Filamin-A Is Essential for Dopamine D2 Receptor Expression and Signaling in Tumorous Lactotrophs

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Context: Dopamine agonists (DA) are the first choice treatment of prolactinomas. However, a subset of patients is resistant to DA, due to undefined dopamine D2 receptor (D2R) alterations. Recently, D2R was found to associate with filamin-A (FLNA), a widely expressed cytoskeleton protein with scaffolding properties, in melanoma and neuronal cells.

Objective: The aim of the study was to investigate the role of FLNA in D2R expression and signaling in human tumorous lactotrophs and rat MMQ and GH3 cells.

Design: We analyzed FLNA expression in a series of prolactinomas by immunohistochemistry and Western blotting. We performed FLNA silencing or transfection experiments in cultured cells from DA-sensitive or -resistant prolactinomas and in MMQ and GH3 cells, followed by analysis of D2R expression and signaling.

Results: We demonstrated reduced FLNA and D2R expression in DA-resistant tumors. The crucial role of FLNA on D2R was demonstrated by experiments showing that: 1) FLNA silencing in DA-sensitive prolactinomas resulted in 60% reduction of D2R expression and abrogation of DA-induced inhibition of prolactin release and antiproliferative signals, these results being replicated in MMQ cells that endogenously express FLNA and D2R; and 2) FLNA overexpression in DA-resistant prolactinomas restored D2R expression and prolactin responsiveness to DA, whereas this manipulation was ineffective in GH3 cells that express FLNA but not D2R. No alteration in FLNA promoter methylation was detected, ruling out the occurrence of epigenetic FLNA silencing in DA-resistant prolactinomas.

Conclusions: These data indicate that FLNA is crucial for D2R expression and signaling in lactotrophs, suggesting that the impaired response to DA may be related to the reduction of FLNA expression in DA-resistant prolactinomas. (***J Clin Endocrinol Metab*** **97: 967–977, 2012)**

Dopamine D2 receptor (D2R) mediates the inhibitory effects on prolactin (PRL) secretion and cell proliferation exerted by dopamine on pituitary lactotrophs (reviewed in Ref. 1). Prolactinomas are the most frequent pituitary tumors, and dopamine agonists (DA) are their first choice treatment because they normalize PRL levels and reduce tumor size in the majority of patients. However, a subset of patients displays resistance to the action of these drugs (2). Consistent with the antiproliferative action of dopamine, the occurrence of resistance to DA has

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2011-2902 Received October 21, 2011. Accepted December 15, 2011.
First Published Online January 18, 2012

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Abbreviations: BrdU, Bromodeoxyuridine; DA, dopamine agonist; D2R, dopamine D2 receptor; FLNA, filamin-A; PRL, prolactin; siRNA, small interfering RNA.

been associated with the shift of a prolactinoma to an invasive tumor or even a carcinoma (2). The molecular events involved in DA resistance are not fully understood. Reduction in D2R expression has been reported (3, 4), particularly in DA-resistant with respect to DA-sensitive prolactinomas (5). Although some D2R polymorphisms have been found to correlate with DA resistance (6), no mutation of the D2R gene has been identified so far in prolactinomas (7), whereas data on possible postreceptorial defects are not conclusive (2, 3).

Several studies identified specific protein-protein interactions as determinant in the regulation of receptor anchoring and signaling. Filamin-A (FLNA) (8), a widely expressed cytoskeleton protein involved in cell morphology regulation and motility, was found to associate with D2R in cell systems, particularly melanoma and neuronal cells (8–10). FLNA is characterized by an N-terminal actin-binding domain followed by 24 tandem repeats of about 96 amino acids and a C-terminal repeat containing a self-assembly sequence that allows for homodimerization. This interaction involves repeat 19 of FLNA and the N-terminal region of the third intracellular loop of D2R (amino acids 211–241) (9). Although previous studies demonstrated that FLNA interaction is crucial for D2R targeting and signaling (8–10), no data are available in lactotroph cells.

In the present study, we investigated the expression of FLNA in prolactinomas with different responsiveness to DA and evaluated the impact of FLNA on D2R expression and signaling. By immunohistochemistry and Western blot, both FLNA and D2R were strongly reduced or absent in DA-resistant prolactinomas in comparison with DA-sensitive tumors. The demonstration that FLNA silencing in DA-sensitive prolactinomas resulted in a significant reduction of D2R expression and abrogation of DA-induced signals, whereas FLNA overexpression in DA-resistant prolactinomas restored D2R expression and responsiveness to DA, strongly points to a crucial role of FLNA in D2R expression and signaling in tumorous lactotrophs.

Subjects and Methods

Immunohistochemistry

Immunohistochemistry was performed on sections from paraffin-embedded prolactinomas present in pathology archives. These samples were related to 14 patients resistant to DA treatment (*i.e.* failure to normalize PRL levels and to shrink tumor mass at a cabergoline dosage of 3 mg/wk; see Ref. 2) and five sensitive patients. Specific antibodies for FLNA (Abnova Corp., Taipei City, Taiwan) and D2R (Santa Cruz Biotechnology, Santa Cruz, CA) were used, and antigen-antibody detection was performed with the Dako ChemMate En Vision detection kit (Dako A/S, Glostrup, Denmark). FLNA and D2R immunoreactivities were graded according to an immunohistochemical score that takes into account both the percentage of positive cells (0–30% = 1; 31–60% = 2; 61–100% = 3) and the staining intensity (0 = absence of immunoreactivity; 1 = weak; 2 = medium intensity; and 3 = strong reactivity) considering at least 400 cells in the main representative high-power field, as described (11).

Pituitary cell culture

The study was previously approved by the local ethics committee. Informed consent was obtained from all subjects involved in the study. Human pituitary cells were obtained by the transsphenoidal route from two patients sensitive to DA who discontinued the treatment due to side effects and three DA-resistant patients. Tissues were enzymatically dissociated in DMEM containing 2 mg/ml collagenase at 37 C for 2 h, as previously described (11). Rat pituitary MMQ cells (ATCC CRL-10609) were grown in RPMI 1640 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and antibiotics. Rat pituitary GH3 cells (ATCC CCL-82.1) were grown in Ham F10 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics.

FLNA silencing and transfection in pituitary cells

FLNA gene silencing was performed in prolactinomas and MMQ cells using species-specific human and rat FLNA predesigned small interfering RNA (siRNA), respectively, and siPORT NeoFX transfection agent (Ambion, Austin, TX) according to manufacturer's instructions. To obtain the best efficiency of FLNA silencing, three different human FLNA silencer select predesigned siRNA and three specific for rat FLNA purchased from Ambion were tested. Preliminary experiments to determine the optimal concentration of siRNA and the kinetics of silencing of FLNA were performed. A negative control siRNA, a nontargeting sequence without significant homology to the sequence of human, mouse, or rat transcripts, was used in each experiment.

The plasmid encoding FLNA (pREP4 filamin-A expression vector) was kindly provided by Dr. Michel Bernier (Biomedical Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD). Transient transfections of FLNA were performed in cultured cells from three DA-resistant prolactinomas and GH3 cells using JetPEI (Polyplus Transfection, San Marcos, CA) according to the instruction of the manufacturer. Mock-transfected cells were used in each experiment as negative control.

Western blotting was performed in each experiment to control the expression level of FLNA in silenced or transiently transfected cells. RT-PCR was performed to verify the FLNA silencing or transfection and the possible effects on D2R transcript.

Total and cell surface D2R expression

Variation in D2R expression levels was evaluated in prolactinomas and in MMQ cells transfected with FLNA siRNA, negative control siRNA, or pREP4-FLNA by Western blot analysis. To determine the degradation pathway, MMQ cells were incubated with 10 μm lactacystin or 50 μm chloroquine for 20 h at 37 C as previously reported (11).

Biochemical analysis of membrane expression of D2R was performed using the biotin method previously described (12). Total cellular protein was immunoprecipitated with D2R antibody and resolved by SDS-PAGE under nonreducing conditions. To detect biotinylated proteins, antibiotin antibody was used.

The resulting bands were evaluated with the image analysis program NIH ImageJ.

PRL secretion and cAMP assay

Prolactinoma cells and MMQ cells were silenced with FLNA or negative control siRNAs or transfected with pREP4 FLNA for 72 h and incubated for 16 h with increasing concentrations of BIM53097. The superselective analog BIM53097, specific for DR2, was kindly provided by Dr. M. Culler (Biomeasure Incorporated/IPSEN, Milford, MA).

Human and rat PRL were measured in culture medium by specific immunoassays (Perkin-Elmer, Turku, Finland; and SPI Bio, Montigny-le-Bretonneux, France, respectively), according to the manufacturers' instructions.

To quantify the inhibition of forskolin-induced cAMP accumulation, MMQ cells transfected with FLNA siRNA or negative control siRNA were preincubated with 0.5 mM 3-isobutyl-1-methylxantine for 30 min, and subsequently with 1 μM forskolin with or without increasing doses of BIM53097 for 30 min at 37 C. Intracellular cAMP was measured by enzymatic immunoassay (Promega, Madison, WI).

Proliferation assay and ERK1/2 phosphorylation

Cell proliferation was assessed as previously reported (11). MMQ cells were treated with or without FLNA siRNA or negative control siRNA for 72 h and then incubated with BIM53097 for 48 h at 37 C and with bromodeoxyuridine (BrdU) for 2 h to allow BrdU incorporation in newly synthesized cellular DNA.

For ERK1/2 analysis, pituitary cells silenced or transfected with pREP4 FLNA were serum-starved for 24 h and then stimulated with serum with or without BIM53097 for 10 min. Analysis of ERK1/2 activation was performed by Western blotting as previously described (13).

Confocal microscopy analysis

Silenced MMQ cells were fixed with 4% paraformaldehyde and treated with 0.1 M glycin in PBS (pH 7.4) and 0.3% Triton X-100 buffer. Cells were incubated overnight at 4 C with anti-DRD2 antibody and stained with Alexa Fluor 488-conjugated secondary antibody and Alexa Fluor 546-Phalloidin (Invitrogen, Carlsbad, CA) for 1 h at room temperature. TOTO-3 iodide (642/660) (Invitrogen) was used for nuclei staining. Confocal microscopy was carried out on a Radiance 2100 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA) equipped with a Krypton/Argon laser and a red laser diode. To reduce bleed-through, images were acquired sequentially. Noise reduction was achieved by "Kalman filtering."

FLNA methylation analysis

Genomic DNA was extracted from paraffin-embedded and fresh samples of six surgically removed prolactinomas (three DA-sensitive and three DA-resistant prolactinomas) by Nucleon BACC2 genomic DNA purification kit (GE Healthcare, Buckinghamshire, UK).

The methylation status of CpG islands localized in FLNA promoter region was assessed by direct sequencing of bisulfite-treated genomic DNA (14, 15). This region was *in silico* analyzed for the localization of transcription factor binding sites and CpG islands using MatInspector (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html), ENSEMBL (http://www.ensembl.org/index.html), University of California Santa Cruz (UCSC) (http://genome.ucsc.edu/), and NCBI (http://www.ncbi.nlm.nih.gov/). Primer sequences were specifically designed to amplify the modified DNA using MethPrimer (http://www.urogene.org/methprimer/index1.html) (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

The methylation status was also analyzed for each considered DNA locus with two software tools, which furthermore allow verifying bisulfite conversion efficiency: BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de/) and BISMA (http://biochem.jacobs-university.de/BDPC/BISMA/). Every sample was tested twice for each CpG island to confirm the reproducibility of our results.

Statistical analysis

The results are expressed as the mean ± SD. A paired two-tailed Student's *t* test was used to detect the significance between two series of data. Analysis of FLNA silencing was tested *vs.* the negative control by two-way ANOVA. Calculations were performed by GraphPad Prism 3.0 software (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was accepted as statistically significant.

Results

FLNA expression is reduced in DA-resistant prolactinomas

Immunohistochemistry was performed on sections of paraffin-embedded prolactinomas surgically removed from 14 patients with resistance to DA treatment (*i.e.* failure to normalize PRL levels and to shrink tumor mass at cabergoline dosage of 3 mg/wk; see Ref. 2) and five DA-sensitive patients. Samples of human DA-resistant prolactinomas showed a weak cytoplasmic staining for FLNA that was significantly reduced in comparison with that of DA-sensitive prolactinomas (mean immunoreactivity score, 2.2 ± 1.8 and 6.3 ± 2.5, respectively; $P < 0.05$) (Fig. 1, A and B). As expected, D2R expression was reduced in DA-resistant with respect to DA-sensitive adenomas (mean immunoreactivity score, 3.1 ± 1.3 *vs.* 5.9 ± 2.1; $P < 0.05$). These data were confirmed by Western blot analysis showing a strong reduction of FLNA and D2R expression in DA-resistant tumors.

D2R expression in prolactinoma cells depends on FLNA expression

To investigate the possible causal association between FLNA and D2R levels, we evaluated D2R expression changes in cells from DA-sensitive prolactinomas after FLNA silencing by siRNA techniques and in cells from DA-resistant prolactinomas overexpressing FLNA by transient transfection. In lactotrophs from DA-sensitive prolactinomas (n = 2), 72-h FLNA silencing induced a significant decrease in the levels of FLNA protein expression with respect to control (45 and 51% reduction, re-

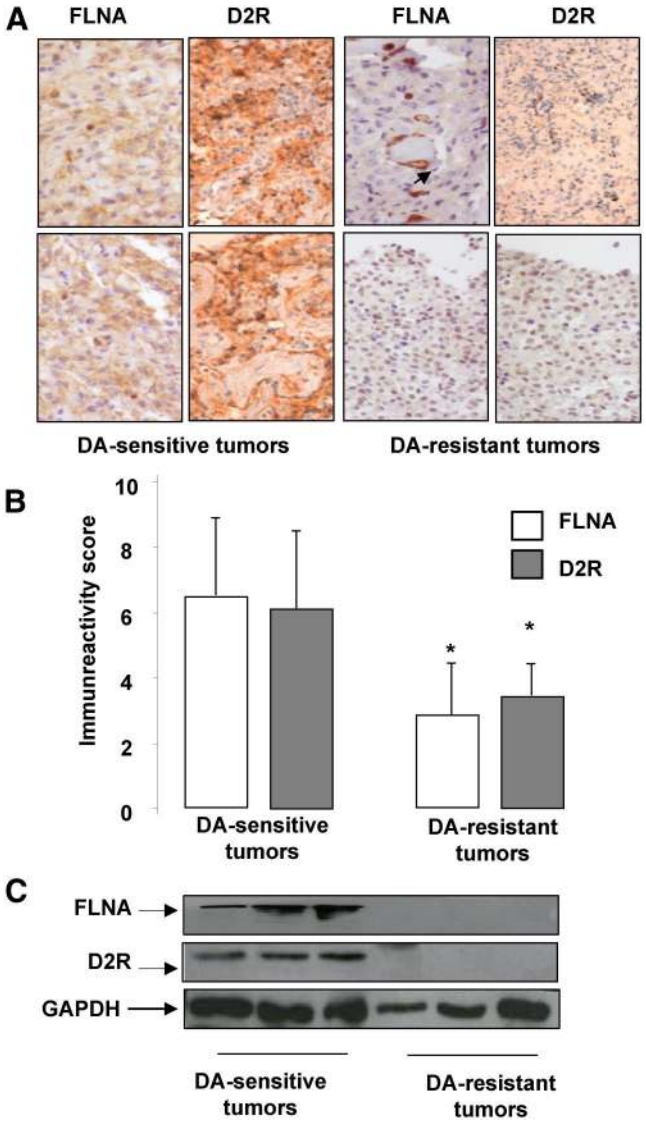

FIG. 1. FLNA expression is reduced in DA-resistant prolactinomas. A, Representative pictures of immunohistochemistry for FLNA and D2R in prolactinomas removed from patients sensitive or resistant to DA (20× magnification). The *arrow* indicates a vessel positive for FLNA shown as internal positive control. B, Immunoreactivity score for FLNA and D2R obtained in five DA-sensitive and 14 DA-resistant prolactinomas. Immunoreactivities were graded according to an immunohistochemical score (range from 0 to 9) that takes into account both the percentage of positive cells and the staining intensity (see *Subjects and Methods*). *, $P < 0.05$ *vs.* DA-sensitive tumors, *t* test. C, Immunoblots of FLNA and D2R performed on DA-sensitive and DA-resistant prolactinoma samples are shown. FLNA and D2R antibodies were from Abnova and Santa Cruz Biotechnology, respectively. The equal amount of protein was confirmed by stripping and reprobing with an anti-GAPDH antibody. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

spectively), associated with a marked reduction of D2R levels (60 and 62%, respectively) (Fig. 2A, *left panel*). Conversely, increased expression of FLNA in cells from DA-resistant prolactinomas (n = 3) transfected with pREP4 expression vector containing cDNA of human FLNA resulted in a significant increase in FLNA expression (about 6.5-fold over control at 72 h), that was associated with enhanced D2R expression level (about 7-fold over control cells) (Fig. 2A, *right panel*).

FLNA is required for D2R-mediated inhibition of PRL release and ERK1/2 activation in prolactinoma cells

To investigate the role of FLNA on the biological responses elicited by D2R activation, we first analyzed the regulation of PRL release. Exposure of DA-sensitive prolactinoma cells transfected with the negative control siRNA to the D2R-selective agonist BIM53097 caused a reduction of PRL release at 100 nM (25 and 28% inhibition, respectively, in two prolactinomas; $P < 0.05$ *vs.* basal), whereas in FLNA-silenced cells BIM53097 was ineffective (two-way ANOVA; $P = 0.0041$; Fig. 2B). We then analyzed DA-induced inhibition of PRL release from DA-resistant prolactinoma cells transfected with FLNA expression vector. As expected, no effect of BIM53097 on PRL release was observed in mock-transfected cells, whereas transfection with FLNA restored PRL responsiveness to BIM53097 (two-way ANOVA; $P = 0.0009$; 34 ± 5% inhibition of PRL release at 100 nM in three prolactinomas, Bonferroni post test; $P < 0.001$ *vs.* basal) (Fig. 2B). Both FLNA silencing and overexpression did not affect basal PRL release (data not shown).

To investigate the role of FLNA in D2R-mediated inhibition of cell proliferation, we analyzed the effects of D2R activation on ERK1/2 phosphorylation, which mediates the antimitotic action of DA. In cells from DA-sensitive prolactinomas, we found about a 2-fold increase of ERK1/2 phosphorylation after 10-min incubation with 100 nM BIM53097, this effect being totally abrogated in FLNA-silenced cells (two-way ANOVA; $P = 0.0006$; Fig. 2C).

Effects of FLNA silencing on D2R expression and targeting in MMQ cells

To further elucidate the effect of FLNA on D2R expression and signaling, we used MMQ cell line, a cell model of prolactinoma endogenously expressing functional D2R and FLNA. Taking advantage of the high levels of FLNA in this cell line, we silenced cells by siRNA techniques. A 72-h transfection with FLNA siRNA resulted in a strong decrease of FLNA transcript and protein expression (90 ± 6% inhibition), which was maintained for 6 d (Fig. 3A). In agreement with data obtained in prolactinoma cells from DA-sensitive prolactinomas, MMQ cells silenced with FLNA siRNA showed a progressive reduction of total D2R protein expression, which reached the maximum at 6 d of silencing (65 ± 12% reduction). On the contrary, no reduction of D2R transcript was observed, confirming the specificity of silencing of FLNA.

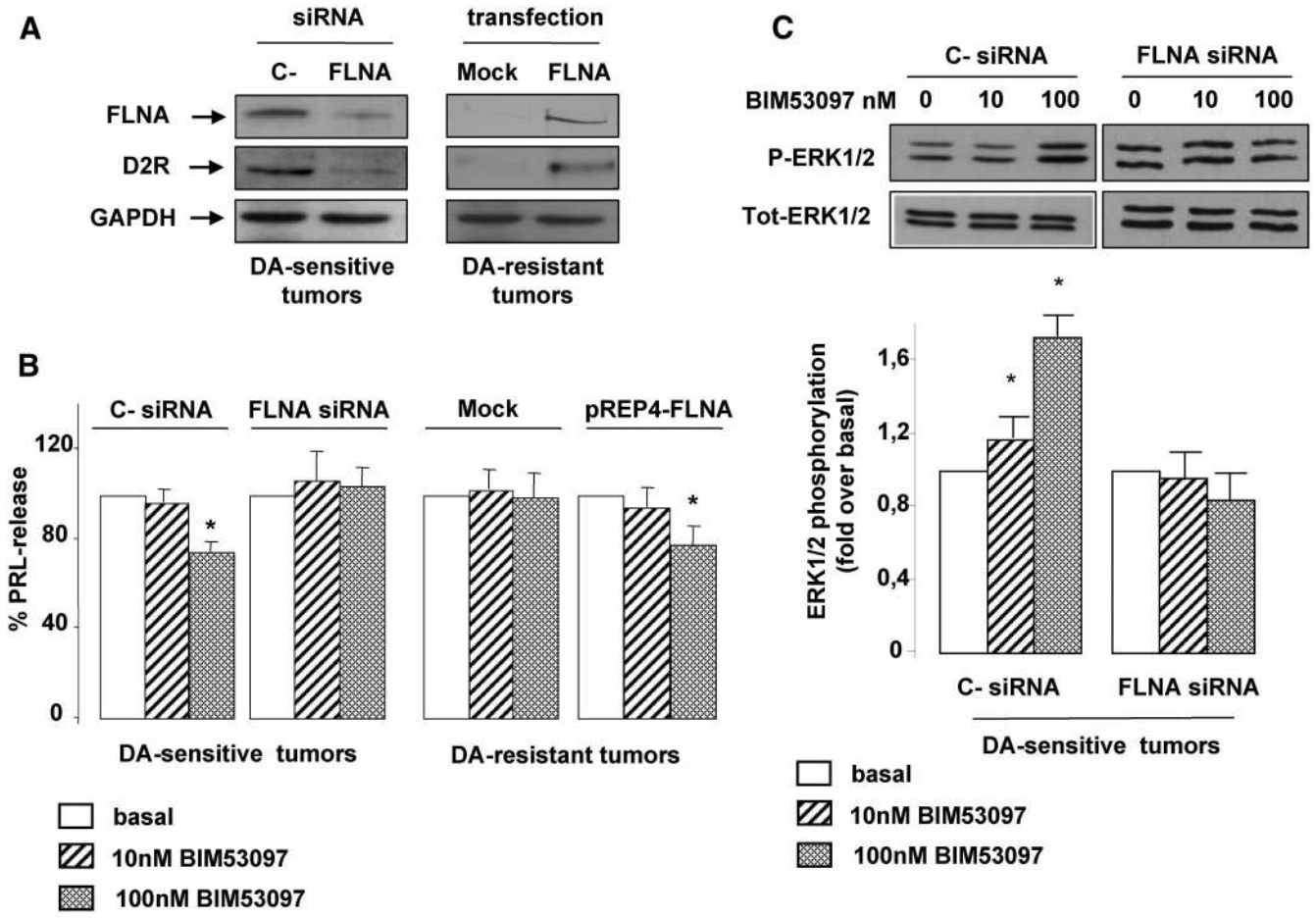

FIG. 2. D2R expression and signaling in prolactinomas depends on FLNA levels. A, Representative immunoblots of FLNA and D2R in DA-sensitive or -resistant prolactinoma cells transiently transfected with negative control or FLNA siRNA or pREP4-FLNA for 72 h. FLNA siRNA-treated cells showed a strong decrease in FLNA protein expression that was associated with a reduction of D2R expression, whereas enhanced FLNA expression in DA-resistant tumors was associated with a significant increase of D2R expression. The equal amount of protein was confirmed by stripping and reprobing with an anti-GAPDH antibody. B, FLNA is required for D2R-mediated inhibition of PRL release. FLNA silencing abolished the inhibitory effect of selective D2R agonist BIM53097 on PRL secretion in DA-sensitive tumors (two-way ANOVA, $P = 0.0041$). On the contrary, in DA-resistant tumors, no effect of BIM53097 on PRL release was observed in mock-transfected cells, whereas transfection with FLNA restored PRL responsiveness to BIM53097 (two-way ANOVA, $P = 0.0009$). Cells were incubated with negative control siRNA or FLNA siRNA or pREP4-FLNA for 72 h and treated with increasing concentrations of BIM53097 for 16 h. PRL was measured in culture medium. Each determination was done in triplicate. Values represent mean (±SD). *, $P < 0.001$ *vs.* corresponding basal, Bonferroni post test. C, FLNA is required for D2R-mediated ERK1/2 activation in DA-sensitive tumors. Representative immunoblot of ERK1/2 phosphorylation demonstrating that D2R-mediated activation of ERK1/2 by BIM53097 (10 nM and 100 nM for 10 min) was present in C− siRNA and abolished in FLNA siRNA-transfected DA-sensitive tumors (two-way ANOVA, $P = 0.0006$). The graph shows the quantification of phospho-ERK1/2 normalized to total ERK1/2 (mean ± SD from three independent experiments). Values represent mean (±SD). *, $P < 0.05$ *vs.* corresponding basal, *t* test. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; C−, negative control.

Therefore, we hypothesized that interaction with FLNA may stabilize D2R protein against degradation. To evaluate D2R degradation pathways, MMQ cells were treated with the lysosome inhibitor chloroquine or the proteasome inhibitor lactacystin. Treatment of MMQ cells after 4 d of siRNA transfection with chloroquine induced a strong increase in D2R levels with respect to untreated cells, whereas lactacystin was ineffective (Fig. 3B, and data not shown).

We then investigated the effects of FLNA on D2R cell surface expression. MMQ cells were analyzed by fluorescence microscopy 72 h after transfection with FLNA siRNA or negative control siRNA. As shown in Fig. 3C, MMQ cells, untransfected or transfected with negative control siRNA, showed D2R mainly expressed at the plasma membrane, frequently displaying membrane clustering, with a small amount located into endocytic vesicles throughout the cytoplasm. On the contrary, in FLNA-silenced cells, D2R redistributed from the plasma membrane to several cytoplasmic vesicles, suggesting that FLNA is required for cell surface expression of D2R.

These data were confirmed by a biochemical assay, by which 72 h after siRNA transfection, biotinylated cell surface proteins and total cellular proteins were immunoprecipitated by D2R antibody and the cell surface D2R was

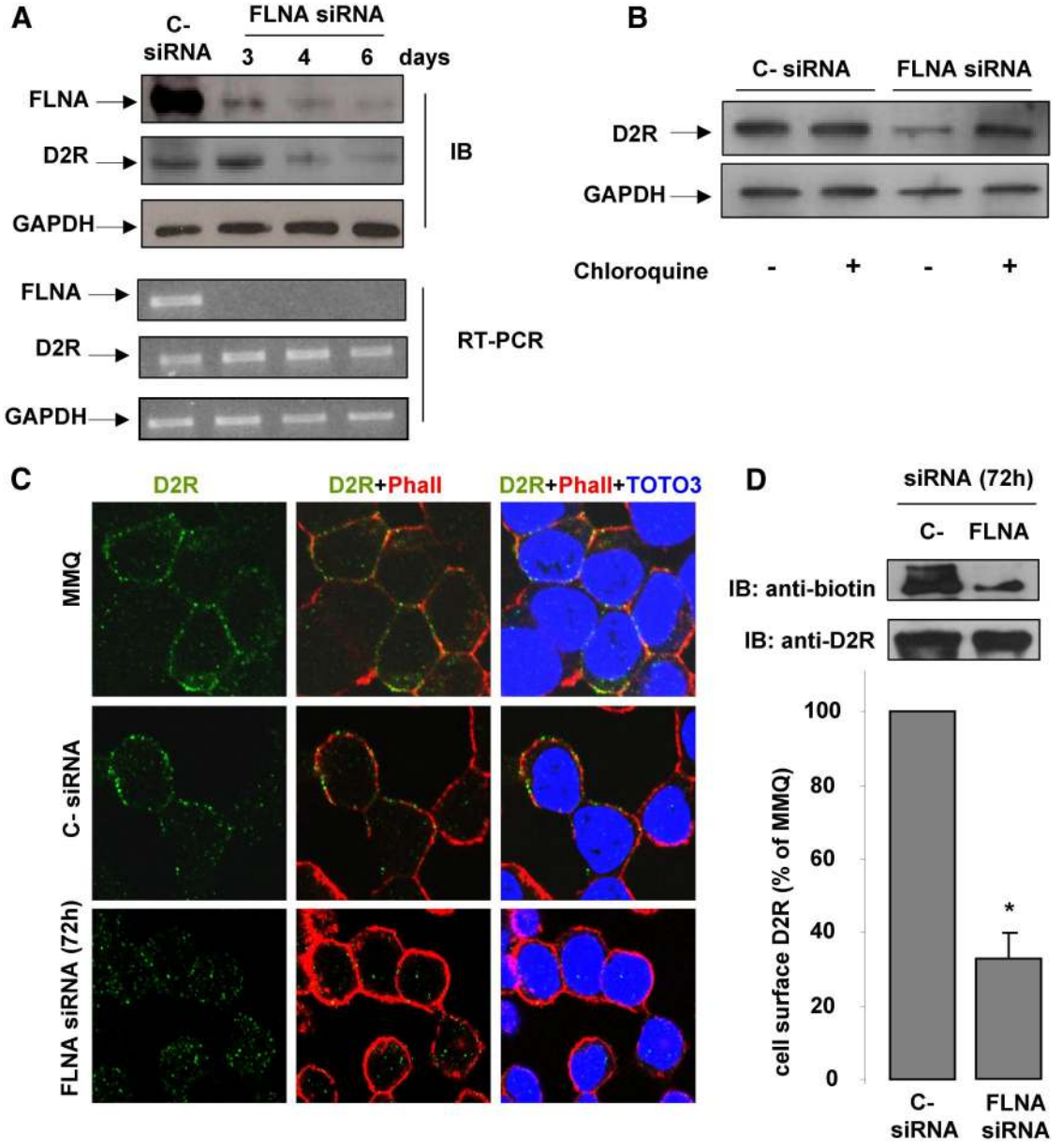

FIG. 3. Effects of FLNA silencing on D2R expression and targeting in MMQ cells. A, Representative immunoblots and RT-PCR analysis of FLNA and D2R in MMQ cells transiently transfected with negative control or FLNA siRNA for 3, 4, and 6 d. FLNA siRNA-treated cells showed a strong decrease in FLNA protein and transcript, which was associated with a reduction of D2R protein expression but not transcript. The equal amount of protein was confirmed by stripping and reprobing with an anti-GAPDH antibody. For RT-PCR, the GAPDH gene was used as internal standard to normalize template concentration. B, Representative immunoblot performed with antibodies raised against D2R protein. The lysosome inhibitor chloroquine (20 h, 50 μM) induced an increase in D2R expression in lysates obtained from cells transfected with FLNA siRNA after 4 d with respect to untransfected cells (MMQ). C, Representative confocal microscopy images of untransfected (MMQ) or transfected with negative control siRNA or FLNA siRNA cells for 72 h stained for D2R (*green*). Fluor 546-Phalloidin (*red*) that stains the actin cytoskeleton was used to facilitate the interpretation of cell structure, and TOTO-3 iodide (*blue*) was used for nuclei staining. In MMQ and negative control cells, D2R was mainly localized at the plasma membrane, with frequent clustering, whereas in FLNA siRNA cells, D2R redistributed to cytoplasmic vesicles. The results shown are representative images of three individual experiments. D, Biochemical analysis of membrane expression of D2R. At 72 h after siRNA transfection, biotinylated cell surface proteins and total cellular proteins were immunoprecipitated by D2R antibody, and cell surface D2R was detected by an antibiotin antibody. Biotinylation assay showed reduced D2R expression at the cell membrane in cells transfected with FLNA siRNA for 72 h. The *graph* shows the quantification of cell surface expression of D2R (mean value $\pm$ SD from three independent experiments). The 72-h FLNA silencing induced a 64 $\pm$ 7% reduction of cell surface D2R compared with control cells. *, $P < 0.05$ *vs.* negative control, *t* test. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; C−, negative control; IB, immunoblot.

detected by an antibiotin antibody. Densitometric analysis revealed that at this time point FLNA silencing induced a 64 $\pm$ 7% reduction of cell surface D2R compared with control cells (Fig. 3D).

Effects of FLNA silencing on D2R signaling in MMQ cells

To confirm the results obtained with prolactinoma cells, D2R-mediated PRL secretion inhibition was tested

in MMQ cells transfected for 72 h with FLNA siRNA or negative control siRNA. As expected, MMQ cells expressing FLNA showed a dose-dependent reduction of PRL release by BIM53097 (32 ± 9% inhibition at 100 nM, Bonferroni post test, $P < 0.01$ *vs.* basal), that was abrogated in FLNA-silenced cells (two-way ANOVA, $P < 0.0001$) (Fig. 4A). No difference in basal PRL release was observed in silenced *vs.* control cells.

To further investigate the role of FLNA in D2R signaling, we evaluated the DA inhibition of forskolin-induced intracellular cAMP accumulation that reflects the reduction in adenylyl cyclase activity in the presence of phosphodiesterase inhibitors. BIM53097 dose-dependently inhibited forskolin-stimulated cAMP accumulation in cells untransfected or transfected with negative control siRNA, with a maximal inhibition of 52 ± 2 and 51 ± 3%, respectively, at 10 nM (Fig. 4B), whereas this effect was significantly reduced in FLNA-silenced cells (two-way ANOVA, $P = 0.0088$) with a maximal inhibition of 28 ± 11% at 10 nM (*t* test, $P < 0.05$ *vs.* negative control).

Finally, we analyzed the effect of D2R activation on cell growth by a proliferation assay based on BrdU incorporation. A 48-h incubation with BIM53097 significantly reduced the proliferation of MMQ cells untransfected or transfected with the negative control siRNA (30 ± 8 and 24 ± 10% inhibition at 100 nM, respectively; *t* test, $P < 0.05$ *vs.* basal). This effect was lost in FLNA-silenced MMQ cells (two-way ANOVA, $P = 0.0026$, siRNA FLNA *vs.* negative control siRNA) (Fig. 4C). Accordingly, we found about 2-fold increase of ERK1/2 phosphorylation after 10-min incubation of control MMQ cells with 100 nM BIM53097, whereas no effect was observed in FLNA-silenced cells (two-way ANOVA, $P < 0.0001$; Fig. 4D).

FLNA expression does not affect D2R expression in GH3 cells

We investigated the expression of FLNA and D2R by Western blot and RT-PCR (Fig. 5) in rat pituitary GH3 cells, a cell line that does not express D2R contrary to MMQ. Our data showed that FLNA was abundantly expressed in this cell line. The absence of relationship between FLNA and D2R expression was further investigated by FLNA overexpression experiments. In fact, in cells transfected with FLNA expression vector, no D2R transcript or protein was detected at 3, 4, or 5 d after transfection. These data suggest that other mechanisms are involved in the lack of D2R expression in this cell line.

FLNA promoter methylation analysis

Searching for possible causes of FLNA-reduced expression in DA-resistant prolactinomas, we investigated the presence of methylation alterations within the FLNA gene promoter regions in prolactinoma samples expressing FLNA (DA sensitive) or not expressing FLNA (DA resistant). Bisulfite genomic sequencing was performed to determine the methylation status of CpG islands localized in the promoter regions of FLNA gene (15). Bisulfite sequencing data did not show methylation in the FLNA promoter, either in FLNA-expressing or in nonexpressing tumor samples, suggesting that the reduced expression of FLNA observed in prolactinomas was not associated with epigenetic silencing.

Discussion

In the present study, we demonstrated that FLNA, a widely expressed cytoskeleton protein found to associate with several G protein-coupled receptors and particularly D2R in melanoma and neuronal cells (8–10), has a crucial role in D2R expression and signaling in lactotrophs. We reported that human prolactinomas, and in particular those removed from patients in whom DA treatment did not achieve PRL normalization and tumor shrinkage, showed reduced FLNA expression together with decreased D2R expression. Based on this novel observation, we investigated the possible causal relationship between these two events. By modulating FLNA expression, we provide evidence that FLNA silencing in cells from DA-sensitive prolactinomas induced a marked reduction of D2R levels, whereas FLNA overexpressing in cells obtained from DA-resistant prolactinomas was associated with enhanced D2R expression level. The modifications of receptor levels resulting from manipulating FLNA expression had a profound impact on D2R signaling because FLNA knockdown prevented the reduction of PRL release induced by D2R activation from DA-sensitive prolactinoma cells, whereas DA-resistant prolactinomas lacking FLNA recovered PRL responsiveness when transfected with FLNA expression vector. Accordingly, intracellular cytostatic signals involved in lactotroph growth arrest, such as DA-induced phosphorylation of ERK1/2 (16, 17), were abrogated in the absence of FLNA expression. Admittedly, FLNA silencing and overexpression experiments were performed in a limited number of tumors because indication to transphenoidal surgery is limited to the small proportion of patients resistant or intolerant to DA (about 5–10% in cabergoline-treated cohorts) (18). However, our data strongly support the view that reduction in D2R expression is the main event involved in DA resistance (3–5) and provide evidence that the loss of FLNA expression may be one of the mechanisms involved in this reduction.

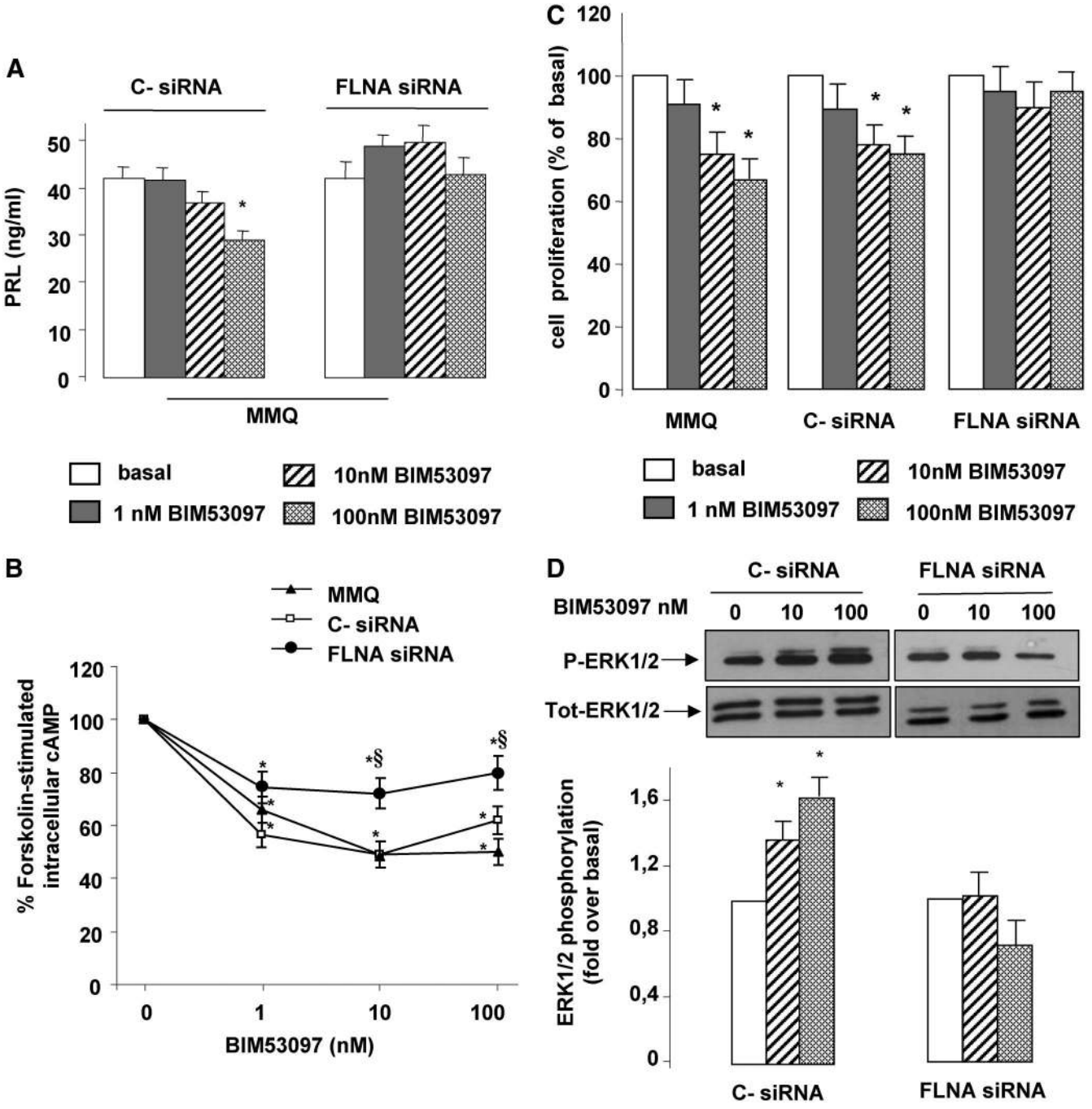

FIG. 4. Effects of FLNA silencing on D2R signaling in MMQ cells. A, FLNA silencing abolished the inhibitory effect of selective D2R agonist BIM53097 on PRL secretion in MMQ cells. Cells were incubated with negative control siRNA or FLNA siRNA for 72 h and treated with increasing concentrations of BIM53097 (1, 10, or 100 nM) for 16 h. PRL was measured in culture medium. Experiments were repeated at least three times, and each determination was done in quintuple. Values represent mean (±SD). Two-way ANOVA, $P < 0.0001$. *, $P < 0.01$ *vs.* corresponding basal, Bonferroni post test. B, Effect of FLNA silencing on D2R-mediated adenylyl cyclase inhibition. In cells untransfected (MMQ) or transfected with negative control siRNA (C− siRNA), the D2R selective agonist BIM53097 dose-dependently inhibited forskolin-stimulated cAMP accumulation. Cells transfected with FLNA siRNA showed a significant reduction of cAMP inhibition with respect to control cells (two-way ANOVA, $P = 0.0088$). Experiments were repeated at least three times, and each determination was done in quintuple. Values represent mean ± SD. *, $P < 0.05$ *vs.* corresponding basal; §, $P < 0.05$ *vs.* C− siRNA, *t* test. C, Effect of BIM53097 treatment (1, 10, or 100 nM) on proliferation of untransfected cells (MMQ), cells transfected for 72 h with negative control siRNA or FLNA siRNA. In MMQ and C− siRNA, BIM53097 caused a dose-dependent inhibition of proliferation, whereas no effect on cell proliferation was mediated by D2R in FLNA siRNA (two-way ANOVA, $P = 0.0026$). Experiments were repeated at least three times, and each determination was done in quintuple. Values represent mean (±SD). *, $P < 0.05$ *vs.* corresponding basal, *t* test. D, Representative immunoblot of ERK1/2 phosphorylation demonstrating that D2R-mediated activation of ERK1/2 by BIM53097 (10 nM, 100 nM for 10 min) was present in C− siRNA and abolished in FLNA siRNA transfected MMQ cells (two-way ANOVA, $P < 0.0001$). The *graph* shows the quantification of phospho-ERK1/2 normalized to total ERK1/2 (mean value ± SD from three independent experiments). In C− siRNA cells, BIM53097 increased ERK1/2 phosphorylation after 10-min incubation, whereas no change was observed in FLNA-silenced MMQ cells. *, $P < 0.05$ *vs.* corresponding basal, *t* test. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; C−, negative control.

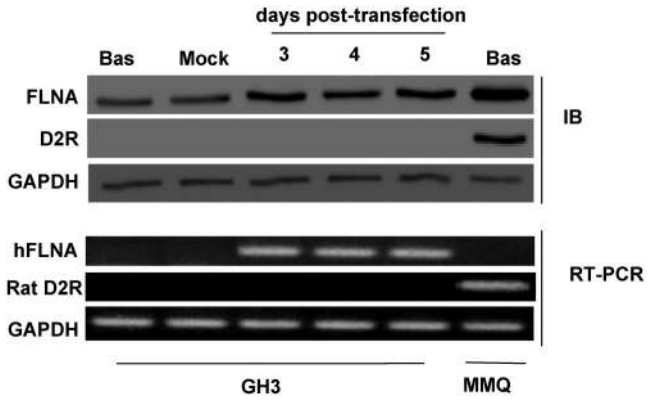

FIG. 5. Effects of FLNA transfection on D2R expression in GH3 cells. Representative immunoblots (*upper panel*) and RT-PCR analysis (*lower panel*) of FLNA and D2R in GH3 cells untransfected (Bas), mock transfected, or transiently transfected with pREP4-FLNA for 3, 4, and 5 d. FLNA, but not D2R, was endogenously expressed in this cell line. RT-PCR analysis was performed with PCR primers that recognize human FLNA to detect the expression of human FLNA transcript in transfected cells. No D2R transcript or protein was detected 3, 4, or 5 d after transfection. The equal amount of protein was confirmed by stripping and reprobing with an anti-GAPDH antibody. For RT-PCR, the GAPDH gene was used as positive control for the template cDNA. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IB, immunoblot; hFLNA, human FLNA.

Because previous studies demonstrated that FLNA has a protective effect from instability of other receptors, such as calcium-sensing receptor and calcitonin receptor, as well as for cystic fibrosis transmembrane conductance regulator and the high-affinity IgG receptor FcγRI (19–22), we evaluated D2R degradation pathways in the MMQ cell line, a cell model of prolactinoma endogenously expressing functional D2R that was shown to express high basal levels of FLNA.

As demonstrated in DA-sensitive prolactinomas, FLNA silencing was associated with reduction of total D2R protein expression. Because no reduction of D2R transcript was observed after FLNA silencing, these data support our hypothesis that interaction with FLNA may stabilize D2R against protein degradation.

Previous works reported that after constitutive and ligand-induced internalization, D2R is recycled back to the plasma membrane (23–25) or trafficked to the lysosomal pathway and degraded (26, 27). Here we demonstrated that D2R undergoes lysosomal degradation in FLNA-silenced cells. These data are consistent with the view that FLNA may control the fate of D2R toward either recycling or lysosomal degradation. In this respect, it is worth noting that FLNA directly interacts with β-arrestins (28) that are involved in D2R trafficking (29), suggesting the formation of a complex receptor-FLNA-arrestin possibly involved in the regulation of D2R stability.

Furthermore, we demonstrated that FLNA was required not only for D2R stability but also for the correct targeting to cell surface. Immunofluorescence analysis carried out on MMQ cells revealed that endogenous D2R was mainly localized to the plasma membrane, with a clustering distribution and to some extent in cytoplasmic vesicles, as previously observed in HEK293 and neuroblastoma cells (25), suggesting that D2R is constitutively internalized to a certain degree. The requirement of FLNA for a correct D2R targeting to the plasma membrane was demonstrated by the redistribution of D2R from cell surface to endocytic vesicles observed in FLNA-silenced cells.

As in prolactinomas, even in MMQ cells the modification of receptor stability and localization resulting from FLNA silencing had a profound impact on D2R signaling. In fact, in the absence of FLNA, the selective D2R agonist was unable to trigger the most relevant biological responses mediated by DA, *i.e.* reduction of PRL release and inhibition of cell proliferation.

These findings deserve some considerations. The loss of D2R effects on PRL release and cell proliferation in prolactinomas and MMQ cells occurred in the presence of a significant reduction (about 60%) of D2R expression as a consequence of FLNA knockdown. However, it is worth noting that the pituitary has a substantial D2R reserve for PRL inhibition, as indicated by previous studies showing that PRL response reaches the plateau at about 40% receptor occupancy in rat pituitary cells (30). Therefore, it is conceivable that the reduction in D2R expression might not entirely account for the loss of D2R signaling. Indeed, in addition to the structural role of FLNA in anchoring D2R to actin cytoskeleton and regulating receptor localization and stability, evidence obtained in other cell systems (8) indicates that FLNA connects signaling molecules in a scaffold complex to enhance the efficiency of signal transduction. Therefore, it is tempting to speculate that FLNA silencing might abrogate the biological responses to DA also by preventing efficient coupling with G proteins and second messengers, which are not randomly distributed in the plasma membrane.

Although we demonstrated the functional relevance of the positive correlation between FLNA and D2R expression in prolactinomas and MMQ cells, our study showed that other events may be involved in the loss of D2R expression in other cell models, such as GH3 cells. In fact, contrary to what was observed in DA-resistant prolactinomas, D2R was not expressed in GH3 cells, although the cells endogenously expressed FLNA. Moreover, D2R was not induced by FLNA overexpression. These data are consistent with the recent observation that epigenetic aberrations, *i.e.* hypermethylation of the D2R CpG island and histone modifications, are responsible for D2R silencing in GH3 cells (31).

As far as the low FLNA levels in DA-resistant prolactinomas are concerned, our study did not reveal the molec-

ular determinants involved in this phenomenon. However, the absence of alterations in the CpG island with the highest probability to have regulatory functions, according to *in silico* analysis and the UCSC database, suggests that the reduced expression of FLNA observed in prolactinomas is not associated with epigenetic silencing.

Considering that low levels of FLNA have been associated with cancer progression, cancer cell migration, and cell invasion in other cell models, it is possible to hypothesize that the shift to invasive or even malignant transformation of human prolactinomas that is frequently associated with resistance to DA drugs might also be related to the reduction in FLNA expression (2, 32–34). Finally, it is at present unknown whether a reduced FLNA expression may also occur in other human tumors characterized by resistance to drugs interacting with other G protein-coupled receptors, such as neuroendocrine tumors resistant to somatostatinergic agents.

In conclusion, our study indicates that FLNA is required to maintain D2R expression and targeting to the plasma membrane as well as D2R-mediated inhibition of PRL release and lactotroph proliferation and provides new evidence for a role of cytoskeleton in cell growth control.

Acknowledgments

We thank Dr. G. Frank, Dr. M. Scanarini, Dr. G. Lasio, and Dr. M. Locatelli for providing us with fresh samples of prolactinomas.

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This work was supported by an Associazione Italiana Ricerca Cancro-Milan grant (MFAG-8972, to G.M.), by a Ministry of Education, University and Research grant (PRIN 2009YJTBAZ_004 to A.G.L.), and by Ricerca Corrente Funds of Fondazione Instituto di Ricovero e Cura a Carattere Scientifico Ca' Granda-Milan.

Disclosure Summary: The authors have nothing to disclose.

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