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Ubc9 promotes breast cell invasion and metastasis in a sumoylation-independent manner

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Abstract

Ubc9 is an E2 conjugating enzyme that transfers the activated SUMO (small ubiquitin-related modifier) to protein substrates, and thus it plays a critical role in sumoylation-mediated cellular pathways. We have previously reported that Ubc9 promotes tumor growth in the xenograft mouse model using breast cancer cell line MCF-7 in part through regulation of Bcl-2 expression. In this study, we show that ectopic expression of wild type Ubc9 (Ubc9-WT) promotes cell invasion and metastasis. Surprisingly, the dominant negative mutant Ubc9 (Ubc9-DN) also causes the same phenotype, indicating that the ability of Ubc9 to promote invasion and metastasis is distinct from its ability to conjugate SUMO to protein substrates. Of considerable interest, several microRNAs such as *miR-224* are regulated by Ubc9. While ectopic expression of Ubc9 causes downregulation of *miR-224*, and suppression of Ubc9 by Ubc9-siRNAs leads to its upregulation. We further show that *miR-224* can inhibit cell invasion and directly targets CDC42 and CXCR4, and that suppression of CDC42 and CXCR4 by RNAi causes inhibition of Ubc9-mediated invasion. Together, these results demonstrate a molecular link between Ubc9 and the metastasis genes such as CDC42 and CXCR4, and thus provide new insight into the mechanism by which Ubc9 promotes tumor invasion and metastasis.

Keywords

Cell invasion; metastasis; miRNA; miR-224; CXCR4; post-transcriptional regulation; tumorigenesis; Ubc9

Introduction

Protein modification involving small ubiquitin-like modifier (SUMO) has been shown to play an important role in regulation of diverse cellular functions (Johnson, 2004; Melchior, 2000). Sumoylation is a multi-step process and requires several enzymes. Among them is the E2 conjugating enzyme Ubc9 which transfers the activated SUMO to protein substrates (Johnson, 2004). Since most of SUMO substrates are nuclear proteins including many

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transcription factors or co-factors, SUMO/Ubc9 impact a variety of cellular pathways such as cell growth, proliferation, apoptosis and even chromatin remodeling (Baek, 2006).

Although it is well known that Ubc9 impacts cellular pathways through sumoylation, Ubc9 can also regulate cellular pathways independent of sumoylation. In this scenario, Ubc9 may function as a regulator of nuclear transport or protein activity or as a co-regulator of transcription. For example, both wild type and sumoylation-defective mutant (or dominant negative) Ubc9 regulate the nuclear localization of Vsx-1, a protein regulating bipolar cell differentiation during zebrafish retinogenesis, by binding to a nuclear localization signal at the N-terminus of the Vsx-1 homeodomain (Kurtzman, Schechter, 2001). Emerging evidence further suggests that Ubc9 can also function as a transcription co-factor independent of sumoylation in mammalian cells. For example, Ubc9 has been shown to modulate transcriptional activity of glucocorticoid receptors (GR) by directly binding to this protein (Kaul et al., 2002). Similarly, like the wild type counterparts, the sumoylation-defective mutant Ubc9 is able to function as a co-activator of the chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI), suggesting that the co-activator ability is distinct from the sumoylation activity. Of interest, chromatin immunoprecipitation (ChIP) assays revealed that ectopically expressed COUP-TFI and Ubc9 were recruited to the endogenous CYP11B2 promoter (Kurihara et al., 2005). Other examples of Ubc9-regulated genes include estrogen receptor α (ERα) (Kobayashi et al., 2004; Sentis et al., 2005) and the gene coding for the immediate-early 2 (IE2) protein of human herpesvirus 6 (Tomoiu et al., 2006).

Ubc9 is a single copy gene and is ubiquitously expressed in all human organs and tissues. However, Ubc9 is frequently upregulated in tumor specimens. For example, Ubc9 mRNA is overexpressed in lung adenocarcinoma, as detected by microarray analysis (McDoniels-Silvers et al., 2002). By semi-quantitative RT-PCR analysis and immunohistochemistry, we detected overexpression of Ubc9 in ovarian carcinoma compared to the matched normal ovarian epithelial cells (Mo et al., 2005) as well as other types of tumors (Wu et al., 2009). Moreover, Ubc9 is the most highly expressed protein in protein extracts from melanoma infiltrated lymph nodes (Moschos et al., 2007). In support of this, we have previously shown that Ubc9 is able to induce Bcl-2 expression in the breast cancer cell line MCF-7 (Lu et al., 2006), which could explain in part why ectopic expression of Ubc9 enhances tumor growth while suppression of Ubc9 function reduces tumor growth in MCF-7 model (Mo et al., 2005). However, little is known whether Ubc9 can promote cell invasion and tumor metastasis.

In this study, we present evidence that Ubc9 promotes invasion and metastasis in a sumoylation independent manner. More importantly, we show that several microRNAs (miRNAs) are regulated by Ubc9. In particular, *miR-224* is negatively regulated by Ubc9. We suggest that Ubc9 promotes invasion and metastasis in part through downregulation of a putative tumor suppressor *miR-224*.

Results

Effect of Ubc9 on cell invasion and metastasis

We have previously shown that ectopic expression of Ubc9 enhances, whereas suppression of Ubc9 function decreases breast tumor growth in the xenograft animal model using MCF-7 cells (Mo et al., 2005), suggesting that Ubc9 plays a causal role in breast tumorigenesis. However, it is not clear whether Ubc9 affects breast tumor cell invasion and metastasis. Therefore, we generated stable clones from the metastatic breast cancer MDA-MB-231 cells expressing Ubc9-WT or Ubc9-DN. After verification of the exogenous gene expression, over 10 stable MDA-MB-231 transfectants for Ubc9-WT or Ubc9-DN or vector control were pooled. S-Fig.1A showed that the exogenous Ubc9-WT or Ubc9-DN level was highly expressed compared to the endogenous counterpart. To confirm that the ectopically expressed Ubc9-WT or Ubc9-DN affects protein sumoylation, Western blot was carried out using anti-SUMO antibody. As expected, Ubc9-DN suppressed while Ubc9-WT enhanced protein sumoylation (s-Fig. 1B). Next, we examined the effect of Ubc9-WT or Ubc9-DN on cell invasion. Matrigel chamber assay revealed that Ubc9-WT increased cell invasion with about as twice invasive cells as the vector control (Fig. 1A and B). This result is consistent with the report that Ubc9 plays a role in prostate cancer cell invasion (Kim et al., 2006). However, to our surprise, Ubc9-DN also increased cell invasion by over 2-fold compared to the vector control (Fig. 1A and B), just like Ubc9-WT. We then tested another two metastatic breast cancer cell lines LM2-4142 (Minn et al., 2005) and MDA-MB-468 with ectopic expression of Ubc9-DN or Ubc9-WT and obtained similar results to those of MDA-MB-231 cells (s-Fig.2). Therefore, these results suggest that Ubc9 promotes breast cancer cell invasion independent of sumoylation.

To further investigate the role of Ubc9 in cell invasion, we suppressed Ubc9 expression by Ubc9 specific siRNAs. As shown in Fig. 1C, both Ubc9-siRNA-1 and Ubc9-siRNA-2 efficiently suppressed Ubc9 expression, as detected by Western blot. Invasion assay indicated that Ubc9-siRNA-1 substantially inhibited invasiveness of MDA-MB-231 cells (Fig. 1C and D). A similar inhibitory result was also seen with Ubc9-siRNA-2, which was derived from a different location of the Ubc9 sequence.

To determine whether Ubc9-mediated invasion is due to increased cell proliferation, we measured cell growth by MTT. As shown in s-Fig.3 A and B, in both MDA-MB-231 and LM2-4142 cells, Ubc9 had no significant effect on cell growth, suggesting that the observed invasion enhanced by Ubc9 is not likely due to cell proliferation differences. However, Ubc9-siRNA inhibited cell growth (s-Fig.3C), which is presumably because the degree of knockdown was severe. It is known that Ubc9 is an essential gene and knockout of Ubc9 causes embryo lethality (Nacerddine et al., 2005).

Effect of Ubc9 on metastasis in an experimental model

To determine whether Ubc9 plays a role in tumor metastasis, we performed experimental metastasis assays by injecting the transfected MDA-MB-231 cells into female nude mice through tail veins. Similar to invasion results, both Ubc9-DN and Ubc9-WT caused more metastases than the vector control. For example, average lung tumor nodules were between

80–100 for Ubc9-DN and Ubc9-WT, whereas there were about 20 for the vector control (Fig. 2A and B). In contrast, suppression of Ubc9 by Ubc9-siRNA-1 caused a substantial reduction of tumor nodules (Fig. 2C and D). These results further suggest that Ubc9 promotes tumor invasion and metastasis independent of its SUMO conjugation activity.

miR-224 is negatively regulated by Ubc9

Given the importance of Ubc9 in tumor invasion and metastasis, next we determined the molecular link between Ubc9 and tumor invasion, and set to profile gene expression for MDA-MB-231 cells expressing Ubc9-WT or vector alone. Since Ubc9-DN had the same effect on invasion and metastasis as Ubc9-WT, it was not included in the profiling assays. We were particularly interested in miRNAs because as non-coding RNAs, miRNAs have been shown to play an important role in tumor metastasis (Ma et al., 2007). We profiled a total of 474 miRNAs by real time PCR. As shown in Fig. 3A, the vast majority of miRNAs were expressed at similar levels between Ubc9-WT and vector control (also see s-Table 1). Only a small fraction of miRNAs were differentially expressed. We selected 35 miRNAs of them which revealed the most differential expression (s-Table 1, highlighted in red) for further verification, and showed that three miRNAs, *miR-224*, *miR-200b* and *miR-559*, were most downregulated or upregulated (Fig. 3B). Initial characterization suggested a role of *miR-224* in cell invasion and the computer-aided algorithms indicated that several metastasis-related genes are potential targets for *miR-224*. Furthermore, suppression of *miR-200b* and *miR-559* by antagomirs revealed only a slight inhibition of invasion. Therefore, we chose *miR-224* in this study. We further confirmed that both Ubc9-WT and Ubc9-DN suppressed *miR-224* by TaqMan real time PCR (Fig. 3C). In contrast, Ubc9-siRNAs increased its expression (Fig. 3D).

Suppression of cell invasion by *miR-224*

Suppression of *miR-224* by Ubc9 suggests that *miR-224* may play a suppressive role in invasion. Thus, we first ectopically expressed *miR-224* in MDA-MB-231 cells and then measured their invasion ability. Fluorescence microscopy confirmed a high transduction rate (over 90%) and real-time PCR revealed a high level of mature *miR-224* compared to vector control (s-Fig. 4A). As expected, *miR-224* significantly reduced the number of invaded cells (Fig. 4A). For example, *miR-224* infected cells revealed only 45% of invaded cells. To further determine the role of *miR-224* in invasion, we suppressed *miR-224* by anti-*miR-224*. As expected, anti-*miR-224* significantly enhanced cell invasion, further supporting a suppressive role of *miR-224* in cell invasion (Fig. 4B). Real time PCR assays confirmed that anti-*miR-224* suppressed the endogenous *miR-224* level (s-Fig. 4B).

To determine whether *miR-224* is an important effector in the Ubc9-mediated cell invasion, we introduced the *miR-224* expression vector into the Ubc9 overexpressing MDA-MB-231 cells by infection and then tested whether *miR-224* can block Ubc9 induced-invasion. As shown in Fig. 4C, *miR-224* significantly suppressed Ubc9-induced invasion. Compared to the pCDH vector control, *miR-224* caused over 50% reduction of invasion in the cells which were previously transfected with the pCMV vector control. In the cells which were previously transfected with Ubc9-WT, we also found a similar reduction of invasion when *miR-224* was compared to the pCDH vector control (Fig. 4C). Consistent with our previous

findings (Fig. 2A), the number of overall invaded cells for the Ubc9-WT cells (both pCDH and *miR-224*) was higher than for the pCMV vector controls (both pCDH and *miR-224*). Therefore, the Ubc9-mediated cell invasion can be partially reversed by ectopic expression of *miR-224*.

miR-224 directly targets CDC42 and CXCR4

To determine how *miR-224* impacts cell invasion, we tested several predicted target genes based on computer-aided analysis, which included several commonly cited miRNA target prediction programs TargetScan4 (13), miRBase Target5 (http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl), PicTar (14) and miRanda (17). This search combined with luciferase reporter assays identified CDC42 and CXCR4 as targets for *miR-224*; on the other hand, *miR-224* had no effect on another two putative targets ATF2 and Jag1 although *miR-224* was predicted to interact with the 3'-UTR of these two genes. As shown in Fig. 5B, *miR-224* suppressed the luciferase activity of Luc-CDC42-UTR by 60% compared to vector control. To determine the importance of the putative *miR-224* binding site in CDC42-UTR (Fig. 5A, top), we deleted this site, generating Luc-CDC42-UTR-d. This deletion abolished the suppression activity of *miR-224* (Fig. 5B, left), suggesting that *miR-224* silences CDC42 expression through interaction with this binding site. To further determine the effect of *miR-224*, we suppressed the endogenous *miR-224* by anti-*miR-224* and this suppression was able to enhance the luciferase activity by about 30% (Fig. 5B, right). Finally, western blot detected a reduced level of the endogenous CDC42 by *miR-224* compared to vector control, further supporting a suppressive role of *miR-224* in CDC42 (Fig. 5C).

Similar to the results of CDC42, we showed that *miR-224* also suppressed CXCR4 (Fig. 5E). Further analysis indicated that *miR-224* exerted its silencing function by directly binding to the *miR-224* site in the 3'-UTR of CXCR4 (Fig. 5E). In contrast, anti-*miR-224* enhanced the luciferase activity of Luc-CXCR4-UTR (Fig. 5E, right). Finally, *miR-224* suppressed the endogenous CXCR4 (Fig. 5F).

Suppression of CDC42 and CXCR4 inhibits Ubc9-mediated cell invasion

To address whether Ubc9 indirectly affects CDC42 and CXCR4 levels, we examined CDC42 and CXCR4 levels in Ubc9-DN or Ubc9-WT cells. As expected, Ubc9-DN and Ubc9-WT upregulated CDC42 and CXCR4 (Fig.6A). Next, we suppressed CDC42 and CXCR4 by RNAi (Fig. 6B). We noted that knockdown of CDC42 also slightly suppressed CXCR4, and vise versa for some reason. We then determined the effect of knockdown of these two genes on cell invasion. As shown in Fig. 6C, the number of invaded cells for CDC42-sh#2 or CXCR4-sh#4 was substantially lower than that for vector control even under different backgrounds (vector, Ubc9-DN or Ubc9-WT). Moreover, double knockdown (CDC42-sh#2 + CXCR4-sh#4) caused further suppression of invasion. Separate knockdown experiments with shRNA (or siRNA) derived from different parts of these two genes also suppressed cell invasion (s-Fig.5). Together, these results are consistent with previous findings that CDC42 and CXCR4 play a role in cell migration and tumor metastasis (Fukata, Kaibuchi, 2001; Hinton et al., 2008), and specific suppression of CDC42 and CXCR4 by RNAi inhibited cell mobility or cell migration (Chen et al., 2003; El-Sibai et al., 2007),

which may provide a molecular explanation as to why Ubc9 is able to promote cell invasion and metastasis.

Discussion

Ubc9 is well known for its key role in protein sumoylation and sumoylation-mediated cellular pathways, ultimately impacting tumor initiation and progression (Mo, Moschos, 2005). However, there is scarce information available in the literature as to whether and how Ubc9 impacts cell invasion and metastasis. In one report, ectopic expression Ubc9 was shown to be able to enhance invasion in prostate cancer cells through regulation of sumoylation of reptin because sumoylation is required for its repressive function, and disruption of sumoylation causes repression of the metastatic suppressor KAI1 (Kim et al., 2006). Our study provides evidence, for the first time to our knowledge, that Ubc9 can promote invasion and metastasis in a sumoylation independent manner.

As a multi-functional protein, Ubc9 has been shown to mediate diverse cellular pathways. Unlike other E2 conjugating enzymes involved in ubiquitination, Ubc9 is the only E2 enzyme essential for sumoylation and thus, it plays a crucial role in determining sumoylation status of many SUMO substrates. Therefore, its expression or activity is believed to be essential for these SUMO pathways. In contrast to the well-known sumoylation associated function of Ubc9, much less is known about its sumoylation independent function. Emerging evidence suggests that Ubc9 can function as either a modulator of enzymatic activity or a transcriptional co-factor. In addition to the previously mentioned proteins such as Vsx-1 (Kurtzman, Schechter, 2001), GR (Kaul et al., 2002) and ERα (Kobayashi et al., 2004; Sentis et al., 2005), a recent report indicates that overexpression of either Ubc9-WT or Ubc9-DN inhibits GLUT4 degradation and promotes its targeting to the unique insulin-responsive GLUT4 storage compartment (Liu et al., 2007). Thus, Ubc9 is implicated in the insulin-responsive glucose transport by a mechanism independent of its catalytic activity (Liu et al., 2007). With regard to the function of Ubc9 as a transcriptional co-factor, Ubc9 was shown to be able to enhance pleomorphic adenoma gene like-2 (PLAGL2), a transactivator of the surfactant protein-C (SP-C), by a similar sumoylation-independent mechanism (Guo et al., 2008). Moreover, ChIP assay identifies the association of PLAGL2 and Ubc9 with the SP-C promoter *in vivo*, demonstrating the function of Ubc9 as a co-factor of PLAGL2 to mediate PLAGL2 interactive SP-C promoter activity (Guo et al., 2008). This finding is consistent with the report that both wild type and mutant Ubc9 can interact with COUP-TFI to activate the CYP11B2 promoter (Kurihara et al., 2005). In agreement with these reports, our study further suggests that Ubc9-mediated invasion could be separated from its ability to conjugate SUMO to the substrates because sumoylation deficient Ubc9 (Ubc9-DN), like Ubc9-WT, is able to promote cell invasion and metastasis. More importantly, both Ubc9-DN and Ubc9-WT have the same negative effect on *miR-224* expression.

The mechanism underlying the Ubc9-mediated invasion and metastasis may be complex. Our study suggests that at least, miRNAs such as *miR-224* could be one of its downstream players. MicroRNAs are small RNA molecules that are capable of regulating coding genes at the posttranscriptional level (Bartel, 2004; Pillai, 2005; Zamore, Haley, 2005). Increasing

evidence indicates that miRNAs are the key players that affect not only tumor growth, but also tumor invasion and metastasis (Calin, Croce, 2006; Ma, Weinberg, 2008). For example, *miR-10b* serves as a breast cancer metastasis initiation factor because ectopic expression of *miR-10b* affects invasion and metastasis, but not tumor growth (Ma et al., 2007). On the other hand, *miR-200* is a metastatic suppressor by directly targeting the mRNA of the E-cadherin transcriptional repressors ZEB1 and ZEB2, leading to up-regulation of E-cadherin (Korpal et al., 2008; Park et al., 2008). These findings prompt us to profile miRNA expression in the MDA-MB-231 cells overexpressing Ubc9. Our study demonstrates that *miR-224* is specifically suppressed by Ubc9.

Therefore, our study establishes a novel role of Ubc9 as a regulator of miRNA expression. However, it is not clear how Ubc9 regulates miRNA expression. Available evidence suggests that regulation of miRNAs can take place at the transcriptional or posttranscriptional level. At the transcriptional level, several miRNAs, such as *miR-17~92*, *miR-34*, *miR-21*, and *miR-223* have been shown to be regulated by transcription factors, such as c-MYC (He et al., 2005), p53 (He et al., 2007), Stat3 (Loffler et al., 2007), REST (Singh et al., 2008), NFI-A and C/EBPα (Fazi et al., 2005). On the other hand, the posttranscriptional regulation could take place at the miRNA processing or miRNA stability. For instance, Lin28, a developmentally regulated RNA binding protein, was shown to selectively block the processing of pri-*let-7* miRNAs in embryonic cells (Viswanathan et al., 2008).

Based on our preliminary results indicating that levels of both pre-*miR-224* and pri-*miR-224* are also downregulated by Ubc9 (Zhu et al, unpublished), we believe that Ubc9-mediated repression of *miR-224* is more likely to occur at the transcriptional level. Since *miR-224* is an intronic gene embedded in the GABRE gene (Wilke et al., 1997), there are at least two possibilities that *miR-224* can be transcriptionally regulated. One possibility is that *miR-224* is co-regulated by the GAGRE gene. Alternatively, *miR-224* may carry its own promoter. These possibilities are currently under investigation. Dissection of Ubc9-mediated miRNA expression will provide a better understanding of how Ubc9 impacts cell invasion and metastasis.

In summary, we demonstrate that Ubc9 is a multi-functional protein that promotes cell invasion and metastasis independent of sumoylation. Moreover, Ubc9 negatively regulates *miR-224*. Finally, identification of CDC42 and CXCR4 as direct targets for *miR-224* provides new insight into Ubc9-mediated cell invasion and metastasis. Therefore, given its important role in these aspects, Ubc9 may prove to be a potential therapeutic target for cancer intervention because Ubc9 is often upregulated in various types of tumors (McDoniels-Silvers et al., 2002; Mo et al., 2005; Moschos et al., 2007; Wu et al., 2009).

Materials and Methods

Reagents

Primary antibody against Ubc9 was custom made; other primary antibodies were from the following vendors: CDC42 from Cell Signal, CXCR from Applied Biological Materials (British Columbia, Canada), SUMO-1 from Invitrogen (Carlsbad, CA). Secondary

antibodies conjugated with IRDye 800CW or IRDye 680 were purchased from LI-COR Biosciences (Lincoln, NE). PCR primers were purchased from Sigma-Genosys (Woodland, TX) or IDT (Coralville, IA) and LNA antagomirs were purchased from IDT. CDC42 shRNAs and CXCR4 shRNAs were purchased from Open Biosystems (Huntsville, AL). Synthetic CDC42 siRNA was from Santa Cruz (Santa Cruz, CA). Ubc9-siRNAs were from Applied Biosystems (Foster City, CA)

Cell culture

All cell lines were purchased from ATCC (Manassas, VA) except LM2-4142 (Minn et al., 2005), a generous gift from Dr. Joan Massagué. MDA-MB-231, LM2-4142 and MDA-MB-468 cells were grown in RPMI 1640 (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Cambrex) supplemented with 10% FBS. All media contained 2 mM glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Cells were incubated at 37 °C and supplemented with 5% CO2 in the humidified chamber.

miRNA profiling

Total RNA was extracted from MDA-MB-231 cells stably overexpressing Ubc9-WT and then was subject to miRNA profiling using QuantiMir kit (System Biosciences, Mountain View, CA). A total of 474 miRNAs registered in Sanger miRBases (version 9.2) were profiled in this study. Three μg total RNA was first anchor-tailed by poly A polymerase, and then annealed by an oligo dT adapter at 60°C for 5 min. For reverse transcription, 10 μL of RT master mix (1.5 μL of water, 4 μL 5 × buffer, 1.5 μL of 0.1 M DTT, 2 μL of 10 mM dNTPs, 1 μL of Reverse transcriptase) were combined with 10 μL template (poly A tailed). The 20 μL RT reaction was incubated at 42°C for 60 min, 95° C for 10 min, cooled to room temperature, and then diluted 6-fold with 100 μL of water. Following reverse transcription, real-time PCR was performed in a 96-well optical PCR plate using 7500 HT PCR instrument (Applied Biosystems). The reaction solution contained 10 μL of 2 × SYBR green PCR master mix (Applied Biosystems), 5.5 μL of water, 0.5 μL of 5 μM universal primer, 2 μL of 5 μM forward primer (the mature miRNA sequence converted to DNA), and 2 μL of the diluted cDNA. Dissociation curves were typically generated post-run for analysis of amplicon species. U6 was used as an internal control.

Plasmids

Wild type Ubc9 (Ubc9-WT) or dominant negative mutant Ubc9 (Ubc9-DN) was constructed in pCMV-Myc as previously described (Lu et al., 2006). The same DNA fragment carrying Myc-Ubc9 or Myc-Ubc9-DN was also cloned into a lentiviral vector pCDH-CMV-MCS-EF1-copGFP (pCDH, System Biosciences). Restriction enzymes sites were introduced by PCR using standard methods.

To construct a plasmid expressing *miR-224*, we first amplified ~0.5 kb DNA fragment covering the pre-microRNA, using genomic DNA from a healthy blood donor as a template. PCR reactions were performed using the high fidelity Phusion enzyme (New England Biolabs Ipswich, MA) and corresponding specific primers: miR-224-5.1 (sense) 5'-

AGTCAGTCTCTGGATGAGGG; miR-224-Not1-3.1 (antisense) 5'-GCGGCCGCGTAAGTATGCTCCAGATGG where Not1 site was underlined. The amplified fragment was first cloned into a PCR cloning vector (PCR8, Invitrogen) and subsequently cloned into the lentiviral vector pCDH at EcoR1 and Not1 sites where the EcoR1 site was from the PCR cloning vector. Expression of the mature *miR-224* was verified by TaqMan real-time PCR.

The luciferase-UTR reporter plasmids, such as Luc-CDC42-UTR and Luc-CXCR4-UTR, were constructed by introducing the CDC42 or CXCR4 3'-UTR carrying a putative *miR-224* binding site into pGL3 control vector (Promega, Madison, WI). We amplified the CDC42 or CXCR4 3'-UTR sequence from MCF-10A cDNA using PCR primers: CDC42-UTR-5.1(sense) 5'-AATTCATTAACCAGTGGTTAGC CDC42-UTR-Not1-3.1 (antisense) 5'-GCGGCCGCACAGATGTCTGTCTTCTAGCAC CXCR4-UTR-5.1 (sense) 5'-CACAGATGTAAAAGACTTTTTTT CXCR4-UTR-Not1-3.1 (antisense) 5'-GCGGCCGCTTTAACATGTACTTTTATTAAC The PCR products were also first cloned into a PCR cloning vector and then subcloned into a modified pGL3 control vector as described previously (Sachdeva et al., 2009). To delete the putative *miR-224* binding site in the 3'-UTR of CDC42 or CXCR4, we used the two step PCR method where two sets of overlapped primers were used to amplify two fragments followed by a second PCR as described previously (Zhu et al., 2008). All the amplified products were verified by DNA sequencing before cloning into the final destination vector.

Transfection

Transfection of Ubc9-siRNAs and anti-*miR-224* locked nucleic acid (LNA) oligo was performed using RNAifectin reagent (Applied Biological Materials) following the manufacturer's protocol. In brief, cells (1×10^5) were seeded in 6-well plates and incubated overnight. About 100 pmol Ubc9-siRNAs or anti-*miR-224* per well was used for transfection.

Transfection of 293T cells was carried out using the calcium phosphate method, as described previously (Mo, Beck, 1999). The transfected cells were cultured overnight before they were harvested and lysed for luciferase assay or for extraction of protein or RNA.

Stable clones

Stable Ubc9-WT or Ubc9-DN expressing clones were established by transfection, followed by selection in the presence of puromycin (1 μg/ml). Individual colonies (over 10) were pooled after verification of the exogenous gene expression by western blot.

Luciferase Assay

Luciferase assay was carried out in 293T cells to determine the effect of *miR-224* on the activity of Luc-CDC42-UTR or Luc-CXCR4-UTR. First, cells were transfected with appropriate plasmids in 12-well plates. Then, the cells were harvested and lysed for luciferase assay 24 h after transfection. The assays were carried out using a luciferase assay kit (Promega) according to the manufacturer's protocol. β-galactosidase or renilla luciferase was used for normalization.

PCR/RT-PCR and real-time RT-PCR

PCR reactions were performed to amplify the 3'-UTR of CDC42 or CXCR4 according to the standard three-step procedure. Annealing temperature varied depending on the primers used. For RT-PCR, we isolated total RNA using Trizol reagent (Invitrogen) per the manufacturer's protocol. To detect mature *miR-224* expression in cell lines, we also used Trizol reagent to isolate total RNA, which was then amplified by TaqMan stem-loop RT-PCR method, as described previously (Chen et al., 2005; Lao et al., 2006).

Western Blot

Cells were harvested and protein was extracted 2 days after transfection as previously described (Si et al., 2007).

Invasion assays

Effect of Ubc9 or *miR-224* on the invasion ability of MDA-MB-231, LM2-4142 or MDA-MB-468 cells was determined using matrigel invasion chambers (BD Biosciences). Cells infected with Ubc9-WT, Ubc9-DN and *miR-224* or cells transfected with Ubc9-siRNAs were seeded into inserts at $2–4 \times 10^4$ per insert in serum-free medium and then transferred to wells filled with the culture medium containing 10% FBS. After 24 h incubation, non-invading cells on the top of the membrane were removed by cotton swabs. Invaded cells on the bottom of the membrane were fixed, followed by staining with 0.05% crystal violet. Invaded cells on the membrane were then counted as follows. Since the distribution of cells on the membrane was not always even, we first took a picture at a low magnificence and then enlarged the image in a computer screen with grids so that all of the cells on the entire membrane were counted.

Experimental metastasis assay

Female athymic nude (nu/nu) mice (4–5 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and were maintained in the Southern Illinois University School of Medicine's accredited animal facility. All animal studies were conducted in accordance with NIH animal use guidelines and a protocol approved by SIU Animal Care Committee. In brief, 1.5×10^6 exponentially growing MDA-MB-231 cells (vector control, Ubc9-WT, Ubc9-DN, scrambled oligo or Ubc9-siRNA-1) were injected into nude mice through tail veins. Four weeks after injection, the animals were sacrificed. The lungs were harvested, fixed in Bouin's solution and tumor nodules were countered.

Statistical analysis

Statistical analysis of data was performed using the Student's *t* test. Differences with p values less than 0.05 are considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

miRNA	microRNA
PCR	polymerase chain reaction
RT	reverse transcription
SUMO	small ubiquitin-like modifier
UTR	untranslated region

Reference

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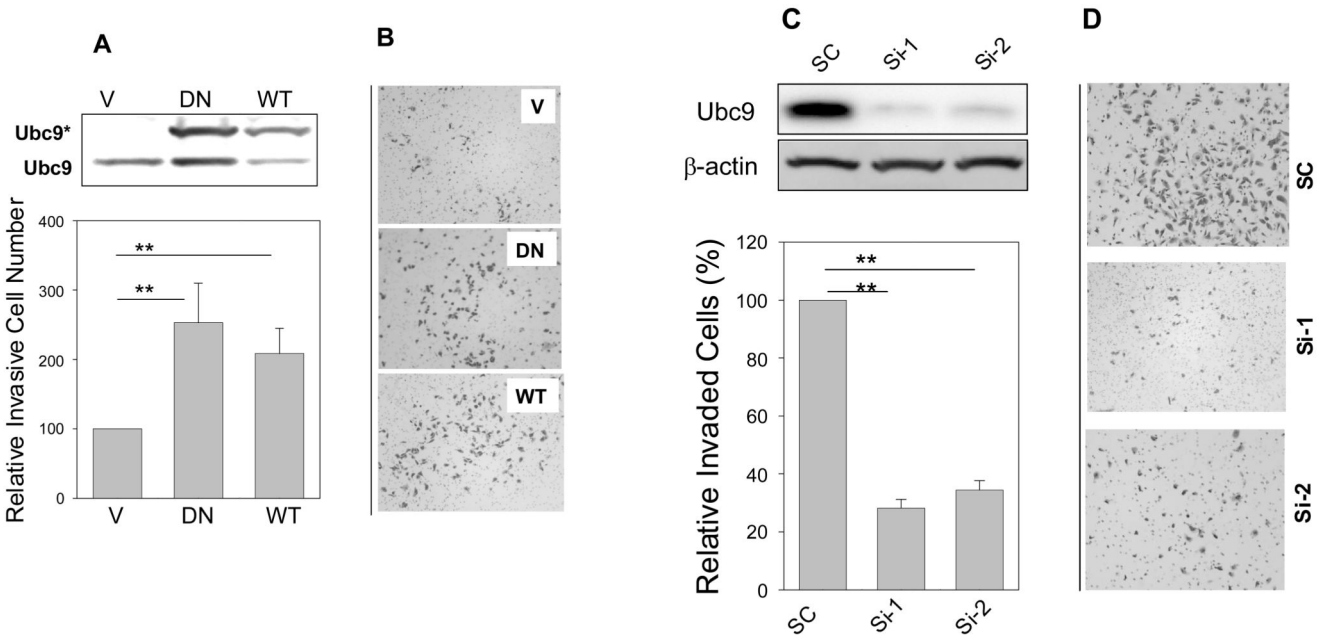

Fig. 1. Effect of Ubc9-WT, Ubc9-DN and Ubc9-siRNAs on cell invasion

Stable transfected or transiently transfected MDA-MB-231 cells (2×10^4 per insert) were subject to cell invasion assays as detailed in Materials and Methods. Cell number on the membrane was counted 24 h later. **A** and **B**, Both Ubc9-WT and Ubc9-DN promote cell invasion. Top panel in A is a western revealing expression of exogenous Ubc9 (DN or WT), as denoted by Ubc9*. **C** and **D**, Suppression of cell invasion by Ubc9-siRNAs. V, vector (pCMV-Myc); DN, Ubc9-DN; WT, Ubc9-WT; SC, scrambled oligo; Si-1, Ubc9-siRNA-1; Si-2, Ubc9-siRNA-2. Values in **A** and **C** are means ± SE of three independent experiments. ** $p<0.01$.

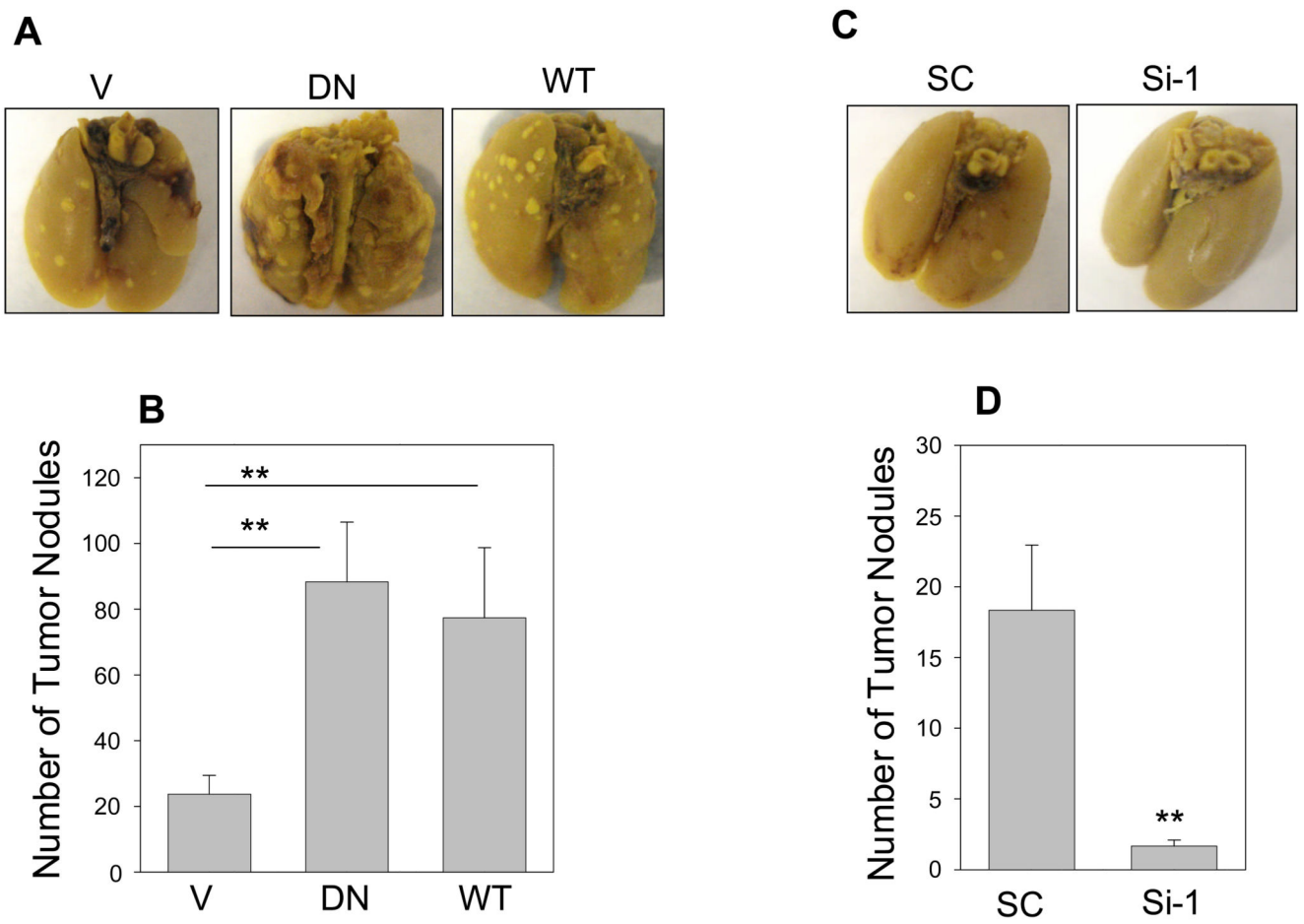

Fig. 2. Effect of Ubc9-WT, Ubc9-DN and Ubc9-siRNA-1 on lung metastasis in an experimental metastasis model

Stable transfected or transiently transfected MDA-MB-231 cells (1.5×10^6) were injected into female nude mice through tail veins as described in Materials and Methods. Mice were sacrificed and tumor nodules in lung were counted 4 weeks after injection. **A** and **C**, Representative tumor bearing lungs; **B** and **D**, Average lung tumor nodules (n = 5). V, vector; WT, Ubc9-WT; DN, Ubc9-DN; SC, scrambled oligo; Si-1, Ubc9-siRNA-1. ** $p<0.01$.

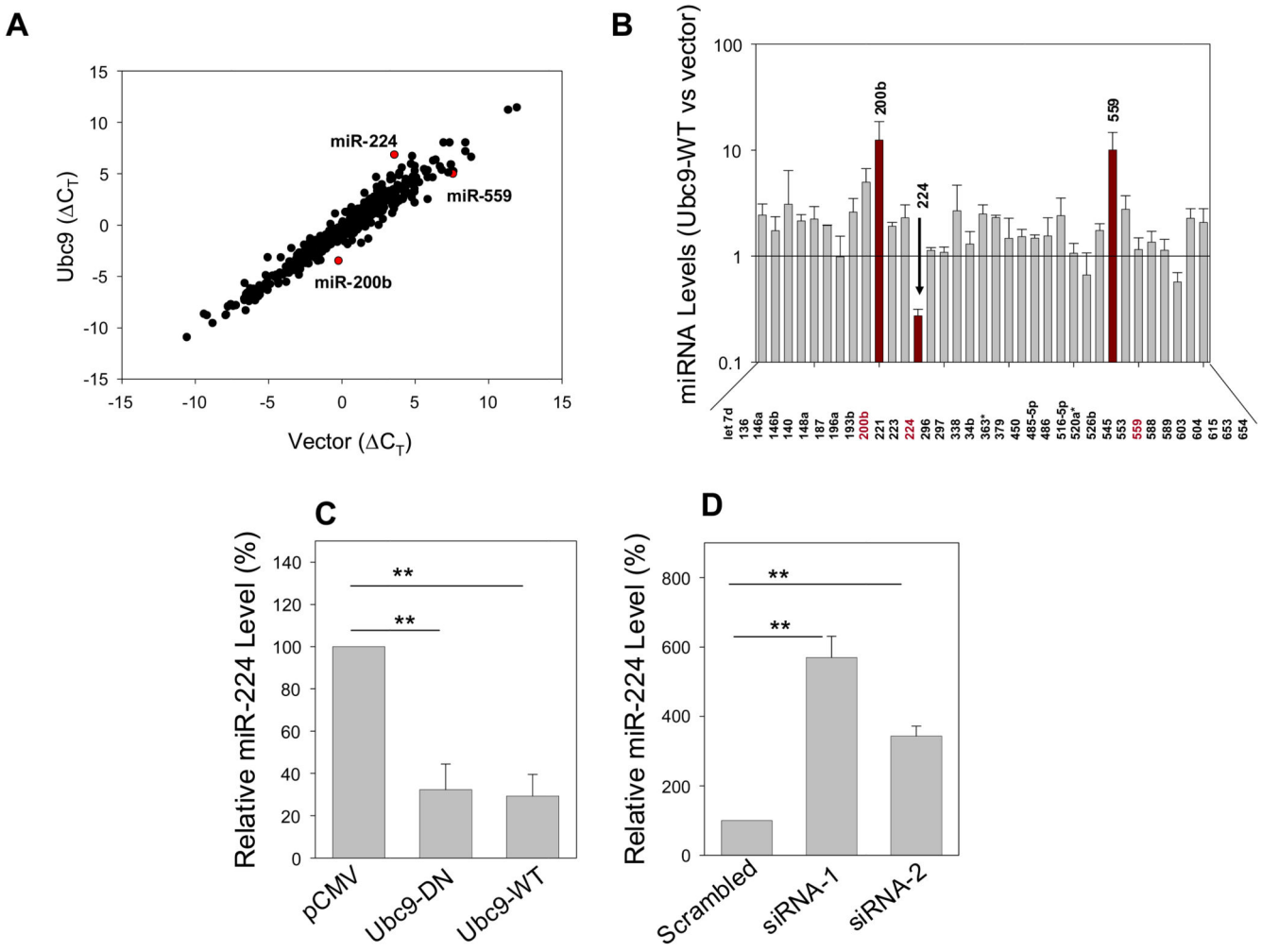

Fig. 3. Effect of Ubc9 on miRNA expression

A, MDA-MB-231 cells transfected with vector alone or Ubc9-WT were subject to miRNA profiling by QuantiMir real-time PCR. Shown here is the initial profiling result based on ΔCt values. **B**, Secondary profiling result for 35 miRNAs which were most differentially expressed. It is obvious that Ubc9 represses *miR-224* (dark red); at the same time, Ubc9 upregulates *miR-200b* and *miR-559* (dark red). Relative miRNA levels were expressed as fold changes based on ΔΔCt values (Ubc9 vs vector control after normalization with U6), **C**, Further confirmation of the negative effect of both Ubc9-WT and Ubc9-DN on *miR-224* by TaqMan real-time PCR. **D**, Ubc9-siRNAs enhances *miR-224* expression, as detected by real-time PCR. The values in **C** and **D** are means ± SE of three independent experiments. **, P< 0.01.

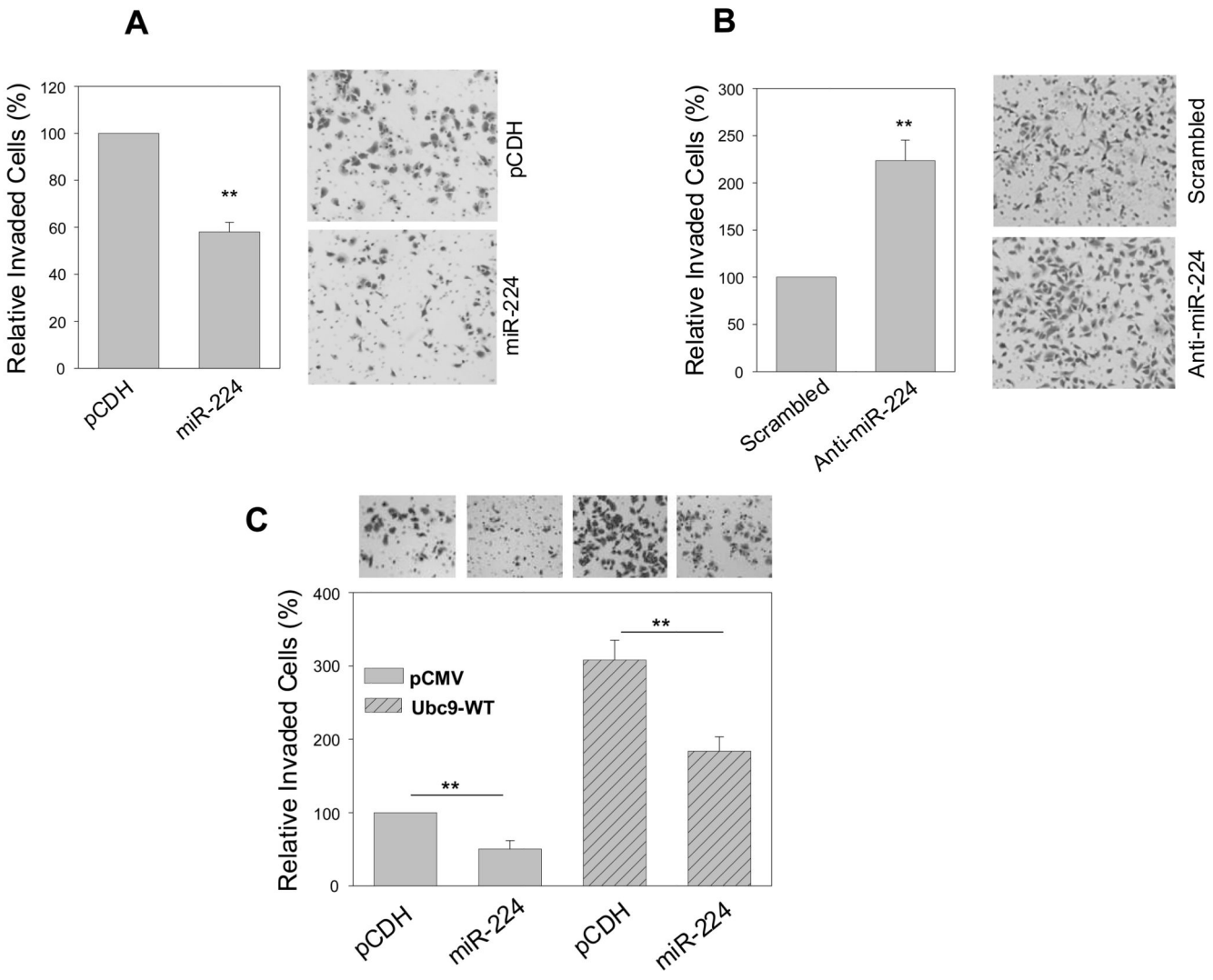

Fig. 4. While *miR-224* suppresses, anti-*miR-224* enhances cell invasion

A, Ectopic expression of *miR-224* suppresses cell invasion. MDA-MB-231 cells were infected with vector (pCDH) or *miR-224* expression vector (*miR-224*) and then were subject to cell invasion assays as described in Materials and Methods. **B**, Anti-*miR-224* enhances cell invasion. MDA-MB-231 cells were transiently transfected with scrambled LNA oligo (scrambled) or anti-*miR-224* LNA oligo. The cells were subjected to invasion assays 2 days after transfection. **C**, Ubc9-mediated invasion can be blocked by *miR-224*. Stable transfected MDA-MB-231 cells (Vector pCMV or Ubc9-WT) were first infected with vector (pCDH) or *miR-224*. Two days later, the cells were subjected to invasion assays as described in Materials and Methods. Values in **A**, **B**, and **C** are means ± SE of three independent experiments. ** $p<0.01$. Representative fields of invaded cells are shown either on the right (**A** and **B**) or above the chart (**C**).

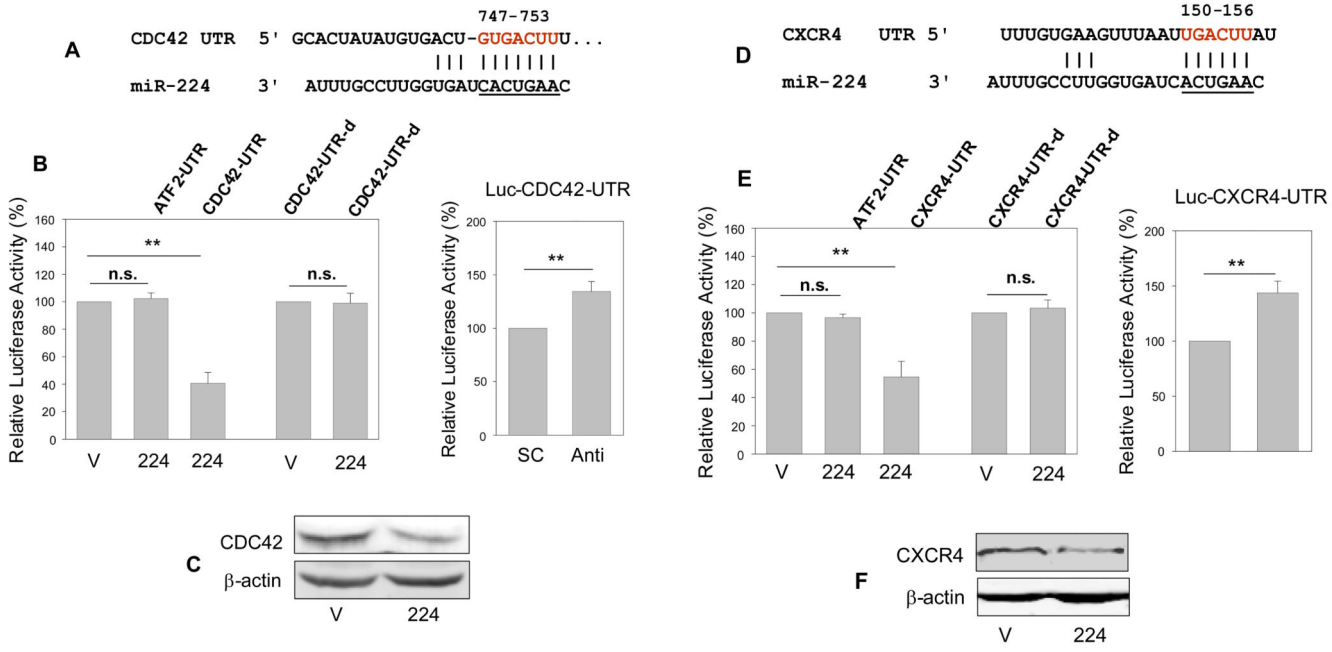

Fig. 5. Identification of CDC42 and CXCR4 as direct targets for *miR-224*

A, A putative *miR-224* binding site in the CDC42-UTR. The sequence in red matched with the *miR-224* seed sequence (underlined) was deleted in Luc-CDC42-UTR-d. **B**, Effect of *miR-224* on the luciferase activity of Luc-CDC42-UTR (CDC42-UTR) and Luc-CDC42-UTR-d (CDC42-UTR-d). 293T cells were transfected with Luc-CDC42-UTR along long with vector control (V) or *miR-224* (224), scrambled LNA oligo (SC) or anti-*miR-224* LNA oligo (Anti). Similarly, 293T cells were transfected with Luc-ATF2-UTR (ATF2-UTR) along with vector control or *miR-224*. Luciferase assays were carried out 24 h after transfection. Only one normalized vector control (100%) is shown here. ATF2-UTR serves as a negative control. **C**, Suppression of the endogenous CDC42 protein by *miR-224* in MDA-MB-231 cells. **D**, A putative *miR-224* binding site in the CXCR4-UTR (in red) which was deleted in Luc-CXCR4-UTR-d. **E**, Effect of *miR-224* on the luciferase activity of Luc-CXCR4-UTR (CXCR4-UTR) and Luc-CXCR4-UTR-d (CXCR4-UTR-d). Luciferase assays were performed same as in **B**. **F**, Suppression of the endogenous CXCR4 protein by *miR-224* in MDA-MB-231 cells. The values in **B** and **D** are means ± SE of three independent experiments. **, P< 0.01; n.s, not significant.

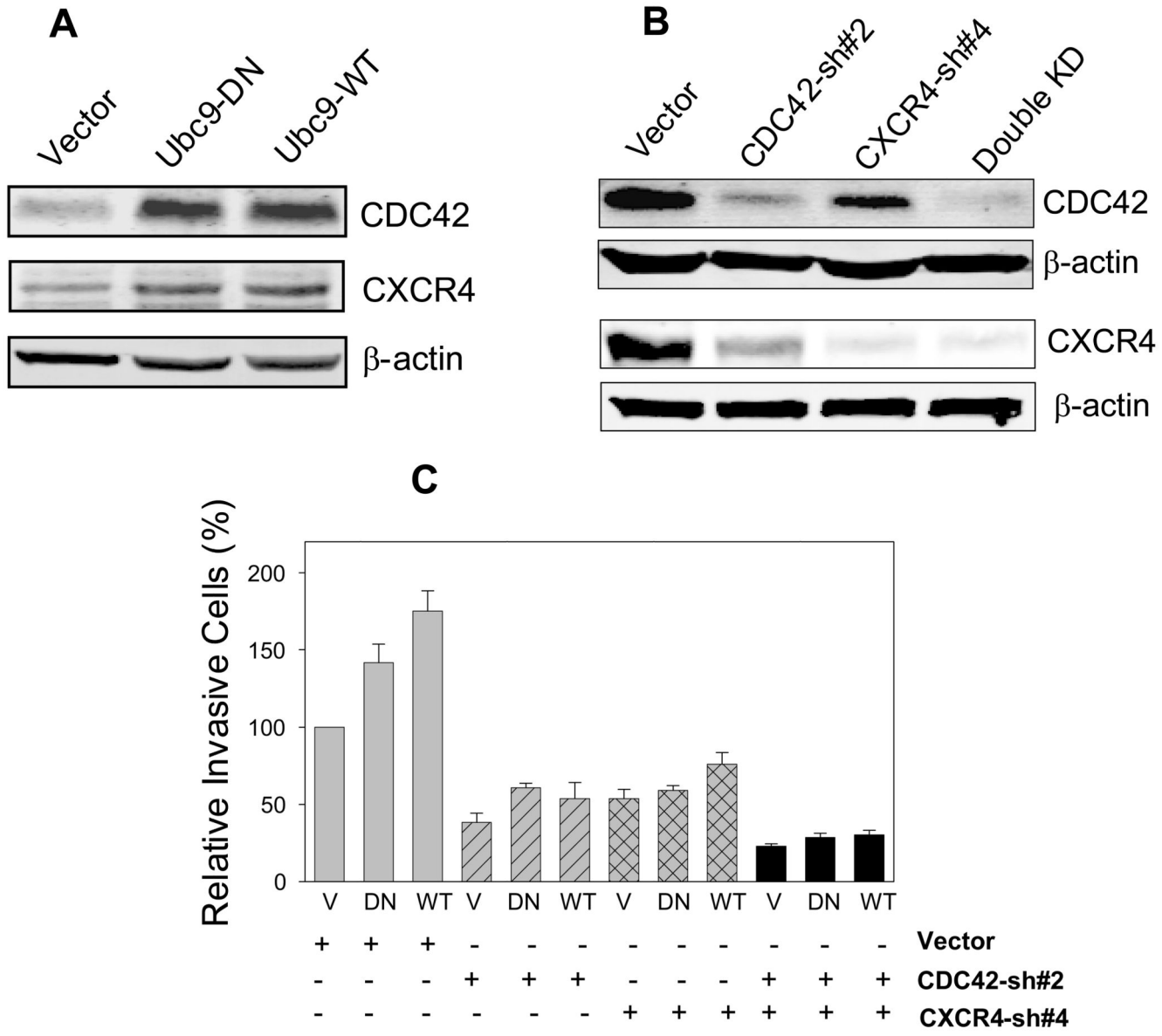

Fig. 6. Suppression of CDC42 and CXCR4 inhibits Ubc9-mediated cell invasion

A, Western blot reveals upregulation of CDC42 and CXCR4 in Ubc9-DN and Ubc9-WT cells, respectively. **B**, Suppression of CDC42 and CXCR4 by RNAi. MDA-MB-231 cells were infected with CDC42 or CXCR4 shRNA and their expression levels were detected by western blot. **C**, Both CDC42 and CXCR4 shRNAs suppress cell invasion. The cells as indicated were subject to Matrigel chamber assays as detailed in Materials and Method. Values were means ± SE of three independent experiments.