CDCA7 finely tunes cytoskeleton dynamics to promote lymphoma migration and invasion

Carla Martín-Cortázar,1 Yuri Chiodo,1 Raul Jiménez-P.,1 Manuel Bernabé,2 María Luisa Cayuela,2 Teresa Iglesias3,4 and Miguel R. Campanero1,5

1Department of Cancer Biology, Instituto de Investigaciones Biomédicas Alberto Sols, Madrid; 2Telemerase, Aging and Cancer Group, Research Unit, Department of Surgery, CIBERehd, Instituto Murciano de Investigación Biosanitaria (IMIB), Murcia; 3Department of Endocrine and Nervous Systems Pathophysiology, Instituto de Investigaciones Biomédicas Alberto Sols, Madrid; 4Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid and 5Centro de Investigaciones Biomédicas en Red en Enfermedades Cardiovasculares (CIBERCV), Madrid, Spain

ABSTRACT

Metastases, the major cause of death from cancer, require cells’ acquisition of the ability to migrate and involve multiple steps, including local tumor cell invasion and basement membrane penetration. Certain lymphoid tumors are highly metastatic, but the mechanisms of invasion by lymphoma cells are poorly understood. We recently showed that CDCA7, a protein induced by MYC, is overexpressed in lymphoid tumors and that its knockdown decreases lymphoid tumor growth without inhibiting the proliferation of normal cells. Here we show that CDCA7 is critical for invasion and migration of lymphoma cells. Indeed, CDCA7 knockdown in lymphoma cells limited tumor cell invasion in matrigel-coated transwell plates and tumor invasion of neighboring tissues in a mouse xenograft model and in a zebrafish model of cell invasion. CDCA7 silencing markedly inhibited lymphoma cell migration on fibronectin without modifying cell adhesion to this protein. Instead, CDCA7 knockdown markedly disrupted the precise dynamic reorganization of actomyosin and tubulin cytoskeletons required for efficient migration. In particular, CDCA7 silencing impaired tubulin and actomyosin cytoskeleton polarization, increased filamentous actin formation, and induced myosin activation. Of note, inhibitors of actin polymerization, myosin II, or ROCK reestablished the migration capacity of CDCA7-silenced lymphoma cells. Given the critical role of CDCA7 in lymphoma genesis and invasion, therapies aimed at inhibiting its expression or activity might provide significant control of lymphoma growth, invasion, and metastatic dissemination.

Introduction

Cancer cells acquire molecular alterations relative to their normal counterparts which confer them endless proliferative activity, resistance to death, and the capacity to metastasize, among other traits. Metastases are the major cause of death from cancer and their biological heterogeneity creates a critical obstacle to treatment.1 Certain lymphoid tumors are highly metastatic, invading the spleen, lymph nodes and central nervous system. Indeed, direct invasion of the central nervous system occurs in 5% of all patients with non-Hodgkin lymphoma.2 The incidence varies with clinical aggressiveness and can be as high as 27% for very aggressive lymphomas3 and as high as 70% in the case of acute lymphoblastic leukemia in the absence of central nervous system-directed prophylactic treatment.3

Metastases of epithelial cancers involve local tumor cell invasion, basement membrane penetration, invasation into blood or lymphatic vessels followed by exit from the circulation, and colonization of distant tissues. Most carcinoma cells produce matrix-degrading enzymes to clear a path for tissue invasion. The matrix metalloproteinase (MMP) family, a diverse group of calcium-dependent zinc-con-
CDCA7 promotes lymphoma invasion

Results

CDCA7 silencing inhibits lymphoma invasion of adjacent tissues

Subcutaneous inoculation of lymphoma cells in immunodeficient mice gives rise to the formation of solid tumors, whose growth is impaired upon CDCA7 knockdown. To further investigate the role of CDCA7 in lymphomagenesis, we screened for histological differences between control- and CDCA7-silenced tumors. We therefore transduced DG-75 Burkitt lymphoma cells with lentivirus encoding CDCA7-specific shRNA (sh-25 and sh-83) to knock down CDCA7 expression. Immunoblot analysis showed that both shRNA markedly decreased CDCA7 levels in these cells relative to the levels in control cells transduced with a non-targeting shRNA (sh-Ctrl) (Figure 1A). As previously reported, CDCA7 silencing decreased tumor growth (Online Supplementary Table S1). Hematoxylin-eosin staining of tumor sections revealed that 100% of tumors formed by control-transduced cells contained muscle or adipose tissue (Figure 1B, C and D). In contrast, 100% of tumors formed by CDCA7-transduced cells did not contain any muscle or adipose tissue (Figure 1B, C). Histological analysis of tumor sections revealed that CDCA7 silencing inhibited the invasion and migration of lymphoma cells towards adjacent tissues (Figure 1D). To further investigate the role of CDCA7 in lymphomagenesis, we screened for histological differences between control- and CDCA7-silenced tumors. We therefore transduced DG-75 Burkitt lymphoma cells with lentivirus encoding CDCA7-specific shRNA (sh-25 and sh-83) to knock down CDCA7 expression. Immunoblot analysis showed that both shRNA markedly decreased CDCA7 levels in these cells relative to the levels in control cells transduced with a non-targeting shRNA (sh-Ctrl) (Figure 1A). As previously reported, CDCA7 silencing decreased tumor growth (Online Supplementary Table S1). Hematoxylin-eosin staining of tumor sections revealed that 100% of tumors formed by control-transduced cells contained muscle or adipose tissue (Figure 1B, C and D). In contrast, 100% of tumors formed by CDCA7-transduced cells did not contain any muscle or adipose tissue (Figure 1B, C). Histological analysis of tumor sections revealed that CDCA7 silencing inhibited the invasion and migration of lymphoma cells towards adjacent tissues (Figure 1D).

Methods

Details of the Methods can be found in the Online Supplementary Appendix.

Lentivirus production, cell transduction, and immunoblotting

Lentiviral particles were produced and cells were transduced, as described elsewhere, employing vectors encoding either a non-targeting short hairpin (sh)RNA or CDCA7-targeting shRNA, sh-25 and sh-83. Cell lysates were prepared, resolved in sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed as described previously.

In vitro and in vivo migration and invasion assays

In vitro transwell migration and invasion assays were carried out in Boyden chambers using filters (3-μm pore size) coated with fibronectin or a matrigel solution. In vivo invasion assays were performed using zebrafish embryos and subcutaneous xenografts in mice. All animal procedures were approved by the CSIC Ethics Committee (ref. PROEX 51/14 and 215/17).

Results

CDCA7 silencing inhibits lymphoma invasion of adjacent tissues

Subcutaneous inoculation of lymphoma cells in immunodeficient mice gives rise to the formation of solid tumors, whose growth is impaired upon CDCA7 knockdown. To further investigate the role of CDCA7 in lymphomagenesis, we screened for histological differences between control- and CDCA7-silenced tumors. We therefore transduced DG-75 Burkitt lymphoma cells with lentivirus encoding CDCA7-specific shRNA (sh-25 and sh-83) to knock down CDCA7 expression. Immunoblot analysis showed that both shRNA markedly decreased CDCA7 levels in these cells relative to the levels in control cells transduced with a non-targeting shRNA (sh-Ctrl) (Figure 1A). As previously reported, CDCA7 silencing decreased tumor growth (Online Supplementary Table S1). Hematoxylin-eosin staining of tumor sections revealed that 100% of tumors formed by control-transduced cells contained muscle or adipose tissue (Figure 1B, C and D). In contrast, 100% of tumors formed by CDCA7-transduced cells did not contain any muscle or adipose tissue (Figure 1B, C). Histological analysis of tumor sections revealed that CDCA7 silencing inhibited the invasion and migration of lymphoma cells towards adjacent tissues (Figure 1D).

Methods

Details of the Methods can be found in the Online Supplementary Appendix.

Lentivirus production, cell transduction, and immunoblotting

Lentiviral particles were produced and cells were transduced, as described elsewhere, employing vectors encoding either a non-targeting short hairpin (sh)RNA or CDCA7-targeting shRNA, sh-25 and sh-83. Cell lysates were prepared, resolved in sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed as described previously.

In vitro and in vivo migration and invasion assays

In vitro transwell migration and invasion assays were carried out in Boyden chambers using filters (3-μm pore size) coated with fibronectin or a matrigel solution. In vivo invasion assays were performed using zebrafish embryos and subcutaneous xenografts in mice. All animal procedures were approved by the CSIC Ethics Committee (ref. PROEX 51/14 and 215/17).

Results

CDCA7 silencing inhibits lymphoma invasion of adjacent tissues

Subcutaneous inoculation of lymphoma cells in immunodeficient mice gives rise to the formation of solid tumors, whose growth is impaired upon CDCA7 knockdown. To further investigate the role of CDCA7 in lymphomagenesis, we screened for histological differences between control- and CDCA7-silenced tumors. We therefore transduced DG-75 Burkitt lymphoma cells with lentivirus encoding CDCA7-specific shRNA (sh-25 and sh-83) to knock down CDCA7 expression. Immunoblot analysis showed that both shRNA markedly decreased CDCA7 levels in these cells relative to the levels in control cells transduced with a non-targeting shRNA (sh-Ctrl) (Figure 1A). As previously reported, CDCA7 silencing decreased tumor growth (Online Supplementary Table S1). Hematoxylin-eosin staining of tumor sections revealed that 100% of tumors formed by control-transduced cells contained muscle or adipose tissue (Figure 1B, C and D). In contrast, 100% of tumors formed by CDCA7-transduced cells did not contain any muscle or adipose tissue (Figure 1B, C). Histological analysis of tumor sections revealed that CDCA7 silencing inhibited the invasion and migration of lymphoma cells towards adjacent tissues (Figure 1D).
These non-lymphoid tissues were extremely disorganized and embedded within the tumor (Figure 1B, C and Online Supplementary Table S1), suggesting that lymphoma cells invaded the neighboring fat or muscle. In contrast, only 40% of tumors formed by CDCA7-silenced cells contained non-tumoral tissues and, when present, these tissues showed a rather well-preserved organization (Figure 1B, C and Online Supplementary Table S1). These results therefore suggest that while control lymphoma cells readily invade and disorganize adjacent tissues, CDCA7-silenced lymphoma cells hardly invade them. We looked for gene expression profiles of metastatic lymphomas using Genevestigator. While we found gene expression profiling data of more than 1,600 cases of lymphoid tumors, we only found data on four metastatic cases (Online Supplementary Figure S1). Of note, CDCA7 levels were high in these cases and in numerous non-metastatic lymphoma/leukemia samples (Online Supplementary Table S1), suggesting that CDCA7 might be clinically relevant.

**CDCA7 silencing restrains lymphoma invasion in vitro and in vivo**

To confirm the contribution of CDCA7 to lymphoma cell invasion, we determined the capacity of CDCA7-silenced cells to invade matrigel-coated transwell plates. CDCA7 knockdown in DG-75 cells transduced with lentivirus encoding sh-hCtI, sh-25 or sh-83 was confirmed by immunoblotting (Figure 2A, left panel). Transduced cells were suspended in serum-free medium and seeded in the top chamber of matrigel-coated transwell plates. We used fetal bovine serum (FBS) as a chemoattractant in the lower chamber of these plates. Quantification of the number of cells capable of crossing the matrigel barrier and reaching the lower chamber showed that the invasive capacity of CDCA7-silenced cells was markedly lower.
than that of control-transduced cells (Figure 2B). To determine whether CDCA7 mediates invasion of other lymphoma cells, we transduced BL-2 (Burkitt lymphoma) and Toledo (diffuse large B-cell lymphoma) cells with sh-25 or sh-83 lentivirus. These shRNA readily silenced CDCA7 expression in these cells (Figure 2A, middle and right panels) and sharply decreased their invasive capacity relative to that of control cells (Figure 2B).

As the zebrafish is a robust model for studying the invasive behavior of human tumor cells,29 we used it to evaluate the contribution of CDCA7 to lymphoma invasion in vivo. Transduced DG-75 cells were stained with live dye DiI and microinjected into the yolk sac of zebrafish embryos. The capacity of these cells to escape the yolk sac and migrate to the embryo tail was quantified as the percent of embryos with >5 labeled lymphoma cells in the tail. While control cells were found in the tail of nearly 60% of the embryos, less than 40% of the embryos inoculated with CDCA7-silenced cells showed lymphoma cells in the tail (Figure 3A, B). To assess whether these results could be extended to other lymphomas, we determined the capacity of control and CDCA7-silenced Toledo cells to migrate from the yolk sac to the embryo tail. We found that CDCA7 knockdown markedly decreased the presence of Toledo cells in the tail (Figure 3C, D). Together, our results strongly suggest that CDCA7 is a key mediator of lymphoma invasion.

**CDCA7 silencing hinders lymphoma migration**

To investigate the mechanisms underlying CDCA7 regulation of cell invasion, we analyzed the expression of MMP2 and MMP9, the major metalloproteinases involved in basement membrane and stromal ECM degradation during invasion.5 The expression of these metalloproteinases was not detected in DG-75, BL-2, and Toledo cells (Online Supplementary Figure S2A), but it was readily detected in breast cancer MCF-7 or colon carcinoma SW480 cells (Online Supplementary Figure S2B). Since these results suggest that ECM degradation is not required for lymphoma invasion, we hypothesized that the migratory capacity of lymphoma cells might be critical for invasion. We therefore assessed the contribution of CDCA7 to lymphoma cell migration using fibronectin-coated transwell plates and FBS as a chemoattractant stimulus. BL-2 and Toledo cells attached poorly to fibronectin, but their binding was stimulated in the presence of the TS2/16 monoclonal antibody (Online Supplementary Figure S3A), an anti-integrin β1 monoclonal antibody that increases the avidity and affinity of β1 integrins for their ligands.30 Of note, we could not detect adhesion of DG-75 cells to fibronectin even in the presence of this antibody (not shown). As lymphoma cells bind poorly to fibronectin, they reach the lower transwell chamber instead of remaining attached to the fibronectin-coated filter. Quantification of the number of cells in the lower chamber showed that CDCA7 silencing markedly decreased the migratory capacity of DG-75, BL2, and Toledo cells (Figure 4).

Although lymphoma cells bind poorly to fibronectin, the ablation of this binding could formally account for the inhibition of cell migration upon CDCA7 silencing. Alternatively, a sharp increase in binding could also slow down migration. However, we found that CDCA7 knockdown did not substantially affect the binding of lymphoma cells to fibronectin (Online Supplementary Figure S3A). Moreover, CDCA7 silencing did not affect the expression of integrins α4 and β1 (Online Supplementary Figure S3B, C), the subunits of the major fibronectin receptor of these cells. Activation of β1 integrin binding activity induces a conformational modification of the β1 subunit that is recognized by the HUTS-21 monoclonal antibody.31 Staining of lymphoma cells with HUTS-21 showed that CDCA7 silencing did not affect the activity of this fibronectin receptor (Online Supplementary Figure S3B, C).

![Figure 2. CDCA7 knockdown inhibits lymphoma invasion in vitro.](image-url)
FBS contains numerous potentially chemoattractant stimuli for lymphoma cells whose receptors could be downregulated upon CDCA7 silencing, thereby accounting for the inhibition of cell migration towards FBS. To challenge this hypothesis, we used the stomal cell-derived factor 1 (SDF1) chemokine as chemoattractant for BL-2 and Toledo cell instead of FBS. SDF1 activated BL-2 and Toledo cells migration in fibronectin-coated transwell plates (Online Supplementary Figure S4A) and this migration was markedly inhibited by CDCA7 silencing (Online Supplementary Figure S4B). However, the expression of CXCR4, the SDF1 receptor, was not modified in these cells upon CDCA7 silencing, as determined by flow cytometry analysis (Online Supplementary Figure S5). CDCA7 knockdown could potentially inhibit migration towards SDF1 by modulating the binding of lymphoma cells to fibronectin. However, SDF1 did not regulate this interaction and CDCA7 silencing also failed to modulate the binding of SDF1-treated lymphoma cells to fibronectin (Online Supplementary Figure S6).

Disruption of the tubulin and actomyosin cytoskeletons by CDCA7 silencing

Since cytoskeleton reorganization is critical for cell migration, we next investigated the role of CDCA7 in the reorganization of the microtubule and actomyosin cytoskeletons in lymphoma cells. Confocal microscopic imaging of lymphoma cells stained with fluorescein-labeled phalloidin showed a polarized distribution of filamentous actin (F-actin) in >40% control-transduced Toledo and BL-2 lymphoma cells (Figure 5A-D). CDCA7 silencing reordered F-actin around the cells, decreasing the percentage of cells with polarized distribution of F-actin (Figure 5A-D). In addition, CDCA7 knockdown markedly increased F-actin levels (Figure 5A, B, E). Staining of the microtubule cytoskeleton revealed its marked polarization in control-transduced lymphoma cells and that CDCA7 knockdown elicited its redistribution around the cells (Figure 5A-D). Moreover, while actin and microtubule cytoskeletons were located in opposite ends of most control-transduced cells, their distribution overlapped in CDCA7-silenced cells (Figure 5A, B, F). Of note, we could not assess the polarization of tubulin and actin cytoskeletons in DG-75 cells because these cells did not attach to the fibronectin-coated coverglasses used for these studies.

The actin-binding protein α-actinin is an important organizer of the actomyosin cytoskeleton.32 Four α-actinin isoforms have been identified (ACTN1-ACTN4), but non-muscle cells express only ACTN1 and ACTN4.32 Staining of lymphoma cells with a monoclonal antibody specific
for ACTN1 barely detected its expression in control-transduced cells (Figure 6A-D). However, ACTN1 was readily detected in CDCA7-knockdown cells (Figure 6A-D). In contrast, a polyclonal antibody that reacts with isoforms 1, 2, and 4 showed a similar staining intensity in control and silenced cells (Figure 6A-D). These results suggested that CDCA7 might inhibit ACTN1 expression. However, immunoblot analysis of these cells with the monoclonal antibody revealed similar ACTN1 levels in control and silenced cells (Figure 6E). Together, these data suggest that CDCA7 silencing unmasks the epitope recognized by the ACTN1-specific monoclonal antibody.

Staining of α-actinin with the polyclonal antibody showed a dotted pattern in control-transduced BL-2 and Toledo cells and, contrary to the actin and tubulin cytoskeletons, its distribution was not substantially affected by CDCA7 silencing (Online Supplementary Figure S7A, B). As one of the roles of α-actinin is to act as a link between integrins and the actin cytoskeleton, we investigated the distribution of active β1 integrins in lymphoma cells. Similar to α-actinin, active β1 integrin staining showed a dotted pattern in control- and CDCA7-knockdown BL-2 and Toledo cells (Online Supplementary Figure S7A, B). The presence of numerous white dots in merged images (Online Supplementary Figure S7A, B) strongly suggested that α-actinin and active β1 integrins do indeed colocalize in these cells. Determination of Pearson and Mander coefficients supported this hypothesis (Online Supplementary Figure S7C, D).

The actomyosin cytoskeleton is constituted by F-actin in association with numerous proteins, including myosins and tropomyosins (TPM). To investigate whether CDCA7 also regulates the cellular distribution of these proteins, we used fluorescence microscopy analysis. We found that TPM3 showed a polarized distribution in nearly 60% of control-transduced BL-2 and Toledo lymphoma cells, which was markedly decreased upon CDCA7 knockdown (Figure 7A-D).

As phosphorylation of the myosin regulatory light chain (MLC) on Ser19 is a marker of NM-II activation, we investigated the distribution of active myosin in lymphoma cells by immunofluorescence using an antibody that specifically recognizes MLC phosphorylated on that residue (pMLC-S19). Similar to TPM3 and F-actin, pMLC-S19 was located in one pole of nearly 40% of control-transduced lymphoma cells and its polarized distribution markedly decreased upon CDCA7 silencing (Figure 7A-D). Of note, this redistribution was accompanied by a substantial increase of pMLC-S19 levels in silenced cells (Figure 7A, B, E). MLC phosphorylation can be induced by RhoA kinase (ROCK). To determine whether ROCK-mediated MLC activation contributed to the inhibition of cell migration imposed by CDCA7 knockdown, we treated control and lymphoma cells with the ROCK inhibitor fasudil. We found that fasudil inhibited MLC phosphorylation (Online Supplementary Figure S8) and neutralized the inhibition of cell migration in CDCA7-silenced lymphoma cells (Figure 8). Similarly, the NM-II inhibitor blebbistatin restored the migration competency of CDCA7-silenced cells (Figure 8), suggesting that ROCK-mediated NM-II activation hindered cell migration upon CDCA7 knockdown. Given the increase of F-actin in CDCA7-silenced cells, we also investigated the contribution of actin polymerization to the inhibition of lymphoma cell migration. We found that treatment of these cells with the actin polymerization inhibitor cytochalasin D overcame the migratory restraint imposed by CDCA7 silencing (Figure 8).

Discussion

While the processes and mechanisms involved in carcinoma invasion and the formation of metastases have been extensively characterized, little is known about the molecular mechanisms involved in lymphoma cell invasion. Here we show that CDCA7 is a critical mediator of lymphoma cell invasion in vivo and in vitro and that CDCA7 knockdown greatly impairs lymphoma migration, through the regulation of tubulin and actomyosin cytoskeleton dynamics.

Metastases involve breaching of numerous histological barriers to move to distant sites. In the case of epithelial cancers, this process involves not only cell motility but also the proteolytic degradation of ECM molecular components. Among hundreds of protease genes, the MMP family has been implicated in carcinoma tumor invasion and metastasis formation. Indeed, MMP are over-expressed in lymphoma cell invasion and migration.
pressed in multiple tumors\(^3\) and their overexpression is critical for carcinoma invasion and metastasis formation.\(^5\) MMP2 and MMP9, in particular, degrade type IV collagen, a major component of the basement membrane, and thus facilitate tumor invasion.\(^7\) MMP9 is also required for intravasation, extravasation, and local migration of tumor cells.\(^5\)

Infiltrating non-tumoral lymphocytes often express elevated MMP levels.\(^3\) In fact, the capacity of these cells to penetrate through basement membrane equivalents in vitro is facilitated by active MMP2 and MMP9.\(^3\) In addition, MMP9 was found on the surface of B-cell chronic lymphocytic leukemia cells, where it is a critical regulator of cell migration.\(^7\) MMP2 or MMP9 is also found in some lymphoma cell lines.\(^3\) Nonetheless, none of the lymphoma cells used in our study expresses these MMP, indicating that CDCA7 does not regulate the capacity for invasion of these cells through these proteins. We cannot rule out however that CDCA7 may potentially promote lymphoma invasion through paracrine stimulation of MMP2/9 production by neighboring stromal cells or through the regulation of other MMP. Alternatively,
CDCA7 may regulate the expression or the activity of other enzymes involved in basement membrane degradation, such as heparanases and sulfatases.41,42

The histological barriers confronting metastasizing cells vary in ECM composition, organization, and biophysical characteristics. Cells might therefore use different means to negotiate these diverse physical barriers. While, as mentioned above, normal lymphocytes are facilitated in their crossing of basement membrane by MMP,38 their migration within three-dimensional collagen matrices was insensitive to a protease inhibitor cocktail targeting MMP, serine proteases, cysteine proteases, and cathepsins.12 In contrast, the invasive behavior of epithelial cancer cells was impaired by pharmacological inhibition of proteases.13 These results suggest that instead of clearing a path for tissue invasion, normal lymphocytes use protease-independent mechanisms to slither through interstices in the stromal ECM. Similarly, ECM degradation is not required for lymphoma cell migration.14

The protease-independent fashion of negotiating physical barriers involves the coordinated adoption of an ameboid type of migration and the use of actomyosin-based mechanical forces to physically displace matrix fibrils.6 Similar to the mesenchymal type of movement adopted by epithelial cells, ameboid migration requires dynamic assembly/disassembly of the actomyosin network.13 However, while mesenchymal migration relies strongly on coordinated cell adhesion to the ECM in the leading

---

**Figure 6.** Increased α-actinin staining in CDCA7-silenced lymphoma cells. BL-2 and Toledo cells were transduced with the indicated short hairpin (sh) RNA, seeded on coverslips coated with 2 μg fibronectin, and stimulated with 10 ng/mL stromal cell-derived factor 1 for 15 min. Representative confocal microscopy images (1 section) of (A) Toledo and (B) BL-2 transduced cells stained with an anti-α-actinin monoclonal antibody (mAb) and DAPI or an anti-α-actinin polyclonal antibody (Poly) and DAPI. Quantification of relative α-actinin staining with the monoclonal and the polyclonal antibodies in (C) Toledo and (D) BL-2 cells transduced as indicated. ns, non-significant; ****P<0.0001 (one-way analysis of variance with the Bonferroni post-test). (E) Representative CDCA7 and α-actinin (probed with the mAb) immunoblot analysis of cell lysates from BL-2 and Toledo cells transduced with the indicated shRNA. Bar, 10 μm.
edge and its detachment at the opposite end of the cell, amoeboid movement is driven by short-lived and relatively weak interactions with the ECM. In amoeboid migration, movement is generated by cortical filamentous actin in the cell front in the absence of focal contacts and stress fibers.

Given that the lymphoma cells used in our study do not express MMP2 and MMP9 and bind fibronectin very weakly, we propose that these cells use an amoeboid type of invasion. In line with a minor role for cell adhesion in the movement of these cells, the inhibition of cell migration and invasion upon CDCA7 silencing was not paralleled by a substantial modification of their binding to fibronectin. Accordingly, the expression and activity of VLA-4, the major fibronectin receptor of these cells, was not affected by CDCA7 knockdown.

Figure 7. CDCA7 silencing decreases tropomyosin 3 polarization and promotes myosin light chain phosphorylation. BL-2 and Toledo cells were transduced with the indicated short hairpin (sh) RNA, seeded on coverslips coated with 2 µg fibronectin, and stimulated with 10 ng/mL stromal cell-derived factor 1 for 15 min. Representative confocal microscopy images (1 section) of (A) Toledo and (B) BL-2 transduced cells stained with anti-tropomyosin 3 (TPM3), anti-phospho-myosin light chain (pMLC) and DAPI. Quantification of the percentage of (C) Toledo and (D) BL-2 cells displaying polarized distribution of TPM3 or pMLC. (E) Quantification of relative pMLC fluorescence intensity. Data are presented as the mean ± standard error of mean of three independent experiments. **P<0.01, ***P<0.001, and ****P<0.0001 (one-way analysis of variance with the Bonferroni post-test). Bar, 10 µm.
A recent report described the association of CDCA7 overexpression in the most aggressive breast cancer subtype with metastatic relapse and that CDCA7 mediates breast cancer migration through transcriptional upregulation of the EZH2 epigenetic modifier. However, CDCA7 knockdown did not substantially modify EZH2 mRNA levels in lymphoma cells (Online Supplementary Figure S9), indicating that CDCA7 regulates migration and invasion through distinct mechanisms in breast cancer and lymphoma.

During cell migration, tubulin and actomyosin cytoskeletons are located in opposite ends of the cells, and cell migration involves their constant and coordinated remodeling.\(^{11,16}\) We propose that CDCA7 is required for the dynamic remodeling of both cytoskeletons and that its absence (as in knockdown cells) elicits depolarization and stabilizes cortical actin filaments, thus preventing the high dynamism of tubulin and actomyosin cytoskeletons required for cell migration. Supporting this hypothesis, we have shown that tubulin and F-actin are grouped in opposite poles of most control lymphoma cells and that both redistribute around the cell in CDCA7-silenced lymphoma cells. Similarly, the polarized distribution of TPM3 and p-MLC observed in most control cells is lost upon CDCA7 knockdown.

In contrast, the dotted distribution of \(\alpha\)-actinin was not lost in CDCA7-silenced cells. It should be noted that besides binding to actin filaments, \(\alpha\)-actinin associates with a number of signaling molecules, ion channels, transcription factors, and transmembrane receptors, including integrins.\(^{44}\) It therefore seems likely that \(\alpha\)-actinin dissociates from F-actin in CDCA7-silenced cells, remaining associated with integrins or other transmembrane receptors. Indeed, our data support the notion that \(\alpha\)-actinin is associated with active integrins in both control and CDCA7-knockdown cells. Among the four \(\alpha\)-actinin isoforms identified, non-muscular cells only express ACTN1 and ACTN4.\(^{46}\) The staining of lymphoma cells with a monoclonal antibody specific to ACTN1 was markedly increased in CDCA7-silenced cells relative to control cells, raising the possibility that CDCA7 knockdown upregulated ACTN1 expression. However, the staining of these cells with a polyclonal antibody common to ACTN1 and ACTN4 showed no substantial differences between control and silenced cells, and ACTN1 immunoblot analysis revealed similar protein levels in both cell populations. Together these results support the notion that instead of regulating ACTN1 levels, CDCA7 regulates, by unknown mechanisms, ACTN1 conformation or its association with other proteins, thus increasing the exposure of the epitope recognized by the monoclonal antibody. We propose a model whereby CDCA7 is required for the dynamic association/dissociation of integrin-bound \(\alpha\)-actinin to/from F-actin. The association of \(\alpha\)-actinin to the actomyosin cytoskeleton would mask the epitope recognized by the monoclonal antibody. The absence of CDCA7 would hamper the association of integrin-bound \(\alpha\)-actinin to this cytoskeleton, exposing the epitope and, more importantly, altering the cytoskeleton dynamics required for efficient migration. Forced CDCA7 downregulation would also hinder migration through the stabilization of F-actin and myosin activation. Of note, both processes can be activated by ROCK\(^{33}\) and we show herein that ROCK inhibition re-established the migratory capacity of silenced cells. These results suggest that CDCA7 silencing might induce ROCK activation in lymphoma cells.

We have shown that CDCA7 is critically involved in the anchorage-independent growth of lymphoid tumors and in lymphomagenesis.\(^{11}\) While CDCA7 is also expressed in normal diploid fibroblasts, its silencing in these cells did not inhibit their anchorage-dependent proliferation.\(^{11}\) Hence, given the essential role of CDCA7 in lymphoma progression and invasion, treatments that inhibit its expression or its activity represent an attractive strategy for controlling lymphoma growth, invasion, and metastatic dissemination.

**Acknowledgments**

The authors thank D. Trono for plasmids; the Spanish Ministerio de Economía y Competitividad for grants SAF2017-88881-R and SAF2017-88885-R (MINECO/AEI/FEDER, UE) to MRC and TIV, respectively; the Comunidad de Madrid for controlling lymphoma growth, invasion, and metastatic dissemination. The cost of this publication was paid in part with FEDER funds.
References

1. Fidler J, Kripke ML. The challenge of targeting metastasis. Cancer Metastasis Rev. 2015;34(4):653-641.
2. Colucci N, Glantz M, Recht L. Prevention and treatment of central nervous system involvement by non-Hodgkin's lymphoma: a review of the literature. Semin Neurol. 2004;24(4):395-404.
3. Kufe DW, Holland JF, Frei E, American Cancer Society. Cancer Medicine. 5th ed. Hamilton, Ont.; Lewiston, NY: BC Decker, 2003.
4. Bonnans C, Chou J, Webz. Remodeling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014;15(12):786-801.
5. Mittal R, Patel AF, Debs LH, et al. Intricate functions of matrix metalloproteases in physiological and pathological conditions. J Cell Physiol. 2016;231(12):2599-2621.
6. Sabeh F, Shimizu-Hirota R, Weiss SJ. Protease-dependent versus -independent cancer cell invasion programs: three-dimensional amoeboid movement revisited. J Cell Biol. 2009;185(1):11-19.
7. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer. 2005;3(5):362-374.
8. Ridley AJ. Life at the leading edge. Cell. 2003;112(3):451-463.
9. Vicente-Manzanares M, Webb DJ, Horwitz AR. Cell migration at a glance. J Cell Sci. 2012;125(15):3623-3635.
10. Parsons JT, Horwitz AR, Schwartz MA. Cell polarization in migrating cells. Cell. 2005;121(3):451-463.
11. Vicente-Manzanares M, Ma X, Adelstein RS, Horwitz AR. Non-muscle myosin II takes centre stage in cell adhesion and migration. Nat Rev Mol Cell Biol. 2009;10(11):778-790.
12. Wolf K, Muller R, Borgmann S, Brocker EB, Friedl P. Amoeboid shape change and contact guidance. T lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. Blood. 2005;106(2):5262-5269.
13. Wolf K, Mazo I, Leung H, et al. Compensatory mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolytic components in the migration of T lymphocytes. J Cell Biol. 2003;160(2):267-277.
14. Michalis UR. Mechanisms of endothelial cell migration. Cell Mol Life Sci. 2014;71(12):2133-2148.
15. Lammermann T, Sott M. Mechanical modes of ‘amoeboid’ cell migration. Curr Opin Cell Biol. 2009;21(5):636-644.
16. Krummel MF, Macara I. Maintenance and modulation of T cell polarity. Nat Immunol. 2007;8(7):715-721.
17. Gomes ER, Jani S, Gundersen GG. Nuclear movement regulated by Cdc42, MRCK, myosin, and actin flow establishes MTOC polarization in migrating cells. Cell. 2005;121(5):451-465.
18. Jimenez FR, Martin-Cortazar C, Kourani O, et al. CDCA7 is a critical mediator of lymphomas that selectively regulates anchorage-independent growth. Haematologica. 2018;103(10):1669-1678.
19. Prescott JE, Oshthu RC, Lee LA, et al. A novel c-Myc-responsive gene, JPO1, participates in neoplastic transformation. J Biol Chem. 2001;276(5):4827-4824.
20. Jeness C, Giunta S, Muller MM, Kimura H, Muir TW, Funahashi H. HELLs and CDCA7 comprise a bipartite nucleosome remodeling complex defective in ICF syndrome. Proc Natl Acad Sci U S A. 2018;115(5):E876-E885.
21. Thijsen FE, Ito Y, Grillo G, et al. Mutations in CDCA7 and HELLs cause immunodeficiency-centromeric instability-facial anomalies syndrome. Nat Commun. 2015;6:7870.
22. Gill RM, Gabor TV, Couzens AL, Scheid MF. The MYC-associated protein CDCA7 is phosphorylated by AKT to regulate MYC-dependent apoptosis and transformation. Mol Cell Biol. 2015;35(3):498-519.
23. Oshthu RC, Kaim B, Prescott JE, et al. The Myc target gene JPO1/CDCA7 is frequently overexpressed in human tumors and has limited transforming activity in vivo. Cancer Res. 2005;65(16):5620-5627.
24. Alvaro-Blanco J, Urso K, Chiodyo Y, et al. MAZ induces MYB expression during the exit from quiescence via the E2F site in the MYB promoter. Nucleic Acids Res. 2017;45(17):9960-9975.
25. Campanero MR, Herrero A, Calvo V. The histone deacetylase inhibitor trichostatin A induces GADD45 gamma expression via Oct and NF-Y binding sites. Oncogene. 2008;27(9):1263-1272.
26. Molina-Privado I, Jimenez FR, Montes-Moreno S, et al. E2F4 plays a crucial role in Burkitt lymphoma tumorigenesis. Leukemia. 2012;26(10):2277-2285.
27. Molina-Privado I, Rodriguez-Martinez M, Rebollo F, et al. E2F1 expression is deregulated in human cancer stem cells in distant metastasis and therapy response. Methods Cell Biol. 2015;125(1):562-570.
28. Hruz T, Laule O, Szabo G, et al. Expression and function of the matrix metalloproteinase-9 is up-regulated by CDCL1/CCR7 interaction via extracellular signal-regulated kinase-1/2 signaling and is involved in CDCL1-driven β-cell chronic lymphocytic leukemia cell invasion and migration. Blood. 2008;111(1):383-388.
29. Vacca A, Ribatti D, Iuliano M, et al. Human lymphoblastoid cells produce extracellular matrix-degrading enzymes and induce endothelial cell proliferation, migration, morphogenesis, and angiogenesis. Int J Clin Lab Res. 1996;26(1):55-66.
30. Arroyo AG, Garcia-Pardo A, Sanchez-Madrid F, et al. Activation of the E2F site in the MYB promoter by phosphorylated MYC induces GADD45 gamma expression. EMBO J. 2009;28(2B):1389-1397.
31. Madri JA, Graesser D. Cell migration in the immune system: the evolving inter-related roles of adhesion molecules and proteases. Dev Immunol. 2005;7(2-4):105-116.
32. Krammer PH, Wauben E, Gallard R, Bunnett NW, Hauser SL. T cell gelatinases mediate basement membrane transmigration in vitro. J Immunol. 1995;154(9):4579-4589.
33. Redondo-Munoz J, Jose Terol M, Garcia-Marco JA, Garcia-Pardo A. Matrix metalloproteinase-9 is up-regulated by CDCL1/CCR7 interaction via extracellular signal-regulated kinase-1/2 signaling and is involved in CDCL1-driven β-cell chronic lymphocytic leukemia cell invasion and migration. Blood. 2008;111(1):383-388.
34. Freije PJ, Carpen O. Alpha-actinin revisited: a fresh look at an old player. Cell Motil Cytoskeleton. 2004;58(2):104-111.
35. Sebolen B, Salzano A, Antonini-Carugo K. Alpha-actinin structure and regulation. Cell Mol Life Sci. 2006;63(17):2688-2701.