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RESEARCH COMMUNICATION

DLC1 is a chromosome 8p tumor suppressor whose loss promotes hepatocellular carcinoma

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Deletions on chromosome 8p are common in human tumors, suggesting that one or more tumor suppressor genes reside in this region. Deleted in Liver Cancer 1 (*DLC1*) encodes a Rho-GTPase activating protein and is a candidate 8p tumor suppressor. We show that DLC1 knockdown cooperates with Myc to promote hepatocellular carcinoma in mice, and that reintroduction of wild-type DLC1 into hepatoma cells with low DLC1 levels suppresses tumor growth in situ. Cells with reduced DLC1 protein contain increased GTP-bound RhoA, and enforced expression a constitutively activated *RhoA* allele mimics DLC1 loss in promoting hepatocellular carcinogenesis. Conversely, down-regulation of RhoA selectively inhibits tumor growth of hepatoma cells with disabled DLC1. Our data validate DLC1 as a potent tumor suppressor gene and suggest that its loss creates a dependence on the RhoA pathway that may be targeted therapeutically.

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Tumor suppressor genes act in signaling networks that protect against tumor initiation and progression, and can be inactivated by deletions, point mutations, or promoter hypermethylation. Although tumor suppressors are rarely considered direct drug targets, they can negatively regulate pro-oncogenic signaling proteins that are amenable to small molecule inhibition. For instance, NF1 inhibits the Ras signaling pathway, which is deregulated in many cancers and has been pursued for its therapeutic potential (Downward 2003). Similarly, PTEN inhibits the PI3–kinase pathway, and inhibitors of PI3K pathway components such as PI3K, AKT, and mTORs have entered clinic trials (Luo et al. 2003).

Recurrent chromosomal deletions found in sporadic cancers often contain tumor suppressor genes. For example, PTEN loss on chromosome 10q23 frequently occurs in various cancers and promotes tumorigenesis by deregulating the PI3 kinase pathway (Maser et al. 2007). Similarly, heterozygous deletions on chromosome 8p22 in many hepatocellular carcinomas (HCC) (Jou et al. 2004) and other cancer types, including carcinomas of the breast, prostate, colon, and lung (Matsuyama et al. 2001; Durkin et al. 2007). Several genes, including *DLC1*, *MTUS1*, *FGL1* and *TUSC3*, have been identified as candidate tumor suppressors in this region (Yan et al. 2004). *Deleted in Liver Cancer 1* (*DLC1*) is a particularly attractive candidate owing to its genomic deletion, promoter methylation, and underexpressed mRNA in cancer (Yuan et al. 1998, 2003a; Ng et al. 2000; Wong et al. 2003; Guan et al. 2006; Seng et al. 2007; Ying et al. 2007; Zhang et al. 2007; Pike et al. 2008; for review, see Durkin et al. 2007).

Despite its potential importance, functional data implicating DLC1 loss in tumorigenesis are lacking. *DLC1* encodes a RhoGAP protein that catalyzes the conversion of active GTP-bound RhoGTPase (*Rho*) to the inactive GDP-bound form and thus suppresses Rho activity (Yuan et al. 1998). DLC1 has potent GAP activity for RhoA and limited activity for CDC42 (Wong et al. 2003; Healy et al. 2008). When overexpressed, DLC1 inhibits the growth of tumor cells and xenografts (Yuan et al. 2003b, 2004; Zhou et al. 2004; Wong et al. 2005; Kim et al. 2007), but whether this requires its Rho-GAP activity or other functions remains unresolved (Qian et al. 2007; Liao et al. 2007). Most functional studies to date have relied on DLC1 overexpression and, as yet, none have documented that loss of DLC1 promotes transformation in vitro or tumorigenesis in vivo. Indeed, homozygous *dlc1* knockout mice die around embryonic day 10.5 (E10.5), and there is no overt phenotype in *dlc1* heterozygous mice (Durkin et al. 2005).

Our laboratory recently developed a "mosaic" mouse model whereby liver carcinomas can be rapidly produced with different genetic alterations by manipulation of cultured embryonic liver progenitor cells (hepatoblasts) followed by transplantation into the livers of recipient mice (Zender et al. 2005, 2006). We previously used this model to identify new oncogenes in HCC, which could be characterized in an appropriate biological and genetic context (Zender et al. 2006). Furthermore, using this system, we showed that shRNAs capable of suppressing gene function by RNAi could recapitulate the consequences of tumor suppressor gene loss on liver carcinogenesis (Zender et al. 2005; Xue et al. 2007). Here we combine this mosaic model and RNAi to validate DLC1 as a potent tumor suppressor gene and study its action in vivo.

Results and Discussion

Studies using low-resolution genome scanning methods have identified chromosome 8p deletions as common lesions in liver carcinoma and other tumor types. To confirm and extend these observations, we examined a se-

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ries of data sets of copy number alterations in HCC obtained using representational oligonucleotide microarray analysis (ROMA), a variation of array-based CGH that enables genome scanning at high resolution (Lucito et al. 2003). In a panel of 86 liver cancers, heterozygous deletions encompassing the *DLC1* were observed in 59 tumors (Fig. 1A,B; data not shown). Consistent with previous reports, these deletions were large (>5 Mb), encompassing >20 annotated genes but invariably included the *DLC1* locus. Indeed, heterozygous deletions of *DLC1* occurred more frequently than those observed for the well-established tumor suppressors such as *INK4a/ARF*, *PTEN*, and *TP53* (Fig. 1C). Furthermore, *DLC1* deletions were nearly as common as those for *TP53* in other major tumor types such as lung, colon, and breast (Fig. 1C). Again, most 8p deletions were large, although in breast cancer *DLC1* resided at a local deletion epicenter reminiscent of that surrounding the *INK4a/ARF* locus on chromosome 9p21 (Fig. 1D,E). Although we did not examine the status of the remaining allele in this tumor cohort, studies suggest that it can be silenced by promoter methylation (Yuan et al. 2003a; for review, see Durkin et al. 2007). Together, these data suggest that DLC1 loss plays an important role in human cancer but, in the absence of functional validation, are not conclusive.

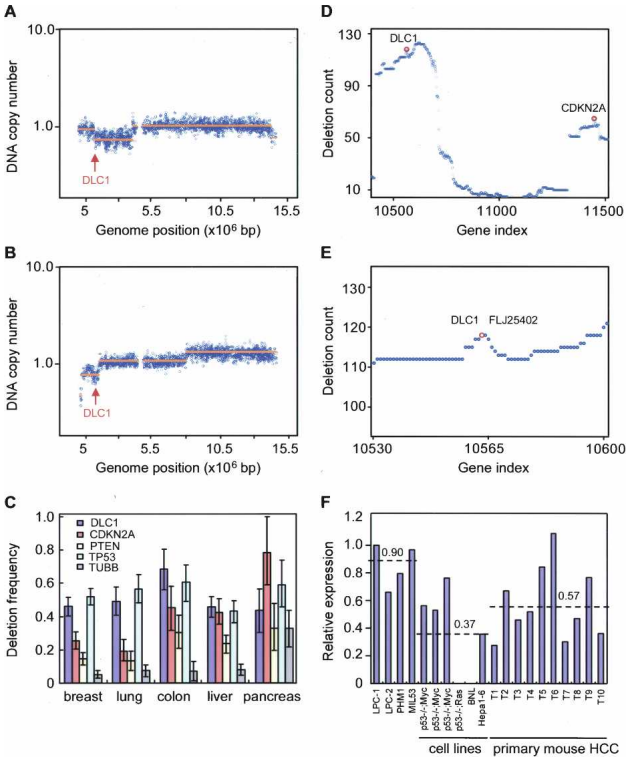

Figure 1. *DLC*1 is a candidate tumor suppressor on chromosome 8p22. (*A*,*B*) DNA copy number profiles for chromosome 8 of two representative human HCC samples reveals chromosome 8p22 deletions containing *DLC1*. The blue data points represent the averaged fluorescent ratio (tumor vs. normal) and the orange lines correspond to the value determined by copy number segmentation. Arrows denote the *DLC1* locus. (*C*) Deletion counts per case for *DLC1*, $p16^{INK4a}$, *PTEN*, *TP53*, and β-*Tubulin* (*TUBB*) in five types of human carcinomas. The counts were obtained from ROMA profiles of 257 breast, 137 colon, 86 liver, 213 lung, and 46 pancreas cancers, respectively. β-*Tubulin* serves as a negative control. The error bars indicate the 90% confidence intervals for each quantity. (*D*) Deletion counts in ROMA profiles of 257 human breast cancers. A deletion count in each profile set was obtained by finding the maximal tier number for 24,719 genes across the genome in each profile in the set and summing the result over the set. The counts are plotted against the gene ordinal number, with the genes sorted by their genomic transcription start position. Shown is chromosome 8 and 9. The points in the vicinity of *DLC1* and $p16^{INK4A}$ are highlighted by red circles. (*E*) High-magnification view of 8p22 region in *D*. *DLC1* and *FLJ25402* reside at a local deletion epicenter. *FLJ25402* is an uncharacterized gene. (*F*) qPCR analysis of DLC1 expression in mouse embryonic liver progenitor cells (LPC), immortalized liver progenitor cells (MIL53 and PHM1), mouse HCC cells, and primary mouse HCC tumors. Numbers indicate the average value of each group.

Human liver tumor cells with 8p deletions invariably expressed low *DLC1* mRNA (Supplemental Fig. S1). We also examined a series of murine liver carcinomas produced from embryonic hepatoblasts (Zender et al. 2006). None of the tumors we analyzed contained deletions on mouse chromosome 8qA4, the region containing the murine *dlc1* gene (data not shown), although many displayed reduced *dlc1* mRNA levels compared with parental hepatoblasts or immortalized liver progenitor cells (Fig. 1F). Thus, down-regulation of DLC1 expression can occur during murine hepatocellular carcinogenesis.

If DLC1 is a bona fide tumor suppressor in humans, we hypothesized that reductions in DLC1 levels should promote HCC in mice. To test this hypothesis and to avoid complications associated with the embryonic lethality of *dlc1* knockout animals, we decided to knockdown DLC1 expression in hepatoblasts using RNAi and test the ability of these cells to form tumors following engraftment into the livers of recipient mice. We therefore generated microRNA-based shRNA capable of efficiently suppressing DLC1 expression at mRNA and protein levels (Fig. 2A,B).

Our genomic analyses, together with previous reports, identify amplifications of the *Myc* oncogene and inactivation of the *p53* tumor suppressor as common events in HCC (Staib et al. 2003; data not shown). We therefore decided to test the impact of DLC1 loss in cells coexpressing Myc and lacking p53, which together are only weakly oncogenic in this model (Zender et al. 2006). p53-deficient liver progenitor cells were cotransduced with one retrovirus expressing Myc and another coexpressing a DLC1 shRNA with a GFP reporter, thus enabling imaging of cells with reduced DLC1 expression (Supplemental Fig. S2A,B). Interestingly, DLC1 knockdown had little impact on the colony forming ability of liver progenitor cells in vitro (Fig. 2C; Supplemental Fig. S3A).

Genetically modified liver progenitors were seeded into the livers of syngeneic recipients to assess their ability to form tumors in situ. In contrast to the modest impact of DLC1 loss in vitro, DLC1 shRNAs significantly accelerated tumor onset in vivo (P value < 0.0001 for shDLC1-1 and P < 0.0005 for shDLC1-2) (Fig. 2D,E). In fact, at 57 d post-transplantation, GFP-positive tumor nodules were observed in the livers of most animals receiving cells harboring DLC1 shRNAs, whereas the control animals showed no macroscopically detectable tumor burden (Fig. 2E). Furthermore, the pathology of tumors derived from DLC1 knockdown resembled aggressive human HCC and displayed a high proliferative index as assessed by Ki67 immunohistochemistry (Fig. 2F). Tumors also expressed the HCC markers α-fetoprotein (AFP) and albumin (Supplemental Fig. S3B). These data demonstrate that loss of DLC1 can efficiently promote the development of HCC.

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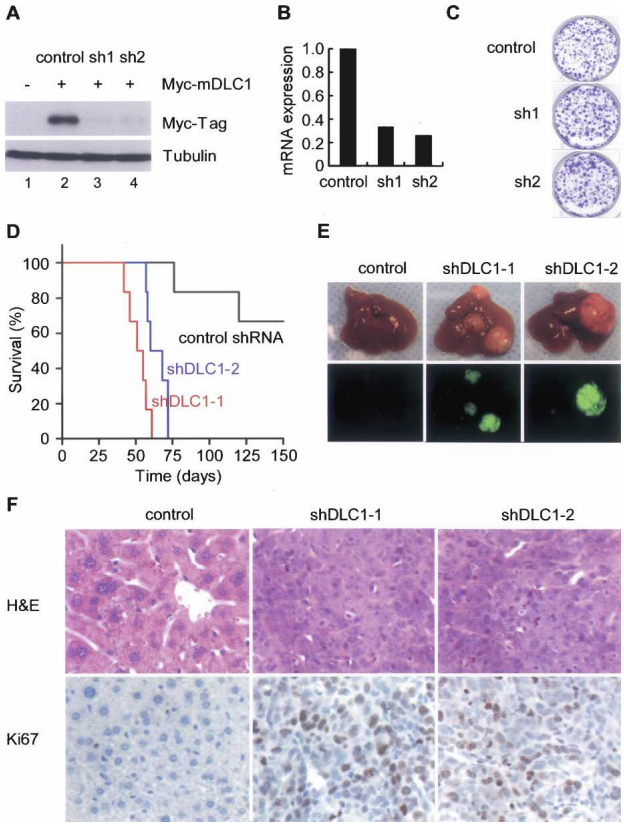

Figure 2. DLC1 loss triggered by in vivo RNAi promotes tumorigenesis in a mouse liver cancer model. (*A*) Immunoblots of 293T cells cotransfected with a 6xMyc-tagged murine *dlc1* cDNA (lanes *2–4*) and a control shRNA (lane *2*) or DLC1 shRNAs (lanes *3,4*). Tubulin serves as a loading control. (*B*) DLC1 qRT–PCR of *p53*-null liver progenitor cells coinfected with Myc and a control shRNA or two DLC1 shRNAs. (*C*) Cells as in *B* were plated at equal number and stained with crystal violet after 8 d. Shown are representative results from three experiments. (*D*) DLC1 loss cooperates with Myc and p53 loss to accelerate liver tumor formation. Kaplan-Meier survival curve of mice transplanted with *p53*-null liver progenitor cells coinfected with Myc and DLC1 shRNAs ($n = 6$ for each group). (*E*) Representative images of explanted livers. GFP imaging identifies shRNA transduced cells. (*F*) Histopathology of representative tumors. Proliferating cells were labeled by Ki67 staining.

We also ectopically expressed the murine *dlc1* gene in mouse hepatoma cells and tested their ability to form tumors orthotopically. To this end, we cloned a Myc-tagged murine *dlc1* cDNA and confirmed its ability to produce a protein of the correct molecular weight (Fig. 3A). A mouse hepatoma cell line harboring a luciferase reporter and expressing oncogenic Ras and undetectable DLC1 (see Fig. 1F, lane 8) was infected with the DLC1-expressing retrovirus or an empty vector. Consistent with the literature (Ng et al. 2000), reintroduction of DLC1 produced a modest effect on proliferation in colony formation assays (Supplemental Fig. S4A,B).

At 2 wk post-liver transplantation, animals receiving control cells developed aggressive liver tumors revealed by whole-body bioluminescence imaging (Fig. 3B, left). Mice transplanted with cells expressing DLC1 had a greatly reduced tumor burden (Fig. 3B [right], C). Accordingly, explanted livers from control animals harbored disseminated tumor nodules that coexpressed both luciferase and GFP (Fig. 3D, top panel), whereas livers from mice receiving cells overexpressing DLC1 showed little evidence of tumor formation even using these sensitive imaging reporters (Fig. 3D, bottom).

Since DLC1 has GAP activity for RhoA (Wong et al. 2003), we tested whether DLC1 knockdown increased RhoA-GTP levels and mimicked activated RhoA in promoting stress fiber formation, a hallmark of RhoA activity (Wong et al. 2005). *p53*-deficient liver progenitor cells harboring Myc and DLC1 shRNAs displayed increased levels of GTP-bound RhoA as assessed by a pull-down assay using a Rhotekin-RBD domain (Kim et al. 2007), which selectively binds RhoA-GTP (Fig. 4A,B). Concordantly, hepatoblasts with reduced DLC1 levels showed a prominent actin stress fiber network as assessed by fluorescent phalloidin labeling actin filaments (Fig. 4C). Thus, loss of DLC1 recapitulates the effects of activated RhoA on liver epithelial cells in vitro.

To test whether activated RhoA, like loss of DLC1, can promote liver carcinoma formation, we infected $p53^{-/-}$ liver progenitor cells with retrovirus expressing Myc and a constitutively active *RhoA* ($RhoA^{V14}$) (Fig. 4D) and transplanted the cells into the livers of syngeneic mice. Indeed, enforced expression of $RhoA^{V14}$ dramatically accelerated tumor formation (Fig. 4E [$P < 0.0001$], F; see also Supplemental Fig. S5), producing tumors with the pathological features of HCC (data not shown). Thus, DLC1 loss and RhoA activation have similar effects on hepatocellular carcinogenesis.

Although RhoA has been identified as a DLC1 effector, overexpression studies suggest that other DLC1 functions can contribute to its anti-proliferative activities (Liao et al. 2007; Qian et al. 2007). To determine whether RhoA is required for maintaining tumorigenesis stimulated by DLC1 loss, we tested whether suppression of RhoA in DLC1-suppressed hepatoma lines would impact their expansion as subcutaneous tumors in immunocompromised mice. shRNAs capable of down-regulating

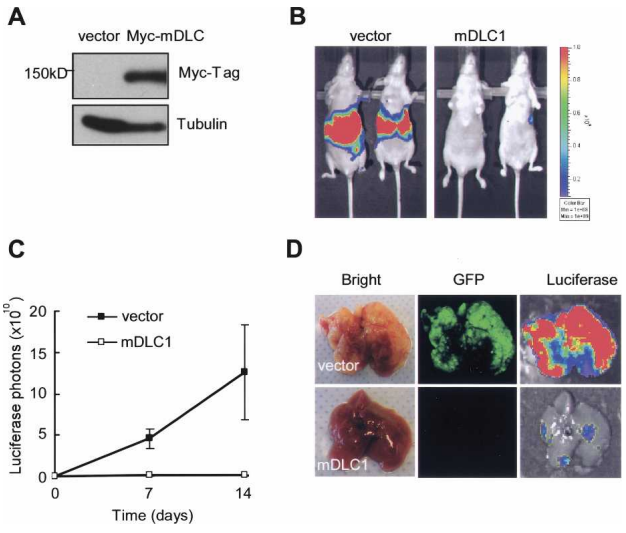

Figure 3. Reintroduction of DLC1 reverts tumorigenesis. (*A*) Immunoblots of Ras-driven hepatoma cells infected with control retrovirus (v) or retrovirus expressing DLC1 cDNA. (*B*) Bioluminescence imaging of in situ liver tumor. Hepatoma cells as in *A* are transplanted into livers of NCR nu/nu mice and imaged at day 14. (*C*) Quantification of luciferase signal as in *B*. Error bars denote SD ($n = 3$). (*D*) Bioluminescence and GFP imaging of explanted livers at day 14.

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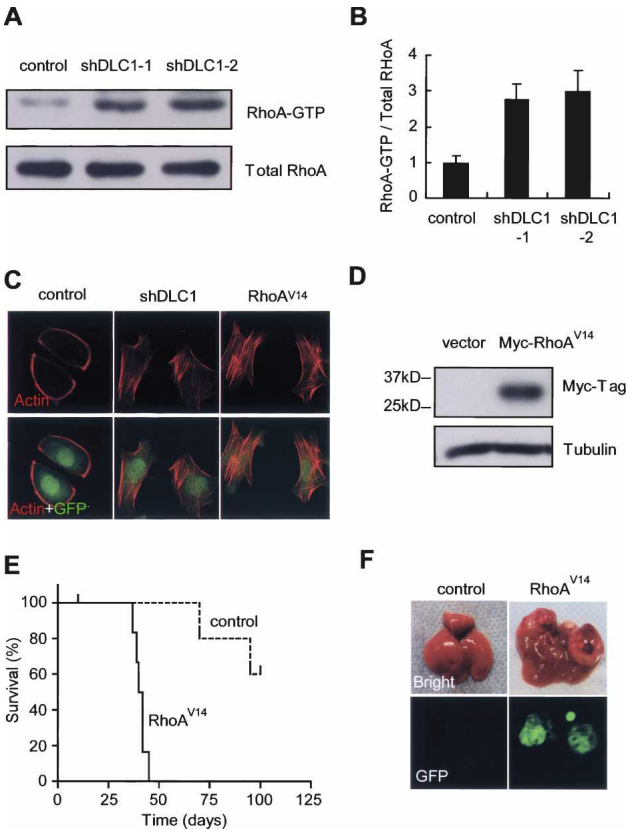

Figure 4. DLC1 knockdown deregulates RhoA activity, which is sufficient to accelerate tumorigenesis. (*A*) RhoA-GTP pull-down assay of *p53*-null liver progenitor cells coinfected with Myc and a control shRNA or DLC1 shRNAs. (*B*) Quantification of *A*. Error bars denote SD ($n = 3$). (*C*) DLC1 knockdown increases actin stress fiber formation. $p53^{-/-}$;*Myc* Liver progenitor cells infected with shDLC1 or $RhoA^{V14}$ were serum starved and stained with fluorescent phalloidin. (*D*) Immunoblots of $p53^{-/-}$-null hepatoblasts infected with Myc and a constitutively active $RhoA^{V14}$ allele with 6xMyc tag at the N terminus. (*E*) Activated RhoA cooperates with Myc and loss of p53 to accelerate liver tumor formation. Kaplan-Meier survival curve of syngeneic mice transplanted with *p53*-null liver progenitor cells coinfected with Myc and $RhoA^{V14}$ ($n = 6$ for each group). (*F*) Representative images of explanted livers at day 40 following cell transplantation. GFP imaging identifies retrovirally transduced cells.

RhoA to varying degrees (Fig. 5A) decreased the in vivo growth of two independent murine hepatoma lines with undetectable DLC1 (Fig. 5B, cell lines 1,2; Supplemental Fig. S6A,B). Of note, none of the shRNAs completely suppressed RhoA expression, and their ability to limit tumor expansion was proportional to their knockdown efficiency (Supplemental Fig. S6A). The impact of these shRNAs was less pronounced in hepatoma cell lines with higher DLC1 levels (Fig. 5B, cell lines 3,4; Supplemental Fig. S6C,D). Although complete inhibition of RhoA activity might be generally cytostatic (see Piekny et al. 2005), these data suggest that RhoA is required for maintaining the growth of tumors with attenuated DLC1 activity.

RhoA signals through ROCK kinase and other mediators to activate a downstream kinase cascade regulating cell mobility and cytoskeleton remodeling (Boettner and Van Aelst 2002; Sahai and Marshall 2002; Benitah et al. 2004; Jaffe and Hall 2005). As DLC1 loss leads to increased RhoA activation and RhoA is important for DLC1-mediated tumorigenesis, we hypothesized that DLC1 loss might sensitize tumor cells to inhibitors of RhoA effectors. As an initial test of this idea, we compared colony formation of $p53^{-/-}$;*Myc* liver progenitor cells infected with control or DLC1 shRNAs in the presence of Y27632 or Fasudil, two distinct small molecule compounds capable of blocking ROCK kinase activity. These agents selectively suppress colony formation in cells harboring a DLC1 shRNA (Fig. 5C,D), although another putative ROCK inhibitor (H1152) was generally toxic (data not shown). Still, these pharmacologic results are consistent with the genetic studies indicating a dependency on Rho signaling in DLC1-deficient cells.

In this study, we combined in vivo RNAi and a mosaic mouse model of HCC to study the impact of DLC1 loss on liver carcinogenesis in mice, which to date has not been possible owing to the embryonic lethality of DLC1 knockout animals. We show that DLC1 loss, when combined with other oncogenic lesions, promotes HCC in vivo and that RhoA activation is both necessary and sufficient for its effects. In our survey of copy number alterations in human tumors, 8p22 deletions encompassing *DLC1* occurred in >60% of heptocellular carcinomas as well as a large portion of human lung, breast, and colon carcinomas (see also Durkin et al. 2007). Similarly, RhoA is up-regulated in HCC and many other tumor types (Sahai and Marshall 2002; Fukui et al. 2006). Although other tumor suppressor genes may also reside in the 8p region, our results demonstrate that DLC1 is functionally important and highlight the potential importance of the RhoA signaling network in epithelial cancers.

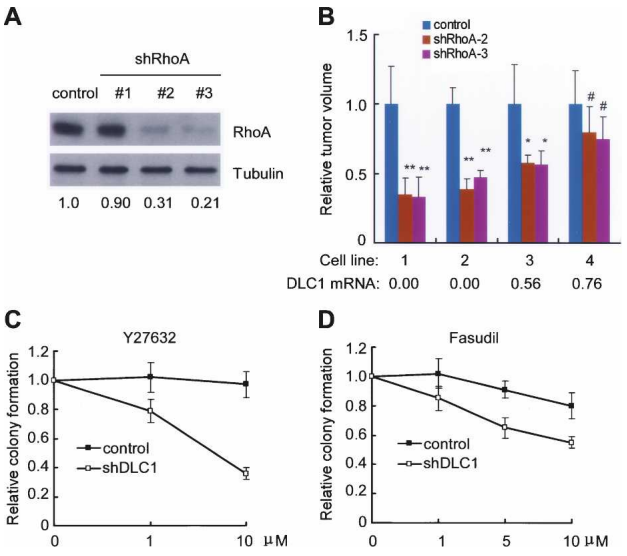

Figure 5. HCC mediated by DLC1 loss requires RhoA and is sensitized to Rho inhibitors. (*A*) Hepatoma cell line BNL is infected by retroviruses expressing control shRNA (control) or three RhoA shRNAs and immunoblotted for RhoA protein. Tubulin serves as a loading control. Numbers denote relative protein abundance. (*B*) RhoA shRNAs selectively suppress tumors harboring low levels of DLC1. Relative tumor volume is the last measurement in Figure S6A-D. (*) $P < 0.05$; (**) $P < 0.0005$; (#) $P > 0.05$. DLC1 mRNA level is measured by qRT–PCR and normalized to control liver progenitor cells. (*C*,*D*) Loss of DLC1 sensitizes liver cells to ROCK kinase inhibitors Y27632 and Fasudil. Error bars denote SD ($n = 3$).

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Molecularly targeted therapies have been devised for inhibiting several oncogenic pathways, including those affected by BCR-ABL, activated Ras and PI3kinase (Downward 2003; Luo et al. 2003). Although tumor suppressors are generally not amenable to direct therapeutic targeting, their mutation may confer a cellular dependency on downstream oncogenic proteins that can be inhibited with small molecule drugs. In this regard, the impact of DLC1 loss may parallel that produced by loss of PTEN, which deregulates the PI3K pathway and can sensitize cells to pharmacological inhibitors of downstream effectors such as mTOR (Maser et al. 2007). Our data indicate that RhoA is required for maintaining at least some tumors driven by DLC1 loss, and that cells with disabled DLC1 are particularly sensitive to inhibitors that target at least one RhoA effector. Clearly, more studies will be required to confirm and extend these observations; nevertheless, the high frequency of DLC1 loss in human cancer implies that pharmacologic intervention of the signaling pathways modulated by DLC1 may have broad therapeutic utility.

Material and methods

Plasmid construction

Two miR30 design shRNAs (codex accession HP_260153 and HP_255554) targeting mouse DLC1 were subcloned from the pSM2 RNAi codex library vector into the MSCV-SV40-GFP vector. The full-length mouse DLC1 was amplified from a RIKEN cDNA (M5C1068G17) and cloned into the MSCV-PGK-PIG vector harboring 6xMyc tag at the N terminus. Constitutive active RhoA ($RhoA^{V14}$) was cloned into the MSCV-IRES-GFP. Myc was cloned into pWZL-Neo.

Generation and characterization of liver carcinomas

Embryonic heptoblasts were isolated from E13.5 embryos, enriched by an E-cadherin immunoselection, and cultured as described (Zender et al. 2005). Cells with transduced with retroviral vectors (Xue et al. 2007), expanded briefly in culture, and transplanted into the spleen of retrorsine pretreated mice where the progenitor cells engraft the liver (Zender et al. 2005). Animals were treated with CCl_4 to stimulate tumor growth (Zender et al. 2006). For subcutaneous tumor growth, 2×10^6 cells were injected into the flanks of nude mice and monitored as described (Zender et al. 2006). Fluorescence and bioluminescence imaging of tumor-bearing mice was as described (Xue et al. 2007). For colony formation assays, 5000 cells were plated; after 6–8 d, colony formation was quantified by the Multigauge software (FujiFilm). Histopathological evaluation of murine liver carcinomas was performed by an experienced pathologist (S.S.). Ki67 staining was performed using standard protocols on paraffin-embedded tumor sections.

Gene activity and expression

RhoA-GTP pull-down assay was performed using EZ-detect GTPase activation kit (Pierce). For immunoblotting, fresh tumor tissue or cell pellets were lysed in Laemmli buffer using a tissue homogenizer. Equal amounts of protein (16 µg) were separated on 10% SDS–polyacrylamide gels and transferred to PVDF membranes. Blots were probed with antibodies against RhoA (1:1000; Santa Cruz Biotechnologies, 26C4), Myc-Tag (1:1000; Abcam, 9E10), or Tubulin (1:5000; B-5-1-2, Sigma). For immunofluorescence, cells were serum-starved for 24 h, fixed, blocked with 5% goat serum, and incubated with 1:1000 Alexa 594-conjugated phalloidin (Invitrogen). Images were captured under a 40× lens of a confocal microscope (Zeiss). To assess mRNA expression, hepatoma cells or tumors were freshly homogenized in Trizol (Gibco) and RNA was purified with Qiagen RNeasy columns and converted to cDNA using TaqMan reverse transcription reagents (Applied Biosystems). qPCR reactions were done in triplicate with SYBR Green PCR Master Mix (Applied Biosystems). The expression level of each gene was normalized to β-actin. Primer sequences can be found in the Supplemental Material.

Gene copy number analysis of human tumors

The ROMA data sets used in this study were obtained by analyzing panels of primary tumors (Hicks et al. 2006; S. Powers, S.W. Lowe, R. Lucito, and M. Wigler, in prep.). Copy-number profiles underwent normalization, segmentation, and masking of frequent copy number polymorphisms (Hicks et al. 2006). Deletion frequencies for DLC1 and other relevant genes were compared with all other genes found in the NCBI Entrez Gene database as described in the Supplemental Material and as well were described in Krasnitz et al. (A. Krasnitz and M. Wigler, in prep.).

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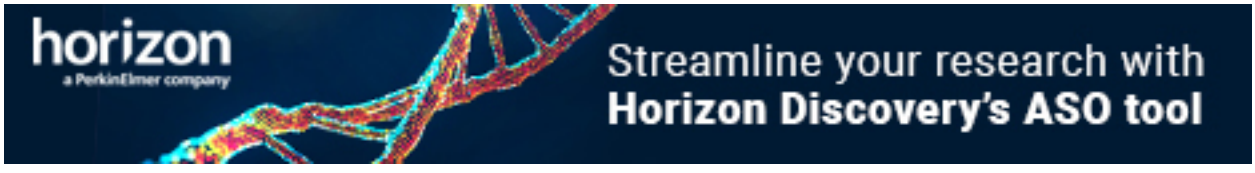

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