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Cover Letter

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Editor

JoVE

Manuscript "*Integration of bioinformatics approaches and experimental validations to understand the role of Notch signaling in ovarian cancer*"

Dear Dr. Ronald Myers,

Thanks a lot for your invitation. We really appreciate your help and the great opportunity. Here, please find the attached manuscript entitled "Integration of bioinformatics approaches and experimental validations to understand the role of Notch signaling in ovarian cancer" for publication as a protocol article in *JoVE*.

Notch signaling is a highly conserved regulatory pathway involved in many cellular processes. Dysregulation of this signaling pathway often leads to interference with proper development and may even result in initiation or progression of cancers in certain cases. Bioinformatics is a useful way to extract smaller pieces of information from large-scale datasets. Through the implementation of various bioinformatics approaches, researchers can quickly, reliably, and efficiently interpret these large datasets, yielding insightful applications and scientific discoveries. Here, we present a protocol for integration of bioinformatics approaches to investigate the role of Notch signaling in ovarian cancer. Furthermore, we demonstrate validation of bioinformatics findings through experimentation.

We believe that this article will help scientists use bioinformatics approaches for their scientific designs and discoveries, it will attract profound interest from scientists and physicians working in the cancer research field and is therefore appropriate for inclusion in a professional protocol journal like *JoVE*.

We certify that none of the material has been published or is under consideration elsewhere.

The manuscript has been seen and approved by all listed authors. We would very much appreciate consideration of our paper for publication in *JoVE*. Thank you for your time and we look forward to your response.

Sincerely yours,

Dongyu Jia

TITLE:

Integration of Bioinformatics Approaches and Experimental Validations to Understand the Role of Notch Signaling in Ovarian Cancer

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KEYWORDS:
NOTCH2, NOTCH3, MAML1, NICD, Notch signaling, ovarian cancer, Drosophila, bioinformatics, protocol

SUMMARY:
Bioinformatics is a useful way to process large-scale datasets. Through the implementation of bioinformatics approaches, researchers can quickly, reliably, and efficiently obtain insightful applications and scientific discoveries. This article demonstrates the utilization of bioinformatics in ovarian cancer research. It also successfully validates bioinformatics findings through experimentation.

ABSTRACT:
Notch signaling is a highly conserved regulatory pathway involved in many cellular processes. Dysregulation of this signaling pathway often leads to interference with proper development and may even result in initiation or progression of cancers in certain cases. Because this pathway serves complex and versatile functions, it can be studied extensively through many different approaches. Of these, bioinformatics provides an undeniably cost-efficient, approachable, and user-friendly method of study. Bioinformatics is a useful way to extract smaller pieces of information from large-scale datasets. Through the implementation of various bioinformatics approaches, researchers can quickly, reliably, and efficiently interpret these large datasets, yielding insightful applications and scientific discoveries. Here, a protocol is presented for integration of bioinformatics approaches to investigate the role of Notch signaling in ovarian cancer. Furthermore, bioinformatics findings are validated through experimentation.

INTRODUCTION:

The Notch signaling pathway is a highly conserved pathway that is important for many developmental processes within biological organisms. Notch signaling has been shown to play a

significant role in cell proliferation and self-renewal, and defects in the Notch signaling pathway can lead to many types of cancers[1-6]. In some circumstances, the Notch signaling pathway has been linked to both tissue growth and cancer as well as cell death and tumor suppression[7]. Multiple Notch receptors (NOTCH 1−4) and co‑activator Mastermind (MAML 1−3), all with diverse functions, add an additional level of complexity. While the Notch signaling pathway is sophisticated in terms of functions, its core pathway is simple on a molecular basis[8]. Notch receptors act as transmembrane proteins composed of extracellular and intracellular regions[9]. A ligand binding to the extracellular region of Notch receptors facilitates proteolytic cleavage, which allows the Notch intracellular domain (NICD) to be released into the nucleus. NICD then binds to co‑activator Mastermind to activate downstream gene expression[10].

In recent years, Notch signaling has been shown to play a variety of roles in the initiation and progression of several types of cancers across different species[6,11]. For instance, Notch signaling has been linked to tumorigenesis involving the human *NOTCH1* gene[12]. Recently, the *NOTCH2, NOTCH3, Delta-like 3 (DLL3), Mastermind‑like protein 1 (MAML1)*, and *a disintegrin and metalloproteinase domain‑containing protein 17 (ADAM17)* genes were shown to be strongly associated with ovarian cancer, especially with the poor overall survival of patients[13].

As the amount of experimental and patient-associated data continuously increases, the demand for analysis of the available data increases as well. The available data are scattered across publications, and they may deliver inconsistent or even contradictory findings. With the development of new technology in recent decades, such as next-generation sequencing, the amount of available data has grown exponentially. Although this represents rapid advancements in science and opportunities for continued biological research, assessing the meaning of publicly available data to solve research questions is a great challenge[14]. We believe bioinformatics is a useful way to extract smaller pieces of information from large-scale datasets. Through the implementation of various bioinformatics approaches, researchers can quickly, reliably, and efficiently interpret these large datasets, yielding insightful discoveries. These discoveries may range from the identification of potential new drug therapy targets or disease biomarkers, to personalized patient treatments[15,16].

Bioinformatics itself is rapidly evolving, and approaches are constantly changing as technological advances sweep medical and biological science. Currently, common bioinformatics approaches include the utilization of publicly accessible databases and software programs to analyze DNA or protein sequences, identify genes of particular relevance or importance, and determine the relevance of genes and gene products through functional genomics[16]. Although the field of bioinformatics is certainly not limited to these approaches, these are significant in helping clinicians and researchers manage biological data for the benefit of patients as a whole.

This study aims to highlight several important databases and their use for research about the Notch signaling pathway. *NOTCH2*, *NOTCH3*, and their co‑activator *MAML1* were used as examples for the database study. These genes were used because the importance of the Notch signaling pathway in ovarian cancer has been validated. Systematic analyses of retrieved data confirmed the importance of Notch signaling in ovarian cancer. In addition, because Notch

signaling is well conserved across species, it was confirmed that overexpression of *Drosophila melanogaster NICD* and *Mastermind* together can induce tumors in *Drosophila* ovaries, supporting the database findings and the significant and conserved role of Notch signaling in ovarian cancer.

PROTOCOL:

1. Prediction of Clinical Outcomes from Genomic Profiles (PRECOG)

NOTE: The PRECOG portal (precog.stanford.edu) accesses publicly available data from 165 cancer expression datasets, including gene expression levels and patient clinical outcomes[17]. It specifically provides the Meta-Z analysis, which incorporates large datasets to provide Z-scores of different genes in 39 cancer types to indicate patient overall survival. Poor and good survival rates are indicated by positive and negative Z-score values, respectively.

1.1. Create an account with an academic affiliated email to access this database. Enter the email address and password associated with the account.

1.2. Click on the **View Details** button located underneath the **Meta-Z analysis** heading.

1.3. Input the gene of interest into the **Search** bar.

1.4. Use the scroll bar located on the bottom of the screen to obtain the survival Z-score for the specific cancer type of interest.

2. CSIOVDB

NOTE: CSIOVDB (csibio.nus.edu.sg/CSIOVDB/CSIOVDB.html) is a microarray database developed by the Cancer Science Institute of Singapore to study ovarian cancer[18]. This database contains data of carcinomas from different tumor sites as well as normal ovary tissue data. In addition, CSIOVDB provides Kaplan-Meier survival plots to assess patient survival with differential gene expression levels. CSIOVDB can be applied to investigate the association between gene expression levels and ovarian cancer stages/grades.

2.1. Input gene of interest, then click the **Search** button.

2.2. Click on the **Disease State** tab.

NOTE: This tab provides summary statistics of gene expression of the target gene of interest in ovarian cancer disease states.

2.3. Click on the **Histology** tab.

NOTE: This tab provides summary statistics of gene expression of the target gene of interest in major ovarian cancer histologies.

2.4. Click on the **Clinico-pathological Parameters** tab.

Note: This tab provides a comparison of the gene expression levels among different ovarian cancer stages, grades, and clinical responses with Mann-Whitney tests.

2.5. Click on the **Survival** tab.

NOTE: This tab provides Kaplan-Meier plots associated with **Overall Survival** and **Disease-Free Survival**. For this database, disease-free survival is considered progression- and recurrence-free survival[18]. Multivariate analyses for **Overall Survival** and **Disease-Free Survival** are also found under this tab. The multivariate analyses compare features that relate to ovarian cancer prognoses (stage, grade, surgical debulking, histology, age) and the gene of interest.

2.6. Click on the **Subtype** tab.

NOTE: This tab provides summary statistics and Mann-Whitney tests for the expression level of the gene of interest in molecular subtypes of ovarian cancer. This tab also provides Kaplan-Meier plots associated with **Overall Survival** and **Disease-Free Survival** of the gene of interest in molecular subtypes of ovarian cancer.

3. Gene Expression across Normal and Tumor tissue (GENT)

NOTE: The GENT portal (medical-genome.kribb.re.kr/GENT) is developed and maintained by the Korea Research Institute of Bioscience and Biotechnology (KRIBB)[19]. It collects 16,400 (U133A; 241 datasets) and 24,300 (U133plus2; 306 datasets) publicly available samples. After standardization, GENT offers gene expression data across diverse tissues, which are further divided into tumor and normal tissues.

3.1. Click on the **Search** tab at the top of the screen.

3.2. In the section labeled **1. Keyword**, select the **Gene symbol** for the **Terms** from the dropdown menu, input the gene symbol of the gene of interest in the blank area of the **Keyword** section, and select **Tissue** for the **Type** option.

3.3. Click the **Search** button at the bottom of the **1. Keyword** section. It shows the summary graphs of gene expression in normal and tumor tissues of different cancer types based on the U133A and U122Plus2 platforms.

NOTE: It is optional to select the **Data Filtering** option on the top of the summary graph to single out a particular database to study.

3.4. Click the link next to **Result Data Download** to access the detailed information about the gene expression values, tissue types, and data sources.

4. Broad Institute Cancer Cell Line Encyclopedia (CCLE)

NOTE: CCLE (portals.broadinstitute.org/ccle) was created by the Broad Institute and provides genomic profiles and mutations of 947 human cancer cell lines[20].

4.1. Input the desired genes into the search bar and then click the **Search** button.

4.2. In the section labeled **Select Dataset**, click the **mRNA expression (RNAseq)** option from the dropdown menu.

NOTE: Other options include **mRNA expression (Affy)**, **Achilles shRNA knockdown**, and **Copy Number**.

4.3. Click on the **Toggle All Traces** button. Select the tissue type of interest from the gray box on the right. Scroll down to the bottom of the screen and click the **Download mRNA expression** button.

4.4. Open the downloaded text document. Copy and paste all the text into **Sheet 1**. Copy all the text in the **Sheet 1**.

4.5. Click on the sheet in the spreadsheet software **Sheet 2** tab on the bottom of the spreadsheet. Right click on the **A** column, select **Paste Special**, and then select the **Transpose** option in Sheet 2.

4.6. Once the text is transposed into two columns on **Sheet 2**, click the dropdown arrow for the **Sort & Filter** option heading and then select the **Filter** option. An arrow will appear in the heading area labeled **Gene**. Click on the arrow and type in the tissue type of interest.

NOTE: This step will filter all the data and only display gene expression levels for the tissue type of interest.

5. cBioPortal

NOTE: cBioPortal (www.cioportal.org) was developed at the Memorial Sloan Kettering Cancer Center (MSK), and accesses, analyzes, and visualizes large scale cancer genomic data[21,22]. Specifically, this portal allows researchers to search for genetic alterations and signaling networks.

5.1. Using the query on the landing page, click the organs/tissues of interest under the section labeled **Select Studies**. Select the particular study of interest.

5.2. In the section labeled **Select Genomic Profiles**, select from the three options: **Mutations**, **Putative copy-number alterations from GISTIC**, or **mRNA Expression**. Further select corresponding data from the dropdown menu for **Select Patient/Case Set**.

5.3. Enter the target gene symbol(s) in the query box of **Enter Genes**. Click the **Submit Query** button.

5.4. Click on the **Network** tab at the top of the page to retrieve the desired gene network.

NOTE: The signaling network is color-coded. The inputted genes are indicated by seed nodes with a thick border. Each gene is represented by a red circle, and the color intensity of the red circle reflects its mutation frequency. Genes are connected by differently colored lines. Brown lines mean "In Same Component", indicating the involvement in the same biological component. Blue lines mean "Reacts With", indicating gene reactions. Green lines mean "State Change", suggesting that one gene might cause a state change of another gene.

5.5. Click on the **File** tab at the top of the image to choose **Save as Image (PNG)** for network image downloading.

6. Dissection of *Drosophila* with desired genotypes and DAPI staining

NOTE: Collect the female *Drosophila* with the desired genotypes, then dissect the fly ovaries to undergo the procedures of DAPI staining for imaging.

6.1. Prepare fly stocks *tj-Gal4, Gal80ts/CyO; UAS-NICD-GFP/TM6B*, *w*; UAS-mam.A;* and *w[1118]* to create flies with *NICD*-overexpression (*tj-Gal4, Gal80ts/+; UAS-NICD-GFP/+*) and *NICD* and *mam*-overexpression (*tj-Gal4, Gal80ts/UAS-mam.A; UAS-NICD-GFP/+*) capability.

6.2. Apply the temporal and regional gene expression targeting (TARGET) technique to control spatiotemporal gene expression[23]. Raise flies at 18 °C until adulthood, then shift to 29 °C for 48 h with yeast before dissection.

NOTE: *tj-Gal4* can only drive *UAS* expression under higher temperatures, when the inhibition by *Gal80ts* is relieved. The addition of yeast prior to dissection enlarges the ovaries for harvesting.

6.3. Place 3 mL of 1x phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) in an embryo collection dish. Use a CO_2 pad to anesthetize the flies.

6.4. Choose a female fly, then carefully grab the lower thorax of the fly using a pair of dissecting forceps and submerge it into the 1x PBS solution in an embryo collection dish. Use a second pair of forceps to pinch the lower abdomen and pull gently to release the internal organs.

6.5. Identify and detach the pair of ovaries from the fly body. Break the muscular sheath located at the posterior end of the ovaries and separate the ovarioles.

NOTE: Separating the ovarioles and breaking the muscular sheath is required in order to achieve higher quality staining results.

6.6. Place the ovaries in a 1.5 mL centrifuge tube that contains 500 µL of 1x PBS. The tube should remain on ice until all ovaries are collected.

6.7. Remove the 1x PBS and place 0.5 mL of fix solution (4% formaldehyde) into the tube. Place the tube on the nutator for 10 min.

6.8. Remove the fix solution from the tube and dispose of it in a suitable waste container. Use 1 mL of 1x PBT (1x PBS supplemented with 0.4% Triton™ X-100) to wash the ovaries 3x for 15 min.

6.9. Discard the final PBT wash and add 1 mL of PBTG (0.2% bovine serum albumin, 5% normal goat serum in 1x PBT) to prevent nonspecific binding.

NOTE: This step could be skipped for DAPI staining, but it is essential for antibody staining. Detailed immunohistochemistry staining can be found in Jia et al.[24].

6.10. Place 150 µL of DAPI (10 µg/mL) in the tube for 10–15 minutes nutation. Discard the DAPI and wash the ovaries 1x for 10 min using 1 mL of 1x PBT. Remove the PBT and wash 2x for 10 min using 1x PBS.

6.11. Remove excess PBS until approximately 300 µL of PBS remains in the tube with the ovaries. Pipet the ovaries up and down several times using a 200 µL pipette, in order to free the egg chambers.

6.12. Gently spin down the tube and carefully remove as much 1x PBS solution as possible without removing the ovaries. Place 120 µL of mounting solution into the tube.

NOTE: Mounting solution is sticky, so it is difficult to transfer exactly 120 µL of mounting solution into a tube. To alleviate this issue, a 1,000 µL pipette tip can be used to add three drops of mounting solution into the tube.

6.13. Remove approximately 0.33 mm from a 200 µL pipette tip and use the newly cut pipette tip to place the mounting solution on a microscope glass slide.

6.14. Gently place the coverslip glass on the mounting solution and seal the edges of the cover slip with transparent nail polish.

NOTE: Sealing the edges of the cover glass is needed to prevent the egg chambers from flowing inside of the mounting solution when taking confocal images.

6.15. Acquire images with a confocal microscope using the following settings: objective lens = 10x magnification; numerical aperture = 0.8; DAPI emission wavelength = 410−513 nm.

REPRESENTATIVE RESULTS:

Using the procedure mentioned in step 1 using the PRECOG portal, the Z-scores of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer were obtained (1.3, 2.32, 1.62, respectively). The negative Z-score values indicate the poor overall survival of patients with high expression levels of the three genes. Using **Conditional Formatting** of the spreadsheet software, the Z-score values are shown in a colored bar graph in **Figure 1**.

The CSIOVDB database was used to confirm the findings. Using the instructions in step 2, *NOTCH2*, *NOTCH3*, and *MAML1* were sequentially inputted in the CSIOVDB database search area, and the patient survival data located under the **Survival** tab was retrieved. In addition to the **Overall Survival** data, CSIOVDB provides **Disease-Free Survival**. CSIOVDB further separates patients to present the survival data based on Q1 vs. Q4 (lower quartile vs. upper quartile) of gene expression levels. Consistent with previous findings, high expression of *NOTCH2*, *NOTCH3*, and *MAML1* correlate with poor overall survival and disease-free survival (**Figure 2A**, **2B**). Meanwhile, the **Clinico-pathological Parameters** tab of CSIOVDB also provides a comparison of the gene expression levels among different ovarian cancer stages, grades, and clinical responses with Mann-Whitney tests. The results show that higher expression levels of *NOTCH2*, *NOTCH3*, and *MAML1* are associated with advanced ovarian cancer stages (**Figure 2C**).

Because *NOTCH2*, *NOTCH3*, and *MAML1* are critical for overall patient survival, the gene expression levels in ovarian tumors and cancer cell lines were investigated further. The expression data of *NOTCH2*, *NOTCH3*, and *MAML1* in normal and tumor ovarian tissues were downloaded from the U133A platform using the step 3 instructions for GENT. Scientists can process the downloaded data according to their own specific research purpose. Here, we utilized the data to produce the box and whisker plots using GraphPad Prism (version 8). Further permutation tests suggested that *NOTCH2*, *NOTCH3*, and *MAML1* are highly expressed in tumor tissues (**Figure 3A**). Next, the expression data of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer cell lines were downloaded according to protocol step 4, using CCLE. Gene expression levels in cancer cell lines are shown by the box and whisker plots (**Figure 3B**). Even though expression levels of *NOTCH2*, *NOTCH3*, and *MAML1* are high in cancer cell lines, conclusions cannot be drawn due to the lack of normal cell line controls in the CCLE database. However, scientists can identify the origin of cancer cell lines, and compare the expression levels based on different grades, stages, and other clinicopathological parameters.

Once the significance of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer were confirmed, the cBioPortal was utilized to study their associated signal network. Using protocol step 5, **Ovary/Fallopian Tube** was selected for **Select Studies**, then the **Ovarian Serous Cystadenocarcinoma (TCGA, Nature 2011)** dataset was chosen for analysis. For the section labeled **Select Genomic Profiles**, the **mRNA Expression** was selected, and finally its profile **mRNA expression Z-scores (all genes)**. For the section **Select Patient/Case Set**, the **Samples with mRNA data (Agilent microarray) (489)** option was chosen from the dropdown menu. At the end, genes

NOTCH2, *NOTCH3*, and *MAML1* were selected to submit the query. Based on the three core genes, a signaling network was created to provide the 50 most frequently altered neighboring genes, which are also in the same pathway with the highest mutation rates (**Figure 4**).

Because Notch signaling is well conserved across species, it was investigated in *Drosophila* ovarian cancer. Notch signaling has been previously reported to regulate follicle cell proliferation[25], differentiation[26,27], and cell cycle regulation[28,29]. Overexpression of NICD alone did not induce tumors in *Drosophila* (**Figure 5A**), as the epithelium of the *Drosophila* egg chambers remained intact with one single layer. However, overexpression of NICD and Mam together induced tumors in *Drosophila* (**Figure 5B**), which is demonstrated by multiple epithelial layers and accumulated cells.

FIGURE LEGENDS:

Figure 1: Expression of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer is associated with poor overall survival. The survival Z-scores of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer patients are presented. Poor survival is indicated by negative Z-score values.

Figure 2: High levels of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer are associated with poor overall survival, poor disease-free survival, and advanced cancer stages. The microarray database CSIOVDB provides Kaplan-Meier overall survival and disease-free survival plots of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer patients, and gene expression levels in different cancer stages.

Figure 3: *NOTCH2*, *NOTCH3*, and *MAML1* are highly expressed in ovarian tumors and cancer cell lines. P values are indicated to compare gene expression in normal ovaries and corresponding ovarian tumors. (Abbreviations: Ovary-N = normal ovary tissues; Ovary-C = ovarian cancer tissues).

Figure 4: *NOTCH2/NOTCH3/ MAML1* genes and their associated signaling network with the 50 most frequently altered neighboring genes. The signaling network is color-coded. The inputted genes are indicated by seed nodes with a thick border. Each gene is represented by a red circle, and the color intensity of the red circle reflects its mutation frequency. Genes are connected by differently colored lines. Brown lines mean “In Same Component”, indicating the involvement in the same biological component. Blue lines mean “Reacts With”, indicating gene reactions. Green lines mean “State Change”, suggesting that one gene might cause a state change of another gene.

Figure 5: *NICD* and *mam* in *Drosophila* also induce ovarian tumors. A. Overexpression of *NICD* alone does not induce tumor formation in *Drosophila*. B. Overexpression of *NICD* and *mam* together induce tumors in *Drosophila*. Scale bar = 50 μm

DISCUSSION:

As there are countless approaches and methods for the utilization of bioinformatics, there are numerous databases available online to the general public. An abundance of information can be extracted from each of these databases, but some are best suited for particular purposes, such

as assessing patient survival based on certain inputs. Systematic analyses of retrieved data from different individual databases can convincingly yield important scientific findings.

The current analysis focuses on the role of Notch signaling in ovarian cancer through the utilization of bioinformatics approaches. For instance, the Meta-Z analysis on the PRECOG portal database was used to obtain Z-scores that indicate patient survival outcomes in clinical cancer studies. CSIOVDB is another meta-analysis database that was used to study survival outcomes of ovarian cancer patients. The CSIOVDB data successfully validated the findings from the PRECOG portal that *NOTCH2*, *NOTCH3*, and *MAML1* are critical for overall patient survival. Later, the applications of the GENT and CCLE databases further demonstrated that *NOTCH2*, *NOTCH3*, and *MAML1* are highly expressed in ovarian tumors and cancer cell lines. The combination of these databases systematically revealed the significant roles of *NOTCH2*, *NOTCH3*, and *MAML1* in ovarian cancer. This use of bioinformatics methods provided an efficient way to do cancer research cost-effectively and shows how it can yield important findings for future experimental and clinical applications.

Bioinformatics provides the public the capability to access results from thousands of experiments all at once. The information derived from public databases provides a cost-effective and efficient way to establish an experimental design prior to performing experiments. In addition, it is important to note that publicly available data can be scattered across publications and may deliver inconsistent or even contradictory findings, which requires meta-analyses to be performed through bioinformatics approaches. Scientists can design and perform experiments based on the data found through large bioinformatics databases to validate specific scientific hypotheses. Results from the *Drosophila* experiment confirmed the findings from the bioinformatics databases and further supported the idea that Notch pathway components should continue to be investigated as potential therapeutic drug targets. The successful validation of bioinformatics findings through experimentation also suggests the importance of bioinformatics approaches for scientific discoveries.

There may be some limitations of bioinformatics. First, some websites/tools might not update their findings due to time efforts or costs associated with maintenance. Second, some websites/tools do constantly update, but the update with additional input might alter previously obtained results. Third, developers of some websites/tools reserve copyrights and restrict the use of their contents. Fourth, analyses or algorithms of certain websites/tools might not always be accurate.

To overcome these limitations, some steps or modifications and troubleshooting for better future applications are suggested. First, some websites/tools do allow researchers to manually load new data for analysis. If not, researchers can download and analyze the most recent data on their own. Second, researchers need to repeatedly run their analyses, and keep record of the dates. If results significantly change, researchers might need to use the additional input of data to figure out the reasons. Third, researchers can find an alternative website/tool to run their analyses to avoid potential copyright issues. Fourth, researchers can get additional websites/tools to validate their important findings. If there are any problems with analyses or algorithms, researchers can

download and re-analyze the data to correct the mistakes or use other websites/tools with the appropriate settings.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

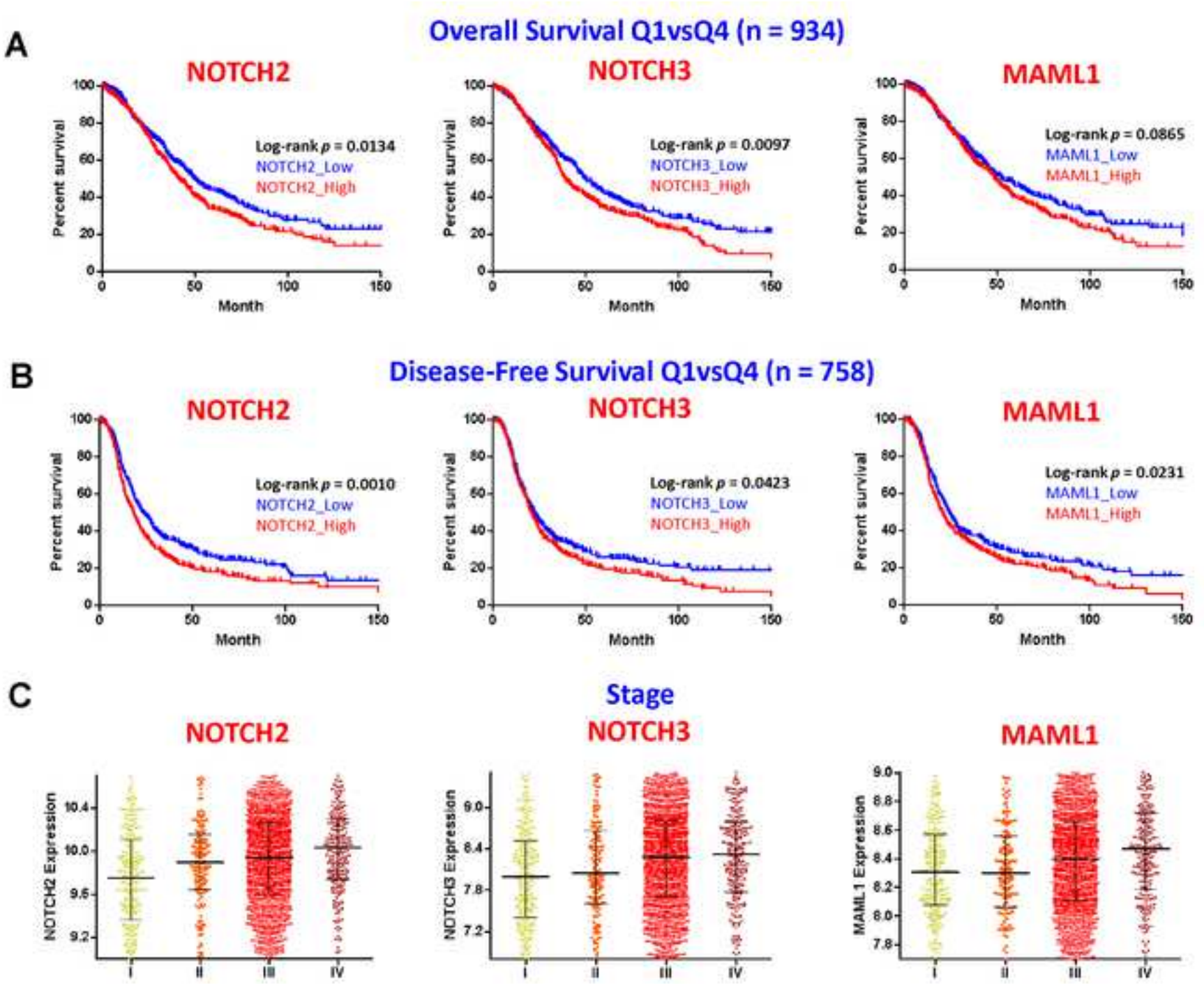

Figure 3

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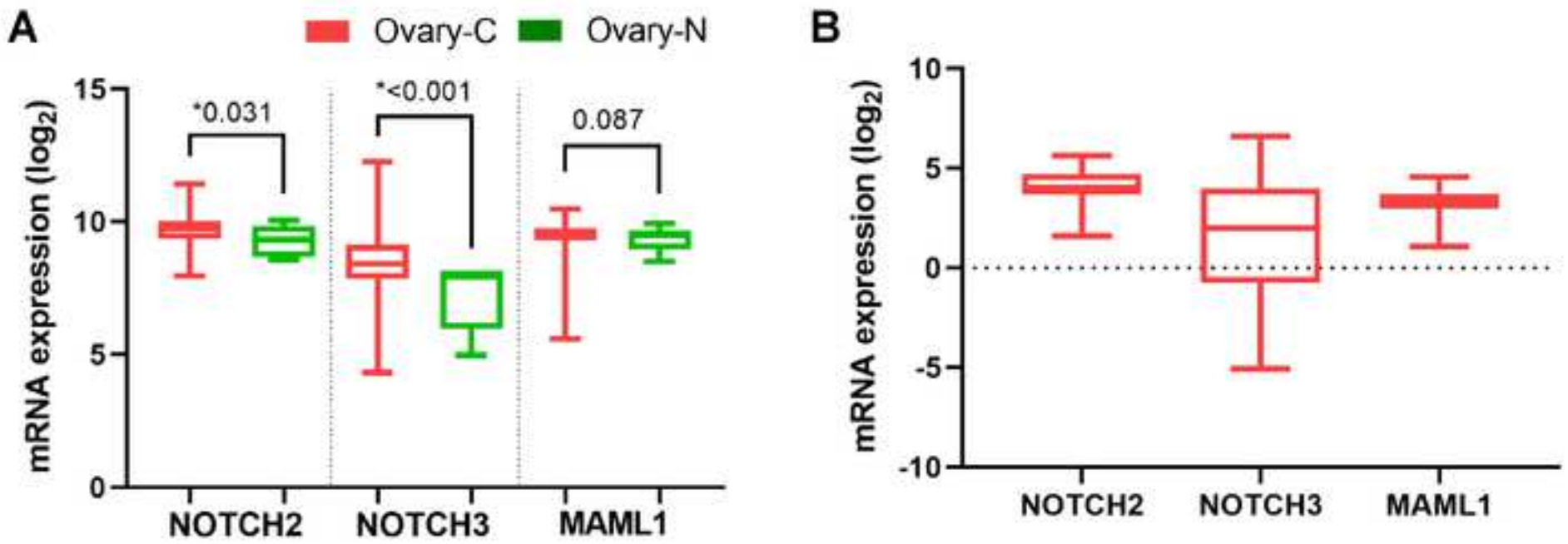


Figure 4

Click here to access/download;Figure;Figure 4.psd

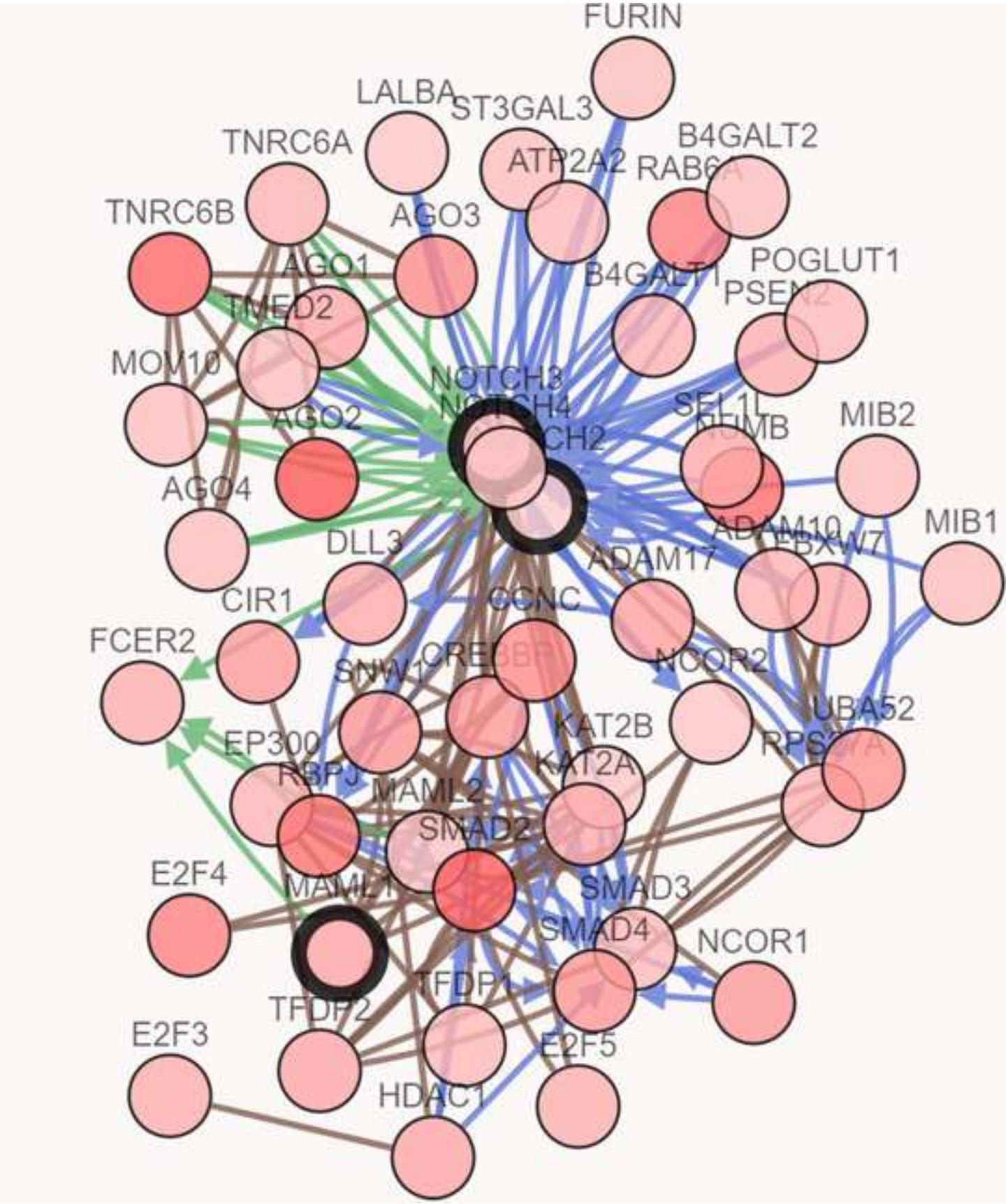
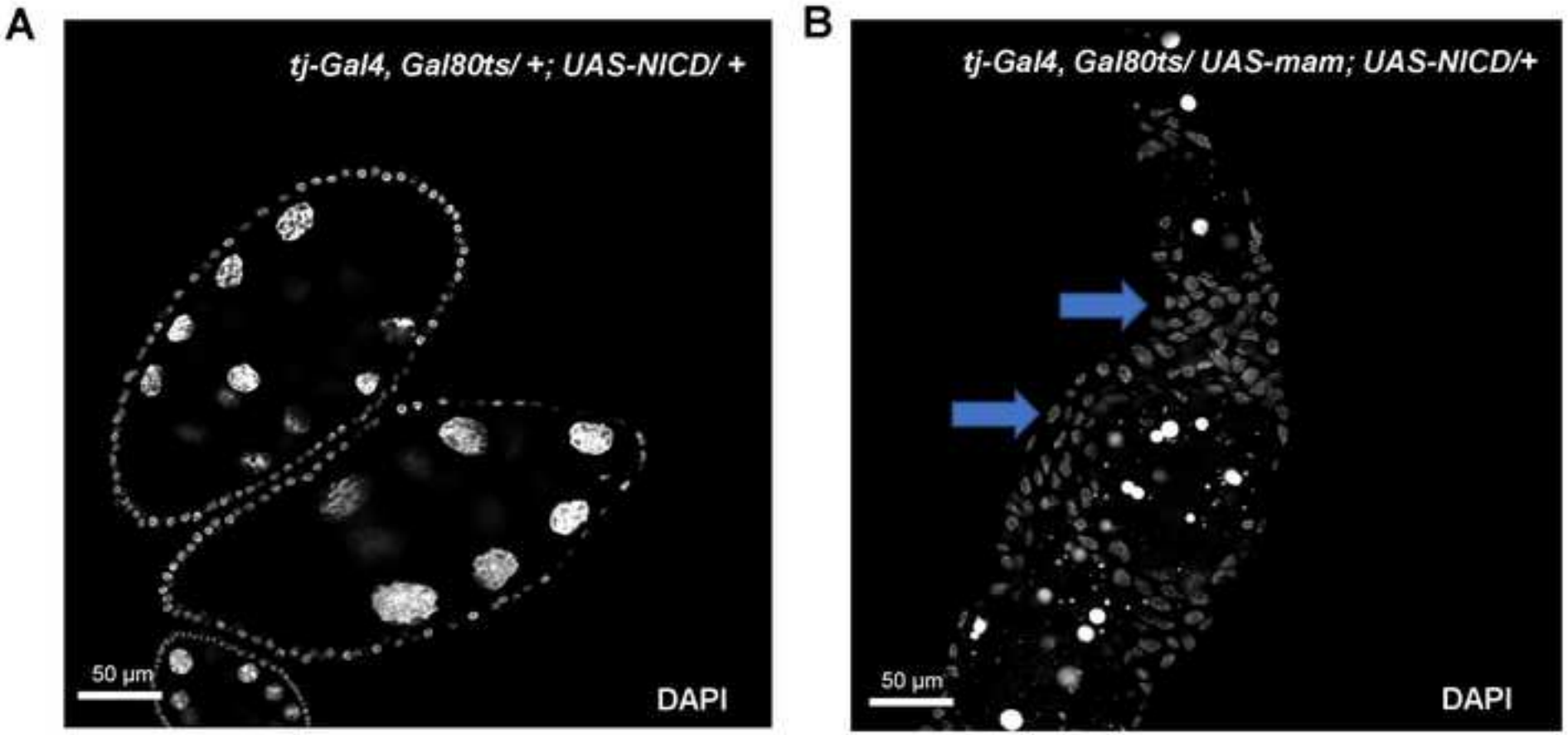


Figure 5



Name of Material/ Equipment

DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)

PBS, Phosphate Buffered Saline, 10X Powder, pH 7.4

Goat serum

Embryo dish

Nutating mixers

tj-Gal4, Gal80ts/ CyO; UAS-NICD-GFP/ TM6B

w*; UAS-mam.A

w[1118]

The PRECOG portal

CSIOVDB

The Gene Expression across Normal and Tumor tissue (GENT) Por

Broad Institute Cancer Cell Line Encyclopedia (CCLE)

cBioPortal

Zeiss 710 Inverted confocal microscope

Company

Invitrogen

ThermoFisher

Gibco

Electron Microscopy Sciences

Fisherbrand

Dr. Wu-Min Deng at Florida State University

Bloomington Drosophila Stock Center

Bloomington Drosophila Stock Center

Stanford University

Cancer Science Institute of Singapore

Korea Research Institute of Bioscience and Biotechnology (KRIBB)

Broad Institute and The Novartis Institutes for BioMedical Research

Memorial Sloan Kettering Cancer Center (MSK)

Carl Zeiss

Catalog Number

D1306

FLBP6651

16210064

70543-45

88861041

N/A

#27743

#5905

https://precog.stanford.edu

csibio.nus.edu.sg/CSIOVDB/CSIOVDB.html

medical-genome.kribb.re.kr/GENT

portals.broadinstitute.org/ccle

www.cioportal.org

ID #M 210491

Comments/Description

1:1000 Dilution

Dissolved with ddH_2O to make 1X PBS

Serum

Dissection Dish

Nutator

Fly stock

Fly stock

Fly stock

Publicly accessible database of cancer expression datasets

Microarray database used to study ovarian cancer

Publicly accessible database of gene expression data across diverse tissues, divided into tumor and noı

Provides genomic profiles and mutations of human cancer cell lines

Portal that allows researchers to search for genetic alterations and signaling networks

Examination and image collection of fluorescently labeled specimens

rmal tissues.

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Title of Article:	Integration of bioinformatics approaches and experimental validations to understand the role of Notch signaling in ovarian cancer
Author(s):	Shawna Defreitas, Molly Rowe, Lily Paculis, Dongyu Jia

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Institution:	Georgia Southern University
Title:	Assistant Professor
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Dear Editor,

Thanks a lot for your careful editorial and peer review.

Based on your nice comments and suggestions, we made edits to our manuscript accordingly. All the changes have been highlighted. We are confident that our manuscript is suitable for publication in terms of quality of science and ethics.

Thank you again for your time and help. If you need further materials, don't hesitate to inform us.

Best regards,

Dr. Dongyu Jia

RESPONSE TO COMMENTS OF EDITORS

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

During the process of revision, we thoroughly proofread the manuscript.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 6.23: Mention magnification, lens NA and other relevant settings.

In the revised manuscript, we provide more specific details as suggested.

• Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.

In the revised manuscript, we change the protocol numbering accordingly.

• Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and

maximum of 4 sentences per step.
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
5) Notes cannot be filmed and should be excluded from highlighting.

In the revised manuscript, we highlight the protocol steps accordingly.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Thanks a lot for the nice suggestions. In the revised manuscript, we are more focused on the methods and protocol, and provide additional paragraphs to cover discussion part in detail.

• Figures: Please add scale bars to fig 5.

Scale bars are added to the revised Figure 5.

• Figure/Table Legends: Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

In the revised manuscript, we expand the legends to adequately describe the figures/tables.

• References: Please spell out journal names.

For the references, we spell out journal names in the revised manuscript.

• Table of Materials:Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as software used, microscope, etc.

In the revised manuscript, we provide more detailed and complete list in the Table of Materials, including software and microscope.

• Please define all abbreviations at first use.

We define all abbreviations at first use in the revised manuscript.

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RESPONSE TO COMMENTS OF REVIEWERS

It's our great honor that both reviewers think highly of our manuscript and agree to publish in the current form.

Reviewers' comments:

Reviewer #1:
In the present protocol, the authors demonstrate a reliable method to identify those potential signaling pathways that might be involved in the progression of ovarian cancer. By integrating a variety of online bioinformatics tools, such as PRECOG, CCLE, cBioPortal, etc., the activation of NOTCH signaling in ovarian cancer was identified. I searched JOVE and could not find a similar protocol describing such an integrated bioinformatics approach. **In short, this integrated bioinformatics analysis is very useful for other researchers and can be considered for publication in JOVE**.

Reviewer #2:
Manuscript Summary:
Bioinformatics is an efficient way to do biomedical research using large-scale datasets. In this study, the authors demonstrated utilization of bioinformatics in ovarian cancer research. The authors systematically analyzed the roles of main components of Notch signaling in ovarian cancer using multiple bioinformatics methods. Also, the authors validated bioinformatics findings through experimentation. **The present study provided efficient and money-saving bioinformatics methods to do cancer research. The research results also provided clinically relevant information for studies regarding Notch signaling in ovarian cancer development and shed light on drug development targeting Notch signaling against ovarian cancer.**