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Characterization of dual PTEN and p53-targeting microRNAs identifies microRNA-638/Dnm2 as a two-hit oncogenic locus

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Summary

Tumor suppressor genes (TSGs) are often concomitantly lost or mutated in human cancers and have been shown to act synergistically to promote tumorigenesis. In addition to genomic alterations, post-transcriptional regulation by microRNAs (miRNAs) represents another mechanism by which TSG expression is dysregulated in cancers. Although miRNAs which target critical TSGs such as *PTEN* or *p53* have been identified, little is known about miRNAs that concomitantly regulate both these key TSGs. In this study, we characterize miR-518c* and miR-638 as dual *PTEN* and *p53*-targeting miRNAs which are upregulated in multiple human cancers. We focus on miR-638 and show that it associates independently with these two tumor suppressor transcripts as well as BRCA1, a known miR-638 target. We find that miR-638 overexpression promotes tumorigenesis and demonstrate cooperativity between miR-638 and its host gene Dnm2, suggesting that the Dnm2 locus encodes two distinct oncogenic components which play important roles in tumorigenesis.

Introduction

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is one of the most frequently lost and mutated tumor suppressor genes (TSGs) in a wide spectrum of human cancers. PTEN encodes a plasma-membrane lipid phosphatase that functions predominantly as an inhibitor of the proto-oncogenic PI3K-AKT signaling pathway which regulates cell growth and survival (Song et al., 2012). *In vivo* models which demonstrate that heterozygous *PTEN* loss and even subtler reduction in its expression lead to increased

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incidence of spontaneous tumors in multiple tissue types provide further support for the key tumor suppressive function of PTEN (Berger et al., 2011; Di Cristofano et al., 1998).

P53, epitomized as the 'guardian of the genome', is one of the most studied and frequently mutated or deleted TSGs. It encodes a transcription factor which regulates the expression of target genes involved in DNA repair, cell cycle arrest, apoptosis, senescence and other cellular processes (Vousden and Lane, 2007). Additional evidence for the importance of *p53* in tumorigenesis was provided by mouse models which demonstrated that both the heterozygous or homozygous loss of p53 leads to an increased risk of cancer in a variety of tissues (Donehower et al., 1992).

The functions of these two TSGs are intricately intertwined. PTEN has been shown to physically associate with p53, regulate its DNA binding activity and protect it from Mdm2-mediated degradation (Freeman et al., 2003; Mayo et al., 2002). Conversely, p53 has been reported to regulate PTEN transcription (Stambolic et al., 2001). Several studies have examined the functional relevance of this reciprocal cooperation between *PTEN* and *p53*: Chen *et al* found that although Pten inactivation leads to non-lethal invasive cancer in the prostate after long latency and p53 inactivation did not lead to cancer, combined Pten and p53 inactivation resulted in invasive prostate cancer in mice with lethality by 7 months (Chen et al., 2005). Puzio-Kuter *et al* observed that Pten or p53 single mutant mice did not develop bladder tumors up to a year of age, but inactivation of both genes led to large tumors with 100% penetrance by 6 months (Puzio-Kuter et al., 2009). Andjelkovic *et al* found that the loss of both these genes occurred frequently in non-small cell lung cancer patients, was associated with increased lymph node invasion and reduced survival (Andjelkovic et al., 2011).

Collectively, these reports suggest that these two TSGs may cross-talk at multiple levels and that their compound inactivation is an event that is strongly selected for in human cancers. This in turn led us to hypothesize that mechanisms which induce the concomitant downregulation of both these tumor suppressors may play key roles in cancer development. Aside from genetic deletions or mutations which result in the heterozygous or homozygous loss of *PTEN* or *p53* function, other mechanisms which contribute to the dysregulation of *PTEN* or *p53* expression in cancers include epigenetic silencing, transcriptional repression, post-translational modifications and post-transcriptional regulation by microRNAs (miRNAs) (Song et al., 2012).

MiRNAs are small non-coding RNAs which modulate gene expression by binding to specific recognition sites on target transcripts (Bartel and Chen, 2004). Several studies have identified miRNAs that target either the *PTEN* or *p53* transcripts (Hermeking, 2012; Tay et al., 2013). However, little is known about miRNAs that concomitantly regulate both these key TSGs. In this study, we characterize miR-518c* and miR-638 as dual *PTEN* and *p53*-targeting miRNAs. We find that overexpression of miR-638, which is encoded in the oncogenic Dnm2 locus, promotes tumorigenic properties including cell proliferation, migration and invasion. These data suggest that the Dnm2 gene comprises a two-hit oncogenic locus which plays an important role in cancer progression.

Results

Identification of potential dual PTEN and p53-targeting miRNAs

We first performed miRNA target prediction analyses to identify miRNAs which may target both full-length *PTEN* and *p53* transcripts (Table S1). As the combined loss of PTEN and p53 has been shown to promote prostate cancer metastasis (Abou-Kheir et al., 2011), we next examined the expression profiles of these miRNAs in prostate cancer. Of the 51 predicted dual-targeting miRNAs, 14 were upregulated in human metastatic prostate cancer samples (Figure 1A and S1A). We selected miRs-518c*, 584 and 638 for experimental validation as their expression levels were also increased in colon and kidney cancers (Figure S1B and C), additional cancers with frequent loss of PTEN and p53.

Next, we investigated their effect on endogenous *PTEN* and *p53* levels. Overexpression of miRs-518c*, 584 and 638 resulted in a significant decrease in PTEN protein levels in both HCT116 colon cancer and U2OS osteosarcoma cell lines which express wild-type *PTEN* and *p53* (Figure 1B and C). This was accompanied by a concomitant increase in Akt activation. Similarly, miRs-518c*, 584 and 638 reduced p53 protein levels and decreased expression of p21, a downstream effector of p53 (Figure 1D and E).

Direct regulation of the PTEN and p53 transcripts by miRs-518c* and 638

As PTEN and p53 have been shown to reciprocally co-regulate each other as discussed above, we next investigated whether these observed effects were due to *bona fide* miRNA targeting or were alternatively a result of protein-mediated crosstalk. PC3 prostate cancer cells were selected as an ideal model system for this purpose as they are PTEN-null and p53-null, and do not express detectable levels of either transcript or protein (Isaacs et al., 1991; Vlietstra et al., 1998).

miRs-518c*, 584 and 638 have predicted target sites in the 5’UTR and/or coding region of *PTEN* and *p53* (Figure S2A). To examine whether these predictions represented functional binding sites, we first constructed vectors expressing the 5’UTR+coding region of *PTEN* and *p53*, and co-transfected them into PC3 cells with miRs-518c*, 584 or 638. Overexpression of miRs-518c* and 638 significantly downregulated the expression of both 5’UTR+CDS constructs, suggesting that their effect on *PTEN* and *p53* is partly 3’UTR-independent (Figure 2A and B). Although miR-584 also downregulated expression of exogenous PTEN, it did not have a significant effect on exogenous p53 expression. This suggests that the predicted miR-584 MREs in *p53* are not *bona fide* MREs, and that its effect on endogenous *p53* is most likely indirect.

Next, we generated luciferase reporter constructs harbouring the various fragments (5’UTR, CDS [-ATG], 3’UTR) of the *PTEN* and *p53* transcripts. Overexpression of miR-638 resulted in a modest but significant decrease in expression of the PTEN-5’UTR, CDS (-ATG) and 3’UTR reporters, as well as the p53-CDS (-ATG) and 3’UTR reporters (Figure 2C and D). It did not affect the p53-5’UTR reporter. These results are consistent with the location of predicted MREs on these transcripts.

miR-518c* overexpression reduced expression of the PTEN-CDS(-ATG) and p53-5'UTR constructs, consistent with the location of several predicted MREs (Figure 2C and D). However, it did not affect expression of the PTEN-5'UTR or p53-CDS(-ATG) reporters, suggesting that these predictions were not *bona fide* MREs. Furthermore, it significantly reduced expression of the PTEN-3'UTR reporter, suggesting the existence of additional MREs.

miR-584 overexpression reduced expression of the PTEN-5'UTR and PTEN-CDS(-ATG) constructs, consistent with the location of its predicted PTEN MREs (Figure 2C). Its ability to reduce expression of the PTEN-3'UTR reporter suggests that it may also have additional target sites there. Consistent with our earlier observations, it did not have a significant effect on any of the *p53* reporters.

Taken together, these data provide evidence that *PTEN* is a *bona fide* target of miR-584 and suggest that both miRs-638 and 518c* are dual *PTEN* and *p53* targeting miRNAs.

miR-638 independently targets PTEN, p53 and BRCA1

Intriguingly, *BRCA1* has been identified as a target of miR-638 (Nicoloso et al., 2010). *BRCA1* is an important TSG involved in DNA damage repair and the maintenance of genomic stability, and the *BRCA1* protein has been shown to reciprocally co-regulate *p53* and Akt signalling (Altiok et al., 1999; Zhang et al., 1998). To disentangle potential miRNA-mediated regulation from protein-dependent crosstalk, we performed biotinylated miRNA pulldowns to examine the direct association of miR-638 with each of these tumor suppressor transcripts. We found that *PTEN*, *p53* and *BRCA1* transcripts were enriched in miR-638 pulldowns, suggesting that they all independently associate with miR-638 (Figure 2E).

miR-638 was shown to regulate *BRCA1* by binding to a single MRE in its coding region. To identify the specific MREs responsible for its regulation of *PTEN* and *p53*, we cloned the predicted miR-638 MREs downstream of a luciferase reporter. Overexpression of miR-638 resulted in a modest but significant reduction in the levels of all these reporter constructs (Figure S2B), except for the PTEN-C2 MRE which appeared to have a cell-type-dependent effect.

Although miRNA binding to MREs in the coding regions and 3'UTRs of target genes generally results in the repression of gene expression, miRNA binding to 5'UTRs may enhance translation (Orom et al., 2008). To investigate the potential context-dependent effect of the miR-638 5'UTR and CDS MREs, we generated reporter constructs with the 5'UTR MREs upstream of the luciferase reporter, and the CDS MREs just upstream and in-frame with the luciferase stop codon. Overexpression of miR-638 resulted in a modest but significant reduction in the levels of these PTEN 5'1, 5'3, C1 and p53 C1 MRE reporter constructs in PC3 cells (Figure S2C). These data are consistent with our previous observations when they were cloned into the luciferase 3'UTR and suggest that these MREs do not function in a context-dependent manner. However, we no longer saw a significant reduction in luciferase activity with the PTEN 5'2 MRE cloned into the luciferase 5'UTR or

the PTEN C2 MRE cloned into the luciferase coding region, suggesting that these two MREs may not be functional in their endogenous contexts.

We next performed site-directed mutagenesis of the seed regions of the CDS and 3'UTR MREs in the luciferase reporter constructs harbouring the corresponding full-length transcript fragments. Mutation of the PTEN C1 and p53 3'1 MREs resulted in complete rescue of the miR-638 mediated suppression of the respective luciferase constructs, while mutation of the PTEN 3'1 and p53 C1 MREs resulted in a partial but significant rescue of the respective luciferase constructs (Figure S2D). These results provide further support for the functional role of these MREs in miR-638 mediated PTEN and p53 regulation.

Overexpression of miRs-638 and 518c* *promotes tumorigenesis*

PTEN and p53 have been shown to function as important regulators of cell migration, invasion and proliferation. We thus hypothesized that miRs-518c* and 638 may be critical modulators of these hallmarks of tumorigenesis. *In vitro* wound healing and Boyden chamber assays demonstrated that overexpression of miR-518c* or miR-638 resulted in a significant increase in both cell migration (Figure 3A, S3A and B) and invasion (Figure 3B and S3C). Additionally, miR-518c* or miR-638 overexpression increased cell proliferation (Figure 3C) and enhanced anchorage-independent growth in soft agar (Figure 3D, S3D and E). Similar effects on target gene expression, cell proliferation and migration were observed with 2nM of transfected mimic (Figures 4A–C). As this amount of transfected mimic is more representative of the fold induction observed in cancers (Figure S3F), these data provide support for the functional relevance of the upregulation of these miRNAs in various pathophysiological conditions.

We subsequently focused our attention on miR-638 due to its ability to target *PTEN*, *p53* and *BRCA1* and as it had a consistently stronger effect on enhancing tumorigenic potential *in vitro*. Notably, we found that miR-638 overexpression increased the growth of colon cancer xenograft tumors in nude mice (Figure S3G). Next, we determined endogenous miR-638 expression levels in a panel of cell lines with wild-type PTEN, p53 and BRCA1 (Figure S4A). All cell lines tested expressed detectable levels of miR-638, with the highest levels observed in U2OS and HCT116 cells. Inhibition of endogenous miR-638 resulted in a significant upregulation of PTEN and p53 protein levels (Figure 4E), with a concomitant decrease in cell proliferation, migration and invasion (Figure 5F and G). These data are complementary to those observed in the overexpression experiments and provide further evidence for the functional role of physiological levels of miR-638 in regulating PTEN and p53 levels.

miR-638 is a functionally relevant component of the oncogenic DNM2 locus

The miR-638 precursor is located in the first intron of the DNM2 gene (Figure 5A). DNM2 is a microtubule-associated, GTP-binding protein and recent reports have demonstrated that its overexpression promotes growth, migration and invasion in various cancers (Eppinga et al., 2012; Feng et al., 2012). On this basis, we hypothesized that the DNM2 locus may represent a two-hit oncogenic locus which produces both the DNM2 protein and miR-638.

To test this hypothesis, we first overexpressed miR-638 and DNM2 independently and in combination in both HCT116 and U2OS cells. As previously reported, DNM2 overexpression led to an increase in cell proliferation (Figure 5B) (Feng et al., 2012). The concurrent overexpression of both miR-638 and DNM2 significantly augmented cell growth above that observed with either miR-638 or DNM2 alone (Figure 5B). Next, we investigated potential cooperativity between miR-638 knockdown and DNM inhibition. U2OS cells were selected for proof-of-principle studies as they expressed the highest levels of endogenous miR-638. Growth curve analyses demonstrated that the combined effect of miR-638 knockdown and either Dynasore or MiTMAB, two small molecule inhibitors of DNM function, led to a reduction in cell proliferation which was significantly greater than that observed with miR-638 knockdown or Dynasore or MiTMAB treatment alone (Figure 5C). These data suggest that miR-638 and the DNM2 protein represent two distinct oncogenic components encoded by the DNM2 locus.

Discussion

In this study, we examine miRNA-mediated post-transcriptional regulation of the full-length *PTEN* and *p53* transcripts and study their role in promoting tumorigenesis. We characterize miR-518c* and miR-638 as *PTEN* and *p53*-targeting miRNAs, and confirm previous reports which identify *BRCA1* as an additional miR-638 target (Li et al., 2012; Nicoloso et al., 2010). These miRNAs may represent a powerful mechanism to simultaneously modulate levels of these key TSGs which are known to cooperate reciprocally to inhibit tumorigenesis.

Existing studies have focused exclusively on the 3'UTRs of *PTEN* and *p53* as sites for miRNA regulation. It has been shown in both cancer cell lines and primary tumors that transcripts often have shorter 3'UTRs in cancer cells compared to untransformed cells and thus may be subject to less 3'UTR-dependent miRNA regulation (Lembo et al., 2012; Mayr and Bartel, 2009). For example, *PTEN*, *p53* and *BRCA1* have 3 (Lianoglou et al., 2013), 3 and 7 validated alternative polyadenylation sites respectively. It is thus conceivable that the ability of miR-638 and 518c* to bind and regulate these transcripts in a partly 3'UTR-independent fashion may enable them to modulate the expression of a larger subset of transcript variants. This may be of particular relevance in cancer cells with reduced 3'UTR-dependent miRNA regulation. Interestingly, the miR-638 binding site in *BRCA1* identified by Nicoloso *et al* was in its coding region.

The role of these miRNAs in tumorigenesis remains largely uncharacterized. miR-518c* is located in the chromosome 19 miRNA cluster (C19MC), which is the largest human miRNA gene cluster discovered to date (Bortolin-Cavaille et al., 2009). This miRNA cluster has been reported to be amplified in aggressive embryonal brain and parathyroid tumors and epigenetically reactivated through DNA demethylation in gastric cancer (Li et al., 2009; Tsai et al., 2009; Vaira et al., 2012). Specifically, elevated levels of miR-518c* were reported to be significantly associated with reduced progression-free survival in bladder carcinoma (Dyrskjot et al., 2009).

The miR-638 precursor is located in the first intron of the Dnm2 gene. Our data suggest that the Dnm2 locus encodes two distinct proto-oncogenic components which play important roles in tumorigenesis. Copy number amplifications at this locus were observed in ovarian and uterine cancers (Figure S4B). In accordance with this, DNM2 transcript expression was found to be significantly elevated in multiple ovarian cancer datasets (Figure S4C). Critically, miR-638 amplification was found to be significantly associated with decreased patient survival in ovarian cancer (Figure S4D). Additional studies reported that high expression levels of miR-638 were significantly associated with shorter overall survival in adrenocortical carcinoma (Ozata et al., 2011) and squamous cell lung carcinoma (Landi et al., 2010). These data provide further support for the functional relevance of miR-638 in human cancers.

Interestingly, it has been reported that miR-638 is one of several miRNAs that is selectively packaged in membrane vesicles released from cancer cells (Jaiswal et al., 2012). A very recent study found that miR-638 was one of only three serum miRNAs inversely associated with overall survival in nasopharyngeal carcinoma patients (Liu et al., 2014). Aside from its function in a cell of origin, miR-638 may be able to regulate gene expression in recipient cells and thus play an important role in the intercellular regulation of cancer development.

Related work from our group and others has demonstrated that transcripts which contain binding sites for shared miRNAs can co-regulate each other by titrating miRNA availability, thus functioning as natural miRNA sponges or competing endogenous RNAs (ceRNAs) (Tay et al., 2014). In particular, much effort has focused on characterization of the PTEN ceRNA network. Our study suggests that in certain conditions whereby the abundance of *PTEN, p53* and *BRCA1* falls within the range for optimal ceRNA crosstalk, their ability to sequester miR-518c* and miR-638 may represent an additional mechanism of reciprocal co-regulation between these TSGs. Furthermore, existing studies which examine ceRNA crosstalk between protein-coding transcripts have been limited exclusively to 3’UTR-dependent regulation. We suggest that expanding ceRNA crosstalk analysis to include 5’UTRs and coding regions of protein-coding transcripts may lead to new insights into this network of competitive miRNA-RNA interactions and to the identification of novel TSGs or oncogenes based on their ceRNA function.

Experimental Procedures

MiRNA target prediction was performed using Rna22 (Miranda et al., 2006). MREs and transcript fragments were cloned into psicheck2 and pcDNA3.1 according to standard protocols. For the psicheck2 constructs, the 5’UTR fragments were cloned upstream of the luciferase coding region while the CDS and 3’UTR fragments were cloned in the luciferase 3’UTR. Site-directed mutagenesis was performed according to manufacturer’s protocols using the QuikChange Lightning Site-Directed Mutagenesis Kit from Agilent Technologies. HCT116, U2OS and PC3 cells were cultured under standard conditions. MiRNA mimics and inhibitors were purchased from Dharmacon. Transient transfections were performed with 2nM or 100nM of miRNA mimic or inhibitor with Dharmafect 1 according to manufacturer’s protocols. Western blot, real-time PCR and luciferase assays were performed as previously described (Tay et al., 2008). Biotinylated miRNA pulldowns were performed

as previously described (Lal et al., 2011). Growth curves and soft agar assays were performed as previously described (Tay et al., 2011). Wound healing, transwell migration and xenograft assays were performed according to standard protocols. Invasion assays were performed using Matrigel Invasion Chambers from BD Biosciences according to manufacturer's protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Identification of microRNAs targeting multiple tumor suppressor genes
- These microRNAs are upregulated in multiple human cancers
- Their overexpression increases cell migration, invasion and proliferation
- miR-638 cooperates with its host gene Dnm2 to promote tumorigenesis

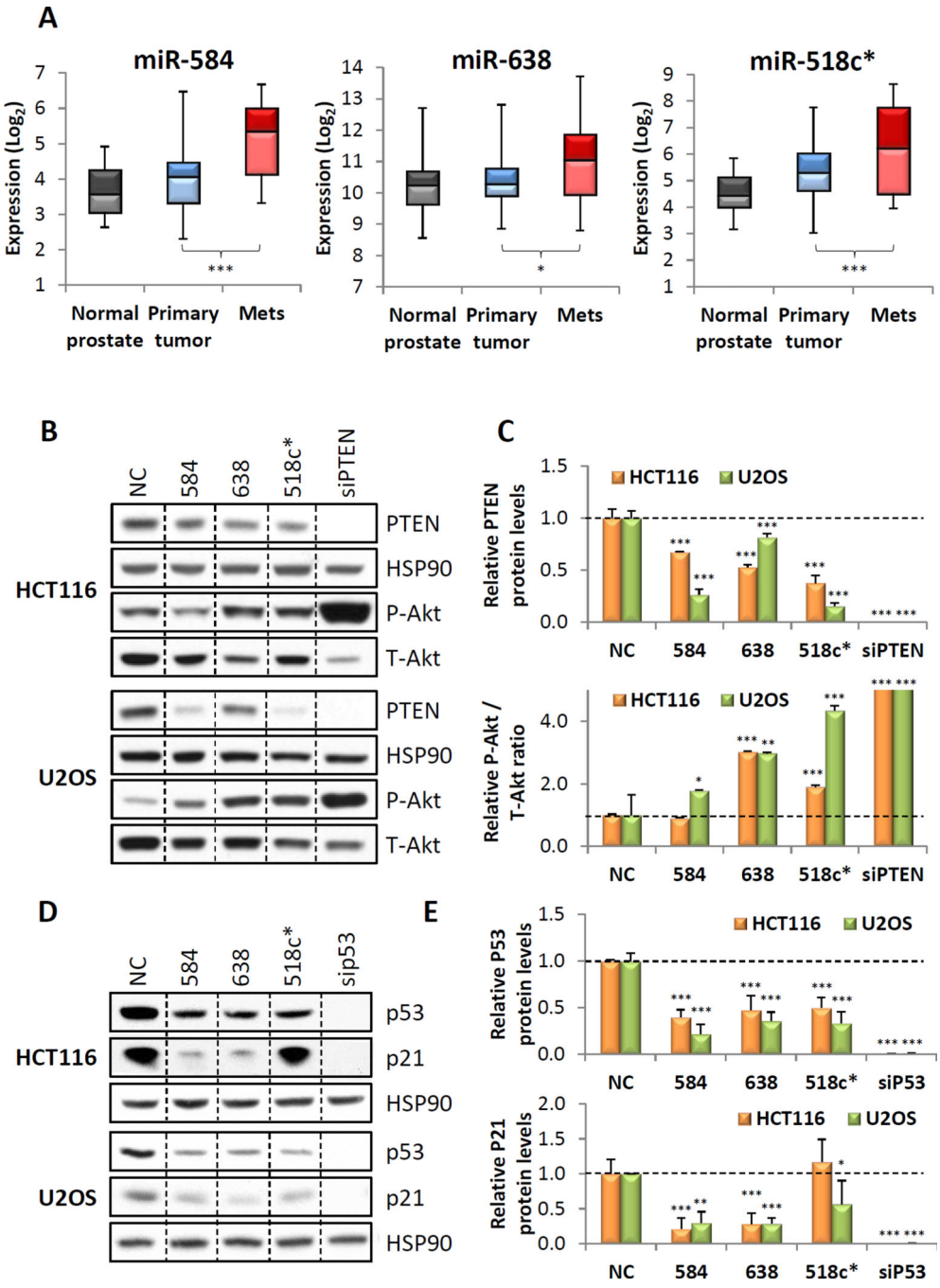

Figure 1. Identification of potential dual PTEN and p53-targeting miRNAs

(A) Expression of miRs-584, 638 and 518c* in primary and metastatic prostate cancer compared to normal prostate. **(B,C)** Effect of miR-584, 638 or 518c* overexpression on endogenous PTEN and P-Akt expression. Representative western blots are shown in (B), and quantitation of all blots is shown in (C). **(D,E)** Effect of miR-584, 638 or 518c* overexpression on endogenous p53 and p21 expression. Representative western blots are shown in (D), and quantitation of all blots is shown in (E). (A,C,E) *p < 0.05; **p , 0.01; ***p < 0.001. See also Figure S1 and Table S1.

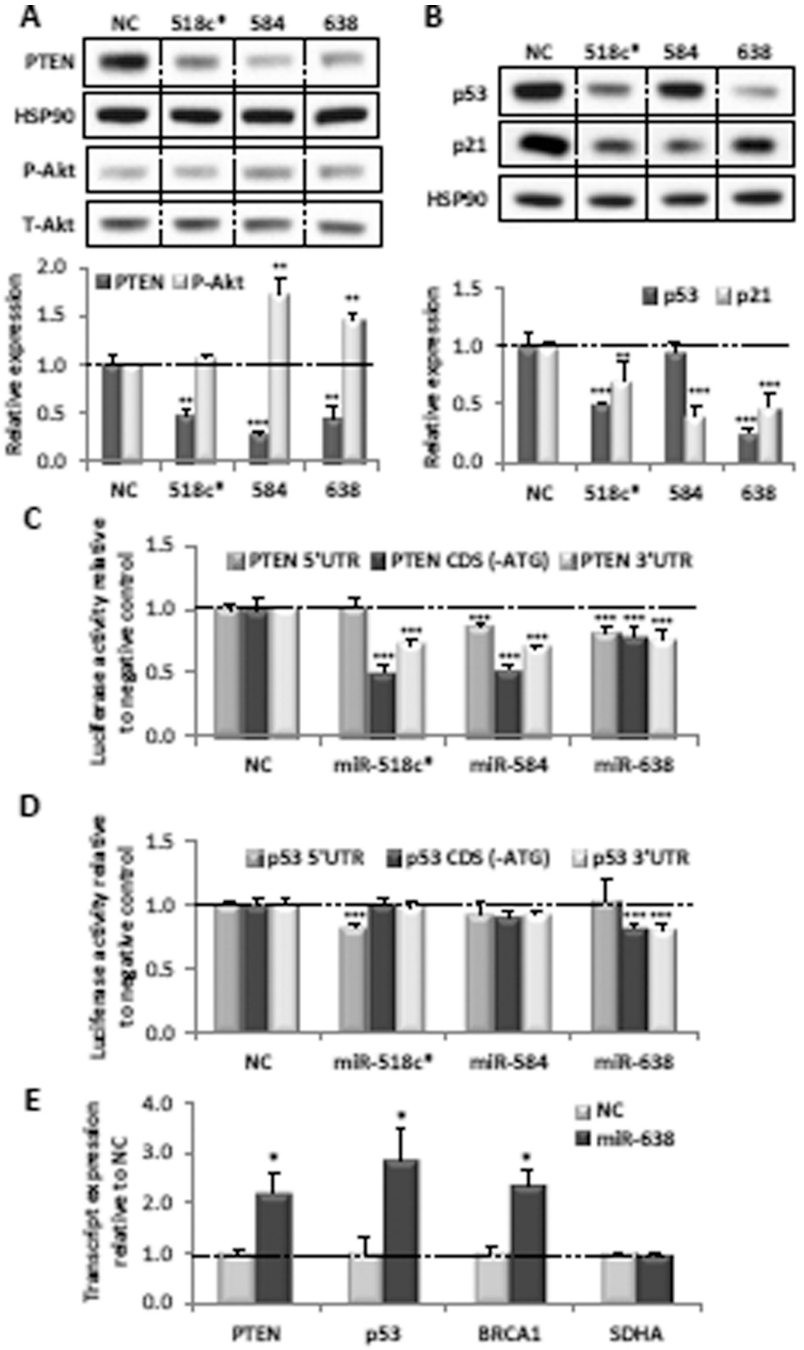

Figure 2. miRs-638 and 518c* directly target PTEN and p53

(A) Expression of exogenous PTEN and P-Akt in PC3 cells after co-transfection of the negative control (NC), miR-518c*, miR-584 or miR-638 with a plasmid construct expressing only the PTEN 5'UTR+CDS. **(B)** Expression of exogenous p53 and p21 in PC3 cells after co-transfection of the NC, miR-518c*, miR-584 or miR-638 with a plasmid construct expressing only the p53 5'UTR+CDS. **(C,D)** Luciferase validation assays demonstrating the effect of miR-518c*, miR-584 or miR-638 overexpression on the 5'UTR, 3'UTR and ATG-less CDS fragments of (C) PTEN and (D) p53 relative to the NC in PC3

cells. **(E)** Expression of PTEN, p53 and BRCA1 mRNAs in biotinylated miR-638 pulldowns from U2OS cells. (A–E) Mean ± SD; n ≥ 4; *p < 0.05; **p , 0.01; ***p < 0.001. See also Figure S2.

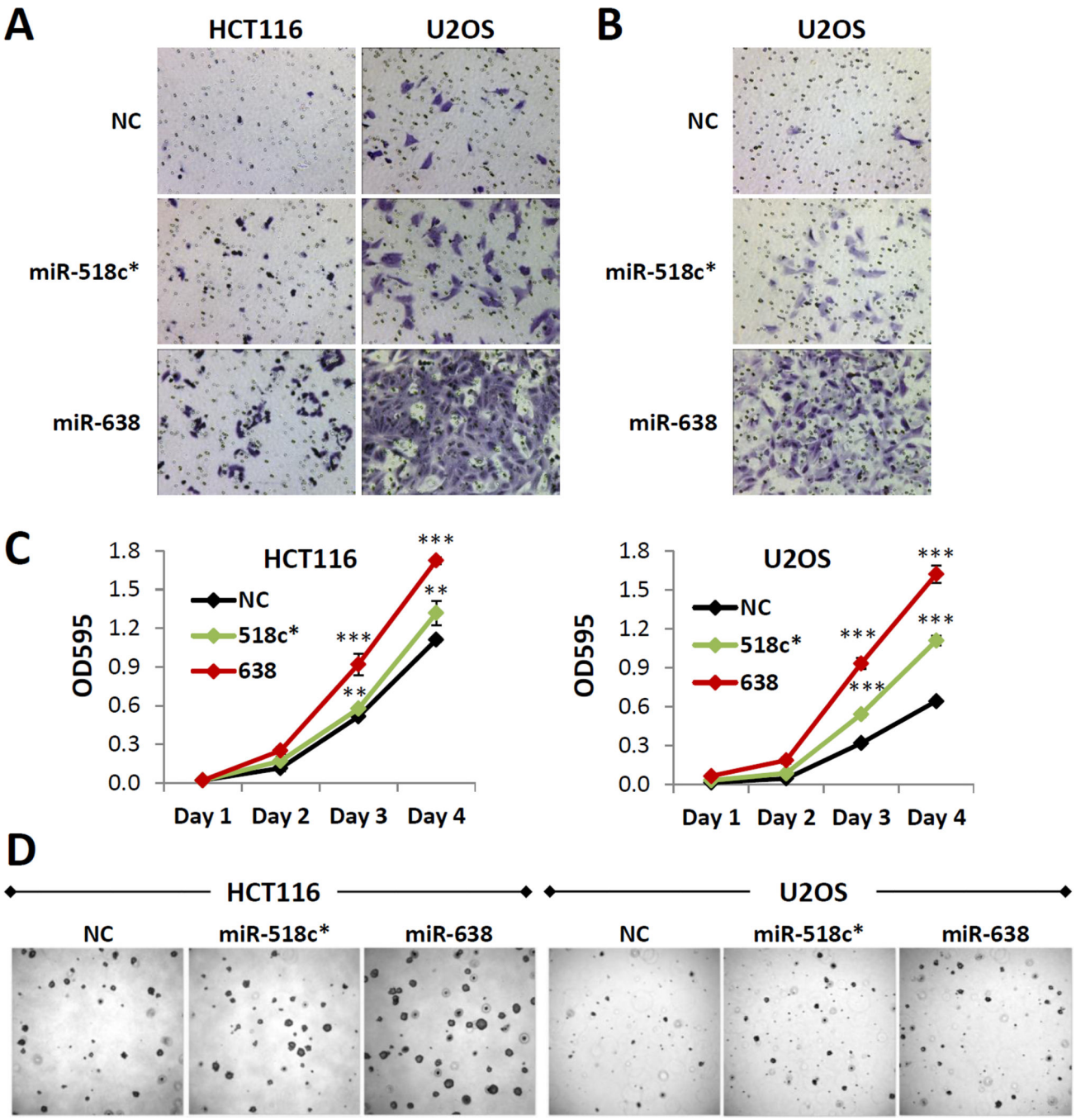

Figure 3. Overexpression of miRs-638 and 518c* promotes tumorigenesis

(A–D) Effect of miR-638 or 518c* overexpression on cell migration (A) and invasion (B), proliferation (C) and anchorage-independent growth (D) *in vitro*. **(E)** Effect of miR-638 overexpression on HCT116 xenograft tumor formation in nude mice. (C) Mean ± SD; n ≥ 4; *p < 0.05; **p , 0.01; ***p < 0.001. See also Figure S3.

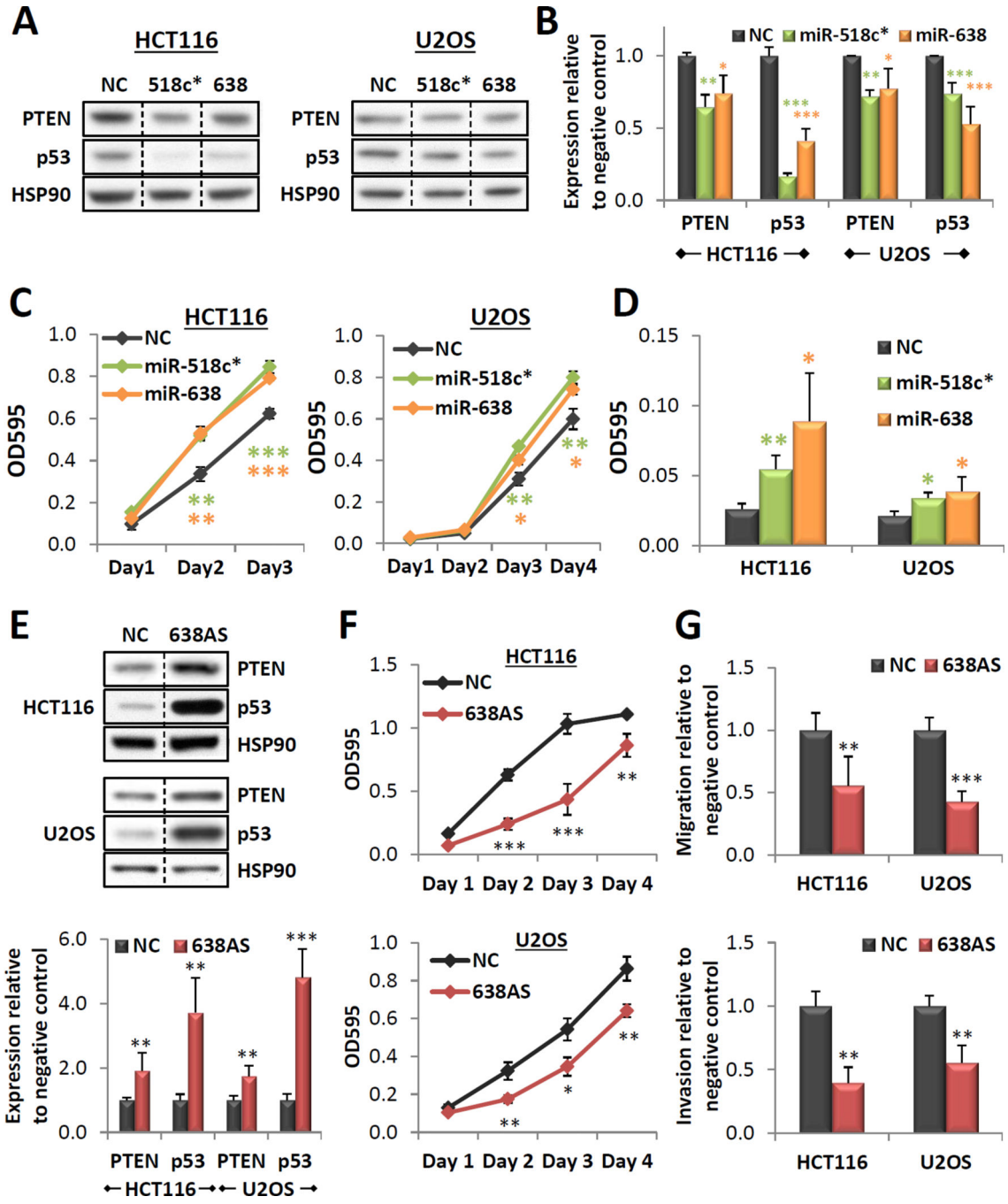

Figure 4. Functional relevance of physiological levels of miRs-638 and 518c*

(A,B) Effect of miR-518c* or 638 overexpression (transfected at 2nM) on endogenous PTEN and p53 expression. Representative western blots are shown in (A) and quantitation of all blots is shown in (B). **(C,D)** Effect of miR-518c* or 638 overexpression (transfected at 2nM) on cell proliferation (C) and migration (D). **(E)** Effect of miR-638 inhibition (638AS) on endogenous PTEN and p53 expression. Representative western blots are shown in the top panel and quantitation of all blots is shown below. **(F,G)** Effect of miR-638 inhibition on

cell proliferation (F), migration (G, top panel) and invasion (G, botton panel). Mean ± SD; n ≥ 4; *p < 0.05; **p , 0.01; ***p < 0.001.

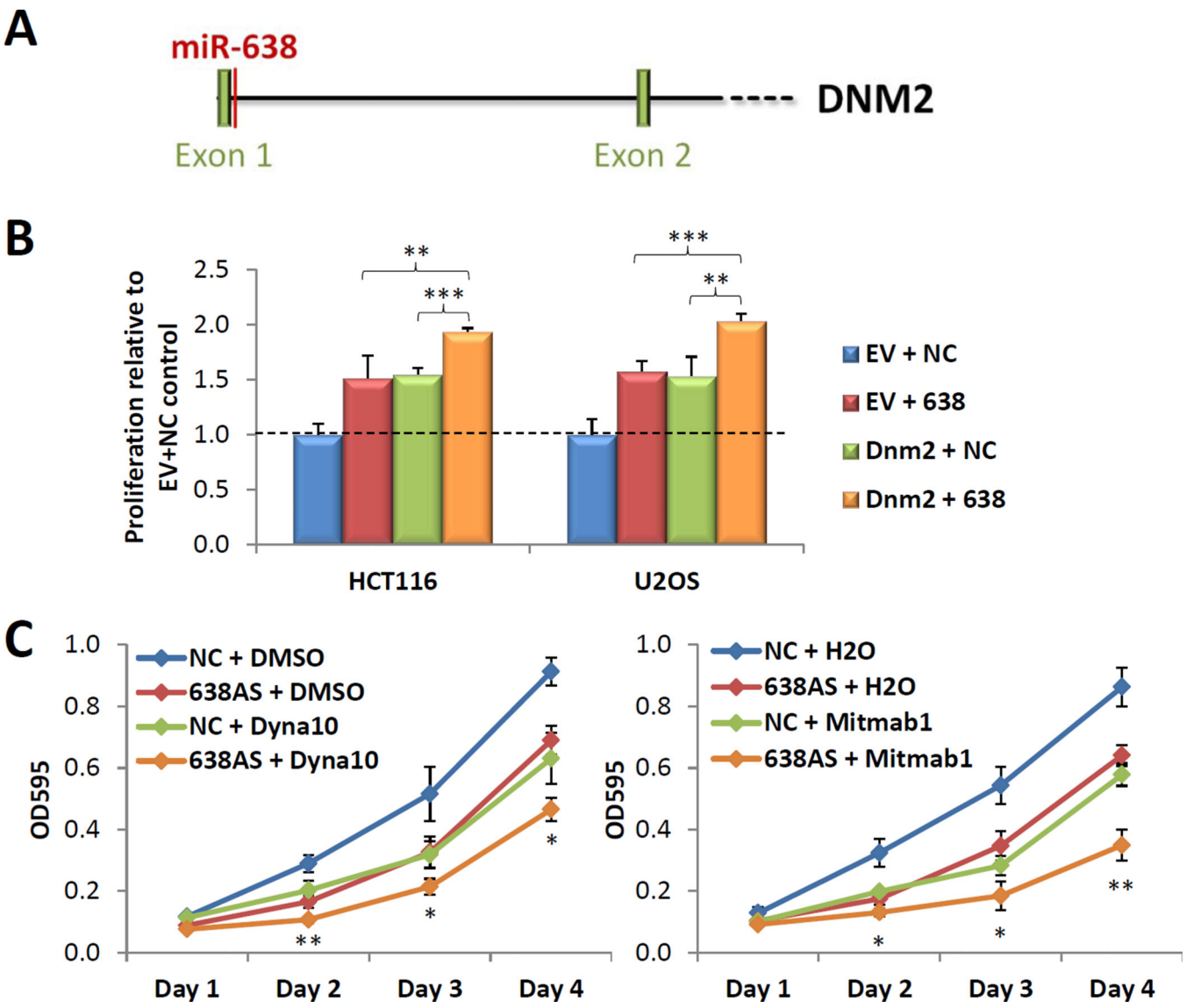

Figure 5. miR-638 is part of the oncogenic DNM2 locus

(A) Schematic depicting the location of the miR-638 precursor in the DNM2 gene. **(B)** Effect of the combinatorial overexpression of miR-638 and Dnm2 on cell proliferation. **(C)** Combined effect of miR-638 knockdown and the Dynamin inhibitors Dynasore (left panel) and Mitmab (right panel) on cell proliferation in U2OS cells. (B,C) Mean ± SD; n ≥ 4; *p < 0.05; **p , 0.01; ***p < 0.001. See also Figure S4.