β-Catenin Inhibitor ICAT Modulates the Invasive Motility of Melanoma Cells

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Abstract

Inhibitor of β-catenin and TCF (ICAT) inhibits β-catenin transcriptional activity by competing with T-cell factor/lymphoid enhancer factor. We documented high ICAT levels in human melanoma cells, in which β-catenin signaling is frequently deregulated, finding a correlation with the capacity to form metastases in nude mice. Ectopic expression of ICAT in melanoma cells did not affect their proliferation but increased cell motility and Matrigel invasion of metastatic cells in a manner relying upon stable ICAT-β-catenin interaction. This effect was associated with conversion of an elongated/mesenchymal phenotype to a round/amoeboid phenotype in the absence of similar effects on elongated morphology of nonmetastatic melanoma cells. Transition from mesenchymal to amoeboid movement was associated with decreased levels of NEDD9 and activated Rac1, a positive regulator of mesenchymal movement. Ectopic ICAT promoted colonization of melanoma cells in the lungs of nude mice, suggesting an increase in metastatic potential. Together, our results showed that by downregulating Rac signaling in metastatic melanoma cells, ICAT increased their invasive motility by promoting a morphologic variation that facilitates a favorable adaptation to their microenvironment. Cancer Res 74(7): 1983–95. © 2014 AACR.

Introduction

β-Catenin is a multifunctional protein exhibiting various functions depending on its cellular localization and interactions with diverse protein ligands. In the cytoplasm and nucleus, β-catenin is the central protein of the canonical WNT signaling pathway. Aberrant WNT signaling is associated with many types of cancer including carcinomas and melanoma. Constitutive nuclear localization of β-catenin in mouse melanocytes promotes their immortalization and favors melanoma progression and invasion in an NRAS-driven melanoma model (1, 2). High nuclear β-catenin levels in melanoma samples have been associated in some patients with improved survival (3); however, conflicting findings for the in vitro invasion of melanoma cells have been reported (4, 5), suggesting that β-catenin may act as a pro- or anti-in vitro invasion factor. Inhibitor of β-catenin and TCF (ICAT) is one of the few direct negative regulators of β-catenin (6). This small protein of 81 amino acids (encoded by the conserved CTNNBI1 gene) is known to repress T-cell factor/lymphoid enhancer factor (TCF/LEF)-β-catenin transcriptional activity in vitro (7–9). Crystallographic analysis of the ICAT/β-catenin complex identified two ICAT domains essential for interaction: (i) an amino-terminal domain composed of three α helices and (ii) a non-structured carboxy-terminal domain that adopts a β-sheet-like conformation upon interaction with β-catenin (7, 8, 10).

Total invalidation of the ICAT mouse gene leads to premature death of newborn animals associated with gut, kidney, and craniofacial anomalies, suggesting an important role in epithelial establishment/maintenance and neural crest cell differentiation (11). This phenotype is consistent with ICAT being involved, through β-catenin regulation, in the fate and migration of neural crest cells giving rise to many cell types including melanocytes (11, 12).

Variable ICAT expression has been detected in metastatic and nonmetastatic human melanoma samples (13, 14). However, whether ICAT plays a role in melanoma progression and/or metastasis formation is still unknown. Formation of metastases is a multistage process involving cell motility. The motility of melanoma cells through three-dimensional (3D) matrices in vitro relies on two interconvertible modes of movements involving Rac and Rho GTPases (15). Elongated cells display a Rac-dependent mesenchymal movement (16), whereas round/ellipsoid cells display Rho/Rho-associated, coiled-coil containing protein kinase (ROCK)-dependent amoeboid motility (17–20).

Mesenchymal movement in melanoma cells seemed to be driven, in vitro, by the neural precursor expressed, developmentally downregulated 9 (NEDD9) protein, a member of the CAS family (15). TCF/LEF binding sites have been found in the human promoter of NEDD9 in colorectal cancer cells, identifying NEDD9 as a novel target of the WNT/β-catenin signaling pathway (21). Although increased NEDD9 expression has been reported in...
human metastatic melanoma samples (22), recent observations on mammary tumors argue that NEDD9 may have a more complex role in tumor formation, invasion, and metastasis than originally thought (23, 24). Here, we show that ICAT-mediated downregulation of NEDD9 expression converts the morphology of human Lu1205 melanoma cells toward a more rounded phenotype, leading to increased cell motility in 2D, invasiveness in 3D, and tumor cell colonization in the lungs of nude mice.

Materials and Methods

Cell lines

The human melanoma cell lines Lu1205 and WM522, obtained from Dr. M. Herlyn (Wistar Institute, Philadelphia, PA), Skmel3 and Mel501 from Dr. CR Gedding (Ludwig Institute, Oxford, UK), and Daun-1 from Florence Faure (Institut Curie, Paris, France) were authenticated in 2011 by comparative genomic hybridization array and transcriptomic analyses. Our results matched with publicly available datasets. Cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Sigma), 5 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/ml streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Plasmids and transfection

Plasmids, DNA constructs, transfections, production of recombinant proteins, dual luciferase assays, and flow cytometry were performed according to standard methods and are detailed in Supplementary Methods. Real-time quantitative PCR (RT-qPCR) and RNA silencing were performed as described previously (2). Primers and siRNA sequences are provided in Supplementary Tables S1 to S4.

Antibodies, immunoblotting, immunofluorescence, affinity precipitation, and in-gel zymography

A polyclonal anti-ICAT antibody was generated by immunizing a rabbit with a synthetic peptide (TQ-AFSSSETEDRRQ-C) corresponding to residues 69-80 of the C-terminal end of human ICAT, coupled to the keyhole limpet hemocyanin carrier protein. Mouse monoclonal against human ICAT (clone 5C6) and phallolidin-FTIC antibodies were from Sigma-Aldrich. Rabbit polyclonal antibody raised against phosphorylated myosin light chain (pMLC, T18/S19) was from Cell Signaling Technology. The Anti-Rac1 mouse monoclonal antibody was part of the Rac1 Activation Assay Biochem Kit (Cytoskeleton, Inc.). NEDD9-specific monoclonal (clone 2G9), and polyclonal antibodies specific to β-catenin were from Abcam. The standard methods used for cell lysis, Western blotting, affinity precipitation, cell immunofluorescence, and in-gel zymography are described in Supplementary Methods.

Wound healing, time-lapse phase-contrast microscopy, and invasion assays

Wound healing assays were performed as described previously (2). For time-lapse microscopy, cells were first transiently transfected with ICAT-Flag cDNA constructs, pGFP empty vector as control or siRNAs. Cells were then seeded sparsely to minimize cell-cell interactions and migration analysis was performed using an inverted microscope equipped for fluorescence imaging (Life Imaging Services). Time-lapse recording started 48 hours after transfection using a 20× lens. Fluorescent and phase-contrast images were collected at 4-minute intervals over 12 hours with a CDD camera (CoolSNAP fx, Photometrics) operated by Metamorph software (Molecular Devices). Analysis of cell migration was carried out by manually tracking individual cells in fluorescent frames, using the ImageJ software and a manual tracking plugin (http://rsb.info.nih.gov/ij/plugins/track/track.html). Inverted Matrigel invasion assays were performed as described previously (25).

Mice, subcutaneous and tail vein injections, and tissue preparation

Female nu/nu mice were housed in specific pathogen-free conditions at Institut Curie and when required they were sacrificed in accordance with French and European Union laws. Eight-week-old mice were tail-vein injected with 2 × 10^6 Lu1205-Luciferase (Lu1205-Luc) or Lu1205-ICAT-Luc cells. After sacrifice, lungs were collected and processed as described (26). Subcutaneous injections were performed as previously described (27).

IVIS analysis

Photon emissions from mice injected with Lu1205-Luc or Lu1205-ICAT-Luc cells were measured using an IVIS Spectrum Imaging System (Caliper Life Sciences). Mice were injected intraperitoneally with 10 μl/g of body weight of the r-Luciferin Firefly solution (15 mg/mL; Caliper Life Sciences) and then anesthetized before imaging. Images were collected using the field of view in the D position, 25 cm, medium binning with an F-stop of 1, and a maximum exposure time of 5 minutes. The luminescent signal was quantified with Living Image 4.3.1 software and expressed as photons/s/cm^2/sr (sr, steradian). Mice were sacrificed when the photon emission reached 10^7 photons/s/cm^2/sr or after a 20% body weight lost.

Statistical analysis

Prism6 software (GraphPad) was used for statistical analyses. Single-cell migration and mRNA content differences were assessed using the Mann–Whitney test (two-tailed), Collective cell migration, cell invasion, cell number, proliferation rates, and luciferase activity differences were studied using two-way ANOVA test followed by Bonferroni posttest. Mice survival curves were drawn as Kaplan–Meier cumulative proportion surviving graphs, and corresponding P values were calculated with the log-rank (Mantel–Cox) test. Melanoma metastases expression profiles were normalized using limma's normalize Quantiles method under R v2.15.2. Hierarchical clustering of CTNNB1P1 expression profiles was performed using Euclidean distances and Ward’s construction method. Differences in distant metastasis-free survival (DMFS) values between the two clusters were assessed using a Wilcoxon rank-sum test. All P values <0.05 were considered significant. All value sets were tested for normality using a Shapiro–Wilk normality test.

Results

High ICAT level expression in human melanoma cell lines is associated with formation of tumor metastases in nude mice

RT-qPCR analysis was used to compare ICAT mRNA levels in a series of 21 human melanoma cell lines. Cell lines
ICAT Modulates Melanoma Cell Motility and Invasion

Figure 1. High ICAT expression in human melanoma cell lines forming metastasis in nude mice. A, analysis of endogenous ICAT expression in a panel of 21 human melanoma cell lines. RT-qPCR was used to assay ICAT mRNA, using TATA box binding protein (TBP) as an internal reference. RNA levels, means ± SEM of three independent experiments. B, average values for ICAT expression in the group of cell lines forming s.c. tumors and metastases (green), compared with the groups of nonmetastatic cell lines forming (white) or not (orange) s.c. tumors. C, Immunoprecipitation of endogenous ICAT (8 kDa) with an anti-ICAT antibody and Western blot analysis of β-catenin and β-actin (loading control) from five melanoma cell lines. D, Immunofluorescence images of Lu1205, WM852, Dauv-1, Mel501, and Skmel3 cells incubated with an anti-ICAT monoclonal antibody (green) and 4',6-diamidino-2-phenylindole (DAPI; blue). *, P < 0.05; **, P < 0.01; ns, not significant. Scale bar, 20 μm.

forming metastases when s.c. injected in nude mice exhibited higher ICAT mRNA levels than nonmetastatic cell lines forming or not s.c. tumors (Fig. 1A and B). Presence and variable expression of the endogenous ICAT protein in five representative melanoma cell lines was visualized after immunoprecipitation and revelation with an anti-ICAT polyclonal antibody (Fig. 1C). Differential expression of ICAT between the cell lines was further illustrated by
immunofluorescence staining of cells grown on collagen-coated supports (Fig. 1D). Although faint staining of the nuclei with an anti-ICAT monoclonal antibody was visible in some cells, the endogenous ICAT protein in the different cell lines was mainly cytoplasmic. Specificity of the ICAT staining pattern was validated by knocking down ICAT with an siRNA (Supplementary Fig. S1A and S1B). Electrophoretic analysis of β-catenin in the same cell lines showed that the main partner for interaction with ICAT was more abundant in Mel501, Skmel3, and Dauv-1 cells than in Lu1205 and WM 852 cells (Fig. 1C and Supplementary Fig. S1C). β-Catenin was located both in the nucleus and cytoplasm of Mel501 and Skmel3 cells, whereas in Lu1205 cells, it was present in the cytoplasm and at the cell–cell contact.

**Transient exogenous ICAT expression increases melanoma cell motility**

Because high ICAT levels in melanoma cells were apparently associated with metastasis formation in nude mice, we analyzed the possible involvement of ICAT in 2D cell motility using time-lapse video microscopy. The movement of single Lu1205, Mel501, and Skmel3 cells was monitored over a 12-hour time period. Both endogenous ICAT expression and ICAT knockdown experiments were performed. First, the different cell types were transiently cotransfected with human control pcDNA3 or ICAT-WT cDNA and plasmid pEGFP-N1 for visualization of transfected cells. Expression of exogenous ICAT was evaluated by RT-qPCR and Western blot analysis of transfected cells (Fig. 2A). Mock Lu1205 cells migrated faster than Mock Mel501, and faster than Skmel3 cells, which was correlated with the level of endogenous ICAT. Exogenous ICAT expression significantly increased the migration speed by 28% for Lu1205, 46% for Mel501, and 54% for Skmel3 cells (Fig. 2B, Supplementary Movies S1–S4). This increased migration speed was independent of cell proliferation and apoptosis as demonstrated by flow cytometry analysis (Supplementary Fig. S2). In a second set of experiments, endogenous ICAT expression was reduced through the use of an siRNA-targeting ICAT expression (si ICAT). ICAT mRNA transcripts were reduced by 80% in Lu1205, 65% in Mel501, and 81% in Skmel3 cells exposed to ICAT siRNA when compared with siScrable-transfected cells (Fig. 2C). The migration speed of these ICAT-depleted Lu1205, Mel501, and Skmel3 cells was approximately half that of the respective controls (Fig. 2D, Supplementary Movies S5 and S6), confirming that ICAT positively regulates motility of these melanoma cells.

**Increased melanoma cell motility through ICAT exogenous expression requires ICAT/β-catenin interaction**

To assess whether ICAT/β-catenin protein interaction was required for ICAT activation of melanoma cell motility, we generated an ICAT cDNA encoding a mutant protein unable to interact with β-catenin. On the basis of the 3D crystal structure of ICAT in complex with β-catenin (7, 8), three conserved ICAT residues among vertebrates (Y15, K19, and V22) predicted to form hydrophobic bonds with β-cate

**Increased ICAT expression enhances melanoma cells invasive capacities in Matrigel**

Because cell motility in 2D is not necessarily equivalent to that in 3D (2, 28, 29), we assessed whether increased ICAT expression affects the invasive capacities of melanoma cells in a 3D Matrigel system. Lu1205, Mel501, and Skmel3 cells transiently transfected with control pcDNA3 (Mock), CMV::ICAT-WT or CMV::ICAT-DQE vectors, were submitted to an inverted Matrigel invasion assay. Mock Lu1205 cells were more invasive than Mel501 and than Skmel3 cells. Matrigel invasion of Lu1205, Mel501, and Skmel3 cells expressing exogenous ICAT-WT was greater than their respective controls (Fig. 3A–C). As expected, the ICAT-DQE mutant did not significantly affect the invasive capacities of transfected cells. Increased invasion potential can be due to the activation of matrix metalloproteinase (MMP). To test this hypothesis, we evaluated the endogenous level of MT1-MMP/MMP14 (30) by RT-qPCR and their activity by zymography in the presence and absence of ICAT-WT and ICAT-DQE in Lu1205, Mel501, and Skmel3 cells (Fig. 3D and E). Lu1205 and Mel501 cells showed similar amount of mRNA transcripts, whereas Skmel3 expressed approximately two times less MT1-MMP transcripts. The expression of pcDNA3 (Mock), CMV::ICAT-WT (ICAT-WT), or CMV::ICAT-DQE (ICAT-DQE) in the different cell lines did not affect the amounts of MT1-MMP transcripts, suggesting that ICAT-mediated invasion is MT1-MMP independent. These results were confirmed by in-gel gelatin zymography of conditioned media. Lu1205 and Mel501 cells transiently transfected with the same expression vectors showed similar amounts of proMMP2 and active MMP2. By contrast and in line with RT-qPCR data, Skmel3 cells had almost undetectable levels of MMPs.
Together, these results indicate that increased exogenous ICAT expression did not affect melanoma cells proteolytic capacities. Hence, the increased invasiveness of Lu1205, Mel501, and Skmel3 cells expressing exogenous ICAT was not a consequence of MMPs-mediated Matrigel degradation.
Exogenous ICAT expression affects Lu1205 cell morphology in 2D

The actin cytoskeleton and regulators of actin dynamics are central to individual cell movement (19). The morphology of Lu1205, Mel501, and Skmel3 cells transiently transfected with pcDNA3 (Mock), CMV::ICAT-WT, and CMV::ICAT-DQE before growing on collagen-coated plates was examined for polymerized actin after immunofluorescence staining. Both Mel501 and Skmel3 cells showed increased invasive capacities when transfected with ICAT-WT compared to Mock and ICAT-DQE. In contrast, Lu1205 cells did not show a significant difference in invasive capacities among the three groups.

Figure 3. Exogenous ICAT expression increases the invasive capacities of Lu1205, Mel501, and Skmel3 cells in Matrigel. A–C, left, comparison of representative Matrigel invasion assays of Lu1205 (A), Mel501 (B), and Skmel3 (C) cells transiently transfected with Mock, ICAT-WT, or ICAT-DQE expression vectors. Right, quantification of transfected cells invading the Matrigel by more than 45 μm. D, RT-qPCR analysis of MT1-MMP mRNA levels in Lu1205, Mel501, and Skmel3 transfected with Mock, ICAT-WT, or ICAT-DQE. E, in-gel zymography of Lu1205, Mel501, and Skmel3 cells transfected with Mock, ICAT-WT, or ICAT-DQE; proMMP2 and activated MMP2 are shown. *, P < 0.05; ns, not significant.
and Skmel3 cells exhibited an elongated morphology that remained unchanged after exogenous ICAT expression (Supplementary Fig. S5A and S5B). By contrast, Lu1205 cells exhibited two types of morphology (Fig. 4A and B): approximately 40% of the cells were elongated (mesenchymal type) and the other 60% exhibited a round morphology (amoeboid type). Exogenous ICAT-WT expression significantly increased the proportion of round cells by 20%, whereas ICAT-DQE exogenous expression had no such effect. A round morphology is often associated with a bleb-driven type of motility (16). To assess the presence of blebs, Lu1205 cells were transiently transfected with a LifeAct-TagGFP2 plasmid allowing visualization of F-actin in living cells. Time-lapse microscopy revealed migrating cells exhibiting spherical protrusions devoid of actin with short lifetimes consistent with a bleb-associated movement (Supplementary Fig. S5C).

Members of the Rho family of small GTPases are key regulators of melanoma cell movement (15, 31). To assess the possible involvement of Rac and Rho in the increased motility of Lu1205 cells expressing exogenous ICAT-WT, two specific inhibitors were used. Treatment with the Rac inhibitor NSC23766 for 24 hours substantially increased the proportion of round cells to 92%, thus mimicking the effect of exogenous ICAT-WT. Inhibition of the Rho/ROCK pathway with the Y27632 inhibitor had the opposite effect, with more than 93% of cells showing an elongated morphology (Fig. 4A and B). ICAT exogenous expression was able to counteract Y27632 inhibitor effects with 26% of the cells showing a round morphology (Supplementary Fig. S6A and S6B). We performed pull-down assays of Rac1-GTP with PAK-PDB beads to test for the effects of increased ICAT expression on Rac activation. In Mel501 and Skmel3, the level of Rac1-GTP (active Rac1) was lower than in Lu1205 cells (Supplementary Table S5) and it was reduced when exogenous ICAT was present (Supplementary Fig. S5D). The amount of Rac1-GTP (active Rac1) in Lu1205 cells was markedly reduced (40%) when the exogenous ICAT-WT protein was present, whereas ICAT-DQE protein had no such effect (Fig. 4C), indicating that increased ICAT-WT expression downregulates Rac signaling. Amoeboid movement is associated with high levels of pMLC II. Strong staining with anti-pMLC antibody of ICAT-WT–transfected cells was observed in round cells, whereas elongated cells showed a faint pMLC staining. Similarly, Rac inhibitor–treated cells showed abundant anti-pMLC labeling, whereas inhibition of Rho/ROCK signaling greatly reduced the staining (Fig. 4A). Hence, NSC23767 treatment of Lu1205 cells recapitulated the effects of increased ICAT expression. Rho and Rac inhibitors did not affect the elongated morphology of Mel501 and Skmel3 cells (Supplementary Table S5). Altogether, these findings suggest that increased ICAT expression in vitro in Lu1205 cells induces a switch toward a round morphology, through downregulation of Rac signaling, but this mechanism does not operate in Mel501 and Skmel3 cells.

**NEDD9 is a downstream effector of ICAT in melanoma cells**

As ICAT modulated the Rho/Rac signaling pathway in Lu1205 cells, we searched for β-catenin target genes involved in the regulation of cell motility. ICAT is a known regulator of NEDD9, a key downstream effector of β-catenin in melanoma cells (15). To assess whether ICAT modulates NEDD9 expression in melanoma cells, we performed qRT-PCR analysis in ICAT-expressing and control cells. As shown in Figure 5, ICAT expression significantly increased the expression of NEDD9 in Lu1205 cells, whereas Skmel3 cells showed no change. These results indicate that ICAT modulates NEDD9 expression in melanoma cells, suggesting a potential role for ICAT in the regulation of cell motility through the β-catenin signaling pathway.
in cell migration and able to regulate the Rho/Rac signaling pathway. **NEDD9**, a regulator of Rac driving mesenchymal movement (15) and promoting migration of colorectal cancer cells (21), emerged as a candidate. To test for a relationship between ICAT and NEDD9 in controlling melanoma cell motility, we quantified NEDD9 mRNA transcripts and proteins in five melanoma cell lines (Fig. 5A). NEDD9 mRNA was abundant in the two cell lines with a low endogenous ICAT content (Mel501 and Skmel3), but it was less expressed in Lu1205, WM852, and Dauv-1 cells exhibiting high endogenous ICAT content. At the protein level, marked differences were noted. In Mel501 and Skmel3 cells, one major band of 105 kDa was present. In Lu1205, WM852, and Dauv-1, at least two bands were visible, a lower band (105 kDa) similar to the one seen in Mel501 and Skmel3, and an upper band (115 kDa; Fig. 5A; Supplementary Table S5) that may correspond to a highly phosphorylated form of NEDD9 (32).

To test whether ICAT was able to inhibit NEDD9 expression by competing with TCFs/Lef1s for binding to β-catenin, Lu1205 and Mel501 cells were transiently transfected with the NEDD9 promoter driving expression of the luciferase gene (21). A marked luciferase activity was observed in Lu1205 and Mel501 cells. Cotransfection with an ICAT-WT vector reduced luciferase activity by 60%, both in Lu1205 and Mel501, whereas the ICAT-DQE mutant had no significant effect (Fig. 5B and C). Importantly, analysis of Lu1205, Mel501, and Skmel3 cells transiently expressing exogenous ICAT-WT showed reduced levels of NEDD9 mRNA transcripts and protein (Fig. 5D). Such reduction was not seen in cells expressing exogenous ICAT-DQE. Accordingly, knockdown of ICAT in Lu1205 cells using an si ICAT increased NEDD9 protein expression (Supplementary Figs. S1B and S5E).

To clarify the role of NEDD9 on melanoma cell motility in 2D, NEDD9 was knocked down in Lu1205, Mel501, and Skmel3 cells using a pool of siRNAs. The resulting decreased expression of NEDD9, confirmed by RT-qPCR and Western blot analysis (Fig. 6A), was associated in Lu1205 cells with reduced Ral1 activation (Supplementary Fig. S5F) and increased migration speeds of transfected cells in tracking assays (Fig. 6B). In Lu1205 cells, ectopic NEDD9 expression by transient transfection with a pcDNA3-NEDD9 expression vector significantly decreased the migration speed of transfected cells (Fig. 6C and D). The modification of Lu1205 cell migration speed was associated with a switch from a round to an elongated morphology.

![Image](cancerres.aacrjournals.org/doi/fig/411x373.png)

**Figure 5.** Increased ICAT expression reduces NEDD9 levels in a panel of five human melanoma cell lines. A, top, RT-qPCR quantification of NEDD9 and ICAT mRNA using TBP as an internal reference. The values reported for RNA levels are means ± SEM of three independent experiments. B, Western blot analysis of NEDD9 in the five melanoma cell lines; bands with a molecular weight of 105 and 115 kDa are visible. β-Actin, loading control. B and C, effect of transient exogenous ICAT expression on the NEDD9 promoter activity. Lu1205 (B) and Mel501 (C) cells were transiently transfected with a NEDD9-luciferase reporter in presence (Lu1205) or absence (Mel501) of CMV:β-catenin. Lu1205 and Mel501 cells were also transfected with Mock, ICAT-WT, or ICAT-DQE expression vectors. Data, means ± SEM of three independent experiments. D, top, RT-qPCR of NEDD9 mRNA levels in Lu1205, Mel501, and Skmel3 transfected with control Mock, ICAT-WT, or ICAT-DQE vectors. Data, means ± SEM of three independent experiments. Bottom, Western blot analysis of NEDD9 levels in the three cell lines (β-actin, loading control). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant.
Figure 6. NEDD9 depletion increases Lu1205, Mel501, and Skmel3 cell migration speed. A, RT-qPCR and Western blot analysis of NEDD9 in Lu1205, Mel501, and Skmel3 cells transfected with control si SCR or si smart pool against NEDD9 (si NEDD9). B, cells were subjected to a single-cell migration assay followed by 2D video microscopy for 12 hours. Fifty cells were studied for each experiment. C and D, similar experiments in cells transfected with Mock or CMV::NEDD9 (NEDD9) expression vectors. RNA levels and migration speeds are expressed as means ± SEM of three independent experiments. TBP, internal reference. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant.
because 76% of cells expressing exogenous NEDD9 exhibited an elongated phenotype (Supplementary Fig. S5G). By contrast, increased NEDD9 expression had no effect on Mel501 and Skmel3 cells migration speed. This might be explained by an absence of impact of NEDD9 overexpression on Mel501 and Skmel3 elongated cellular morphology.

**Increased ICAT expression accelerates tumor cell colonization in the lungs of nude mice**

Because cell motility and invasive behavior are essential steps of the metastatic process (33), we next investigated whether increased ICAT expression could enhance the invasiveness of melanoma cells. Lu1205 cells were chosen as they were previously shown to form metastases in the lungs of nude mice after tail vein injection (26), whereas Mel501 and Skmel3 did not. Bioluminescent imaging was used to follow tumor formation. Representative luciferase-positive Lu1205-Luc (−ICAT) and Lu1205-ICAT-Luc (+ICAT) stable clones were generated. Two clones were selected based on their equivalent bioluminescent signal (in the range of 2 x 10^7 p/s/cm^2/sr for 10^4 cells), and injected intravenously. Presence of luciferase-expressing cells was monitored with the IVIS spectrum system (Fig. 7A). Bioluminescence was detected in the lungs of 2 of 8 injected mice expressing exogenous ICAT (+ICAT), 5 weeks after injection. By week 9, a luminescent signal was detectable in 7 of 8 animals. In the control group (−ICAT), luminescence was detected after 5 weeks in 1 of 8 mice. This number increased to 7 of 8 mice by week 15. The Kaplan–Meier analysis disclosed a significant difference between the survival time of mice injected with control cells and those injected with exogenous ICAT-expressing cells (Fig. 7B). Immunofluorescence staining with an anti-ICAT antibody (green) and DAPI (blue), D, RT-qPCR and Western blot analysis of ICAT and NEDD9 in the lungs of nude mice. **, P < 0.05; ***, P < 0.001. Scale bar, 50 μm.

**High ICAT expression is associated with reduced survival time of patients with melanoma**

To assess whether our in vitro and in vivo findings had any relevance to human melanoma, we took advantage of publicly available transcriptomic data and clinical parameters from 22 patients with metastatic melanoma (34). On the basis of an unsupervised hierarchical clustering of ICAT/CTNNB1 expression, the cohort was divided into two clusters. Cluster 1 (n = 8) comprised metastases with high ICAT/CTNNB1 levels and cluster 2 (n = 14) comprised metastases with intermediate and low ICAT/CTNNB1 levels (Supplementary
Fig. S7A). Importantly, a significant 4-fold reduction in the DMFS time was observed in cluster 1 when compared with cluster 2, suggesting that high ICAT/CTNNBIP1 expression could be associated with decreased survival of some metastatic patients (Supplementary Fig. S7B). Using the same transcriptomic data, we investigated whether high ICAT levels correlated with low NEDD9 levels, as we observed in vitro. NEDD9 mRNA expression in patients with metastases and high β-catenin/ICAT levels was apparently lower than in patients with low β-catenin/ICAT levels. The correlation coefficient \( r = 0.381 \) between β-catenin/ICAT levels and NEDD9 showed a trend suggesting that ICAT might also downregulate NEDD9 levels in some human melanoma samples (Supplementary Fig. S7C).

Discussion

WNT signaling is mediated through β-catenin, which serves as a hub for the recruitment of positive and negative regulators of transcription (35, 36). From a recent large-scale study of melanoma exomes, 7% of patients exhibited β-catenin/CTNNB1 “driver” mutations (37). However, it remains unclear whether β-catenin acts as an inhibitor, a promoter, or both during melanomagenesis and metastasis formation because of conflicting in vitro and in vivo results (1–5, 38, 39). It was recently inferred that a subtle balance in the level and activity of β-catenin is required in normal melanocytes to prevent melanoma formation (40). One of the regulators of this balance might be ICAT, a small acidic protein that sterically competes with members of the TCF/LEF family for binding to β-catenin (6, 7). Although no mutations in the ICAT/CTNNB1P1 gene have been identified in human melanoma samples (13, 37, 41), the ability of ICAT to regulate β-catenin transcriptional activity may modulate the clinical outcome of patients depending on its level of expression.

In this study, we observed that human melanoma cell lines forming metastases in nude mice exhibited high endogenous ICAT expression, whereas most nonmetastatic melanoma cell lines expressed moderate or low levels of ICAT. Furthermore, based on publicly available transcriptomic data (34), we found that the highest ICAT transcriptional levels in tumor samples from patients with melanoma were associated with low distant metastasis-free survival times, thus emphasizing the putative role of ICAT in metastasis progression. Using three human melanoma cell lines expressing variable levels of endogenous ICAT, we demonstrate for the first time that ICAT when stably bound to β-catenin acts as a positive regulator of melanoma cell motility in a 2D system and increases their invasive capacities in 3D. These findings are consistent with our recent demonstration that presence of nuclear β-catenin in melanoma cells reduces their in vitro motility (2). Presence of blebs and increased MLC phosphorylation were observed in Lu1205 cells, whereas secretion of MMPs was unaffected. Together, this suggests that actomyosin contraction in Lu1205 cells expressing exogenous ICAT could generate hydrostatic pressure, causing the plasma membrane to detach from the actin cortex, resulting in bleb formation (42). Round blebbly cells would squeeze through the impeding collagen fibers present in Matrigel (43–45), thereby facilitating matrix invasion independently of proteases. Although widely accepted in vitro (16), tumor invasion through protease-independent mechanisms remains controversial in vivo (33, 46) but was recently supported by the visualization of bleb-driven tumor cell migration in primary explants of human carcinoma (42).

In vivo, tumor cell extravasation and tissue colonization are both characteristics of the metastatic process (33). Tail vein injection of melanoma cells leads to extravasation and lung colonization, partially mimicking metastasis formation. Using bioluminescence imaging, we visualized increased tumor colonization and accelerated formation of nodules in the lungs of nude mice grafted with Lu1205 cells overexpressing ICAT. The capacity of melanoma cells to metastasize may rely on their ability to change morphology and adapt their movement to the microenvironment so as to respond to signals transmitted by mediators emerging from the stroma (15, 47, 48). This model, implicating a Rho/Rac-mediated switch between mesenchymal and amoeboid movement, is supported by the high invasiveness of Lu1205 cells expressing exogenous ICAT. The absence of morphologic changes in elongated Mel501 or Skmel3 cells expressing ectopic ICAT and their poor invasiveness may account for their inability to form lung metastases in nude mice (not shown). In keeping with this result, Rac activity associated with mesenchymal movement inhibited lung colonization by circulating tumors cells (15) and amoeboid movement favored lung colonization by A375M melanoma cells in nude mice (43).

The molecular mechanisms by which ICAT increases melanoma cell motility and promotes tumor colonization are not fully elucidated. One of the downstream effectors of ICAT/β-catenin, involved in cell motility (32), might be the scaffold protein NEDD9 previously reported to be overexpressed in 30% to 50% of metastatic melanomas samples (22). Surprisingly, high NEDD9 levels in Mel501 and Skmel3 cells were not associated with metastasis formation, whereas NEDD9 expression was lower in the metastatic Lu1205 and WM852 cells. Lu1205 cells mainly exhibited a round shape, which in the presence of ectopic NEDD9 expression shifted to an elongated morphology. By contrast, Mel501 and Skmel3 cells were almost exclusively elongated with numerous protrusions and NEDD9 overexpression had no morphologic incidence. Similar results have been previously reported in other melanoma cell lines in which NEDD9 overexpression increased the number of elongated cells in 3D, whereas NEDD9 reduction had the opposite effect (15). Likewise, involvement of NEDD9 in the motility of cell lines originating from different organs/tissues seemed to be cell-type and system dependent (29, 32, 49). Results with Lu205 cells implicate NEDD9 as a downstream effector of ICAT/β-catenin activity. NEDD9 in complex with the guanine nucleotide exchange factor DOCK3 activates Rac1 to promote mesenchymal movement (15, 50). Reduction of NEDD9 level through increased ICAT activity results in less Rac1-GTP, thus favoring Rho/ROCK-driven amoeboid movement. This process does not seem to operate in the poorly motile Mel501 or Skmel3 cells that remain elongated even in the presence of Rac inhibitor. In these cells, ICAT/β-catenin...
targets (such as M-MITF) may play a more critical role than NEDD9.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: M.J. Domingues, L. Larue, J. Bonaventure
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References


Acknowledgments
The authors thank Drs. C. Gottardi (University of Chicago, Chicago, IL), E. Golemis (Fox Chase Cancer Center, Philadelphia, PA), M. Herlyn (Wistar Institute, Philadelphia, PA), and M. Piel (Institut Curie) for providing biologic materials. They also thank M.-C. Lienafa (Institut Curie) and A. Campbell (Beatson Institute, Glasgow, UK) for technical assistance. The authors also thank members of the animal colony and imaging facilities of the Institut Curie.

Grant Support
This work was supported by grants from "La Fondation ARC contre le Cancer" and INCa. M.J. Domingues is recipient of a fellowship from the Ministère de l’Éducation Nationale de la Recherche et de la Technologie (MENRT).

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Received March 28, 2013; revised January 7, 2014; accepted January 21, 2014; published OnlineFirst February 10, 2014.


β-Catenin Inhibitor ICAT Modulates the Invasive Motility of Melanoma Cells

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