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Jagged1-Fluc: A Luminescent Recombinant Protein as a Sensitive Tool for High-Throughput Screening of Colorectal Cancer

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Jagged1-Fluc: a luminescent recombinant protein as a sensitive tool for high-throughput screening of colorectal cancer

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Abstract

Colorectal cancer (CRC) onset is closely linked to dysregulated Notch signaling, particularly involving receptors like Notch1 and Notch3, and ligands such as Jagged1 (Jag1), which are overexpressed in aggressive subtypes. To address the need for quantitative assessment of Notch expression in clinical settings, we developed a luminescent recombinant probe (Jag1-Fluc) by fusing the extracellular domain of a high-affinity Jag1 mutant with red-emitting firefly

luciferase. The Jag1-Fluc probe can bind to Notch, thus correlating the intensity of emitted light with the concentration of Notch in the sample.

In cell-free assays, Jag1-Fluc demonstrated a linear luminescent signal across different concentrations (detection limit: 0.20 ± 0.03 μg/mL). When applied to living and fixed human colorectal cancer cells, the probe showed a dose-dependent light emission (5–50 μg/mL), confirming its ability to bind endogenous Notch receptors. Competitive binding assays using soluble human Jag1 chimera demonstrated concentration-dependent signal inhibition (IC_{50} = 0.55 ± 0.06 μg/mL), validating probe specificity. Notably, in a small cohort of human biopsies, Jag1-Fluc enabled stratification of Notch expression, with luminescence intensity progressively increasing from hyperplastic polyps to low- and high-grade adenomas. These findings reinforce prior evidence linking Jag1 overexpression to CRC progression. The assay's simplicity and sensitivity highlight its potential for early CRC screening, paving the way for personalized medicine by enabling tailored therapeutic monitoring and strategies based on Notch signaling profiles. This approach potentially enhances treatment efficacy and reduces side effects, representing a valuable tool for precision oncology.

Keywords: colorectal cancer, Notch pathway, Jagged1, red firefly luciferase, recombinant protein, luminescence, imaging.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related mortality in the Western population [1]. According to data registered by the

Abbreviations: APC, adenomatous polyposis coli; ATCC, American Type Culture Collection; AUC, area under the curve; BL, bioluminescent/bioluminescence; BLI, bioluminescence imaging; BRAF, B-Raf proto-oncogene serine/threonine kinase; BSA, bovine serum albumin; Caco-2, human colon carcinoma cell line; CMS4, consensus molecular subtype 4; CRC, colorectal cancer; DFS, disease-free survival; ECD, extracellular domain; FAP, familial adenomatous polyposis; FBS, fetal bovine serum; Fluc, firefly luciferase; HaCaT, human adult low calcium temperature (skin keratinocyte cell line); HBSS, Hanks' balanced salt solution; HUVEC, human umbilical vein endothelial cells; IARC, International Agency for Research on Cancer; ICN, intracellular Notch domain; IPTG, isopropyl β-D-1-thiogalactopyranoside; Jag1,

International Agency for Research on Cancer (IARC), almost 1.9 million cases were diagnosed just in 2022, with approximately 1 million deaths annually [2]. The gradual progression of CRC offers the possibility of diagnosing the disease early before the malignant transformation occurs, so a deeper mechanistic understanding is highly needed to address new targeted therapeutic approaches. Indeed, CRC development is a multi-step process involving several morphological, histological, genetic, and epigenetic alterations that accumulate over time [3]. From 90 to 95% of CRCs are adenocarcinomas, starting with benign adenomatous polyps, which can evolve into adenomas and eventually CRC over 10-15 years [4]. However, only a small fraction of polyps acquires malignant features. Risk factors for CRC include genetic predisposition (12–35% of cases), such as familial or personal histories of colorectal polyps or CRC, and syndromes like Lynch syndrome, familial adenomatous polyposis (FAP), Peutz-Jeghers, and Cowden syndromes [5]. Environmental factors, such as gut microbiota dysbiosis [6] and bile acid pool alterations [7], are also demonstrated to contribute to CRC development.

Progression from intestinal adenoma to advanced CRC in mice and humans correlates with the accumulation of specific mutations and dysregulated signaling in additional pathways.

Key mutations, particularly in the adenomatous polyposis coli (APC) gene, Kirsten rat sarcoma virus (KRAS) gene, B-Raf proto-oncogene serine/threonine kinase (BRAF), mutL homolog 1 (MLH1), and other mismatch repair genes, drive tumorigenesis by disrupting the regulation of critical signaling pathways. These include Wnt/β-catenin, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), transforming growth factor-beta (TGF-β), and tumor protein p53 (P53) [8–11]. Recent studies suggest a pivotal role of altered Notch signaling activation in CRC, partially due to cross-talks of Notch with other pathways.

Jagged1; KRAS, Kirsten rat sarcoma virus; LFNG, lunatic fringe; LOD, limit of detection; LOQ, limit of quantification; MAPK, mitogen-activated protein kinase; MCF-7, Michigan Cancer Foundation-7 (human breast cancer cell line); MFNG, manic fringe; MLH1, MutL homolog 1; OS, overall survival; o/n, overnight; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PI3K, phosphatidylinositol 3-kinase; RBPJ, recombination signal binding protein for immunoglobulin kappa J region; RPMI, Roswell Park Memorial Institute; T-ALL, acute lymphoblastic leukemia; TGF-β, transforming growth factor-beta.

Indeed, Notch and its downstream effectors stand out as particularly compelling targets for further investigation, as this pathway plays a crucial role in tumor progression, stemness, and therapy resistance. Despite its recognized relevance, the Notch signaling network remains an underexplored yet promising target, warranting deeper investigation to uncover potential therapeutic opportunities [12–15]. Indeed, aberrant Notch activity is a hallmark of a high percentage of colorectal tumors, particularly involving receptors like Notch1 and -3, and ligands such as Jagged1 (Jag1), which are overexpressed in aggressive subtypes like consensus molecular subtype 4 (CMS4) and serrated lesions [16, 17]. Notch signaling is a crucial pathway that controls various cellular processes, including stem cell self-renewal, cell fate specification, and differentiation [18]. The pathway consists of five ligands (Jag1,2 – Dll1,3,4) and four receptors (Notch1-4) and is activated by ligand-receptor interactions between adjacent cells, with the extracellular domain (ECD) of the receptor mediating ligand binding [19]. These interactions trigger sequential proteolytic cleavages catalyzed by ADAM10/17 (a disintegrin and metalloprotease) and the γ-secretase complex. The intracellular Notch domain (ICN), once liberated, translocates to the nucleus, where it interacts with recombination signal binding protein for Immunoglobulin kappa J region (RBPJ), to activate the transcription of target genes [20].

Receptor–ligand interactions are regulated by receptor glycosylation, with the Fringe family of N-acetylglucosaminyltransferases, primarily Lunatic Fringe (LFNG) and Manic Fringe (MFNG), enhancing Delta-induced signals and decreasing responsiveness to Jag ligands [21]. The Espinosa team demonstrated that high Notch activity in intestinal tumors expressing Jag1 but lacking MFNG is predictive of poor survival rates in CRC patients [22]. In line with this observation, many reports have highlighted that Jag1 is frequently overexpressed in human CRC tissues compared to normal tissues, which correlates with cancer progression and poor prognosis [23–25].

It is widely known that the dysregulation of Notch signaling results in developmental deficits in humans, such as Alagille Syndrome, and is involved in different types of neoplasia [26],

among which are acute lymphoblastic leukemia (T-ALL) [27], breast cancer, lung adenocarcinoma, and ovarian cancer [28, 29].

In addition to Notch1, deregulated Notch3 signaling has also been suggested to play a key role in T-ALL, due to the oncogenic potential of the Notch3 ICD [30]. Indeed, the role of Notch3 has garnered attention due to its association with poor disease-free survival (DFS) and overall survival (OS) in CRC patients [31]. Clinical and experimental evidence indicate that Jag1 amplifies Notch signaling, promoting a Notch3/Jag1 feedback loop that supports cancer cell survival, proliferation, and invasion in various tumors, such as T-ALL, ovarian, and breast cancers [32]. Jag1 ligand is not strictly required for intestinal homeostasis, exerting pro-oncogenic function in the gut in Mfng-low tumors, thus opening the possibility of targeting Jag1 in human CRC patients with less or no intestinal side effects [21, 33].

To investigate the interaction between Jag1 and Notch receptors in precancerous stages, we developed a recombinant protein combining the ECD of a high-affinity mutated form of Jag1 [34] with red-emitting firefly luciferase (Fluc). This construct was designed to assess if Jag1-Fluc binds to Notch receptors and whether its light emission upon binding correlates with increasing severity of precancerous colonic lesions.

Common analytical methods used to detect Notch receptors and ligands in polyps, gut adenomas, and CRCs are mRNA expression analysis by quantitative reverse transcription PCR (qRT-PCR), *in situ* hybridization, western blot, immunohistochemistry (IHC), and transcriptomic approaches (RNA-Seq, microarrays). Almost all these approaches are time and cost-consuming, while western blot and IHC, even if used as routine analysis in clinical practice, don't allow reliable quantification of biomarkers mainly because they are semi-quantitative techniques with several limitations (*i.e.,* subjective interpretation and scoring, non-linear signal and saturation, lack of absolute quantification standards). To overcome these issues, we developed a fast and cost-effective quantitative approach using light emission as the analytical strategy, focusing on the binding of Jag1 with Notch receptors.

2. Experimental Procedures

2.1. Reagents

Phosphate-buffered saline (PBS) tabs (giving a 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH 7.4), LB broth powder (Lennox), agar powder, dimethylsulfoxide (DMSO), sodium and potassium chloride, tris base, acetic acid, EDTA, trypsin-EDTA, antibiotic solution 100X (10,000 U/mL penicillin and 10 mg/mL streptomycin), methanol, protease inhibitor cocktail, kanamycin sulfate, ampicillin (sodium salt), isopropyl β-D-1- thiogalactopyranoside (IPTG), β-mercaptoethanol, Tween 20, bovine serum albumin (BSA), H_2O_2 solution, horse serum, sodium-deoxycholate and Triton X-100 were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) high glucose, Roswell Park Memorial Institute (RPMI) 1640 medium, Hanks' balanced Salt solution 10X (HBSS), and fetal bovine serum (FBS) were purchased from Microgem (Naples, Italy). JAG1 ORF Vector (human) was purchased from ABM (Richmond, BC, Canada), while the pIDTSmart (Amp) for JAG1_Mut_High_Affinity (320 bp) was from IDT (Coralville, Iowa, US). pCMVRed FLuc, Pierce Firefly Luc One-Step glow assay kit as substrate, Bac-to-Bac™ HBM TOPO™ Secreted Expression System, MAX Efficiency™ DH10Bac Competent Cells, ExpiFectamine™ Sf Transfection Reagent, One Shot™ TOP10 Chemically Competent E. coli, Phusion Site-Directed Mutagenesis Kit, Pure Link™ HiPure Plasmid Miniprep kit, Ni-NTA Purification System, BacPAK™ qPCR Titration Kit, Zero Blunt® TOPO® PCR Cloning Kit, restriction enzymes, Phusion U Hot Start PCR Master Mix, Pierce BCA Protein Assay, Hoechst 33342 Solution, Hank's Balanced Salt Solution (HBSS), paraformaldehyde (PFA), and western blotting detection reagents were purchased from Thermo Fischer Scientific (Waltham, MA, USA). PCR extraction kit and gel Purification Kit were obtained from Qiagen (Hilden, Germany). M200 and LSGS kit were purchased from Life Technologies (Waltham, MA, USA). Laemmli sample buffer, Protein Markers, and Mini-Protean TGX (4–20%) gels were obtained from Bio-Rad (Hercules, CA, USA). Mouse-anti-His mAb was purchased from GenScript (Piscataway, NJ, US). Antibodies used were rabbit monoclonal to Notch3 (#5276)

from Cell Signalling (Danvers, MA, US), rabbit polyclonal to β-tubulin (bs-4511R) from BIOSS (Woburn, MA, US), and Goat anti-rabbit IgG(H+L) HRP conjugate (31466) from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant Human Jag1 Fc Chimera Protein was purchased by Biotechne (Minneapolis, MN, USA). Primers for RT-PCR were purchased from IDT (Coralville, IA, USA) and Sigma-Aldrich. All other chemicals and solvents were of the highest analytical grade.

2.2 Fusion gene design, transformation, and protein expression

The coding region for the ECD of the JAG1_WT gene was cloned into pTOPO. Next, a fragment containing S32L, R68G, D72N, and T87R mutations (Figure S1), which confer a higher binding strength for Notch receptors [34], was inserted in the end terminal part of JAG1_WT, obtaining the resulting plasmid pTopo_JAG1_Mut, according to the manufacturer's instructions (Thermo Fisher).

Then, JAG1mut and R-Firefly luciferase (Fluc) coding genes were combined in a single fusion construct, which was inserted into the pFast-Bac expression vector to generate pFast-Bac JAG1mut_Fluc (Figure S2). Following the insertion of pFast-Bac JAG1mut_Fluc into a Bacmid, the resulting construct was transfected into Sf-9 insect cells. Then, the High Five insect cells were infected with the recombinant Baculovirus to produce the JAG1mut_Fluc soluble protein (according to the manufacturer's instructions, Bac-to-Bac® TOPO® Cloning Kits, Thermo Fisher Scientific, Waltham, MA, USA). Then, JAG1mut-Fluc protein was isolated from cell lysates using immobilized metal affinity chromatography (Ni-NTA). Analysis by SDS-PAGE and western blot indicated that isolated JAG1mut_Fluc exhibited an approximate size of 150 kDa, following the predicted size (Figures S3 and S4).

2.3 Insect cell culture

Commercially available serum-free insect cell lines *Spodoptera frugiperda* Sf-9 and High Five™ (BTI-TN-5BI-4 from *Trichoplusia ni*), and Sf900-II medium were acquired from Thermo Fisher Scientific (Waltham, MA, US) and maintained in Sf900-II with continuous shaking at 120 rpm at 27 °C. For expression culture, High Five™ cells were grown in Grace's Insect Cell Culture Medium without supplements (Thermo Fisher Scientific, Waltham, MA, US) and incubated at 27 °C before infection.

2.4 Human cell culture

Human colorectal adenocarcinoma cells (Caco-2) and human adult low calcium temperature keratinocytes cell line (HaCaT) were obtained from the American Type Culture Collection (ATCC). Both cell lines were grown in DMEM high glucose, supplemented with 10% heat-inactivated FBS, 2.5 mM L-glutamine, and penicillin/streptomycin. Michigan Cancer Foundation-7 (MCF-7) cells, a breast cancer cell line, were kindly gifted by Prof. Natalia Calonghi (Department of Pharmacy and Biotechnology-FABIT, University of Bologna). Cells were maintained in RPMI 1640 containing 10% heat-inactivated FBS and 2.5 mM penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs), purchased from Life Technologies (Waltham, MA, US), were plated on gelatin-coated tissue culture dishes and maintained in phenol red-free basal medium M200 (Thermo Fisher Scientific, Waltham, MA, US) containing 10% heat-inactivated FBS and growth factors (LSGS). HUVECs at passages 2 to 7 (70–90% confluency) were harvested and analyzed. All cell lines were maintained at 37°C in an atmosphere of 5% CO_2. These cell lines were selected because they had already been studied for the Notch pathway [35–38].

2.5 Calibration curve of Jag1-Fluc in a cell-free system

The -spectrum of Jag1-Fluc was obtained using Spectramax iD3 (Molecular Devices, San Jose, CA, US), setting bandpass filters ranging from 580 to 800 nm, for 1000 ms. The spectrum was collected after adding the substrate cocktail from the Pierce Firefly Luc One-

Step glow assay kit. Different dilutions of Jag1-Fluc, starting from 10 µg/mL (range 0.25-10 µg/mL), were prepared by diluting the 400 µg/mL stock solution with a working solution made of PBS, 2% FBS, and 100 µg/mL of $CaCl_2$. 100 µL of each different concentration (working solution for the blank) were dispensed in 96-well black microtiter plate wells. The reaction was triggered by adding 100 µL of substrate, and the resulting light emission was monitored for 90 min using a luminometric plate reader. The calibration curve was obtained by plotting the luminescent signal versus the different concentrations of Jag1-Fluc and fitting the experimental data to a straight line using the least squares method. The limits of detection (LOD) and quantification (LOQ) of the assay were evaluated as the concentrations of Jag1-Fluc giving luminescent signals corresponding to those of the blank plus three and ten times its standard deviation, respectively.

2.6 Cell-based calibration curve of Jag1-Fluc in living and fixed human colorectal adenocarcinoma cells (Caco-2)

The day before the experiment, Caco-2 cells (1×10^5 cells $well^{-1}$) were plated in 96-well black microtiter plate wells and kept for 24h at 37 °C, with 5% CO_2. The day after the medium was removed, cells were washed twice with PBS at room temperature and incubated with different concentrations of Jag1-Fluc (range: 5-50 µg/mL) in a solution made of HBSS and 0.1% BSA for 1h. The bioluminescent (BL) emission signal was monitored using a luminometric plate reader. The temperature was maintained at 37°C during the measurement. Then, to assess if the BL signal is detectable at lower incubation times, living Caco-2 cells (1×10^5 cells $well^{-1}$) were plated in a 96-well black microtiter plate, harvested for 24h, and then incubated with Jag1-Fluc (50 µg/mL) for 1h, 30 min, and 10 min. After two PBS washes, the kinetic profile of BL emission was monitored using the IVIS Spectrum (PerkinElmer) with the following parameters: open for total bioluminescence, exposure time = 20 min, binning = medium: 8, field of view = 12.6 × 12.6 cm, and f/stop = 1. Analysis of BLI images was performed with Living Image 4.3 software (PerkinElmer).

The same procedure was also performed in fixed cells. One day before the experiment, Caco-2 cells (1×10^5 cells well^{-1}) were seeded in a 96-well black microtiter plate. The day after the cell medium was removed, the cells were washed twice with PBS, fixed with 4% PFA, and incubated with 100 µL of a blocking solution (PBS, 5% BSA, and 100 µg/mL of $CaCl_2$) for 1h. Then, 100 µL of Jag1-Fluc (5-50 µg/mL) were added for 1h and overnight (o/n) to assess the best incubation time. The following day, after the protein removal, cells were washed twice with PBS at room temperature, and subsequently, 100 µL of the substrate solution was added. Light emission was monitored for 90 min using a luminometric plate reader. The dose-response curve was obtained by plotting the luminescent signal versus Jag1-Fluc concentrations and fitting the experimental data to a straight line using the least squares method.

2.7 Jag1-Fluc selectivity assay for Notch receptors

The day before the experiment, Caco-2 (1×10^5 cells well^{-1}) and MCF-7 (5×10^4 cells well^{-1}) cells were plated in a 96-well black microtiter plate and kept for 24h at 37 °C with 5% CO_2. The day after the medium was removed, the cells were washed twice with PBS, fixed with 4% PFA, and incubated with 100 µL of a blocking solution for 1h (PBS, 5% BSA, and 100 µg/mL of $CaCl_2$). Then, cells were treated o/n (for about 12h) with serial dilutions of human soluble Jag1 chimera (range 0.1-25 µg/mL) able to bind Notch receptors in a solution of PBS, 2% FBS, and 100 µg/mL of $CaCl_2$. On the day of the experiment, 100 µL of Jag1-Fluc (50 µg/mL) were added to each well. After 12h of incubation, the supernatant containing the two different solutions was removed from each well, and the cells were washed twice with PBS at room temperature. A luminometric plate reader monitored the light emission signal after substrate addition. The same procedure was performed in living Caco-2 cells.

2.8 *In Vitro* Cell-based luminescence Imaging

Imaging experiments were performed using an Olympus IX73 inverted microscope (Olympus Corporation, Tokyo, Japan) equipped with an ultrasensitive EM-CCD camera (ImagEM X2, Hamamatsu Photonics KK, Shizuoka, Japan) controlled by the proprietary software HCImage v.4.2.6.1 and enclosed in a dark box to prevent interference from ambient light. Caco-2 cells (1×10^5 cells $well^{-1}$) were grown in a 96-well black microtiter plate for 24h at 37 °C with 5% CO_2. The day after, cells were fixed with 4% PFA as described above and incubated overnight with three different concentrations of Jag1-Fluc (400, 200, 50 µg/mL). Negative control was performed by just adding PBS to the cells. Before each imaging session, Jag1-Fluc was gently removed, and cells were washed twice with PBS. Images of Caco-2 cells were acquired using a 10X objective with an integration time of 5, 8, and 12 min, at a gain level set to 200, after adding 100 µL of substrate cocktail to trigger the luminescent reaction. All experiments were performed in triplicate and repeated at least three times.

The same procedure was performed by adding the nuclei staining step to the protocol. Caco-2 cells were fixed and incubated overnight with Jag1-Fluc at the maximum concentration (400 µg/mL). The day after the Jag1-Fluc protein was removed, cells were washed twice with PBS and stained with bisbenzimide at 1 µg/mL (Hoechst 33342- Thermo Fisher Scientific). Images of Caco-2 cells were acquired using a 20X objective with an integration time of 5, 8, and 12 min, at a gain level set to 200, after adding 100 µL of substrate cocktail to trigger the luminescent reaction.

Image processing was performed using the freely available Java-based software ImageJ v. 1.52d.

2.9 Study population

The study, named ALMA0118, was conducted with approval from the Local Ethics Committee of the Emilia Romagna region (601/2018/Sper/AOUBo) and adhered to the Declaration of Helsinki and its later amendments. This was an exploratory, controlled, single-center human study, performed at the Sant'Orsola Malpighi Hospital in Bologna (Italy), which

identified three groups of subjects: n=4 subjects with hyperplastic polyps (mean age: 63.3±8.6, males), n= 3 with low- (mean age: 60.3±9.7, males) and n= 3 high-grade (mean age: 68.3±0.5, males) dysplasia adenomas. Enrolled subjects, who provided written informed consent, were patients undergoing routine colonoscopy and receiving standard clinical care. Specifically, the analyzed specimens included fragments from polyps removed via endoscopic polypectomy during standard procedures or a portion of the excised tissue from surgical resections.

2.10 *Ex-vivo* luminescence imaging on human specimens

Luminescence imaging was performed on formalin-fixed, paraffin-embedded samples from 10 patients in total with hyperplastic polyps (4) and low- and high-grade dysplasia adenomas (6), enrolled in a clinical study performed at the Sant'Orsola-Malpighi University Hospital in Bologna (Italy). After paraffin removal by xylene and rehydration through graded ethanol, antigen retrieval was carried out by heating sections in 95°C water for 25 min in the presence of 1X IHC select citrate buffer pH 6.0 (Sigma-Aldrich, St Louis, MO, USA). Tissue sections were then treated with a solution made of 3% H_2O_2 at 4°C for 30 min to inhibit the peroxidases and then incubated for 1h at room temperature with the blocking solution containing 3% horse serum and Triton 100X (final concentration: 0.1X in PBS). Then the samples were incubated with Jag1-Fluc 200 and 50 µg/mL in a solution of PBS, 2% FBS, and 100 µg/mL of $CaCl_2$ in a humidified chamber o/n at 4°C (negative control: only PBS). On the day of the experiment, Jag1-Fluc was removed, and the samples were washed twice with PBS. After substrate addition, images were taken using the IVIS Spectrum (PerkinElmer) with the following parameters: open for total bioluminescence, exposure time = 1, 10, 20, 30, 40, 50, 60,120 sec, binning = medium: 8, field of view = 12.6 × 12.6 cm, and f/stop = 1. Analysis of the images was performed with Living Image 4.3 software (PerkinElmer). Once the tissue location was identified on the slide, regions of interest (ROIs) of identical size (0.545 cm^2) were selected for all samples, ensuring that the tissue area of interest was consistently

analyzed. Background signals were measured from two random points (0.545 cm^2) in the surrounding area, and overlays of the luminescence signal with the tissue image were used to confirm that the signal observed was derived from the tissue.

2.11 Statistical analysis

The existence of differences between any pairs of means was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test to assess the pairwise significance of differences between groups in real-time PCR analysis. A t-test was used to evaluate differences between hyperplastic polyps and adenomas in luminescence imaging. GraphPad Prism v. 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot experimental data for the least-squares fitting of calibration and dose-response curves (for evaluation of IC50 values). A $p < 0.05$ was considered statistically significant. Each experiment was carried out with three technical replicates (i.e., three wells per sample) and repeated at least two times for statistical power.

3. Results

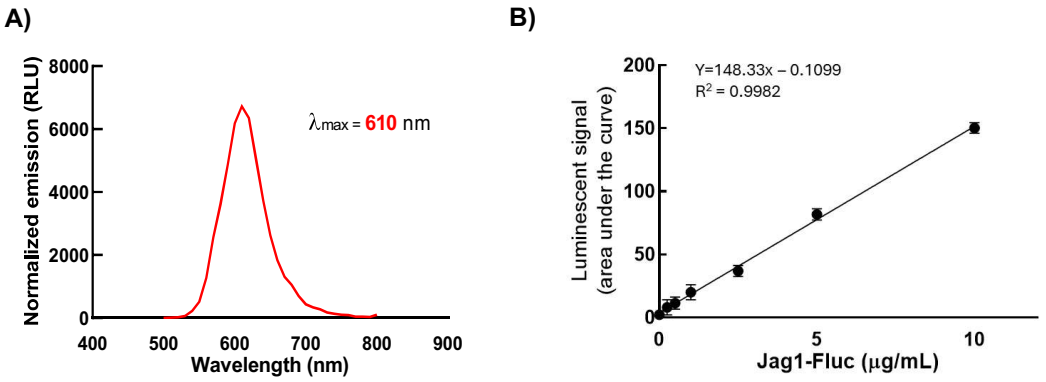
3.1 Production and Characterization of High-Affinity Jag1-Fluc Using the Baculovirus-Insect Cell Expression System

Firstly, to obtain a high-affinity Jag1-derived probe for Notch receptors [34], a construct coding the fragment containing the ECD (N terminal region) of the JAG1 gene with the S32L, R68G, D72N, and T87R substitutions was first isolated and then fused to the gene of the red-emitting firefly luciferase (Figure S1, S2).

The protein was expressed from this construct in insect cells to have proper glycosylation [39]. Once the light emission signal was confirmed at 610 nm (Figure 1A), the correlation

between the luminescent output and the concentration of Jag1-FLuc was investigated in a cell-free system to test the sensitivity of the protein. Luminescent measurements were carried out for 90 min after triggering the light emission , and the Area Under the Curve (AUC), referred to as the light signal ~~emission~~ between 0 and 90 minutes, was analyzed. The calibration curve showed a good linear correlation between the luminescent signal and the concentration of the high-affinity Jag1-Fluc up to 10 µg/mL (Figure 1B) with a LOD and LOQ of 0.20 ± 0.03 and 0.50 ± 0.03 µg/mL, respectively.

Figure 1*: (**A**) Normalized light emission spectra of Jag1-Fluc. (**B**) Calibration curve showing the correlation between luminescent signal and concentration of the Jag1-Fluc protein in a cell-free model. Each point represents the mean ± SD of three independent measurements.*



3.2 Enhanced Performance of Jag1-Fluc in Caco-2 Cells Through Optimized Incubation Protocols

We tested in Caco-2 immortalized cell line the efficiency of the Jag1-Fluc probe to bind Notch receptors. Caco-2 cells were chosen because they represent an invaluable tool for modeling a non-aggressive phenotype of CRC and express high levels of Notch receptor 3, as confirmed through western blot and real-time PCR analysis (Figure S5). BL emission was first measured in living Caco-2 cells exposed to Jag1-Fluc at concentrations ranging from 5 to 50 µg/mL for different times (i.e., 10 min, 30 min, and 1h). A linear correlation between the BL signal and Jag1-Fluc concentration was found (Figure 2A), with a LOD and LOQ of 0.4 ±

0.2 µg/mL and 3.0 ± 0.2 µg/mL, respectively. A 1h exposure time was chosen for subsequent experiments, as it yielded the highest sensitivity in respect to 15 and 30 min of incubation (data not shown). We didn't expose cells to Jag1-Fluc for more than 1h to avoid cell survival issues. To evaluate the probe's sensitivity in samples that better mimic human biopsies, we investigated the light emission in PFA-fixed Caco-2 cells. We compared the probe sensitivity after 1h incubation, as performed in living cells, to an o/n exposure (5-50 µg/mL). Figure 2B showed a linear correlation between the luminescent signal and Jag1-Fluc concentration up to 50 µg/mL for both incubation times. However, o/n incubation was chosen for subsequent experiments, as it not only yielded the highest luminescent signal but also resulted in lower LOD and LOQ values compared to 1h (LOD and LOQ of 0.8 ± 0.2 µg/mL and 6.0 ± 0.2 µg/mL, respectively).

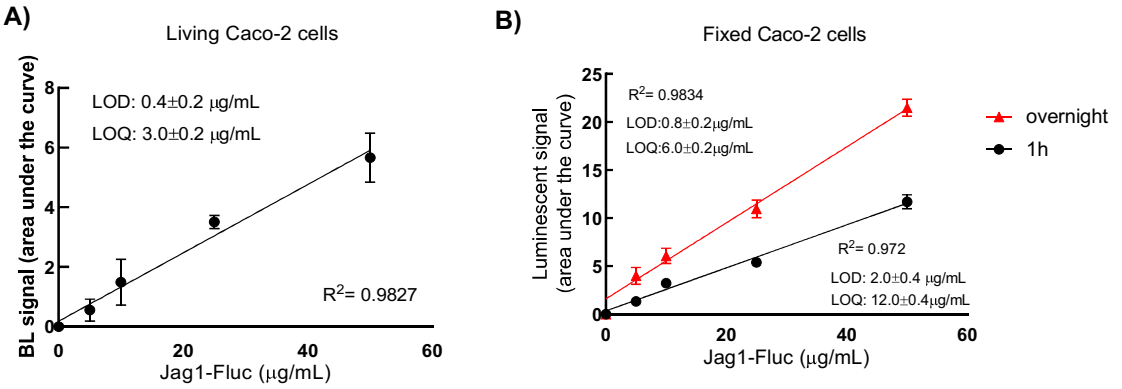


Figure 2. *(**A**) Calibration curves showing the correlation between luminescent signal and concentration of the Jag1-Fluc protein in living and (**B**) fixed Caco-2 cells after 1h and o/n incubation with Jag1-Fluc (range 5–50 µg/ml). Each point represents the mean ± SD of three independent measurements.*

3.3 Assessment of Jag1-Fluc selectivity in Caco-2 cells through a competitive assay using a wild-type Jag1-IgG1 Fc chimera

The Jag1-Fluc probe selectivity was assessed using a wild-type soluble human Jag1-IgG1 Fc chimera as a competitive agonist on fixed Caco-2 and MCF-7 cells, the latter a breast cancer cell line that expresses high levels of all Notch receptors [40, 41].

PFA-fixed cells were first incubated o/n with increased concentrations of the human soluble Jag1-IgG1 Fc chimera (range 0.1-25 µg/mL). Then, the probe was added to reach a final concentration of 50 µg/mL for 12h. A decrease in luminescent signal was observed, proportional to the concentration of the Jag1 chimera in Caco-2 cells and MCF-7, obtaining an IC50 of 0.55 ± 0.06 µg/mL (Figure 3A) and 0.45 ± 0.04 µg/mL, respectively (Figure 3B). These results were confirmed in Caco-2 living cells, treated for 1h with Jag1-Fluc (50 µg/mL) in the presence or absence of o/n pretreatment with the human soluble Jag1-Fluc chimera (25 µg/mL) (Figure S6).

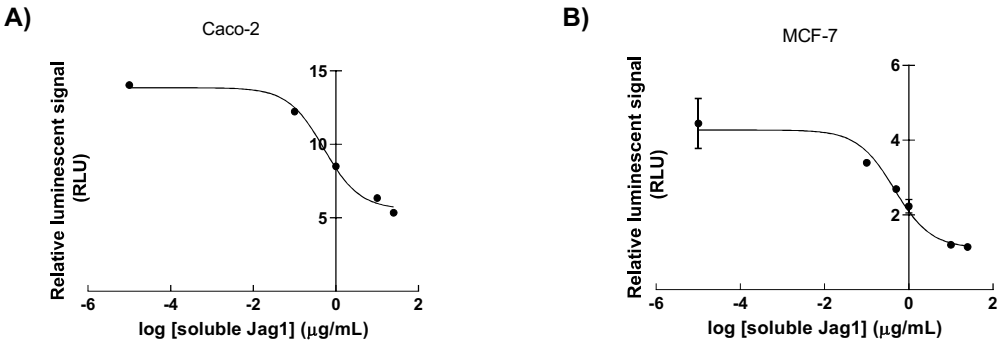


Figure 3: *(**A**) IC50 graph of fixed Caco-2 (1x10^5 cells well $^{-1}$) and (**B**) MCF-7 cells (5x10^4 cells well $^{-1}$) treated with the human soluble Jag-1 chimera (range 0.1-25 µg/mL) and Jag1-Fluc at the fixed concentration of 50 µg/mL. Each point represents the mean ± SD of three independent measurements.*

3.4 Binding localization of Jag1-Fluc to Notch receptors in Caco-2 cells through luminescence imaging

To investigate the binding localization of Jag1-Fluc to the ECD of Notch receptors, luminescence imaging was performed on fixed Caco-2 cells (1 × 10^5 cells/well). After the fixing step, cells were incubated o/n with different concentrations of Jag1-Fluc (50–400 µg/mL) and imaged with a 10X objective magnification, using Hoechst 33342 dye (1 µg/mL)

to stain cell nuclei. Increasing the concentration from 50 to 400 µg/mL, more and sharper appreciable luminescent signals were observed (Figure 4). Negative control didn't exhibit any light emission (data not shown). Pictures showed that the accessibility and/or the number of exposed Notch receptors in Caco-2 cells is heterogeneous due to the differences in membrane protein accessibility, associated with distinct cellular phases, consistent with previous findings [42], also considering that PFA fixation would most probably hinder the Notch receptor and probe interaction to some degree.

The overlay of Hoechst 33342 fluorescence and luminescence images acquired demonstrated that the signal doesn't overlap with nuclei (Figure S7).

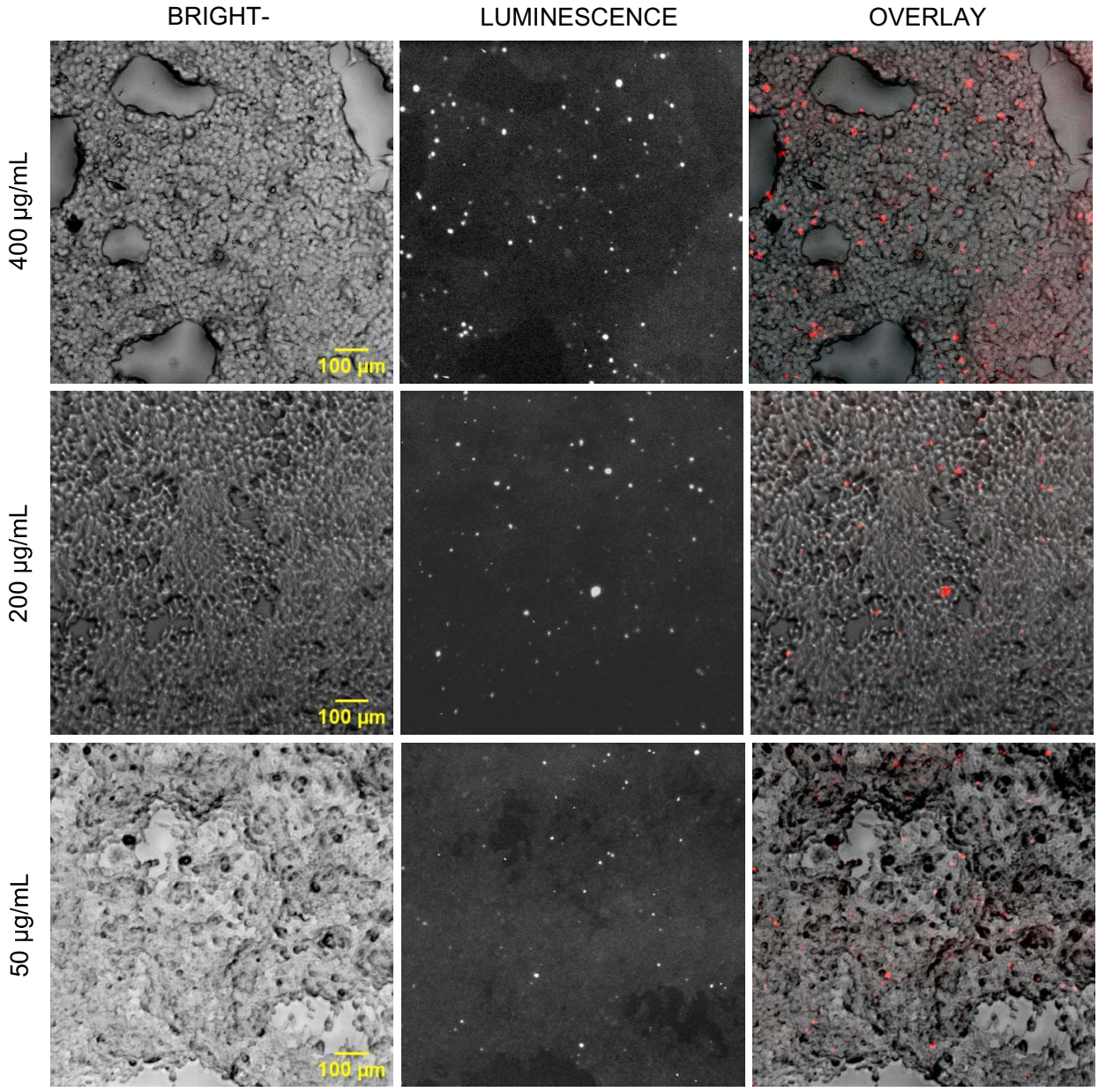


Figure 4: *Bright-field, luminescence, and pseudocolor overlay images of fixed Caco-2 cells (10X objective, 12 min acquisition) incubated overnight with Jag1-Fluc (400-200-50 µg/mL). Scale bar: 100 µm.*

3.5 Preliminary luminescence imaging analysis of Jag1-Fluc in human biopsies reveals differential luminescent signal in hyperplastic polyps, low- and high-grade adenomas

To explore the potential of Jag1-Fluc as a diagnostic tool for human tissues, we conducted a preliminary analysis of biopsies obtained from 10 enrolled subjects: 4 with hyperplastic polyps and 6 with low- (n=3) and high-grade dysplasia (n=3) adenomas. Following deparaffination and rehydration, the biopsies were incubated o/n with Jag1-Fluc (range 50-200 µg/mL), and the detection by the IVIS imager was performed at various time points (1, 5,10, 20, 30, 60, and 120 sec). A detectable luminescent signal was observed after just 1 second of acquisition using 50 µg/mL, whereas no light emission was detected in a section from the same subject treated with PBS, used as the negative control (Figures 5A, 5B, and 5C). Quantitative analysis of the total luminescent flux revealed a statistically significant difference ($p< 0.05$) between hyperplastic polyps and high-grade dysplasia adenomas. Specifically, the adenoma samples exhibited higher luminescent signals compared to the hyperplastic polyps, as shown in Figure 5D. For each sample, the acquisition image at 5 seconds was analyzed to ensure a strong and more stable light emission. Notably, IHC analyses performed on the same samples revealed an increased expression of Notch3, consistent with the observation of increased luminescent signal between the three groups (Figure S8).

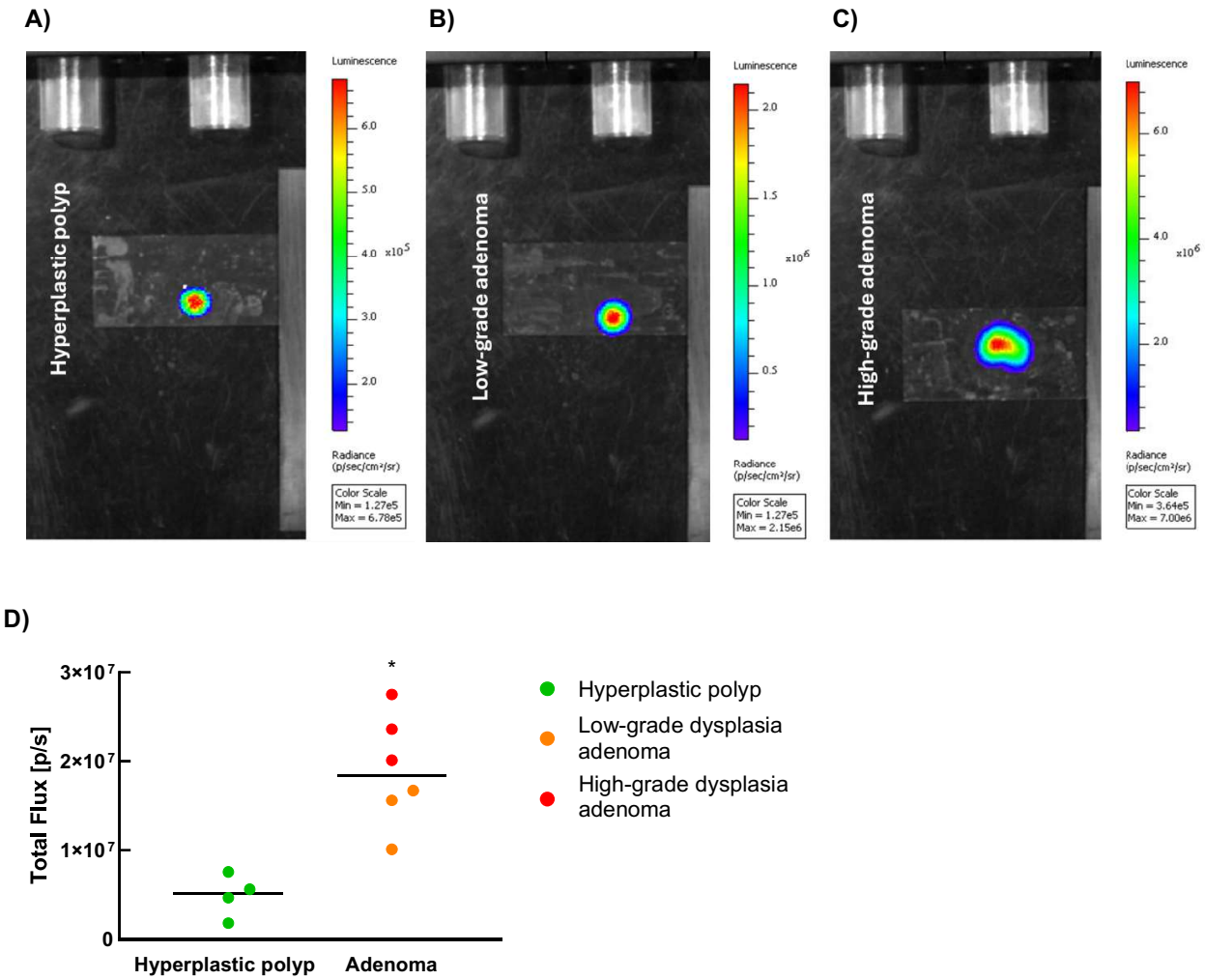


Figure 5: *Luminescence imaging of human biopsies of patients with (**A**) hyperplastic polyp and (**B**) low- and (**C**) high-grade dysplasia adenomas in the presence of Jag1-FLuc 50 µg/mL at IVIS imager with an exposure time of 1s. **D**) Scatter plot showing the total flux (expressed as photons per second, p/s) detected in samples of hyperplastic polyps (green), and low- (orange) and high-grade adenomas (red). Each dot represents individual sample values, while horizontal lines indicate the median for each group. Statistical analysis revealed a significant difference between the two groups (*p < 0.05).*

4. Discussion

Notch signaling is crucial for intestinal cell differentiation, promoting absorptive over secretory fate, stem cell maintenance, immune modulation, and overall tissue homeostasis, playing a fundamental role in the integrity and function of the gastrointestinal tract [19, 43–45]. Altered

activation of this pathway is associated with a variety of pathologies, including different types of cancer such as breast, ovarian, and lung cancer, and hematological malignancies [46]. In CRC, the deregulation of Notch signaling is well-documented due to its crucial role in maintaining intestinal homeostasis. Recent studies suggest that aberrant Notch activation can drive tumor progression by promoting various cellular processes, including cell survival, proliferation, and migration [16]. Consequently, this contributes to a poorer prognosis and a higher metastatic potential in CRC. Indeed, Shaik et al. reported Notch signaling deregulation in 86% of CRC biopsies from a cohort of 66 patients [47]. Notably, the activation of Notch1 and Notch3 has been specifically linked to CRC initiation and progression, with high expression levels correlating with poor OS and increased recurrence [29, 48]. The activation of Notch signaling is closely linked to the interaction of receptors with ligands; thus, Notch ligands are involved in this process. For example, increased Dll4 levels have been observed in CRC biopsies compared with normal tissue [49], and studies have shown that the deletion of just one *Jag1* allele in APC mutant mice ($APC^{Min/+}$) significantly reduces tumor size and active Notch1 levels [15]. Interestingly, Jag1 ligand is not strictly required for intestinal homeostasis, and its expression level is significantly associated with CRC recurrence [25], exerting pro-oncogenic function in the gut, thus opening the possibility of targeting Jag1 in human CRC patients with less or no intestinal side effects [21, 33].

Given this strong association between Notch signaling and CRC, the Notch ligand Jag1 could serve as a promising biomarker for different stages of the disease. In this context, we developed a luminescent assay using a recombinant protein combining the ECD of a high-affinity mutant of the Notch ligand Jag1 with a red-emitting firefly luciferase [34]. The red-emitting firefly luciferase was chosen for its superior ability to penetrate living tissue compared to other variants, thereby enhancing the sensitivity of BL imaging for both *in vitro* and *in vivo* applications [50, 51]. Moreover, the mutated form of Jag1 developed by Luca et al. enables stronger and more prolonged interactions with Notch receptors [34]. The emission of the Jag1-Fluc construct was measured by acquiring its spectrum, and then its luminescent signal was tested in a cell-free system. After obtaining a strong light emission, an *in vitro* cell

model was used for further investigation. Caco-2 cells were selected for this purpose due to their established use in Notch signaling studies [35, 52]. After confirming, through real-time PCR and western blot, that Caco-2 cells express high levels of the Notch3 receptor, data which are in line with previous reports, compared to other cell lines (Figure S5), we investigated the binding ability of Jag1-Fluc. A strong luminescent signal was detected in both living and fixed cells. To evaluate the probe's ability to avoid off-target effects, a soluble Jag1-IgG1 Fc chimera was used as a competitor, as several studies have already characterized soluble forms of Notch ligands that can mimic their natural counterparts [53, 54]. As expected, we observed a reduced light emission in the presence of high concentrations of the soluble Jag1 chimera in both Caco-2 and MCF-7, a breast cancer cell line, which expresses all Notch isoforms and, in particular, high levels of Notch1 [55, 56]. Indeed, MCF-7 cells are involved in Notch signaling, with Notch1 playing a critical role in their proliferation, survival, and resistance to apoptosis [57]. Data obtained confirmed that the wild-type soluble Jag1-IgG1 Fc chimera effectively competes with the probe for the same target, suggesting a lack of target binding by the probe. Furthermore, we confirmed the Jag1-Fluc possible use for diagnostic purposes by performing imaging on human biopsies obtained from subjects with different intestinal lesions (hyperplastic polyp, low- and high-grade dysplasia adenomas). Jag1-Fluc demonstrated progressively increasing light output from hyperplastic polyps to high-grade adenomas. To the best of our knowledge, this is the first attempt to specifically focus on and quantify the binding of Jag1 with Notch receptors in CRC initiation. Even with the caveat of the low number of patients, this luminescent gradient may suggest a correlation between Notch receptor expression and, consequently, Notch signaling activation and the progression of intestinal lesions. These findings align with the well-documented role of Notch signaling in CRC pathogenesis, supporting its involvement in tumor initiation and progression [23, 58]. The increase in Notch-related luminescence in high-grade dysplasia adenomas is consistent with the pathway's involvement in advanced disease stages, which drives tumor proliferation, survival, and metastasis. These findings highlight the potential of Jag1-Fluc as a diagnostic tool for stratifying lesion stages.

5. Conclusions

Given the expected increase in CRC incidence and mortality rates, understanding the molecular and cellular mechanisms underlying CRC becomes pivotal for improving diagnostics, therapy, and prevention strategies. In this work, we reported the development of a new Jag1-Fluc luminescent recombinant protein probe and its characterization for detecting ECD Notch receptors in Caco-2 cells. Due to its involvement in both physiological and cellular processes in numerous disease states, the opportunity to fully understand the existing roles and interactions between Jag1 and Notch receptors in CRC progression is critically essential [36, 59]. Thus, the findings of this study provide compelling evidence for the utility of Jag1-Fluc as a tool to investigate Notch signaling, particularly in the context of CRC.

The increasing luminescent signal observed in biopsies suggests a correlation between Notch signaling activation and the progression of CRC, emphasizing its potential as a biomarker for early detection and disease monitoring. Additionally, the ability of Jag1-Fluc to differentiate between lesion types further underscores its value as a potential diagnostic tool for CRC and other cancers, where aberrant Notch signaling is a key driver of tumorigenesis. This study lays the groundwork for future research to refine the application of our newly developed Jag1-Fluc probe in live animal models and clinical settings, providing a novel approach for real-time monitoring of Notch signaling in cancer progression and therapy response.

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Authors contributions

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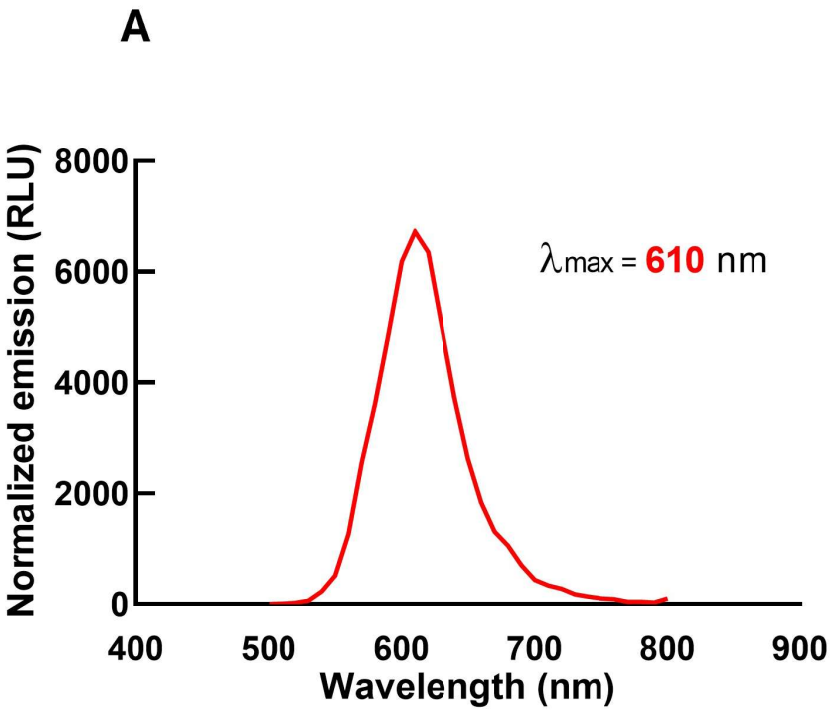
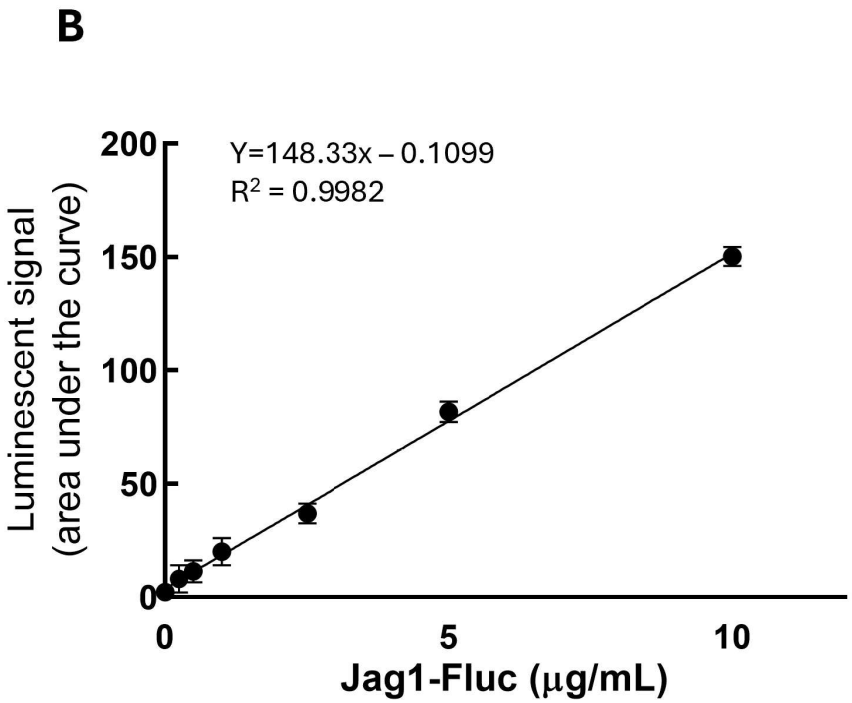
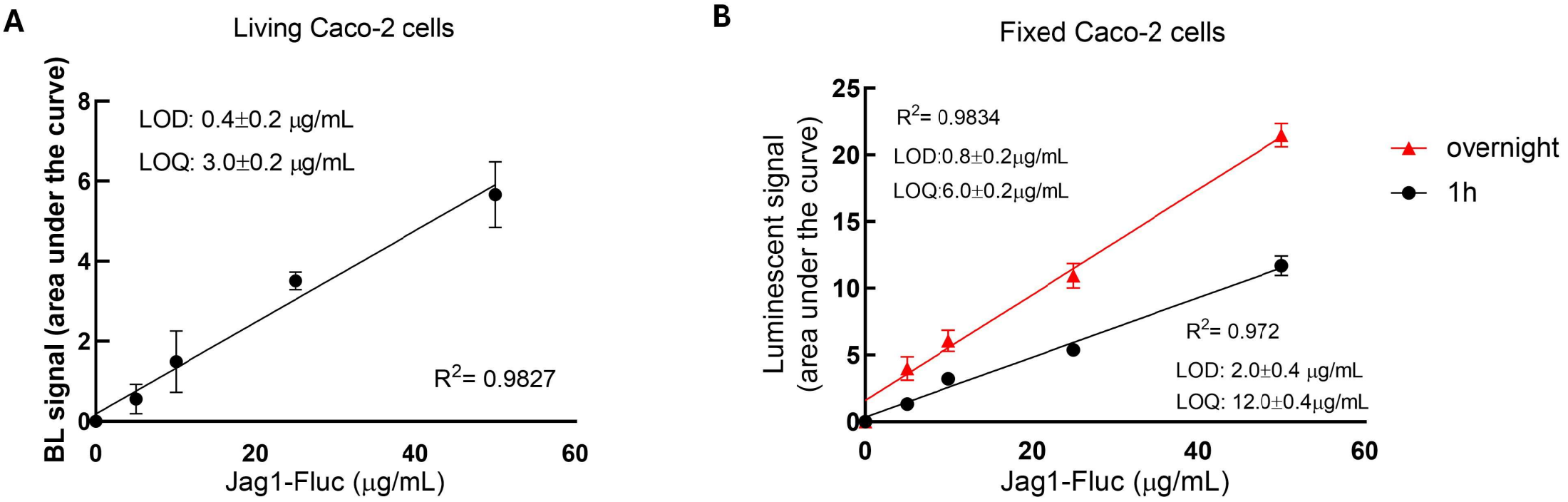
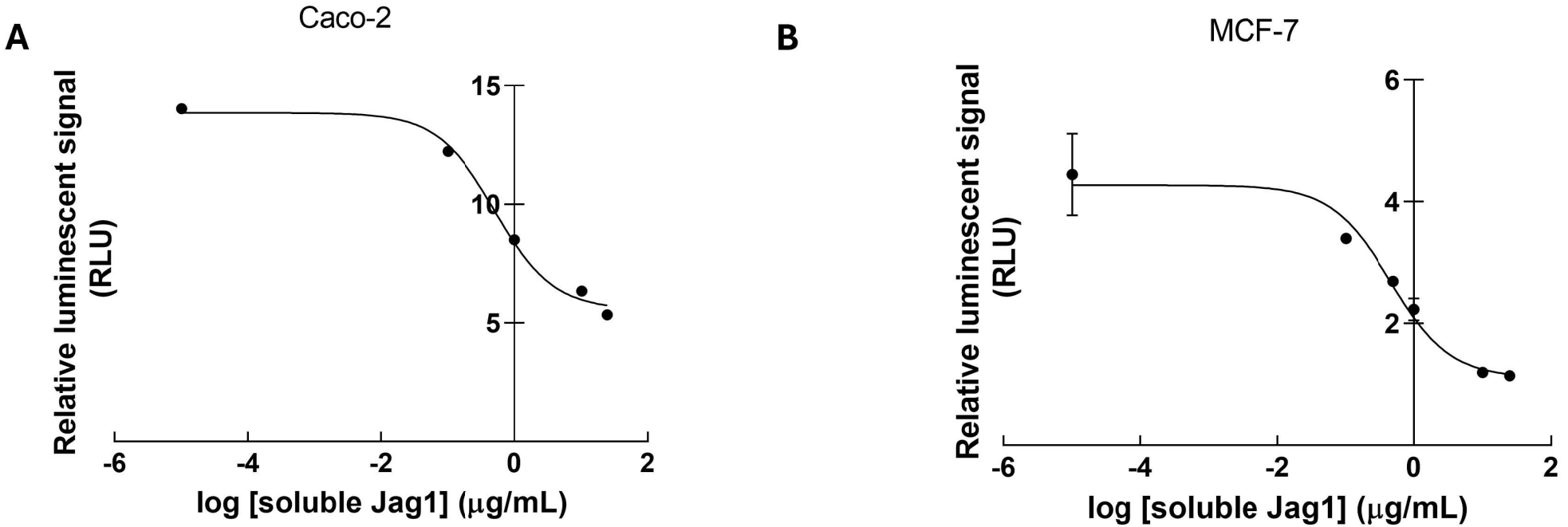
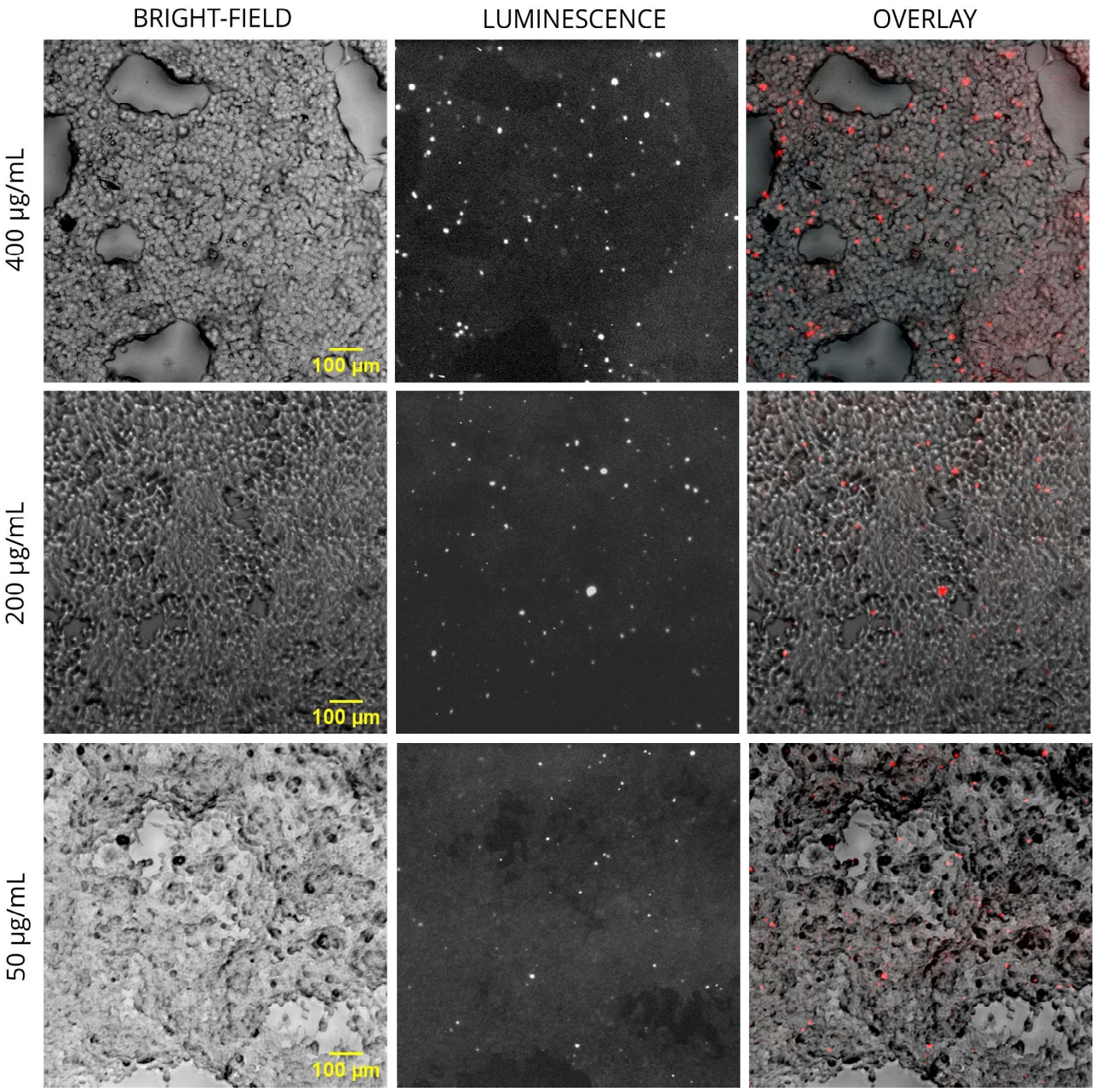
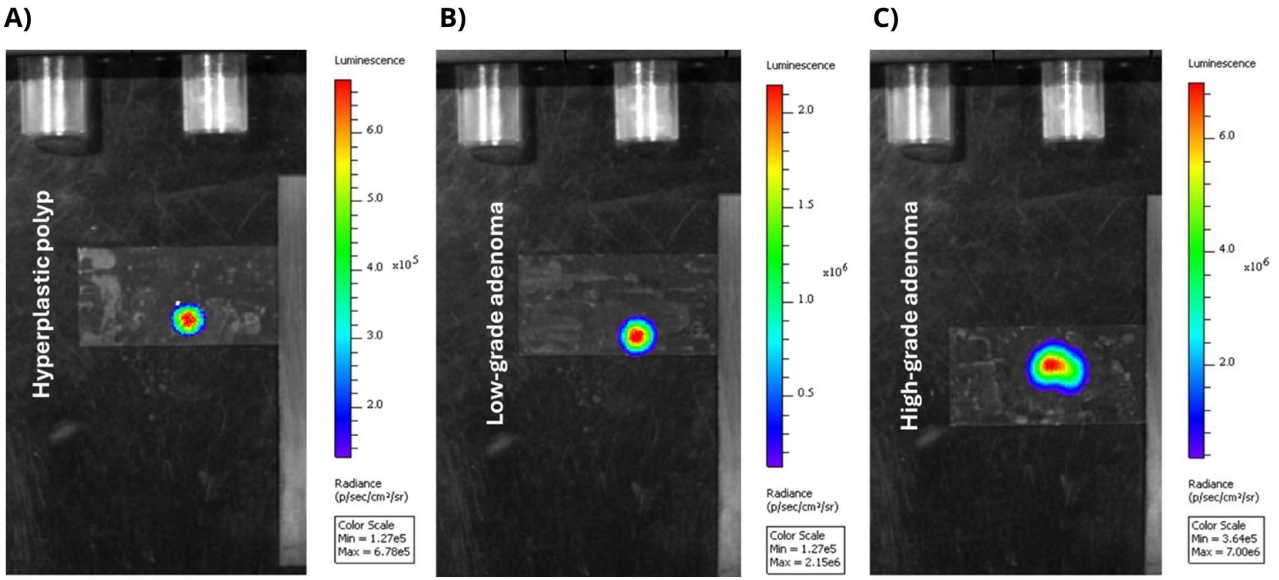

A
Normalized emission (RLU)
8000
6000
4000
2000
0
400
500
600
700
800
900
Wavelength (nm)
λmax = 610 nm

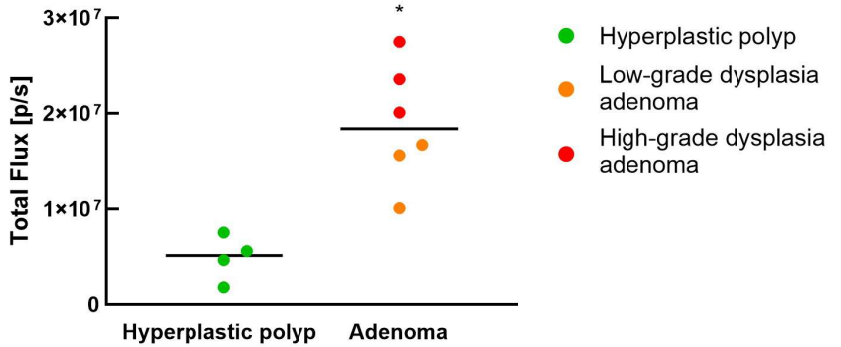

B
Luminescent signal
(area under the curve)
200
150
100
50
0
0
5
10
Jag1-Fluc (μg/mL)
Y=148.33x – 0.1099
R2 = 0.9982


A
Living Caco-2 cells
BL signal (area under the curve)
LOD: 0.4±0.2 μg/mL
LOQ: 3.0±0.2 μg/mL
R2= 0.9827
Jag1-Fluc (μg/mL)
B
Fixed Caco-2 cells
Luminescent signal (area under the curve)
R2= 0.9834
LOD:0.8±0.2μg/mL
LOQ:6.0±0.2μg/mL
R2= 0.972
LOD: 2.0±0.4 μg/mL
LOQ: 12.0±0.4μg/mL
overnight
1h
Jag1-Fluc (μg/mL)


A
Caco-2
Relative luminescent signal (RLU)
15
10
5
-6
-4
-2
0
2
log [soluble Jag1] (μg/mL)
B
MCF-7
Relative luminescent signal (RLU)
6
4
2
-6
-4
-2
0
2
log [soluble Jag1] (μg/mL)


BRIGHT-FIELD
LUMINESCENCE
OVERLAY
400 µg/mL
200 µg/mL
50 µg/mL
100 µm
100 µm
100 µm


A)
Hyperplastic polyp
Luminescence
6.0
5.0
4.0
x10^5
3.0
2.0
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.27e5
Max = 6.78e5
B)
Low-grade adenoma
Luminescence
2.0
1.5
x10^6
1.0
0.5
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.27e5
Max = 2.15e6
C)
High-grade adenoma
Luminescence
6.0
4.0
x10^6
2.0
Radiance (p/sec/cm²/sr)
Color Scale
Min = 3.64e5
Max = 7.00e6


3×10^7
2×10^7
1×10^7
0
Total Flux [p/s]
*
Hyperplastic polyp
Adenoma
Hyperplastic polyp
Low-grade dysplasia adenoma
High-grade dysplasia adenoma

Jagged1-Fluc: a ~~bio~~luminescent recombinant protein as a sensitive tool for high-throughput screening of colorectal cancer

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Abstract

Colorectal cancer (CRC) onset is closely linked to dysregulated Notch signaling, particularly involving receptors like Notch1 and Notch3, and ligands such as Jagged1 (Jag1), which are overexpressed in aggressive subtypes. To address the need for quantitative assessment of Notch expression in clinical settings, we developed a ~~bio~~luminescent ~~(BL)~~ recombinant probe (Jag1-Fluc) by fusing the extracellular domain of a high-affinity Jag1 mutant with red-emitting

firefly luciferase. The Jag1-Fluc ~~BL~~ probe can bind to Notch, thus correlating the intensity of emitted light with the concentration of Notch in the sample.

In cell-free assays, Jag1-Fluc demonstrated a linear ~~BL~~ luminescent signal across different concentrations (detection limit: 0.20 ± 0.03 µg/mL). When applied to living and fixed human colorectal cancer cells, the probe showed a dose-dependent ~~luminescent signal~~ light emission (5–50 µg/mL), confirming its ability to bind endogenous Notch receptors. Competitive binding assays using soluble human Jag1 chimera demonstrated concentration-dependent signal inhibition (IC_{50} = 0.55 ± 0.06 µg/mL), validating probe specificity. Notably, in a small cohort of human biopsies, Jag1-Fluc enabled stratification of Notch expression, with luminescence intensity progressively increasing from hyperplastic polyps to low- and high-grade adenomas. These findings reinforce prior evidence linking Jag1 overexpression to CRC progression. The assay's simplicity and sensitivity highlight its potential for early CRC screening, paving the way for personalized medicine by enabling tailored therapeutic monitoring and strategies based on Notch signaling profiles. This approach potentially enhances treatment efficacy and reduces side effects, representing a valuable tool for precision oncology.

Keywords: colorectal cancer, Notch pathway, Jagged1, red firefly luciferase, recombinant protein, ~~bio~~luminescence, imaging.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related mortality in the Western population [1]. According to data registered by the

Abbreviations: APC, adenomatous polyposis coli; ATCC, American Type Culture Collection; AUC, area under the curve; BL, bioluminescent/bioluminescence; BLI, bioluminescence imaging; BRAF, B-Raf proto-oncogene serine/threonine kinase; BSA, bovine serum albumin; Caco-2, human colon carcinoma cell line; CMS4, consensus molecular subtype 4; CRC, colorectal cancer; DFS, disease-free survival; ECD, extracellular domain; FAP, familial adenomatous polyposis; FBS, fetal bovine serum; Fluc, firefly luciferase; HaCaT, human adult low calcium temperature (skin keratinocyte cell line); HBSS, Hanks'

International Agency for Research on Cancer (IARC), almost 1.9 million cases were diagnosed just in 2022, with approximately 1 million deaths annually [2]. The gradual progression of CRC offers the possibility of diagnosing the disease early before the malignant transformation occurs, so a deeper mechanistic understanding is highly needed to address new targeted therapeutic approaches. Indeed, CRC development is a multi-step process involving several morphological, histological, genetic, and epigenetic alterations that accumulate over time [3]. From 90 to 95% of CRCs are adenocarcinomas, starting with benign adenomatous polyps, which can evolve into adenomas and eventually CRC over 10-15 years [4]. However, only a small fraction of polyps acquires malignant features. Risk factors for CRC include genetic predisposition (12–35% of cases), such as familial or personal histories of colorectal polyps or CRC, and syndromes like Lynch syndrome, familial adenomatous polyposis (FAP), Peutz-Jeghers, and Cowden syndromes [5]. Environmental factors, such as gut microbiota dysbiosis [6] and bile acid pool alterations [7], are also demonstrated to contribute to CRC development.

Progression from intestinal adenoma to advanced CRC in mice and humans correlates with the accumulation of specific mutations and dysregulated signaling in additional pathways. Key mutations, particularly in the adenomatous polyposis coli (APC) gene, Kirsten rat sarcoma virus (KRAS) gene, B-Raf proto-oncogene serine/threonine kinase (BRAF), mutL homolog 1 (MLH1), and other mismatch repair genes, drive tumorigenesis by disrupting the regulation of critical signaling pathways. These include Wnt/β-catenin, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), transforming growth factor-beta (TGF-β), and tumor protein p53 (P53) [8–11]. Recent studies suggest a pivotal role of altered

balanced salt solution; HUVEC, human umbilical vein endothelial cells; IARC, International Agency for Research on Cancer; ICN, intracellular Notch domain; IPTG, isopropyl β-D-1-thiogalactopyranoside; Jag1, Jagged1; KRAS, Kirsten rat sarcoma virus; LFNG, lunatic fringe; LOD, limit of detection; LOQ, limit of quantification; MAPK, mitogen-activated protein kinase; MCF-7, Michigan Cancer Foundation-7 (human breast cancer cell line); MFNG, manic fringe; MLH1, MutL homolog 1; OS, overall survival; o/n, overnight; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PI3K, phosphatidylinositol 3-kinase; RBPJ, recombination signal binding protein for immunoglobulin kappa J region; RPMI, Roswell Park Memorial Institute; T-ALL, acute lymphoblastic leukemia; TGF-β, transforming growth factor-beta.

Notch signaling activation in CRC, partially due to cross-talks of Notch with other pathways. Indeed, Notch and its downstream effectors stand out as particularly compelling targets for further investigation, as this pathway plays a crucial role in tumor progression, stemness, and therapy resistance. Despite its recognized relevance, the Notch signaling network remains an underexplored yet promising target, warranting deeper investigation to uncover potential therapeutic opportunities [12–15]. Indeed, aberrant Notch activity is a hallmark of a high percentage of colorectal tumors, particularly involving receptors like Notch1 and -3, and ligands such as Jagged1 (Jag1), which are overexpressed in aggressive subtypes like consensus molecular subtype 4 (CMS4) and serrated lesions [16, 17]. Notch signaling is a crucial pathway that controls various cellular processes, including stem cell self-renewal, cell fate specification, and differentiation [18]. The pathway consists of five ligands (Jag1,2 – Dll1,3,4) and four receptors (Notch1-4) and is activated by ligand-receptor interactions between adjacent cells, with the extracellular domain (ECD) of the receptor mediating ligand binding [19]. These interactions trigger sequential proteolytic cleavages catalyzed by ADAM10/17 (a disintegrin and metalloprotease) and the γ-secretase complex. The intracellular Notch domain (ICN), once liberated, translocates to the nucleus, where it interacts with recombination signal binding protein for Immunoglobulin kappa J region (RBPJ), to activate the transcription of target genes [20].

Receptor–ligand interactions are regulated by receptor glycosylation, with the Fringe family of N-acetylglucosaminyltransferases, primarily Lunatic Fringe (LFNG) and Manic Fringe (MFNG), enhancing Delta-induced signals and decreasing responsiveness to Jag ligands [21]. The Espinosa team demonstrated that high Notch activity in intestinal tumors expressing Jag1 but lacking MFNG is predictive of poor survival rates in CRC patients [22]. In line with this observation, many reports have highlighted that Jag1 is frequently overexpressed in human CRC tissues compared to normal tissues, which correlates with cancer progression and poor prognosis [23–25].

It is widely known that the dysregulation of Notch signaling results in developmental deficits in humans, such as Alagille Syndrome, and is involved in different types of neoplasia [26],

among which are acute lymphoblastic leukemia (T-ALL) [27], breast cancer, lung adenocarcinoma, and ovarian cancer [28, 29].

In addition to Notch1, deregulated Notch3 signaling has also been suggested to play a key role in T-ALL, due to the oncogenic potential of the Notch3 ICD [30]. Indeed, the role of Notch3 has garnered attention due to its association with poor disease-free survival (DFS) and overall survival (OS) in CRC patients [31]. Clinical and experimental evidence indicate that Jag1 amplifies Notch signaling, promoting a Notch3/Jag1 feedback loop that supports cancer cell survival, proliferation, and invasion in various tumors, such as T-ALL, ovarian, and breast cancers [32]. Jag1 ligand is not strictly required for intestinal homeostasis, exerting pro-oncogenic function in the gut in Mfng-low tumors, thus opening the possibility of targeting Jag1 in human CRC patients with less or no intestinal side effects [21, 33].

To investigate the interaction between Jag1 and Notch receptors in precancerous stages, we developed a recombinant protein combining the ECD of a high-affinity mutated form of Jag1 [34] with red-emitting firefly luciferase (Fluc). This construct was designed to assess if Jag1-Fluc binds to Notch receptors and whether its light emission upon binding correlates with increasing severity of precancerous colonic lesions.

Common analytical methods used to detect Notch receptors and ligands in polyps, gut adenomas, and CRCs are mRNA expression analysis by quantitative reverse transcription PCR (qRT-PCR), *in situ* hybridization, western blot, immunohistochemistry (IHC), and transcriptomic approaches (RNA-Seq, microarrays). Almost all these approaches are time and cost-consuming, while western blot and IHC, even if used as routine analysis in clinical practice, don't allow reliable quantification of biomarkers mainly because they are semi-quantitative techniques with several limitations (*i.e.,* subjective interpretation and scoring, non-linear signal and saturation, lack of absolute quantification standards). To overcome these issues, we developed a fast and cost-effective quantitative approach using light emission as the analytical strategy, focusing on the binding of Jag1 with Notch receptors.

2. Experimental Procedures

2.1. Reagents

Phosphate-buffered saline (PBS) tabs (giving a 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH 7.4), LB broth powder (Lennox), agar powder, dimethylsulfoxide (DMSO), sodium and potassium chloride, tris base, acetic acid, EDTA, trypsin-EDTA, antibiotic solution 100X (10,000 U/mL penicillin and 10 mg/mL streptomycin), methanol, protease inhibitor cocktail, kanamycin sulfate, ampicillin (sodium salt), isopropyl β-D-1- thiogalactopyranoside (IPTG), β-mercaptoethanol, Tween 20, bovine serum albumin (BSA), H_2O_2 solution, horse serum, sodium-deoxycholate and Triton X-100 were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) high glucose, Roswell Park Memorial Institute (RPMI) 1640 medium, Hanks' balanced Salt solution 10X (HBSS), and fetal bovine serum (FBS) were purchased from Microgem (Naples, Italy). JAG1 ORF Vector (human) was purchased from ABM (Richmond, BC, Canada), while the pIDTSmart (Amp) for JAG1_Mut_High_Affinity (320 bp) was from IDT (Coralville, Iowa, US). pCMVRed FLuc, Pierce Firefly Luc One-Step glow assay kit as ~~BL~~ substrate, Bac-to-Bac™ HBM TOPO™ Secreted Expression System, MAX Efficiency™ DH10Bac Competent Cells, ExpiFectamine™ Sf Transfection Reagent, One Shot™ TOP10 Chemically Competent E. coli, Phusion Site-Directed Mutagenesis Kit, Pure Link™ HiPure Plasmid Miniprep kit, Ni-NTA Purification System, BacPAK™ qPCR Titration Kit, Zero Blunt® TOPO® PCR Cloning Kit, restriction enzymes, Phusion U Hot Start PCR Master Mix, Pierce BCA Protein Assay, Hoechst 33342 Solution, Hank's Balanced Salt Solution (HBSS), paraformaldehyde (PFA), and western blotting detection reagents were purchased from Thermo Fischer Scientific (Waltham, MA, USA). PCR extraction kit and gel Purification Kit were obtained from Qiagen (Hilden, Germany). M200 and LSGS kit were purchased from Life Technologies (Waltham, MA, USA). Laemmli sample buffer, Protein Markers, and Mini-Protean TGX (4–20%) gels were obtained from Bio-Rad (Hercules, CA, USA). Mouse-anti-His mAb was purchased from GenScript (Piscataway, NJ, US). Antibodies used were rabbit monoclonal to Notch3 (#5276)

from Cell Signalling (Danvers, MA, US), rabbit polyclonal to β-tubulin (bs-4511R) from BIOSS (Woburn, MA, US), and Goat anti-rabbit IgG(H+L) HRP conjugate (31466) from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant Human Jag1 Fc Chimera Protein was purchased by Biotechne (Minneapolis, MN, USA). Primers for RT-PCR were purchased from IDT (Coralville, IA, USA) and Sigma-Aldrich. All other chemicals and solvents were of the highest analytical grade.

2.2 Fusion gene design, transformation, and protein expression

The coding region for the ECD of the JAG1_WT gene was cloned into pTOPO. Next, a fragment containing S32L, R68G, D72N, and T87R mutations (Figure S1), which confer a higher binding strength for Notch receptors [34], was inserted in the end terminal part of JAG1_WT, obtaining the resulting plasmid pTopo_JAG1_Mut, according to the manufacturer's instructions (Thermo Fisher).

Then, JAG1mut and R-Firefly luciferase (Fluc) coding genes were combined in a single fusion construct, which was inserted into the pFast-Bac expression vector to generate pFast-Bac JAG1mut_Fluc (Figure S2). Following the insertion of pFast-Bac JAG1mut_Fluc into a Bacmid, the resulting construct was transfected into Sf-9 insect cells. Then, the High Five insect cells were infected with the recombinant Baculovirus to produce the JAG1mut_Fluc soluble protein (according to the manufacturer's instructions, Bac-to-Bac® TOPO® Cloning Kits, Thermo Fisher Scientific, Waltham, MA, USA). Then, JAG1mut-Fluc protein was isolated from cell lysates using immobilized metal affinity chromatography (Ni-NTA). Analysis by SDS-PAGE and western blot indicated that isolated JAG1mut_Fluc exhibited an approximate size of 150 kDa, following the predicted size (Figures S3 and S4).

2.3 Insect cell culture

Commercially available serum-free insect cell lines *Spodoptera frugiperda* Sf-9 and High Five™ (BTI-TN-5BI-4 from *Trichoplusia ni*), and Sf900-II medium were acquired from Thermo Fisher Scientific (Waltham, MA, US) and maintained in Sf900-II with continuous shaking at 120 rpm at 27 °C. For expression culture, High Five™ cells were grown in Grace's Insect Cell Culture Medium without supplements (Thermo Fisher Scientific, Waltham, MA, US) and incubated at 27 °C before infection.

2.4 Human cell culture

Human colorectal adenocarcinoma cells (Caco-2) and human adult low calcium temperature keratinocytes cell line (HaCaT) were obtained from the American Type Culture Collection (ATCC). Both cell lines were grown in DMEM high glucose, supplemented with 10% heat-inactivated FBS, 2.5 mM L-glutamine, and penicillin/streptomycin. Michigan Cancer Foundation-7 (MCF-7) cells, a breast cancer cell line, were kindly gifted by Prof. Natalia Calonghi (Department of Pharmacy and Biotechnology-FABIT, University of Bologna). Cells were maintained in RPMI 1640 containing 10% heat-inactivated FBS and 2.5 mM penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs), purchased from Life Technologies (Waltham, MA, US), were plated on gelatin-coated tissue culture dishes and maintained in phenol red-free basal medium M200 (Thermo Fisher Scientific, Waltham, MA, US) containing 10% heat-inactivated FBS and growth factors (LSGS). HUVECs at passages 2 to 7 (70–90% confluency) were harvested and analyzed. All cell lines were maintained at 37°C in an atmosphere of 5% CO_2. These cell lines were selected because they had already been studied for the Notch pathway [35–38].

2.5 Calibration curve of Jag1-Fluc in a cell-free system

The ~~BL~~ spectrum of Jag1-Fluc was obtained using Spectramax iD3 (Molecular Devices, San Jose, CA, US), setting bandpass filters ranging from 580 to 800 nm, for 1000 ms. The spectrum was collected after adding the ~~BL~~ substrate cocktail from the Pierce Firefly Luc

One-Step glow assay kit. Different dilutions of Jag1-Fluc, starting from 10 µg/mL (range 0.25-10 µg/mL), were prepared by diluting the 400 µg/mL stock solution with a working solution made of PBS, 2% FBS, and 100 µg/mL of $CaCl_2$. 100 µL of each different concentration (working solution for the blank) were dispensed in 96-well black microtiter plate wells. The reaction was triggered by adding 100 µL of ~~BL~~ substrate, and the resulting ~~BL~~ light emission was monitored for 90 min using a luminometric plate reader. The calibration curve was obtained by plotting the ~~BL~~ luminescent signal versus the different concentrations of Jag1-Fluc and fitting the experimental data to a straight line using the least squares method. The limits of detection (LOD) and quantification (LOQ) of the assay were evaluated as the concentrations of Jag1-Fluc giving ~~BL~~ luminescent signals corresponding to those of the blank plus three and ten times its standard deviation, respectively.

2.6 Cell-based calibration curve of Jag1-Fluc in living and fixed human colorectal adenocarcinoma cells (Caco-2)

The day before the experiment, Caco-2 cells (1×10^5 cells $well^{-1}$) were plated in 96-well black microtiter plate wells and kept for 24h at 37 °C, with 5% CO_2. The day after the medium was removed, cells were washed twice with PBS at room temperature and incubated with different concentrations of Jag1-Fluc (range: 5-50 µg/mL) in a solution made of HBSS and 0.1% BSA for 1h. The bioluminescent (BL) emission signal was monitored using a luminometric plate reader. The temperature was maintained at 37°C during the measurement. Then, to assess if the BL signal is detectable at lower incubation times, living Caco-2 cells (1×10^5 cells $well^{-1}$) were plated in a 96-well black microtiter plate, harvested for 24h, and then incubated with Jag1-Fluc (50 µg/mL) for 1h, 30 min, and 10 min. After two PBS washes, the kinetic profile of BL emission was monitored using the IVIS Spectrum (PerkinElmer) with the following parameters: open for total bioluminescence, exposure time = 20 min, binning = medium: 8, field of view = 12.6 × 12.6 cm, and f/stop = 1. Analysis of BLI images was performed with Living Image 4.3 software (PerkinElmer).

The same procedure was also performed in fixed cells. One day before the experiment, Caco-2 cells (1×10^5 cells well^{-1}) were seeded in a 96-well black microtiter plate. The day after the cell medium was removed, the cells were washed twice with PBS, fixed with 4% PFA, and incubated with 100 µL of a blocking solution (PBS, 5% BSA, and 100 µg/mL of $CaCl_2$) for 1h. Then, 100 µL of Jag1-Fluc (5-50 µg/mL) were added for 1h and overnight (o/n) to assess the best incubation time. The following day, after the protein removal, cells were washed twice with PBS at room temperature, and subsequently, 100 µL of the ~~BL~~ substrate solution was added. ~~BL~~ Light emission was monitored for 90 min using a luminometric plate reader. The dose-response curve was obtained by plotting the ~~BL~~ luminescent signal versus Jag1-Fluc concentrations and fitting the experimental data to a straight line using the least squares method.

2.7 Jag1-Fluc selectivity assay for Notch receptors

The day before the experiment, Caco-2 (1×10^5 cells well^{-1}) and MCF-7 (5×10^4 cells well^{-1}) cells were plated in a 96-well black microtiter plate and kept for 24h at 37 °C with 5% CO_2. The day after the medium was removed, the cells were washed twice with PBS, fixed with 4% PFA, and incubated with 100 µL of a blocking solution for 1h (PBS, 5% BSA, and 100 µg/mL of $CaCl_2$). Then, cells were treated o/n (for about 12h) with serial dilutions of human soluble Jag1 chimera (range 0.1-25 µg/mL) able to bind Notch receptors in a solution of PBS, 2% FBS, and 100 µg/mL of $CaCl_2$. On the day of the experiment, 100 µL of Jag1-Fluc (50 µg/mL) were added to each well. After 12h of incubation, the supernatant containing the two different solutions was removed from each well, and the cells were washed twice with PBS at room temperature. A luminometric plate reader monitored the ~~BL~~ light emission signal after substrate addition. The same procedure was performed in living Caco-2 cells.

2.8 *In Vitro* Cell-based ~~Bio~~luminescence Imaging

Imaging experiments were performed using an Olympus IX73 inverted microscope (Olympus Corporation, Tokyo, Japan) equipped with an ultrasensitive EM-CCD camera (ImagEM X2, Hamamatsu Photonics KK, Shizuoka, Japan) controlled by the proprietary software HCImage v.4.2.6.1 and enclosed in a dark box to prevent interference from ambient light. Caco-2 cells (1×10^5 cells $well^{-1}$) were grown in a 96-well black microtiter plate for 24h at 37 °C with 5% CO_2. The day after, cells were fixed with 4% PFA as described above and incubated overnight with three different concentrations of Jag1-Fluc (400, 200, 50 µg/mL). Negative control was performed by just adding PBS to the cells. Before each imaging session, Jag1-Fluc was gently removed, and cells were washed twice with PBS. Images of Caco-2 cells were acquired using a 10X objective with an integration time of 5, 8, and 12 min, at a gain level set to 200, after adding 100 µL of ~~BL~~ substrate cocktail to trigger the ~~BL~~ luminescent reaction. All experiments were performed in triplicate and repeated at least three times.

The same procedure was performed by adding the nuclei staining step to the protocol. Caco-2 cells were fixed and incubated overnight with Jag1-Fluc at the maximum concentration (400 µg/mL). The day after the ~~BL~~ Jag1-Fluc protein was removed, cells were washed twice with PBS and stained with bisbenzimide at 1 µg/mL (Hoechst 33342- Thermo Fisher Scientific). Images of Caco-2 cells were acquired using a 20X objective with an integration time of 5, 8, and 12 min, at a gain level set to 200, after adding 100 µL of substrate cocktail to trigger the ~~BL~~ luminescent reaction.

Image processing was performed using the freely available Java-based software ImageJ v. 1.52d.

2.9 Study population

The study, named ALMA0118, was conducted with approval from the Local Ethics Committee of the Emilia Romagna region (601/2018/Sper/AOUBo) and adhered to the Declaration of Helsinki and its later amendments. This was an exploratory, controlled, single-center human study, performed at the Sant'Orsola Malpighi Hospital in Bologna (Italy), which

identified three groups of subjects: n=4 subjects with hyperplastic polyps (mean age: 63.3±8.6, males), n= 3 with low- (mean age: 60.3±9.7, males) and n= 3 high-grade (mean age: 68.3±0.5, males) dysplasia adenomas. Enrolled subjects, who provided written informed consent, were patients undergoing routine colonoscopy and receiving standard clinical care. Specifically, the analyzed specimens included fragments from polyps removed via endoscopic polypectomy during standard procedures or a portion of the excised tissue from surgical resections.

2.10 *Ex-vivo* ~~Bio~~luminescence imaging ~~(BLI)~~ on human specimens

~~Bio~~Luminescence imaging ~~(BLI)~~ was performed on formalin-fixed, paraffin-embedded samples from 10 patients in total with hyperplastic polyps (4) and low- and high-grade dysplasia adenomas (6), enrolled in a clinical study performed at the Sant'Orsola-Malpighi University Hospital in Bologna (Italy). After paraffin removal by xylene and rehydration through graded ethanol, antigen retrieval was carried out by heating sections in 95°C water for 25 min in the presence of 1X IHC select citrate buffer pH 6.0 (Sigma-Aldrich, St Louis, MO, USA). Tissue sections were then treated with a solution made of 3% H_2O_2 at 4°C for 30 min to inhibit the peroxidases and then incubated for 1h at room temperature with the blocking solution containing 3% horse serum and Triton 100X (final concentration: 0.1X in PBS). Then the samples were incubated with Jag1-Fluc 200 and 50 µg/mL in a solution of PBS, 2% FBS, and 100 µg/mL of $CaCl_2$ in a humidified chamber o/n at 4°C (negative control: only PBS). On the day of the experiment, Jag1-Fluc was removed, and the samples were washed twice with PBS. After substrate addition, ~~BLI~~ images were taken using the IVIS Spectrum (PerkinElmer) with the following parameters: open for total bioluminescence, exposure time = 1, 10, 20, 30, 40, 50, 60,120 sec, binning = medium: 8, field of view = 12.6 × 12.6 cm, and f/stop = 1. Analysis of the ~~BLI~~ images was performed with Living Image 4.3 software (PerkinElmer). Once the tissue location was identified on the slide, regions of interest (ROIs) of identical size (0.545 cm^2) were selected for all samples, ensuring that the

tissue area of interest was consistently analyzed. Background signals were measured from two random points (0.545 cm^2) in the surrounding area, and overlays of the luminescence signal with the tissue image were used to confirm that the signal observed was derived from the tissue.

2.11 Statistical analysis

The existence of differences between any pairs of means was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test to assess the pairwise significance of differences between groups in real-time PCR analysis. A t-test was used to evaluate differences between hyperplastic polyps and adenomas in ~~BL~~ luminescence imaging. GraphPad Prism v. 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot experimental data for the least-squares fitting of calibration and dose-response curves (for evaluation of IC50 values). A $p < 0.05$ was considered statistically significant. Each experiment was carried out with three technical replicates (i.e., three wells per sample) and repeated at least two times for statistical power.

3. Results

3.1 Production and Characterization of High-Affinity Jag1-Fluc Using the Baculovirus-Insect Cell Expression System

Firstly, to obtain a high-affinity Jag1-derived probe for Notch receptors [34], a construct coding the fragment containing the ECD (N terminal region) of the JAG1 gene with the S32L, R68G, D72N, and T87R substitutions was first isolated and then fused to the gene of the red-emitting firefly luciferase (Figure S1, S2).

The protein was expressed from this construct in insect cells to have proper glycosylation [39]. Once the ~~BL~~ light emission signal was confirmed at 610 nm (Figure 1A), the correlation between the ~~BL emission~~ luminescent output and the concentration of Jag1-FLuc was investigated in a cell-free system to test the sensitivity of the protein. ~~BL~~ Luminescent measurements were carried out for 90 min after triggering the light emission ~~BL reaction~~, and the Area Under the Curve (AUC), referred to as the ~~BL~~ light signal ~~emission~~ between 0 and 90 minutes, was analyzed. The calibration curve showed a good linear correlation between the ~~BL~~ luminescent signal and the concentration of the high-affinity Jag1-Fluc up to 10 µg/mL (Figure 1B) with a LOD and LOQ of 0.20 ± 0.03 and 0.50 ± 0.03 µg/mL, respectively.

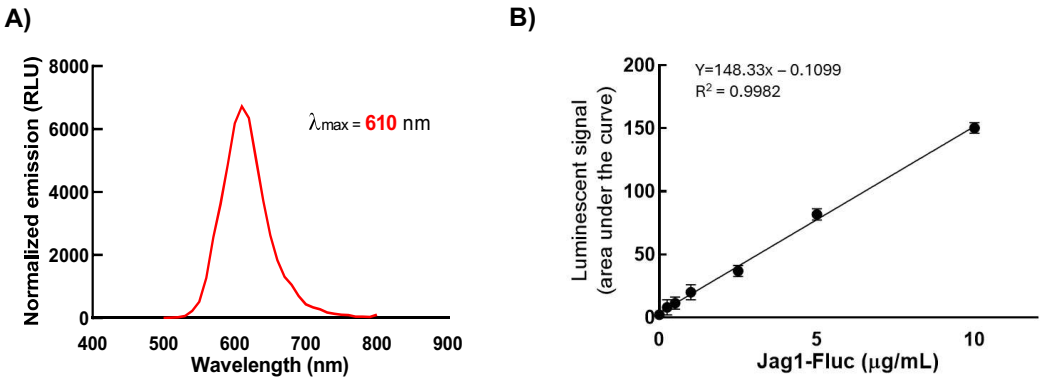

Figure 1: *(**A**) Normalized ~~bioluminescence~~ light emission spectra of Jag1-Fluc. (**B**) Calibration curve showing the correlation between ~~BL~~ luminescent signal and concentration of the Jag1-Fluc protein in a cell-free model. Each point represents the mean ± SD of three independent measurements.*

3.2 Enhanced Performance of Jag1-Fluc in Caco-2 Cells Through Optimized Incubation Protocols

We tested in Caco-2 immortalized cell line the efficiency of the Jag1-Fluc probe to bind Notch receptors. Caco-2 cells were chosen because they represent an invaluable tool for modeling a non-aggressive phenotype of CRC and express high levels of Notch receptor 3, as confirmed through western blot and real-time PCR analysis (Figure S5). BL emission was first measured in living Caco-2 cells exposed to Jag1-Fluc at concentrations ranging from 5

to 50 µg/mL for different times (i.e., 10 min, 30 min, and 1h). A linear correlation between the BL signal and Jag1-Fluc concentration was found (Figure 2A), with a LOD and LOQ of 0.4 ± 0.2 µg/mL and 3.0 ± 0.2 µg/mL, respectively. A 1h exposure time was chosen for subsequent experiments, as it yielded the highest sensitivity in respect to 15 and 30 min of incubation (data not shown). We didn't expose cells to Jag1-Fluc for more than 1h to avoid cell survival issues. To evaluate the probe's sensitivity in samples that better mimic human biopsies, we investigated the ~~bioluminescence~~ light emission in PFA-fixed Caco-2 cells. We compared the probe sensitivity after 1h incubation, as performed in living cells, to an o/n exposure (5-50 µg/mL). Figure 2B showed a linear correlation between the ~~BL~~ luminescent signal and Jag1-Fluc concentration up to 50 µg/mL for both incubation times. However, o/n incubation was chosen for subsequent experiments, as it not only yielded the highest ~~BL~~ luminescent signal but also resulted in lower LOD and LOQ values compared to 1h (LOD and LOQ of 0.8 ± 0.2 µg/mL and 6.0 ± 0.2 µg/mL, respectively).

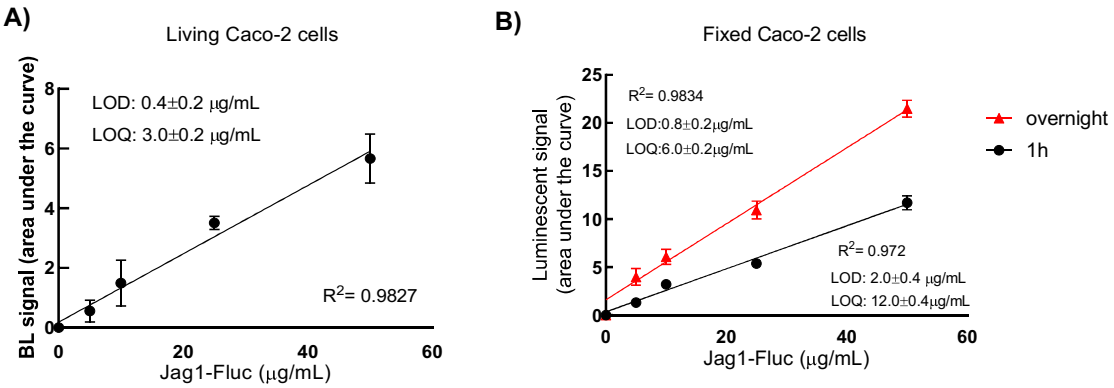

Figure 2. *(**A**) Calibration curves showing the correlation between ~~BL~~ luminescent signal and concentration of the Jag1-Fluc protein in living and (**B**) fixed Caco-2 cells after 1h and o/n incubation with Jag1-Fluc (range 5–50 µg/ml). Each point represents the mean ± SD of three independent measurements.*

3.3 Assessment of Jag1-Fluc selectivity in Caco-2 cells through a competitive assay using a wild-type Jag1-IgG1 Fc chimera

The Jag1-Fluc probe selectivity was assessed using a wild-type soluble human Jag1-IgG1 Fc chimera as a competitive agonist on fixed Caco-2 and MCF-7 cells, the latter a breast cancer cell line that expresses high levels of all Notch receptors [40, 41].

PFA-fixed cells were first incubated o/n with increased concentrations of the human soluble Jag1-IgG1 Fc chimera (range 0.1-25 µg/mL). Then, the probe was added to reach a final concentration of 50 µg/mL for 12h. A decrease in ~~BL~~ luminescent signal was observed, proportional to the concentration of the Jag1 chimera in Caco-2 cells and MCF-7, obtaining an IC50 of 0.55 ± 0.06 µg/mL (Figure 3A) and 0.45 ± 0.04 µg/mL, respectively (Figure 3B). These results were confirmed in Caco-2 living cells, treated for 1h with Jag1-Fluc (50 µg/mL) in the presence or absence of o/n pretreatment with the human soluble Jag1-Fluc chimera (25 µg/mL) (Figure S6).

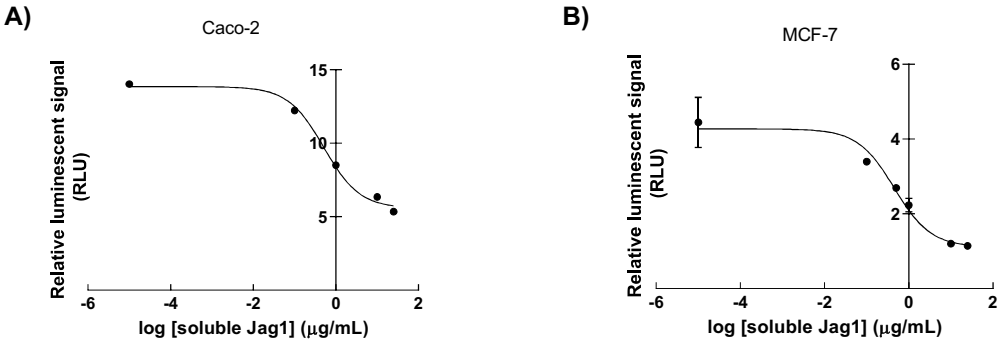

Figure 3: *(**A**) IC50 graph of fixed Caco-2 (1x10^5 cells well $^{-1}$) and (**B**) MCF-7 cells (5x10^4 cells well $^{-1}$) treated with the human soluble Jag-1 chimera (range 0.1-25 µg/mL) and Jag1-Fluc at the fixed concentration of 50 µg/mL. Each point represents the mean ± SD of three independent measurements.*

3.4 Binding localization of Jag1-Fluc to Notch receptors in Caco-2 cells through ~~BLI~~ luminescence imaging

To investigate the binding localization of Jag1-Fluc to the ECD of Notch receptors, ~~bio~~luminescence imaging ~~(BLI)~~ was performed on fixed Caco-2 cells (1 × 10^5 cells/well). After the fixing step, cells were incubated o/n with different concentrations of Jag1-Fluc (50–400 µg/mL) and imaged with a 10X objective magnification, using Hoechst 33342 dye (1 µg/mL) to stain cell nuclei. Increasing the concentration from 50 to 400 µg/mL, more and sharper appreciable ~~BL~~ luminescent signals were observed (Figure 4). Negative control didn't exhibit any ~~BL signal~~ light emission (data not shown). Pictures showed that the accessibility and/or the number of exposed Notch receptors in Caco-2 cells is heterogeneous due to the differences in membrane protein accessibility, associated with distinct cellular phases, consistent with previous findings [42], also considering that PFA fixation would most probably hinder the Notch receptor and probe interaction to some degree.

The overlay of Hoechst 33342 fluorescence and ~~BL~~ luminescence images acquired demonstrated that the signal doesn't overlap with nuclei (Figure S7).

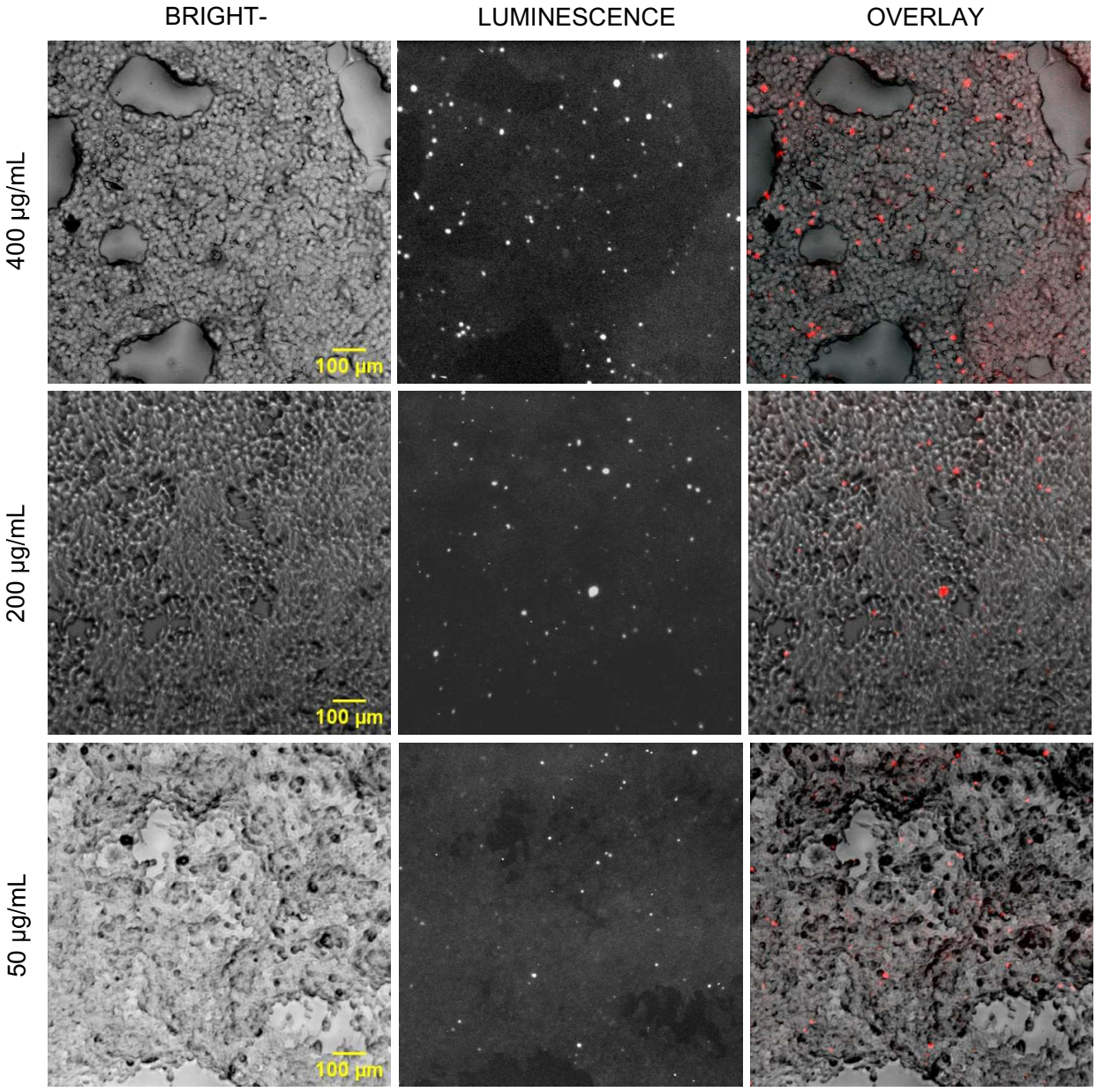

Figure 4: *Bright-field, ~~bio~~luminescence, and pseudocolor overlay images of fixed Caco-2 cells (10X objective, 12 min acquisition) incubated overnight with Jag1-Fluc (400-200-50 µg/mL). Scale bar: 100 µm.*

3.5 Preliminary ~~BLI~~ luminescence imaging analysis of Jag1-Fluc in human biopsies reveals differential ~~BL~~ luminescent signal in hyperplastic polyps, low- and high-grade adenomas

To explore the potential of Jag1-Fluc as a diagnostic tool for human tissues, we conducted a preliminary analysis of biopsies obtained from 10 enrolled subjects: 4 with hyperplastic polyps and 6 with low- (n=3) and high-grade dysplasia (n=3) adenomas. Following deparaffination and rehydration, the biopsies were incubated o/n with Jag1-Fluc (range 50-200 µg/mL), and the detection by the IVIS imager was performed at various time points (1, 5,10, 20, 30, 60, and 120 sec). A detectable ~~BL~~ luminescent signal was observed after just 1 second of acquisition using 50 µg/mL, whereas no light emission was detected in a section from the same subject treated with PBS, used as the negative control (Figures 5A, 5B, and 5C). Quantitative analysis of the total ~~BL~~ luminescent flux revealed a statistically significant difference ($p< 0.05$) between hyperplastic polyps and high-grade dysplasia adenomas. Specifically, the adenoma samples exhibited higher ~~BL~~ luminescent signals compared to the hyperplastic polyps, as shown in Figure 5D. For each sample, the acquisition image at 5 seconds was analyzed to ensure a strong and more stable ~~BL signal~~ light emission. Notably, IHC analyses performed on the same samples revealed an increased expression of Notch3, consistent with the observation of increased ~~BL~~ luminescent signal between the three groups (Figure S8).

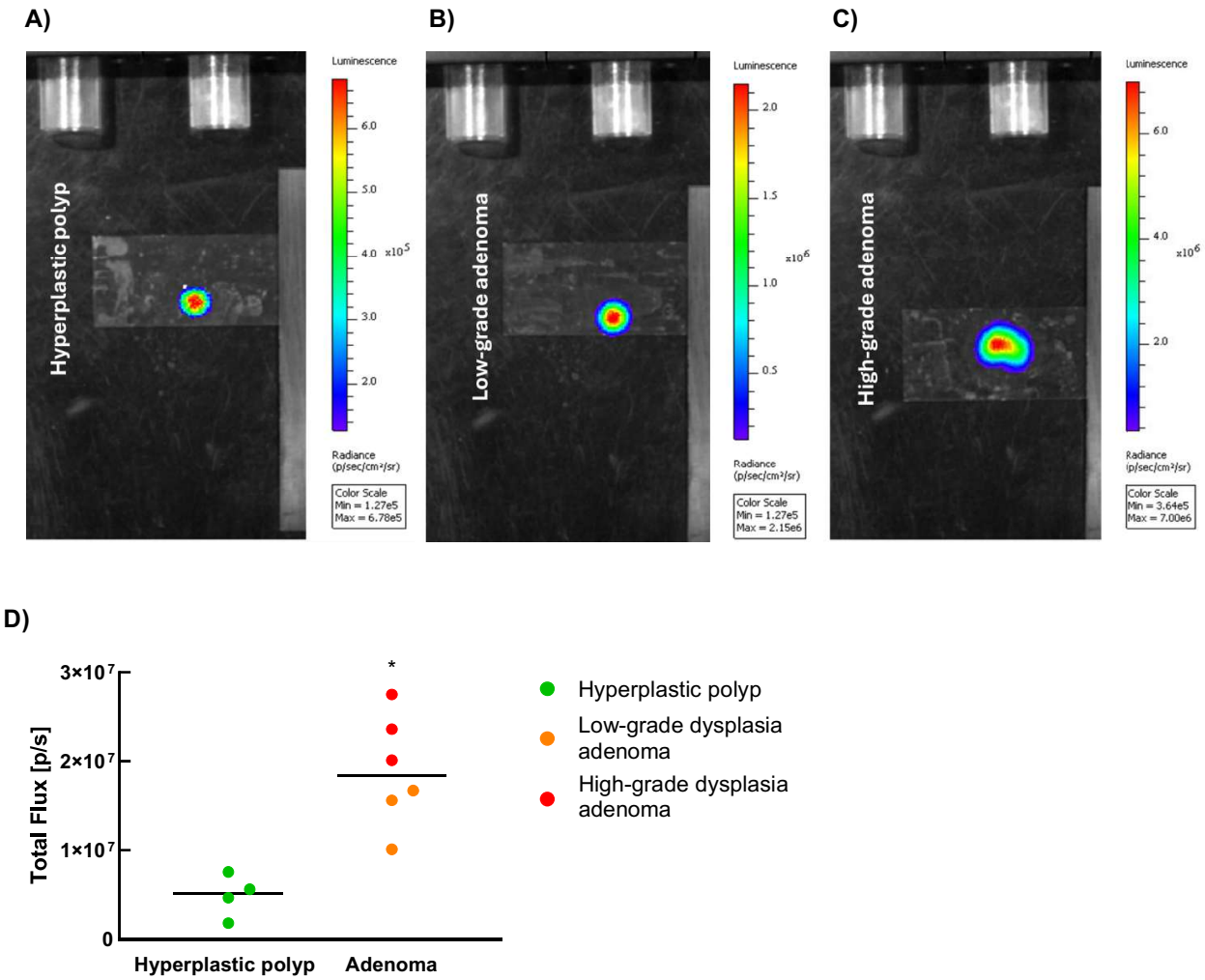

Figure 5: ~~*BLI*~~ *Luminescence imaging of human biopsies of patients with (**A**) hyperplastic polyp and (**B**) low- and (**C**) high-grade dysplasia adenomas in the presence of Jag1-FLuc 50 µg/mL at IVIS imager with an exposure time of 1s. **D**) Scatter plot showing the total flux (expressed as photons per second, p/s) detected in samples of hyperplastic polyps (green), and low- (orange) and high-grade adenomas (red). Each dot represents individual sample values, while horizontal lines indicate the median for each group. Statistical analysis revealed a significant difference between the two groups (*p < 0.05).*

4. Discussion

Notch signaling is crucial for intestinal cell differentiation, promoting absorptive over secretory fate, stem cell maintenance, immune modulation, and overall tissue homeostasis, playing a fundamental role in the integrity and function of the gastrointestinal tract [19, 43–45]. Altered

activation of this pathway is associated with a variety of pathologies, including different types of cancer such as breast, ovarian, and lung cancer, and hematological malignancies [46]. In CRC, the deregulation of Notch signaling is well-documented due to its crucial role in maintaining intestinal homeostasis. Recent studies suggest that aberrant Notch activation can drive tumor progression by promoting various cellular processes, including cell survival, proliferation, and migration [16]. Consequently, this contributes to a poorer prognosis and a higher metastatic potential in CRC. Indeed, Shaik et al. reported Notch signaling deregulation in 86% of CRC biopsies from a cohort of 66 patients [47]. Notably, the activation of Notch1 and Notch3 has been specifically linked to CRC initiation and progression, with high expression levels correlating with poor OS and increased recurrence [29, 48]. The activation of Notch signaling is closely linked to the interaction of receptors with ligands; thus, Notch ligands are involved in this process. For example, increased Dll4 levels have been observed in CRC biopsies compared with normal tissue [49], and studies have shown that the deletion of just one *Jag1* allele in APC mutant mice ($APC^{Min/+}$) significantly reduces tumor size and active Notch1 levels [15]. Interestingly, Jag1 ligand is not strictly required for intestinal homeostasis, and its expression level is significantly associated with CRC recurrence [25], exerting pro-oncogenic function in the gut, thus opening the possibility of targeting Jag1 in human CRC patients with less or no intestinal side effects [21, 33].

Given this strong association between Notch signaling and CRC, the Notch ligand Jag1 could serve as a promising biomarker for different stages of the disease. In this context, we developed a ~~BL~~ luminescent assay using a recombinant protein combining the ECD of a high-affinity mutant of the Notch ligand Jag1 with a red-emitting firefly luciferase [34]. The red-emitting firefly luciferase was chosen for its superior ability to penetrate living tissue compared to other variants, thereby enhancing the sensitivity of BL imaging for both *in vitro* and *in vivo* applications [50, 51]. Moreover, the mutated form of Jag1 developed by Luca et al. enables stronger and more prolonged interactions with Notch receptors [34]. The emission of the Jag1-Fluc construct was measured by acquiring its spectrum, and then its ~~BL~~ luminescent signal was tested in a cell-free system. After obtaining a strong ~~BL signal~~ light

emission, an *in vitro* cell model was used for further investigation. Caco-2 cells were selected for this purpose due to their established use in Notch signaling studies [35, 52]. After confirming, through real-time PCR and western blot, that Caco-2 cells express high levels of the Notch3 receptor, data which are in line with previous reports, compared to other cell lines (Figure S5), we investigated the binding ability of Jag1-Fluc. A strong ~~BL~~ luminescent signal was detected in both living and fixed cells. To evaluate the probe's ability to avoid off-target effects, a soluble Jag1-IgG1 Fc chimera was used as a competitor, as several studies have already characterized soluble forms of Notch ligands that can mimic their natural counterparts [53, 54]. As expected, we observed a reduced ~~BL signals~~ light emission in the presence of high concentrations of the soluble Jag1 chimera in both Caco-2 and MCF-7, a breast cancer cell line, which expresses all Notch isoforms and, in particular, high levels of Notch1 [55, 56]. Indeed, MCF-7 cells are involved in Notch signaling, with Notch1 playing a critical role in their proliferation, survival, and resistance to apoptosis [57]. Data obtained confirmed that the wild-type soluble Jag1-IgG1 Fc chimera effectively competes with the probe for the same target, suggesting a lack of target binding by the probe. Furthermore, we confirmed the Jag1-Fluc possible use for diagnostic purposes by performing ~~BL~~ imaging on human biopsies obtained from subjects with different intestinal lesions (hyperplastic polyp, low- and high-grade dysplasia adenomas). Jag1-Fluc demonstrated progressively increasing ~~BL signal~~ light output from hyperplastic polyps to high-grade adenomas. To the best of our knowledge, this is the first attempt to specifically focus on and quantify the binding of Jag1 with Notch receptors in CRC initiation. Even with the caveat of the low number of patients, this ~~BL~~ luminescent gradient may suggest a correlation between Notch receptor expression and, consequently, Notch signaling activation and the progression of intestinal lesions. These findings align with the well-documented role of Notch signaling in CRC pathogenesis, supporting its involvement in tumor initiation and progression [23, 58]. The increase in Notch-related ~~bio~~luminescence in high-grade dysplasia adenomas is consistent with the pathway's involvement in advanced disease stages, which drives tumor proliferation, survival, and

metastasis. These findings highlight the potential of Jag1-Fluc as a diagnostic tool for stratifying lesion stages.

5. Conclusions

Given the expected increase in CRC incidence and mortality rates, understanding the molecular and cellular mechanisms underlying CRC becomes pivotal for improving diagnostics, therapy, and prevention strategies. In this work, we reported the development of ~~the~~ a new ~~BL~~ Jag1-Fluc luminescent recombinant protein probe and its characterization for detecting ECD Notch receptors in Caco-2 cells. Due to its involvement in both physiological and cellular processes in numerous disease states, the opportunity to fully understand the existing roles and interactions between Jag1 and Notch receptors in CRC progression is critically essential [36, 59]. Thus, the findings of this study provide compelling evidence for the utility of Jag1-Fluc as a ~~bioluminescent~~ tool to investigate Notch signaling, particularly in the context of CRC.

The increasing ~~BL~~ luminescent signal observed in biopsies suggests a correlation between Notch signaling activation and the progression of CRC, emphasizing its potential as a biomarker for early detection and disease monitoring. Additionally, the ability of Jag1-Fluc to differentiate between lesion types further underscores its value as a potential diagnostic tool for CRC and other cancers, where aberrant Notch signaling is a key driver of tumorigenesis. This study lays the groundwork for future research to refine the application of our newly developed Jag1-Fluc probe in live animal models and clinical settings, providing a novel approach for real-time monitoring of Notch signaling in cancer progression and therapy response.

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Authors contributions

Conceptualization, C.C. and A.P. (Antonio Pannuti); methodology, A.P. (Angela Punzo), A.S., A.P. (Antonio Pannuti), G.G., G.Z., L.M., E.B., ML.T., and C.C.; software, A.P. (Angela Punzo), A.S. and A.P. (Antonio Pannuti).; validation, A.P. (Angela Punzo), A.S and E.B., formal analysis, A.P. (Angela Punzo), A.S., A.P. (Antonio Pannuti), and C.C., investigation, A.P. (Angela Punzo), A.S. G.Z. E.D. SK.D: and E.B.; resources, F.B., ML.T., A.D., D.C., L.M., S.R., L.R., A.R., S.D., and C.C.; writing—original draft preparation, A.P. (Angela Punzo), A.P. (Antonio Pannuti), and C.C.; writing—review and editing, A.P. (Angela Punzo), A.P. (Antonio Pannuti), A.S., L.M., S.D., and C.C.; visualization, A.P. (Angela Punzo), A.P. (Antonio Pannuti), and C.C.; supervision, A.P. (Antonio Pannuti), L.M., S.R., A.D., L.R., S.D. and C.C.; project administration, C.C.; funding acquisition, S.D. and C.C. All authors have read and agreed to the published version of the manuscript.

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Supplementary materials

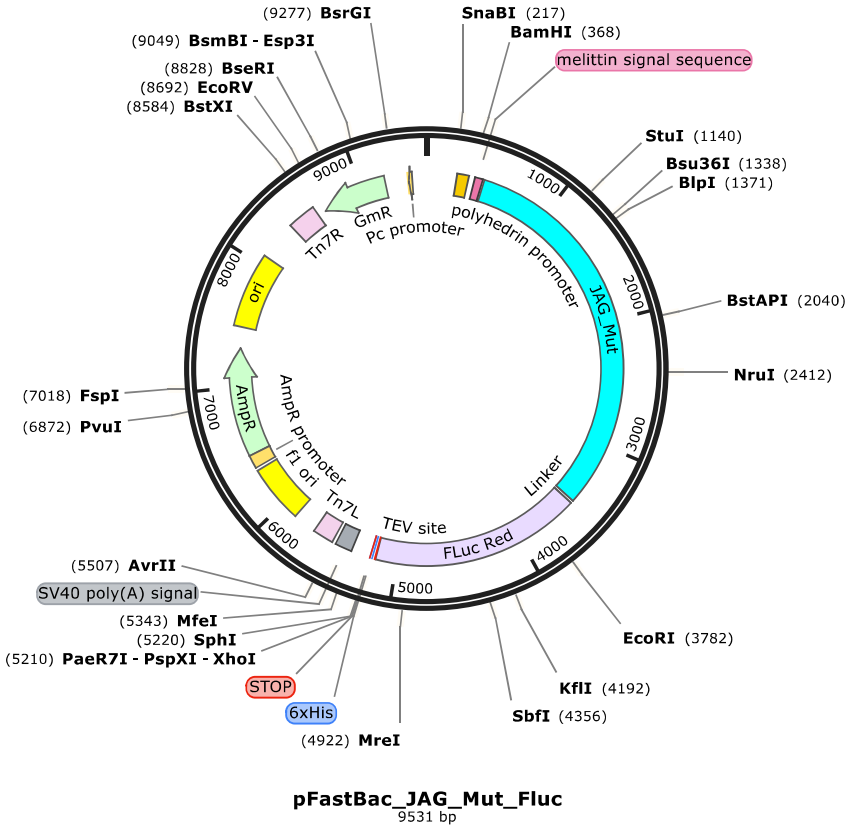
Supplementary Figure S1

Minigene sequence of JAG1_Mut_High_Affinity (ECD domain), 320 bp

GTCTAGAGGATCCGGGGCCCTGGGTCAGTTCGAGTTGGAGATCCTGTCCATGCAGAACGTGAA
CGGGGAGCTGCAGAACGGGAACTGCTGCGGCGGCGCCCGGAACCCGGGAGACCGCAAGTGC
ACCGGTGACGAGTGTAACACATACTTCAAAGTGTGCCTCAAGGAGTATCAGTCCCGCGTCCGTG
CCGGGGGGCCCTGCAGCTTCGGCTCAGGGTCCACGCCTGTCATCGGGGGCAACACCTTCAACC
TCAAGGCCAGCCGCGGCAACGA

Supplementary Figure S2

pFast-Bac expression vector illustrating the structural organization of the Jag1-Fluc fusion construct



Supplementary Figure S3

SDS-PAGE and Western blot analysis of Jag1-Fluc

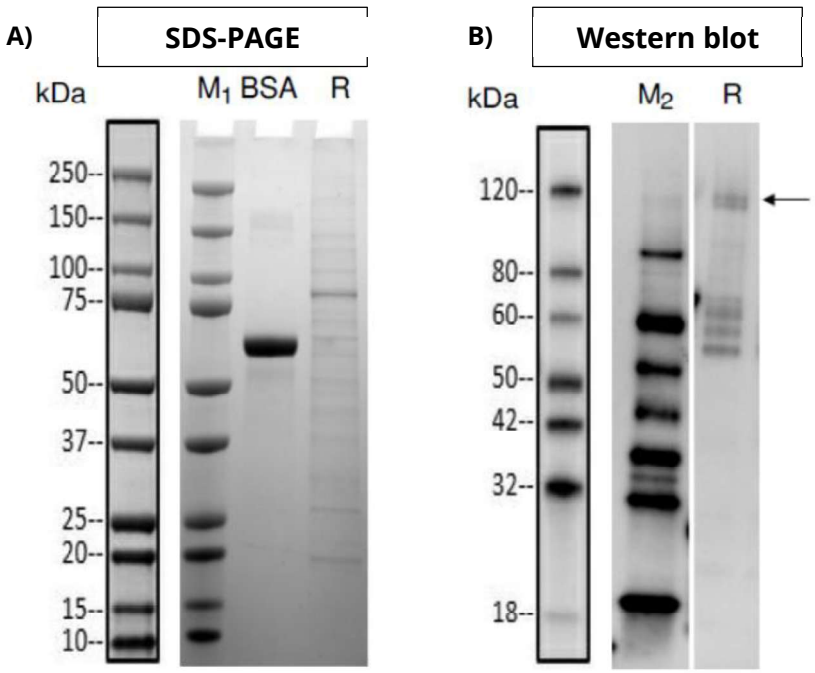


Figure S3: *SDS-PAGE (**A**) and Western blot (**B**) analysis of the target protein Jag1-Fluc (A, B). Lane M_1: protein marker (Bio-rad, 1610374S). Lane M_2: protein marker (GenScript, M00521). BSA: 2.0 µg. R: reducing condition. Primary antibody: Mouse- anti-His mAb (GenScript, A00186). The target protein is indicated by the arrow.*

Supplementary Figure S4

Aminoacidic sequence of Jag1-Fluc recombinant protein

Number of amino acids: 1609 Molecular weight: 175897.63 Theoretical pI: 5.59

MKFLVNVALVFMVVYISYIYADRSLALGQFELEILSMQNVNGELQNGNCCGGARNPGDRKCTGDECNTYFKVCLKEYQSRVRAGGPCSFG
SGSTPVIGGNTFNLKASRGNDRNRIVLPFSFAWPRSYTLLVEAWDSSNDTVQPDSIIEKASHSGMINPSRQWQTLKQNTGVAHFEYRIRVTC
DDYYYGFGCNKFCRPRDDFFGHYACDQNGNKTCMEGWMGPECNRAICRQGCSPKHGSCKLPGDCRCQYGWQGLYCDKCIPHPGCVH
GICNEPWQCLCETNWGGQLCDKDLNYCGTHQPCLNGGTCSNTGPDKYQCSCPEGYSGPNCEIAEHACLSDPCHNRGSCKETSLGFECE
CSPGWTGPTCSTNIDDCSPNNCSHGGTCQDLVNGFKCVCPPQWTGKTCQLDANECEAKPCVNAKSCKNLIASYYCDCLPGWMGQNCDI
NINDCLGQCQNDASCRDLVNGYRCICPPGYAGDHCERDIDECASNPCLNGGHCQNEINRFQCLCPTGFSGNLCQLDIDYCEPNPCQNGAQ
CYNRASDYFCKCPEDYEGKNCSHLKDHCRTTPCEVIDSCTVAMASNDTPEGVRYISSNVCGPHGKCKSQSGGKFTCDCNKGFTGTYCHE
NINDCESNPCRNGGTCIDGVNSYKCICSDGWEGAYCETNINDCSQNPCHNGGTCRDLVNDFYCDCKNGWKGKTCHSRDSQCDEATCNN
GGTCYDEGDAFKCMCPGGWEGTTCNIARNSSCLPNPCHNGGTCVVNGESFTCVCKEGWEGPICAQNTNDCSPHPCYNSGTCVDGDNW
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KYMERYAKLGAIAFTNAVTGVDYSYAEYLEKSCCLGKALQNYGLVVDGRIALCSENCEEFFIPVIAGLFIGVGVAPTNEIYTLRELVHSLGISKP
TIVFSSKKGLDKVITVQKTVTTIKTIVILDSKVDYRGYQCLDTFIKRNTPPGFQASSFKTVEVDRKEQVALIMNSSGSTGLPKGVQLTHENTVT
RFSHARDPIYGNQVSPGTAVLTVVPFHHGFGMFTTLGYLICGFRVVMLTKFDEETFLKTLQDYKCTYVILVPTLFAILNKSELLNKYDLSNLV
EIASGGAPLSKEVGEAVARRFNLPGVRQGYGLTETTSAIIITPEGDDKPGASGKVVPLFKAKVIDLDTKKSLGPNRRGEVCVKGPMLMKGYV
NNPEATKELIDEEGWLHTGDIGYYDEEKHFFIVDRLKSLIKYKGYQVPPAELESVLLQHPSIFDAGVAGVPDPVAGELPGAVVVLESGKNMTE
KEVMDYVASQVSNAKRLRGGVRFVDEVPKGLTGKIDGRAIREILKKPVAKMKGENLYFQGHHHHHH

Supplementary Figure S5

Notch3 expression in Caco-2 cells

Caco-2 cells were harvested for 24h, and then the Notch3 expression was analyzed through Western blot and Real Time-PCR analysis. HaCaT cells and HUVECs were used as positive controls. Protein bands corresponding to the transmembrane (TM) domain of Notch3 were recognizable at 97 kDa; β-tubulin (50 kDa) was used as a reference protein. As shown in Figure S5A, Notch3 is highly expressed in Caco-2 cells compared with HUVECs but is lower than in HaCaT cells. These data were also confirmed through Real-Time PCR analysis, showing a significant difference in Notch3 expression in Caco-2 cells compared with HUVEC ($p < 0.0001$) and HaCaT cells ($p < 0.001$) (Figure S5B).

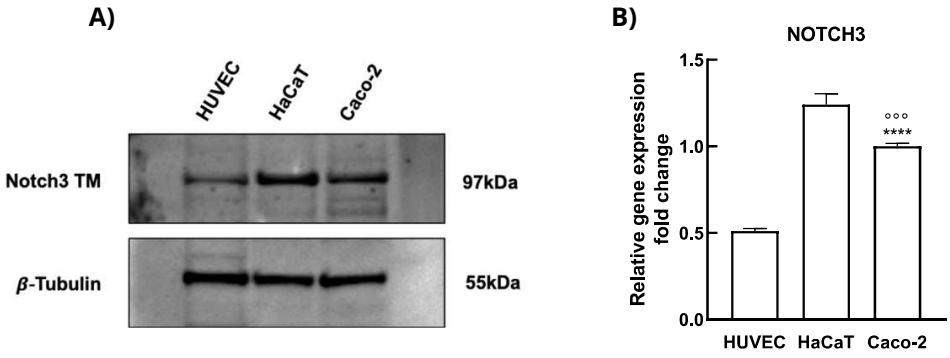

Figure S5: *The protein and gene expression of Notch3 was evaluated through western blot analysis (**A**) and Real time-PCR (**B**) in HUVEC, HaCaT, and Caco-2 cells. ****p< 0.0001 Caco-2 vs HUVEC, °°°p<0.001 Caco-2 vs HaCaT.*

Supplementary Figure S6

Jag1-Fluc selectivity in living Caco-2 cells

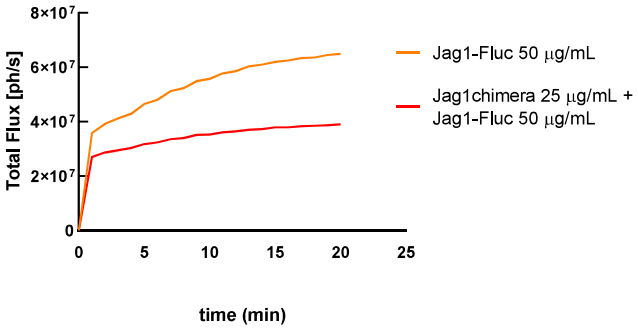

Figure S6: *Bioluminescence kinetic profiles of living Caco-2 cells (1 x10^5 cells $well^{-1}$) obtained in the presence or absence of previous o/n incubation with human soluble Jag-1 chimera (25 µg/mL), and Jag1-Fluc at a fixed concentration (50 µg/mL) for 1h.*

Supplementary Figure S7

Jag1-Fluc ~~BLI~~ luminescence imaging and nuclei staining

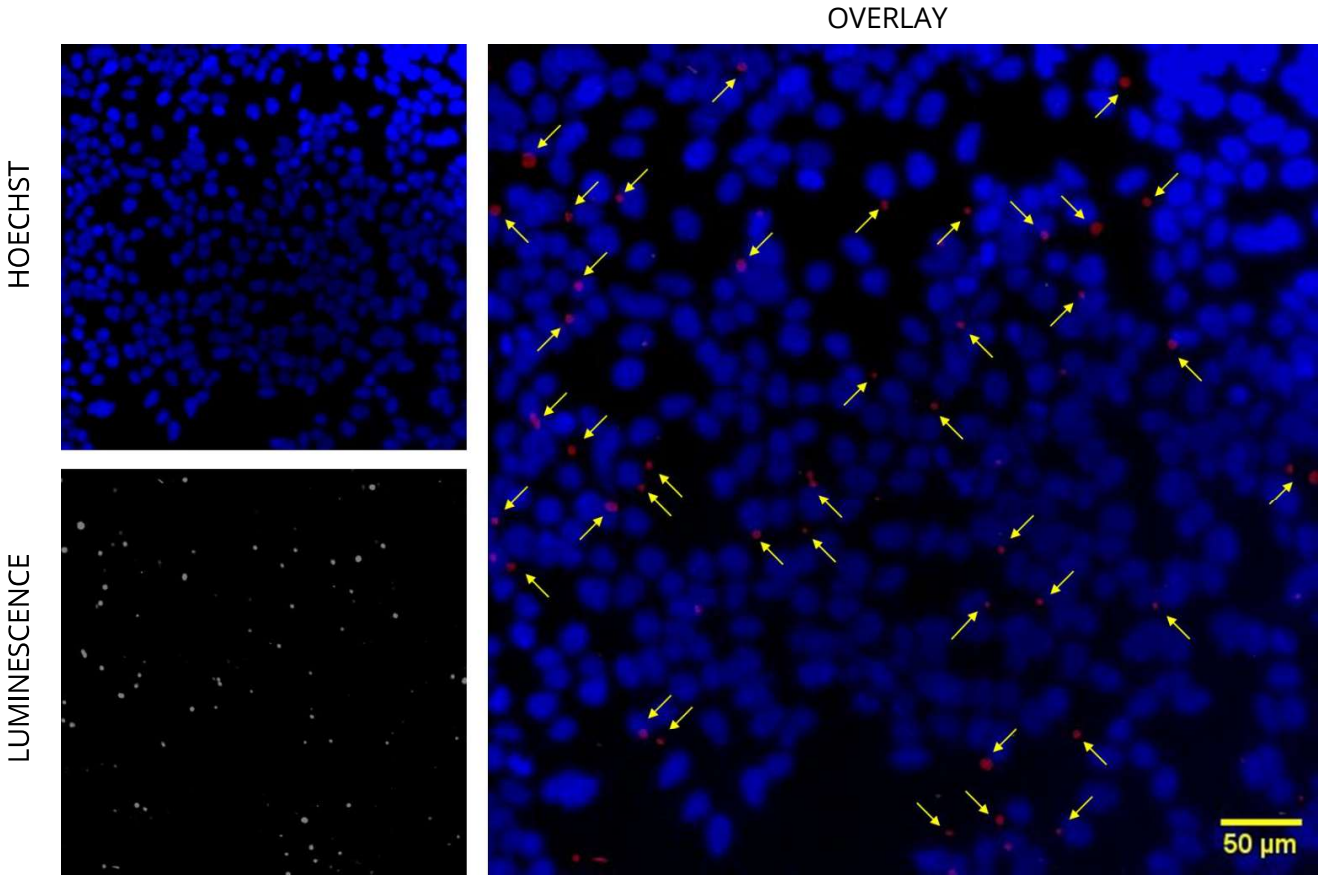

Figure S7: *Hoechst 33342 staining, ~~bio~~luminescence, and pseudocolor overlay images of fixed Caco-2 cells (20X objective, 12 min acquisition) incubated overnight with Jag1-Fluc (400 µg/mL). Jag1-Fluc ~~BL~~ light emission is marked with yellow arrows. Scale bar: 50 µm.*

Supplementary Figure S8

Notch3 expression in human specimens

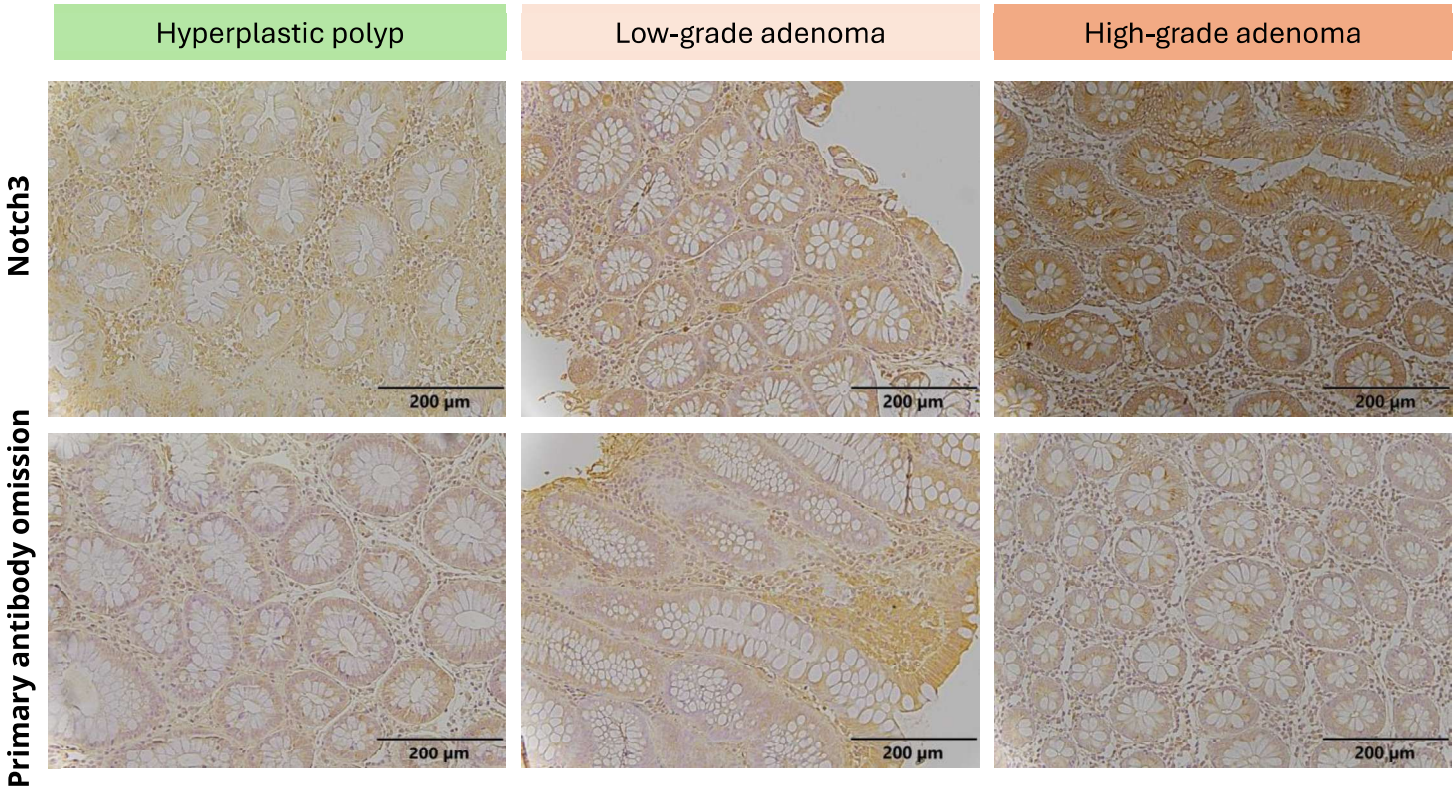

Figure S8: *Specimens from different subjects stained with Notch3 antibody show an increased expression from a hyperplastic polyp to low and high-grade dysplasia adenoma. Corresponding sections without primary antibody staining are shown as negative controls (bottom panels).*

Supplementary Experimental Procedures

Western Blotting Analysis

Caco-2, HaCaT (1×10^6 cells well^{-1}), and HUVEC cells (0.7×10^6 cells well^{-1}) were harvested for 24h in a 6-well plate. Western blot analyses were performed to detect Notch3 transmembrane (TM), and β-tubulin expression, using the corresponding antibodies. Cells were lysed in RIPA Buffer (0.05% sodium-deoxycholate was freshly added) containing 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 mM PMSF and 1 mM sodium orthovanadate on ice for 30 minutes. Protein samples were denatured by incubation at 70°C for 10 minutes in sample buffer (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) containing 0.5 M DTT added and separated on 7.5% NuPAGE gels (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Proteins were transferred from the gel into a pre-made membrane pack (Invitrogen,

iBlot 2NC regular Stacks) using the iBlotTM 2 Dry Blotting system (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 9min at 20V. Nonspecific binding was blocked by incubating membranes with Tris-buffered saline (TBS), 0.1% Tween, pH 7.6, containing 3% BSA for 1h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies, washed 3 times in TBS, 0.1% Tween, and incubated for 1h at room temperature with secondary peroxidase-conjugated antibodies in TBS, 0.1% Tween containing 3% BSA. The following primary and secondary antibodies were used: rabbit anti-Notch 3 (#5276, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-β-tubulin (bs-4511R, Bioss Inc., Woburn, MA, USA), and Goat anti-rabbit IgG(H+L) HRP conjugate (31466, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer instructions. Membranes were washed 3 times in TBS, 0.1% Tween, and developed using PierceTM ECL Western blotting substrate (Thermo Scientific, Waltham, MA, USA), following the manufacturer's instructions. Images of the blots were obtained by exposing them to Fluor-STM MultiImager (Bio-Rad, Hercules, CA, USA), and Quantity One 1-D Analysis Software - Bio-Rad was used for the image analysis.

RNA Extraction and Real-Time PCR

Caco-2, HaCaT (1×10^6 cells well^{-1}), and HUVEC cells (0.7×10^6 cells well^{-1}) were harvested for 24h in a 6-well plate. Total RNA was extracted using a commercially available kit (Qiagen, Hilden, Germany) following manufacturer instructions. RNA concentration and purity were determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 25 ng of total RNA was reverse transcribed using the SuperScript® III First-Strand Synthesis SuperMix (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and amplified using the EXPRESS SYBR® GreenER™ qPCR SuperMix (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol in a final volume of 25 µl. Real-time PCR reactions were conducted on a Rotor-Gene Q QIAGEN Real-Time PCR System (QIAGEN, Hilden, Germany), with an initial step of 5 min incubation at 60°C, then 2 min at 95°C, followed by 40 cycles of amplification: 95°C for 15 s and 60°C for 1 min, and examined on by Rotor-Gene Real-Time Analysis Software 6.0 (QIAGEN, Hilden, Germany). Primer concentration was 50 µM. The following primers were used: RPL13A forward 5'-CACCCTGGAGGAGAAGAGGA-3', reverse 5'-CCGTAGCCTCATGAGCTGTT-3' (Integrated DNA Technologies). NOTCH3 forward 5'- TGTATGCATTCCTTTGTGTC- 3', reverse 5'-AGGAAGAGAAAGTACCACTC- 3´ (Sigma-Aldrich, St Louis, MO, USA). Changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ formula using RPL13A as a reference gene.

Notch 3 immunohistochemical analysis

Notch3 was assessed on specimens from subjects enrolled in the clinical trial ALMA0118

performed at the Sant'Orsola Malpighi Hospital in Bologna (10 subjects with hyperplastic polyps, 20 with low- and high-grade adenomatous polyps, and 20 subjects with CRC). Specifically, the specimens analyzed are fragments taken from polyps removed by endoscopic polypectomy during the normal course of care or a portion of the removed tissue from surgical resections. Before use, slides were deparaffinized in xylene, dehydrated through graded ethanol, and washed thoroughly in PBS. Notch3 antibody (ab23426, Abcam) was used to identify Notch 3 expression in the colon tissue through HRP/DAB standard method using the immunoperoxidase secondary detection system IHC Select HRP/DAB (Merck) using a 1:1000 dilution in a solution made of PBS, 3% horse serum and TritonX100 (0,1). The antigen retrieval step was carried out by heating sections in 90°C water for 20 min in the presence of 10 mmol/l sodium citrate buffer pH 6.0 (Carlo Erba, Milan, Italy) for the staining. Both the immunostaining with the Notch3 antibody and the corresponding negative controls (omission of the primary antibody) were processed simultaneously during the same staining session to ensure experimental consistency. Images have been captured with a Nikon Dxm 1200 camera connected to an Olympus AX 70 microscope, with 4X, 10X, and 20X magnification. In most of the subjects enrolled, it has been observed that the Notch3 protein is highly expressed. Notch3 is localized in enterocytes, immunocytes, and mainly in highly proliferating cells. Moreover, among the three groups, there is a sort of grading showing that low- and high-grade samples have a higher expression of the Notch3 receptor, compared to hyperplastic polyp specimens.

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SCIENZE BIOMEDICHE E NEUROMOTORIE

Bologna, September 25th, 2025

Replies to Reviewers' Comments

Dear Editor, we would like to thank you and the Reviewers for the careful reading of our manuscript and for the constructive comments and suggestions. We have made every effort to address all points raised within the available time. We believe the manuscript has been greatly improved thanks to the Reviewers' insightful feedback.

Our detailed, point-by-point replies to the Reviewers' comments are provided below. The responses are given in ***italic and bold***, and the corresponding changes are highlighted in yellow in the revised version of the manuscript.

Reviewer #1

In this manuscript, Punzo et al. describe the design and characterization of Jag1-Fluc, a recombinant chemiluminescent probe developed to quantify Notch receptor expression in colorectal cancer. The authors present data from cell-free assays, cultured cells, and human biopsies, and report that luminescence intensity varied across lesion types and stages. They propose this approach as a potential tool for investigating Notch signaling and for applications in CRC detection and monitoring. The manuscript is clearly written, easy to follow, and presents promising results. I read the manuscript carefully. It is clear that the Jag1-Fluc probe binds to the Notch receptor and that light emission can be detected through the activity of the covalently linked firefly luciferase. As mentioned in the introduction, Notch receptors are overexpressed in aggressive subtypes of maligant cells, but this does not necessarily mean they are absent in non-malignant cells, correct? I understand that the aim of the assay is to enable the detection and quantification of malignant cells. However, the strategy was not entirely clear to me. From what I understood, the procedure would involve removing a polyp from the patient and performing the assay with Jag1-Fluc. In this case, the result would likely be positive, since Notch is generally expressed. Or is it not always expressed? If it is, how could the assay distinguish between basal expression and the overexpression associated with malignancy? I believe a comment on this direction should be included in the Discussion.

We thank the Reviewer for this important comment. Indeed, even if the overexpression of some Notch signaling components is correlated with CRC progression and metastasis** [1]**, Notch signaling is crucial for intestinal cell differentiation promoting absorptive over secretory fate, stem cell maintenance, immune modulation, and overall tissue homeostasis, playing a fundamental role in the integrity and function of the gastrointestinal tract** [2–4]**. As mentioned in the manuscript, the Notch family is formed by four different receptors (Notch1–4), three Delta ligands (Dll1, 2, and 4), and two Jagged ligands (Jag1 and 2). The ligand binding to the receptor and its subsequent activation is tightly regulated by Fringe glycosyl-transferases (Mfng, Lfng and Rfng). When Notch receptors are glycosylated by Mfng and Lfng, they reduce their affinity for Jagged and become more responsive to Delta-like ligands instead** [5]**. So, differential usage of Notch ligands has been associated with expression of Fringe glycosyltransferases.

Interestingly, Jag1 ligand is not strictly required for intestinal homeostasis, exerting pro-oncogenic function in the gut in Mfng-low tumors, thus opening the possibility of targeting Jag1 in human colorectal cancer patients with less or no intestinal side effects** [6, 7]**. In this context, we developed the Jag1-Fluc recombinant protein, to specifically quantify the binding of Jag1 with Notch receptors. To the best of our knowledge, this is the first attempt to specifically focus on and quantify the binding of Jag1 with Notch receptors. Indeed, even if a very small subset of patient samples were analyzed, Figure 5 showed a significant increase in light emission from polyps to adenomas.

We discussed this important point in the "discussion" paragraph, highlighting the text in yellow.

Minor corrections:

p.8, l. 7: Do not initiate the sentence with numbers. I suggest: "From 90 to 95%…".

We thank the Reviewer for this suggestion. We have modified the sentence accordingly.

p. 9, l. 22: "…poor survival" of CRC patients?

We thank the Reviewer for pointing out this unclear expression. We have revised the sentence to clarify that it refers to the overall poor survival rate of CRC patients. The corrected text now reads: "…which is associated with poor survival rates in CRC patients"

p. 13, section 2.5: I fully understand that the term bioluminescence can, in a broader sense, be applied to any chemical process that emits light, but I recommend that in this

manuscript it be used exclusively to describe light produced by living cells through a chemical reaction. For other cases, such as in vitro assays with cell-free or other "dead-cell" components, I suggest using the terms chemiluminescence or light emission instead. In the specific case of section 2.5 and 2.10 it is not bioluminescence, but chemiluminescence, luminescence or simply light emission. The same is true for sections 3.1 and 3.5: the term "BL" can be simply omitted or replaced by "light mission" in the text and in Figure 1. There are more cases along the text. In summary, living cells = bioluminescence.

We thank the Reviewer for this important clarification. We revised the manuscript to consistently use the term "bioluminescence" only when referring to light emission from living cells, while "light emission" or "luminescence" are now used for cell-free and other in vitro assays. The changes were made throughout the text, figures, and supplementary material.

p. 19, l. 7 from bottom: Figure 2 shows a linear correlation between the emitted light and the concentration of Jag1-Fluc, rather than a saturation profile. Hence, in this sentence "A 1h exposure time was chosen for subsequent experiments, as it yielded the highest BL signal.", if the criterion were to reach maximum intensity, a study with a longer incubation period would have been required until saturation was observed. I also acknowledge that excessively long incubation times would not be appropriate. I recommend that the sentence be reformulated to take these points into account: maximum intensity at appropriated incubation time.

We thank the Reviewer for the helpful comment, since the term "highest BL signal" wasn't appropriate in this context. We rephrased as follows "A 1h exposure time was chosen for subsequent experiments, as it yielded the highest sensitivity in respect with 15 and 30 min of incubation (data not shown). We didn't expose cells to Jag1-Fluc for more than 1h to avoid cell survival issues" (text highlighted in yellow in the manuscript).

p. 25, l. 11 from bottom: In this context, we developed a chemiluminescent assay using a recombinant protein combining the ECD…

We thank the Reviewer for this observation. In the new version of the manuscript, the sentence has been revised as follows: "In this context, we developed a luminescent assay using a recombinant protein combining the ECD …"

Reviewer #2

The manucript by A. Punzo and collaborators is well written, original and methodologically supported. They describe the use of a new fusion protein involving firefly luciferase and Jag1 to detect and investigate Notch receptors disregulation in tumor cells. The method using bioluminescence is quite sensitive and promising for early diagnosis of some type of cancers, especially colorectal cancer which shows overexpression of Jag1 and Notch receptors. One thing that I missed, however, is whether there are alternative methods that were already proposed to detect Notch receptor or Jag1, using fluorescence or other optical methods. If there are, the authors should comment them in the Introduction, and discuss the advantages of their method in relation to other ones. Otherwise, the authors should emphasyze their new approach to investigate and early diagnosis of cancer. Overall, I recommend to accept the manuscript.

We thank the Reviewer for this important comment. Indeed, even if the overexpression of Notch signaling components is correlated with CRC progression and metastasis *[1]****, the involvement of the interaction between Jag1 and Notch receptors in precancerous stages is less explored. For this reason, we developed a recombinant protein combining the ECD of a high-affinity mutated form of Jag1 with red-emitting firefly luciferase (Fluc). This construct was designed to specifically assess if Jag1-Fluc binds to Notch receptors and whether its light emission upon binding correlates with increasing severity of precancerous colonic lesions.***

Common analytical methods used to detect Notch receptors and ligands in polyps, gut adenomas, and CRCs are mRNA expression analysis by quantitative reverse transcription PCR (qRT-PCR), in situ hybridization, western blot, immunohistochemistry (IHC), and transcriptomic approaches (RNA-Seq, microarrays). Almost all these approaches are time and cost-consuming, while Western Blot and IHC, even if used as a routine analysis in clinical practice, don't allow reliable quantification of biomarkers mainly because they are semi-quantitative techniques with several limitations (i.e., subjective interpretation and scoring, non-linear signal and saturation, lack of absolute quantification standards). To overcome these issues, we developed a fast and inexpensive quantitative approach using light emission as the analytical strategy. In the new version of the manuscript, we added some comments in the "Introduction" paragraph, highlighted in yellow.

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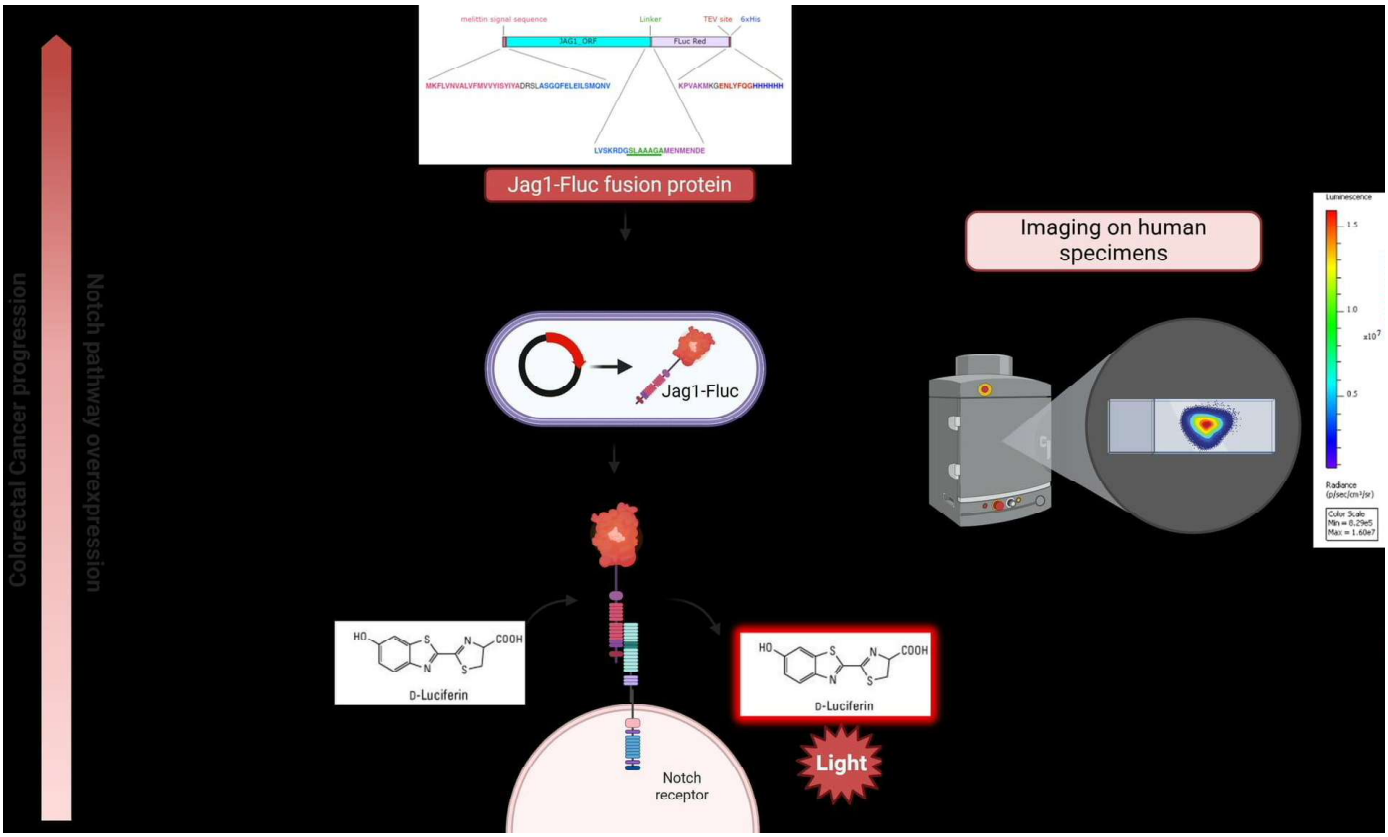
[6] Li, D. (2014) The Notch ligand Jagged1 as a target for anti-tumor therapy. *Front Oncol* **4** https://doi.org/10.3389/fonc.2014.00254

[7] López-Arribillaga, E., Rodilla, V. and Espinosa, L. (2019) Could JAG1 Protein Inhibition Prevent Colorectal Cancer? *Future Oncology* **15**, 345–347 https://doi.org/10.2217/fon-2018-0760


Colorectal Cancer progression
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melittin signal sequence
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Jag1-Fluc
Imaging on human specimens
Luminescence
Radiance (p/sec/cm²/sr)
Color Scale
Min = 8.29e5
Max = 1.60e7
HO
S
N
COOH
D-Luciferin
Notch receptor
Light