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The role of Notch 2 in primary human breast epithelial cells: a preliminary *ex vivo* study

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S u m m a r y*: Breast cancer represents a major form of cancer affecting around one out of eight women in resource-rich countries. A hallmark of cancer, including breast cancer, is the dysregulation of intracellular signalling, a notably example of which is the family of Notch (Notch 1 to 4) receptor. While the effects of Notch 1 intracellular signalling have been significantly studied in breast cancer, those of Notch 2, −which shares 37% similarity to Notch1 receptor−, remain far from completely understood. Here, we studied Notch 2 intracellular signalling in human primary breast epithelial cells. Our results showed that: a) Notch 2 signalling may activate different transcription factors than Notch 1 signalling does, b), Notch 2 signalling causes downregulation of ΔNp63 expression levels, and c) Notch 2 signalling causes distinct changes in the expression levels of integrin proteins in our samples examined. Therefore, if the differences in Notch 1 and Notch2 result in distinct genetic or phenotypic signatures, −an issue to be verified in future studies−, it could be anticipated that targeting Notch 1 and Notch 2 with specific antibodies might lead to distinct therapeutic approaches.*

1. INTRODUCTION

Breast cancer represents a major form of cancer affecting around one out of eight women in resource-rich countries (1); thus, reducing the rates of its incidence and mortality is of paramount public health importance. The biological/ biochemical and,

in particular the genetic basis of breast cancer is now widely established, and it includes alterations in transcription factors-mediated pathways, and previously underappreciated effects of steroid molecules, such as androgens, as well as the dysregulation of cellular signalling leading to aberrant cellular proliferation among others (2-7).

Notch signalling is mediated by the Notch receptors that *a)* are transmembrane receptors conveying intercellular communication, *b)* exist in the vast majority of the animal kingdom, and *c)* consist of four paralogues in mammals, *Notch1*, *Notch2*, *Notch3*, and *Notch4* (8) (9) (10) [for further description on Notch receptor's phylogeny, see: (11)]. The target genes of Notch signaling are not limited to basic helix-loop-helix transcription factors, −i.e., Hairy/Enhancer-of-Split family genes (*HES1*, *HES5*), Hairy/enhancer-of-split related with YRPW motif protein 1 (*HEY1*), Homocysteine-response endoplasmic reticulum-resident ubiquitin- like domain member 1 protein (*HERP1*) - but also include proteins such as PTCRA, GATA3, CDKN1A, and CCND1, which act as Notch ligands as well; however, not all the same target genes are relevant to each Notch paralogue, especially in the context of different cellular processes and diseases. (8) (12) (13).

Overall, besides other cellular contexts (such as ageing (14)), Notch signalling appears to have a cellular context-specific function with both tumor-enhancing (e.g., T cell leukemia (15)) and tumor-suppressing properties (e.g., keratinocytes, bladder urothelium (16, 17)). In particular, Notch signalling is considered a *key regulator* in both breast physiology and breast cancer pathophysiology through a number of cellular processes. These notably include: *(a)* control of the cellular fate between luminal and myoepithelial cells, *(b)* the stem cells' self-renewal from those cells harbored in the mammary gland, *(c)* the cellular interactions between the mammary stem cells and the tissue microenvironment-based macrophages, as well as the transforming growth factor-beta (TGF-β)-mediated epithelial-to-mesenchymal transition (18) (19, 20) (21). However, some (minor) methodological concerns about the study by Chakrabarti *et al.* (20) have been previously expressed (see our comment in: (22)); in brief, these concerns referred to the lack of *a)* validation in human specimens and, in turn, presence of clinical relevance, *b)* measuring Edu in parallel to Ki67 in order to provide a quantitative approach to tissue proliferation (23), and *c)* assessing how Wnt signalling affects Notch receptors, despite the already well-shown close associations between Notch and Wnt signalling pathways (24).

Similarly to other proteins with several paralogues (e.g., PIWI proteins (25)), not all Notch paralogues are implicated in the same manner and to same extent in breast cancerogenesis, neither have they been equally explored. Indeed, *Notch1* and *Notch4*/Int-3 have been first linked to breast cancer in Mouse mammary tumor virus (MMTV) mouse models and represent the main focus of research in breast cancer, even though studies on *Notch3* and *Notch4* exist, as well (26, 27). For instance, it has been shown that *Notch1* intracellular domain (N1ICD) ectopic expression in primary human breast epithelial cells (HBECs) leads to the appearance of spherical structures that grow in suspension (24). Intriguingly, initial studies highlighted an opposing role of *Notch1* and Notch2 in breast physiology and breast cancer pathogenesis, i.e., that *NOTCH 2* receptors were not found expressed in normal mammary gland of the human (28) and that high *Notch1* and low Notch2 levels are linked to poorer tumor differentiation and reduced survival rates (27, 29). Nonetheless, later studies showed that *NOTCH 2* expression is significantly elevated in samples from patients with breast cancer, with *TP53* wild-type/ ER+ tumours, in particular those that carry a specific variant (30). However, *Notch 2* is the least investigated paralogue in human breast cancerogenesis; thus, our study aimed to address this research gap.

2. MATERIAL & METHODS

2.1. RNA isolation and quantitative real-time PCR

We performed isolation of total RNA obtained from human breast epithelial cells which were derived from purification of cellular populations from reduction mammoplasties (as previously described in (31) (32)) (HBECS) using RNeasy (Qiagen AG, Hombrechtikon, Switzerland), and we conducted the synthesis of cDNA using random p(dN)6 primers (Roche Diagnostics AG, Rotkreuz, Switzerland) and MMLV reverse transcriptase (Carlsbad, California, United States, Invitrogen). We performed semi-quantitative real-time RT-PCRs (QRT-PCR) based on the SYBR Green PCR Core Reagents System (Qiagen, Hilden, Germany) on an iCycler real-time PCR detection system (Bio-Rad Laboratories AG, Reinach, Switzerland). Overall, we conducted two independent experiments (i.e., two different patients). For each patient's samples, qPCR reagents were mixed well-enough for 3 reactions and split into 3 wells; these represented the three technical repeats to assess technical reproducibility. Expression levels for individual genes were normalized to 36B4 (i.e., acidic ribosomal phosphoprotein) housekeeping gene (33), which was used as the endogenous control gene. To that end, the relative standard curve technique was applied, and delta Ct (dCt) value was computed by subtracting 36B4 Ct value from the given gene's Ct value in the same sample. Log2 fold change of a given gene's expression between Notch ICD expressing vector and empty vector was calculated as negative difference in their dCt values (-ddCt

value). The resulting log2 fold change value was visualized as bar plot for each gene across experimental groups and patients. Moreover, the primer pairs used to assess each gene on previous literature (i.e., primers for *NOTCH1, ΔNp63, HEY1, ITGA6, ITGB1,* and *ITGB4* were based on (32), for *NOTCH2* on (34), for *HERP1* on (35), and for *HES5* on (36)), and we compared these findings with those in human *N1ICD*-infected HBECs.

3. RESULTS

3.1. Effect of *Notch 2* intracellular domain (N2ICD) overexpression on Notch receptor-downstream genes

We found that N2ICD expression was significantly increased in N2ICD infected HBECS, and N1ICD expression was significantly increased in N1ICD infected HBECS **(Figure 1).** Moreover, we observed that *Herp1* expression levels were significantly increased in N1ICD-infected cells compared to controls, and in N2ICD-infected HBECs compared to controls, and in N1ICD-infected HBECS compared to N2ICD-infected HBECS. However, *Hes5* expression was significantly increased in N1ICD infected HBECS but remained unaltered in N2ICD infected HBECS. However, *Hes5* and *Hey1* did not present consistent patterns between patients' breast primary cells under examination **(Figure 2).**

3.2. Effect of *Notch 2* intracellular domain (*N2ICD*) overexpression on *ΔNp63* and genes expressing extracellular matrix proteins

We observed an inverse association between *ΔNp63* and both *N1ICD* and *N2ICD* expression levels in HBECs of both patients **(Figure 3).** However, the patterns of expression levels for genes encoding extracellular matrix proteins, notably integrin proteins, were not consistent between patients' breast primary cells under examination. On the one hand, in patient AC, *ITGA6*, *ITGB1*, and *ITGB4* remained either unaltered or were upregulated, mostly when *N2ICD* was overexpressed On the other hand, in patient AF, *ITGA6*, *ITGB1*, and *ITGB4* were downregulated when either *N2ICD* or *N1ICD* was overexpressed **(Figure 4)**.

4. DISCUSSION

By having explored the N2ICD effects, we have chosen to focus only on the Notch receptor-downstream effects, and not on the potential differences in ligands binding to N1ICD or N2ICD; thus, our results reflect Notch-mediated signalling rather than Notch receptor interactions. Interestingly, the differences in the expression levels of downstream genes between N1ICD and N2ICD suggest that N1ICD and N2ICD might activate different members of the *HES* family. Given that Notch signalling is implicated in cell fate decisions and differentiation as a result of communication between neighbouring cells, its association with p63 implies a role of the latter in this process in regulating HBECS, similarly to previous observations with Notch1 (32). Similarly, loss of effective Notch signalling resulted in aberrant expression of p63 in mouse mammary luminal cells (37), as well as in non-mammary gland tissues (e.g., keratinocytes) (38).

Moreover, integrins such as ITGB1, ITGB4, and ITGA6, are proteins of the extracellular matrix serving as "connectors" with the cytoskeleton (39). Previous results indicate that N1ICD activates ITGB1 (40), which in turn causes a negative internal loop (41). Interesting is also the fact that rare cellular populations with high Notch receptor expression levels and ITGB4^{+} can lead to tumor promotion (42). Whereas the above findings are consistent with those observed in patient AC, the patient AF demonstrated the inverse pattern. This difference might possibly reflect individual differences, and it underscores a need for increased number of samples of primary human breast cells during experimental practice, e.g., through increasing their availability by relevant biobanks. Therefore, the distinct role of Notch1 and Notch2 in the expression of extracellular matrix proteins remains obscure at least in our study and, thus, needs to be further explored; however, this comparative approach could offer broader explanations on how Notch receptors transform HBECs, in general.

Of note, the similarity between N1ICD and N2ICD is 37% (i.e., 137 out of 427 amino acids). In particular, while high evolutionary conservation has been observed in certain domains (such as the RAM (RBP-jk/CSL associated molecule domain) domain and the ankyrin repeats, the same does not hold true on other regions (e.g., TAD) or the 3'-end aminoacids' phosphorylation motif of the ankyrin repeats; thus, these differences in aminoacids sequences or phosphorylation patterns can exercise profound differences in downstream cellular signalling (43) [as observed also even in human pathogens (44)].

Based on previous studies, the Notch2 receptor is involved in epithelial cell fate in the mammary gland, as it affects cells tied to both the ductal/ alveolar cells (*L cells*) and the luminal/ myoepithelial cells (*S cells*) (45); however, to our knowledge, whether such effects are mediated by *ΔNp63* remain to be yet explored. Notch2 appears also to be involved in the cellular dormancy of breast cancer and the potential for metastasis to the bones (46). Intriguingly, Notch2 expression appears to both affect and be affected by phytochemicals with claimed anti-cancer properties (47) (48) (49) (50). Of note, part of Notch2 effects are mediated in

association with other molecules, e.g., Presenilin-1(47), the binding protein MINAR1, which is also an intrinsically disordered protein (51), and potentially proteins that contribute to the extracellular matrix (52). Moreover, STAT-5 leads to upregulation of the Notch ligands Jagged-1 and DLL4 which, in turn, activate Notch2 in the basal type breast cancer; this action is enhanced by a non-receptor protein tyrosine kinase, named FYN (53).

Future phenotype analyses are expected to assess effects on growth rate, on morphological changes, on markers of epithelial cell differentiation (such as on Keratin 14 & 18 expression) and other cell adhesion molecules (such as cadherins), as well as validate the present findings using orthogonal approaches (i.e, Western blot, immunocytochemistry, etc). Likewise, future studies should assess if and how the aberrant expression of N2ICD lead to differences in the phenotypical features of HBECS that are transformed to a three-dimensional organoid, as the latter reflects more accurately a tissue morphology (54). Ideally, assessing N2ICD effects on the single-cell level (through single-cell RNA-sequencing) could help deciphering the N2ICD-mediated cellular heterogeneity.

Collectively, as in many similar cases with protein paralogues, the comparison between N2ICD and N1ICD downstream signalling can offer broader insights into the critical Notch receptors domains that mediated downstream signalling pathways, with potential therapeutic impact for breast cancer subcategories with pronounced Notch signalling. Theoretically, if the differences in N1ICD and N2ICD result in distinct genetic or phenotypic signatures, targeting Notch1 and Notch2 with specific antibodies might lead to distinct therapeutic approaches. However, caution is needed before extrapolating clinically meaning results; for instance, observations from clinical trials assessing the efficacy of monoclonal antibodies targeting the Notch2/Notch3 receptors (tarextumab) failed to be promising despite initial encouraging preclinical results (55)(56).

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Authors' contributions
AFAM: conceived and designed the study, performed the experiments, and analyzed the data. AFAM, PT, PP, SZ: drafted the first version of the manuscript. AFAM, PT, PP, SZ: revised the draft for important intellectual content. AFAM, PT, PP, SZ: read and approved the final version of the manuscript.

Disclosure of Conflicts of Interest
No financial or other conflict of interest to be declared.

Data Availability
Raw data are available upon reasonable request.

Ethical Publication Statement
We here-in confirm that: *a)*. we have read the Journal's position on issues that are involved in ethical publication, and *b).* affirm that this report is consistent with those guidelines.

Conflicts of Interest: The author declares no conflicts of interest regarding the publication of this paper.

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FIGURES

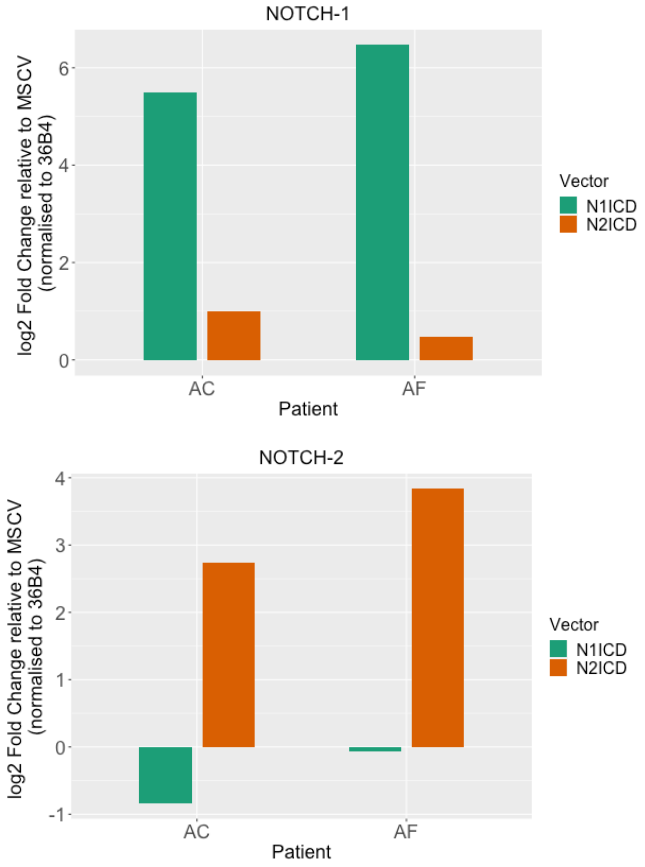

Figure 1. *Expression levels of Notch 1 Intracellular Domain (N1ICD) and Notch 2 Intracellular Domain (N2ICD) in MSCV-N1ICD versus MSCV-N2ICD HEBCs in patient AC and AF.*

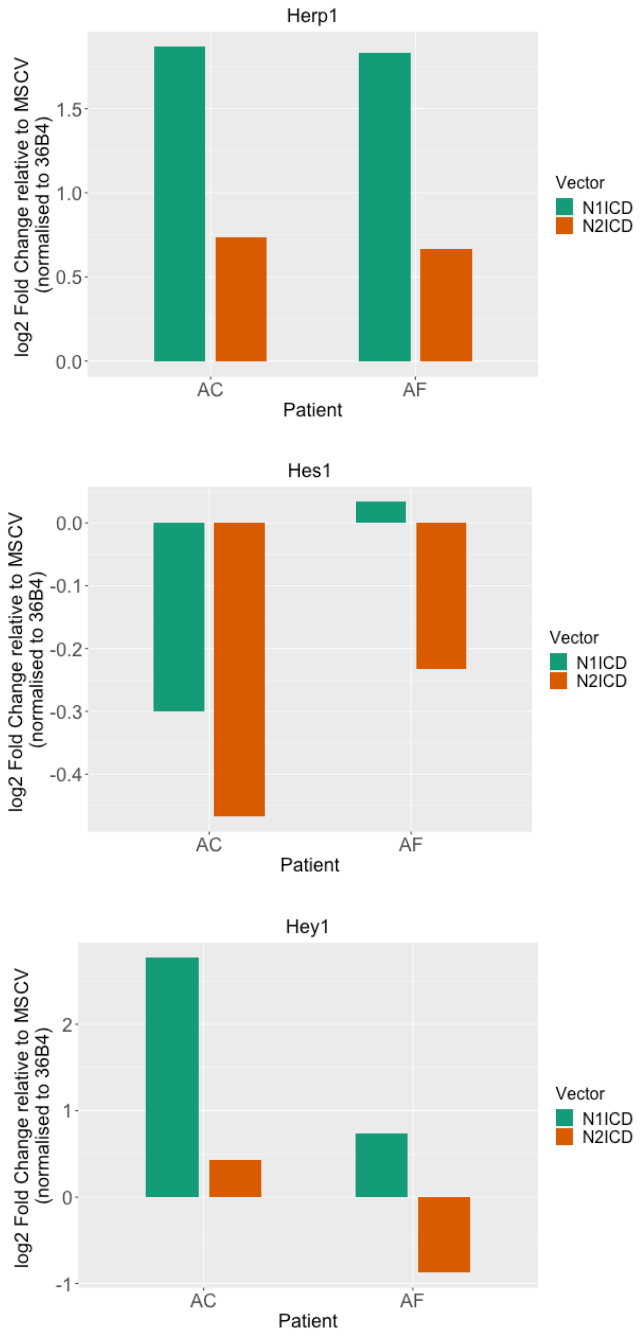

Figure 2. *Expression levels of Notch 2 downstream genes, Herp1, Hes5, and Hey1, in MSCV-N1ICD versus MSCV-N2ICD HEBCs in patient AC and AF.*

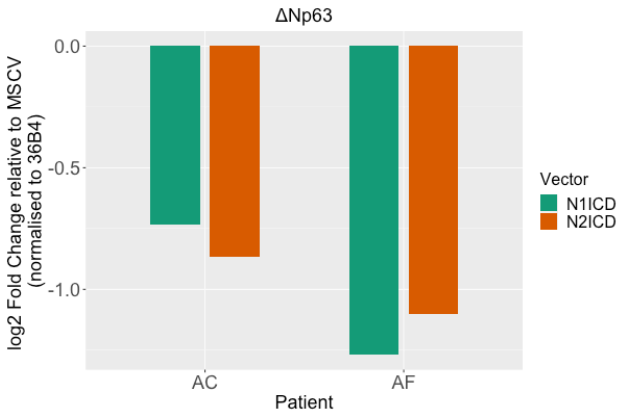

Figure 3. *ΔNp63 expression levels in MSCV-N1ICD versus MSCV-N2ICD HEBCs in patient AC and AF.*

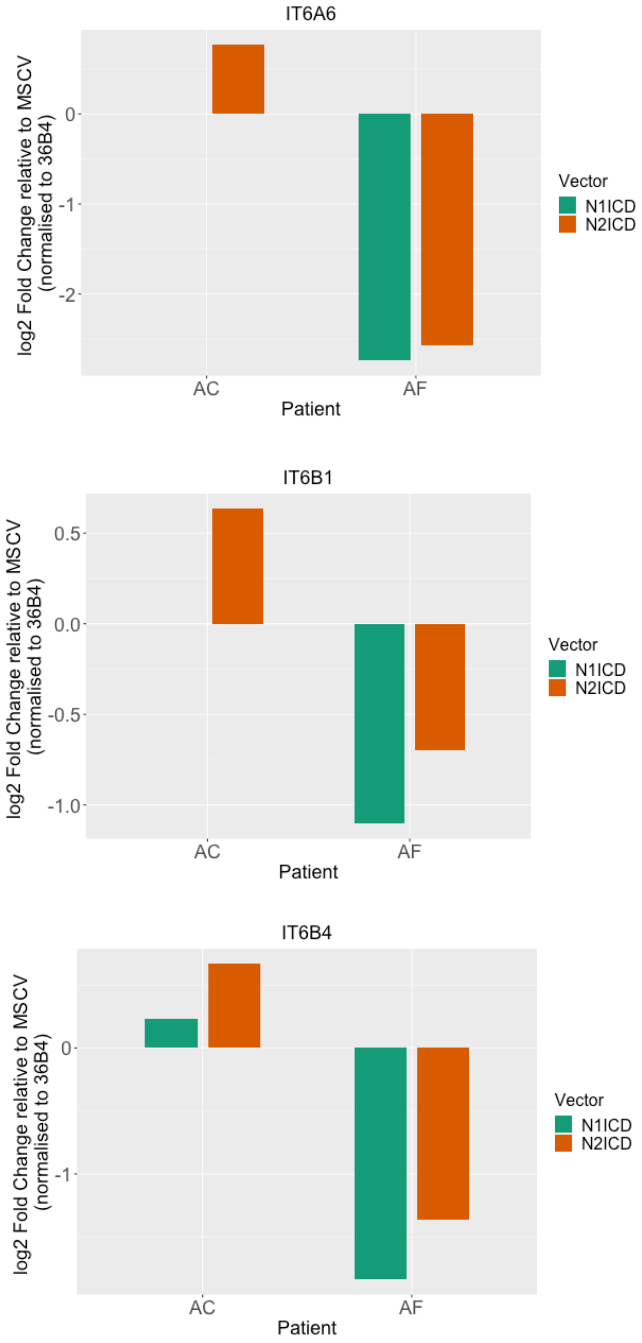

Figure 4. *ITGA6, ITGB1, and ITGB4 levels in MSCV-N1ICD versus MSCV-N2ICD HEBCs in patient AC and AF.*

SUPPLEMENTARY MATERIAL

Supplementary File 1. Cell culture and gene expression.
Supplementary Figure 1. Effective expression of N2ICD in 293 T cells.
Supplementary Figure 2. Ligation and orientation control.

Supplementary File 1. Cell culture and gene expression.
As Notch receptor's intracellular domains are constitutively active and, thus, mimic the ligand-activated form (1), we transfected the expression vector pcDNA4.V5 for human *Notch2* intracellular domain (*N2-ICD*) to 293T cells, which consist of a human renal epithelial cell line having the rare characteristic of becoming largely transfectable by $Ca_3(PO4)_2$ transfection protocol (after having produced the expression vector in large quantities through bacterial transformation). Then, we controlled for the efficient expression of *N2ICD*-downstream genes (*Hes1*, *Hes5*, *Hey1*, *Herp1*) 48-hours after transfection by reverse-quantitative PCR **(Supplementary Figures 1 & 2).** Following this, we proceeded with cloning *N2ICD* into retroviral vectors (i.e., MSCV-neo 1 retroviral vector). Then, we co-transfected human *N2ICD*-ligated MSCV-neo vector and MSCV proteins-containing vector to 293T cells, and we infected the produced virus to primary HBECs, which stemmed from clinical samples of two patients undergoing reduction mammoplasties (as in previous studies (2, 3)).

SUPPLEMENTARY REFERENCES

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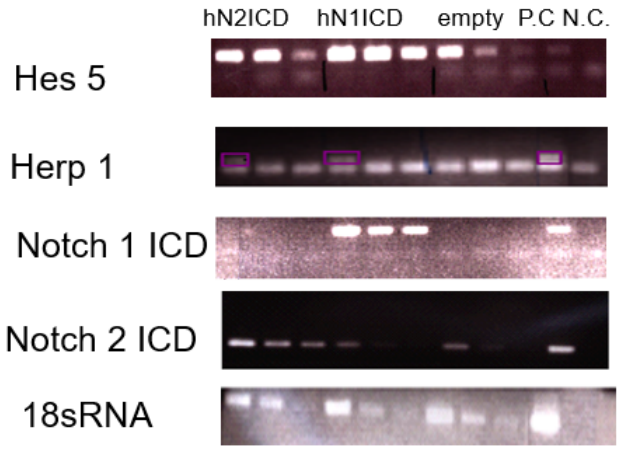
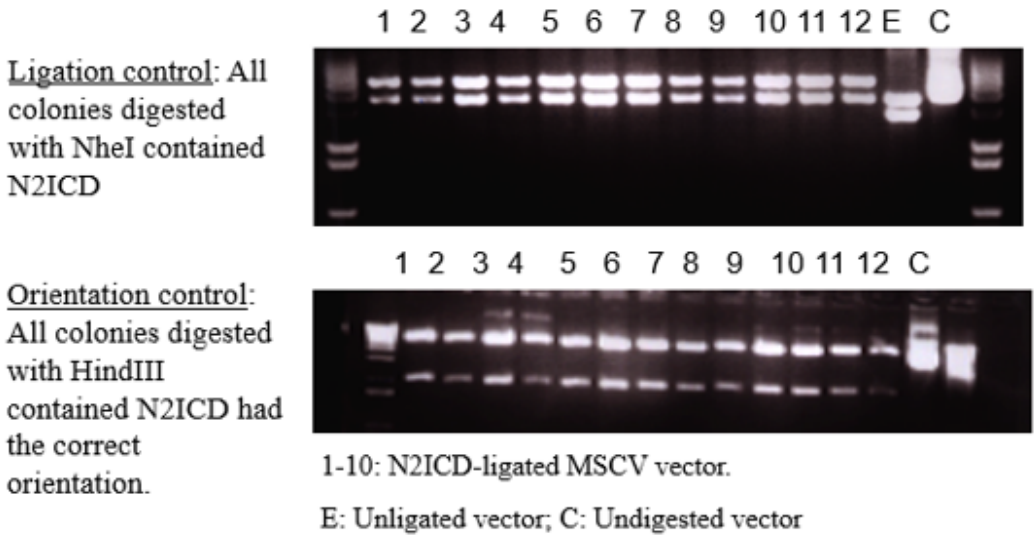


Supplementary Figure 1. *Effective expression of N2ICD in 293 T cells.*



Supplementary Figure 2. *Ligation and orientation control.*