# ROR1 Contributes to Melanoma Cell Growth and Migration by Regulating N-Cadherin Expression via the PI3K/Akt Pathway

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The Receptor tyrosine kinase-like Orphan Receptor 1 (ROR1) is primarily expressed by neural crest cells during embryogenesis. Following a complete downregulation after birth, ROR1 was shown to re-express in various types of cancers. Little is known about ROR1 expression and function in melanoma. Here we show that ROR1 is aberrantly expressed in both melanoma cell lines and tumors and that its expression associates with poor Post-Recurrence Survival of melanoma. Using gain- and loss-of-function approaches we found that ROR1 enhances both anchorage-dependent and -independent growth of melanoma cells. In addition, ROR1 decreases cell adhesion and increases cell motility and migration. Mechanistically, ROR1 was found to induce upregulation of Akt and the mesenquimal markers N-cadherin and vimentin. The regulation of N-cadherin by ROR1 relies on both Akt dependent and independent mechanisms. ROR1 does not affect Wnt canonical pathway but was found to be engaged in a positive feedback loop with Wnt5a. In summary, we show that ROR1 contributes to melanoma progression and is a candidate biomarker of poor prognosis. Although further studies are needed to confirm this possibility, the present work indicates that ROR1 is a good prospective target for melanoma cancer therapy. 

Key words: ROR1; melanoma; N-cadherin; Akt; migration

## INTRODUCTION

Melanoma is the most deadly form of skin cancer and is responsible for 60% of deaths from this type of cancer [1]. Molecular targeted therapies have shown promise in the management of melanoma. However, acquired resistance to therapy is a common problem, highlighting the need of new molecular targets and more effective combinational therapies [2]. Receptor tyrosine kinases (RTK) play a critical role in normal development and their aberrant expression and activation leads to the progression of various types of cancer [3]. RTK-like Orphan Receptor (ROR) 1 and 2 share an overall 58% homology in the amino acid sequences and are evolutionarily conserved among different species [4]. ROR proteins consists of an extracellular region containing an immunoglobulin (Ig)-like domain, a cysteine rich domain (CRD), a kringle (KNG) domain, and a cytoplasmatic region which includes a serine/threonine domain and a TK domain with protein kinase activity [5]. ROR proteins are primarily expressed during embryogenesis, mainly in migrating neural crest and mesenchymal cells, and play a critical role in development and organogenesis [6,7]. Studies in rodents suggest that ROR1

Abbreviations: Akt, RAC8alpha serine/threonine 8 protein kinase; CIP, Calf intestinal alkaline phosphatase; CLL, Chronic lymphocytic leukemia; CM, Conditioned medium; CRD, Cysteine rich domain; CREB, Cyclic AMP-responsive element-binding; Dvl, Dishevelled; E-cadherin, Epithelial cadherin; EMT, Epithelial mesenchymal transition; FoxO, Forkhead box protein O1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HuMel, Human immortalized melanocytes; Ig, Immunoglobulin; KNG, Kringle; MFS, Metastasis-Free Survival; mTORC1, Mammalian target of rapamycin complex 1; N-cadherin, Neural cadherin; NF-kb, Nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, Phosphoinositide-3-kinase; PRS, Post-Recurrence Survival; ROR, RTK-like orphan receptor; RTK, Receptor tyrosine kinases; qRT-PCR, quantitative reverse transcriptase – polymerase chain reaction; TCF, T-cell factor; TGFb, Transforming growth factor beta; shRNA, short hairpin RNA; STAT3, Signal transducer and activator of transcription 3.

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and ROR2 play both redundant and nonredundant roles in the body [8]. Both proteins are strongly downregulated afterbirth and are virtually absent in normal adult tissues [6,7,9,10].

Interestingly, ROR1 was shown to be expressed in different types of leukemia [11–16], lung adenocarcinoma [17], renal cell carcinoma [18], breast cancer [19], and melanoma [20]. In breast adenocarcinomas, high levels of ROR1 were associated with aggressive disease progression and shown to contribute to tumor-cell growth through activation of the PI3K/Akt/CREB pathway [19]. Furthermore, ROR1 was associated with Epithelial Mesenchymal Transition (EMT) and positively correlated with higher rates of relapse and metastatic capacity in breast cancer cells [21]. Accordingly, antibodies targeting ROR1 can inhibit breast cancer progression and metastasis [21]. Wnt5a has been suggested to be a ROR ligand for tumor cells and many of the processes regulated by ROR1 have been associated with Wnt5a [12,19]. However, the link between ROR1 and Wnt5a needs to be better understood since ample evidence shows that Wnt5a mediates tumor suppressor signals in breast cancer [22].

There is little information about the role of ROR1 in melanoma. Targeting of ROR1 in melanoma cells was shown to induce apoptosis [20]. On the other hand, O'Connell et al. in 2013 showed that ROR1 determines a poorly invasive phenotype and inhibits melanoma metastasis [23]. In the current study, we show that ROR1 is aberrantly expressed in melanoma and contributes to several malignant features of melanoma cells.

## MATERIALS AND METHODS

### Cell Culture

Melanoma cell lines were provided by Dr. Zeev Ronai (The Sanford-Burnham Institute, La Jolla) except for the cell lines 888Mel, 501Mel, WM983A, WM983B that were provided by Dr. Alain Mauviel (Institute Curie, Orsay, France). Immortalized melanocytes were kindly provided by Dr. David Fisher (Massachusetts General Hospital, Boston). The L cells expressing Wnt3a and Wnt5a were kindly provided by Dr. Stuart Aaronson (Mount Sinai School of Medicine, New York). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, PAA) 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen), at 37°C and 5% $CO_2$. The conditioned medium (CM) containing Wnt3a, Wnt5a, or control was prepared as previously described [24]. Briefly, L cells were seeded at a density of $1 \times 10^6$ cells in a 100 mm dish containing DMEM with 1% FCS and cultured for 4 days. Then CM was harvested, centrifuged at 1000*g* for 10 min, filtered, and stored at −20°C.

### shRNA Constructs, Over-Expression System and Viral Infection

The oligonucleotides targeting ROR1 (5′-GCAAGCATCTTTACTAGGA-3′ and 5′-AAGTGTTCTCAGTTCGCCATT-3′) and scramble (5′-GAAACTGCTGACCGTTAAT-3′) were cloned into pRetroSuper vector. Human ROR1 was cloned into the lentiviral vector VIRSP. To generate the viral particles, HEK-293T producer cells were cotransfected with the retro or lenti vectors and the packaging plasmids. Viral supernatants were harvested, filtered and used to transduce A375, Lu1205, and UACC903 cells. Cells were selected with 3 μg/ml puromycin for one week and then maintained with 1 μg/ml puromycin.

### Western Blotting

For the Western blotting analysis, cell lysates were collected by addition of lysis buffer supplemented with protease and phosphatase inhibitors for 10 min on ice [25]. The cell lysates were centrifuged at 13000 rpm for 15 min at 4°C, and the supernatants were collected and quantified using the Bradford method [26]. Between 20–50 μg of proteins were diluted in 6× Laemmli buffer, boiled at 95°C for 5 min, separated on 10–12% SDS–PAGE gels and then transferred to nitrocellulose membrane. The membranes were blocked with 5% milk in 0.05% Tween-PBS at room temperature for 1 h and then incubated with the primary antibodies: Akt1 (cs-5298), pAkt1/2/3 (sc-7985), Dvl-2 (sc-13974), N-cadherin (sc-7939), GAPDH (sc-25778), RhoA/C (sc-179), ROR1 (cs-4102, sc-130386 and R&D-AF2000), STAT3 (sc-482), pSTAT3 (sc-8059), Tubulin (SIGMA-T9026), Vimentin (AMF-17b), and Wnt5a/b (cs-2530), at 4°C overnight. The corresponding HRP-conjugated secondary antibodies: anti-mouse (GE NA931V), anti-rabbit (GE NA934), or anti-goat (sc-2020) were incubated for 1 h at room temperature. Immunoreactive bands were detected by an ECL system (Amersham Biosciences).

### Validation of ROR1 Antibodies

To validate our data regarding the expression of ROR1 we used three ROR1 antibodies along this work (AF2000 from R&D, sc-130386 from Santa Cruz and cs-4102 from Cell Signaling). The three of them were tested and compared in every technique employed to determine ROR1 protein expression. Antibodies AF2000 and cs-4102 were selected for use in Western blots, whereas antibodies sc-130386 and cs-4102 were used for immunohistochemistry. AF2000 was the better antibody for flow cytometry analysis.

### Adhesion Assays

Cells were detached with 2 mM EDTA, counted and suspended in DMEM 10% FBS at a concentration of $2 \times 10^5$ cells/ml. 200 μl of the cell suspension was seeded in 96-well flat bottom plates for 30 m at 37°C. Then the plates were washed with PBS three times and the remaining adherent cells were fixed and stained with 0.5% crystal violet in 20% methanol for 10 min at room temperature. The cells were washed in PBS and the dye was dissolved with 100 μl of 10% methanol and 5% acetic acid. The absorbance was

measured at 590 nm using a microplate reader (Labsystems Multiskan). The results are expressed as the ratio of control cell's absorbance and indicate mean ± s.e. from three independent experiments.

### Wound Healing Assays

Cells were cultured to confluency in six-well plates previously coated with 5 μl/ml fibronectin and then scratch-wounded using a 200 μl pipette tip. The cells were washed three times with PBS and incubated with DMEM 10% FBS for 16 h at 37°C. Initial and final wound widths were photographed and the cell-free area was measured. The results are expressed as the percentage of migration in each case and indicate mean ± s.e. from three independent experiments.

### Transwell Migration Assays

Cells were detached with 2 mM EDTA, counted and suspended in DMEM 1% FSB at a concentration of $2 \times 10^5$ cells/ml. 200 μl of the cell suspension was seeded on the upper chamber of a 24-well 8.0 μm pore size Cell Culture Insert (BD). The bottom side of each insert was previously coated with 5 μg/ml fibronectin and the bottom chamber was filled with 600 μl of DMEM 10% FSB. After 16 or 20 h (depending on the experiment), inserts were removed, washed, and cells that had migrated to the bottom side of the inserts were stained with 0.1% crystal violet in 20% methanol and counted in an inverted microscope.

### Survival Analysis

Gene expression and survival data were retrieved from NCBI GEO database. Metastasis-Free Survival (MFS) was measured in months from the date of initial surgery to the date of initial detection of recurrent disease or last follow-up without evidence of recurrence. Post-Recurrence Survival (PRS) was defined as the time from the date of initial detection of recurrent disease to the date of melanoma-related death or the last date the patient was known to be alive. Patients known to be alive at last follow-up were censored. MFS and PRS curves were plotted using the Kaplan–Meier method and survival differences in variables were determined using the log-rank test. A value of $P < 0.05$ was regarded as statistically significant.

### Statistics

All experiments were performed at least three times. A mean and standard error was derived from all repeated experiments. Student's *t*-test was performed to compare treated groups to vehicle control. Values of $P < 0.05$ were considered statistically significant.

## RESULTS

### ROR1 Is Expressed in Melanoma and Associates With Short Post-Recurrence Survival.

To evaluate the expression of ROR1 we performed Western blot and quantitative Real-Time PCR (qRT-PCR) analysis in five primary and eight metastatic melanoma cell lines. Though at variable levels, ROR1 was detected in all cell lines analyzed and showed similar expression levels in both primary and metastatic cell lines (Figure 1A and Supplementary Figure S1). ROR1 mRNA and protein levels in five of the cell lines that express lower amounts of ROR1 were compared to those of human immortalized melanocytes (HuMel, Figure 1B). ROR1 levels markedly increased compared to that seen in HuMel with a fold increase ranging from 3.1 in Lu1205 to 11.8 in A375 and 14.3 in UACC903. These three cell lines will be used throughout this work. Unlike ROR1, Wnt5a, and ROR2 expression was undetectable in many cell lines (Figure 1A). ROR2 was expressed in some metastatic cell lines but the three cell lines displaying the highest levels of ROR2 expression corresponded to cell lines derived from primary tumors (WM115, WM35, and 888-Mel). Expression of ROR1 did not associate with either ROR2 or Wnt5a levels (Figure 1A). Post hoc analysis revealed that most of the cells lines we used have a "proliferative" phenotype according to the Phenotype-Specific Expression database (an algorithm that characterizes melanoma cell lines as either "proliferative" or "invasive" based on the expression of a 97-gene signature) [27]. The Lu1205 cell line, which displayed the lowest level of ROR1, was the only one having an "invasive" phenotype raising the possibility that ROR1 level might associate with these phenotypes [23]. The analysis of ROR1 expression in the 220 cell lines in the database determined that, unlike Wnt5a, ROR1 expression does not associate with neither of these two phenotypes (Supplementary Figure S2). From our experiments and microarray data we concluded that ROR1 is aberrantly expressed in melanoma cell lines but it is not associated neither with a given progression stage nor with proliferative or invasive phenotypes.

We next determined ROR1 expression in samples from melanoma patients. Immunohistochemical analyzes revealed that eight out of thirteen (62%) samples were positive for ROR1 staining (Figure 1C, D and Supplementary Table S1). These results were confirmed by an independent analysis of a second cohort that revealed 82% of positive samples for ROR1 (Supplementary Table S2). Since many of these patients did not have a prolonged or well-documented follow-up, we examined published DNA microarrays to study the relevance of ROR1 expression in melanoma tumors. We first performed Kaplan–Meier survival analysis using the dataset GSE46517, composed of 39 metastatic melanoma tissue samples. We segregated patients into two groups based upon their relative expression of ROR1. We observed a lower MFS in patients with high (upper 50th percentile) versus patients with low

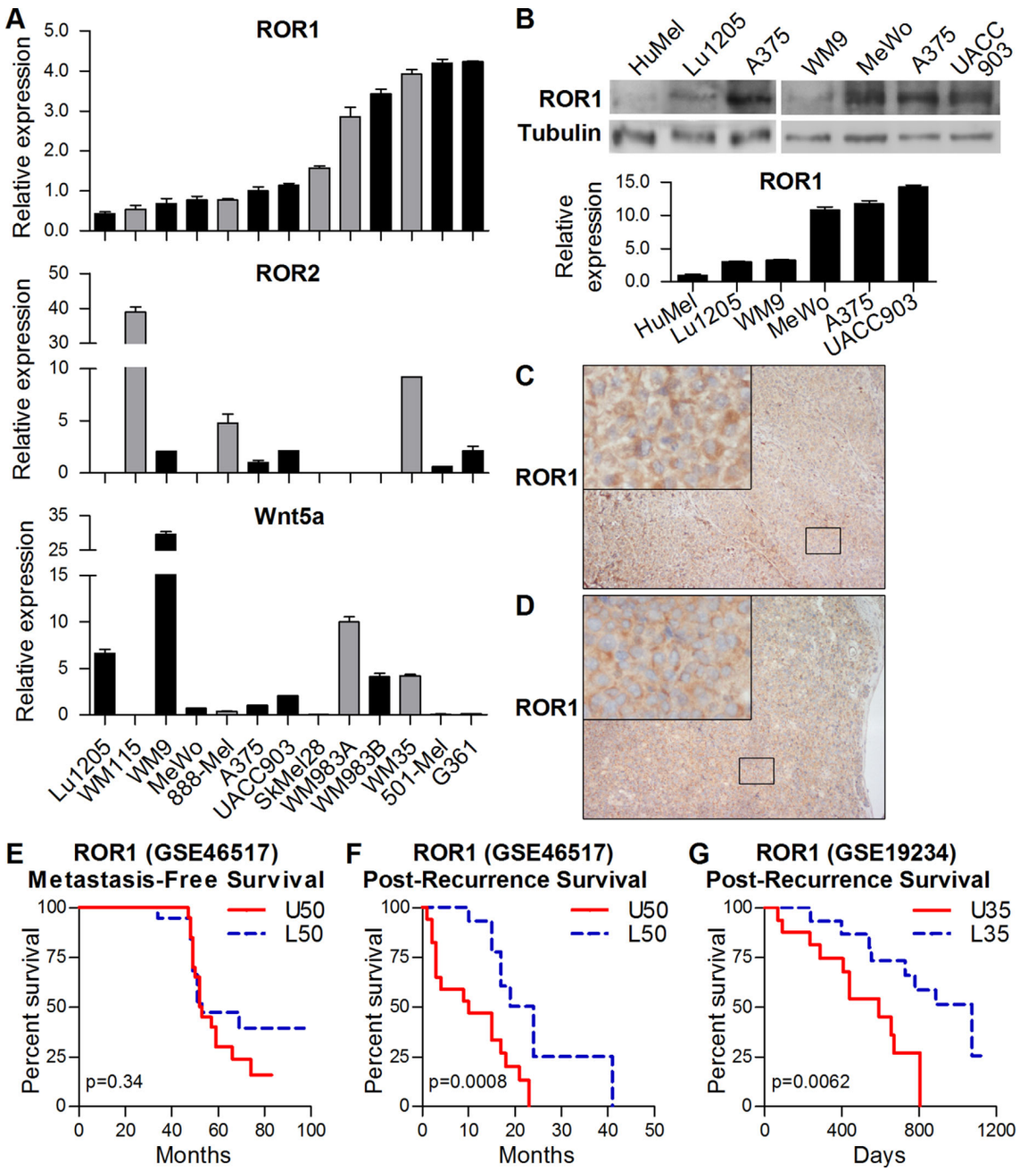


Figure 1. Expression of ROR1 in human melanoma cell lines and tumor samples. (A) ROR1, ROR2, and Wnt5a mRNA relative levels in melanoma cell lines. Black and gray bars indicate metastatic and primary cell lines, respectively. mRNA levels were normalized to internal RNPII levels and expressed as relative to A375 cell line. (B) Top panel: Western blot analyzes of ROR1 expression in human immortalized melanocytes (HuMel) and melanoma cell lines. Tubulin was used as loading control. Bottom panel: ROR1 mRNA relative levels in HuMel and melanoma cell lines. mRNA levels were normalized to internal RNPII levels and expressed as relative to HuMel. (C, D) Representative images of immunohistochemistry staining for ROR1 with hematoxylin counterstaining from two ROR1 positive melanoma patients. Inset shows 500× magnification of the indicated area. Patient ID: 12–8,149 (C) and 09–1,1521 (D). (E, F, G) Kaplan–Meier survival analysis for 39 metastatic melanoma tissue samples from the dataset GSE46517 (E, F) or 44 metastatic melanoma tissue samples from the dataset GSE19234 (G) using defined cut-off values for ROR1 expression. For the GSE46517 dataset, Metastasis-Free Survival (E) or Post-Recurrence Survival (F) was compared segregating the patients into two groups: lower 50th percentile (L50, n = 19) and upper 50th percentile (U50, n = 20). For the GSE19234 dataset Post-Recurrence Survival (G) was compared segregating the patients into two groups: lower 35th percentile (L35, n = 18) and upper 35th percentile (U35, n = 19). Statistical differences were determined by log-rank test, *P* values are shown.

(lower 50th percentile) ROR1 levels, although the difference was not statistically significant ($P = 0.34$, Figure 1E). However, we observed a significant correlation between higher expression levels of ROR1 and shorter PRS (median survival 10 vs. 24 months, Hazard Ratio = 4.640, 95%CI = 1.890 –11.39, $P = 0.0008$, Figure 1F). On the contrary, neither ROR2 nor Wnt5a showed any correlation with either MFS or PRS (Supplementary Figure S3). To confirm these observations we analyzed the dataset GSE19234, composed of 44 metastatic melanoma tissue samples from stage III and stage IV patients [28]. Again, we observed a significant association between higher expression levels of ROR1 and shorter PRS (median survival 440 vs. 1073 days, Hazard Ratio = 3.818, 95%CI = 1.463–9.959, $P = 0.0062$, Figure 1G). MFS could not be analyzed in this dataset. Together, these results indicate that ROR1, a protein normally not expressed in adult tissues, is frequently expressed in melanoma and may serve as a potential prognostic marker in clinical practice.

### Stable ROR1-Overexpressing and -Deficient Cell Lines

To investigate the biological and biochemical mechanisms regulated by ROR1, both gain- and loss-of-function approaches were employed. The A375 cell line was chosen for these experiments since these cells display moderate levels of ROR1 that were suitable for both silencing and overexpression (Figure 1A). In addition, this cell line also presents moderate levels of Wnt5a (Figure 1A). We transduced A375 cells with retroviral particles encoding short-hairpin RNAs (shRNAs) specific for ROR1 or a scramble sequence (control shRNA) and the stable cell lines A375-shROR1 and A375-scramble were established. ROR1 was efficiently silenced in A375-shROR1 cells compared with A375-scramble cells as assessed via qRT-PCR, Western blot and flow cytometry (Figure 2A, B and Supplementary Figure S4A). To rule out nonspecific effects of the shRNA, we designed a second shRNA for ROR1 (called shROR1 II) which also silenced ROR1 expression albeit slightly less efficiently (Figure S4B, C). ROR1 was also efficiently silenced both in Lu1205 and UACC903 melanoma cells (Supplementary Figure S5, S6A). A375 cells were also transduced with either an expression vector encoding human ROR1 or an empty vector and stable A375-ROR1 and A375-empty cells were established. Expression of exogenous ROR1 in A375-ROR1 cells was confirmed by various methods (Figure 2C, D, and Supplementary Figure S7). Flow cytometry analysis indicated that exogenous ROR1

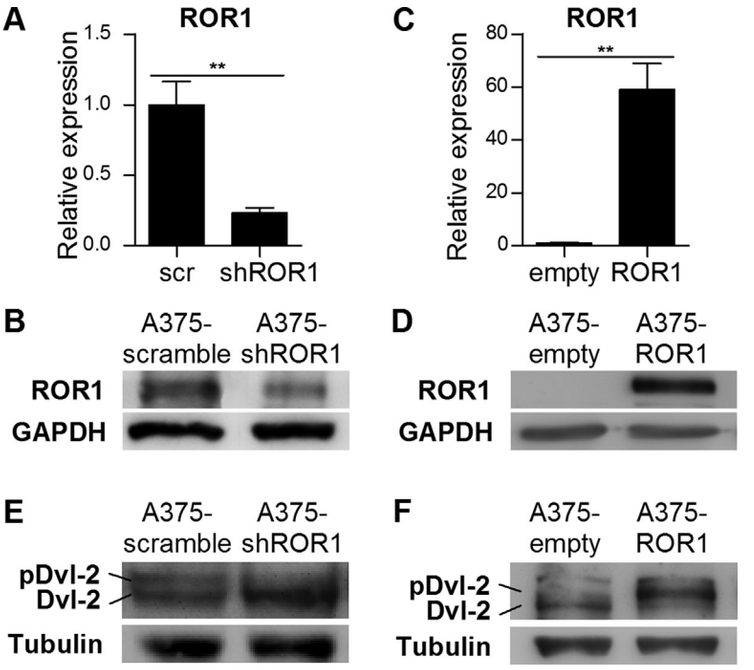


Figure 2. Silencing and overexpression of ROR1 in A375 human melanoma cell line. (A, B) A375-scramble and A375-shROR1 cells were analyzed for ROR1 expression by qRT-PCR (A) and Western blot (Antibody AF2000) (B). Similar analyses were performed in A375-empty and A375-ROR1 cells (C, D). In D we used the ROR1 antibody cs-4102 which has a weak reactivity against endogenous ROR1 but strongly recognized exogenous ROR1. mRNA levels were normalized to internal RNPII levels and expressed as relative to control cells (A375-scramble or A375-empty) in qRT-PCR. The mean ± s.e. from three independent experiments is shown. GAPDH was used as loading control in Western blot. (E, F) ROR1 regulates Dvl-2 phosphorylation. Western blot analysis of Dvl-2 in A375-scramble and A375-shROR1 (E) or A375-empty and A375-ROR1 (F) protein lysates. Tubulin was used as loading control. $^{**}P<0.01$.

localized to the plasma membrane (Supplementary Figure S7).

### ROR1 Regulates Dvl-2 Phosphorylation

Dvl-2 is a key upstream component of the Wnt pathway and its phosphorylation is a common marker of the activation of both canonical and noncanonical Wnt pathways. Upon Wnt stimulus, Dvl proteins are phosphorylated by CK1εd, leading to a shift of the apparent molecular weight on SDS-PAGE (pDvl) [29]. Western blot of protein extracts from melanoma cells with Dvl-2-specific antibodies reveals two bands, corresponding to non-phosphorylated (lower band) and phosphorylated (upper band) Dvl-2, respectively (Dvl-2 and pDvl-2, Supplementary Figure S8A) [30]. Both A375 and Lu1205 cells present some degree of Dvl-2 phosphorylation in the absence of Wnt stimulation, indicating that this pathway is partially activated in a constitutive way (Figure 2E, F, and Supplementary Figure S8). Silencing of ROR1 partially abrogated basal Dvl-2 phosphorylation as evidenced by the relative increase in the abundance of the non-phosphorylated Dvl-2 band (Figure 2E and Figure S8B). The inhibition of Dvl-2 phosphorylation is not complete because inputs mediated by other Wnt receptors are not affected by ROR1 shRNA and stimulate Dvl-2 phosphorylation. This shortcoming was circumvented by the gain of function approach. ROR1 overexpression induced a marked increase in pDvl-2 levels at expenses of a decrease in the levels of Dvl-2 (Figure 2F). These results confirm that both ROR1 shRNA and cDNA are functional and establish that ROR1 regulates Dvl-2 phosphorylation in melanoma.

### ROR1 Silencing Inhibits Both Anchorage-Dependent and -Independent Growth of Melanoma Cells

We next wanted to assess whether ROR1 can influence key aspects of transformed cells such as cell proliferation and anchorage-independent growth. As determined by crystal violet and MTT assays, the proliferation of A375-shROR1 cells showed a significant reduction ($P < 0.05$) compared to A375-scramble cells (Figure 3A and Supplementary Figure S9A). Similar results were obtained in Lu1205 cells upon silencing of ROR1, confirming the results observed in A375 cells and ruling out non-specific effects of the shRNA on a particular cell line (Supplementary Figure S10A). On the other hand, ROR1 overexpression significantly ($P < 0.05$) increased cell proliferation compared to control cells

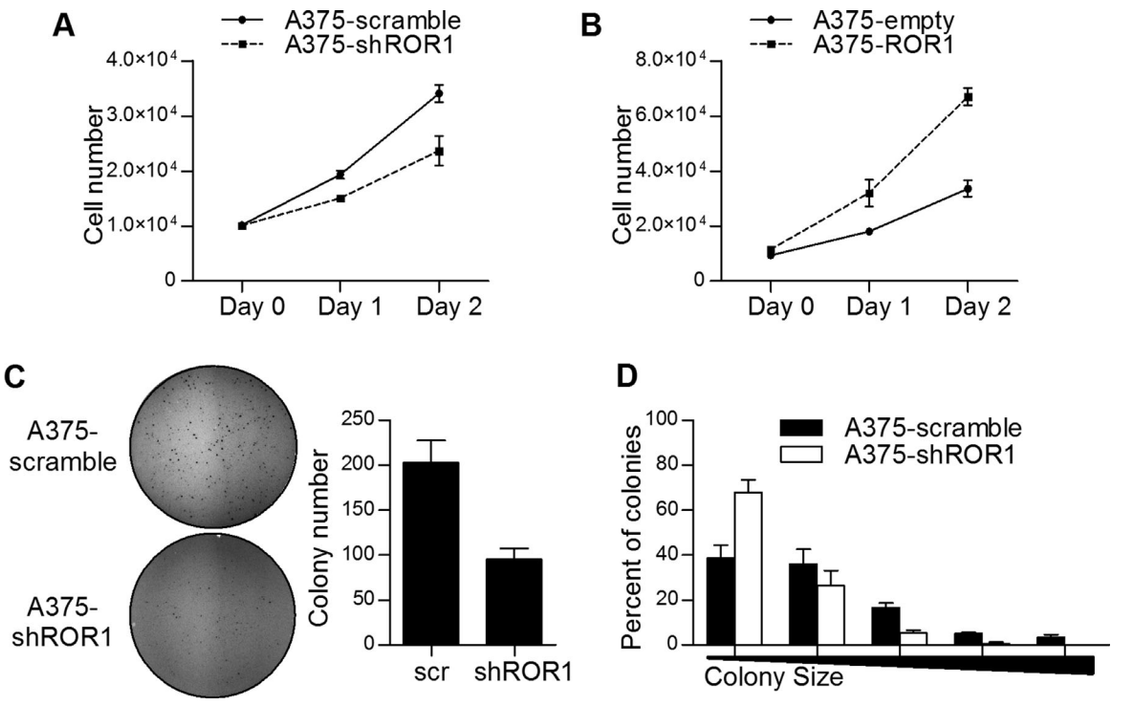


Figure 3. ROR1 increases anchorage-dependent and -independent growth. (A, B) Crystal violet assays to assess cell growth were performed in A375-scramble and A375-shROR1 (A) or A375-empty and A375-ROR1 (B). The results are expressed as the number of cells grown at each time point. A representative experiment of two performed is shown. (C, D) A375-scramble and A375-shROR1 cells were grown in soft agar for 21 days. Images of representative plates are shown. Colonies were counted and plotted as the mean ± s.e. from triplicates. A representative experiment of two performed is shown (C). Total colonies were divided based upon their size in five categories and the percentage of total colonies corresponding to each size was plotted (D).

(Figure 3B and Figure S9B). Using a soft-agar assay we observed that ROR1 silencing reduced the colony forming capacity of A375 cells (Figure 3C). In addition, individual colony size was much smaller in A375-shROR1 cells than in A375-scramble cells (Figure 3D). Silencing of ROR1 in Lu1205 cells markedly reduced colony size with no apparent effect in colony number (Figure S10B, S10C). These results indicate that ROR1 contributes to both anchorage-dependent and -independent cell growth in melanoma cells.

### ROR1 Is Implicated in Loss of Cell Adhesion and Increased Motility and Migration of Melanoma Cells

The aforementioned association between ROR1 expression and aggressive clinical behavior may reflect an underlying functional contribution of ROR1 in melanoma progression. We then determined whether manipulation of ROR1 levels in A375 cells affects cell adhesion to tissue culture plates. Silencing of ROR1 significantly increased adhesion of cells by about 40% after 30 min incubation compared to A375-scramble cells (Figure 4A). To control the

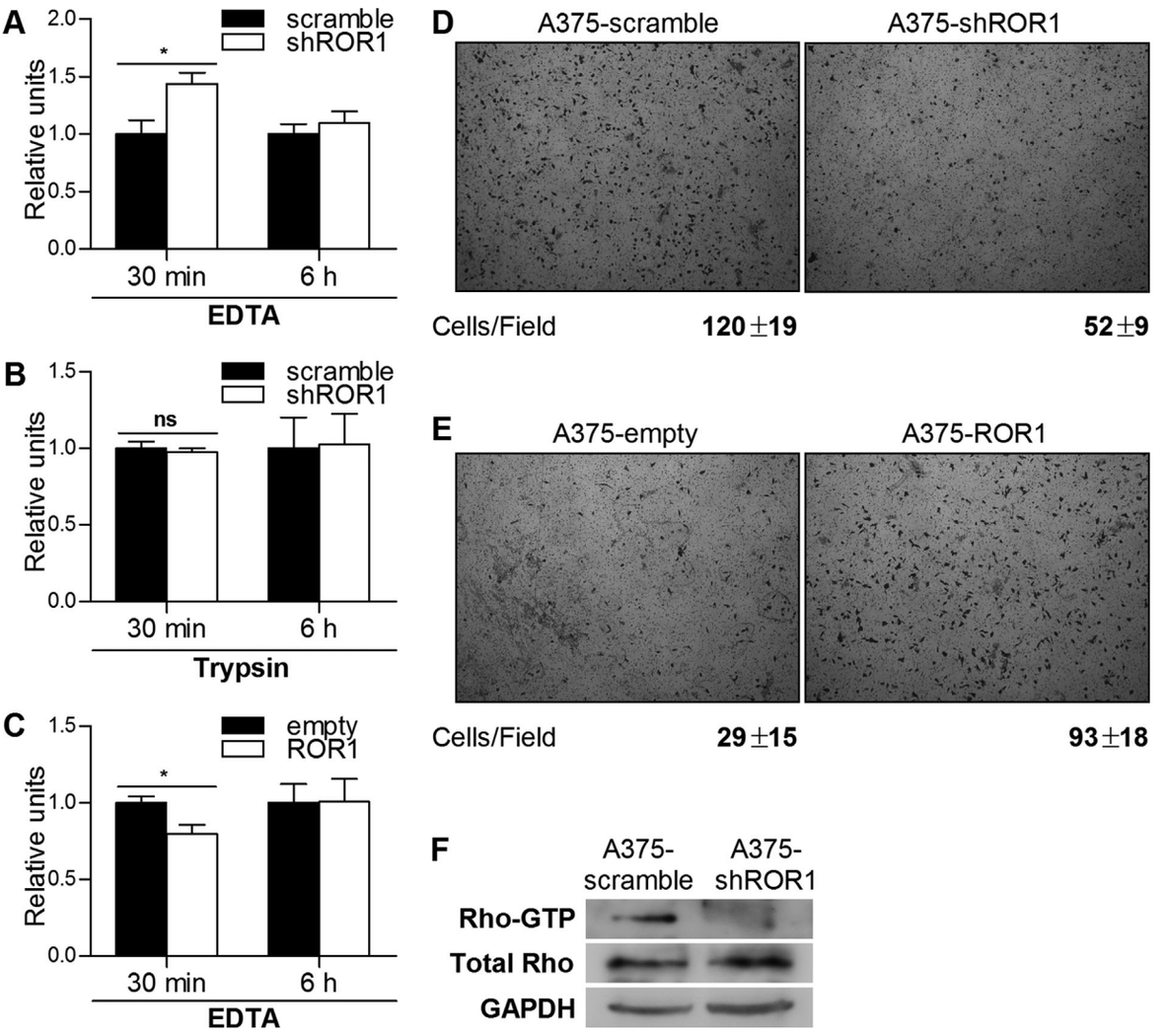


Figure 4. ROR1 affects adhesion and migration of A375 melanoma cells. (A, B, C) Cell adhesion assays were performed in A375-scramble and A375-shROR1 (A, B) or A375-empty and A375-ROR1 (C). Cells were harvested using EDTA (A, C) or trypsin (B) and cell adhesion after 30 min and 6 h was quantified by the absorbance at 590 nm after crystal violet staining. The results are expressed as the ratio of the absorbance relative to the control cells (A375-scramble or A375-empty). The mean ± s.e. from three independent experiments is shown. (D, E) Transwell migration assays were performed in A375-scramble and A375-shROR1 (D) or A375-empty and A375-ROR1 (E). Cells were allowed to migrate for 20 h in D and 16 h in E. Representative images of cells that migrated through the pores of the transwell chamber are shown (top panel). The results are expressed as the number of cells per field, and the mean ± s.d. are indicated (bottom panel). (F) GST-RBD pull-down assay in A375-scramble and A375-shROR1. The amount of Total and active RhoA/C (Rho-GTP) in protein extracts were determined by Western blot before and after pull down with GST-RBD, respectively. GAPDH levels in protein lysates are also shown. ns: not significant, $^{*}P < 0.05$.

number of cells plated for each cell line, we determined the number of cells attached to the plate after 6 h. We did not find any difference at this time point where all the cells eventually adhere. The difference in adhesion seen at 30 min was not observed in a control experiment using cells harvested with trypsin instead of EDTA (Figure 4B). On the other hand, A375-ROR1 cells showed a significant reduction on cell adhesion after 30 min but not after 6 h, compared to A375-empty cells (Figure 4C). These results indicate that ROR1 expression negatively regulates the rate of cell adhesion. Both, a decrease in cell adhesion and an increased colony forming ability, are generally associated with the gain of migrating and invading capabilities of cancer cells [31]. To determine whether ROR1 plays a role in melanoma cell migration we performed transwell migration assays. ROR1 silencing decreased (Figure 4D) while ROR1 overexpression increased (Figure 4E) cell migration, respectively. Wound healing assays confirmed that ROR1 positively regulates cell motility and migration (Supplementary Figure S11). It is known that RhoA and RhoC play key roles in the regulation of actomyosin contractility and in cell locomotion. Silencing of ROR1 in both A375 (Figure 4F) and Lu1205 cells (Supplementary Figure S12) markedly reduced the activation of RhoA/C as determined by pull-down experiments. These results indicate that ROR1 expression in melanoma cells increases features associated with EMT, such as loss of adhesion and increased motility and migration.

### ROR1 Regulates the Expression of Mesenchymal Markers in Melanoma

Cadherin isoform switch (cadherin switching) occurs during normal developmental processes to allow cell types to segregate from one another. This process called EMT, implicating loss of E-cadherin and upregulation of N-cadherin, also occurs in tumor cells that acquire an aggressive and metastatic phenotype characterized by decreased adhesion and increased motility and invasion. Since some of these phenotypic changes were observed in melanoma cells after altering expression of ROR1, we wanted to assess whether ROR1 is implicated in cadherin switching. Western blot and qRT-PCR analysis in A375-shROR1 cells showed a significant reduction on the expression of both N-cadherin and the mesenchymal marker vimentin compared to A375-scramble cells (Figure 5A, B). Similar results were obtained in UACC903 cells (Supplementary Figure S6B). In contrast, ROR1 overexpression promotes a significant increase in both protein and mRNA levels of N-cadherin and vimentin (Figure 5C, D). E-cadherin levels were undetectable both by qRT-PCR and Western blot in most of the cell lines analyzed and silencing of ROR1 was not sufficient to revert this phenotype (data not shown). These data indicate that ROR1 is implicated in the upregulation of mesenchymal markers in melanoma.

### ROR1 Regulates Akt Expression and Participates in a Positive Feedback Loop With Wnt5a

We have determined that ROR1 regulates phosphorylation of Dvl-2, an upstream component of both canonical and non-canonical Wnt pathways (Figure 2). To determine the Wnt pathway specificity of ROR1 we evaluated its effect on the activity of a TCF reporter induced by Wnt3a, a prototypical canonical Wnt ligand. Serial dilutions of Wnt3a CM similarly induced reporter activity in both A375-shROR1 and A375-scramble cells (Figure 6A). These results indicate that ROR1 does not affect Wnt canonical signaling. We then turn our attention to signaling cascades activated by Wnt non-canonical signals, particularly the PI3K/Akt pathway. We found that stable overexpression of ROR1 induced a significant increase in Akt1 level as determined by qRT-PCR and Western blot (Figure 6B). ROR1 silencing significantly reduced the expression of Akt1 and Akt3 (Figure 6C and Supplementary S13), and prevented the phosphorylation of Akt in response to Wnt5a treatment (Figure 6D) but had a minimal effect on basal pAkt levels (Figure 6E). In line with these observations, overexpression of ROR1 impairs the transcriptional activity of FoxO, a transcription factor negatively regulated by Akt (Figure 6F). In addition of being a Wnt5a receptor and signaling intermediate, ROR1 positively regulates Wnt5a expression in a statistically significant way as demonstrated by both gain- and loss-of-function approaches (Figure 6G, H). Furthermore, Wnt5a CM significantly increased ROR1 gene expression (Figure 6I). This observation is in agreement with the finding that Wnt5a induced phosphorylation of STAT3 (Supplementary Figure S14), a major transcriptional regulator of ROR1 [32]. These results indicate that expression of ROR1 in melanoma is implicated in regulating Akt levels and FoxO activity and feeding a positive feedback loop with Wnt5a.

### Both Akt and ROR1 Signals Are Required for Regulation of N-Cadherin

We have shown that expression of ROR1 in melanoma contributes to a less adhesive and more motile phenotype likely associated to the upregulation of mesenchymal markers. Our last goal was to determine whether the upregulation of N-cadherin by ROR1 is mediated by the Akt pathway. Treatment with the PI3K/Akt inhibitor LY-294002 (LY) markedly reduced N-cadherin levels both in control and in A375-ROR1 cells (Figure 7A). After 20 h of LY treatment, N-cadherin expression in A375-ROR1 cells completely leveled to those in A375-empty cells (Figure 7A, lanes 5 and 6). This result indicates that the basal level of N-cadherin as well as the added amount by effect of ROR1 overexpression (lanes 1 and 2) requires the participation of the PI3K/Akt pathway. The role of this pathway in the

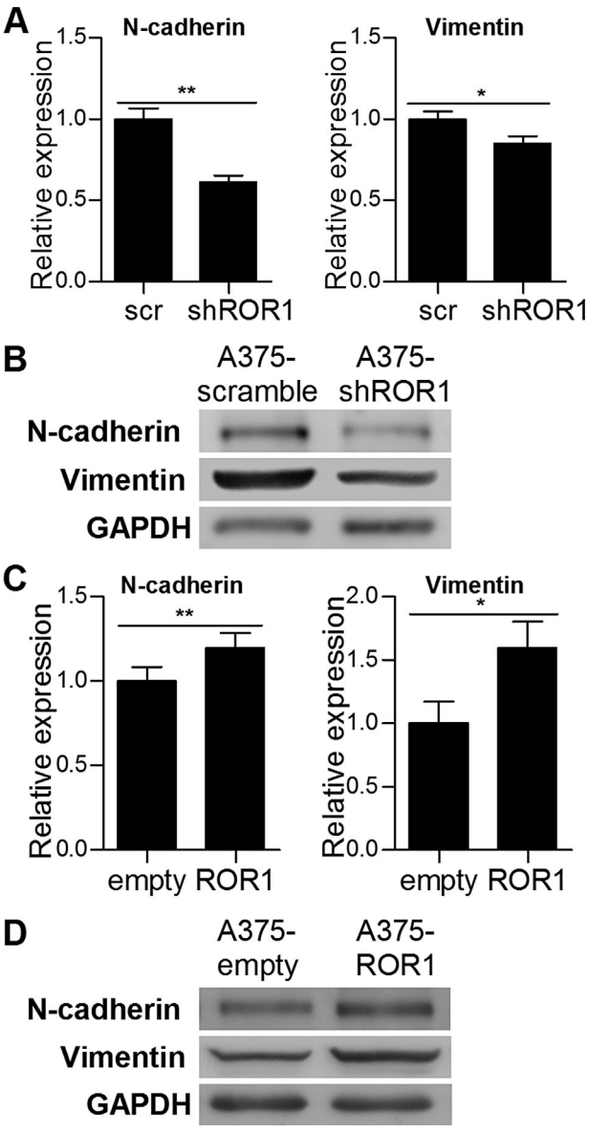


Figure 5. ROR1 regulates N-cadherin and vimentin expression. (A, B) A375-scramble and A375-shROR1 cells were analyzed for N-cadherin and vimentin expression by qRT-PCR (A) and Western blot (B). Similar analyses were performed in A375-empty and A375-ROR1 cells (C, D). mRNA levels were normalized to internal RNPII levels and expressed as relative to control cells (A375-scramble or A375-empty) in qRT-PCR. The mean ± s.e. from three independent experiments is shown. GAPDH was used as loading control. $^{*}P < 0.05$, $^{**}P < 0.01$.

regulation of N-cadherin was further assessed by using the loss of function approach combined with Rapamycin, an mTORC1 inhibitor that induces feedback activation of the PI3K/Akt pathway. As expected, Rapamycin increased N-cadherin levels in A375-scramble cells (Figure 7B, compare lanes 3 and 5 vs. lane 1) but failed to do so in A375-shROR1 cells (Figure 7B, lanes 2, 4, and 6), indicating that Akt activation is unable to regulate N-cadherin if ROR1 is silenced. Stimulation of A375-scramble cells with Wnt5a also upregulated N-cadherin in an Akt-dependent manner (Figure 7C). However, Wnt5a treatment barely increased N-cadherin in A375-shROR1 cells (Figure 7C, lane 4 vs. lane 2). These results demonstrate that both Akt and ROR1 are required for regulation of N-cadherin upon either ROR1 overexpression or Wnt5 stimulation.

## DISCUSSION

The available information about ROR1 in melanoma is limited and conflicting. Whereas Zhang et al. in 2012 described ROR1 expression in some melanoma cell lines of metastatic origin [33], O'Connell et al.

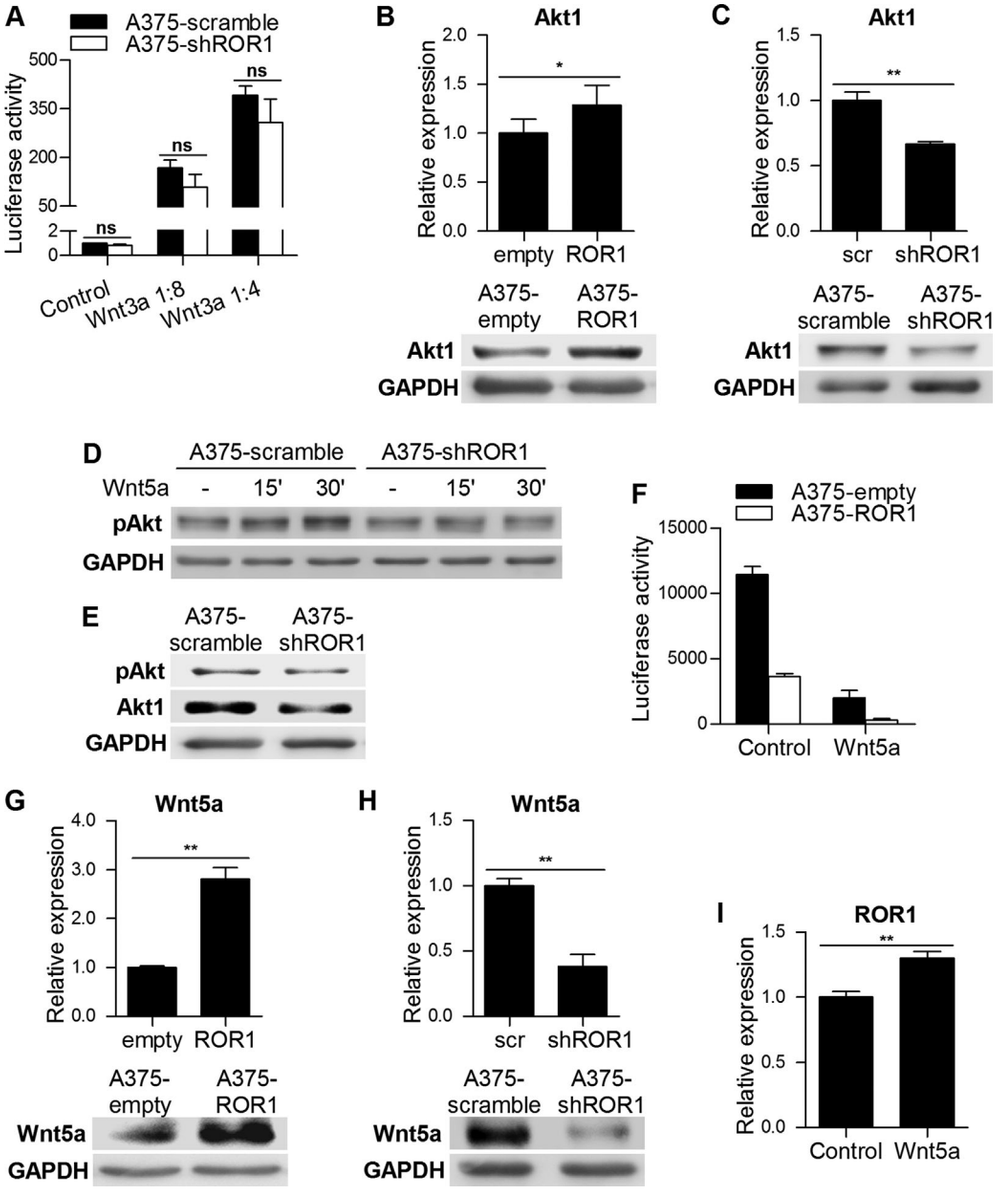


Figure 6. ROR1 regulates Akt/FoxO and participates in a positive feedback loop with Wnt5a. (A) ROR1 silencing does not affect Wnt canonical signaling. A375-scramble and A375-shROR1 cells were transfected with TOP/FOP luciferase plasmids and stimulated with Control or Wnt3a CM (dilution 1:4 or 1:8). Luciferase activity was measured at 24 h. The data were normalized to A375-scramble cells treated with Control CM. The mean ± s.e. from three independent experiments is shown. (B, C) ROR1 upregulates Akt1. Akt1 expression in A375-empty and A375-ROR1 (B) or A375-scramble and A375-shROR1 (C) was determined via qRT-PCR (top panel) and Western blot (bottom panel). mRNA levels were normalized to internal RNPII levels and expressed as relative to control cells (A375-empty or A375-scramble) in qRT-PCR. The mean ± s.e. from three independent experiments is shown. GAPDH was used as loading control in Western blot. (D) ROR1 silencing inhibits Akt activation by Wnt5a. A375-scramble and A375-shROR1 were stimulated with Wnt5a CM for the indicated times and phosphorylation of Akt in Ser 473 (pAkt) was measured by Western blot. GAPDH was used as loading control. (E) ROR1 does not affect basal pAkt levels. Total Akt and pAkt levels in A375-scramble and A375-shROR1 were determined by Western blot. GAPDH was used as loading control. (F) ROR1 inhibits FHRE (ForkHead box Response Element) reporter activity. A375-empty and A375-ROR1 cells were transfected with FHRE-Luc plasmid and treated with Control or Wnt5a CM. Luciferase activity was measured after 24 h and normalized using Renilla activity. The mean ± s.d. from triplicate is shown. A representative experiment of two performed is shown. (G, H, I) Wnt5a and ROR1 are involved in a positive feedback loop. Wnt5a expression in A375-empty and A375-ROR1 (G) or A375-scramble and A375-shROR1 (H) was determined via qRT-PCR (top panel) and Western blot (bottom panel). mRNA levels were normalized to internal RNPII levels and expressed as relative to control cells (A375-empty or A375-scramble) in qRT-PCR. The mean ± s.e. from three independent experiments is shown. GAPDH was used as loading control in Western blot. (I) ROR1 mRNA relative levels in A375 cells after treatment with Control or Wnt5a CM for 10 h. mRNA levels were normalized to internal RNPII levels and expressed as relative to Control treatment. The mean ± s.e. from three independent experiments is shown. ns: not significant, $^{*}P < 0.05$, $^{**}P < 0.01$.

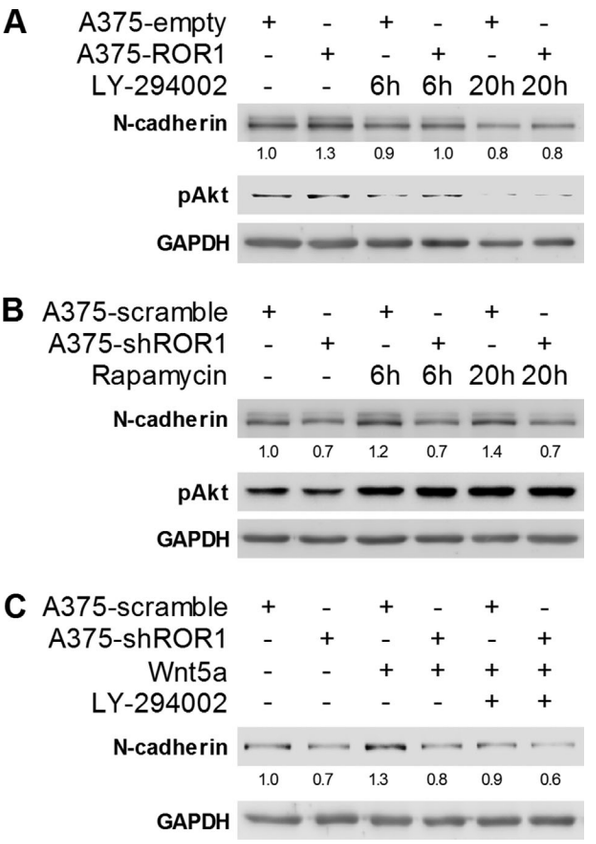


Figure 7. ROR1 regulates N-cadherin expression via the Akt pathway. (A, B, C) Western blot analyzes of N-cadherin expression in A375-empty and A375-ROR1 (A) or A375-scramble and A375-shROR1 (B, C) treated with either 50 μM of LY-294002 (A), 1 μM of Rapamycin (B) for 6 or 20 h or with Wnt5a CM plus/minus 50 μM of LY-294002 for 20 h (C). The intensity of each band was quantified by densitometry. N-cadherin levels were normalized to GAPDH and expressed as the mean ratio relative to control cells (A375-scramble or A375-empty) with no treatment from three (A, B) or two (C) independent experiments. Western blots of pAkt were included to monitor LY and Rapamycin treatments. GAPDH was used as loading control.

showed that ROR1 expression is abundant in primary cells but markedly decreases in metastatic cell lines [23]. After studying a large panel of cell lines we concluded that ROR1 similarly express both in primary and metastatic melanoma cell lines (Figure S1). This finding is supported by microarray analysis of ROR1 expression on a much larger dataset (29 cell lines, Philadelphia dataset, GSE4841, Supplementary Figure S15). The disagreement with the data presented by O'Connell et al. do not arise from errors in assessing ROR1 expression since the relative levels of ROR1 in the four cells lines included in both studies matched well [23]. More importantly, we here show for the first time that ROR1 is frequently expressed in melanoma tumors and that high level of ROR1 expression correlates with shorter Post-Recurrence Survival in melanoma patients. A similar observation regarding the prognostic value of ROR1 was made in ovarian cancer [34], breast cancer [21] and erlotinib-treated non-small-cell lung cancer patients (Karachaliou et al., 2013 ASCO Annual Meeting). Also, phosphorylated ROR1 was associated with progressive disease in chronic lymphocytic leukemia (CLL) [35].

Along with its potential clinical relevance as prognostic marker, ROR1 was shown to contribute to progression of several tumor types [17,19,21,34,36]. To investigate the role of ROR1 in melanoma we performed functional studies in melanoma cells using both gain- and loss-of-function approaches. One of the conclusions we drawn from our experiments is that ROR1 increased colony formation in soft agar and cell proliferation. This, together with the observation that ROR1 inhibition promotes apoptosis of melanoma cells as determined by Hojjat-Farsangi et al. [20] and ourselves (unpublished data), strongly indicates that ROR1 is a positive regulator of cell growth. These findings are consistent with the observed activation of the PI3K/Akt pathway by ROR1 (this article [19,37]).

ROR proteins have been extensively studied in *C. elegans* for its important role in neuronal cell migration [38]. More recently, ROR1 was shown to increase the number of actin stress fibers and cellular

migration in HeLa cells [39]. In agreement with these evidences we found that ROR1 promotes a less adhesive and a more motile and migratory phenotype in melanoma cells. Altogether, the results presented here suggest that ROR1 would contribute both to early (proliferation) and late (adhesion, migration) processes of melanoma progression. The similar expression of ROR1 in both "proliferative" and "invasive" cell lines as well as in both primary and metastatic cells would be consistent with this possibility.

The results presented here are in apparent disagreement with those of O'Connell et al. who described that ROR1 negatively regulates melanoma cell invasion and metastasis [23]. Yet, the two articles are not entirely comparable since O'Connell et al. studied invasion and metastasis and our work focused on earlier events (adhesion and migration). It is striking however, that unlike O'Connell et al. we do not see an increased expression of ROR1 associated with an invasive phenotype when analyzing the Melanoma Phenotype-Specific Expression database (Figure S2). More so because O'Connell et al. used the same database to support their conclusion. We believe that the reason for this difference is that O'Connell et al. just analyzed around 60 cell lines whereas we included the total of 220 cell lines in the database (Figure S2). Also, whereas we analyzed the three ROR1 probes available (Figure S2), O'Connell et al. just considered one [23]. This is particularly risky since overall expression data from one of the probes (probe 211057_at) do not correlate well with the other two ROR1 probes (data not shown), possibly leading to erroneous conclusions.

The regulation of adhesion, motility and migration by ROR1 described in this work suggests that ROR1 influences melanoma progression by inducing and EMT-like process. Similar results were obtained in breast cancer cells, where silencing of ROR1 induced a full array of changes in EMT markers such as decreased expression of vimentin and SNAIL-1/2 [21]. In melanoma, we observed that ROR1 silencing decreased vimentin and N-cadherin expression. Unlike breast cancer cells [21], melanoma cells do not show a recovery on E-cadherin expression upon ROR1 silencing suggesting different regulatory mechanisms. It is plausible that this difference is related with the critical role of active BRAF in melanoma, reorganizing the EMT-transcription factor network [40] and repressing E-cadherin expression [41]. Mechanistically, we determined that ROR1 would exert its effects on melanoma progression by activating non-canonical Wnt signals including phosphorylation of Dvl-2, upregulation of Akt and N-cadherin. Our data show that Akt is required but not sufficient to regulate N-cadherin levels, requiring of additional signals regulated by ROR1 functions. Whether these additional functions of ROR1 are related to its reported ability to translocate to the nucleus needs to be further investigated [39]. Interestingly, we found that ROR1 and Wnt5a form a regulatory feedback loop by positively regulating each other transcription. However their levels in melanoma cell lines usually do not correlate (Figure 1) likely because the major transcriptional regulators of Wnt5a are the NF-kB, Hedgehog, TGFb, and Notch pathways [42].

It is interesting to discuss the connection between ROR1 and its ligand Wnt5a. Wnt5a was shown to play a dual role in cancer acting either as a tumor suppressor or as an oncogene. In the latter case, as occurs in melanoma, Wnt5a contributes to the high motility and invasiveness of cancer cells by activating non-canonical pathways including Akt [43,44]. Mechanistically, we show that ROR1 overexpression has similar effects than Wnt5a stimulation by modulating Dvl-2 phosphorylation and regulating Akt/FoxO activities and N-cadherin expression. Although it is tempting to assume that ROR1 simply mediates Wnt5a signals, we refrain to do so. The recent observation that ROR1 promotes cell growth, EMT and metastasis in breast cancer, which is difficult to reconcile with the dominating notion that Wnt5a is a tumor suppressor in this type of cancer, indicates that we should be cautious when analyzing the link between these proteins [22]. In this regard, the ability of Wnt5a to signal via various receptors including ROR1, ROR2, Ryk, and Fzd7 [23,43] not only might be a critical regulatory process in different cancers but also indicates that Wnt5a and ROR1 signals are not equivalent. The data presented here together with the observation that ROR1 is constitutively phosphorylated in melanoma [20] support the notion that ROR1 expression can contribute to melanoma progression without requiring a pre-existent Wnt functioning circuit driven by Wnt5a. Of course, the effects can be more robust in the presence of autocrine or paracrine Wnt5a signals since Wnt5a and ROR1 signals appear not to be antagonistic in melanoma.

One of the current challenges in melanoma is to identify new targets to develop combinatorial therapies. ROR1 is a tyrosine kinase receptor whose restricted expression in cancer and its pro-tumorigenic role make it a novel and promising therapeutic target. Hence, a number of tools targeting ROR1 have already been developed including small molecule inhibitors [45], T-cells expressing ROR1-specific chimeric antigen receptor [9] and immunotoxins [46]. Biochemical studies have identified two ROR1 inhibitors that selectively killed CLL and pancreatic adenocarcinoma cells [45]. The selective cell surface expression of this protein is particularly suitable for the use of monoclonal antibodies that have been shown to induce apoptosis in CLL [47] and melanoma cells [20]. More recently, UC-961, a humanized anti-ROR1 monoclonal antibody that is being evaluated in a clinical trial for chronic lymphocytic leukemia (Clinical Trial Identifier: NCT02222688), showed biologic activity against

ROR1$^+$ tumor cells and lack of toxicity in preclinical studies [48]. Although further studies are needed to confirm this possibility, the present study indicates that ROR1 is a good prospective target for melanoma cancer therapy.

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