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CDCA7 finely tunes cytoskeleton dynamics to promote lymphoma migration and invasion

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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 lymphomagenesis and invasion, therapies aimed at inhibiting its expression or activity might provide significant control of lymphoma growth, invasion, and metastatic dissemination.

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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 lymphomas[2] 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, intravasation 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-

taining endopeptidases, is the most common group of extracellular matrix (ECM) proteases involved in tumor invasion and metastasis.[4] MMP-2 and MMP-9, in particular, are highly expressed in metastatic cancer cells and contribute to the progression of tumors and formation of metastases.[5] *Ex vivo* studies suggest that carcinoma cells may also use a protease-independent scheme of invasion, whereby cells either squeeze through existing interstices in the ECM or displace ECM components.[6]

To invade surrounding tissues and vessels, cells must acquire the ability to migrate. Indeed, cell migration is required for the initial scattering of cells, egress from the primary tumor, basement membrane penetration, intravasation, and extravasation. Single carcinoma cells can migrate in mesenchymal or amoeboid manners.[7] Mesenchymal migration involves formation of protrusions and their adhesion to the substrate at the cell front, and loss of adhesion at the opposite end. During directional cell migration, actin polymerization drives protrusion formation, whereas the tension generated by non-muscle myosin II (NM-II) retracts the rear end of the cell.[8] The adhesion of the cell to the ECM at the protrusion end is as important as its dissociation at the opposite end of the cell.[9] The interaction with the substrate is mediated mainly by integrins, which have binding-motifs for ECM proteins. The connection between integrins and the actin cytoskeleton is mediated by actin-binding proteins such as talin, vinculin and α-actinin.[10] NM-II molecules are actin-binding proteins comprised of two heavy chains that have ATPase activity, two regulatory light chains that regulate NM-II activity, and two essential light chains that stabilize the heavy chain structure.[11] A major factor that determines cell migration is the cell's intrinsic contractility capacity,[10] which is modulated through the coordinated regulation of myosin activity and actin polymerization.[9] Myosin activity is exquisitely regulated through phosphorylation by signaling complexes and scaffold proteins to finely tune migration.[10] In particular, phosphorylation of Ser19 in the regulatory light chain induces the ATPase activity of NM-II.[11]

The mechanisms of invasion by lymphoma cells are poorly understood, but various reports suggest that the capacity of normal blood T lymphocytes and the lymphoma T-cell line SupT1 to move through three-dimensional collagen lattices does not require ECM degradation.[7,12] Instead, the motile capacity of these cells might be critical for migration through these gels.[7,13] While various migration modes have been described in normal non-lymphoid cells,[14] normal lymphocytes appear to migrate mainly in an amoeboid fashion. Amoeboid migration is characterized by weak adhesion to the substrate[9,10] and rapid cycles of actin polymerization and actomyosin contraction at the front and rear edges, respectively.[15]

Cell migration also involves the reorganization of the microtubule cytoskeleton. Microtubules are organized around the microtubule-organizing center and, similar to the actin filaments, are polarized.[16] The microtubule-organizing center is reoriented between the nucleus and the leading edge in migrating cells and contributes to directional cell migration.[17] The mechanisms that regulate the reorganization of the tubulin cytoskeleton in migrating lymphocytes do, however, remain unknown.

We recently showed that CDCA7 is a critical mediator of lymphomagenesis.[18] *CDCA7* was identified as a MYC-target gene.[19] Its encoded protein associates with the Helicase, lymphoid-specific (HELLS) SNF2 family member and is required for nucleosome remodeling by HELLS and for DNA methylation maintenance.[20,21] AKT-mediated phosphorylation of CDCA7 promotes its nuclear exclusion and sequestration to the cytoplasm.[22] *CDCA7* mRNA was found to be deregulated in several tumor types, including lymphoid tumors,[23] and we recently showed that CDCA7 protein is also overexpressed in lymphoid tumors and that its silencing markedly impairs lymphoid tumor growth without inhibiting the proliferation of normal cells.[18]

As lymphoid tumors can be highly invasive,[2,3] we investigated the potential role of CDCA7 in lymphoid tumor invasion. Here we show that CDCA7 is critical for invasion and migration of lymphoma cells and for the reorganization of the tubulin and actomyosin cytoskeletons.

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,[24] 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.[25]

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 xengrafts in mice. All animal procedures were approved by the CSIC Ethics Committee (ref. 054/2014 and 634/2017) and by the Madrid Regional authorities (ref. PROEX 31/14 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[26,27] whose growth is impaired upon *CDCA7* knockdown.[18] 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-Ctl) (Figure 1A). As previously reported,[18] 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

Online Supplementary Table S1). 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.[28] 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 Figure 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-Ctl, 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

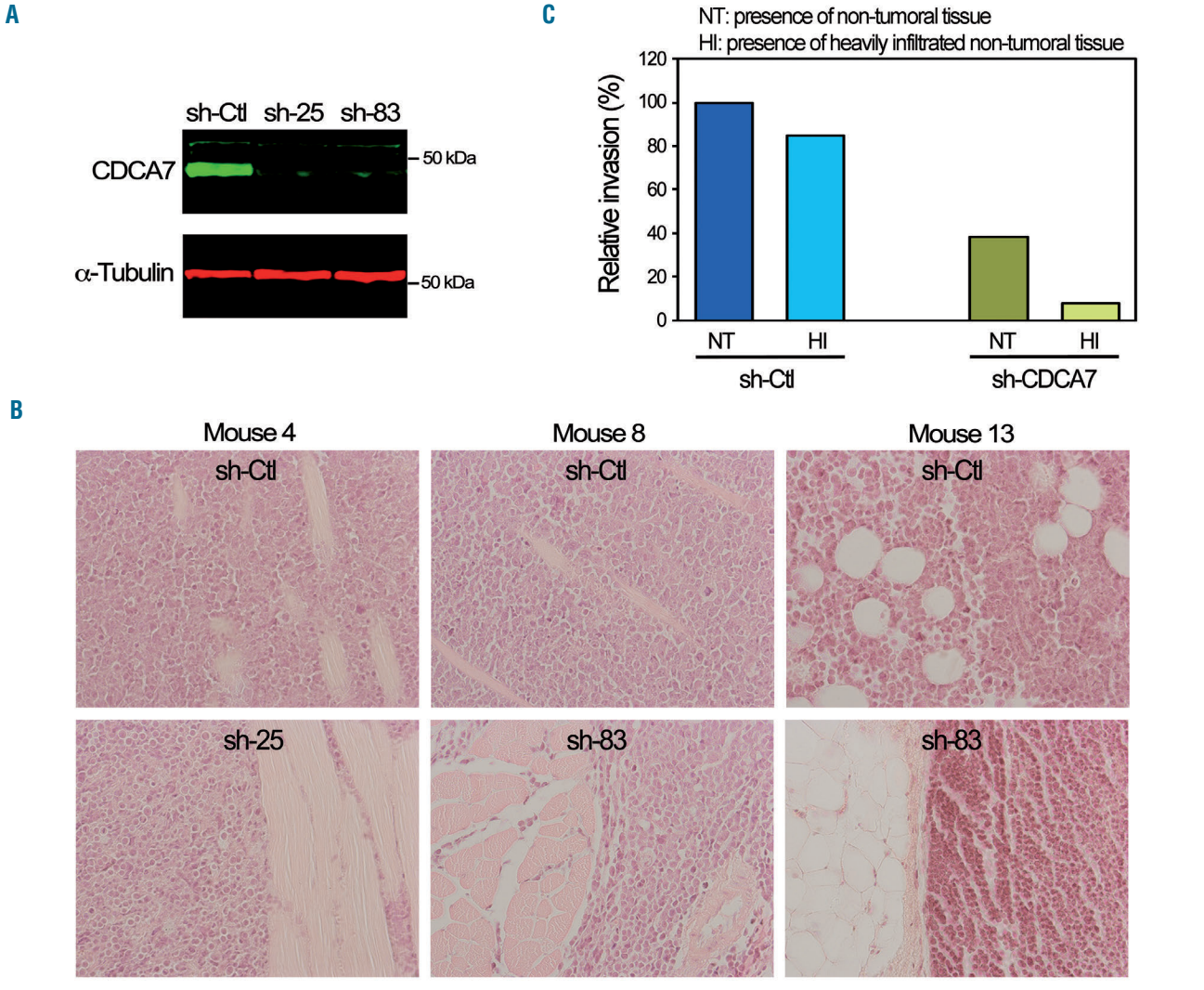

Figure 1. *CDCA7* silencing limits lymphoma dissociation of neighboring tissues. DG-75 cells were transduced with lentivirus encoding short hairpin (sh) control (Ctl) RNA or the *CDCA7*-specific shRNA, sh-25 and sh-83, and selected in the presence of puromycin for >5 days. (A) Representative CDCA7 and α-tubulin (loading control) immunoblot analysis of these cells. (B, C) Transduced DG-75 cells were inoculated subcutaneously in immunodeficient NOD-SCID mice and tumors grown after 3 weeks were embedded in paraffin. All mice were inoculated with control cells in one flank (n=13) and sh-25 (n=7) or sh-83 (n=6) cells in the opposite flank. (B) Representative images of tumor sections from indicated mice stained with hematoxylin. Massively infiltrated (top panels) and poorly or non-infiltrated muscle and fat tissues (bottom panels) are shown. (C) Percentage of tumor masses with presence of non-tumoral tissues (NT) and percentage of tumor masses with heavily infiltrated non-tumoral tissues (HI). Additional information on these tumors is shown in *Online Supplementary Table S1*.

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*).

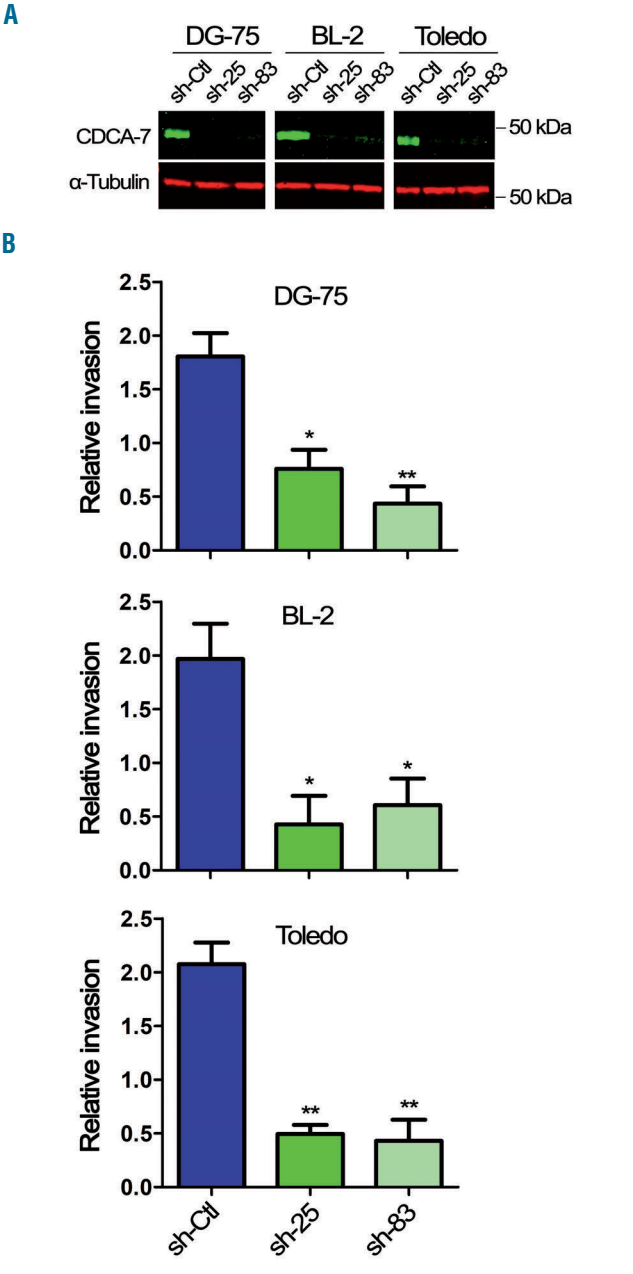

Figure 2. *CDCA7* knockdown inhibits lymphoma invasion *in vitro*. (A) Representative CDCA7 and α-tubulin immunoblot analysis of cell lysates from DG-75, BL-2, and Toledo cells lentivirally transduced with the indicated short hairpin (sh) RNA. (B) These cells were seeded on the upper surface of the matrigel-coated polycarbonate membrane of transwell chambers containing 10% fetal bovine serum in the lower chamber. Quantification of the relative invasive capacity is shown as the mean + standard error of mean. of three independent transductions. **P*<0.05 and ***P*<0.01 (one-way analysis of variance with the Bonferroni post-test).

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 fluorescently-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

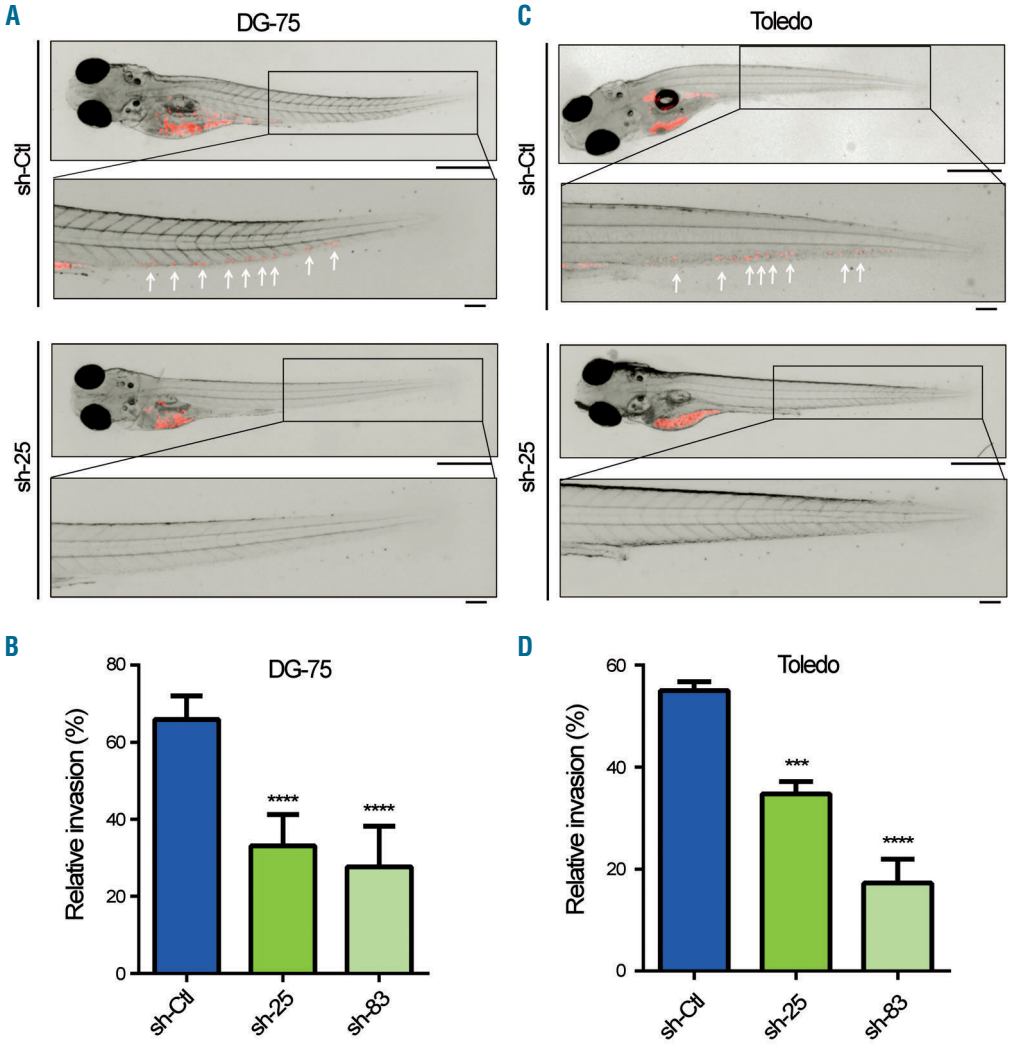

Figure 3. *CDCA7* silencing impairs lymphoma invasion *in vivo*. (A,B) DG-75 and (C,D) Toledo cells lentivirally transduced with short hairpin (sh) control (Ctl) or the CDCA7-specific shRNA, sh25 and sh83, were stained and injected into the yolk sac of zebrafish embryos. Images of representative zebrafish embryos in which sh-Ctl- or sh-25-transduced (A) DG-75 or (C) Toledo cells have invaded (sh-Ctl) or not (sh-25) their caudal region. Long scale bars, 500 μm; short scale bars, 100 μm. Percentage of embryos with >5 (B) DG-75 or (D) Toledo cells in the caudal region shown as the mean + standard error of mean of four independent experiments. ***P<0.001 and ****P<0.0001 (χ^2 and two-tailed Fisher exact test). More than 100 embryos were examined for each condition, in four independent experiments.

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,[11] 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).[33] 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). Together, these results strongly support the notion that CDCA7 modulation of myosin activation and actin polymerization is critical for the regulation of cell migration.

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 proteinase genes, the MMP family has been implicated in carcinoma tumor invasion and metastasis formation.[4] Indeed, MMP are overex-

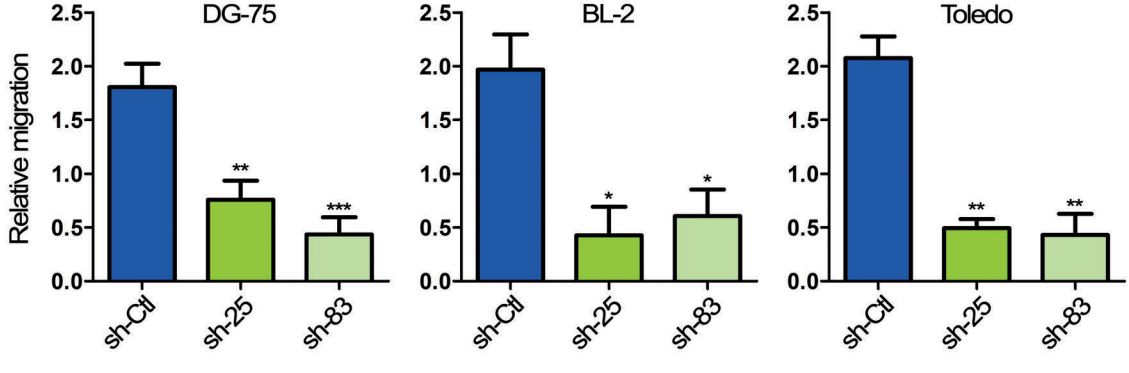

Figure 4. *CDCA7* knockdown markedly inhibits serum-induced lymphoma migration. DG-75, BL-2, and Toledo cells were transduced with lentivirus encoding the indicated short hairpin (sh) RNA and seeded on the upper surface of the fibronectin-coated polycarbonate membrane of transwell chambers containing 10% fetal bovine serum in the lower chamber. Quantification of the relative migration capacity is shown as the mean + standard error of mean of three independent transductions. *P<0.05, **P<0.01 and ***P<0.001 (one-way analysis of variance with the Bonferroni post-test).

pressed in multiple tumors[34] and their overexpression is critical for carcinoma invasion and metastasis formation.[35] MMP2 and MMP9, in particular, degrade type IV collagen, a major component of the basement membrane, and thus facilitate tumor invasion.[4] MMP9 is also required for intravasation, extravasation, and local migration of tumor cells.[36]

Infiltrating non-tumoral lymphocytes often express elevated MMP levels.[37] In fact, the capacity of these cells to penetrate through basement membrane equivalents *in vitro* is facilitated by active MMP2 and MMP9.[38] In addition, MMP9 was found on the surface of B-cell chronic lymphocytic leukemia cells, where it is a critical regulator of cell migration.[39] MMP2 or MMP9 is also found in some lymphoma cell lines.[40] 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,

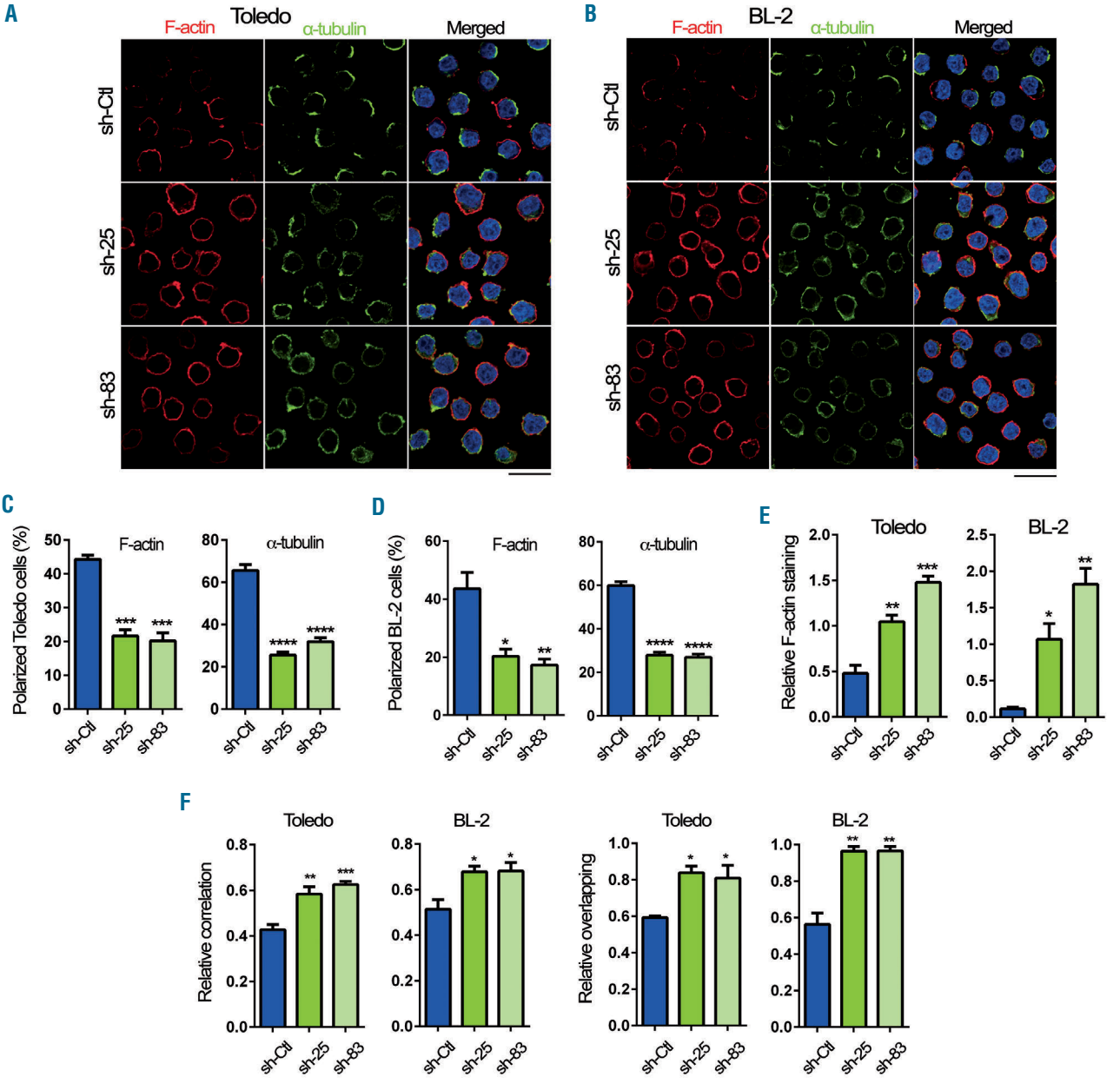

Figure 5. CDCA7 knockdown impairs actin and tubulin cytoskeletons polarization. BL-2 and Toledo cells were transduced with the indicated short hairpin (sh) RNA, seeded on coverslips coated with 2 μg fibronectin, and stimulated with 10 ng/mL stromal cell-derived factor 1 for 15 min. Representative confocal microscopy images (1 section) of (A) Toledo and (B) BL-2 transduced cells stained with anti-α-tubulin, phalloidin (F-actin), and DAPI. Quantification of the percentage of (C) Toledo and (D) BL-2 cells displaying polarized distribution of F-actin and α-tubulin. (E) Quantification of relative F-actin fluorescence intensity and (F) α-tubulin and F-actin colocalization measured as relative correlation (Pearson coefficient) and overlapping (Mander coefficient) of signals. Data are presented as the mean + standard error of mean of three independent experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 (one-way analysis of variance with the Bonferroni post-test). Scale bar, 10 μm.

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.[43]

The protease-independent fashion of negotiating physical barriers involves the coordinated adoption of an amoeboid 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, amoeboid migration requires dynamic assembly/disassembly of the actomyosin network.[15] However, while mesenchymal migration relies strongly on coordinated cell adhesion to the ECM in the leading

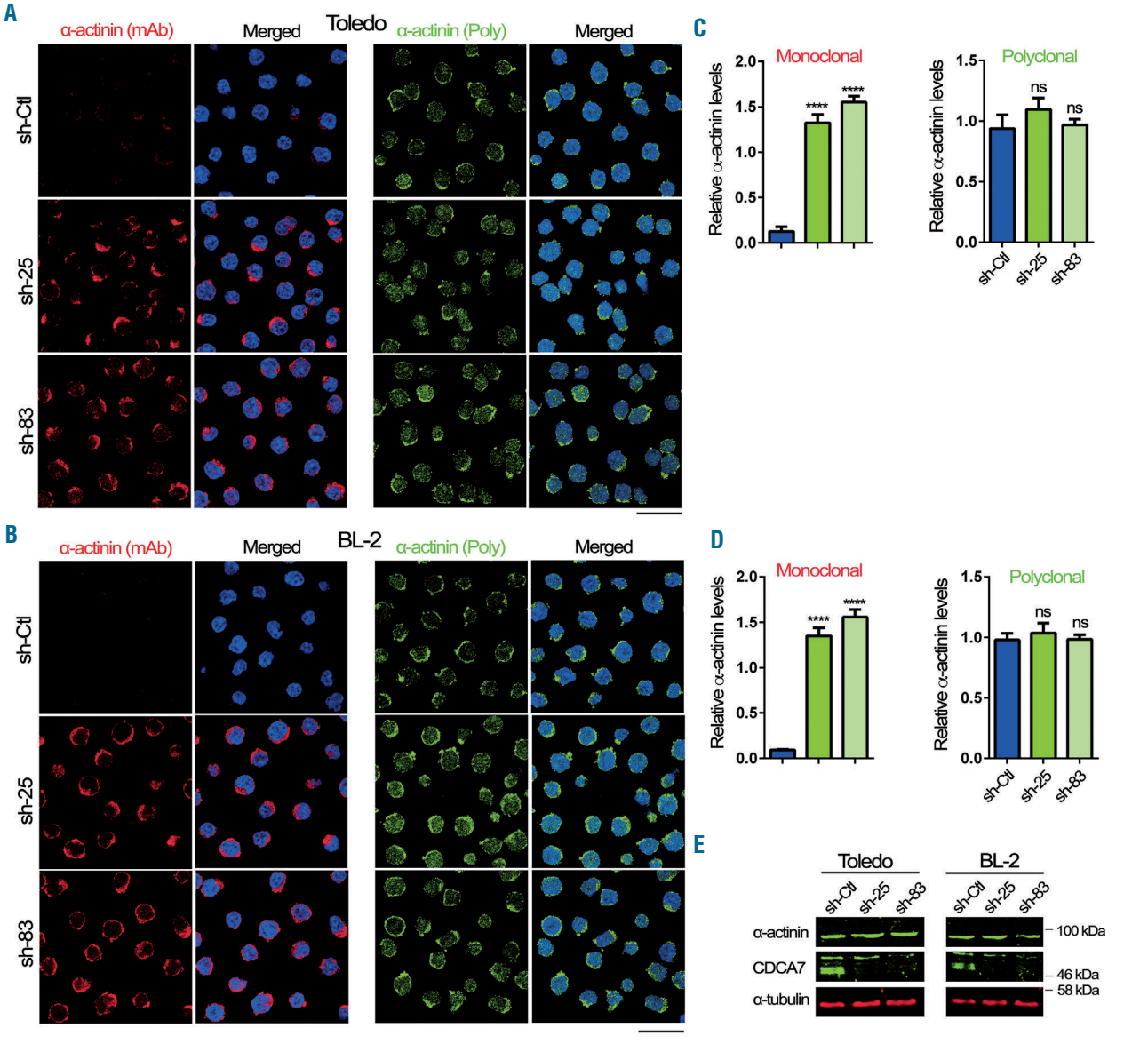

Figure 6. Increased α-actinin staining in *CDCA7*-silenced lymphoma cells. BL-2 and Toledo cells were transduced with the indicated short hairpin (sh) RNA, seeded on coverslips coated with 2 μg fibronectin, and stimulated with 10 ng/mL stromal cell-derived factor 1 for 15 min. Representative confocal 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.[7] In amoeboid migration, movement is generated by cortical filamentous actin in the cell front in the absence of focal contacts and stress fibers.[7]

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.

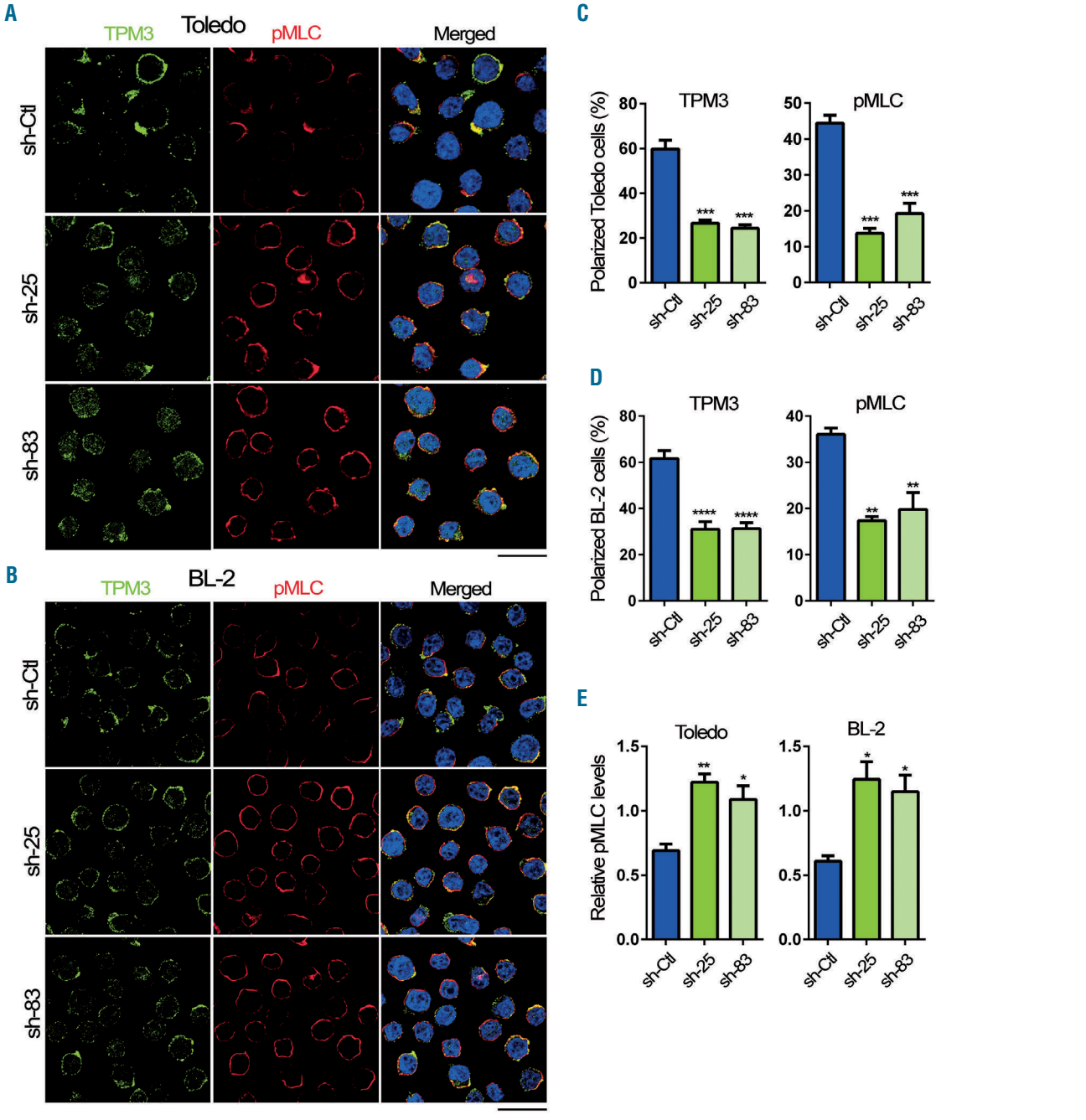

Figure 7. *CDCA7* silencing decreases tropomyosin 3 polarization and promotes myosin light chain phosphorylation. BL-2 and Toledo cells were transduced with the indicated short hairpin (sh) RNA, seeded on coverslips coated with 2 µg fibronectin, and stimulated with 10 ng/mL stromal cell-derived factor 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*<0.05, ***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.[44] 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 α-actinin was not lost in CDCA7-silenced cells. It should be noted that besides binding to actin filaments, α-actinin associates with a number of signaling molecules, ion channels, transcription factors, and transmembrane receptors, including integrins.[45] It therefore seems likely that α-actinin dissociates from F-actin in CDCA7-silenced cells, remaining associated with integrins or other transmembrane receptors. Indeed, our data support the notion that α-actinin is associated with active integrins in both control- and CDCA7-knockdown cells. Among the four α-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 α-actinin to/from F-actin. The association of α-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 α-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.

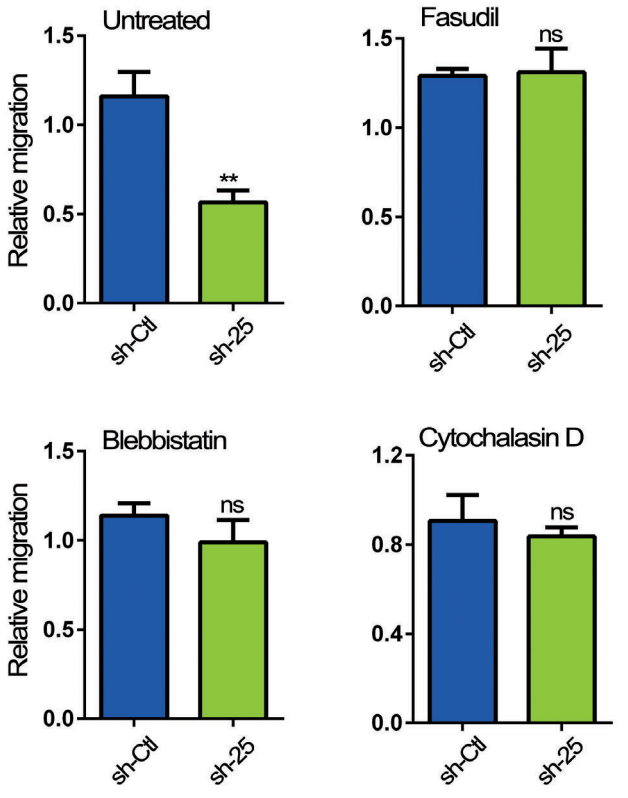

Figure 8. The inhibition of myosin activation or actin polymerization reestablish the migration capacity in CDCA7-silenced cells. Toledo cells were transduced with the indicated short hairpin (sh) RNA, and seeded on the upper surface of the fibronectin-coated polycarbonate membrane of transwell chambers in the presence of 20 μM fasudil, 25 μM blebbistatin, or 0.1 μg/mL cytochalasin D; 10% fetal bovine serum was used in the lower chamber as a chemoattractant. Quantification of the relative migration capacity is shown as the mean + standard error of mean of three independent transductions. ***P*<0.01; ns, non-significant (one-tailed *t*-test).

We have shown that CDCA7 is critically involved in the anchorage-independent growth of lymphoid tumors and in lymphomagenesis.[18] While CDCA7 is also expressed in normal diploid fibroblasts, its silencing in these cells did not inhibit their anchorage-dependent proliferation.[18] 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.

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