability to bind a huge number of intracellular signaling molecules, kinases and transcription factors (Stossel *et al.* 2001, Nakamura *et al.* 2011). FLNA is implicated in the regulation and integration of multiple cellular processes, including cell adhesion, migration,

maintenance of cell shape, differentiation, proliferation and transcription. Moreover, in different human tumors, FLNA may play opposite roles in regulating growth, invasion and metastasis (Shao *et al.* 2016).

FLNA functions are tightly regulated by several mechanisms, including FLNA phosphorylation, mechanical force, intramolecular inhibition, competition with other molecules and proteolysis, as discussed in ‘Mechanisms regulating FLNA expression and function in pituitary tumors’ section.

Cofilin: structure and function

The ADF/cofilin family is a key regulator of actin dynamics. It includes cofilin 1 (a non-muscle type of cofilin), cofilin 2 (a muscle type of cofilin) and ADF (actin-depolymerizing factor or destrin). Cofilin 1 (hereafter referred to as cofilin) is the most abundant and ubiquitous member of this family. Deletion of the cofilin gene *CFL1* is embryonic lethal in mice owing to defects in proliferation, polarization and migration of neural crest cells (Gurniak *et al.* 2005, Bellenchi *et al.* 2007).

Cofilin is a small protein of 19 kDa composed of one actin-depolymerizing factor homology (ADF-H) domain and able to bind both globular (G) actin and filamentous (F) actin. It also contains a NLS that confers to cofilin the ability to carry G-actin to nucleus (Abe *et al.* 1993, Pendleton *et al.* 2003), where it regulates chromosome organization and gene activity (Percipalle 2013).

Cofilin exerts two main biochemical functions: first, it depolymerizes ADP-bound actin filaments near the pointed ends to supply a pool of free G-actin monomers for polymerization; second, it severs actin filaments and initiates actin polymerization by increasing the number of actin-free barbed ends, from which F-actin polymerizes (Bravo-Cordero *et al.* 2013).

Since actin cytoskeleton reorganization is crucial for a number of cellular processes, it is not surprising that cofilin activity is tightly regulated. The main mechanism regulating cofilin activity is phosphorylation at Ser3 (Agnew *et al.* 1995), that prevents its ability to bind actin. Small GTPases of the Rho family are able to promote this posttranslational modification, by triggering a cascade of kinases, including PAK and ROCK, that activate LIMK1 and LIMK2, which ultimately target cofilin. On the other hand, cofilin is dephosphorylated by different phosphatases, such as slingshot 1L, chronophin and phosphatases type 1, 2A and 2B. Even though phosphorylated cofilin (P-cofilin) is generally considered the inactive form of cofilin, it can exert specific functions, such as to promote

Figure 1

Schematic representation of a FLNA. A FLNA monomer is composed of 2647 amino acids and weights 280 kDa. At the N-terminus there is an actin-binding domain that consists of two calponin homology domains, followed by 24 immunoglobulin (Ig)-like β -sandwich fold repeats of about 96 aminoacidic residues each. Repeats 1–15 (rod-1 domain) and 16–23 (rod-2 domain) are separated by a flexible hinge region (H1), and another hinge (H2) separates repeats 23 and 24. FLNA can be cleaved by calpain at both H1 and H2. Repeat 24 is the self-association domain that mediates FLNA homodimerization. A secondary actin-binding domain of lower affinity is located in the rod-1 domain, whereas rod-2 does not interact with actin filaments, remaining free for interactions with multiple partner proteins. Indeed, the majority of FLNA interactions with receptors and signaling proteins are mediated by repeats 16–24. Repeats interacting with DRD2 and SST2, as well as FLNA scaffold region, are shown. Repeat 20 contains a putative nuclear localization signal (NLS) and the residue serine 2152 target of PKA phosphorylation. The deletion of 41 amino acids between repeat 19 and 20, present in the splice variant var-1 of FLNA, is indicated.

the translocation of phospholipase D1 to the plasma membrane and to stimulate its activity (Han *et al.* 2007). Beside phosphorylation, that remains the most important and the most studied system of cofilin regulation, other mechanisms have been described, such as cofilin ubiquitination, pH alterations, oxidation, binding to PtdIns(4,5)P₂ or to tropomyosins, cortactin, CAP1/Srv2p, coronins and Aip1 (Bernstein & Bamburg 2010), that might contribute to finely tune cofilin activity.

Cofilin has been demonstrated to be involved in cancer development, progression, invasion and metastasis. Both an increase of cofilin expression and a reduction of cofilin phosphorylation has been found in human malignant cells, suggesting its potential use as diagnostic/prognostic tumor biomarker (reviewed in Shishkin *et al.* 2016).

Cytoskeleton role in pituitary tumors drug responsiveness

FLNA role in PRL-secreting tumors responsiveness to DRD2 agonists

FLNA involvement in DRD2 regulation is well established. FLNA repeat 19 directly binds DRD2 third intracellular

loop (Lin *et al.* 2001), with a strong impact on DRD2 coupling to adenylate cyclase, receptor clustering and expression on the plasma membrane in human melanoma cells (Li *et al.* 2000, Lin *et al.* 2002) (Fig. 1).

Concerning PRL-secreting pituitary tumors, Peverelli *et al.* showed that the presence of FLNA is essential for DRD2 expression and intracellular transduction of dopamine inhibitory signals (Peverelli *et al.* 2012). The authors demonstrated that tumor tissues from patients resistant to DRD2 agonists treatment showed a reduced expression of both DRD2 and FLNA, by both immunohistochemistry and Western blot. Moreover, they showed by *in vitro* experiments that the absence of FLNA caused the loss of DRD2. Indeed, FLNA gene silencing or overexpression in primary cultured prolactinoma cells from drug-sensitive or -resistant tumors produced corresponding decrease or increase, respectively, of DRD2 (Peverelli *et al.* 2012). The molecular mechanism involved has been elucidated in MMQ, a rat cell model of prolactinoma endogenously expressing functional DRD2 and FLNA. In these cells, FLNA was required for both DRD2 targeting to the cell membrane and DRD2 protection against lysosomal degradation (Peverelli *et al.* 2012) (Fig. 2). In agreement

Figure 2

The figure schematically represents the proposed model of cytoskeleton involvement in regulating DRD2 and SST2 in pituitary tumor cells, and the interplay between FLNA, cofilin and receptors in regulating tumor cell motility. (A) FLNA stabilizes DRD2 expression by preventing its lysosomal degradation, and functions as scaffold in mediating DRD2 signal transduction. (B) DRD2 activates ROCK/LIMK/cofilin pathway, promoting an increase of cofilin phosphorylation (inactivation), with consequent reduction of cell migration and invasion. (C) FLNA interaction with SST2 is required for SST2 clusters formation and alignment along actin fibers, prevents SST2 lysosomal degradation upon agonist challenge and functions as scaffold for inhibitory G proteins allowing SST2 signal transduction. Activated SST2 recruits a protein complex that includes FLNA, RhoA, ROCK, LIMK and cofilin, promoting cofilin phosphorylation and a consequent inhibition of cell migration. (D) FLNA phosphorylation regulates SST2 signal transduction. cAMP/PKA pathway activation induces FLNA phosphorylation. P-FLNA constitutively binds SST2, and hampers its coupling with inhibitory G proteins after agonist activation. SST2, when bound to dephosphorylated FLNA, inhibits adenylate cyclase activity, with a consequent reduction of PKA-mediated FLNA phosphorylation.

with a role for FLNA in the control of DRD2 fate after endocytosis, it has been shown in HEK293 cells that FLNA regulates DRD2 internalization and recycling (Zheng *et al.* 2016).

These data support the notion that FLNA is a trafficking adaptor, which allows an efficient recycling of GPCRs, as also showed for chemokine receptor CCR2 and β 2-adrenergic receptor (Pons *et al.* 2017), and a protection against degradation, as demonstrated for calcium-sensing receptor (Zhang & Breitwieser 2005), calcitonin receptor (Seck *et al.* 2003), cystic fibrosis transmembrane conductance regulator (Thelin *et al.* 2007) and Fc γ RI (Beekman *et al.* 2008).

However, the role of FLNA in DRD2 regulation is not limited to guarantee the appropriate levels of the receptor on the plasma membrane, but extends to a scaffold function that enable DRD2 signal transduction. The work of Peverelli *et al.* demonstrated that in primary cultured human PRL-secreting cells, FLNA silencing prevented the inhibitory effects of DRD2 on PRL secretion and ERK1/2 phosphorylation (Peverelli *et al.* 2012). On the other hand, DA-resistant prolactinoma cells transfected with FLNA become able to respond to dopaminergic drugs (Peverelli *et al.* 2012).

Due to this function of FLNA in DRD2 expression and signaling in lactotrophs, the loss of FLNA expression may be one of the mechanisms involved in resistance of prolactinomas to dopaminergic drugs. Overall, these data reveal FLNA as a novel potential target to modulate the amount of active DRD2 at the cell membrane in PRL-secreting pituitary tumor cells (Fig. 2).

FLNA role in GH-secreting tumor responsiveness to SSAs

In GH-secreting tumors, SSTR2 is the main target of pharmacological therapy. A positive correlation of SSTR2 expression with tumor response to medical therapy with SSA is well documented in literature, but resistance to SSAs has been observed also in the presence of SSTR2, suggesting post-receptor mechanisms involved (revised in Peverelli *et al.* 2015, Gadelha *et al.* 2017, Paragliola *et al.* 2017, Marazuela *et al.* 2018). The efficacy of SSA can be reduced due to an altered expression of beta arrestins, scaffold proteins involved both in desensitization and signal transduction of several GPCRs, including SSTRs (Tulipano *et al.* 2004, Peverelli *et al.* 2008). Indeed, low expression of beta arrestin 1, but not beta arrestin 2, correlated with a reduced recycling rate of SSTR2 and a better biochemical response to SSA, both *in vitro* and *in vivo* (Gatto *et al.*

2013). Alterations in SSTRs signal transduction might be due to aryl hydrocarbon receptor-interacting protein (AIP) tumor-suppressor gene mutations (Daly *et al.* 2010), typically correlating with octreotide resistance (Ibáñez-Costa & Korbonits 2017). Moreover, tumor resistance has been associated with alterations of the expression of Raf kinase inhibitory protein, which regulates MAPK signaling, a pathway involved in mediating the antiproliferative effects of somatostatin (Fougner *et al.* 2008).

Recently, a role for FLNA in both SSTR2 expression after prolonged agonist stimulation and signal transduction has been described. FLNA can directly bind SSTR2 first intracellular loop by its repeats 19–20 (Najib *et al.* 2012) (Fig. 1). In GH-secreting tumors tissues, FLNA protein is expressed at variable levels, with no correlation with SSTR2 expression (Peverelli *et al.* 2014), in striking contrast with DRD2 in prolactinomas (Peverelli *et al.* 2012). In addition, FLNA is not required for a proper localization of SSTR2 on the plasma membrane. Indeed, FLNA silencing in primary cultures of somatotropinomas does not affect SSTR2 intracellular localization on the plasma membrane neither its expression levels (Peverelli *et al.* 2014), in agreement with the evidence that in melanoma cell lines SSTR2 membrane localization does not depend on the presence of FLNA (Najib *et al.* 2012).

At higher level of detail, single-molecule microscopy analysis recently showed that dynamic SSTR2–FLNA interactions control SSTR2 spatial arrangement and mobility at the plasma membrane and are required for the formation of SSTR2 clusters and their alignment along actin fibers in CHO cells (Treppiedi *et al.* 2018). In addition, the transfection of a dominant negative FLNA fragment (FLNA 19–20), which prevents FLNA–SSTR2 binding (Peverelli *et al.* 2014), impaired the coupling of SSTR2 clusters to clathrin-coated pits, with a consequent decrease of SSTR2 internalization (Treppiedi *et al.* 2018) (Fig. 3).

A role for FLNA in dictating the fate of agonist-activated SSTR2, in analogy with DRD2, is supported by the observation that in GH-secreting pituitary tumor cells SSTR2–FLNA interaction is required to prevent activated SSTR2 lysosomal degradation and to maintain SSTR2 stability after prolonged agonist stimulation (Peverelli *et al.* 2014).

At the same time, FLNA is required for SSTR2 signal transduction. In human GH-secreting tumor primary cultured cells, FLNA silencing prevented SSTR2-induced reduction of cyclin D1 and activation of caspase 3/7, required for the antiproliferative and pro-apoptotic effects of SSTR2 (Peverelli *et al.* 2014). The FLNA

Figure 3

Selected frames from a representative image sequence acquired with a total internal reflection fluorescence (TIRF) microscope showing a portion of the cell surface of a CHO cell co-expressing single molecules of SSTR2 (green) and FLNA (magenta) and actin filaments (blue). TIRF microscopy is a powerful tool to visualize cell plasma membrane associated events, such as lateral movements of receptor and protein–protein interactions. Thanks to the higher signal-to-noise ratio compared to epifluorescence, tracking algorithms can be applied to link detected particles in each frame of the acquired image sequence and reconstruct particles trajectories. This example shows the dynamics of a typical FLNA–SSTR2 interaction and the involvement of FLNA in SSTR2 clusters formation and internalization after SSTR2 stimulation with a specific agonist. Two cell surface SSTR2 molecules are indicated by arrows at the beginning of the image sequence (0.06 s). A FLNA molecule, indicated by the arrowhead, appears at the cell surface (0.36 s) and subsequently co-localizes with the SSTR2 particle at the bottom (0.48 s). The FLNA–SSTR2 complex gets in close proximity to the other SSTR2 particle (0.78 s) and a receptor cluster is then formed (1.32 s and 3.36 s). SSTR2 cluster disappears from cell surface (3.66 s), due to its internalization. Scale bar, 1 μ m.

scaffold domain involving repeats 21–24 seems to play a paramount role in facilitating SSTR2 signaling. Indeed, overexpression of dominant negative mutant FLNA 21–24, that does not abolish the interaction of SSTR2 with endogenous FLNA, eliminated SSTR2 effects on apoptosis and ERK1/2 inhibition. These data suggest that the FLNA scaffold properties are required for the recruitment of signal transduction complexes to activated SSTR2. Another mechanism by which FLNA mediates SSTR2 antiproliferative action has been demonstrated in pancreatic neuroendocrine tumors, where a competition of FLNA with PI3K regulatory subunit p85 for the binding to SSTR2 occurs (Najib *et al.* 2012), and where FLNA is required for SSTR2 expression and signaling (Vitali *et al.* 2016). FLNA is also required to mediate the inhibitory effects of SSTR2 on cell migration and invasion in GH-secreting pituitary tumors (Peverelli *et al.* 2018a), by mediating the recruitment to activated SSTR2 of components of the cofilin pathway, as discussed below.

Altogether, these data support the idea that FLNA functions as a molecular platform able to connect SSTR2 with components of the machinery of intracellular trafficking and of the signal transduction cascade (Fig. 2).

Thus, low levels of FLNA in GH-secreting pituitary tumors, by causing loss of coupling of SSTR2 with downstream signal transduction molecules, might cause loss of responsivity of the patient to SS analogs even if in the presence of appropriate levels of SSTR2. Further studies in a large series of patients are required to investigate a possible correlation between FLNA expression levels and clinical behavior of GH-secreting tumors.

Despite the amino acidic sequence of SSTR2 required for FLNA binding is conserved in SSTR5, no data are found in literature about a FLNA role in regulating SSTR5.

This receptor subtype is highly expressed in GH-secreting tumors and is the most expressed SSTRs in ACTH-secreting tumors. Since the multi-ligand SSA pasireotide represents the only pituitary-directed drug approved by regulatory authorities to treat Cushing's disease, at least in Europe, it would be of great interest to evaluate a possible FLNA role in molecular mechanisms underlying the resistance to pasireotide, occurring in 50–70% of patients with Cushing's disease (Guelho & Grossman 2015).

Cytoskeleton role in pituitary tumors invasiveness

Cofilin promotes NFPT invasiveness and is regulated by DRD2

Non-functioning pituitary tumors (NFPTs) frequently show local invasiveness that strongly reduces the success of transsphenoidal neurosurgery, the treatment of choice for this type of tumor, and increases the incidence of tumor recurrence (Meiji *et al.* 2002, Losa *et al.* 2008). No diagnostic molecular markers predictive of the invasive behavior and recurrence of NFPTs are available. Moreover, the molecular mechanisms underlying the invasive behavior of these mostly benign tumors are unknown.

Overexpression of cofilin has been associated with tumor cell proliferation, invasion and metastasis in astrocytoma (Nagai *et al.* 2011), breast (Wang *et al.* 2006, Zhang & Tong 2010), colon (Popow-Woźniak *et al.* 2012), pancreatic (Wang *et al.* 2015), prostate (Collazo *et al.* 2014) and gallbladder (Yang *et al.* 2013) tumors, and cofilin dephosphorylation was observed in human malignant cells (Nagai *et al.* 2011).

A role for cofilin in promoting invasion of NFPTs has recently been demonstrated. Peverelli *et al.* showed that overexpression of cofilin induced an increase of cell migration of human non-functioning pituitary tumor HP75 cells, an effect reproduced by the constitutively active cofilin phosphodeficient mutant S3A, but not phosphomimetic S3D (Peverelli *et al.* 2016). Moreover, the pro-migratory effect of active S3A cofilin was supported by its intracellular co-localization with F-actin in membrane protrusions in HP75 cells, in contrast to S3D cofilin, which is diffusely distributed in the cytoplasm.

These *in vitro* data are well supported by the analysis of cofilin phosphorylation in human NFPT tissues. Indeed, Western blot analysis demonstrated higher phosphorylated cofilin (P-cofilin)/total cofilin ratio in non-invasive than in invasive NFPTs, and immunohistochemistry analysis showed a low or absent P-cofilin staining in invasive tumors, in contrast with high immunoreactivity for P-cofilin found in non-invasive tumors (Peverelli *et al.* 2016). From these experiments, cofilin emerges as a potential new biomarker predictive of NFPTs invasiveness and recurrence, which could provide additional information for prognosis influencing the management of patients and the use of adjuvant therapies.

DRD2 is expressed in most NFPTs (Vieira Neto *et al.* 2015) but medical therapy with DRD2 agonists is still under debate (Delgado-López *et al.* 2018). Beside antiproliferative activity (Colao *et al.* 2000, Florio *et al.* 2008, Peverelli *et al.* 2010, Gagliano *et al.* 2013), DRD2 was recently demonstrated able to exert anti-migratory and anti-invasive activity in cultured cells from NFPTs and HP75 cells (Peverelli *et al.* 2016). This unprecedented effect of DRD2 in a tumor cell model is a consequence of the DRD2 ability to modify the intracellular pool of P-cofilin. Indeed, in these cells, DRD2 agonist promoted a Rho-associated protein kinase ROCK-dependent LIMK phosphorylation, which in turn induced cofilin phosphorylation (Fig. 2). To date, DRD2 coupling with RhoA/ROCK signaling pathway has been described only in mouse striatal neurons (Deyts *et al.* 2009, Galan-Rodriguez *et al.* 2017), but the molecular mechanism involved has never been investigated. As described below, FLNA may function as scaffold linking together GPCRs and components of the cofilin pathway (Peverelli *et al.* 2018a), suggesting that DRD2 effect on P-cofilin may be mediated by FLNA. However, G proteins coupled to DRD2, or beta arrestins, involved in DRD2 internalization (Kim *et al.* 2001) and able to activate Rho (Barnes *et al.* 2005, Ma *et al.* 2012), may be potential molecular players connecting DRD2 to cofilin pathway.

These data first suggest a new role for DRD2 agonists in the control of invasive properties of NFPTs. Indeed, DRD2 actively participates in actin fibers remodeling by controlling cofilin activation, with important consequences on cell migration and invasion.

Despite DRD2 being the main target of PRL-secreting tumors therapy, no data about DRD2 agonists effects on invasion and cofilin phosphorylation in prolactinomas are available in literature. However, it was demonstrated that exposure of lactotrophs to dopamine stabilized the cortical actin cytoskeleton (Carbajal & Vitale 1997), consistently with an increase of P-cofilin.

Finally, the recent observation that the coupling of DRD2 to RhoA/ROCK pathway is specific of the short spliced version of the receptor (D2S) (Galan-Rodriguez *et al.* 2017) deserves further considerations. This DRD2 isoform differs from the long one (D2L) for the loss of 29 amino acids in the third intracellular loop, resulting in different physiological functions. The differential expression of these two splice variants has been associated with the biological behavior of PRL-secreting tumors. Indeed, lower expression of D2S transcript correlated with resistance to dopaminergic drugs (Caccavelli *et al.* 1994, Wu *et al.* 2010) and tumor invasiveness (Wu *et al.* 2010), consistent with a reduced or absent coupling of D2L with cofilin pathway.

GH-secreting tumors invasiveness is regulated by SSTR2 through FLNA-mediated cofilin inactivation

Invasion of the cavernous sinus, reported in 20–50% of patients with GH-secreting tumors, represents a clinical issue since the outcomes of transsphenoidal surgery are less favorable for the lesions invading surrounding tissues (Sarkar *et al.* 2014, Anik *et al.* 2017).

Recently, an anti-migratory effect of SSTR2 in GH-secreting tumor cells has been discovered, in addition to its recognized antiproliferative and pro-apoptotic actions (Peverelli *et al.* 2018a). This previously unknown SSTR2 ability to actively participate to cytoskeleton remodeling is mediated by FLNA and cofilin.

The specific SSTR2 agonist BIM23120 inhibited migration and invasion on collagen IV in both primary cultured cells from human GH-secreting tumors and GH3 cell line (Peverelli *et al.* 2018a). It is of clinical relevance noting that these effects are reproduced by the two SSAs used in the pharmacological therapy of pituitary tumors: octreotide, with high preferential binding affinity for SSTR2, and pasireotide, with a broader spectrum of affinity for different receptor subtypes and high binding affinity to SST5.

The investigation about the molecular machinery involved identified the RhoA/ROCK/cofilin pathway as a new intracellular signaling cascade activated by SSTR2 (Peverelli *et al.* 2018a). Indeed, in GH3 cells, selective SSTR2 activation by BIM23120 strongly activated RhoA and induced a ROCK-mediated increase of P-cofilin/total cofilin ratio, which was required for the anti-invasive effects of SSTR2, in analogy with the mechanisms employed by DRD2 to reach the same effect in NFPT cells (Fig. 2).

In addition, the phosphorylation status of cofilin appeared to be an important regulator of GH-secreting tumor cells motility, since transfection of constitutively inactive phosphomimetic S3D cofilin was able to reduce GH3 cell invasion independently from SSTR2 activation, whereas no effect was observed after transfection of S3A or WT cofilin.

Thus, S3D cofilin functions in a dominant negative manner in this type of tumor, in line with the observation that it may compete with endogenous phosphorylated cofilin for binding to specific phosphatases, with a consequent increase of cofilin phosphorylation status (Chua *et al.* 2003).

The effects of overexpression of active S3A cofilin depend on the cell line. Indeed, S3A cofilin increased NFPT, but not GH-secreting, cell invasion (Peverelli *et al.* 2016, 2018a), supporting the hypothesis that multiple, cell-specific mechanisms, other than phosphorylation, are required to control the initial activation of cofilin (Song *et al.* 2006).

In line with the function of FLNA as scaffold for SSTR2 signal transduction molecules (Peverelli *et al.* 2014), the activation of cofilin pathway required FLNA, as demonstrated by both FLNA genetic silencing and transfection of FLNA dominant negative mutants preventing FLNA binding to SSTR2 (FLNA 19–20) or to signaling molecules (FLNA 21–24) (Peverelli *et al.* 2018a). Moreover, by confocal microscopy and coimmunoprecipitation assays, the authors demonstrated that upon agonist challenge SSTR2 colocalized with FLNA and cofilin at the plasma membrane, but cofilin recruitment to SSTR2 was completely lost in cells silenced for FLNA.

These data suggest that activated SSTR2 recruits a macromolecular complex that through FLNA anchors SSTR2 to actin fibers and connects molecular components of the cofilin pathway, enabling a direct effect of SSTR2 on actin cytoskeleton dynamics (Fig. 2).

Intriguingly, the overexpression of FLNA19–20 and 21–24 in GH3 cells reduced cell invasion, independently

from SSTR2 selective stimulation, first revealing a role of these peptides as inhibitors of tumor cell invasiveness (Peverelli *et al.* 2018a).

The molecular mechanism involved might be related to the ability of FLNA to bind molecules that participate in the regulation of cell adhesion and migration processes. Indeed, the FLNA regions contained in both these dominant negative mutants are required for cell spreading and initiation of cell migration (Baldassarre *et al.* 2009) and integrin binding (Kiema *et al.* 2006, Ithychanda *et al.* 2009).

Mechanisms regulating FLNA expression and function in pituitary tumors

FLNA expression

The molecular events responsible for a low or absent FLNA expression in pituitary tumors remain an open question. A possible role for epigenetic silencing has been ruled out, at least in PRL-secreting tumors, since no methylation in the FLNA promoter regions was found neither in FLNA expressing nor in non-expressing prolactinomas (Peverelli *et al.* 2012).

Despite several germline FLNA mutations that lead to reduced or absent FLNA expression have been identified in human disease (see below), a possible presence of FLNA somatic mutations in pituitary tumors has never been investigated.

FLNA degradation is promoted by ubiquitination triggered by ASB2 proteins, subunits of E3 ubiquitin ligase complexes (Razinia *et al.* 2011). No data about FLNA ubiquitination and degradation process in pituitary tumors are available, but it is of interest to note that alterations of ubiquitin system due to somatic mutations of ubiquitin-specific peptidase 8 (USP8) gene play a pathogenetic role in the development of ACTH-secreting pituitary tumors (Reincke *et al.* 2015).

FLNA splicing variants

It is known that FLNA mRNA is subjected to alternative splicing. Among the described splice variants, of interest is the FLNA splice variant-1 (var-1), containing an internal deletion of 41 amino acids between C-terminal part of repeat 19 and the N-terminal part of repeat 20 (residues 2127–2167) (Pentikäinen *et al.* 2011) (Fig. 1). This deletion increased FLNA/integrins binding compared with nonspliced FLNA (van der Flier *et al.* 2002), suggesting a general role for this alternative splicing in regulating

FLNA binding to its interaction partners. Since FLNA regions that directly interact with SSTR2 and DRD2 are located in repeats 19–20, an altered binding of FLNA var-1 to these receptors can be hypothesized. Moreover, it is worth noting that the aminoacidic residues spliced out in FLNA var-1 include serine 2152, target of phosphorylation (see below), and the putative NLS (2146–2149), with possible important implications for FLNA functions.

FLNA var-1 is widely expressed at low levels, but up to now no data about the expression of this variant in normal and tumoral pituitary is known. It is possible to hypothesize that the alternative splicing of FLNA may have implications in pituitary tumors responsiveness to SSAs and DRD2 agonists.

Mutations in FLNs splice sites have been previously associated with human diseases, such as PVNH (Oegema *et al.* 2013) and dilated cardiomyopathy (Begay *et al.* 2016), involving FLNA and FLNC, respectively.

FLNA phosphorylation

FLNA is target of phosphorylation of different kinases, such as PKA, PKC, CaM-kinase II, Pak1 (p21-activated kinase 1), RSK (ribosomal S6 kinase) and cyclin B1/Cdk1 (Chen & Stracher 1989, Jay *et al.* 2004, Woo *et al.* 2004, Cukier *et al.* 2007, Zhang *et al.* 2012, Hammer *et al.* 2013).

This posttranslational modification critically modulates FLNA functions, since it has been implicated in cell migration (Woo *et al.* 2004, Ravid *et al.* 2008, Zhang *et al.* 2012, Hammer *et al.* 2013, Li *et al.* 2015, Sato *et al.* 2016) focal adhesion formation (Sato *et al.* 2016), integrin binding (Chen *et al.* 2009, Sato *et al.* 2016), calpain-mediated FLNA proteolysis (Zhang *et al.* 1988, Chen & Stracher 1989, Wu *et al.* 1994, Jay & Stracher 1997, García *et al.* 2006, Bedolla *et al.* 2009) and chemokine receptor 2 recycling (Pons *et al.* 2017).

The N-terminal region of FLNA contains a PKA site probably involved in F-actin interaction (Jay & Stracher 1994), whereas the only PKA phosphorylation site in the C-terminal region of FLNA is serine 2152 in the repeat 20 of FLNA (Jay *et al.* 2000) (Fig. 1). cAMP/PKA pathway activation induced FLNA phosphorylation at S2152 in different cell systems (Chen & Stracher 1989, Jay *et al.* 2000, 2004), and recently, the same effect has been reported in GH-secreting pituitary tumors.

Indeed, in both GH3 and GH4C1 cell lines and in primary cultured cells from GH-secreting pituitary tumors, forskolin increased, and SSTR2 agonist reduced, FLNA phosphorylation at S2152, with dramatic effects on FLNA binding to SSTR2 and SSTR2 signal transduction

(Peverelli *et al.* 2018b) (Fig. 2). The authors demonstrated that the phosphomimetic S2152D FLNA mutant constitutively bound to SSTR2, but precluded inhibitory G proteins coupling to SSTR2, and completely abolished antiproliferative, pro-apoptotic and anti-migratory effects of selective SSTR2 activation by BIM23120 (Fig. 2) (Peverelli *et al.* 2018b). In this scenario, phosphorylation seems to be a mechanism to switch FLNA function from a scaffold that allows SSTR2 signal transduction, to a signal termination protein that hampers all SSTR2 antitumoral effects (Fig. 2).

In a broader perspective, further studies are needed to investigate whether this mechanism may control the activity of other GPCRs that bind FLNA. In the field of pituitary tumors, the study of the FLNA phosphorylation offers new insights into the molecular determinants underlying SSA resistance of pituitary tumors and suggests phosphorylated FLNA as a novel biomarker predicting GH-secreting tumor responsiveness to SSA.

Conclusions

Cell cytoskeleton proteins, clearly far from being only structural cell components, are critically involved in the complex molecular machinery that determines the biological behavior of pituitary tumors. In particular, the multifunctional protein FLNA appears to be a molecular platform that by facilitating the interplay with several partners orchestrates DRD2 and SSTR2 expression, localization, internalization, intracellular trafficking, signal transduction and signal termination.

Therefore, its proper function is a mandatory requirement for the antitumoral action of dopaminergic and somatostatinergic drugs in pituitary tumors.

The biological responses to these currently used drugs do not only include the well-established inhibitory effects on hormone secretion and tumor growth, but also include the ability to restraint tumoral cell migration and invasion. It is not surprising that FLNA acts as an intermediary between the receptors of these drugs on the cell surface and the actin filaments of the cell cytoskeleton, also allowing the recruitment of the components of the pathway of cofilin, a protein specifically involved in actin filaments remodeling.

In this scenario, further studies aimed to deeply investigate the mechanisms of regulation of FLNA and cofilin might suggest new pharmacological strategies for DA or SSA resistant and invasive pituitary tumors. One important direction for future studies is to determine a possible crosstalk between the different cytoskeletal

components identified as crucial in pituitary biology, including FLNA, actin, cofilin, keratins and E-cadherin.

Understanding the molecular basis underlying the different biological behaviors of pituitary tumors will be a key milestone in reaching a personalized approach to treatment of this disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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