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Original Article

A peptide derived from *D. melanogaster* Hairless protein promotes the negative regulation of Notch Aberrant Constitutive Signaling on human breast cancer cells

Germán Saucedo-Correa, Rosa E Nuñez-Anita, Ulises Maciel-Ponce, Humberto Contreras-Cornejo, Alejandro Bravo-Patiño

Facultad de Medicina Veterinaria y Zootecnia UMSNH, Michoacán, México

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Abstract: The Notch Signaling Pathway (NSP), by a Notch intracellular domain (NICD) constitutive overexpression, has been related to many cancer types. In breast cancer, the constitutively activated NSP plays a principal role in aberrant cell cycle progression, poor cellular differentiation and apoptosis inhibition. It is well known the high conservation of Notch proteins through the metazoarians. Hairless is a negative regulator of NSP in *D. melanogaster*, but a homologous Hairless protein in mammals is unclear. The design of an expression plasmid, pReNegAID, which encodes a peptide based on the CSL binding domain of the Hairless protein of *D. melanogaster* will be used for analyzing the ReNegAID peptide participation it the negative regulation of NSP in the mammary gland cancer context. Both the pReNeg-AID plasmid and the mock plasmid were transfected into breast cancer cells in order to analyze cell proliferation (MTT assay) and gene expression pattern related to the NSP common genes between Hedgehog and Wingless pathways and genes related to apoptosis, cell differentiation and cell cycle. Moreover, control expression of Luciferase reported plasmid was performed and data showed that ReNeg-AID peptide induces a switch in the gene expression pattern related to the NSP and induce G1/S cell cycle arrest by the negative regulation of the Notch-1 receptor expression and it suggests cross talking between Hedgehog pathway (Hh) and NSP in mammary cancer cells to avoid the molecular machinery of initial epithelial to mesenchymal transition (EMT).

Keywords: Breast cancer, MCF-7, negative regulation, cancer therapy, notch-1, notch signaling pathway

Introduction

The Notch Signaling Pathway (NSP) (Figure S1) is an ancestral cell communication circuit highly conserved in all metazoan. Their transcription activation complex evolution through time has been minimal, however, the transcription repression complex presents more variability and specific tissue behavior in different organisms where it has been described [1, 2]. In the last years the NSP activation mechanism has been elucidated and in recent years the Notch mechanism of negative regulation, mainly in different cell populations and different cellular context, as well as its implications in different diseases such as cancer [3-5].

In the cancer context, it has been known that NSP has a principal role in both promoting the cancer cells appearing and maintaining the disease state [6, 7]. The role of the NSP in cancer has been firstly described in acute lymphoblastic leukemia. Today, the NSP is considered as a primordial target for the strategy against cancer disease, especially for breast cancer [6, 8, 9].

The NSP exerts control over different events essential for the proper function of the cell, such as differentiation, apoptosis, signaling pathways cross talking with both Hedgehog (Hh) and Wingless (Wnt) pathways. It is an essential regulator of the cell cycle G1/S phases transitions and it has been shown that if the Notch signaling pathway is not regulated properly, aberrations arise in its information flow, leading the cell to a cancer state [10, 11].

It is known that in breast cancer the NSP presents a constitutive activation mainly through Notch1 and Notch2 receptors and rarely through Notch4 receptor, promoting a strike on the mammary gland cell differentiation, an epithelial to mesenchymal transition (EMT) through deregulating the cross talking between Hh and NSP, promoting high levels of cell migration and more aggressive metastasis. Also, this constitutive activation of the NSP promotes the stop of the cell cycle checkpoints performed by cyclin D1 and cyclin E1, as well as changes in the transcriptional rates of *Fos* and *FosL* genes promoting the apoptosis evasion [11-15].

The strategies focused against breast cancer related to NSP distorted activity are based mainly on the γ-secretase inhibitors (GSI's) to block the NICD release from the cellular membrane avoiding its transport into the nucleus. Also, antibodies directed against ligands and receptors interactions are used to prevent the NSP cascade. Antibodies against co-activator Mastermind are also used, to prevent the transcriptional activation complex inhibiting the CSL/Mastermind interaction [16, 17]. However, these strategies still have collateral damage to the patients since the total inhibition of the NSP on healthy cells can have contradictory and lethal effects in the long-term [18-20].

Due to the high conservation of the NSP through the metazoan kingdom, inter species experiments have been conducted to corroborate the correct interaction between proteins from *D. melanogaster* NSP and proteins from the *Mus musculus* and human NSP, mainly between proteins involved in the transcriptional negative regulation. It has been demonstrated that *D. melanogaster* Hairless protein, responsible for negative regulation of the NSP at the fruit fly early embryo development, is capable of bounding, with high affinity, to mammal CSL transcription factor and downregulating the NSP activity [21-23].

The aim of this study was to demonstrate that the ReNeg-AID (Regulation Negative-AID) peptide (Figure S2), derived from the CSL binding domain of the *D. melanogaster* Hairless protein (**Figure 1A**), is capable of modifying the transcriptional pattern of genes related to the NSP which are involved in both proliferation inhibition and cell cycle arrest in mammary cancer cells. Interestingly, the transcriptional pattern gene in non-cancer mammary cells (MCF-12F) has not shown changes, but it caused changes in MCF7 and MDA-MB-231 cells. In summary, the result supported the hypothesis that pReNeg-AID could be employed as an adjuvant together with other anti-cancer therapies for mammary cancer or other cancer related to aberrant expression of Notch-1 cancer which is opening the opportunity to propose a new strategy against breast cancer where the Notch pathway is involved.

Material and methods

For cell culture: Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Cat. No. 12100046) for MCF-7 and MDA-MB-231 cells, DMEM-F12 Ham (Gibco, Cat. No. 12500096) for MCF12-F cells, fetal bovine serum (FBS, Gibco, Cat. No. 10437028), penicillin, streptomycin, and trypsin-EDTA (supplied by GIBCO-USA). For DMEM-F12 Ham complete growth medium 20 ng/ml of epidermal growth factor (Gibco, Cat. No. PHG0315), 0.01 mg/ml of human insulin and 500 ng/ml of hydrocortisone were added. The plasmid used for cell transfection (pFN21K HaloTag® CMV Flexi® Vector) was supplied by Promega™.

ReNeg-AID peptide

The ReNeg-AID peptide (patent pending) comes from the Hairless (H) protein of *Drosophila melanogaster*, specifically from the binding domain of the Hairless protein to the transcriptional factor Su(H) [CSL]. The amino acid sequence of pReNeg-AID was determined from position 1987aa-2782aa sequence for the Hairless protein. The ReNeg-AID DNA sequence was cloned into the vector pFN21K HaloTag CMV (pReNeg-AID, patent pending) following the manufacturer's specifications (PROMEGA™) (**Figure 1A**).

Cell culture

The non-cancer cell line of human epithelial breast MCF-12F (ATCC Cat# CRL-10783, RRID:CVCL_3745) (CRL-10783), tumorous estrogen receptor-positive MCF-7 (CLS Cat# 300-273/p2720_MCF-7, RRID:CVCL_0031) (HTB-22) and tumorous triple negative MDA-MB-231 (ATCC® HTB-26™) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in

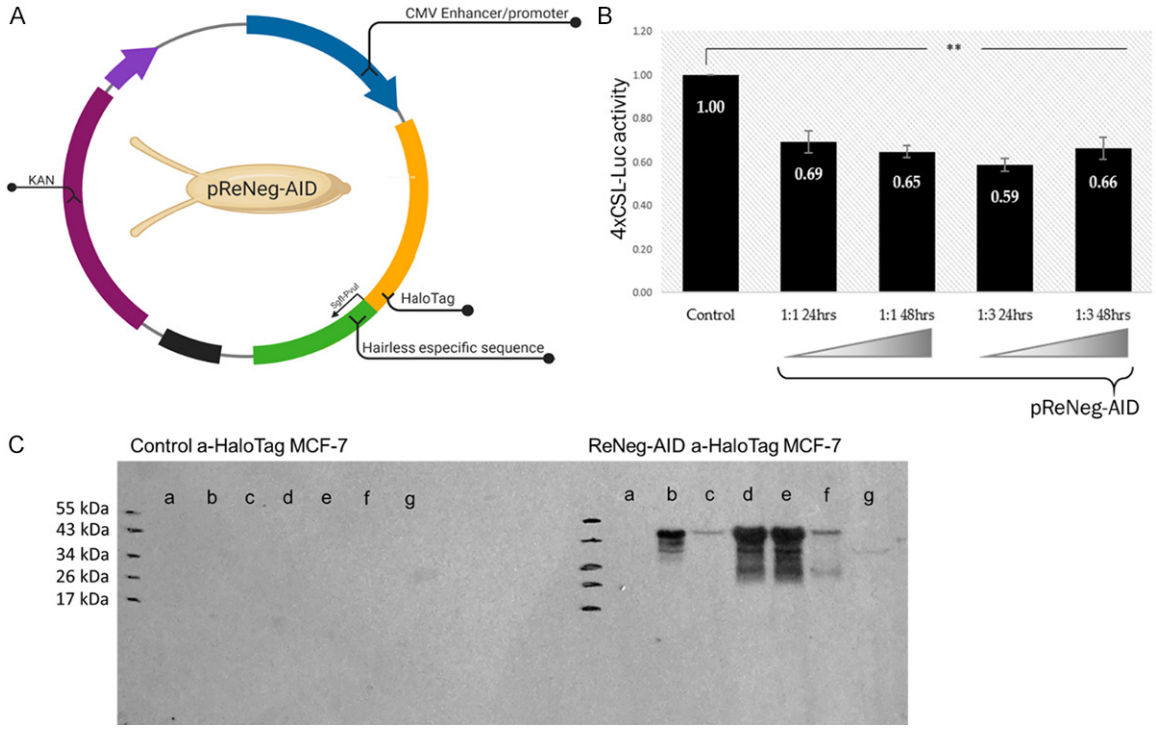

Figure 1. A. Plasmid map of pReNeg-AID. The pFN21K HaloTag® CMV Flexi® Vector (Promega) was used to design the pReNeg-AID. From *Drosophila melanogaster* larvae, the Hairless sequence that joins the CSL transcriptional factor to form a protein repression complex of NSP was obtained and tagged with the HaloTag flag. B. The vector pReNeg-AID decreases luciferase activity. MCF-7 cells were transfected in a molar ratio 1:1 and 1:3, respect to pGL4xCSL and pReNeg-AID vectors at 24 and 48 hours. Relative activity of luciferase was calculated as the luciferase activity of firefly against luciferase activity of Renilla. Each bar represents ± EST of average for triplicated experiments. Values for *P* were determined by student t test (**P ≤ 0.01 vs control. n=4). C. pReNeg-AID Western. The ReNeg-AID peptide is bound to a HaloTag flag (Promega) that uses monoclonal antibodies to be detected, the peptide bound to the tag has a relative weight of 45 kDa, the molecular protein marker used was the 10 to 180 kDa PageRuler Prestained Protein Ladder (Thermo Scientific, # 26616). Control a-HaloTag MCF-7 panel shows the controls without transfection of the ReNeg-AID peptide in MCF-7 cells. ReNeg-AID a-HaloTag MCF-7 panel shows the following data: (a) Control without transfection; (b) Positive control with HaloTag mock plasmid; (c) MCF-7 cells transfected with the pReNeg-AID, 12 hours after transfection; (d) MCF-7 cells transfected with the pReNeg-AID, 24 hours after transfection; (e, f and g) show the transfection at 48, 72 and 96 hours respectively.

DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (basal medium) at 5% CO_2 and 37°C for 48 h before transfections. The cell lines were obtained by the ATCC provider at the beginning of the experiments; therefore, these cell lines were new.

Transfection & electroporation

The cell culture was incubated with 5% of CO_2 at 37°C until reaching 80% of confluent. The culture was then tripzinated and an 1 × 10^6 cells were harvested, centrifuged until the pill was formed. Then, a 100 µl nucleofection solution (Cell Line Nucleofector® Kit V, protocol number T/C-28a2, supplied by AMAXA®) was prepared with 2 ng of the plasmid pReNeg-AID and 400 µl of OptiMEM medium (supplied by GIBCO-USA, Cat. No. 31985062). The nucleofection solution was added to the cells and the cells were placed in a 4 mm electroporation cuvette in an electroporator system (supplied by BTX® cuvettes & electroporation systems-Harvard Apparatus ECM 630 exponential decay wave electroporation system, item 45-2051) and the following conditions were applied: 140 V, 70 ms with one pulse; then the cuvette with the cell solution was incubated for five minutes at room temperature between 18°C to 25°C and the cells were cultured in a six-well plates with 1.5 ml of supplemented culture media in a humidified 37°C/5% CO_2 incubator for 48 h.

MTT assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay. 48 hours before transfection cells were cultured at 3×10^3 cells/well in DMEM medium and incubated at 37°C in 96-well plates with 5% CO_2. Then, 20 µL of MTT (5 mg/mL in PBS) was added to each well, and cells were incubated for 4 hours at 37°C. The supernatants were removed, 200 µL of dimethyl sulfoxide (DMSO) was added, and the absorbance (595 nm) was determined by a microplate reader (Bio-Rad, Hercules, CA, USA). For each cell line there were 3 trials.

Luciferase assay

One day before transfection, 450,000 cells were seeded on a petri dish. Later, the cells were co-transfected with the pGL4xCSL and the pReNeg-AID vectors, following the protocol of Xfect™ (Clontech). The luciferase activity was measured 24 and 48 hours post transfection following the Dual-Luciferase® Reporter Assay System (Promega) protocol.

Quantitative real time PCR (RT-qPCR)

RT^2 Profiler PCR (Qiagen, Cat. PAHS-059Z, No. 330231) Notch related gene arrays: Total cell RNA was isolated from MCF12-F, MCF-7 and MDA-MB-321, subsequently treated with DNase I and purified using RNeasy Mini Kit (Qiagen, Cat. No. 74034) according to manufacturer's instructions. 25 µg of high-quality total RNA was then reverse transcribed using the First Strand Synthesis Kit (Qiagen Cat. No. 330401) and subsequently loaded onto Human Notch RT2 profiler array (PAHS-059Z) according to manufacturer's instructions. The real time PCR was performed by using SYBR Green as a marker for DNA amplification on a thermocycler StepOnePlus™ System (Applied BioSystems, Thermo Fisher Scientific).

Flow cytometry

For flow cytometry the control cells and ReNeg-AID cells were harvested 48 hours post-transfection. A total of 1×10^6 cells in PBS were dyed with 400 µl of IP followed by adding 50 µl of RNAsa and incubated for 30 minutes to 1 hour. Data were collected on the Attune™ NXT Flow Cytometer (Thermo Fischer Scientific) using the BL2-H (lin)/Histogram channel to obtain the cell cycler phases graphics with the following parameters: FCS-260 V, SSC-280 V and BL2-360 V. Analysis was performed with FlowJo software V. 10 (Tree Star, Inc).

Statistical analysis

MTT proliferation, luciferase assay and cytometric analysis were analyzed by student's t-test, and values with $P < 0.05$ were considered statistically significant. All experiments were performed at least three times with n=4 for each of them.

For gene expression Qiagen's online web analysis tool was utilized to produce comparative scatter plots and fold change was calculated by determining the ratio of mRNA levels to control values using the ΔCt method (2-ΔΔCt). All data were normalized to an average of two of this housekeeping genes, ACTB and GAPDH. All experiments were performed at least three times with n=4 for each of them.

Results

Luciferase assay and western blot

The reporter vector pGL4xCSL was designed for controlling the gene transcription of the luciferase enzyme under the control of one promoter that contains regulatory binding elements from the CSL transcription factor. The co-transfection results showed that MCF-7 cells with the pGL4xCSL and pReNeg-AID vectors decreased the enzymatic activity of luciferase to 41% ($P \leq 0.01$).

The molar relation between the transfection vector pGL4xCSL and pReNeg-AID was 1:1 and 1:3 respectively. The luciferase activity was measured 24- and 48-hours post-transfection. However, there was no significant difference between the readings of luciferase enzyme activity taken at 24 and 48 hours after transfection. The decrease in the luciferase enzyme activity was not drastic, although H is the largest NSP antagonist in *D. melanogaster*, possibly because the polypeptide coded by the pReNeg-AID vector lacks the co-binding domains for Groucho and CtBP co-repressors. However, the polypeptide coded by the pReNeg-AID vector was able to decrease the luciferase activity (**Figure 1B**). The Anti-HalgoTag monoclonal antibody (Promega) was used to detect the ReNeg-AID peptide expression (**Figure 1C**).

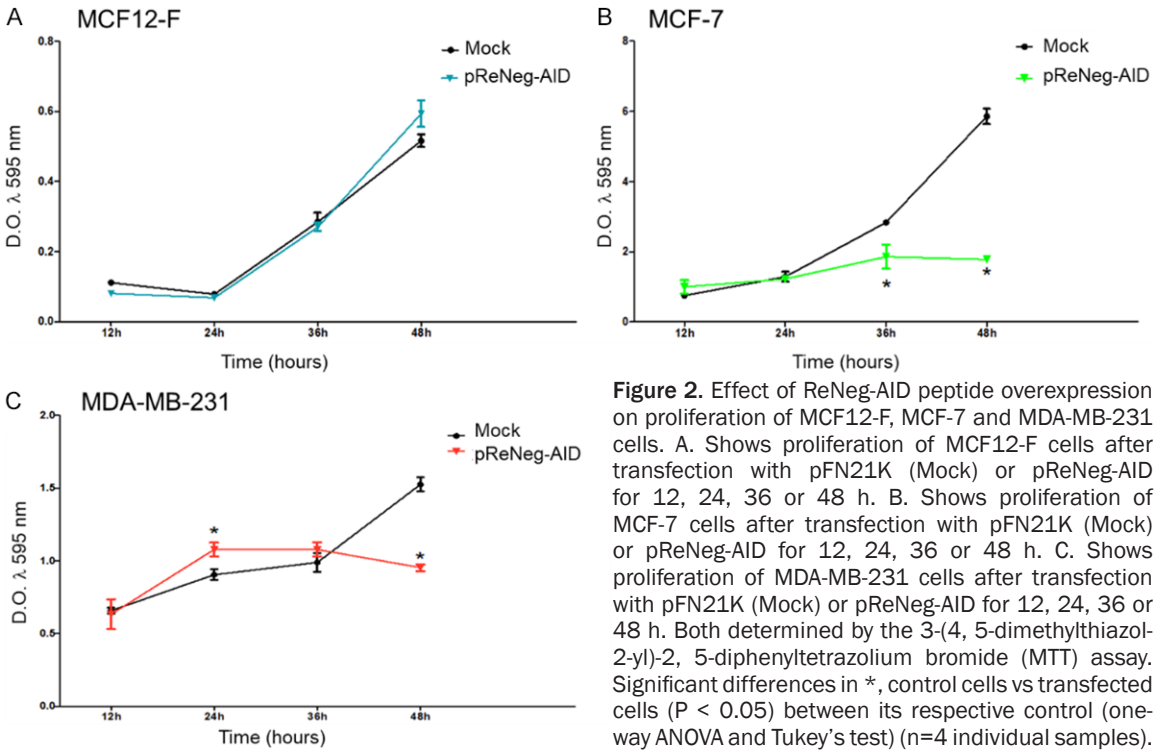

Figure 2. Effect of ReNeg-AID peptide overexpression on proliferation of MCF12-F, MCF-7 and MDA-MB-231 cells. A. Shows proliferation of MCF12-F cells after transfection with pFN21K (Mock) or pReNeg-AID for 12, 24, 36 or 48 h. B. Shows proliferation of MCF-7 cells after transfection with pFN21K (Mock) or pReNeg-AID for 12, 24, 36 or 48 h. C. Shows proliferation of MDA-MB-231 cells after transfection with pFN21K (Mock) or pReNeg-AID for 12, 24, 36 or 48 h. Both determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Significant differences in *, control cells vs transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey's test) (n=4 individual samples).

MTT proliferation assay

Figure 2A shows the proliferation behavior of MCF-12F cells after transfection with mock vector pFN21K (black line) and pReNegAID vector (blue line), at 12, 24, 36- and 48-hours post-transfection (hpt). The statistical analysis indicated that there are no significant differences between the pReNeg-AID transfected and mock transfected MCF12-F cells. This result means that the ReNeg-AID peptide activity has no effect on non-cancerous mammary gland cells. **Figure 2B** shows the behavior of MCF-7 cells proliferation after transfection with both mock vector pFN21K (black line) or pReNeg-AID vector (green line) at 12, 24, 36 or 48 hpt. The statistical analysis indicated significant differences (*) at 36 hpt ($P \leq 0.005$) and 48 hpt ($P \leq 0.005$), causing a decrease in cell proliferation when the ReNeg-AID peptide was over expressed in this cancer cells. As shown in **Figure 2C**, the MDA-MB-231 cell line presented an irregular proliferation, due to its genetic background with respect to the NSP that is activated in its non-canonical way. For this reason, the peptide used is not able to control the constitutive activation of the Notch pathway in triple negative cancer cells.

Fold-change gene expression related to inhibition of the NSP and cell differentiation process mediated by the NSP in mammary gland cells

For gene related to inhibition of the NSP, the comparative analysis of the transcriptional rate fold-change of *AES, CTNNB1, DTX1, GLI1, NCOR2, POFUT1* and *SMO* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt (**Figure 3A**) showed that the over expression of the ReNeg-AID peptide caused down regulation of the expression pattern for *CTNNB1* ($P \leq 0.00004$, -15.22 times), *NCOR2* ($P \leq 0.00004$, -37.14 times), *POFUT1* ($P \leq 0.000002$, -24.54 times) and *SMO* ($P \leq 0.000104$) genes. Also, it caused an up-regulation for the expression pattern of *AES* ($P \leq 0.000105$, 7.05 times), and there were no changes in *DTX1* and *GLI1* genes.

For gene related to differentiation a comparative analysis of the transcriptional rate fold-change of *DLL4, DTX1, HES1, HES5, HEY1, HEY2, HEYL, JAG1, NOTCH1* and *NOTCH4* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt (**Figure 3B**) showed that the over expression of the ReNeg-AID peptide caused a down-regulations of the expression pattern for

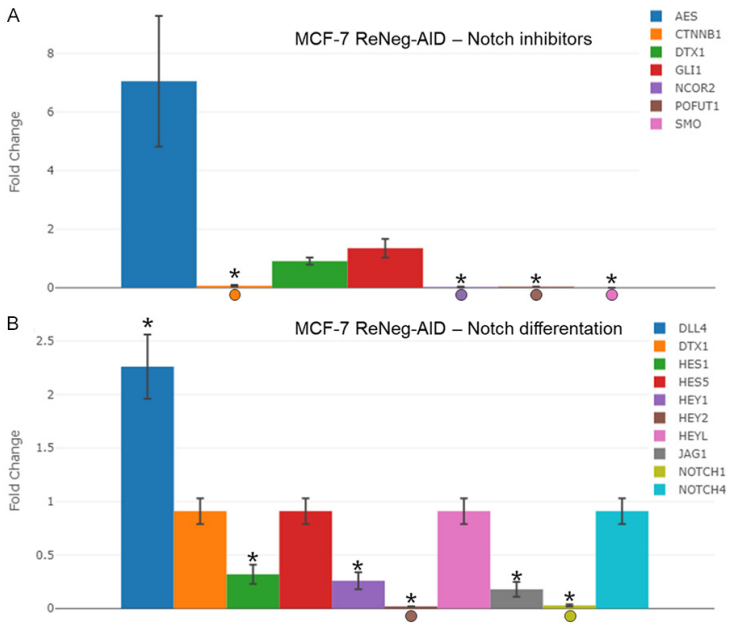

Figure 3. Profile of gene expression related to differentiation and Inhibition of Notch pathway after transfection with pReNeg-AID in MCF-7 cells in contrast with Mock transfected cells. A. Fold change of the expression of genes related to the inhibition of NSP. mRNA for *AES, CTNNB1, DTX1, GLI1, NCOR2, POFUT1* and *SMO* were quantified by qPCR method. B. Fold change of the expression of genes related to cellular differentiation. mRNA for *DLL4, DTX1, HES1, HES5, HEY1, HEY2, HEYL, JAG1, NOTHC1* and *NOTCH4* were quantified by qPCR. In all cases mRNA was quantified using a real-time PCR method (RT^2 Profile™ PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* genes served as internal control and was used to normalize for differences in input RNA, and the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey's test) (n=4 individual samples).

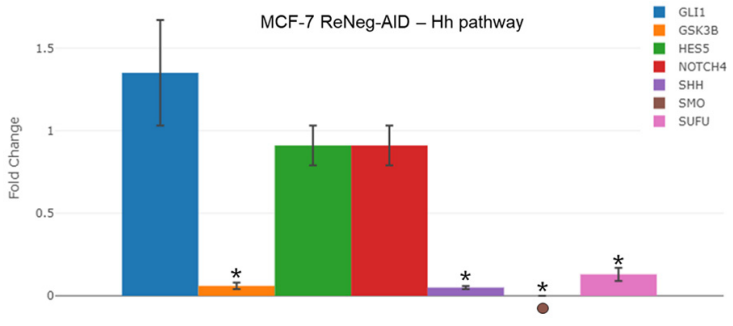

Figure 4. Profile of gene expression of genes interconnected between Notch and Hh pathways, after transfection with pReNeg-AID in MCF-7 cells in contrast with Mock transfected cells. Fold change of the expression of genes interconnected between Notch and Hh signaling pathways. mRNA for *GLI1, GSK3B, HES5, NOTCH4, SSH, SMO* and *SUFU* were quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT^2 Profile™ PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control used to normalize for differences in input RNA, and the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey's test) (n=4 individual samples).

HES1 (P ≤ 0.000553, -3.08 times), *HEY1* (P ≤ 0.002341, -3.86 times), *HEY2* (P ≤ 0.00002, -58.61 times), *JAG1* (P ≤ 0.0012, -5.53 times) and *NOTCH1* (P ≤ 0.00005, -30.41 times) genes. Also, it caused an up-regulation of the expression pattern for *DLL4* (P ≤ 0.000004, 2.26 times). For *DTX1, HES5, HEYL* and *NOTCH4* genes, there were no changes.

Transcriptional rate fold-change of genes related to the cross talk between Hh and NSP pathways in mammary gland cells

A comparative analysis (**Figure 4**) of the transcriptional rate fold-change of *GLI1, GSK3B, HES5, NOTCH4, SHH, SMO* and *SUFU* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells, 48 hpt, showed that the over expression of the ReNeg-AID peptide caused a down-regulation of the transcriptional expression pattern for *GSK3B* (P ≤ 0.000069, -17.01 times), *SHH* (P ≤ 0.000017, -21.82 times), *SMO* and *SUFU* (P ≤ 0.000286, -7.47 times). Also, it caused an up-regulation of the transcriptional expression pattern for *GLI1,* but there were no changes for *HES5* and *NOTCH4* genes.

Transcriptional rate fold change of genes involved in the cross talk between Wnt and NSP pathways in mammary gland cells

A comparative analysis (**Figure 5**) of the transcriptional rate fold-change of *AXIN1, CTNNB1, FZD2, FZD3, FZD4, FZD7, GSK3B, LRP5, WISP1* and *WNT11* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells at 48 hpt was performed. The over expression of the ReNeg-AID peptide caused a down-regulation of the gene expression pattern for *AXIN1* (P ≤ 0.000041), *CTNNB1* (P ≤ 0.00004, -15.02 times), *FZD2* (P ≤ 0.00247, -2.02 times), *FZD3* (P

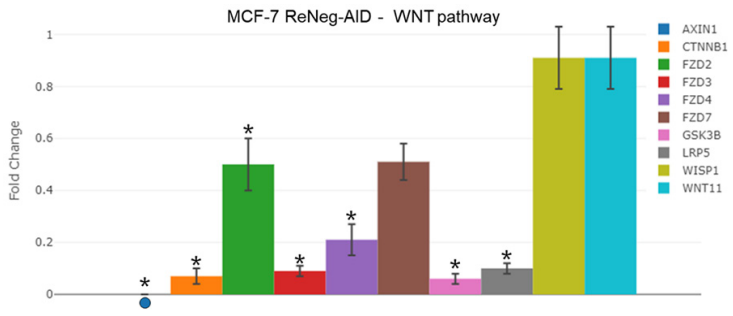

Figure 5. Profile of gene expression of genes interconnected between Notch and Wnt pathways, after transfection with pReNeg-AID in MCF-7 cells in contrast to Mock transfected cells. Fold change of the expression of genes interconnected between Notch and Hh signaling pathways. mRNA for *AXIN1, CTNNB1, FZD2, FZD3, FZD4, FZD7, GSK3B, LRP5, WISP1* and *WNT11* were quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT^2 Profile™ PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control used to normalize for differences in input RNA, and the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey's test) (n=4 individual samples).

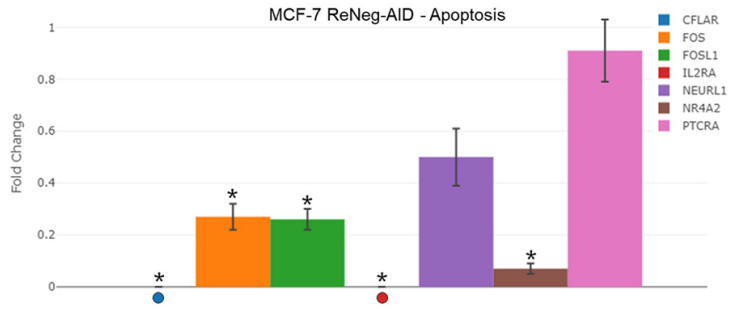

Figure 6. Profile of gene expression of genes related to regulation of the apoptosis by the control of Notch Signaling Pathway. Fold change of the expression of genes related to the regulation of the apoptosis. mRNA for *AXIN1, CFLAR, CTNNB1, FOS, FOSL1, IL2RA, NEURL1, NR4A2* and *PTCRA* genes were quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT^2 Profile™ PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control used to normalize for differences in input RNA, and the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey's test) (n=4 individual samples).

≤ 0.00006, -10.61 times), *FZD4* (P ≤ 0.00026, -4.81 times), *GSK3B* and *LRP5* (P ≤ 0.000073, -10.33 times). *FZD7* showed no statistically significant change. For *WISP1* and *WNT11* genes, there were no changes.

Transcriptional rate fold change of genes related to apoptosis regulation under the control of the NSP in mammary gland cells

A comparative analysis (**Figure 6**) of the transcriptional rate fold-change of *AXIN1, CFLAR, CTNNB1, FOS, FOSL1, IL2RA, NEURL1, NR4A2* and *PTCRA* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt was performed. The over expression of the ReNeg-AID peptide caused a down-regulation of the expression pattern for *AXIN1* and *CFLAR* (P ≤ 0.00004, -1348.80 times), *CTNNB1* and *FOS* (P ≤ 0.000176, -3.74 times), *FOSL1* (P ≤ 0.00004, -3.83 times), *IL2RA* (P ≤ 0.000193), *NEURL1* and *PTCRA* (no significant change).

Transcriptional rate fold change of genes related to cell cycle under NSP control

A comparative analysis (**Figure 7A**) of the transcriptional rate fold change of *AXIN1, CCND1, CCNE1, CDK1A, JAG2 and NOTCH2* genes, MCF-7 control cells vs MCF-7 ReNeg-AID cells 48 hpt was performed. The ReNeg-AID peptide over expression caused a down-regulation of the expression pattern for *AXIN1, CCND1* (P ≤ 0.0005, -392.06 times), *CCNE1* (P ≤ 0.00005), *CDK1A* (P ≤ 0.00006, -50.59 times), *JAG2* (P ≤ 0.000184, -12.77 times), *NOTCH1* (P ≤ 0.00005, -30.41 times), *NOTCH2* (P ≤ 0.000007, -34.62 times) genes.

Flow cytometry and cell cycle

Figure 7B shows the cell cycle histograms of MCF-7 control cells compared with MCF-7 ReNeg-AID cells, 3n experiments with 3 repeats were performed for each condition. G1 phase peak on MCF-7 control showed an average percentage of 39.9 ± 2.6 vs an average of 34.6 ± 2.5 for MCF-7 ReNeg-AID without statistical significance. S phase valley on MCF-7 control showed an average percentage of 26.4 ± 3.6 vs an average of 37.7 ± 0.98 for MCF-7 ReNeg-AID with statistical significance (P ≤ 0.0382). The G2/M phases peak on MCF-7 control showed an average percentage of 18.1 ± 3.6 vs an average of 6.91 ± 1.4 for MCF-7 ReNeg-AID with statistical significance (P ≤ 0.0183).

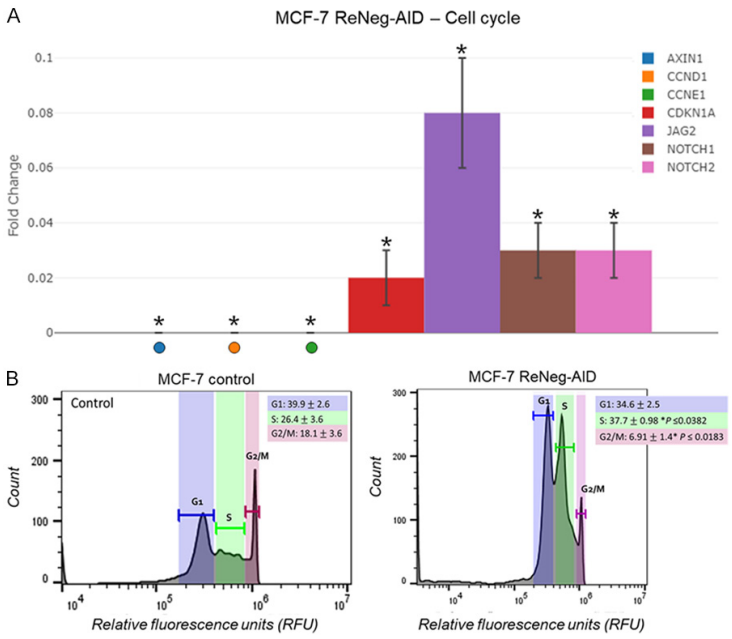

Figure 7. Profile of gene expression of genes related to cell cycle control and analysis of cell cycle arrest. A. Fold change of the expression of genes related to the cell cycle control. mRNA for *AXIN1, CCND1, CCNE1, CDKN1A, JAG2* and *NOTCH2* were quantified by qPCR method. mRNA was quantified using a real-time PCR method (RT^2 Profile™ PCR Array Human Notch Signaling Pathway, Qiagen). *ACTB* and *GAPDH* served as internal control used to normalize for differences in input RNA, and the fold change threshold was cut-off in 2. Significant differences in *, MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey's test). (n=4 individual samples). B. Cell cycle analysis. After 48 h cell transfection with Mock and pReNeg-AID respectively, the cells were pooled, stained with Propidium Iodide (PI), and analyzed by flow cytometry as described in the Materials and Methods section. Each histogram shows a flow cytometric plot of 10,000 cells per sample and is representative of three independent experiments. The percentage of cells (mean ± S.D.) in G1, S, and G2/M phases is listed. Significant differences in *, MCF-7 control vs MCF-7 transfected cells ($P < 0.05$) between its respective control (one-way ANOVA and Tukey's test).

Discussion

Cellular proliferation. It has been demonstrated that the ReNeg-AID peptide has no any effect on non-cancerous mammary gland cells at any of the tested time, and cell proliferation remains unaltered if we compare transfected (blue line) and not transfected (black line) MCF-12F cells (**Figure 2A**). On the other hand, the ReNeg-AID peptide presents an effect over the MCF-7 cells proliferation (**Figure 2B**, green line). Cells proliferation began to diminish at 36 hpt, and at 48 hpt it is clear that the cell proliferation is severely compromised, compared to MCF-7 cells transfected with the mock plasmid (**Figure 2B**, black line). All these together suggest that the ReNeg-AID peptide is capable of stopping cancer cell proliferation and, moreover, it seems that this peptide somehow is capable of affecting cell proliferation, and does not generate changes in gene expression in the MCF-12F cell line (Figure S3).

Gene related to the NSP inhibition: Expression of the ReNeg-AID peptide in MCF-7 cells promoted that the *AES* gene, which participates in NSP inhibition, will show an increase in its transcription rate, which codifies for the Groucho protein. This protein is one of the main pleiotropic corepressors of the NSP at nuclear level and has been related to the correct expression of the NSP target genes. The binding activation complex structured by the CSL transcriptional factor, Mastermind (MAML) and NICD proteins, is inhibited by the Groucho protein which recruits histone deacetylases and inhibits the NSP target genes [5, 21, 24]. No changes in *DTX1* gene expression, the gene codifies to a protein named Deltex, were reported This protein has been reported to be able to physically interact with the Notch-1 receptor avoiding its translocation to the nucleus, and, as a consequence, the NSP target genes will not be expressed (**Figure 3A**) [6, 25, 26]. The *GLI1* gene, which codes for GLI1 protein, in the context of breast cells can negatively regulate NSP by direct physical interaction or cross-talk with the HIF1α factor [27-29]. This prevents NICD1 from entering the nucleus and promotes vacuolar proteolysis when the SMO gene, which encodes the Smoothness protein, is negatively regulated by not presenting changes of these proteins normally function in the context of NSP [15, 30, 31].

Also, the ReNeg-AID peptide expression in MCF-7 cells promotes that three genes experience a reduction in their transcriptional rate. The first one, *NCOR2* gene, which codes for the NCoR2 protein, shows a negative regulation; and it has been known that this is a specific tis-

sue repressor for NSP in mammary gland cells. In contrast to its negative regulation, the *AES* gene positive regulation is capable of compensating the regulatory protein complexes mechanisms involved in the inhibition of NSP (**Figure 3A**) [32-34]. The second gene, *CTNNB1*, that codes for the β-catenin protein, shows a negative regulation in its transcriptional rate, which could be indicating that the physical interaction reported between Notch-1 and β-catenin was happening when the ReNeg-AID peptide was expressed; this interaction of Notch-1/β-catenin promotes the half-live for Notch-1 and Notch-2 in the mammary gland (**Figure 3A**) [35, 36]. The third is the *POFUT1* gene, which encodes the OFUT1 protein, is responsible for a correct ligand-receptor interaction in the NSP transduction mechanism; its negative regulation promotes control at the membrane level to the NSP in a negative way when an interaction between the Jagged-1 ligand and the Notch-1 receptor is established [37-39].

Gene related to NSP and cell differentiation: A negative regulation was observed for the *JAG1* and *NOTCH1* genes, which encode Jagged-1 and Notch-1 proteins respectively. Overexpression of ReNeg-AID promoted the decrease of ligand/receptor interaction, so that the NSP target genes such as *HES1*, *HEY1* and *HEY2* have a negative regulation. This phenomena would suggest that the mammary gland cancer cells come to a dedifferentiated state in order to recover the normal control of cell cycle and apoptosis [2, 40]. On the other hand, a positive regulation of *DLL4* gene has been observed. *DLL4* codes for Delta-4 ligand proteins; this positive regulation at mammary gland cancer cells seems to be promoting the establishment of Delta-4/Notch-4 (ligand/receptor) interaction, in order to take control in the absence of Jagged-1/Notch-1 interaction, although it has been shown that Notch-4/Delta-4 is not able to recover the whole cell process mediated by Jagged-1/Notch-1 interaction [26, 41].

When the ReNeg-AID peptide was over expressed in breast cancer cells, *Hes5* gene did not present changes, promoting the direct cross talking between vessel cells and epithelial cells through Notch-4/Delta-4 interaction which, in consequence, promotes a normal *de novo* angiogenesis process [42, 43] but the new vessels will not be functional because the Notch-1/Jagged-1 interaction is not occurring and it is necessary to complete the whole vessel differentiation process. This change in the expression pattern of *Notch4* and *Notch1* genes interrupts the differentiation of the Tip/Stalk cells, where the expression of Notch-1 gene is required to get a fully functional vessel cells (**Figure 3B**) [42, 44-46].

Cross talking between NSP and Hh pathways: It had been observed that ReNeg-AID peptide overexpression promotes a negative regulation of *GSK3B*, *SHH*, *SMO* and *SUFU* genes (**Figure 4**), which encode for GSK-3β, Sonic Hedgehog, Smoothened and SUFU proteins, respectively. The negative regulation of Smoothened and Sonic Hedgehog proteins has been related to the progenitor cancer cells population reduction [31, 47, 48]. This phenomena, together with the negative regulation of SUFU and GSK-3β proteins, two negative regulators of the Hh pathway, prevents the GLI1 and GLI2 proteins degradation, which is essential for the correct differentiation process of progenitor mammary gland cells [49-51]. As a consequence, the GLI1 protein cytoplasm accumulation increases the amount of HIF1α protein in cytoplasm which avoids the NICD1 translocation to the nucleus [15, 52]. All this together has been related to a diminished cell migration and EMT decrease on mammary cancer cells led by Hh pathway and would suggest a cross talk with the γ-secretase activity by the Notch-1 overexpression [53, 54].

Cross talk between NSP and WNT pathways: A diminished transcriptional rate of those genes encoding AXIN and β-catenin proteins has been observed (**Figure 5**). It has been known that the interaction between these proteins results in a transcriptional complex that triggers the expression of genes dependent on the WNT pathway activity, which is involved in cell cycle and cell growth. When the ReNeg-AID peptide is overexpressed, the complex AXIN/β-catenin is not formed. In normal cells, when the β-catenin protein is released from the AXIN/β-catenin complex, it interacts with the NICD present at cell cytoplasm, avoiding its degradation. In these cancer cells the β-catenin in cytoplasm is also diminished by the ReNeg-AID peptide overexpression, which shrinks its interaction with NICD, causing its degradation which, instead, causes a negative transcriptional regulation of the NSP target genes [35, 55, 56].

It had been observed that the ReNeg-AID peptide overexpression promotes a negative regu-

lation of *FZD2, FZD3, FZD4*, and *LRP5* genes. Therefore, a WNT signaling pathway inactivation occurs because the receptors Frizzled 2 (FZD2), Frizzled 3 (FZD3) and Frizzled 4 (FZD4) are diminished in their membrane concentration. In contrast, the *WNT11* and *WISP1* genes did not show changes, which do not have any receptors to interact with. This effect is potentiated by the lower LRP-5 protein concentration, which has the important role in stabilizing ligand/receptor interaction in the WNT pathway context. All this together could be meaning that the WNT pathway is not activated by the deficiency of receptors. Nevertheless, at the same time the ReNeg-AID peptide overexpression on MCF-7 cell promotes a deficiency of the *CTNNB1* gene transcription, avoiding the presence of β-catenin in cell cytoplasm, which instead prevents its translocation into the nucleus, promoting the expression of WNT pathway target genes. The decrease of β-catenin in the cytoplasm promotes a cytoplasmic NICD half-live decrement, because NICDs are caught by proteasomes [35, 36, 44, 57].

NSP and apoptosis: A negative regulation of *CFLAR*, *FOS*, *FOSL*, *NR4A2* and *IL2RA* genes was observed (**Figure 6**) by the ReNeg-AID peptide overexpression in MCF-7 cells. Those genes encode for FADD-L1, C-Fos, Fra1, Nurr1 and CD25 proteins, respectively. The ReNeg-AID peptide overexpression suggests that FADD-L1/C-Fos/Fra1 interaction is able to positively regulate the activation mechanism of FAS/FADD apoptotic receptors by the extrinsic way, together with the Nurr1 and CD25 regulation on MCF-7 cells [54, 58]. However, it is known that NSP is able to regulate the intrinsic apoptotic pathway by the expression of *NEURL1* and *PTCRA* genes. It is also known that these proteins are involved in the differentiation processes and in the negative regulation of the NSP, promoting the regulation of the apoptotic processes mediated by PUMA and Bcl-2 proteins. Nevertheless, it is clear that more experimental data is necessary to elucidate the participation of these proteins in the apoptotic processes regulation in MCF-7 cells carried out by NSP [14, 59].

Cell cycle and NSP: As shown in **Figure 7A**, a negative regulation of *AXIN1*, *CNND1*, *CNNE1*, *CDK1A*, *JAG2*, *NOTCH1* and *NOTCH2* genes, which are involved in cell cycle, has been observed. These genes encode for Axin1, Cyclin D1, Cyclin E1, CDK1, Jagged-2, Notch-1 and Notch-2 proteins. It has been reported that in MCF-7 cells the Jagged-2/Notch-2 interaction promotes the cell cycle initiation mediated by the activity of Cyclin D1 [11]. In breast cancer the Notch-1 constitutive overexpression promotes the Notch-2 receptor overexpression and, as a consequence, the expression of the NSP target genes. One of these genes is *CNND1* (Cyclin D1) which is responsible of the cell cycle G1/S phase transition. It is suggested that Cyclin D1 overexpression could promote a G1/S checkpoint malfunction and, as a consequence, a loop cell cycle that will eventually lead the mammary gland cells to an uncontrolled proliferation. The ReNeg-AID peptide overexpression in MCF-7 cells negatively regulates the overexpression of both Notch-2 and Jagged-2 proteins, and therefore promotes the negative regulation of Cyclin D1 and Cyclin E1 [60, 61]. This negative regulation of those proteins arrests the cell cycle at G1/S phase in MCF-7 cells (**Figure 7B**), where the cell population is mainly arrested in the S phase, as a consequence causing a diminished proliferation. Finally, it had been observed that a down regulation of *CDK1A* gene transcription, caused by the ReNeg-AID peptide overexpression, in MCF-7 cells should cause an instability of the cell cycle associated with an early apoptotic activation process. This could mean that the combined down regulation of both *CDK1A* and *CNND1* genes promotes the cell cycle arrest of mammary gland cancer cells [12, 62, 63].

Overexpression of ReNeg-AID peptide in MCF-7 cells regulates negatively the constitutive expression of Notch-1 receptor at different levels. On cellular membrane level it negatively regulates the Jagged1/Notch-1 pathway by negative regulation of POFUT1 and the normal expression of DTX1. At cytoplasmic level, it negatively regulates the half-lives of the Notch receptor by negative regulation of β-catenin and Axin1 and by the normal expression of the GLI1 protein. At nuclear level, the activation complex protein between CSL/MAML/NICD is negatively regulated by the positive expression of Groucho protein and by the very nature of the ReNeg-AID peptide. The most significant effect of the ReNeg-AID overexpression occurs in the cell cycle. The MCF-7 cells were arrested in the G1/S phase by the negative regulation of Cyclin D1 and Cyclin E1, and by the effect of ReNeg-AID peptide expression on normal cells (MCF-

12F) that does not cause significant effects on the pattern genes related to the Notch pathway. The effect of the ReNeg-AID peptide overexpression opens the doors for future research based on the negative regulation at nuclear level in cancer cells that present a constitutive activation of the Notch signaling pathway and that it can be used as an alternative adjuvant strategy against breast cancer. It remains to analyze the possible routes of administration and/or action, by which the peptide ReNeg-AID can have an effect in vivo. The results of the MDA-MB-231 cell line were performed as with the MCF-7 and MCF-12F cell line, however, the data are not shown as conclusive results due to the nature of expression of the Notch pathway since it presents a non-canonical signaling of the Notch pathway and merits more detailed studies to answer that question.

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Disclosure of conflict of interest

None.

Address correspondence to: Alejandro Bravo-Patiño, Facultad de Medicina Veterinaria y Zootecnia UMSNH, Michoacán, México. Tel: (52) 443-2 95 8029; E-mail: brapal68@gmail.com; alejandro.bravo@umich.mx

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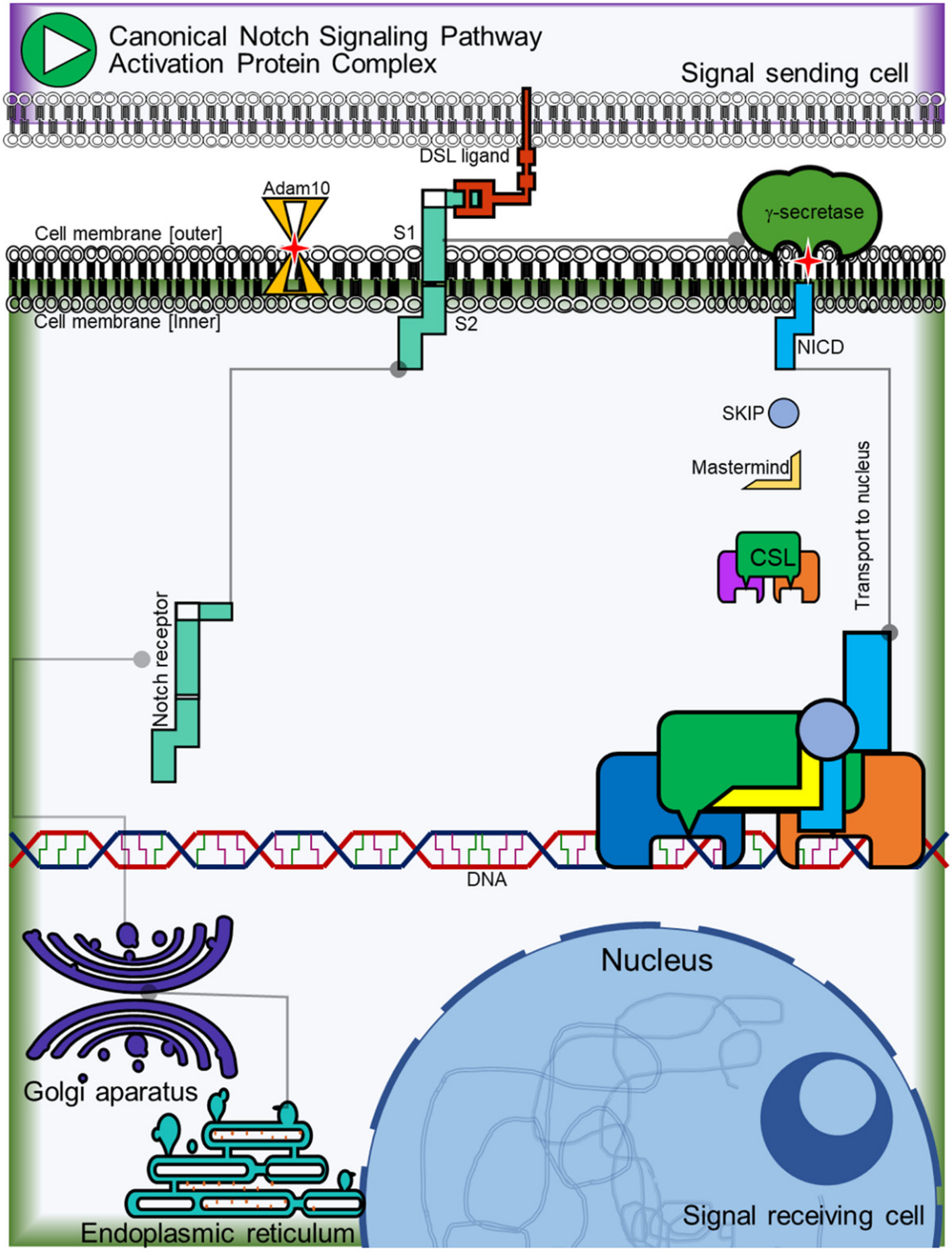

Figure S1. NSP canonical activation protein complex. The Notch receptor in mammals (Notch1-4) undergoes post-transcriptional modifications in the endoplasmic reticulum and in the Golgi apparatus where it is finally granted the specificity of binding by its ligands of the DSL family (Delta, Serrate and Lag-1). Once the Notch receptor is found on cell membrane it is recognized by the ADAM10/TACE metallopreotease that makes a proteolytic cut by separating the extracellular domain of the Notch protein once it is bound to its ligand (S1). The Notch intracellular domain (NICD) is then released from the cell membrane by the action of γ-secretase (S2). Once NICD is released from the cell membrane it is directed to the nucleus where it forms an activation complex by recruiting the Mastermind and SKIP co-activators to bind to the CSL transcriptional factor and initiate the expression of genes dependent on the Notch pathway like *Hes* and *Hey* family genes, CCND1, CCND3, Notch receptor and Notch ligands.

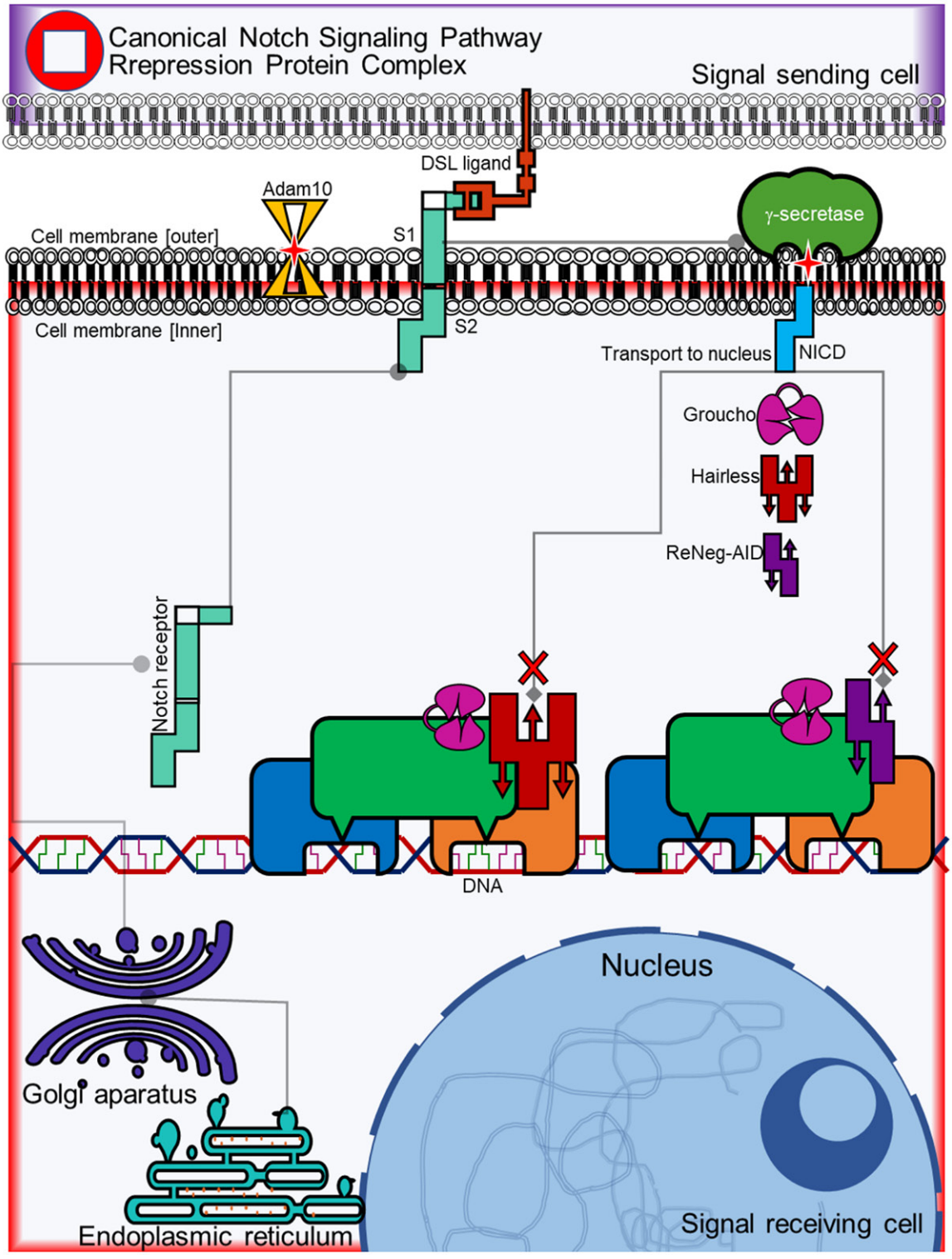

Figure S2. *NSP repression protein complex*. The Hairless protein competes for the union of the CSL transcriptional factor against NICD with a similar binding affinity to form a repression complex with the help of co-repressor Groucho. The ReNeg-AID peptide having part of the CSL binding domain of the Hairless protein competes in the same way against NICD to form a repression complex and change the expression pattern of genes dependent on the Notch pathway.

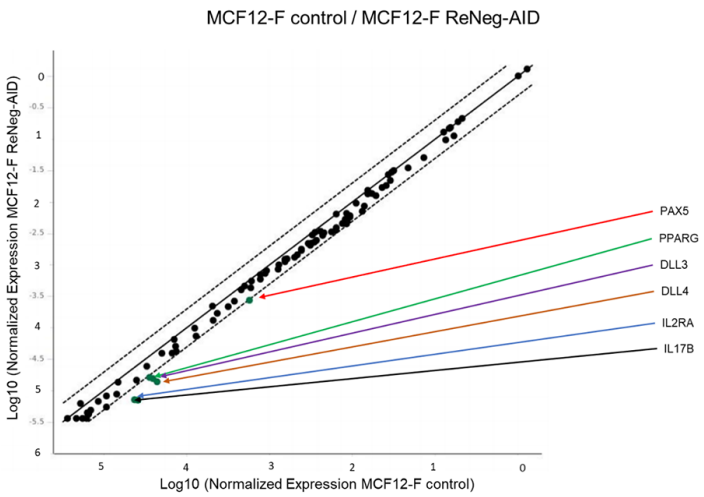

Figure S3. Scatter plot profile of the gene expression related to Notch Signaling Pathway on MCF-12F cells. mRNA was quantified using a real-time PCR method (RT^2 Profile™ PCR Array Human Notch Signaling Pathway, Qiagen). *GAPDH* served as internal control and was used to normalize for differences in input RNA. No significant differences were detected in MCF-12F control vs MCF-12F transfected cells between its respective control (one-way ANOVA and Tukey's test) (n=4 individual samples). Only *PAX5, PPARG, DLL3, DLL4, IL2RA* and *IL17B* genes were regulated negatively without significant differences.