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ORIGINAL ARTICLE

Induction of the intestinal stem cell signature gene SMOC-2 is required for L1-mediated colon cancer progression

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Overactivation of Wnt-β-catenin signaling, including β-catenin-TCF target gene expression, is a hallmark of colorectal cancer (CRC) development. We identified the immunoglobulin family of cell-adhesion receptors member L1 as a β-catenin-TCF target gene preferentially expressed at the invasive edge of human CRC tissue. L1 can confer enhanced motility and liver metastasis when expressed in CRC cells. This ability of L1-mediated metastasis is exerted by a mechanism involving ezrin and the activation of NF-κB target genes. In this study, we identified the secreted modular calcium-binding matricellular protein-2 (SMOC-2) as a gene activated by L1-ezrin-NF-κB signaling. SMOC-2 is also known as an intestinal stem cell signature gene in mice expressing Lgr5 in cells at the bottom of intestinal crypts. The induction of SMOC-2 expression in L1-expressing CRC cells was necessary for the increase in cell motility, proliferation under stress and liver metastasis conferred by L1. SMOC-2 expression induced a more mesenchymal like phenotype in CRC cells, a decrease in E-cadherin and an increase in Snail by signaling that involves integrin-linked kinase (ILK). SMOC-2 was localized at the bottom of normal human colonic crypts and at increased levels in CRC tissue with preferential expression in invasive areas of the tumor. We found an increase in Lgr5 levels in CRC cells overexpressing L1, p65 or SMOC-2, suggesting that L1-mediated CRC progression involves the acquisition of a stem cell-like phenotype, and that SMOC-2 elevation is necessary for L1-mediated induction of more aggressive/invasive CRC properties.

INTRODUCTION

Hyperactivation of Wnt-β-catenin target gene expression is considered a characteristic feature of human colorectal cancer (CRC) development.[1–3] In previous studies, we identified members of the immunoglobulin-like cell-adhesion receptor family (L1 and Nr-CAM), known for their major function in brain development[4,5] as target genes of the β-catenin-TCF transcription complex in human CRC cells.[6,7] L1 was detected preferentially in cancer cells at the invasive front of human CRC tissue and its expression in CRC cells lacking this protein conferred enhanced motility, tumorigenesis and metastasis to the liver in a mouse model.[7,8] Analysis of gene expression patterns in CRC cells overexpressing L1 revealed a number of genes whose expression is also altered in a large set of human CRC tissue samples from patients.[8,9] L1 confers these tumorigenic capacities in CRC cells by mechanisms involving the recruitment (by L1) of the cytoskeletal protein ezrin and the NF-κB regulator IκB into a submembranal complex resulting in increased phosphorylation and proteasomal degradation of IκB, followed by the activation of NF-κB target gene transcription.[10] The analysis of one such target gene, insulin-like growth factor-binding protein 2,[11] revealed that besides its increased expression in CRC tissue, it is specifically localized in cells at the bottom of normal colonic crypts,[11] reminiscent of the site where Lgr5^{+} intestinal stem cells were detected in the mouse intestine.[12] In this study, we have compared the gene expression patterns conferred by L1, ezrin and NF-κB in human CRC cells to that of the Lgr5^{+} stem cell signature genes in the mouse intestine[12] and studied the role of secreted modular calcium-binding protein-2 (SMOC-2), a secreted matricellular calcium-binding protein,[13] that was present at increased levels in these gene expression patterns. We show that induction of SMOC-2 is necessary for the increased motility and liver metastasis conferred by L1 in human CRC cells, we detected SMOC-2 at the bottom of normal colonic crypts and preferentially in cells at the more invasive areas of CRC tissue. We found that Lgr5 expression is elevated in L1 and SMOC-2 overexpressing cells, and that SMOC-2 expression confers a more mesenchymal phenotype with decreased E-cadherin and an increase in Snail levels by a mechanism involving integrin-linked-kinase (ILK). We propose that the induction (by L1) of the intestinal stem cell signature gene *SMOC-2* may have a role in conferring a motile/invasive and dedifferentiated phenotype that can contribute to the development of more aggressive CRC.

RESULTS

L1 induces SMOC-2 expression in CRC cells by an ezrin and NF-κB-dependent mechanism

In a previous study of L1-mediated signaling in CRC cells, we detected the L1-ezrin-NF-κB-activated gene insulin-like growth factor-binding protein 2 at the bottom of colonic crypts in normal human colon mucosa and at increased levels throughout the tumorigenic CRC tissue.[11] This localization of insulin-like growth factor-binding protein 2 in normal colonic crypts was reminiscent of the recently reported localization of a number of gene products in Lgr5^{+} mouse intestinal stem cells.[12] We have therefore compared the list of L1-ezrin-NF-κB regulated genes that are expressed at increased levels in CRC cells with that of genes preferentially expressed in Lgr5^{+} intestinal stem cells[12]

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(Supplementary Tables 1 and 2). We identified, among others, the secreted matricellular calcium-binding protein 2 gene SMOC2, as a gene expressed at increased levels in all these gene expression patterns. Therefore, we wished to determine the role of SMOC-2 in L1-mediated human CRC development.

The analysis of SMOC-2 RNA levels in clones of human Ls174T CRC cells overexpressing either L1 (Figure 1a, lanes 3, 4), or the NF-κB subunit p65 (Figure 1a, lanes 5, 6), revealed that SMOC-2 RNA is induced in such cell clones compared with CRC cells transfected with an empty vector (Figure 1a, lanes 1, 2). Moreover, by interfering with NF-κB signaling by using the IκB-SR inhibitor (Figure 1a, lanes 7, 8), or by suppressing the level of the NF-κB subunit p65 by using an shRNA to p65 (Figure 1a, lanes 9, 10), the ability of L1 to induce SMOC-2 RNA expression in CRC cells was blocked (Figure 1a, compare lanes 3, 4 to lanes 9, 10). Furthermore, the involvement of ezrin in this L1-mediated induction of SMOC-2 was demonstrated by using CRC cells expressing L1, in which the endogenous ezrin levels were suppressed by shRNA to ezrin (Figure 1a, lanes 11, 12) that displayed no induction of SMOC-2 RNA.

Since SMOC-2 is a secreted protein,[13,14] we have also analyzed the levels of SMOC-2 protein in the conditioned medium of the cell clones described in Figure 1a. We found essentially the same result as with analyzing RNA levels, namely that L1 induced the

level of secreted SMOC-2 in CRC cells (Figure 1b, lanes 3 and 4) by a mechanism depending on NF-κB signaling (Figure 1b, lanes 5 and 6, compare with lanes 1 and 2) and ezrin (Figure 1c, lane 2, compare with lanes 3 and 4). Interference with either NF-κB signaling using IκB-SR (Figure 1b, lanes 7 and 8), or with shRNA to p65 (Figure 1b, lanes 9 and 10), or the suppression of ezrin levels (Figure 1c, lanes 3 and 4), all lead to the blocking of the ability of L1 to induce SMOC-2 levels (compare with SMOC-2 levels in control, Figure 1b, lanes 3 and 4, and Figure 1c, lane 2).

Analysis of the transcriptional activity of a SMOC2 gene promoter construct[15] demonstrated an increase in its activation by L1 and NF-κB (Figure 1d, p65) indicating that the activation of SMOC-2 transcription driven by L1 involves NF-κB activation. We examined in chromatin immunoprecipitation experiments the possibility that a mechanism involving the binding of NF-κB to the SMOC2 gene promoter increases SMOC-2 transcription. The experiments summarized in Figure 1e demonstrate the binding of p65 to NF-κB consensus sequences in the SMOC2 gene promoter (Figure 1e, lane 2, upper panel). The IκB promoter containing a consensus p65-binding sequence served as positive control for p65 binding (Figure 1e, lane 2, bottom panel). Together, these studies suggest that L1 induces the expression of SMOC-2 in CRC cells by a mechanism involving ezrin and the
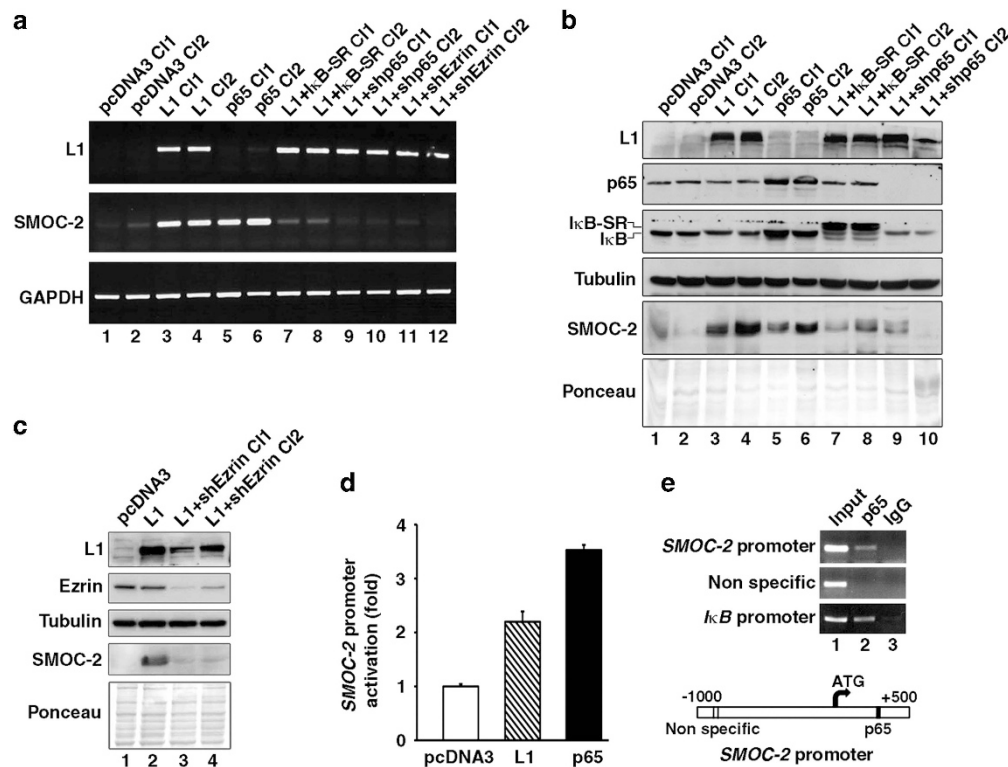

Figure 1. SMOC-2 expression is induced by L1 in CRC cells by a mechanism involving Ezrin and NF-κB-mediated transactivation of the SMOC2 gene. (**a**) Semiquantitative RT-PCR of RNA levels in individually isolated Ls174T CRC cell clones expressing the empty vector pcDNA (lanes 1 and 2), L1 (lanes 3 and 4), the NF-κB p65 subunit (lanes 5 and 6), L1 plus the NF-κB inhibitor IκB-SR (lanes 7 and 8), L1 and an shRNA to p65 (lanes 9 and 10) and L1 and shRNA to Ezrin (lanes 11 and 12). The levels of L1, SMOC-2 and GAPDH RNA (as loading control) were determined. (**b**) The level of SMOC-2 protein was determined in the conditioned medium (CM) of the cell clones described in (**a**), after collecting the CM from cells grown for 2 days in the absence of serum. The levels of p65, IκB and IκB-SR were determined in the cell layer by western blotting. Tubulin immunoblotting and Ponceau staining served as loading controls. (**c**) The level of SMOC-2 protein in the CM of CRC cell lines expressing the empty vector pcDNA (lane 1), L1 (lane 2) and L1 plus shRNA to Ezrin (lanes 3 and 4) was determined as described in (**b**) for SMOC-2. The levels of Ezrin, L1 and tubulin were determined in the same cell layer. (**d**) Activation of the SMOC2 gene promoter was determined in 293 T cells transfected with the SMOC2 promoter construct and an empty vector (pcDNA3), or in cells co-transfected with either a plasmid expressing L1 or the p65 NF-κB subunit. The fold promoter activation was determined after normalizing for transfection efficiency with a β-galactosidase construct. (**e**) Chromatin immunoprecipitation of SMOC2 promoter DNA sequences containing an NF-κB-binding site and a non-specific sequence in the SMOC2 promoter. Non-immune IgG served as a negative control for the anti-p65 antibody and an IκB gene promoter sequence containing a p65-binding site served as positive control.

transcriptional activation of the *SMOC2* gene promoter by binding of NF-κB to *SMOC-2* promoter sequences.

SMOC-2 regulates the motility, proliferation and metastasis of CRC cells

To examine the effect of changes in SMOC-2 levels on CRC cell behavior, we isolated Ls174T CRC cell clones stably overexpressing SMOC-2 (Figure 2a, lanes 3 and 4). Since we observed that in L1-expressing cells the SMOC-2 protein was localized at increased levels in the pericellular area (outside the cell) near the tips of cellular protrusions (Figure 3a, compare L1-transfected to pcDNA-transfected cells), we hypothesized that SMOC-2 is involved in regulating the motility of these cells. We found that clones of SMOC-2-overexpressing CRC cells were even more effective than L1-expressing cell clones in promoting the motile processes involved in closing an artificial wound introduced in a confluent cell monolayer in 'scratch wound' experiments (Figure 2b). CRC cell clones also proliferated faster under stressful conditions (in the absence of serum) than empty vector-transfected cell clones, but were significantly less effective than L1 in conferring this proliferative property (Figure 2c). To analyze the requirement for SMOC-2 in the L1-mediated increase in CRC cell motility and proliferation under stressful conditions, we isolated L1-expressing cells in which the levels of endogenous SMOC-2 were suppressed by shRNA to SMOC-2 (Figure 2d, lanes 2–4). In such CRC cells, cell motility was reduced and was similar to that of empty vector-transfected cells, and the presence of L1 was insufficient to confer increased motility (Figure 2e, L1+shSMOC-2). Similarly, the ability to proliferate in the absence of serum that is induced by L1 expression was also dramatically suppressed in L1+shSMOC-2 CRC

cell clones (Figure 2f), implying that these important features conferred by L1 in CRC cells require an increase in SMOC-2 levels.

A key property of L1-transfected human CRC cells is their ability to metastasize to the liver upon injection into the tip of the spleen of immunodeficient mice.[8,10,11] We have therefore analyzed the metastatic capacity of L1-expressing CRC cells in which the level of endogenous SMOC-2 was suppressed and found that L1 expression was unable to confer liver metastasis by CRC cells in which SMOC-2 levels were reduced (Figure 3b, L1+shSMOC-2), although such cells formed tumors at the site of injection (Figure 3b, arrows). Together, these results suggest that the induction of SMOC-2 in L1-transfected cells is a necessary step that enables such cells to display an increase in cell motility, proliferation under stressful conditions and metastasis to the liver after injection into the spleen.

SMOC-2 promotes dispersed colony morphology by increasing the expression of Snail and reducing E-cadherin levels by a mechanism involving ILK

To begin to understand the molecular mechanisms whereby SMOC-2 confers its effects on CRC cells, we examined the possibility that the increase in cell motility and metastatic capacities observed after SMOC-2 expression in CRC cells may result from the induction of properties characteristic of epithelial-to-mesenchymal like transition (EMT).[16] We found that CRC cells expressing SMOC-2 displayed a more elongated morphology compared with control cells and did not form tight colonies even at higher cell densities (Figure 4b), while control CRC cells formed small colonies when contacting each other (Figure 4a). These morphological features conferred by SMOC-2 transfection were associated with a decrease in E-cadherin protein levels (Figure 4e)
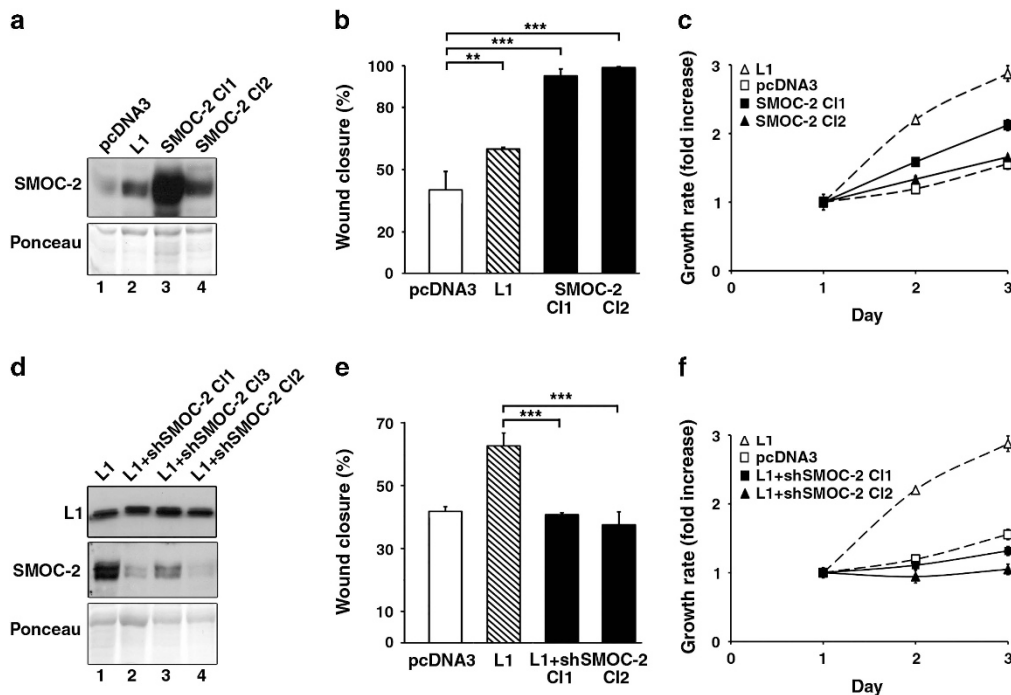

Figure 2. SMOC-2 overexpression induces CRC cell motility and proliferation under stress, whereas SMOC-2 suppression in L1-expressing cells inhibits these cellular properties. (**a**) Individual Ls174T CRC cell clones overexpressing SMOC-2 were isolated and the level of SMOC-2 protein was determined in the conditioned medium from the cultures of such cell clones (by western blotting) and compared with that in L1 overexpressing Ls174T cells and to empty vector (pcDNA3) transfected CRC cell clones. (**b**) The motility in 'scratch wound closure' experiments was compared between CRC cells stably transfected with either pcDNA3, L1, or SM'OC-2 constructs. (**c**) The growth in the absence of serum was compared in tetraplicates of the cell clones described in (**a**) for 3 days. (**d**) CRC cell clones overexpressing L1 in which the level of endogenous SMOC-2 was suppressed with shRNA to SMOC-2 were isolated (lanes 2–4) and the levels of SMOC-2 and L1 in these clones was determined by western blotting. (**e**) The motility of the clones described in (**d**) and their rate of growth (**f**) were determined as in (**b**) and (**c**), respectively. **P < 0.01, ***P < 0.001.

a

SMOC-2   SMOC-2-β1-integrin-DAPI

b

L1 Cl1   L1 Cl2   L1+shSMOC-2 Cl1   L1+shSMOC-2 Cl2

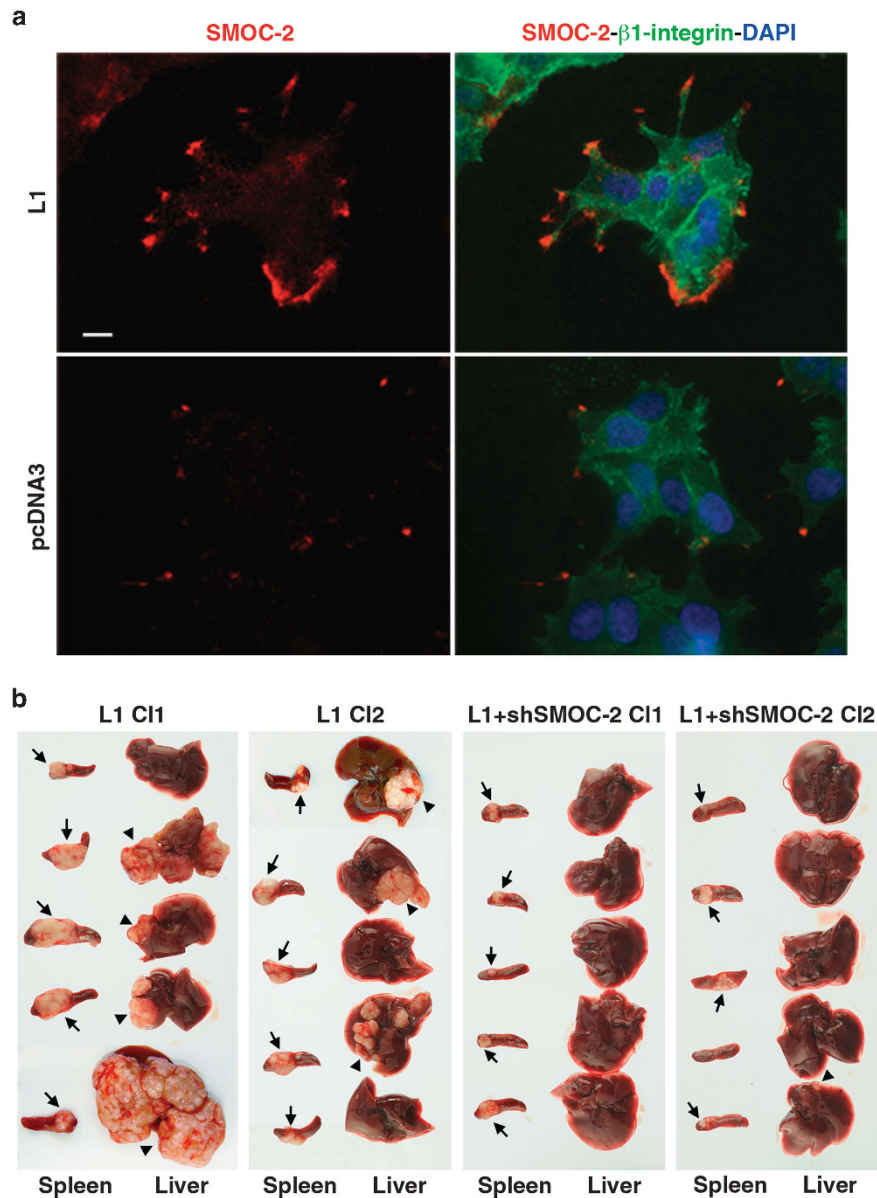Spleen Liver   Spleen Liver   Spleen Liver   Spleen Liver

Figure 3. SMOC-2 is localized outside the cells near the tip of CRC cell protrusions and the suppression of SMOC-2 in L1-expressing CRC cells blocks their metastatic capacities. (**a**) Empty vector (pcDNA3) and L1-transfected (L1) CRC cells were doubly stained for SMOC-2 and β_1-integrin and viewed by immunofluorescence microscopy. The nuclei were stained with DAPI. SMOC-2 is in red, β_1-integrin in green and DAPI in blue. The white bar represents 10 µm. (**b**) Clones of CRC cells stably expressing L1 (L1 Cl1 and L1 Cl2) and clones of L1-expressing cells in which SMOC-2 levels were suppressed (as described in Figure 2d) were injected into the tip of the spleen of immunodeficient mice. After 6 weeks the spleens and livers were excised and the growth of CRC cells in the spleen at the site of injection (arrows) and the metastases in the liver (arrowheads) was determined.

and RNA (Figure 4f) and with an increase in the RNA level of the transcription factor Snail (Figure 4g), known as a master EMT regulator that suppresses E-cadherin transcription.[16]

L1 expression in CRC cells results in the growth of such cells in small colonies and also in three-dimensional aggregates that are mediated through L1-L1 cell–cell adhesions (Figure 4c).[7,9] The suppression of endogenous SMOC-2 levels in L1-expressing cells resulted in a flatter, more epithelial colony morphology (Figure 4d, compare with Figure 4c) and an increase in E-cadherin levels (Figure 4h), suggesting that SMOC-2 expression in CRC cells confers a more mesenchymal and motile phenotype by a mechanism reminiscent of EMT.

Since SMOC-2 was shown to affect 3T3 cell growth by a mechanism involving ILK,[15] we wished to determine whether the effects conferred by SMOC-2 on CRC cell morphology,

E-cadherin expression and cell motility involve ILK activity. We have used the ILK inhibitor QLT0267 and siRNA against ILK[17] to inhibit ILK and analyzed their effect on SMOC-2 overexpressing cells. The results summarized in Figure 5 show that an 8 h treatment with the ILK inhibitor was sufficient for changing the colony morphology of SMOC-2 overexpressing CRC cells (Figure 5a). When ILK was inhibited, the cells regained a more compact colony morphology characteristic of control Ls174T cells (Figure 5a left panel, compare with Figure 4a). Concomitantly, ILK inhibition resulted in increased E-cadherin levels in the various CRC cell clones, including L1-transfected cells, and SMOC-2 overexpressing Ls174T cell clones (Figure 5b). Importantly, inhibition of ILK also reduced the motility of SMOC-2 over-expressing cells (Figure 5c). By using a different approach of transfecting two different ILK siRNA sequences that suppressed
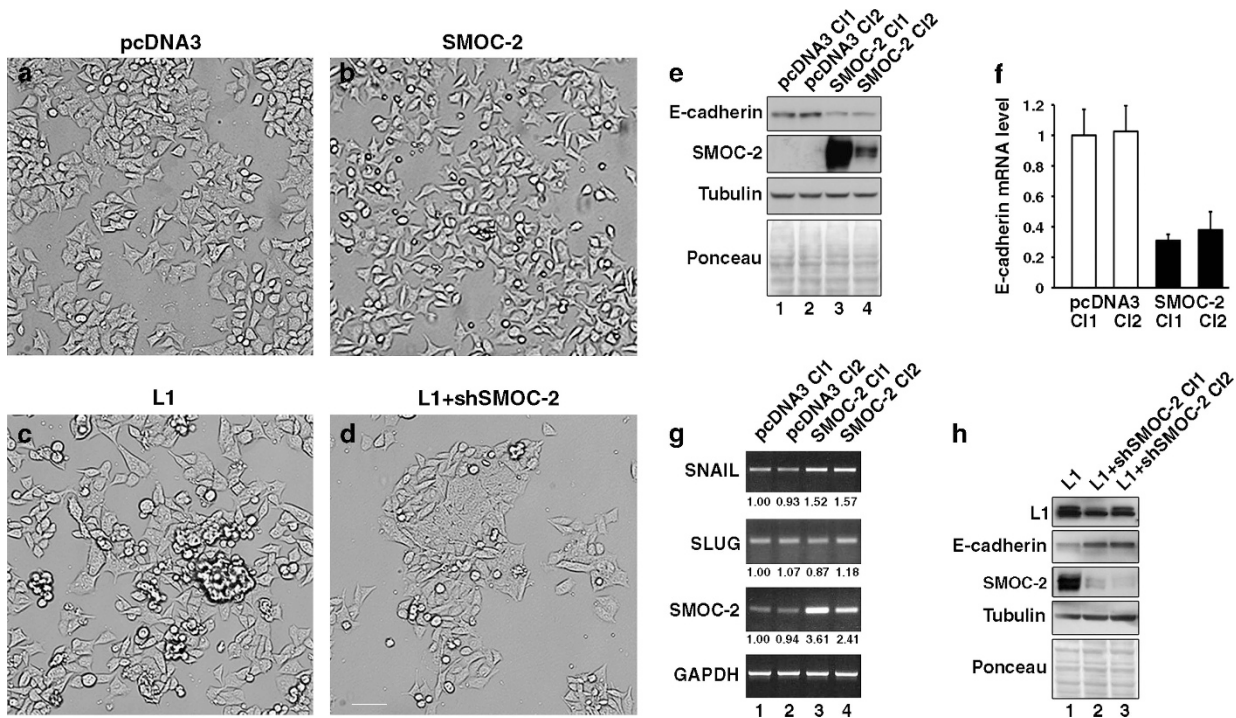
Figure 4. SMOC-2 confers a dispersed colony morphology in CRC cells, decreases E-cadherin and increases SNAIL expression. (**a**) The morphology of Ls174T CRC cells stably transfected with empty vector (pcDNA3), (**b**) SMOC-2, (**c**) L1 and (**d**) L1 plus shRNA to SMOC-2. (**e**) The expression of E-cadherin protein in CRC cell clones expressing pcDNA3 or SMOC-2. (**f**) The levels of E-cadherin RNA determined by quantitative real-time PCR in the CRC cell clones described in (**e**). (**g**) The levels of SNAIL, SLUG and SMOC-2 RNA in CRC cell clones stably expressing pcDNA3 (lanes 1 and 2), or SMOC-2 (lanes 3 and 4) were determined by RT-PCR. The values for fold changes in RNA levels under each lane were normalized to the level of GAPDH RNA in each sample. (**h**) The levels of E-cadherin, L1 and SMOC-2 protein in CRC cell clones stably expressing L1 and two L1+shSMOC-2 cell clones. Tubulin immunoblotting and Ponceau staining served as loading controls.

ILK expression (Figure 5d), we found that the motility of SMOC-2 overexpressing cells was similarly reduced (Figure 5e). Taken together, these results suggest that the effects conferred by SMOC-2 on CRC cell morphology and motility involve signaling by ILK that affects E-cadherin expression.

SMOC-2 is localized at the bottom of normal colonic crypts and preferentially in invasive areas of CRC tissue

In a recent study, using an *Lgr5* promoter-SMOC-2 construct for knock-in studies in transgenic mice, SMOC-2 was detected at the bottom of intestinal crypts in the Lgr5+ stem cell compartment of such mice.[12] Interestingly, in CRC cells overexpressing L1 or SMOC-2 we observed a higher level of Lgr5 RNA compared with control (empty vector-transfected) CRC cells (Figure 6a) and also in p65-transfected CRC cells (Supplementary Table 2), consistent with the notion that these CRC cells might display more 'stem cell-like' properties. We therefore examined, by immunohistochemistry, the localization of SMOC-2 in normal human colonic tissue and in CRC tissue and detected SMOC-2 in cells exclusively localized at the bottom of normal colonic crypts (Figures 6e and f, arrows). Human CRC tissue displayed an increased and diffuse expression of SMOC-2 throughout the tumor tissue (Figures 6b and c), but a preferentially increased immunostaining of SMOC-2 was observed in the more invasive areas of the tumor (Figure 6d, arrowheads). Taken together, these studies suggest that SMOC-2 may have an important role in CRC progression toward a more motile/invasive and 'stem cell-like' phenotype conferred in L1-expressing cells by the ezrin-NF-κB pathway.

DISCUSSION

SMOC-2 is a matricellular protein[13–15,18] reported to be involved in various developmental processes,[15,18,19] in regulating the cell cycle and cyclin D1 expression in 3T3 cells,[15] angiogenic growth factor activity,[20] cell-adhesion and migration of keratinocytes (possibly via integrins),[21] and Ran GTPase-modulated 3T3 cell transformation and SKBR3 breast cancer cell invasion.[22] Recently, SMOC-2 was identified as a marker of intestinal stem cells in mice, based on its increased expression and exclusive localization in epithelial cells at the bottom of intestinal crypts in Lgr5+ stem cells.[12] In the present study, we detected SMOC-2 also among the genes induced in human CRC cells by L1 through the involvement of an ezrin-NF-κB signaling pathway. We demonstrated that L1-mediated SMOC-2 induction is necessary for promoting a more invasive-metastatic phenotype in CRC cells by L1, since suppression of endogenous SMOC-2 levels in L1-expressing CRC cells blocked their capacity to form liver metastases. Moreover, we found that SMOC-2 is exclusively localized in cells at the bottom of normal human colonic crypts, in agreement with its localization in Lgr5+ cells at the bottom of mouse intestinal crypts,[12] suggesting that SMOC-2 is expressed at elevated levels in stem cells of the human colonic epithelium and could possibly serve as a marker for such cells. The increased Lgr5 RNA expression that we detected in L1, p65 and SMOC-2 overexpressing CRC cells is in line with a study that found an increase in Lgr5 in more metastatic CRC cell lines and in CRC tissue.[23,24] The more intense immunostaining of SMOC-2 in CRC tissue, especially in the invasive areas of the tumor tissue front, is indicative of the important role that SMOC-2 plays in CRC progression. In line with this notion, we found a higher motility in CRC cells overexpressing
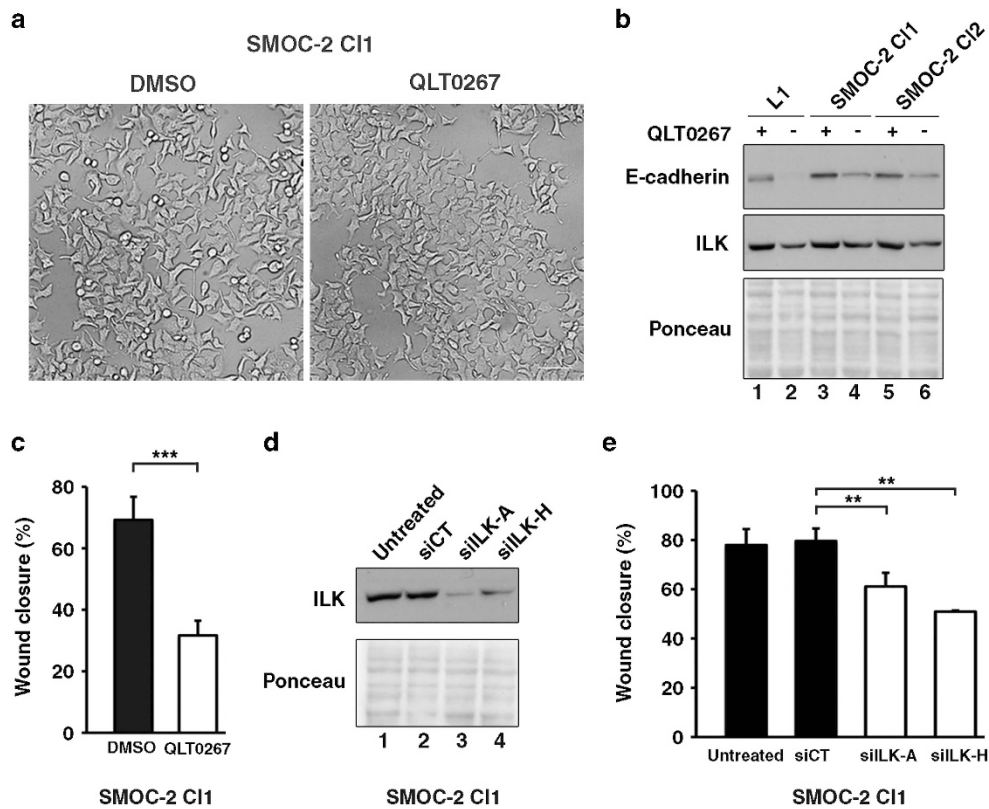
Figure 5. Inhibition of ILK in SMOC-2 overexpressing cells induced the formation of compact colony morphology, an increase in E-cadherin and reduced CRC cell motility. (**a**) The morphology of SMOC-2 overexpressing CRC cells treated for 8 h with either DMSO or the ILK inhibitor QLT0267. (**b**) Ls174T CRC cell clones transfected with L1 or SMOC-2 (2 clones), were either treated for 8 h with QLT0267, or left untreated and cell lysates were analyzed by western blotting for E-cadherin and ILK protein levels. The blots were stained with Ponceau to demonstrate equal loading. (**c**) Cell motility was determined by the artificial wound closure method in SMOC-2 overexpressing cells treated (for 24 h) with either DMSO or QLT0267. (**d**) The level of ILK was determined by western blotting in SMOC-2 overexpressing Ls174T CRC cells transfected with either control siRNA (siCT), or with two different siRNA sequences to ILK (silk-A and silk-H). Ponceau staining of the western blots served as loading control. (**e**) The motility of the cells described in (**d**) was determined by the 'scratch wound' closure experiment. $**P < 0.01$, $***P < 0.001$.

SMOC-2 and its requirement for L1-mediated CRC cell metastasis to the liver in a mouse model. A recent study also reported on a correlation between Lgr5 expression and SMOC-2 in human CRC tissue and showed that such cells hold a high tumor initiating potential and self-renewal capacity.[23]

The possibility that L1 expression induces some stem cell-like properties in CRC cells (exemplified by increased Lgr5 and SMOC-2 levels) is in agreement with studies in transgenic mice where an inducible enhancement of NF-κB signaling and Wnt activation were shown to be involved in the dedifferentiation of nonstem cells and the acquisition of tumor-initiation capacities by intestinal cells into tumor initiating stem cells of mice.[25] In addition, we have shown that the induction of higher Lgr5 levels and more invasive capacities by CRC cells transfected with either L1, p65, or SMOC-2 is associated with the acquisition of a more motile phenotype with decreased E-cadherin levels and an increase in Snail expression in SMOC-2 overexpressing cells. Together, these findings on CRC cells overexpressing SMOC-2 are in agreement with the hypothesis that the acquisition of stem cell-like properties and EMT occur in parallel during certain stages of cancer progression, as suggested in several studies.[26–28] In a previous study we reported that the expression of other markers suggested to serve for the isolation of human CRC stem cells, such as CD131 and EpCAM, remains unchanged in L1 overexpressing CRC cells.[29] In later studies it was reported that these stem cell markers (contrary to Lgr5) are found in a variety of epithelial cells and are not unique to stem cells.[30]

The mechanism(s) by which SMOC-2 confers these effects on CRC cells properties most probably involve ILK since the inhibition of ILK activity with QLT0267, or the suppression of ILK levels with siRNA to ILK blocked the effects conferred by SMOC-2. The involvement of ILK was also demonstrated in the induction of cyclin D1 and DNA synthesis in 3T3 cells by SMOC-2,[15] and in SW480 CRC cells transfection with ILK resulted in the induction of EMT-like properties (enhanced migration, decrease in E-cadherin and increase in vimentin, Snail and Slug) via the NF-κB pathway.[31]

Stem cells residing at the bottom of the intestinal and colonic crypts are responsible for the rapid self-renewal of these tissues by cell division, upward movement and progressive differentiation.[32] By studying the role of SMOC-2 that is both a colonic stem cell marker and a contributor to L1-mediated CRC progression, we expect to learn more about the functional significance of stem cell signature genes (such as *SMOC-2* and others) in intestinal and colonic homeostasis and disease.

MATERIALS AND METHODS

Cell culture, proliferation, artificial wound closure and transfections

Ls174T human CRC cells were grown as described.[8] Ls174T-SMOC-2, Ls174T-L1, Ls174T-p65 and Ls174T-control (pcDNA3-transfected) cells were maintained in medium containing neomycin (800 µg/ml), Ls174T-L1 +shp65, Ls174-L1+IκB-SR and Ls174T-L1+shSMOC-2 cells in medium with both neomycin (800 µg/ml) and puromycin (10 µg/ml), Ls174T-L1+shEzrin cells in medium with both neomycin (800 µg/ml) and zeocin (500 µg/ml).
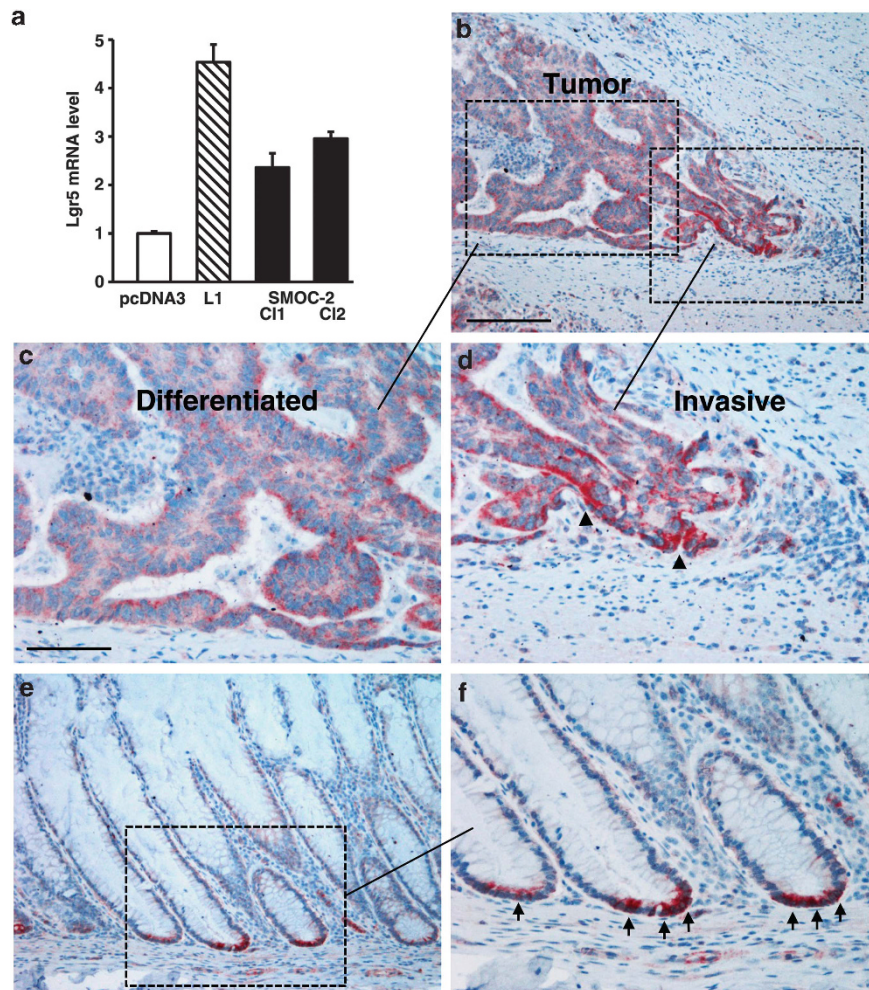
Figure 6. L1 and SMOC-2 induce Lgr5 expression in CRC cells and SMOC-2 is localized at the bottom of colonic crypts in normal mucosa and in invasive areas of human CRC tissue. (**a**) Lgr5 RNA levels in CRC cells transfected with either empty vector (pcDNA3), L1, or SMOC-2 (SMOC-2 Cl1 and Cl2). (**b**) Immunohistochemical staining of SMOC-2 in human CRC tissue. (**c**) SMOC-2 immunostaining of an enlarged area of the more central part of the tumor shown in (**b**). (**d**) SMOC-2 staining showing an invasive area (marked by arrowheads) in the enlarged CRC tissue area shown in (**b**). (**e**) Immunostaining for SMOC-2 at the bottom of normal human colonic crypts. (**f**) Enlargement of the boxed area shown in (**e**). The arrowheads in (**f**) point to SMOC-2 positive cells at the bottom of the normal colonic crypts. The bar represents 100 μm in (**b**) and (**e**), and 50 μm in (**c**), (**d**) and (**f**).

For cell growth assays, 10^4 cells/well were seeded in 96-well dishes and cell number determined in triplicates for 3 days. An artificial wound was introduced into confluent cell cultures by using a micropipette. The medium was replaced with fresh medium containing 0.35 μg/ml Mitomycin-C to inhibit cell proliferation. Pictures were taken at 0 and 24 h after introducing the wound and percent wound closure was determined. Ls174T cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). ILK was inhibited in Ls174T-control, Ls174T-L1 and Ls174T-L1+shSMOC-2 with 50 μM of QLT0267 as previously described.[17] Cell images were taken after 6 h treatment and protein lysates were collected for immunoblotting after 8 h. In the artificial wound closure experiments, 25-μM of QLT0267 was added to Ls174T-L1+shSMOC--2 cells upon the introduction of wounds. For ILK siRNA transfection, Ls174T-L1+shSMOC-2 cells were transiently transfected by using Darma-FECT (Dharmacon, Lafayette, CO, USA) according to the instructions of the manufacturer. Cells were treated with 25-nM of either control siRNA (siCT-non silencing control), or the ILK targeting sequences siILK-A and siILK-H as described[17] (Supplementary Table 3).

Plasmids

The wt L1, p65 and IκB-SR cDNA, were previously described.[10] SMOC-2 cDNA was obtained from Dr S Milano and Dr M Cerione (Cornel University, Ithaca, New York, NY, USA). The SMOC-2 responsive gene promoter reporter plasmid was provided by Dr C Vaziri (University of North Carolina,

Chapel Hill, NC, USA). Ezrin shRNA was prepared as described.[10] SMOC-2 shRNA was prepared in pSUPER.puro according to the manufacturer's instructions (pSUPER.puro RNAi System, OligoEngine, Seattle, WA, USA) by using the target sequences shown in Supplementary Table 3.

Luciferase reporter assays

Ls174T-L1, Ls174T-p65 and Ls174T-control cells were transfected by using Lipofectamine 2000 in triplicate plates together with 0.25 μg β-galactosi-dase plasmid and 0.5 μg SMOC-2 promoter reporter plasmids in pGL2b, or with 0.25 μg of empty pGL2b plasmid. The cells were lysed 24 h after transfection and luciferase and β-galactosidase levels were determined by the luciferase assay system (Promega, Fitchburg, WI, USA). Luciferase activity was normalized to β-galactosidase activity for transfection efficiency.

RT-PCR

RNA was isolated using the EZ-RNA kit (Biological Industries, Kibbutz Beit-Haemek, Israel). Semiquantitative RT-PCR and quantitative real-time PCR (qRT-PCR) was conducted using the sequences shown in Supplementary Table 3. Quantitative real-time PCR was performed on the ABgene thermocycler with the ABsolute SYBR green ROX mix (ABgene, Epsom, UK). Triplicates of 1-ng cDNA template and 500-nM gene-specific primers were used. GAPDH cDNA sequences served to normalize for RNA levels. Primers

were examined for efficiency, displaying an amplification slope of -3.33 ± 0.3 and $r^2 > 0.98$. Quantitative real-time PCR was started by incubating the samples at 95 °C for 10 min followed by PCR amplification cycles (95 °C for 20 s and 60 °C for 1 min for 40 cycles). Data analysis was conducted with the $\Delta\Delta C_T$ method with the ABgene thermocycler software. For semiquantitative RT-PCR the products were analyzed by agarose gel electrophoresis.

Gene arrays

Gene expression patterns for CRC cells overexpressing L1, L1+shRNA to ezrin, SMOC-2 and the NF-κB p65 subunit were obtained as previously described[8–10] and these gene expression patterns were compared with that obtained for Lgr5^{+} mouse intestinal stem cells.[12] The results of these comparisons are presented in Supplementary Tables 1 and 2.

Chromatin immunoprecipitation assays

Rabbit anti-p65 (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the immunoprecipitation and rabbit anti-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as control antibody. Chromatin immunoprecipitation was carried out as described,[11] with the exception that the DNA was purified by using the PCR Purification Kit (Promega) and was subjected to PCR with the specific primers shown in Supplementary Table 3.

Immunoblotting and immunofluorescence

Immunoblotting was carried out using the following antibodies: Rabbit anti-SMOC-2 provided Dr U. Hartmann (University of Cologne, Cologne, Germany, at 1:500), mouse anti-IκBα/MAD-3 (BD Biosciences, Franklin Lakes, NJ, USA, at 1:1000), goat Ab against NF-κB p65 (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA, at 1:1000), mouse anti-E-cadherin (BD Biosciences, at 1:1000), rabbit anti-L1 (gift from Dr V Lemmon, University of Miami, FL, USA, at 1:5000), mouse anti-Ezrin (Sigma-Aldrich, St Louis, MO, USA, at 1:5000) and mouse anti-α-tubulin (Sigma-Aldrich, at 1:200 000). Western blots were developed by using the ECL method (Amersham Biosciences, Buckinghamshire, UK). For immunofluorescence, cells cultured on glass coverslips were permeabilized with 0.5% Triton X-100 and fixed with 3% PFA. Immunostaining for β1-integrin was carried out using a mouse anti-β1-integrin antibody provided by Dr B Geiger (Weizmann Institute of Science, Rehovot, Israel). The secondary antibodies were Alexa-488-conjugated goat anti-mouse IgG (Invitrogen) and Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Immunohistochemistry

Immunohistochemistry was carried out on 25 paraffin-embedded human colorectal adenocarcinomas as described.[11] For SMOC-2 detection, polyclonal mouse anti-SMOC-2 antibody was used. The streptomycin/AB system was employed to detect antibody binding according to the manufacturer's protocol (Dako, Hamburg, Germany). Sections were counterstained with hemalaun (Merck, Darmstadt, Germany).

Metastasis assays

For metastasis assays, groups of 5–10 nude mice (5–7 weeks old) were injected with 1.5×10^6 cells in 20-μl PBS into the distal tip of the spleen. After 6–7 weeks the spleens and livers were removed as described.[8,11]

Statistical analysis

Statistical significance was determined by the Fisher's exact test for mouse metastasis experiments. The significance of quantitative real-time PCR comparisons for RNA levels was determined by ANOVA. In wound closure and luciferase reporter assay studies the significance was determined by ANOVA. A P-value of < 0.05 was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)