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Notch4 Signaling Induces a Mesenchymal–Epithelial–like Transition in Melanoma Cells to Suppress Malignant Behaviors

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No potential conflicts of interest were disclosed.

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Abstract

The effects of Notch signaling are context-dependent and both oncogenic and tumor-suppressive functions have been described. Notch signaling in melanoma is considered oncogenic, but clinical trials testing Notch inhibition in this malignancy have not proved successful. Here, we report that expression of the constitutively active intracellular domain of Notch4 (N4ICD) in melanoma cells triggered a switch from a mesenchymal-like parental phenotype to an epithelial-like phenotype. The epithelial-like morphology was accompanied by strongly reduced invasive, migratory, and proliferative properties concomitant with the downregulation of epithelial–mesenchymal transition markers Snail2 (*SNAI2*), Twist1, vimentin (*VIM*), and MMP2 and the reexpression of E-cadherin (*CDH1*). The N4ICD-induced phenotypic switch also resulted in significantly reduced tumor growth *in vivo*. Immunohistochemical analysis of primary human melanomas and cutaneous metastases revealed a significant correlation between Notch4 and E-cadherin expression. Mechanistically, we demonstrate that N4ICD induced the expression of the transcription factors Hey1 and Hey2, which bound directly to the promoter regions of Snail2 and Twist1 and repressed gene transcription, as determined by EMSA and luciferase assays. Taken together, our findings indicate a role for Notch4 as a tumor suppressor in melanoma, uncovering a potential explanation for the poor clinical efficacy of Notch inhibitors observed in this setting.

Introduction

During recent years, emerging evidence suggests that Notch signaling plays an important role in the development and progression of several cancers including melanoma (1–4). The majority of these studies report an oncogenic function of Notch1 while a thorough understanding of other Notch receptors is still sparse. Considering the profound evidence about the oncogenic role of Notch in melanoma, a phase II trial using broad Notch signaling inhibitors surprisingly only showed minimal clinical activity against metastatic melanoma (5). Therefore, more in-depth knowledge about the detailed functions of Notch signaling in melanoma is required to assess the potential of Notch as a therapeutic target. In this study, we report a novel tumor-suppressive function of Notch4 in melanoma by triggering a mesenchymal–epithelial–like transition that leads to a low motile, proliferative, and invasive phenotype that shows reduced tumorigenicity *in vivo*.

Materials and Methods

Cell culture

The primary human melanoma cell line WM35, and metastatic cell lines WM9, WM164, and 451Lu were kindly provided by Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA) and grown in RPMI1640 medium (Sigma-Aldrich), supplemented with 2% FBS (PPA) and 2% L-glutamine (PPA). All cells were maintained at 37° C in a humidified atmosphere containing 5% CO_2. Cells were harvested for individual experiments after washing with PBS (pH 7.4; Life Technologies) using trypsin (Life Technologies). Cell lines received in 2005 were tested for authenticity in 2011 (WM9 and WM35), 2012 (WM164), and 2014 (451Lu) using short tandem repeat (STR) genotyping.

In vivo study

Female mutant inbred, 3- to 8-week-old C.B-17/IcrHanHsdArcPrkdcscid mice were purchased from the Animal Resources Centre (Canning Vale, Australia) and randomly separated into two groups of 5 mice and injected with 1×10^6 451Lu cells transduced with pLNCX-empty vector or pLNCX-N4ICD. Tumor growth was monitored twice weekly for 4 weeks, measured using a caliper. Tumor volume was calculated according to the formula: (width × length × height)/2.

Plasmid and siRNA transfection

Transfection experiments were performed as described previously (6) using 150 pmol siRNAs or 2 μg of plasmid DNA (Supplementary Table S1).

Viral vector production and transduction

Experiments were performed as described previously (6) using vectors pLNCX-empty vector and pLNCX-N4ICD.

Immunoblotting

Immunoblotting was performed as described previously (6) using specific primary and corresponding peroxidase-conjugated secondary antibodies (Supplementary Table S2). Protein lysates from frozen tissue samples were generated using 500 μL RIPA buffer per 10 mg of tissue and sonicated at 180 watts 10 × 10 seconds.

qRT-PCR

qRT-PCR was performed as described previously (7) using gene-specific primers (Supplementary Table S3). The qPCR array (PAHS-090Z, Qiagen) was performed according to the manufacturer's protocol using mRNA extracted from WM9.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (6) using Cy3-labeled oligonucleotides (Supplementary Table S4).

Luciferase assay

Luciferase assay was performed as described previously (6) in WM164 using the respective plasmids (Supplementary Tables S1 and S5) cotransfected with pSV-β-Galactosidase control vector for normalization.

Migration assay

Migration assay was performed as described previously (6) using pLNCX-empty vector or pLNCX-N4ICD–transduced cells.

Invasion assay

Previously described migration assay (6) was modified by coating FluoroBlok cell culture inserts with Matrigel (Corning).

Wound healing assay

Wound healing assay was performed as described previously (6) using pLNCX-empty vector or pLNCX-N4ICD–transduced cells.

Proliferation assay

Proliferation assay was performed as described previously (6) using pLNCX-empty vector or pLNCX-N4ICD–transduced cells.

Adhesion assay

Adhesion assay was performed as described previously (6) using pLNCX-empty vector or pLNCX-N4ICD–transduced cells and E-cadherin–expressing keratinocytes (HaCaT).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed as described previously (6) using specific antibodies (Supplementary Table S2) and promoter-specific primers (Supplementary Table S3).

IHC

Formalin-fixed, paraffin-embedded tissues from the archives of the Department of Dermatology and the Department of Pathology, University Hospital St. Poelten, Karl Landsteiner University of Medical Sciences (St. Poelten, Austria) were deparaffinized and stained using the BenchMark XT automated immune-staining platform and the Ultra View Universal DAB Detection System (Ventana Medical Systems). The collection and storage of samples were performed according to the local ethical guidelines (EK-Votum number: GS4-EK-3/063–2011). Antigen retrieval was performed before using prediluted antibodies (Supplementary Table S2). Scoring of tissue slides was performed independently by two investigators (0, no staining; 1+, weak positive staining; 2+, moderate positive staining; 3+, strong positive staining). The stained sections were examined with an Olympus BX53 microscope and photographed with an Olympus DP73 camera (Olympus Electronics).

Statistical analysis

Unpaired t test was used to determine statistical significance (not significant, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Spearman ρ was calculated using IBM SPSS Statistics, version 20.

Results

Overexpression of the constitutively active N4ICD results in an epithelial-like phenotypic switch in human melanoma cells

Overexpression of intracellular domain of Notch4 (N4ICD), but not empty vector, caused a phenotypic switch from a parental mesenchymal-like to an epithelial-like phenotype (Fig. 1A and Supplementary Fig. S1A). These epithelial-like cells exhibited strongly reduced chemokinesis (Fig. 1B, left), reduced migration (Fig. 1B, right and Supplementary Fig. S1B), invasiveness (Fig. 1C and Supplementary Fig. S1C), and proliferation (Supplementary

Fig. S1D), but increased adhesion to keratinocytes (Supplementary Fig. S1E). Subcutaneous injection of 1×10^6 empty vector control or N4ICD-transduced 451Lu cells into the right flank of C. B-17/IcrHanHsdArcPrkdcscid mice resulted in palpable tumor formation of empty vector cells 14 days after injection. In comparison, N4ICD-transduced cells showed a delayed tumor onset of more than 4 days. Tumors generated by parental cells resulted in significantly higher tumor volume throughout the observation period (Fig. 1D). Taken together, the observed alterations of cell mobility following N4ICD overexpression combined with the significantly reduced tumor growth *in vivo* suggest a possible tumor-suppressive role of Notch4 in melanoma.

Notch4-mediated molecular changes resemble a mesenchymal–epithelial transition involving noncanonical Notch signaling

On the molecular level, the phenotypic switch is accomplished by changes of several characteristic markers of epithelial–mesenchymal transition. Investigating the expression pattern of 50 EMT-related genes in N4ICD-transduced WM9 using a qPCR array revealed 17 significantly changed genes (Supplementary Table S6), of which 11 showed an expression pattern corresponding to a MET (Fig. 2A). Changes of selected EMT markers, including E-cadherin (*CDH1*), vimentin (*VIM*), MMP-2 (*MMP2*), Snail2 (*SNAI2*), and Twist1 (*TWIST1*) were confirmed by immunoblotting and qRT-PCR (Fig. 2B and Supplementary Fig. S2A). Immunohistochemical analyses of 30 primary human melanomas and 30 cutaneous and subcutaneous metastases revealed a significant positive correlation between Notch4 and E-cadherin expression (Fig. 2C, top; Supplementary Table S7). Interestingly, areas, especially melanoma nests, with high Notch4 staining matched with E-cadherin positivity (Fig. 2C, bottom and Supplementary Fig. S2B), but Notch4 was also found in E-cadherin–negative tissue. Snail2 and Twist1 are downregulated in N4ICD-overexpressing cells and both have been shown to be activated by Notch ICD through its main DNA-bound mediator CSL (*RBPJ*; refs. 8,9). Silencing experiments using sequence-specific siRNA targeting CSL showed that both EMTRs are upregulated following CSL knockdown on the protein and mRNA level (Fig. 2D and Supplementary Fig. S3A), suggesting that CSL acts as a transcriptional repressor. Analysis of the promoter regions of Snail2 and Twist1 revealed the presence of potential CSL-binding sites (Supplementary Fig. S3B; as reported by refs. 10–12). EMSA and ChIP assays showed binding of CSL to these sequences on Snail2 and Twist1 promoters (Supplementary Fig. S3C and S3D). Considering canonical Notch signaling, in which binding of Notch ICD to the DNA-bound transcriptional repressor CSL results in target gene activation (13), these results indicate that Notch4 induced suppression of Snail2 and Twist1 is mediated in a noncanonical fashion, despite CSL binding to promoters of Snail2 and Twist1.

Notch downstream targets Hey1 and Hey2 regulates Snail2 and Twist1

Next, we confirmed the specificity of the observed changes after N4ICD overexpression by silencing Notch4, which led to the upregulation of Snail2 and Twist1, suggestive of Notch4 acting as a transcriptional repressor (Supplementary Fig. S4A and S4B). In search of a noncanonical Notch signal transduction mechanism, we proposed that the Notch target genes Hey1 (*HEY1*) and Hey2 (*HEY2*) might be transcriptional repressors of Snail2 and Twist1. As expected, both Hey proteins are regulated by Notch4 (Fig. 3A and Supplementary Fig.

S4C). Silencing and overexpression experiments identified Hey1 as a transcriptional repressor of Snail2 and Twist1 (Fig. 3B). Knockdown and overexpression experiments showed that Hey2 regulates Snail2 but not Twist1 (Fig. 3C). These observations were confirmed in another melanoma cell line (Supplementary Fig. S4D and S4E). Taken together, these results demonstrate that Hey1 and Hey2 as downstream targets of Notch4 are involved in the regulation of Snail2 and Twist1.

Hey1 and Hey2 directly bind to and suppress Snail2 and Twist1 promoter activity

To investigate the hierarchy of N4 signal transduction, we performed double transfection experiments. Silencing of CSL in N4ICD-overexpressing cells resulted in decreased expression of Hey1 and Hey2, confirming that both Hey proteins are direct targets of Notch (14,15). In addition, CSL silencing in N4ICD-overexpressing cells led to reexpression of Snail2 and Twist1, which can be attributed to decreased expression of Hey1/2 and the diminished suppressive activity of CSL itself (Fig. 3D). Furthermore, melanoma cells were simultaneously transiently transfected with specific siRNA targeting Notch4 and plasmids encoding Hey1 or Hey2 cDNA, respectively. Cells overexpressing Hey1 showed suppressed expression levels of Snail2 and Twist1 despite a significant decrease in Notch4 protein levels (Fig. 4A, top), indicating that Hey1 overrules Notch4. Cotransfection of siRNA targeting Notch4 and Hey2 cDNA resulted in the suppression of Snail2 but upregulated Twist1 (Fig. 4A, bottom), which again indicates that Hey2 overrides Notch4 in the regulation of Snail2 but has no effect on Twist1. Silencing of Hey1/2 in Notch4-overexpressing cells confirmed these findings (Supplementary Fig. S5A). Sequence analysis of Snail2 and Twist1 promoter regions revealed several possible Hey protein–binding motives (E-Boxes as reported by ref. 16). EMSA of two randomly chosen E-Boxes (Supplementary Fig. S5B) showed that Hey1 and Hey2 bind to these sequences while showing reduced binding to mutated control sequences (Fig. 4B). To corroborate the direct regulation of Snail2 and Twist1 by Hey proteins, we performed luciferase assays. Overexpression of Hey1 caused a strongly reduced luciferase activity of Snail2 or Twist1 promoter constructs while Hey2 only reduced the luciferase activity of Snail2 constructs (Fig. 4C). The luciferase activity was recovered by mutating E-Boxes in the promoter regions. To conclude, these experiments show that Hey1 and Hey2 directly suppress Snail2 and Hey1 Twist1 expression.

Discussion

Overexpression of the constitutively active N4ICD results in the emergence of an epithelial-like phenotype characterized by strongly reduced migratory, invasive, and proliferative properties *in vitro*. MET is described by increased E-cadherin expression, whereas several mesenchymal markers like vimentin, MMP-2, Snail2, and Twist1 are downregulated, which, at least partially, is mediated through Hey1 and Hey2 (Fig. 4D). E-cadherin is well known to have tumor-suppressive functions and reexpression in melanoma has been shown to reduce invasive behavior (17). Opposed to E-cadherin, vimentin (18), MMP-2 (19), Snail2 (20), and Twist1 (21) are reported to facilitate migration, invasiveness, and metastasis formation, suggesting that the observed molecular changes following N4ICD overexpression correspond to the observed alterations in cellular functionality. Moreover, N4ICD overexpression resulted in a significant increase of epithelial markers such as *DSP, OCLN,*

SPP1, FGFBP-1, and *IL1RN* indicating the induction of an epithelial gene signature. These observations combined with reduced tumor growth of N4ICD-overexpressing cells *in vivo* strongly suggest a tumor-suppressive function of Notch4 in melanoma by promoting the formation of a less aggressive epithelial-like phenotype. This finding is further strengthened by immunohistochemical analyses of primary human melanoma samples showing a significant positive correlation between E-cadherin and Notch4 in melanoma nests and areas of E-cadherin expression in cutaneous and subcutaneous metastases. Nevertheless, the detailed mechanisms and circumstances that determine the subset of Notch-activated genes are highly context dependent and still need to be examined in more detail. One of the possible consequences of a mesenchymal–epithelial transition, the reexpression of E-cadherin, has been linked to cell survival at metastatic sites in breast carcinoma (22) and several studies reported the involvement of MET in promoting metastatic colonization (reviewed in ref. 23). Taken together, our data identified Notch4 as a tumor suppressor in melanoma, suggesting a possible explanation for the clinical ineffectiveness of γ-secretase inhibitors for the treatment of metastatic melanoma. However, it cannot be ruled out that the observed phenotypic switch results in dormant cells with capabilities of increased survival. More detailed understanding of the mechanisms how active Notch4 induces a MET-like phenotype in melanoma could open new avenues to interfere with tumor dormancy contributing to overcome drug resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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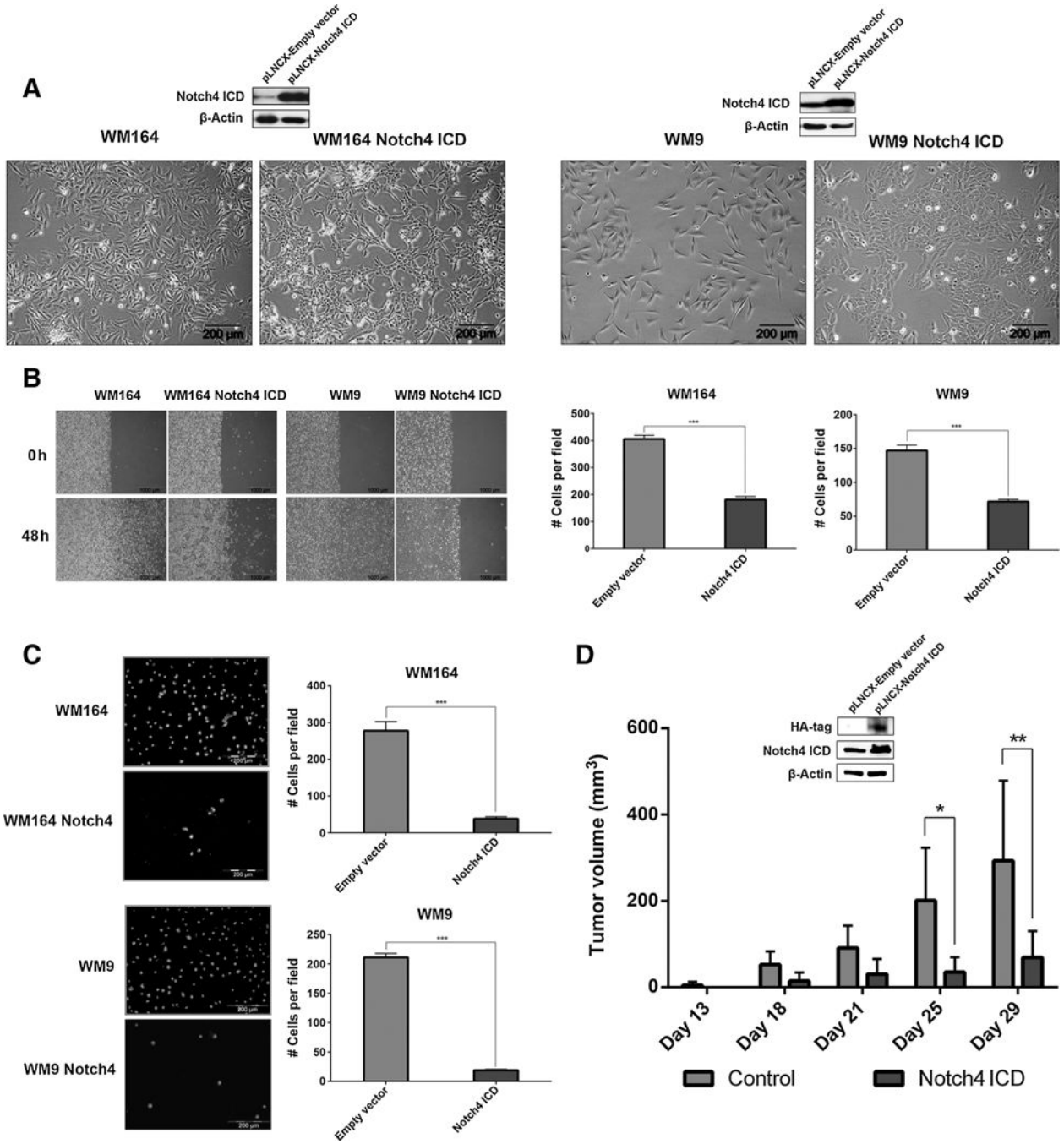

Figure 1.

Phenotypic switch following N4ICD overexpression in human melanoma. A, morphology of N4ICD-transduced cells. Expression of N4ICD was confirmed by immunoblotting. β-Actin was used as a loading control. B, chemokinesis (left) and migration (right) of N4ICD-transduced cells. C, invasion of N4ICD-transduced cells. D, tumor volume induced by N4ICD or empty vector–transduced 451Lu. Expression of N4ICD was confirmed by immunoblotting. β-Actin was used as a loading control. n.s., not significant; *, $P \leq 0.05$; ***, $P \leq 0.01$; ***, $P \leq 0.001$.

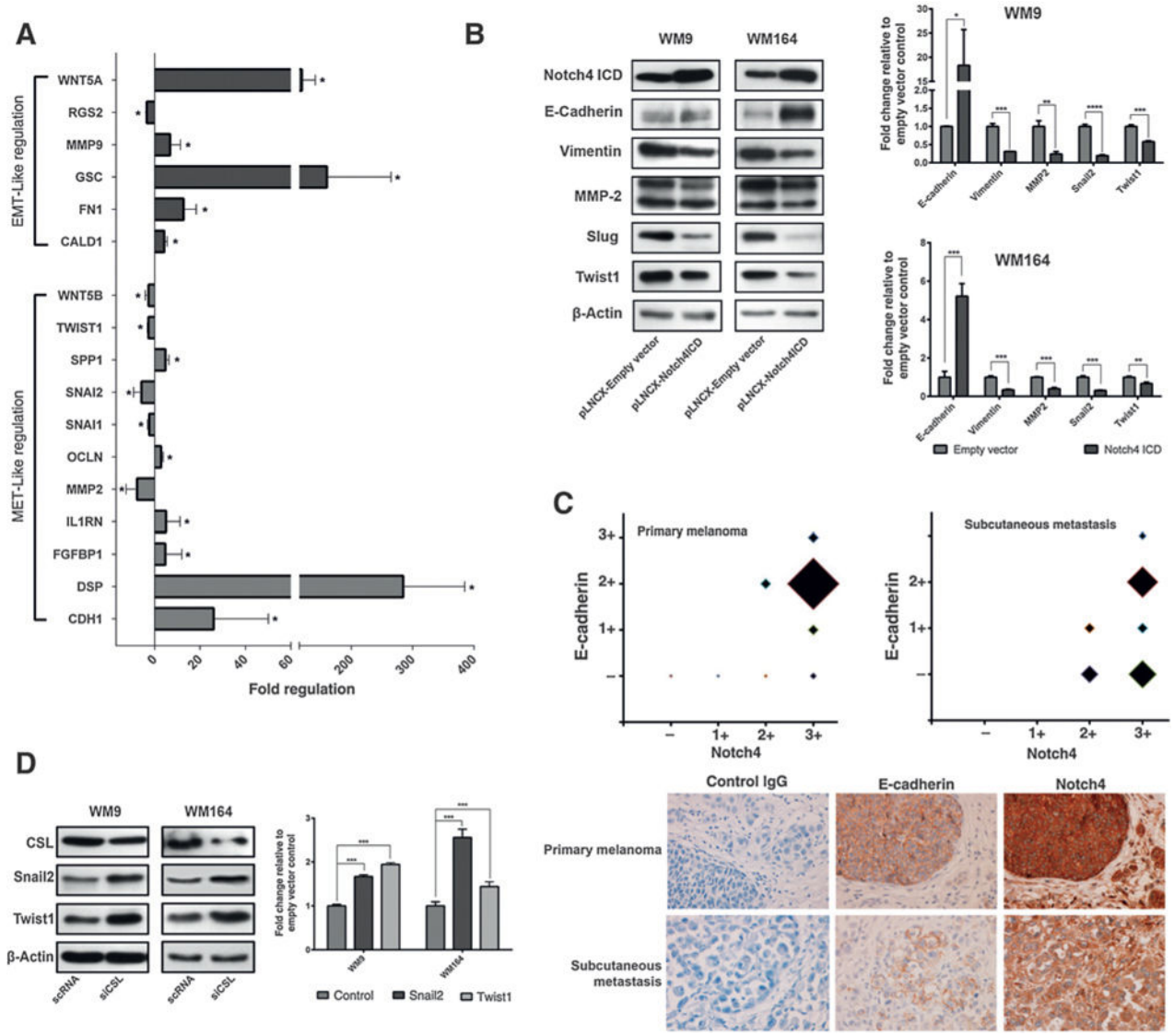

Figure 2.

Notch4 regulates EMT markers. A, mRNA levels of WM9 transduced with pLNCX-empty vector or pLNCX-N4ICD were analyzed using a qPCR array. Significant results are relative to empty vector–transduced cells and plotted as fold increase. B, immunoblots (left) and mRNA levels (right) of WM9 and WM164 transduced with pLNCX-empty vector or pLNCX-N4ICD C, E-cadherin and Notch4 staining of primary ($n = 30$) and metastatic ($n = 30$) melanoma samples plotted against each other (top). The size of the square indicates the number of samples, showing the respective expression levels. IHC of a primary melanoma and a subcutaneous metastasis for E-cadherin and Notch4 and unspecific secondary IgG antibody (×40; bottom). D, immunoblots (left) and mRNA levels (right) of WM9 and WM164 transfected with siRNA targeting CSL. The error bars represent SDs from the mean. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

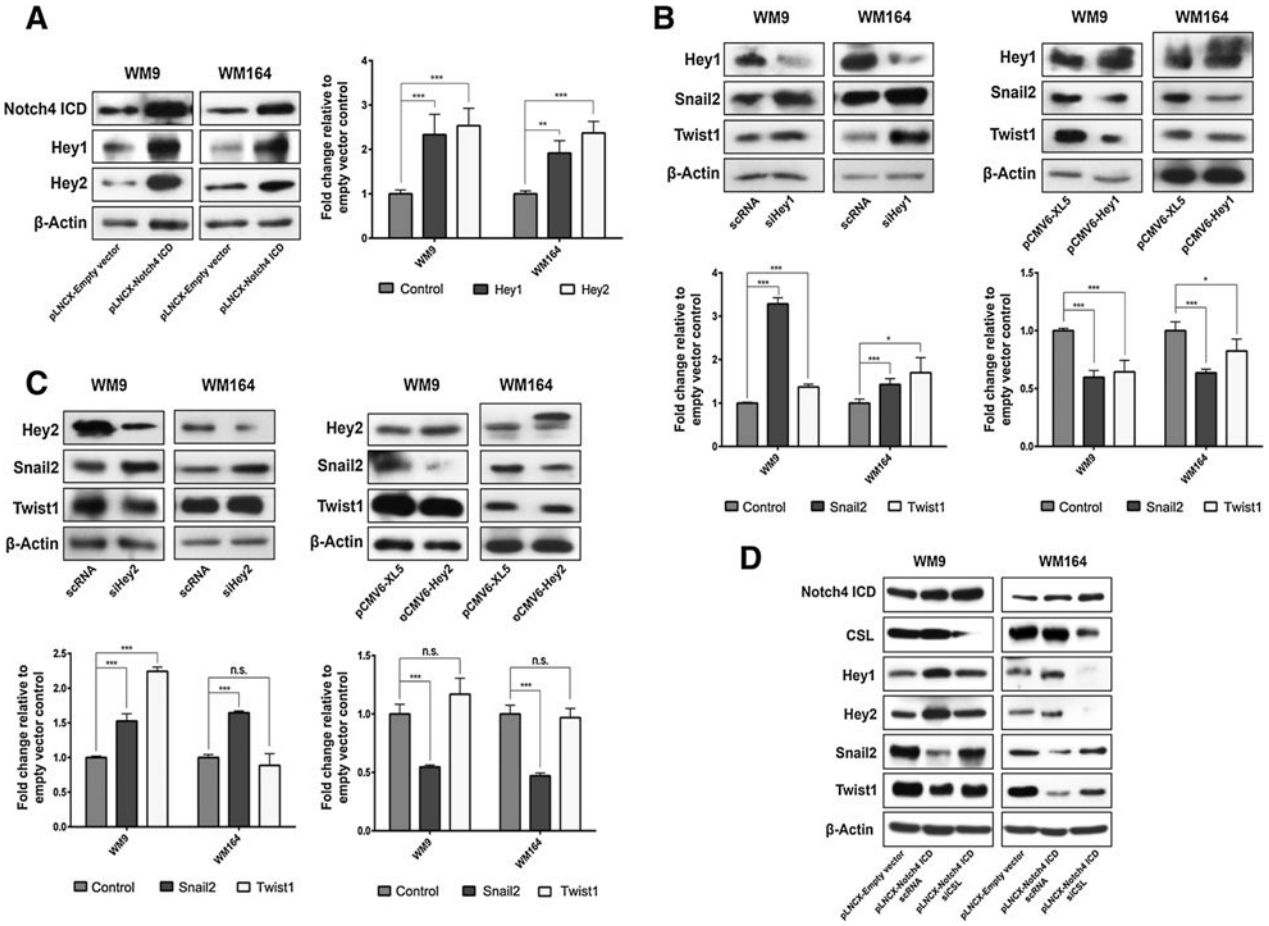

Figure 3.

Notch effector genes Hey1/2 suppress Snail2 and Twist1 expression. A, immunoblots (left) and mRNA levels (right) of WM9 and WM164 transduced with pLNCX-empty vector or pLNCX-N4ICD. B, immunoblots (top) and mRNA (bottom) of WM9 and WM164 transfected with siRNA-targeting Hey1 (left) or plasmids encoding Hey1 cDNA (right). C, immunoblots (top) and mRNA levels (bottom) of WM9 and WM164 transfected with siRNA targeting Hey2 (left) or plasmids encoding Hey2 cDNA (right). D, immunoblots of WM9 and WM164 transduced with pLNCX-empty vector, pLNCX-N4ICD, or pLNCX-N4ICD and siRNA targeting CSL. Error bars, SDs from the mean. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

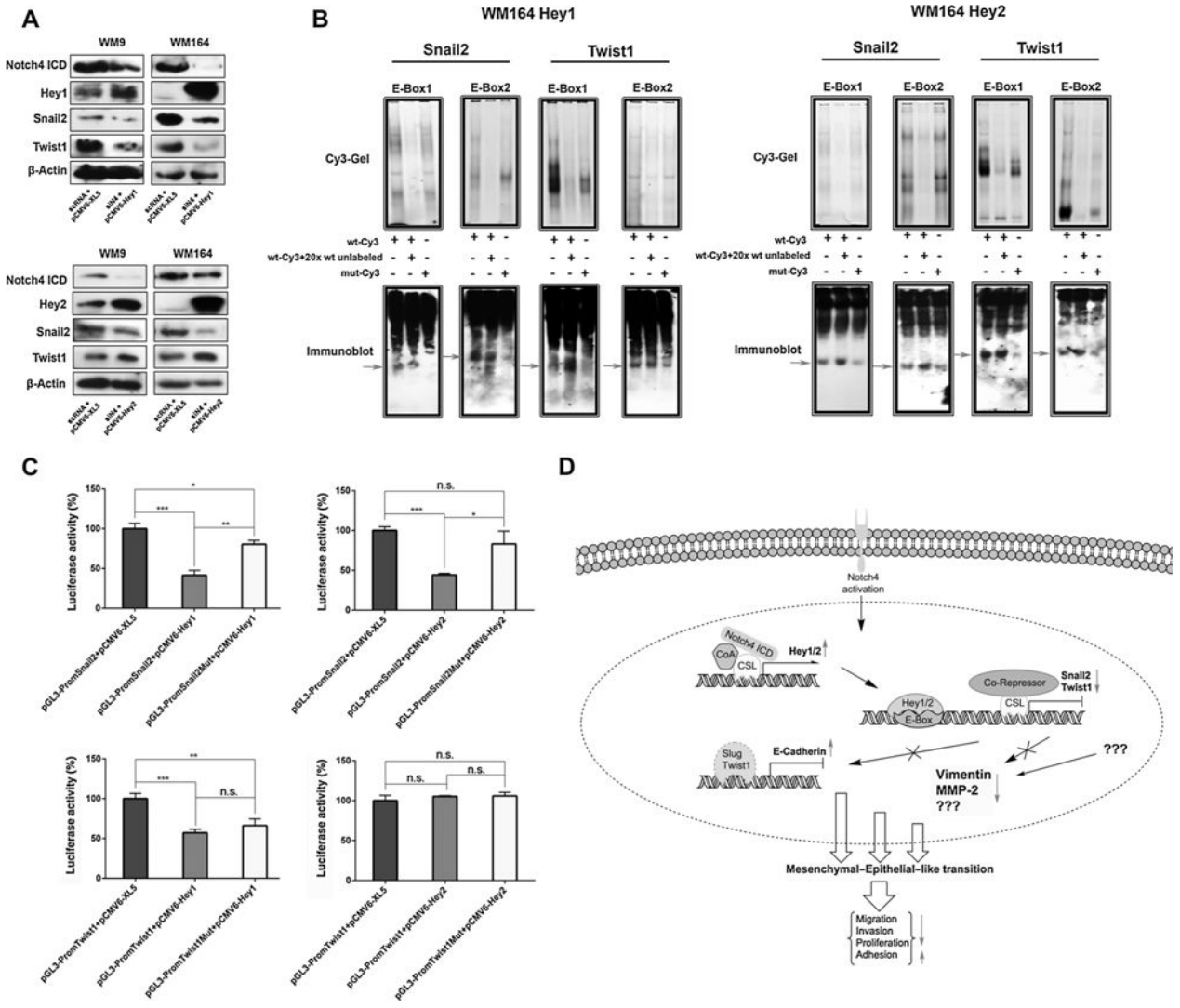

Figure 4.

Notch4-mediated activation of Hey1/2 suppress Snail2 and Twist1. A, immunoblots of WM9 and WM164 cotransfected with siRNA targeting Notch4 and plasmids encoding Hey1 or Hey2 cDNA. B, EMSA of WM164 incubated with Cy3-labeled promoter fragments and probed for Hey1 or Hey2. C, luciferase assay of WM164 cells cotransfected with the indicated plasmids. Error bars, SDs from the mean. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. D, proposed model of Notch4 signal transduction leading to a MET-like transition.