Pro-metastatic functions of Notch signaling is mediated by CYR61 in breast cells

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ABSTRACT

Metastasis is the main cause of cancer related deaths, and unfolding the molecular mechanisms underlying metastatic progression is critical for the development of novel therapeutic approaches. Notch is one of the key signaling pathways involved in breast tumorigenesis and metastasis. Notch activation induces pro-metastatic processes such as migration, invasion and epithelial to mesenchymal transition (EMT). However, molecular mediators working downstream of Notch in these processes are not fully elucidated. CYR61 is a secreted protein implicated in metastasis, and its inhibition by a monoclonal antibody suppresses metastasis in xenograft breast tumors, indicating the clinical importance of CYR61 targeting. Here, we aimed to investigate whether CYR61 works downstream of Notch in inducing pro-metastatic phenotypes in breast cells. We showed that CYR61 expression is positively regulated by Notch activity in breast cells. Notch1-induced migration, invasion and anchorage independent growth of a normal breast cell line, MCF10A, were abrogated by CYR61 silencing. Furthermore, upregulation of core EMT markers upon Notch1-activation was impaired in the absence of CYR61. However, reduced migration and invasion of highly metastatic cell line, MDA MB 231, cells upon Notch in-hibition was not dependent on CYR61 downregulation. In conclusion, we showed that in normal breast cell line MCF10A, CYR61 is a mediator of Notch1-induced pro-metastatic phenotypes partly via induction of EMT. Our results imply CYR61 as a prominent therapeutic candidate for a subpopulation of breast tumors with high Notch activity.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer related deaths among women (Ferlay et al., 2013). Metastasis is the main reason of the deaths, decreasing five-year survival rates from 85 % for patients with regional metastasis to 27 % in case of distant metastasis (Howlader et al., 2017). Uncovering the molecular mechanisms underlying metastatic progression is of utmost interest to define targetable molecules for the development of novel therapeutic approaches.

Notch signaling, which is long known for its importance in devel-opment and tissue homeostasis, is also one of the key signaling path-ways involved in metastasis in several cancer types including breast (Siebel and Lendahl, 2017). Upon interaction of Notch receptors (Notch1-4) with ligands (Delta-like-ligand1, -3, -4 and Jagged1, -2), two subsequent cleavages release Notch intracellular domain, which translocates to the nucleus to activate its target genes. High expression levels of Jagged1 and Notch1 in gastric cancer (Yeh et al., 2009) and both Jagged1 and Jagged2 in breast cancer patients (Sethi et al., 2011; Xing et al., 2011) were correlated with invasiveness and metastasis, pointing to the clinical importance of Notch signaling in metastatic progression. At the cellular level, several cell line studies revealed that Notch activation induces pro-metastatic cellular processes, including migration, invasion, and epithelial to mesenchymal transition (EMT) in glioma (Zhang et al., 2012), hepatocellular carcinoma (Wang et al., 2012; Zhou et al., 2012), lung (Matsuno et al., 2012; Xie et al., 2012), stomach (Hsu et al., 2012) and pancreas (Bao et al., 2011) cancers. Specifically in breast cancer cell lines, Notch signaling activation by different factors such as hypoxia (Xing et al., 2011), IL6 signaling (Studebaker et al., 2008) or KLF4 expression (Yu et al., 2011), increased migration and invasion abilities of the cells, while its inhibition reduced brain metastasis of breast cancer cell line MDA MB 231 (McGowan et al., 2011). In parallel, Notch activation at the downstream of hypoxia or TGFβ signaling induced EMT, along with upregulation of the core

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EMT regulators such as SNAI1 and SNAI2 (also known as Slug, a direct target of Notch pathway) (Chen et al., 2010; Zhang et al., 2010). NFκB (Wang et al., 2006; Zhang et al., 2012), GATA3 (Yang et al., 2011) and STAT3 (Hsu et al., 2012) were implicated in downstream of Notch signaling in inducing metastatic phenotypes in glioblastoma, pancreas, lung and stomach cancers. In breast cancer cell lines, Jagged2 expression and consequent Notch activation was shown to induce invasion through AKT phosphorylation (Xing et al., 2011). Apart from the few molecules mentioned above, downstream mediators of Notch during induction of metastasis are not fully elucidated. Distinct functions of different Notch receptors in breast cancer and metastasis await further investigation. Here, we mainly focused on Notch1, which is one of the first receptors to be associated with breast cancer. It has been widely studied in different cancer types and was shown as an important player for disease progression in different contexts (Guo et al., 2011; Ranganathan et al., 2011).

CYR61 (also known as CCN1) is a secreted protein that has a well-established pro-tumorigenic role in breast cancer. Its high expression is correlated with poor prognosis and lymph node metastasis (Jiang et al., 2004; Tsai et al., 2000). CYR61 overexpression increased migration and invasion, while its silencing decreased invasion and transendothelial migration of breast cancer cells (Huang et al., 2017; Li et al., 2012; Sanchez-Bailon et al., 2015; Sarkissyan et al., 2014). In xenograft models, CYR61 was implicated in metastasis of breast cancer cells mainly through generation of a pro-angiogenic tumor microenvironment and increased vascularization, which correlate with its ability to promote vascular endothelial growth factor secretion (Espinoza et al., 2014; Harris et al., 2012; Tsai et al., 2002). Silencing of CYR61 reduced extravasation of breast cancer cells to lung and subsequent lung metastasis (Huang et al., 2017). Furthermore, induction of Vimentin, Twist and N-cadherin in response to CYR61 overexpression in pancreatic cancer and osteosarcoma cells suggest that CYR61 might exert its metastatic functions through regulation of EMT (Haque et al., 2012, 2011).

In this study, we investigated whether CYR61 plays a role in the downstream of Notch signaling to develop a pro-metastatic phenotype in breast cells. We found that CYR61 expression is regulated by Notch activity both in normal breast (MCF10A) and breast cancer (MDA MB 231) cell lines. We showed that in MCF10A cells, CYR61 is required for Notch1-induced migration, invasion and anchorage independent growth. Furthermore, Notch1 activation was only able to upregulate mesenchymal markers in the presence of CYR61, suggesting that EMT could be the underlying mechanism in the manifestation of pro-metastatic phenotypes exerted by Notch1 – CYR61 interaction.

2. Materials and methods

2.1. Cell lines, viral infections and treatments

MCF10A and MDA MB 231 cells were obtained from ATCC. MCF10A were cultured in high glucose containing DMEM-F12 (GIBCO) supplemented with 5 % Horse Serum (Biological Industries), 20 ng/ml EGF (Sigma), 0.5 µg/mL Hydrocortisone (Sigma), 100 ng/ml Cholera Toxin (Sigma), 10 µg/mL Insulin (Sigma) and 1 % Penicillin/Streptomycin (GIBCO). MDA MB 231 breast cancer cell line was cultured in high glucose containing DMEM (GIBCO) supplemented with 10 % Fetal Bovine Serum (FBS) (GIBCO) and 1 % Penicillin/Streptomycin (GIBCO). All cell lines were maintained in a humidified incubator with 5 % CO_2 at 37 °C. Notch signaling was activated by overexpression of Notch1 intracellular domain using either MSCV-NICD retrovirus (Yalcin-Ozuysal et al., 2010) or pLenti-GFP-NICD lentivirus. NICD cDNA from MSCV-NICD was subcloned into pLenti-GIII-CMV-GFP-2A-Puro (abm) with BamHI and XbaI for GFP labeling in addition to Notch activation. Notch activity was inhibited by silencing of canonical Notch mediator CSL via shRNA expressed from pLKO.1 vector (shCSL) (Procopio et al., 2015; Zengin et al., 2015) or 90 µM DAPT treatment. CYR61 was silenced by expression of shRNA from pLKO.1 vector

(shCYR61) and CYR61 cDNA was overexpressed using lentiviral vector (Huang et al., 2017). Empty MSCV or pLenti-GFP vectors for NICD and CYR61 expression, and shRNA against GFP for shCSL and shCYR61 were used as controls. Equal volume of DMSO was used as control for DAPT treatments. Viruses were prepared and cells were infected as described previously (Yalcin-Ozuysal et al., 2010; Zengin et al., 2015). Stable cell lines were generated by 10 µg/ml puromycin selection. Viral titrations were assessed on NIH-3T3 cells, which were seeded on 6-well plates and infected with serial virus dilutions. 48 h after infection the cells were transferred to 10 cm plates in the presence of 10 µg/ml puromycin. Puromycin selection continued until uninfected cells die. Then colonies formed in every virus dilution were counted and viral preps that have equal colony forming capacity were used in the experiments.

2.2. RNA isolation and qRT-PCR

Total RNA was isolated by using Pure-link RNA Mini Kit (Ambion) and treated with PureLinkTM DNase (Invitrogen) to prevent DNA contamination. cDNA was synthesized with Fermantas First Strand cDNA Synthesis Kit (Thermo Scientific) from 1 µg total RNA using random hexamer primers. PCR amplification and detection was done on Roche- LightCycler 96 Real Time PCR Detection System using Maxima SYBR Green qPCR Master Mix (Thermo Scientific). TATA box binding protein (TBP) was used for normalization as housekeeping gene and relative mRNA levels were calculated using delta-delta Ct method. Each experiment is normalized to its own control condition. At least three independent experiments were done and Student's *t*-test method was used for statistical analysis. Following primer pairs were used: CYR61 5′-AAGGGGCTGGAATGCAACTT-3′, 5′-CTGCCCGGTAACTTTGACCA-3′; HEY2 5′-AAGATGCTTCAGGCAACAGG-3′, 5′-GCACTCTCGGAATCCTA TGC-3′; Jagged1 5′-GACTCATCAGCCGTGTCTCA-3′, 5′-GGGGAACACT CACACTCAAA-3′; Jagged2 5′-CAATGGTGGCATCTGTGTTG-3′, 5′-GCG ATACCCGTTGATCTCAT-3′; Notch1 5′-CACTGCGAGGTCAACAC AGA-3′, 5′-GCACACTCGTCCACATCGTA-3′; Notch2 5′-TGTGCCTCAAA TCCATGCCT-3′, 5′-ATGGTACACCGCTGACCTTG-3′; Notch3 5′-GCAGC GATGGAATGGGTTTC-3′, 5′-CTGCCAGGTTGGTGCAGATA-3′; Notch4 5′-TTCCACTGTCCTCCTGCCAGAA-3′, 5′-TGGCACAGGCTGCCTTGGA ATC-3′; SNAI1 5′-CTAGGCCCTGGCTGCTACAA-3′,5′-TGTGGAGCAGGG ACATTCG-3′; SNAI2 5′-CTCCTCATCTTTGGGGCGAG-3′, 5′-TTCAATGG CATGGGGGTCTG-3′; TBP 5′-TAGAAGGCCTTGTGCTCACC-3′, 5′-TCTG CTCTGACTTTAGCACCTG-3′; ZEB15′−CCCAGGTGTAAGCGCAGAAA-3, 5′-GTCTGGTCTGTTGGCAGGTC-3′; ZEB2 5′-ATAAGGGAGGGTGGA GTGGAA-3′, 5′-GTTAATTGCGGTCTGGATCGTG-3′.

2.3. Protein isolation and western blot analysis

Total protein was isolated by freshly prepared RIPA Lysis Buffer and homogenized with 26 G syringe. Protein concentrations were determined with Bradford assay. 60 µg of protein sample were run on SDS-Gel and transferred to PVDF membranes for detection by chemiluminescence by using Vilber Fusion SL Imaging System. Images were quantified by ImageJ Gel Analysis Tool. Intensity values of related bands were normalized to values of Beta-actin housekeeping protein. Student's *t*-test was used for statistical calculations. Following primary antibodies were used: anti-β-actin (Abcam, AB75186, 1/3000), anti-CYR61 (Abcam, AB127988, 1/400), anti-HEY2 (Abcam, AB184246, 1/500), anti-SNAI2 (Cell Signaling Technology, 9585S, 1/1000), anti-Vimentin (Cell Signaling Technology, 5741 P, 1/1000).

2.4. Wound healing migration assay

24 h after seeding 1×10^6 MCF10A and 7.5×10^5 MDA MB 231 cells/well on 12-well plates, cells were treated with a final concentration of 10 µg/ml mitomycin C for two hours. Then, scratch was introduced with a 10 µl pipette tip and mitomycin C containing medium

was replaced with serum free medium. Cells were observed under Leica DMI8 confocal microscope in incubation chamber for 3 days at 37 °C with 5 % CO_2. Three positions per well were monitored and the open areas were quantified by MRI Wound Healing Tool macro on ImageJ. Open area percentages were calculated for each position in three independent experiments and Student's *t*-test was used for statistical analysis.

2.5. Invasion analysis

Three channel lab-on-a-chip system (Initio) was used for invasion analysis (Fig. S4). Growth factor reduced matrigel (BD) was mixed with pre-cooled serum free medium in 1:1 ratio, loaded into the middle channel and polymerized at 37 °C for 30 min. Then, lower channel was loaded with 20 % serum containing medium and upper channel with GFP-labeled 1×10^6 MCF10A or MDA MB 231 cells/ml in serum free medium. Chips were incubated vertically at 37 °C, 5 % CO_2 in humidified incubator for three days. Each day chips were placed flat and visualized under Leica DMI8 confocal microscope. Each position was scanned throughout the matrigel for 500 μm and a Z-stack image was generated. For quantification, a specific threshold value for each day was applied to images of all conditions on the same experiment. Then, the distance of each bright pixel to the starting line segment, which defines the border of matrigel channel, was calculated by a Python program we developed. For each position, data are transformed in order to standardize Day 1 distribution to have a mean of zero and standard deviation of 1. Then, the distribution of distances were displayed using box plots on RStudio (RStudio Team, 2016). Student's *t*-test was used for statistical analysis.

2.6. Soft agar colony formation assay

30,000 cells/well in 0.35 % noble agar (BD Difco) were plated on top of a solidified layer of 0.5 % noble agar prepared in growth medium in 6-well plates. Cells were fed with fresh growth medium twice a week for 8 weeks. Then, colonies were stained with 0.005 % crystal violet and analyzed under Leica DMI8 confocal microscope. For each condition, five fields per well were analyzed. From each field, three images (each focused on a different Z layer) were taken. Only the colonies that were in focus and bigger than 30 μm in diameter were counted. Total colony numbers were normalized to control condition in each of three independent experiments. Student's *t*-test was used to calculate statistical significance.

3. Results

3.1. CYR61 expression is regulated by Notch signaling in breast cell lines

In order to understand whether CYR61 expression is controlled by Notch signaling, we activated this pathway in normal breast epithelial cell line MCF10A, which has very low levels of endogenous Notch activity (Mazzone et al., 2010; Rustighi et al., 2009; Shao et al., 2015; Stylianou et al., 2006). Overexpression of the active form of the Notch1 receptor is known to induce transformation, resistance to apoptosis, epithelial to mesenchymal transition, migration and invasion in MCF10A cells (Mazzone et al., 2010; Rustighi et al., 2009; Stylianou et al., 2006; Zhang et al., 2015). So, we activated Notch signaling via overexpression of Notch1 intracellular domain (NICD) as well and used expression of one of the well-known Notch target genes, HEY2, as a readout for pathway activation. HEY2 mRNA expression was increased by 250 fold (Fig. 1A), while its protein expression was upregulated by 3.6 fold (Fig. 1B and C), indicating that Notch1 is activated successfully in MCF10A cells. In response to Notch1 activation, CYR61 expression was increased by 9 fold at the mRNA level (Fig. 1A) and by 26 fold at the protein level (Fig. 1B and C). CYR61 was previously shown to positively regulate Jagged1 expression and Notch1 intracellular domain in

pancreatic cancer cell lines (Haque et al., 2012; Kim et al., 2015). Thus, we overexpressed CYR61 to test whether it regulates Notch pathway components in MCF10A cells as well. We observed that overexpression of CYR61 did not affect the mRNA expression of Notch receptors or Jagged ligands (Fig. S1). Although our data do not exclude the possibility that CYR61 acts upstream of Notch signaling in MCF10A cells, it indicates that there is no regulation at the transcriptional level.

In order to elucidate whether transcriptional control of CYR61 by Notch signaling could be extended to different breast cell types, in MDA MB 231 cells we silenced CSL (also called RBPjκ), the transcriptional mediator of canonical Notch signaling that works downstream of all the Notch receptors. MDA MB 231 is a metastatic basal type breast cancer cell line that expresses all the Notch receptors and has high endogenous Notch activity (Azzam et al., 2013; Bolos et al., 2013; Harrison et al., 2010; Nagamatsu et al., 2014; Stylianou et al., 2006). Infection of MDA MB 231 cells with shRNA against CSL (shCSL) decreased HEY2 mRNA expression by 70 % (Fig. 1 D) and protein expression by 63 % (Fig. 1E and 1 F), indicating the inhibition of Notch activity. Then, we analyzed expression of CYR61 and showed that both mRNA (Fig. 1D) and protein (Fig. 1E and 1 F) levels were decreased by 55 %.

Taken together, our data showed that mRNA and protein expression of CYR61 is positively regulated by Notch signaling activity in both normal breast and breast cancer cell lines.

3.2. CYR61 is a mediator of Notch1-induced migration and invasion

Since both Notch signaling and CYR61 are implicated in metastasis of breast cancer, next we investigated whether CYR61 plays a functional role downstream of Notch signaling in pro-metastatic behaviors of breast cells. First, we generated two conditions in which Notch signaling was activated in the absence or presence of CYR61. For this purpose, we co-infected MCF10A cells with one type of virus for overexpression of Notch1 Intracellular domain (NICD) and another one for silencing of CYR61 (shCYR61). We showed that Notch1-induced CYR61 upregulation was abrogated by the expression of shRNA against CYR61 (shCYR61) (Fig. S2A and S2B). In NICD overexpressing cells, CYR61 protein levels decreased to 31 % in response to shCYR61 infection (Fig. S2C).

We analyzed the effects of CYR61 silencing on Notch1-induced migration by wound healing assay. At all time points we analyzed after scratching, MCF10A cells overexpressing NICD (NICD – Control) closed the wound better than control infected cells (Fig. 2A). On the other hand, cells with active Notch1 receptor failed to close the gap as quickly when CYR61 was silenced (NICD – shCYR61) (Fig. 2A). Open area of the control cells (Control – Control) declined slowly over time, to 84 %, 57 % and 30 % at 12, 24 and 36 h, respectively (Fig. 2B). Cells with active Notch1 signaling in the presence of endogenous CYR61 (NICD – Control) closed the gap faster, showing 69 %, 28 % and 12 % of open area at the respective time points (Fig. 2B). However, percentage of open area was significantly increased in the absence of CYR61 (NICD – shCYR61) to 85 %, 68 % and 44 % at the same time points, regardless of Notch1 activation (Fig. 2B). These results indicate that CYR61 upregulation is required for Notch1-induced migration in MCF10A cells.

Next, we investigated whether Notch1-induced invasion is affected by the absence of CYR61. For this purpose, we utilized a three channel lab-on-a-chip system, which allows day by day visualization of cell invasion through 3D matrigel environment towards serum rich medium (Fig. S3). As expected, control MCF10A cells (Control – Control), non-invasive per se, did not move towards matrigel considerably, while Notch1 activation in the presence of endogenous CYR61 (NICD – Control) resulted in the invasion of MCF10A cells within matrigel towards serum (Fig. 2C and Fig. S4). Silencing of CYR61 (NICD – shCYR61) reduced Notch1-induced invasion to a level comparable to the control condition (Figs. 2C and S4). In order to quantify invasiveness of the cells, we applied threshold to the z-stack images, and then measured the distance of all the bright pixels to the border line between
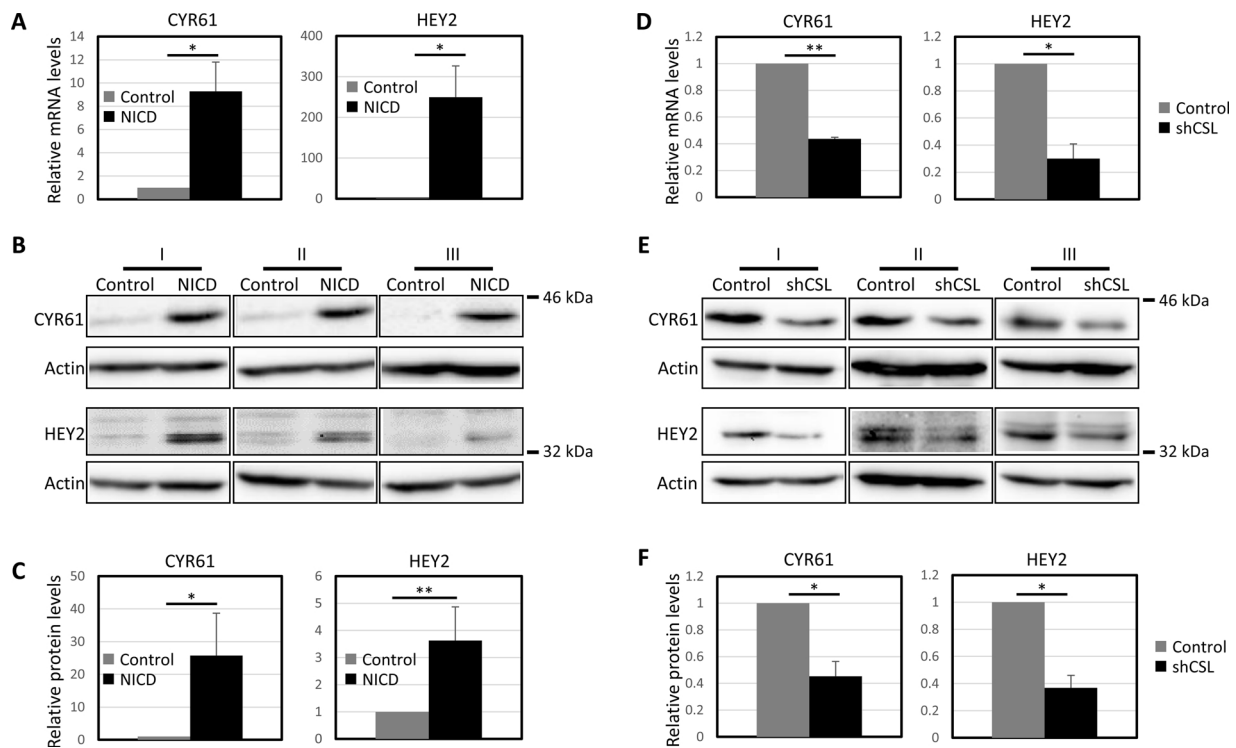
Fig. 1. CYR61 expression is positively regulated by Notch signaling in breast cells. (**A**) mRNA and (**B**) protein expressions of CYR61 and Notch target gene HEY2 were analyzed 48 h after infection of MCF10A cells with control or Notch1 intracellular domain (NICD) overexpressing virus. (**C**) Densitometric analysis of the above three western blots are shown. (**D**) mRNA and (**E**) protein expression of CYR61 and HEY2 were analyzed 48 h after infection of MDA MB 231 cells with virus expressing shRNA against Notch mediator CSL (shCSL) or GFP (Control). (**F**) Densitometric analysis of the above three western blots are shown. Relative expression levels were normalized to control. Data is represented as mean ± S.D. of three independent experiments. (I, II and III stand for three independent infection experiments.) (*$p < 0.05$, **$p < 0.009$).

matrigel and cell channels (Fig. 2C, vertical white dashed line segments). The quantification of three independent experiments (images shown in Figs. 2C and S4) together demonstrates that cells with Notch1 activity (NICD – Control) invaded longer distances than the control (Control - Control), while silencing of CYR61 significantly decreased the distance invaded even though Notch1 signaling was activated (Fig. 2D). Altogether, our data suggest that CYR61 is required for Notch1-induced invasion of MCF10A cells.

Migration and invasion of MDA MB 231 cells are impaired by γ-secretase inhibitors that prevent the cleavage of all the Notch receptors, thus inhibiting their activity. (Azzam et al., 2013; Harrison et al., 2010). We next asked whether these phenotypes could be attributed to reduced CYR61 expression upon Notch inhibition. For the migration and invasion assays, we treated MDA MB 231 cells with DAPT, a γ-secretase inhibitor. Similar to silencing of CSL, DAPT also works as a pan-Notch inhibitor. HEY2 mRNA expression was significantly reduced in response to DAPT treatment, suggesting that Notch signaling is inhibited (Fig. S5, right panel).For our purpose, we infected MDA MB 231 cells with either control or CYR61 overexpressing virus. Then, stably infected cells were treated with DAPT. As intended, in response to DAPT treatment CYR61 expression was downregulated by 50 % in control infected cells and upregulated by 2.9 fold in the overexpression group (Fig. S5, left panel).

Migration capacity of MDA MB 231 cells treated with DAPT in the presence or absence of CYR61 overexpression was assessed by wound healing assay (Fig. 3A). DAPT treatment (DAPT – Control) resulted in a significant increase in the percentage of open area 27 h after the treatment (Fig. 3B). Notably, CYR61 overexpression (DAPT – CYR61) did not rescue the wound closure defect in DAPT treated cells, but on the contrary, it reduced the closure rate significantly at all the analyzed time points (Fig. 3B). Conceivably, invasion of MDA MB 231 cells was reduced in response to DAPT treatment (DAPT – Control) (Figs. 3C and S6). In the presence of CYR61 overexpression (DAPT – CYR61), the

inhibitory effect of DAPT treatment was slightly reduced but an apparent rescue was not observed (Fig. 3D).

Altogether, our data show that Notch1-induced migration and invasion depend on CYR61 upregulation in normal cells. However, restoration of CYR61 expression is not sufficient to rescue anti-migratory or anti-invasive effects of Notch inhibition in metastatic breast cancer cells.

3.3. CYR61 is a mediator of Notch1-induced anchorage independent growth

Anchorage independent growth is considered as another pro-metastatic trait (Mori et al., 2009). Both Notch activity and CYR61 overexpression were independently shown to induce anchorage independent growth of cancer cells (Guo et al., 2011; Lin et al., 2004). Therefore, to further elucidate the role of CYR61 in pro-metastatic functions of Notch, we analyzed anchorage independent growth capacity of MCF10A clones in soft agar (Fig. 4A). In order to make sure that the stable clones maintained the expression patterns during soft agar assay, relative mRNA expression of HEY2 and CYR61 were analyzed. Notch activity remained high in NICD infected cells as demonstrated by increased HEY2 expression (Fig. S7, right panel). Furthermore, cells stably expressing NICD had increased CYR61 levels compared to control cells, while shCYR61 infection kept CYR61 levels lower (Fig. S7, left panel). Colony formation capacity in soft agar was increased by 1.9 fold in MCF10A cells with Notch1 activation (NICD – Control), while CYR61 silencing (NICD – shCYR61) reduced it significantly (Fig. 4B). Overall, our data indicate that CYR61 is a downstream mediator of Notch1 signaling in inducing anchorage independent growth.
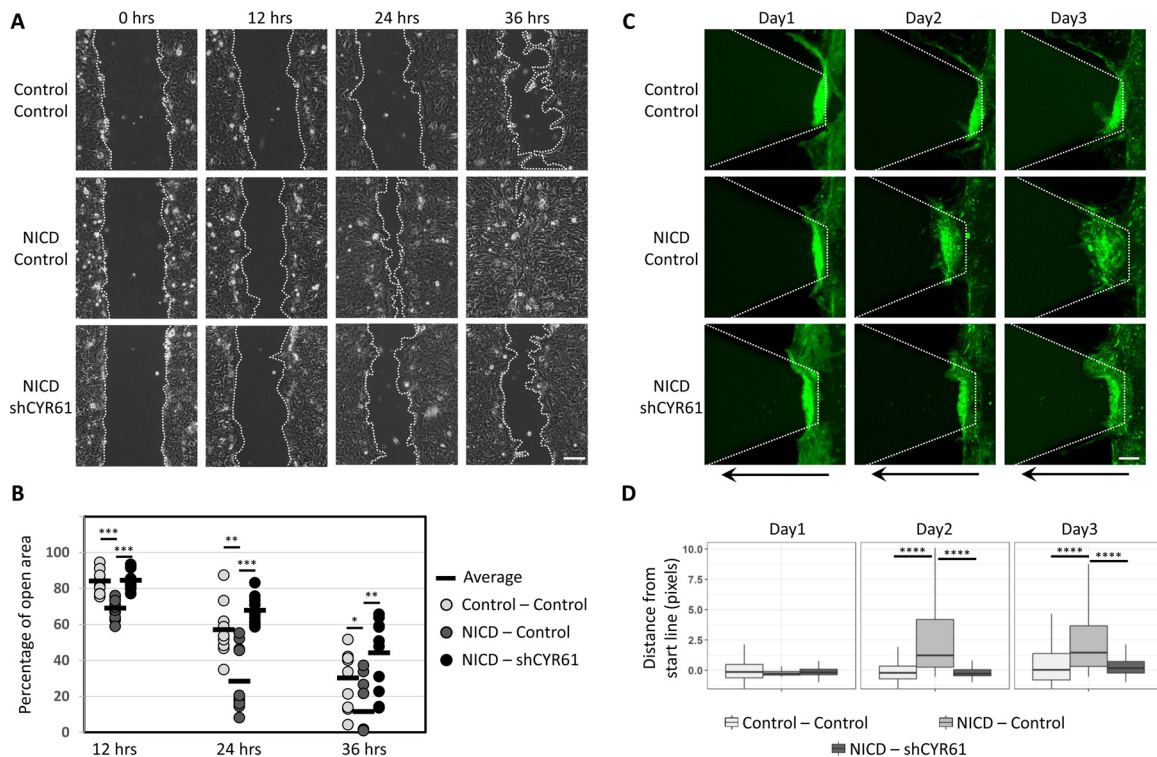
Fig. 2. CYR61 silencing impairs Notch1-induced migration and invasion of MCF10A cells. (**A**) Representative images of MCF10A cells with Notch1 activation in the absence (NICD – shCYR61) or presence (NICD – Control) of CYR61 at 0, 12, 24 and 36 h after scratching are shown. (**B**) Percentage of open area for each condition are plotted for 12, 24 and 36 h after scratching. Each dot represents one area analyzed and line segment represents average of three independent experiments. (**C**) Z-stack images of GFP labeled MCF10A cells with Notch1 activation in the absence (NICD – shCYR61) or presence (NICD – Control) of CYR61 at day 1, 2 or 3 after loading are shown. Dashed lines mark the middle channel filled with matrigel. Black arrows show direction of invasion. The images of one representative experiment out of three are shown. (**D**) Following thresholding of the Z-stack images, distance of each bright pixel to starting line (vertical dashed line indicating the beginning of matrigel channel) was calculated in three independent experiments. All the distance values from three independent experiments were plotted after the data were normalized to day 1 for each condition. (Scale bar: 100 μm) (*$p < 0.02$, **$p < 0.07 \times 10^{-2}$, *** $p < 0.03 \times 10^{-3}$, **** $p < 0.05 \times 10^{-10}$).

3.4. CYR61 is required for Notch1-induced upregulation of mesenchymal markers

Epithelial to mesenchymal transition (EMT) is a process that plays a critical role in many stages of cancer progression including migration, invasion, stem cell traits and metastasis (Lamouille et al., 2014). Therefore, we investigated whether the deficiency in Notch1-induced migration, invasion and anchorage independent growth upon CYR61 silencing could be explained by impaired EMT. We analyzed the expression of EMT core regulators, SNAI1, SNAI2 (also known as Slug), ZEB1 and ZEB2 in response to Notch1 activation in the presence or absence of CYR61. mRNA expression levels of all the core regulators were increased upon Notch1 activation, but reduced significantly when CYR61 was silenced (Fig. 5A). The same pattern was observed for protein expression level of SNAI2, which was increased by approximately 3 fold upon Notch1 activation and decreased significantly in the absence of CYR61 (Fig. 5B, and 5C). In addition, protein expression of Vimentin increased 3-fold upon Notch1 induction and decreased with CYR61 inhibition (Fig. S8A and S8.B).

In MDA MB 231 cells, CYR61 mRNA and protein expression levels were significantly reduced in cells treated with DAPT (DAPT – Control) (Fig. S9A right panel, Fig. S9B and Fig. S9C right panel). EMT core regulator SNAI2 protein levels were strikingly downregulated in response to Notch pathway inhibition by DAPT treatment (DAPT – Control) and remained low despite CYR61 overexpression (DAPT – CYR61) (Fig. 5D and 5E). However, SNAI2 mRNA expression was not significantly altered upon either DAPT treatment or CYR61 overexpression (Fig. S9A left panel). A similar expression pattern was also observed in mRNA and protein expression level of Vimentin (Fig. S9A middle panel, Fig. S9B and Fig. S9C left panel).

Overall our data suggest that transcriptional regulation of CYR61 by Notch signaling activity is conserved between normal and cancer breast cell lines. However, functional requirements for upregulation of CYR61 expression in Notch induced migration and invasion depends on the cellular context, which correlates with the ability to elevate SNAI2 protein levels.

4. Discussion

Small molecule inhibitors that target γ-secretases and monoclonal antibodies against Notch receptors and ligands are being tested in a significant number of clinical trials against several cancer types including breast and pancreatic cancers (Lamy et al., 2017; Takebe et al., 2014). Thus, it is crucial to understand the downstream molecular networks that control the outcome of Notch activation or inhibition in different contexts. In this respect, to the best of our knowledge, we showed for the first time that CYR61 is a mediator of Notch1 induced pro-metastatic phenotypes in normal breast cells partly via induction of EMT. CYR61 – Notch interaction has been previously investigated in endothelial cells and pancreatic cancer. In endothelial cells, CYR61 activated Notch signaling in an integrin-dependent manner (Chintala et al., 2015). In pancreatic cancer cell lines, silencing of CYR61 reduced Jagged1 expression and Notch1 intracellular domain protein levels placing CYR61 upstream of Notch signaling as a positive regulator (Haque et al., 2012; Kim et al., 2015). In the present study, we showed that CYR61 did not affect mRNA expression of Notch receptors or ligands in MCF10A cells (Fig. S1), failing to suggest that it acts upstream of Notch. Furthermore, in pancreatic cancer cells inhibition of Notch signaling by DAPT (γ-secretase inhibitor) treatment had no effect on CYR61 expression (Haque et al., 2012), contrary to the substantial
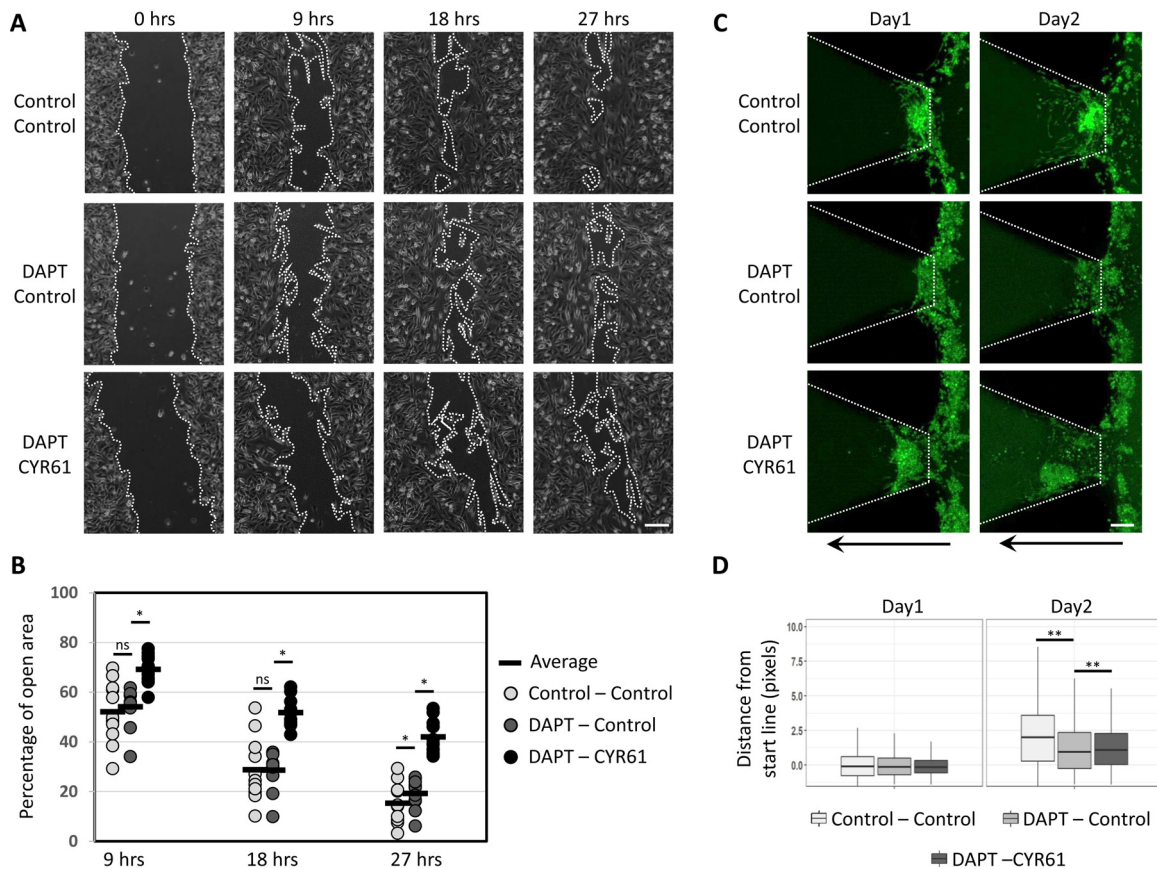
Fig. 3. Overexpression of CYR61 does not rescue impaired migration and invasion of MDA MB 231 cells upon DAPT treatment. (**A**) Representative images of MDA MB 231 cells treated with Notch inhibitor DAPT in the absence (DAPT – Control) or presence (DAPT – CYR61) of CYR61 overexpression at 0, 9, 18 and 27 h after scratching are shown. (**B**) Percentage of open area for each condition are plotted for 9, 18 and 27 h after scratching. Each dot represents one area analyzed and line segment represents average of three independent experiments. (**C**) Z-stack images of GFP labeled MDA MB 231 cells treated with Notch inhibitor DAPT in the absence (DAPT – Control) or presence (DAPT – CYR61) of CYR61 overexpression at day 1 or 2 after loading are shown. Dashed lines mark the middle channel filled with matrigel. Black arrows show direction of invasion. The images of one representative experiment out of three are shown. (**D**) Following thresholding of the Z-stack images, distance of each bright pixel to starting line (vertical dashed line indicating the beginning of matrigel channel) was calculated in three independent experiments. All the distance values from three independent experiments were plotted after the data were normalized to day 1 for each condition. (Scale bar: 100 μm) (*$p < 0.05 \times 10^{-3}$, ** $p < 0.05 \times 10^{-10}$).

evidence we provide here that both DAPT treatment and CSL silencing reduced CYR61 mRNA and protein levels in breast cancer cell line MDA MB 231. Overall, the way Notch signaling and CYR61 interact should be considered as a tissue specific process, which can go in both directions.

Here, we focused on Notch1 receptor in MCF10A cells, but it is plausible to consider that Notch – CYR61 interaction might not be limited to a single Notch receptor. In MDA MB 231 cells, CYR61 expression was downregulated in response to Notch inhibition via silencing of CSL or treatment of DAPT, both of which inhibit the Notch pathway through all four receptors. In recent years, Notch3 was implicated in aggressive phenotypes such as resistance to therapy (Diluvio et al., 2018; Sansone et al., 2016) and invasiveness in both breast cancer (Leontovich et al., 2018) and T-cell acute lymphoblastic leukemia (T-ALL) (Franciosa et al., 2016). In T-ALL, both Notch1 and Notch3 act through the same mediator, CXCR4, which is associated to hypoxic tumor environment that enhances metastasis, same as CYR61 (Ferrandino et al., 2018; Kuonen et al., 2012; Pitt et al., 2015). T-ALL tumor cells that overexpress active Notch1 receptor were positive for CXCR4 that kept them in contact with vascular endothelial cells in the bone marrow niche, and increased infiltration of tumor cells to distant tissues such as liver and lung (Pitt et al., 2015). In another T-ALL model that was generated by overexpression of Notch3 ICD, tumor cells also expressed high levels of CXCR4 (Ferrandino et al., 2018). In parallel with the Notch1 T-ALL model, migration of Notch3 ICD positive cells from thymus to bone marrow and infiltration to spleen were dependent

on CXCR4 function (Ferrandino et al., 2018). Therefore, whether CYR61 acts downstream of Notch3, similar to Notch1, in induction of pro-metastatic traits in breast cancer is a legitimate question that requires further investigation.

The pro-tumorigenic role of CYR61 in breast cancer was mainly established in cancer cell lines. Specifically, inhibition of CYR61 suppressed migration, invasion and metastasis of MDA MB 231 cells (Huang et al., 2017; Sanchez-Bailon et al., 2015). Reduced CYR61 also sensitized MDA MB 231 cells to anoikis (Huang et al., 2017), which is consistent with our observation showing that Notch1-induced anchorage independent growth being impaired upon CYR61 silencing in MCF10A cells. Overall, our findings demonstrate that the pro-metastatic role of CYR61 also applies to an immortalized yet normal breast cell line, MCF10A, downstream of Notch signaling. Thus, the data we present expand the implications of CYR61 inhibition to the earlier stages of tumorigenesis, which is modeled here by a normal cell line, MCF10A, acquiring pro-metastatic traits by Notch1 activation.

In MDA MB 231 cell line, which models advanced stages of breast cancer and is highly metastatic, CYR61 overexpression failed to rescue anti-migratory and anti-invasive phenotypes observed upon Notch inhibition. This result suggests that at the advanced stage of breast cancer, Notch activity regulates a range of downstream molecules and its overall function could not be attributed to CYR61 alone. This observation could also be associated with a negative feedback loop that regulates molecules acting in migration. In addition to the downstream
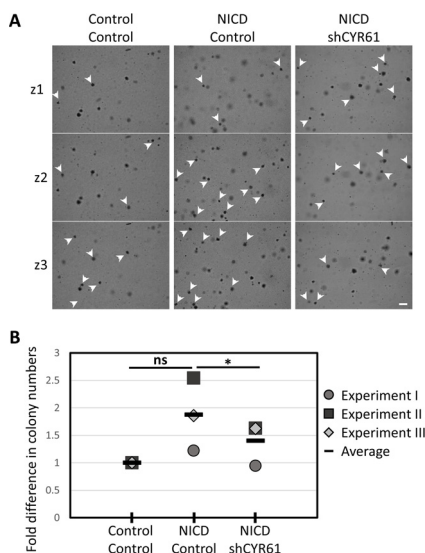
Fig. 4. CYR61 silencing reduces the number of soft agar colonies formed by MCF10A cells with active Notch1 signaling. (**A**) Representative images from three different focal planes of the same area showing soft agar colonies of MCF10A with Notch1 activation in the absence (NICD – shCYR61) or presence (NICD – Control) of CYR61. Only colonies in focus and are larger than 30 μm in diameter were counted. Arrows indicate the counted colonies in each focal plane. (Scale bar: 100 μm). (**B**) Quantification of three independent experiments are shown. Total colony numbers were normalized to control condition for each experiment. (I, II and III stand for three independent experiments.) (*p < 0.05).

role of CYR61 in the Notch pathway shown here, CYR61 is also a target of YAP/TAZ complex, which is involved in the regulation of tissue homeostasis and cancer development as the major effectors of Hippo pathway (Zhang et al., 2011). YAP/TAZ was shown to activate its own negative regulators LATS1/2, which in turn limit cell migration induced

by YAP activation (Moroishi et al., 2015). Overexpression of CYR61 in MDA MB 231 cells could trigger a similar negative feedback loop, which would result in reduced migration.

Our results suggest that SNAI2 could have a central role in EMT deregulation at the early stages of Notch induced breast tumorigenesis. Although we observed a positive correlation between expression of CYR61 and SNAI2 in MCF10A cells, decrease in SNAI2 expression upon Notch inhibition could not be rescued by CYR61 overexpression in MDA MB 231 cells. Since failure of CYR61 overexpression to restore SNAI2 protein levels in MDA MB 231 cells correlates with inability to rescue impaired migration and invasion phenotypes, SNAI2 emerges as a key player in Notch induced pro-metastatic traits. Apart from its core role in EMT, it was also reported and consistent with our findings that SNAI2 is an indispensable player for migration, invasion and metastasis of different cancer cell types including breast; acting downstream of different signals such as Notch, NK-κB, AKT, VEGF, RUNX2 and p63 (Chimge et al., 2011; Dang et al., 2015; Fenouille et al., 2012; Ferrari-Amorotti et al., 2014; Kim et al., 2017; Liu et al., 2018). Therefore, potential role of Slug in Notch-mediated migration and invasion, particularly focusing on downstream of Notch- CYR61 axis in early stage, and other potential players taking over the role of CYR61 at the later stages of breast cancer with high Notch activity should be further investigated.

Along with the experimental data, correlation of high CYR61 expression with increased recurrence, poor prognosis and lymph node metastasis in breast cancer patients (Jiang et al., 2004; Mayer et al., 2017) highlight CYR61 as a prominent candidate for the development of therapeutic strategies. In this respect, it is promising that primary tumor growth and lymph node metastasis in MDA MB 231-derived xenograft tumors were suppressed by a monoclonal antibody against CYR61 (Li et al., 2012). In light of our findings that place CYR61 as a crucial mediator of Notch induced pro-metastatic traits, we could speculate that a subpopulation of breast cancer patients at the early stages of the disease could benefit from a combination of both Notch and CYR61 inhibitors. Moreover, a tight regulation of Notch signaling is
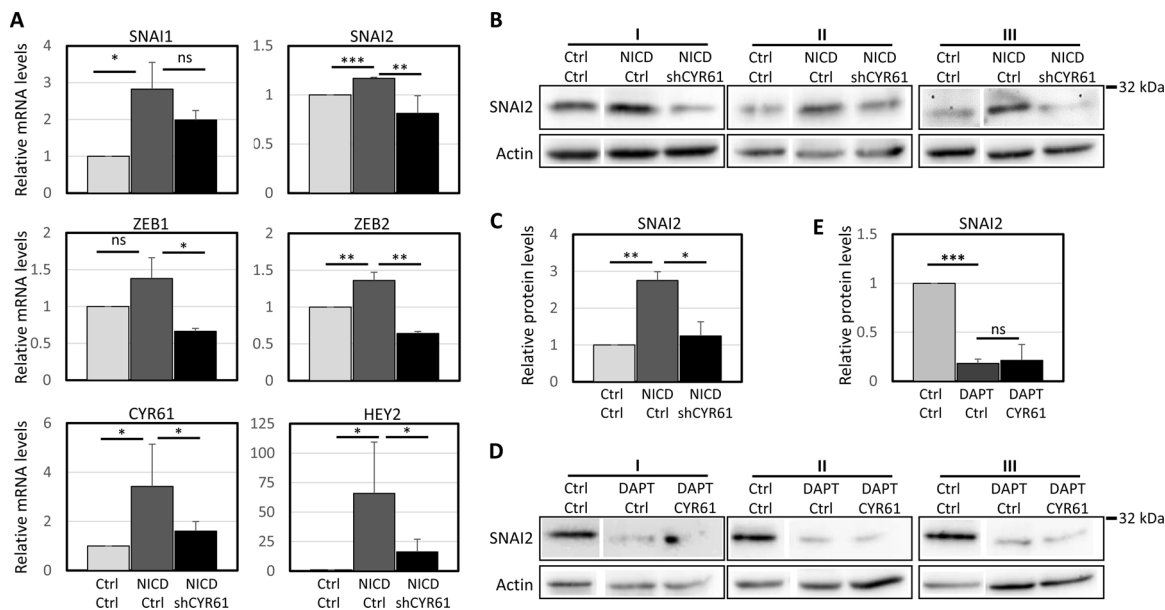
Fig. 5. CYR61 silencing impairs Notch1-induced upregulation of mesenchymal markers. (**A**) mRNA expressions of mesenchymal marker genes, CYR61 and HEY2, (**B**) protein expression of SNAI2 were analyzed 48 h after co-infection of MCF10A cells with two viruses; one for activating Notch1 signaling (NICD), and the other for silencing CYR61 (shCYR61). (**C**) Densitometric analysis of three SNAI2 western blots shown in (B). (**D**) SNAI2 protein expression were analyzed 48 h after treatment of MDA MB 231 cells with Notch inhibitor DAPT in the presence or absence of CYR61 overexpression. (**E**) Densitometric analysis of three SNAI2 western blots shown in (D). Data is represented as mean ± S.D. of three independent experiments. Relative expression levels were normalized to control condition (Ctrl-Ctrl). Ctrl-Ctrl represents infection with two control viruses in MCF10A experiments (A–C), and one control virus infection and DMSO treatment in MDA MB 231 experiments (D and E). TBP and Actin were used as endogenous controls for mRNA and protein analysis, respectively. (I, II and III stand for three independent infection experiments.) (First condition (Ctrl-Ctrl) in all the western blot images were cropped from a nonadjacent lane on the same gel with the other two conditions.) (*p < 0.05, **p < 0.008, ***p < 0.02 × 10^{-5}).

crucial for the maintenance of tissue homeostasis, and thus side effects of Notch inhibitors are relevant concerns. In this respect, CYR61 inhibitors could also be considered as a feasible alternative in the clinic for patients who suffer from side effects of or do not respond to Notch inhibitors.

Author contributions statement

O.Y.O. conceived the project; M.I. and O.Y.O. designed the experiments; M.I., C.K., E.E., Z.E.G., B.F., H.D. and O.Y.O. performed the experiments and analyzed the data; M.O. developed the image analysis program on Python; O.Y.O. drafted the manuscript. All the authors reviewed the manuscript.

Declaration of Competing Interest

Dr. Yalcin-Ozuysal is a share-holder of Initio as of May 2018.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejcb.2020.151070.

References

Azzam, D.J., Zhao, D., Sun, J., Minn, A.J., Ranganathan, P., Drews-Elger, K., Han, X., Picon-Ruiz, M., Gilbert, C.A., Wander, S.A., Capobianco, A.J., El-Ashry, D., Slingerland, J.M., 2013. Triple negative breast cancer initiating cell subsets differ in functional and molecular characteristics and in gamma-secretase inhibitor drug responses. EMBO Mol. Med. 5, 1502–1522.

Bao, B., Wang, Z., Ali, S., Kong, D., Li, Y., Ahmad, A., Banerjee, S., Azmi, A.S., Miele, L., Sarkar, F.H., 2011. Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells. Cancer Lett. 307, 26–36.

Bolos, V., Mira, E., Martinez-Poveda, B., Luxan, G., Canamero, M., Martinez, A.C., Manes, S., de la Pompa, J.L., 2013. Notch activation stimulates migration of breast cancer cells and promotes tumor growth. Breast Cancer Res. 15, R54.

Chen, J., Imanaka, N., Griffin, J.D., 2010. Hypoxia potentiates Notch signaling in breast cancer leading to decreased E-cadherin expression and increased cell migration and invasion. Br. J. Cancer 102, 351–360.

Chimge, N.O., Baniwal, S.K., Little, G.H., Chen, Y.B., Kahn, M., Tripathy, D., Borok, Z., Frenkel, B., 2011. Regulation of breast cancer metastasis by Runx2 and estrogen signaling: the role of SNAI2. Breast Cancer Res. 13, R127.

Chintala, H., Krupska, I., Yan, L., Lau, L., Grant, M., Chaqour, B., 2015. The matricellular protein CCN1 controls retinal angiogenesis by targeting VEGF, Src homology 2 domain phosphatase-1 and Notch signaling. Development 142, 2364–2374.

Dang, T.T., Esparza, M.A., Maine, E.A., Westcott, J.M., Pearson, G.W., 2015. DeltaNp63alpha promotes breast Cancer cell motility through the selective activation of components of the epithelial-to-Mesenchymal transition program. Cancer Res. 75, 3925–3935.

Diluvio, G., Del Gaudio, F., Giuli, M.V., Franciosa, G., Giuliani, E., Palermo, R., Besharat, Z.M., Pignataro, M.G., Vacca, A., d'Amati, G., Maroder, M., Talora, C., Capalbo, C., Bellavia, D., Checquolo, S., 2018. NOTCH3 inactivation increases triple negative breast cancer sensitivity to gefitinib by promoting EGFR tyrosine dephosphorylation and its intracellular arrest. Oncogenesis 7, 42.

Espinoza, I., Menendez, J.A., Kvp, C.M., Lupu, R., 2014. CCN1 promotes vascular endothelial growth factor secretion through alphavbeta 3 integrin receptors in breast cancer. J. Cell Commun. Signal. 8, 23–27.

Fenouille, N., Tichet, M., Dufies, M., Pottier, A., Mogha, A., Soo, J.K., Rocchi, S., Mallavialle, A., Galibert, M.D., Khammari, A., Lacour, J.P., Ballotti, R., Deckert, M., Tartare-Deckert, S., 2012. The epithelial-mesenchymal transition (EMT) regulatory factor SLUG (SNAI2) is a downstream target of SPARC and AKT in promoting melanoma cell invasion. PLoS One 7, e40378.

Ferlay, J., S.I, Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., Bray, F., 2013. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. International Agency for Research on Cancer, Lyon, France.

Ferrandino, F., Bernardini, G., Tsaouli, G., Grazioli, P., Campese, A.F., Noce, C., Ciuffetta,

A., Vacca, A., Besharat, Z.M., Bellavia, D., Screpanti, I., Felli, M.P., 2018. Intrathymic Notch3 and CXCR4 combinatorial interplay facilitates T-cell leukemia propagation. Oncogene 37, 6285–6298.

Ferrari-Amorotti, G., Chiodoni, C., Shen, F., Cattelani, S., Soliera, A.R., Manzotti, G., Grisendi, G., Dominici, M., Rivasi, F., Colombo, M.P., Fatatis, A., Calabretta, B., 2014. Suppression of invasion and metastasis of triple-negative breast cancer lines by pharmacological or genetic inhibition of slug activity. Neoplasia 16, 1047–1058.

Franciosa, G., Diluvio, G., Gaudio, F.D., Giuli, M.V., Palermo, R., Grazioli, P., Campese, A.F., Talora, C., Bellavia, D., D'Amati, G., Besharat, Z.M., Nicoletti, C., Siebel, C.W., Choy, L., Rustighi, A., Sal, G.D., Screpanti, I., Checquolo, S., 2016. Prolyl-isomerase Pin1 controls Notch3 protein expression and regulates T-ALL progression. Oncogene 35, 4741–4751.

Guo, S., Liu, M., Gonzalez-Perez, R.R., 2011. Role of Notch and its oncogenic signaling crosstalk in breast cancer. Biochim. Biophys. Acta 1815, 197–213.

Haque, I., Mehta, S., Majumder, M., Dhar, K., De, A., McGregor, D., Van Veldhuizen, P.J., Banerjee, S.K., Banerjee, S., 2011. Cyr61/CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and promotes pancreatic carcinogenesis. Mol. Cancer 10, 8.

Haque, I., De, A., Majumder, M., Mehta, S., McGregor, D., Banerjee, S.K., Van Veldhuizen, P., Banerjee, S., 2012. The matricellular protein CCN1/Cyr61 is a critical regulator of Sonic Hedgehog in pancreatic carcinogenesis. J. Biol. Chem. 287, 38569–38579.

Harris, L.G., Pannell, L.K., Singh, S., Samant, R.S., Shevde, L.A., 2012. Increased vascularity and spontaneous metastasis of breast cancer by hedgehog signaling mediated upregulation of cyr61. Oncogene 31, 3370–3380.

Harrison, H., Farnie, G., Howell, S.J., Rock, R.E., Stylianou, S., Brennan, K.R., Bundred, N.J., Clarke, R.B., 2010. Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor. Cancer Res. 70, 709–718.

Howlader, N.N.A., Krapcho, M., Neyman, N., Aminou, R., Altekruse, S.F., Kosary, C.L., et al., 2017. SEER Cancer Statistics Review, 1975-2014. National Cancer Institute, Bethesda, MD.

Hsu, K.W., Hsieh, R.H., Huang, K.H., Fen-Yau Li, A., Chi, C.W., Wang, T.Y., Tseng, M.J., Wu, K.J., Yeh, T.S., 2012. Activation of the Notch1/STAT3/Twist signaling axis promotes gastric cancer progression. Carcinogenesis 33, 1459–1467.

Huang, Y.T., Lan, Q., Lorusso, G., Duffey, N., Ruegg, C., 2017. The matricellular protein CYR61 promotes breast cancer lung metastasis by facilitating tumor cell extravasation and suppressing anoikis. Oncotarget 8, 9200–9215.

Jiang, W.G., Watkins, G., Fodstad, O., Douglas-Jones, A., Mokbel, K., Mansel, R.E., 2004. Differential expression of the CCN family members Cyr61, CTGF and Nov in human breast cancer. Endocr. Relat. Cancer 11, 781–791.

Kim, K.H., Chen, C.C., Alpini, G., Lau, L.F., 2015. CCN1 induces hepatic ductular reaction through integrin alphavbeta(5)-mediated activation of NF-kappaB. J. Clin. Invest. 125, 1886–1900.

Kim, M., Jang, K., Miller, P., Picon-Ruiz, M., Yeasky, T.M., El-Ashry, D., Slingerland, J.M., 2017. VEGFA links self-renewal and metastasis by inducing Sox2 to repress miR-452, driving Slug. Oncogene 36, 5199–5211.

Kuonen, F., Secondini, C., Ruegg, C., 2012. Molecular pathways: emerging pathways mediating growth, invasion, and metastasis of tumors progressing in an irradiated microenvironment. Clin. Cancer Res. 18, 5196–5202.

Lamouille, S., Xu, J., Derynck, R., 2014. Molecular mechanisms of epithelial-mesenchymal transition. Nature reviews. Mol. Cell Biol. 15, 178–196.

Lamy, M., Ferreira, A., Dias, J.S., Braga, S., Silva, G., Barbas, A., 2017. Notch-out for breast cancer therapies. N. Biotechnol. 39, 215–221.

Leontovich, A.A., Jalalirad, M., Salisbury, J.L., Mills, L., Haddox, C., Schroeder, M., Tuma, A., Guicciardi, M.E., Zammataro, L., Gambino, M.W., Amato, A., Di Leonardo, A., McCubrey, J., Lange, C.A., Liu, M., Haddad, T., Goetz, M., Boughey, J., Sarkaria, J., Wang, L., Ingle, J.N., Galanis, E., D'Assoro, A.B., 2018. NOTCH3 expression is linked to breast cancer seeding and distant metastasis. Breast Cancer Res. 20, 105.

Lin, H., Huo, R., Wang, L., Zhou, Z., Sun, Y., Shen, B., Wang, R., Li, N., 2012. A novel anti-Cyr61 antibody inhibits breast cancer growth and metastasis in vivo. Cancer Immunol. Immunother. 61, 677–687.

Lin, M.T., Chang, C.C., Chen, S.T., Chang, H.L., Su, J.L., Chau, Y.P., Kuo, M.L., 2004. Cyr61 expression confers resistance to apoptosis in breast cancer MCF-7 cells by a mechanism of NF-kappaB-dependent XIAP up-regulation. J. Biol. Chem. 279, 24015–24023.

Liu, S., Shi, L., Wang, Y., Ye, D., Ju, H., Ma, H., Yang, W., Wang, Y., Hu, J., Deng, J., Zhang, Z., 2018. Stabilization of slug by NF-kappaB is essential for TNF-alpha -Induced migration and epithelial-mesenchymal transition in head and neck squamous cell carcinoma cells. Cell. Physiol. Biochem. 47, 567–578.

Matsuno, Y., Coelho, A.L., Jarai, G., Westwick, J., Hogaboam, C.M., 2012. Notch signaling mediates TGF-beta1-induced epithelial-mesenchymal transition through the induction of Snai1. Int. J. Biochem. Cell Biol. 44, 776–789.

Mayer, S., Erbes, T., Timme-Bronsert, S., Jaeger, M., Rucker, G., Kuf, F., Stickeler, E., Gitsch, G., Hirschfeld, M., 2017. Clinical relevance of Cyr61 expression in patients with hormone-dependent breast cancer. Oncol. Lett. 14, 2334–2340.

Mazzone, M., Selfors, L.M., Albeck, J., Overholtzer, M., Sale, S., Carroll, D.L., Pandya, D., Lu, Y., Mills, G.B., Aster, J.C., Artavanis-Tsakonas, S., Brugge, J.S., 2010. Dose-dependent induction of distinct phenotypic responses to Notch pathway activation in mammary epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 107, 5012–5017.

McGowan, P.M., Simedrea, C., Ribot, E.J., Foster, P.J., Palmieri, D., Steeg, P.S., Allan, A.L., Chambers, A.F., 2011. Notch1 inhibition alters the CD44hi/CD24lo population and reduces the formation of brain metastases from breast cancer. Mol. Cancer Res. 9, 834–844.

Mori, S., Chang, J.T., Andrechek, E.R., Matsumura, N., Baba, T., Yao, G., Kim, J.W., Gatza, M., Murphy, S., Nevins, J.R., 2009. Anchorage-independent cell growth signature identifies tumors with metastatic potential. Oncogene 28, 2796–2805.

Moroishi, T., Park, H.W., Qin, B., Chen, Q., Meng, Z., Plouffe, S.W., Taniguchi, K., Yu,

F.X., Karin, M., Pan, D., Guan, K.L., 2015. A YAP/TAZ-induced feedback mechanism regulates Hippo pathway homeostasis. Genes Dev. 29, 1271–1284.

Nagamatsu, I., Onishi, H., Matsushita, S., Kubo, M., Kai, M., Imaizumi, A., Nakano, K., Hattori, M., Oda, Y., Tanaka, M., Katano, M., 2014. NOTCH4 is a potential therapeutic target for triple-negative breast cancer. Anticancer Res. 34, 69–80.

Pitt, L.A., Tikhonova, A.N., Hu, H., Trimarchi, T., King, B., Gong, Y., Sanchez-Martin, M., Tsirigos, A., Littman, D.R., Ferrando, A.A., Morrison, S.J., Fooksman, D.R., Aifantis, I., Schwab, S.R., 2015. CXCL12-producing vascular endothelial niches control acute t cell leukemia maintenance. Cancer Cell 27, 755–768.

Procopio, M.G., Laszlo, C., Al Labban, D., Kim, D.E., Bordignon, P., Jo, S.H., Goruppi, S., Menietti, E., Ostano, P., Ala, U., Provero, P., Hoetzenecker, W., Neel, V., Kilarski, W.W., Swartz, M.A., Brisken, C., Lefort, K., Dotto, G.P., 2015. Combined CSL and p53 downregulation promotes cancer-associated fibroblast activation. Nat. Cell Biol. 17, 1193–1204.

Ranganathan, P., Weaver, K.L., Capobianco, A.J., 2011. Notch signalling in solid tumours: a little bit of everything but not all the time. Nat. Rev. Cancer 11, 338–351.

RStudio Team, 2016. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA.

Rustighi, A., Tiberi, L., Soldano, A., Napoli, M., Nuciforo, P., Rosato, A., Kaplan, F., Capobianco, A., Pece, S., Di Fiore, P.P., Del Sal, G., 2009. The prolyl-isomerase Pin1 is a Notch1 target that enhances Notch1 activation in cancer. Nat. Cell Biol. 11, 133–142.

Sanchez-Bailon, M.P., Calcabrini, A., Mayoral-Varo, V., Molinari, A., Wagner, K.U., Losada, J.P., Ciordia, S., Albar, J.P., Martin-Perez, J., 2015. Cyr61 as mediator of Src signaling in triple negative breast cancer cells. Oncotarget 6, 13520–13538.

Sansone, P., Ceccarelli, C., Berishaj, M., Chang, Q., Rajasekhar, V.K., Perna, F., Bowman, R.L., Vidone, M., Daly, L., Nnoli, J., Santini, D., Taffurelli, M., Shih, N.N., Feldman, M., Mao, J.J., Colameco, C., Chen, J., DeMichele, A., Fabbri, N., Healey, J.H., Cricca, M., Gasparre, G., Lyden, D., Bonafe, M., Bromberg, J., 2016. Self-renewal of CD133(hi) cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer. Nat. Commun. 7, 10442.

Sarkissyan, S., Sarkissyan, M., Wu, Y., Cardenas, J., Koeffler, H.P., Vadgama, J.V., 2014. IGF-1 regulates Cyr61 induced breast cancer cell proliferation and invasion. PLoS One 9, e103534.

Sethi, N., Dai, X., Winter, C.G., Kang, Y., 2011. Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. Cancer Cell 19, 192–205.

Shao, S., Zhao, X., Zhang, X., Luo, M., Zuo, X., Huang, S., Wang, Y., Gu, S., Zhao, X., 2015. Notch1 signaling regulates the epithelial-mesenchymal transition and invasion of breast cancer in a Slug-dependent manner. Mol. Cancer 14, 28.

Siebel, C., Lendahl, U., 2017. Notch signaling in development, tissue homeostasis, and disease. Physiol. Rev. 97, 1235–1294.

Studebaker, A.W., Storci, G., Werbeck, J.L., Sansone, P., Sasser, A.K., Tavolari, S., Huang, T., Chan, M.W., Marini, F.C., Rosol, T.J., Bonafe, M., Hall, B.M., 2008. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. Cancer Res. 68, 9087–9095.

Stylianou, S., Clarke, R.B., Brennan, K., 2006. Aberrant activation of notch signaling in human breast cancer. Cancer Res. 66, 1517–1525.

Takebe, N., Nguyen, D., Yang, S.X., 2014. Targeting notch signaling pathway in cancer: clinical development advances and challenges. Pharmacol. Ther. 141, 140–149.

Tsai, M.S., Hornby, A.E., Lakins, J., Lupu, R., 2000. Expression and function of CYR61, an angiogenic factor, in breast cancer cell lines and tumor biopsies. Cancer Res. 60, 5603–5607.

Tsai, M.S., Bogart, D.F., Castaneda, J.M., Li, P., Lupu, R., 2002. Cyr61 promotes breast tumorigenesis and cancer progression. Oncogene 21, 8178–8185.

Wang, Z., Banerjee, S., Li, Y., Rahman, K.M., Zhang, Y., Sarkar, F.H., 2006. Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res. 66, 2778–2784.

Wang, X.Q., Zhang, W., Lui, E.L., Zhu, Y., Lu, P., Yu, X., Sun, J., Yang, S., Poon, R.T., Fan, S.T., 2012. Notch1-Snail1-E-cadherin pathway in metastatic hepatocellular carcinoma. Int. J. Cancer 131, E163–172.

Xie, M., Zhang, L., He, C.S., Xu, F., Liu, J.L., Hu, Z.H., Zhao, L.P., Tian, Y., 2012. Activation of Notch-1 enhances epithelial-mesenchymal transition in gefitinib-acquired resistant lung cancer cells. J. Cell. Biochem. 113, 1501–1513.

Xing, F., Okuda, H., Watabe, M., Kobayashi, A., Pai, S.K., Liu, W., Pandey, P.R., Fukuda, K., Hirota, S., Sugai, T., Wakabayashi, G., Koeda, K., Kashiwaba, M., Suzuki, K., Chiba, T., Endo, M., Mo, Y.Y., Watabe, K., 2011. Hypoxia-induced Jagged2 promotes breast cancer metastasis and self-renewal of cancer stem-like cells. Oncogene 30, 4075–4086.

Yalcin-Ozuysal, O., Fiche, M., Guitierrez, M., Wagner, K.U., Raffoul, W., Brisken, C., 2010. Antagonistic roles of Notch and p63 in controlling mammary epithelial cell fates. Cell Death Differ. 17, 1600–1612.

Yang, Y., Ahn, Y.H., Gibbons, D.L., Zang, Y., Lin, W., Thilaganathan, N., Alvarez, C.A., Moreira, D.C., Creighton, C.J., Gregory, P.A., Goodall, G.J., Kurie, J.M., 2011. The Notch ligand Jagged2 promotes lung adenocarcinoma metastasis through a miR-200-dependent pathway in mice. J. Clin. Invest. 121, 1373–1385.

Yeh, T.S., Wu, C.W., Hsu, K.W., Liao, W.J., Yang, M.C., Li, A.F., Wang, A.M., Kuo, M.L., Chi, C.W., 2009. The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. Cancer Res. 69, 5039–5048.

Yu, F., Li, J., Chen, H., Fu, J., Ray, S., Huang, S., Zheng, H., Ai, W., 2011. Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. Oncogene 30, 2161–2172.

Zengin, T., Ekinci, B., Kucukkose, C., Yalcin-Ozuysal, O., 2015. IRF6 is involved in the regulation of cell proliferation and transformation in MCF10A cells downstream of notch signaling. PLoS One 10, e0132757.

Zhang, Z., Wang, H., Ikeda, S., Fahey, F., Bielenberg, D., Smits, P., Hauschka, P.V., 2010. Notch3 in human breast cancer cell lines regulates osteoblast-cancer cell interactions and osteolytic bone metastasis. Am. J. Pathol. 177, 1459–1469.

Zhang, H., Pasolli, H.A., Fuchs, E., 2011. Yes-associated protein (YAP) transcriptional coactivator functions in balancing growth and differentiation in skin. Proc. Natl. Acad. Sci. U. S. A. 108, 2270–2275.

Zhang, X., Chen, T., Zhang, J., Mao, Q., Li, S., Xiong, W., Qiu, Y., Xie, Q., Ge, J., 2012. Notch1 promotes glioma cell migration and invasion by stimulating beta-catenin and NF-kappaB signaling via AKT activation. Cancer Sci. 103, 181–190.

Zhang, X., Zhao, X., Shao, S., Zuo, X., Ning, Q., Luo, M., Gu, S., Zhao, X., 2015. Notch1 induces epithelial-mesenchymal transition and the cancer stem cell phenotype in breast cancer cells and STAT3 plays a key role. Int. J. Oncol. 46, 1141–1148.

Zhou, L., Wang, D.S., Li, Q.J., Sun, W., Zhang, Y., Dou, K.F., 2012. Downregulation of the Notch signaling pathway inhibits hepatocellular carcinoma cell invasion by inactivation of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor. Oncol. Rep. 28, 874–882.