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The adapter SASH1 acts through NOTCH1 and its inhibitor DLK1 in a 3D model of lumenogenesis involving CEACAM1

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Abstract

CEACAM1 transfection into breast cancer cells restores lumen formation in a 3D culture model. Among the top up-regulated genes that were associated with restoration of lumen formation, the adaptor protein SASH1 was identified. Furthermore, SASH1 was shown to be critical for lumen formation by RNAi inhibition. Upon analyzing the gene array from CEACAM1/MCF7 cells treated with SASH1 RNAi, DLK1, an inhibitor of NOTCH1 signaling, was found to be down-regulated to the same extent as SASH1. Subsequent treatment of CEACAM1/MCF7 cells with RNAi to DLK1 also inhibited lumen formation, supporting its association with SASH1. In agreement with the role of DLK1 as a NOTCH1 inhibitor, NOTCH1, as well as its regulated genes HES1 and HEY1, were down-regulated in CEACAM1/MCF7 cells by the action of DLK1 RNAi, and up-regulated by SASH1 RNAi. When CEACAM1/MCF7 cells were treated with a γ-secretase inhibitor known to inhibit NOTCH signaling, lumen formation was inhibited. We conclude that restoration of lumen formation by CEACAM1 regulates the NOTCH1 signaling pathway via the adaptor protein SASH1 and the NOTCH1 inhibitor DLK1. These data suggest that the putative involvement of NOTCH1 as a tumor-promoting gene in breast cancer may depend on its lack of regulation in cancer, whereas its involvement in normal lumen formation requires activation of its expression, and subsequently, inhibition of its signaling.

INTRODUCTION

Lumen formation, an intrinsic property of mammary epithelial cells, can be observed in vitro in 3D cultures containing extracellular matrix (ECM) (1–3). Since one of the hallmarks of cancer is the lack of these cells to polarize and form lumena, the 3D culture system provides an in vitro approach to study the mechanisms required to restore lumen formation of cancer cells. Previously, we have shown that while normal breast epithelia highly express CEACAM1 at their lumenal surface, this expression is lost in breast cancer cells (4). Using

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the 3D culture system, we have shown that phenotypically normal breast cells such as MCF10 express CEACAM1 and form lumena (4), while most breast cancer cells such as MCF7 neither express CEACAM1 nor form lumena (5). Since transfection of MCF7 cells with CEACAM1 restores lumen formation both in vitro (5) and in vivo (6), we have been interested in analyzing the mechanism involved. It is clear from numerous studies that the mechanism of lumen formation involves a complex orchestration of signaling events starting from the ECM via integrin beta-1, leading to cytoskeletal reorganization and new gene expression (7, 8). In spite of this complexity, it appears that the lack of expression of a single gene, such as CEACAM1, is sufficient to abrogate lumen formation in the 3D culture model, making it possible to ask which new genes are expressed by forcing the re-expression of CEACAM1. Our approach to this analysis was to compare MCF7 cells transfected with either wild type CEACAM1 or a phosphorylation null mutant version of CEACAM1 that fails to form lumena in 3D culture. The double mutation (T457A, S459A) in the cytoplasmic domain of the short form splice variant of CEACAM1 is phosphorylation defective (9), rendering it unable to convey necessary signals for lumen formation even though the extracellular domains were unchanged and presumably capable of interacting with other cells and/or the ECM. The results of this analysis revealed changes in over 300 genes (10), emphasizing the complexity of lumen formation. As a next step, we have selected the adaptor protein SASH1, one of the top up-regulated genes in CEACAM1/MCF7 cells for further analysis. SASH1 was selected for a critical role based on the study by Zeller et al. (11) that showed down-regulation of SASH1 in 74% of breast cancers. Thus, SASH1 has been termed a tumor suppressor in breast and other cancers (12–18). Since the low expression of SASH1 in MCF7 cells is restored by forced expression of CEACAM1, we thought it likely that the two proteins were involved in a common lumen formation pathway.

SASH1 is a scaffold protein containing SAM (sterile alpha motif) and SH3 domains (19–21). The SAM domain, originally observed in drosophila, can dimerize with other SAM or non-SAM domain containing proteins yielding oligomeric platforms for cell signaling involving translocation from the cytoplasm to the nucleus (20, 21). In terms of our interest in lumen formation, SASH1 has a role in apoptosis (22), a critical step in lumen formation (3). In the case of SASH1, apoptosis requires its cleavage by caspase-3 producing a C-terminal fragment that associates with chromatin (22). In our 3D model of lumenogenesis, we show that inhibition of SASH1 expression by RNAi inhibits lumen formation and is associated with down-regulation of the NOTCH1 inhibitor DLK1. Supporting a role for both DLK1 and SASH1, inhibition of DLK1 by RNAi also inhibited lumen formation. Taken together, these data implied a role for NOTCH1 in lumen formation, but given the putative role of NOTCH1 as an oncogene in breast cancer (23), it was unclear if its signaling acted in a positive or negative manner. Importantly, NOTCH1 signaling requires activation by γ-secretase (24), and inhibitors of γ-secretase have been used to treat breast cancer (25). Since we show that inhibition of NOTCH1 signaling by an inhibitor of γ-secretase inhibited lumen formation, we conclude that some positive NOTCH1 expression and signaling is required for lumen formation. However, since expression of DLK1, a NOTCH1 signaling inhibitor (26, 27), is also required, NOTCH1 signaling must be terminated at some stage of lumen formation. In fact, our current understanding of lumen formation in the 3D model system is consistent with this possibility, since during the early stages, the cells must aggregate and divide to

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form an acinus, and in the later stages, cease cell division, polarize and form a lumen. We conclude that NOTCH1 signaling, a well know pathway for integration of signals from the microenvironment (28), is positively regulated during the early stages involving cell division in the ECM, and is negatively regulated during the cell polarization stages that ultimately produce the lumen. This idea is compatible with the down-regulation of SASH1 (or DLK1) in cancer, resulting in the uncontrolled regulation of NOTCH1 signaling. Conversely, normal gland formation requires careful regulation of NOTCH1 signaling by the combined functions of SASH1 and DLK1.

MATERIALS AND METHODS

Materials.

Anti-SASH1 antibody was from Bethyl Laboratories, anti-DLK antibody from Millipore, anti-HDAC1 and anti-Lamin A/C antibodies from Cell Signaling Technology, anti-NOTCH1 and anti-beta-actin from Santa Cruz Biotechnologies, anti-NICD from Abcam, and anti-GAPDH from Gene Tex. The MCF7 cell line was obtained from ATCC (HTB-22) and cultured in MEM supplemented with 10% fetal bovine serum, 0.15% sodium bicarbonate, 1 mM of sodium pyruvate, 1x of non-essential amino acids and 1x of antibiotics. Transfection of CEACAM1–4S in the pHβ-actin vector into MCF7 cells was previously described (9).

Western Blotting.

Cells were lysed with NP-40 lysis buffer (10mM Tris-HCL, pH 7.4, 100 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 % NP-40, 10% glycerol, 0.05% sodium deoxycholate and proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN)) on ice for 30 minutes, centrifuged at 4^{o}C for 20 minutes at 13,000 rpm. The supernatant was collected and protein concentration was determined using the Bradford protein assay. Fifty micrograms of protein from each sample were resolved by SDS gel electrophoresis, transferred to PVDF and Western blotted was performed using SuperSignal Western Blot Enhancer from ThermoFisher (Grand Island, NY) with appropriate antibodies and LI-COR WesternSure HRP conjugated secondary antibodies (Lincoln, NE).

Cellular Fractionation.

Method 1: The M-PER Mammalian Protein Extraction and the NE-PER Nuclear and Cytoplasmic Extraction kits (Thermo Fisher) were used per manufacturer's instruction. Method 2: A subcellular fractionation protocol was followed from bio-protocol.org/e754 (vol3, issue 9, May 5, 2013). In brief, cells were lysed using subcellular fractionation buffer (250 mM Sucrose, 20 mM HEPES, 7.4 pH, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1mM EGTA, 1 mM DTT and proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN)) and passed through 21 G needle, left on ice for 20 minutes and centrifuged at 3000 rpm for 5 minutes. Nuclear pellet was washed once with fractionation buffer, the pellet were resuspended in NL buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhbitor cocktail, 10% glycerol). The pellet was sonicated on ice (2× 3sec, each with 3 sec in between, under 30% full amplitude power) and pelleted at 14,000 rpm for 30 min. This pellet includes the nuclear fraction and nuclear membranes. The original supernatant, including the cytosol

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and membrane fraction was further centrifuged at 14,000 rpm for 30 min. The supernatant (cytosolic fraction) was removed and placed in a separate 1.5 ml Eppendorf tube. The pellet was the membrane fraction.

RT-PCR and qRT-PCR.

Total RNA was extracted from cells using the Qiagen RNeasy Plus Mini Kit (Valencia, CA) according to the manufacturers protocol. From 2 μg of each total RNA, 20 μl of cDNA was synthesized with an Ommiscript RT Kit (Qiagen, Valencia, CA). mRNA expression levels of SASH1, DLK1, NOTCH1, HES1, HEY1 and GAPDH was quantified with the iQ5 Mulitcolor Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primers are shown in (Supplemental table, Table S1) and used at a concentration of 20 pM. cDNA (0.05 ug) from the reverse transcription reaction, primers and the Sense Mix Plus SYBR (Quantace Inc, Norwood, MA) were mixed in a total volume of 25 ul and the qRT-PCR reaction was carried out as previously described (10). Levels of mRNA, performed in triplicate, were compared to GAPDH.

RNAi treatment.

Cells were transfected with RNAi oligonucleotides (Supplemental table, Table S2) using the Lipofectamine 2000 transfection agent (Invitrogen, Thermo Scientific, Grand Island, NY). Cells were split, seeded at 50% confluence in T25 flasks overnight, washed twice with 1X PBS, and treated with RNAi oligos (100 nM) and transfection agent (1:100 dilution) in Opti-MEM® I reduced serum medium for 6 hours without removing the transfection solution, after which, cells were returned to MEM medium with 10% fetal bovine serum overnight, harvested and transferred to 3D culture.

3D culture.

Matrigel sandwich assays were performed by coating 12-well cell culture plates with 150 μL of Matrigel (Corning, Bedford, MA) and incubated at 37 degrees Celsius for 30 minutes to let the Matrigel solidify. One hundred thousand cells in 500 μL of mammary epithelial basal medium (MEBM) (Lonza, Walkersville, MD) were added to Matrigel coated wells. Cells were allowed to attach to Matrigel for three hours at 37^{o} C. Medium with floating cells were removed and the bound cells were overlaid with 150 μL of 50% Matrigel and 50% MEBM. After the top layer solidified, 2 mL of MEBM plus bovine pituitary gland extract (BPE) (MEGM SingleQuots, Lonza, Walkersville, MD) were added to the wells and culture medium changed every other day. Lumen formation was assessed after 6 days by light microscopy. Cells were isolated from Matrigel using Cell Recovery Solution by Corning (Bedford, MA) according to manufactures protocol.

Microarray analysis.

Microarray analysis of mRNA from CEACAM1/MCF7 transfected cells versus the double mutant (T457A, S459A) version were grown in Matrigel for 4 days was performed as previously described (9). mRNA isolated from SASH1 RNAi treated vs untreated CEACAM1/MCF7 cells grown in Matrigel for 4 days was used for microarray analysis

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(Functional Genomics Core: Microarray Service at City of Hope). The array used was the GeneChip Human Genome U133 Plus 2.0 array manufactured by Affymetrix.

γ-Secretase Treatment.

CEACAM1/MCF7 cells were grown to 60% confluence in 6-well plates and incubated for 24 hours with either 0.5 μM or 1 μM of the γ-secretase inhibitor LY411575 (Selleckchem, Houston, TX). The stock solution of LY411575, 100 μM in DMSO, was diluted 500 or 100-fold in cell culture media. For lumen formation analysis, CEACAM1/MCF7 cells were grown in the Matrigel sandwich assay and incubated with the γ-secretase inhibitor LY411575 for 24 hours before lumen analysis or 6 days with either 0.5 μM or 1 μM of the γ-secretase inhibitor. Control cells were incubated in 0.1% DMSO for the same time periods.

Confocal microscopy.

CEACAM1/MCF7 cells with or without SASH1 RNAi treatment were grow on a 8 chamber slide and fixed with 4% paraformaldehyde for 20 minutes followed by permeabilization with 0.5% (v/v) Triton X-100 for 10 minutes at room temperature. Following blocking with 5% fetal calf serum (FCS) for 1 hour, the cells were incubated with mouse monoclonal antibody anti-DLK1 for 1 hr at room temperature. The cells were subsequently washed three times with 1x PBS and incubated with anti-mouse secondary antibody conjugated with Alexa 488 for 1 hour. The nuclei were counterstained with DAPI dye. Confocal laser scanning immunofluorescence microscopy (CLSM) was carried out using a Zeiss LSM 510 META confocal microscope. Image analysis was performed using LSM510 META software (Carl Zeiss, USA).

RESULTS

Induction of SASH1 by CEACAM1 during lumenogenesis

We have previously shown that MCF7 breast cancer cells that fail to express CEACAM1, a lumenal marker in normal breast (4, 29), regain lumen formation when transfected with CEACAM1 and grown in 3D culture (5). Similarly, MCF7 cells form tumors in humanized mammary glands in NOD/SCID mice, but form mammary glands capable of secreting milk when transfected with CEACAM1 (6). Strikingly, the cytoplasmic domain of CEACAM1 found in mammary epithelial cells has only twelve amino acids, two of which can be phosphorylated and are necessary for lumen formation, as shown by mutation to the phosphorylation null mutant Thr457Ala, Ser459Ala (9). When a gene chip analysis was performed comparing the gene expression of wild type CEACAM1 to the phosphorylation null mutant in MCF7 cells, over 300 genes were found to change by more than four fold (10). Among the top ten (Supplemental table S3), we identified SASH1 with a log2 ratio of 6.4 (84-fold) as a gene highly induced by forced expressed of CEACAM1 (10). Since SASH1 is highly down-regulated in breast cancer (11) similar to CEACAM1 (29), we asked if it played a role in lumen formation.

Although we had previously shown the mRNA expression levels of SASH1 increased over 84-fold during lumen formation in CEACAM1/MCF7 cells (10), we had not measured the

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protein expression levels of SASH1. As shown in Figure 1A, the protein expression levels of SASH1 increased by 2-fold over the course of lumen formation. In addition, it was possible to significantly knock-down expression of SASH1 by RNAi (Figure 1B).

SASH1 is Necessary for Lumen Formation

In order to determine if SASH1 expression played a role in lumen formation, we grew the SASH1 RNAi treated CEACAM1/MCF7 cells in 3D culture and compared the percent lumen formation vs untreated, lipofectamine treated, and control RNAi treated cells. Representative micrographs of the results are shown in Figure 1C and the overall results tabulated in Table 1. When compared to controls, inhibition of SASH1 by RNAi reduced lumen formation by 80%, in which untreated controls exhibited 93% lumen formation compared to only 13% for SASH1 RNAi treated cells. Thus, SASH1 is a critical protein downstream from CEACAM1 and is involved in lumenogenesis in this model system.

Inhibition of SASH1 expression identifies DLK1

Since we found that SASH1 expression is necessary for CEACAM1/MCF7 cells to form a lumen, we asked if a subset of genes identified in the first screen would be affected by SASH1 RNAi treatment compared to untreated CEACAM1/MCF7 cells grown in 3D culture. The top 9 hits from this analysis are shown in Table 2 according to their fold-change where negative values refer to down-regulation and positive values depict up-regulation. As expected, the top down-regulated hit was SASH1 itself, followed by DLK1 and a number of genes previously found in the first gene chip analysis (10). Interestingly, the top up-regulated gene was RUNX2, also a top hit in the previous gene chip analysis (10). Since we were interested in down-regulated genes, we further investigated DLK1, a gene that exhibited a log2 reduction of 4.1 (17-fold) in the original analysis (10). DLK1, an inhibitor of NOTCH signaling caught our attention due to the role of NOTCH1 in mammary gland formation (30) and in breast cancer (23).

When the mRNA expression levels of DLK1 in CEACAM1/MCF7 cells vs SASH1 RNAi treated cells were analyzed by qRT-PCR, we found that DLK1 mRNA expression was reduced by 88% compared to the RNAi control treated cells (Figure 2A). Similarly, the protein levels of DLK1 were reduced 2-fold compared to control treated cells (Figure 2B).

Having provided evidence that DLK1 is downstream of SASH1, we asked if a knock-down of DLK1 expression would effect SASH1 expression. The inhibition of DLK1 expression by RNAi is shown in Figure 2C (4-fold) and its effect on SASH1 expression is shown in Figure 2D (2-fold). Since DLK1 inhibition has little or no affect on SASH1 expression, we conclude that DLK1 is indeed downstream of SASH1 and there is no evidence of a feedback effect.

SASH1 regulates both DLK1 and DLK2 expression

Although we showed that inhibition of SASH1 expression had its primary affect on DLK1, there is a report that inhibition of DLK1 results in de novo expression of DLK2 as a compensatory effect (31). Given these findings, it was important to determine if DLK1 inhibition by RNAi affected DLK2 expression. As expected qRT-PCR analysis of DLK1

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and DLK2 in CEACAM1/MCF7 cells showed high levels of DLK1 and barely detectable levels of DLK2 (Figure 3A). However, when CEACAM1/MCF7 cells were treated with DLK1 RNAi, the expression of DLK2 was significantly up-regulated (>7-fold compared to the no treatment control, Figure 3B). Since DLK2 expression increased in response to DLK1 inhibition, DLK2 expression was also analyzed in cells treated with SASH1 RNAi. Although DLK2 expression levels were barely detectable in the CEACAM1/MCF7 cells, SASH1 inhibition reduced DLK2 expression even further (Figure 3C). Thus, SASH1 expression can affect the expression levels of both DLK1 and DLK2. Furthermore, when CEACAM1/MCF7 cells were transfected with a SASH1-GFP plasmid and analyzed for DLK2 mRNA expression levels, DLK2 expression was significantly up-regulated in SASH1 compared to non-transfected controls (Figure 3D), providing evidence that SASH1 can positively regulate both DLK1 and DLK2.

Both DLK1 and DLK2 are necessary for lumen formation in CEACAM1/MCF7 cells

Probing the role of DLK1/2 in lumen formation, CEACAM1/MCF7 cells were treated with various RNAi targeting DLK1 and/or DLK2 (RNAi 1 and 2 for DLK1, and RNAi 3 and 4 for DLK1/2. Given the high degree of sequence homology between DLK1 and DLK2, it is not surprising that all four RNAi inhibited DLK1 expression (Figure 4A). Furthermore, RNAi 1 and 2 that were designed with a higher specificity for DLK1 caused an increase in DLK2 expression (Figure 4B), while RNAi designed with broad specificity for DLK1/2 inhibited both DLK1 and DLK2 expression (Figure 4B). These results are in agreement with the study that showed down regulation of DLK1 can lead to up regulation of DLK2 (31).

When CEACAM1/MCF7 cells were transfected with the broadly specific RNAi (RNAi 3 and 4) that inhibited both DLK1/2, and grown in 3D culture, significant reductions in lumen formation (about 70%) were observed (Figure 4C and 4D). These results demonstrate that both DLK1 and DLK2 are necessary for lumen formation since even a two-fold increase in DLK2 expression after DLK1 inhibition can compensate for the loss of DLK1 and still maintain lumen formation. Consequently, the inhibition of both DLK1 and DLK2 are required to inhibit lumen formation. Furthermore, these results confirm that DLK1/2 are down-stream from SASH1 in lumenogenesis.

SASH1 regulates the expression of DLK1 protein and its cleaved product

DLK1 is a cell surface, transmembrane protein that can be cleaved by proteases at the juxtamembrane extracellular domain generating a cleaved soluble form (32–35). Since we showed that SASH1 regulates the transcription of DLK1, we asked if SASH1 expression affected the protein levels of either the full length or cleaved forms of DLK1. Furthermore, both cytosolic and nuclear fractions were analyzed to determine if DLK1, like the NOTCH1 cleavage product NICD (36) was translocated to the nucleus. Immunoblot analysis revealed that both the intact and cleavage product of DLK1 were found in both the nuclear and cytosolic fractions (Figure 5A). Since the finding of DLK1 in the nuclear fraction has not been reported, we compared two methods of cellular fractionation. In method 1 about 10% of DLK1 was found in the nuclear fraction, while in method 2 more than 90% was found in the nuclear fraction. The integrity of the methods was assessed by immunoblotting for HDAC1 (nuclear) and GAPDH (cytosolic). It can be seen that method 2 has a substantial

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contamination of the nuclear fraction with GAPDH, calling into question those results. In both cases, the inhibition of SASH1 by SASH1 RNAi increased the total amount of DLK1 detected, including the accumulation of more of the cleaved form of DLK1 (Figure 5A). Since the detection of DLK1 in the nucleus was unanticipated, confocal staining of DLK1 in CEACAM1/MCF7 cells was performed (Figure 5B). The results show strong cytoplasmic staining with a higher intensity staining in the perinuclear region. As shown by a confocal slice taken through two cells, the DLK1 staining intensity is compatible with the cell fractionation immunoblot analysis of Method 1. We conclude that while some DLK1 can be found in the nucleus, the majority is cytoplasmic.

SASH1 regulates NOTCH1 and its target genes expression

The connection of SASH1 to DLK1, a NOTCH inhibitor, turned our attention to NOTCH expression and the possibility that changes in the level of SASH1 expression that affected DLK1 expression would, in turn, regulate NOTCH signaling. Since parental MCF7 cells are known to express high levels of NOTCH1 (37) we first determined the expression of levels of NOTCH1 in CEACAM1/MCF7 cells before and after treatment with SASH1 RNAi (Figure 6A). The results demonstrate a significant reduction (about 3-fold) in the expression levels of NOTCH1 compared to a no treatment control. Conversely, when NOTCH1 expression was measured in SASH1 over-expressing CEACAM1/MCF7 cells (Figure 6B), NOTCH1 expression increased more than 3-fold. Taken together, we conclude that SASH1 positively regulates NOTCH1 expression, but since SASH1 also positively regulates DLK1 expression, it is likely that these opposing effects are sequential.

Since high expression of NOTCH1 is associated with a poor outcome in breast cancer (23), it may seem logical to assume its expression in normal breast should be low corresponding to the low numbers of cell divisions in a quiescent breast. However, in our model system where single cells are cultured in 3D leading to the formation of acini forming lumena over a 4–6 day period, the cells undergo cell division prior to differentiation. Thus, the activation of NOTCH1, associated with signaling leading to cell division, may be an essential step. In order to explore this possibility, we measured the levels of several NOTCH1 target genes (HES1 and HEY1) and the effects of SASH1 and DLK1 expression on these genes.

Since the expression of HES1 and HEY1 were reduced by SASH1 RNAi in CEACAM1/MCF7 cells (Figure 6C) to the same levels as NOTCH1 itself (Figure 6A), we conclude that SASH1 regulation of NOTCH1 in turn affects the levels of NOTCH1 target genes. As expected, the reverse is true for over-expression of SASH1 in CEACAM1/MCF7 cells where an increase of the expression of NOTCH1 target genes is observed (Figure 6D).

Protein levels of NOTCH1 and NICD are affected by the SASH1 expression

The usual mechanism of NOTCH1 signaling requires its activation by ligands such as Delta or Jagged at the membrane followed by cleavage to generate NICD and its subsequent translocation into the nucleus (38, 39). Since DLK1 can inhibit this pathway (32), and we have shown DLK1 expression is regulated by SASH1 expression, it was important to measure NOTCH1 activation in CEACAM1/MCF7 cells over-expressing SASH1 or in the presence of SASH1 RNAi. As expected, over expression of SASH1 increased protein levels

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of NOTCH1 in whole cell lysates (Figure 7A) and NICD in the nuclear fraction (Figure 7B), while inhibition of SASH1 expression with RNAi, inhibited expression of full length NOTCH1 and NICD (Figures 7A and B). Thus, the protein levels of NOTCH1 and its cleavage product NCID correspond to the changes in mRNA expression of NOTCH1, that are in turn, regulated by SASH1 expression (Figure 6).

Gamma secretase inhibitor LY411575 inhibits lumen formation

Having shown that inhibition of SASH1 or DLK1 expression inhibits lumen formation, we were presented with a possible contradiction. If SASH1 positively regulates both DLK1 and NOTCH1 expression, but DLK1 inhibits NOTCH1 signaling, what is the ultimate effect of NOTCH1 signaling on lumen formation? To answer this question, we treated CEACAM1/MCF7 cells with a γ-secretase inhibitor, since this class of inhibitors block the formation of NICD, the proteolytic fragment of NOTCH1 formed by the action of γ-secretase on NOTCH1 (40). In addition, a γ-secretase inhibitor is in phase 1 trial in breast cancer (25), based on the idea that Notch signaling contributes to breast cancer. As shown in Figure 8A–D, and quantitated in Figure 8E, LY411575 inhibits lumen formation whether administered to cells for one day in 3D culture and removed or administered continuously for 6 days over the course of lumen formation. In addition, when NOTCH1 expression was measured by qRT-PCR, levels fell by 50%, suggesting that the inhibitor had a direct effect on NOTCH1 expression. We conclude that NOTCH1 expression is required for lumen formation. Thus, it appears that the positive regulation of NOTCH1 expression by SASH1 supports lumen formation, and that the subsequent induction of the NOTCH1 inhibitor DLK1 is also critical, suggesting the two activities are temporarily regulated.

DISCUSSION

Lumen formation, a hallmark of the mammary epithelium phenotype, can be disrupted by the lack of expression of even a single gene. This phenomenon is illustrated by CEACAM1, expressed in the lumen of normal breast, and down-regulated in breast cancer. In the case of MCF7 breast cancer cells lacking CEACAM1, transfection with CEACAM1 reinstates lumen formation when the cells are grown in 3D culture. In this study, we asked if genes up-regulated in the CEACAM1 transfected cells also played a critical role. Indeed, inhibition of SASH1, one of the most highly up-regulated genes, also blocked lumen formation. Next, DLK1 was identified as the most down-regulated gene (besides SASH1 itself) in SASH1 RNAi treated cells. Since DLK1 is a known inhibitor of NOTCH1 signaling, we asked if NOTCH1 inhibition was a critical step in lumen formation. To our surprise, inhibition of NOTCH1 signaling with a γ-secretase inhibitor, inhibited lumen formation. Thus, the downstream signaling from CEACAM1 through SASH1 does not occur in a linear fashion from SASH1 to DLK1 to NOTCH1, but instead is branched, since SASH1 can up-regulate both DLK1 and NOTCH1, and the action of DLK1 on NOTCH1, while required, must not interfere with a critical stage of NOTCH1 signaling. A schematic showing the gene network consistent with these data is shown in Figure 9.

These results are consistent with a role for NOTCH1 signaling in alveolar progenitors that expand during pregnancy to form luminal epithelial cells (41, 42). The expansion of cell

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numbers during pregnancy is analogous to the expansion of cell numbers we observe after the plating of single cells in 3D culture. After expansion, the cells must cease cell division in order to become functional alveolar cells. Thus, NOTCH1 signaling is activated during the cell expansion phase and inhibited during the cell differentiation phase. However, if this control on the activation/inhibition of NOTCH1 signaling is lost, cell division continues, a hallmark of cancer. Thus, the uncontrolled or aberrant expression of NOTCH1 is commonly associated with breast tumor formation and subsequent inhibition of lumen formation (39, 42, 43). Therefore, NOTCH1 is necessary for lumen formation, but when disregulated, prevents lumen formation. From our study, we propose that a key regulator of NOTCH1 is DLK1, which in turn, requires activation of SASH1.

SASH1 is an ideal candidate for regulating this pathway. It is an adaptor protein that interacts with many signaling partners, followed by translocation to the nucleus, effecting new gene expression. Supporting evidence for a role in mammary gland development includes its high expression in normal breast, and its low expression in breast and other cancers (11). Since lumen formation requires apoptosis of the central acinar cells (3, 5), and apoptosis is one of the ascribed functions of SASH1 (22), its expression during lumenogenesis also makes sense. Its connection to CEACAM1, while established by our genetic analysis, was not shown by direct association assays such as immunopreciptation (data not shown). However, we have shown that CEACAM1 interacts with the actin cytoskeleton (9), and it is likely that the reorganization of the cytoskeleton that occurs during lumenogenesis provides ample opportunity for the two proteins to interact indirectly. More importantly, we have connected CEACAM1 expression to apoptosis of the central acinar cells (5), further connecting the functions of the two proteins. Indeed, the lack of apoptosis is a hallmark of cancer cells, perhaps explaining why SASH1 has been proposed to be a tumor suppressor gene in so many malignancies (11–18).

Some mention should be made of recent work on murine mammary gland development where Notch signaling has been connected to ELF5 (44), the master transcription factor for mammary gland development (45). In our companion paper, we show that ELF5, downstream of prolactin receptor signaling, is able to substitute for CEACAM1 in MCF7 cells, enabling lumen formation. The work by Chakrabati et al. (44) in the murine system demonstrates that *lack* of ELF5 leads to hyperactivation of Notch signaling. This situation may have a parallel in human breast cancer where lumen formation is largely absent and hyperactivation of notch signaling is common (46). Since we overexpressed ELF5 in a breast cancer cell line (MCF7) that cannot form lumen in 3D culture, we asked if overexpression of ELF5 led to down-regulation of Notch1? Indeed, preliminary data suggests that Notch1 and its activation product NICD are not detectable in MCF7 cells overexpressing ELF5. However, the opposite situation, more aligned to the work of Chakrabarti et al., where ELF5 was silenced in CEACAM1/MCF7 cells, we found lumen formation was blocked, but had no effect on Notch signaling. Thus, even in the presence of CEACAM1 that enables lumen formation, ELF5 is required for lumen formation. Given the complexity of the interactions among the three genes, CEACAM1, ELF5, and Notch1, we are reluctant to claim a direct connection between ELF5 and Notch signaling in the human system until more studies become available.

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Overall, this study demonstrates the utility of the in vitro 3D culture system for unraveling the genetic steps involved in lumenogenesis, and in particular, the role of common genes disregulated in breast cancer. It can be seen that NOTCH1 signaling, when properly regulated, is critical for lumenogenesis, and by extension, to mammary gland expansion. This result suggests that inhibition of NOTCH1 signaling for the treatment of breast cancer may not be the best approach if the goal is to revert the cancer cells to a normal phenotype. Rather, it may be more prudent to reestablish proper control of NOTCH1 signaling by induction of upstream effectors that are associated with lumenogenesis. Moreover, the loss of CEACAM1 expression, a common event in cancers of epithelial origin, leads to down-regulation of the tumor suppressor SASH1, that in turn, regulates the proper level of NOTCH1 expression and signaling.

Supplementary Material

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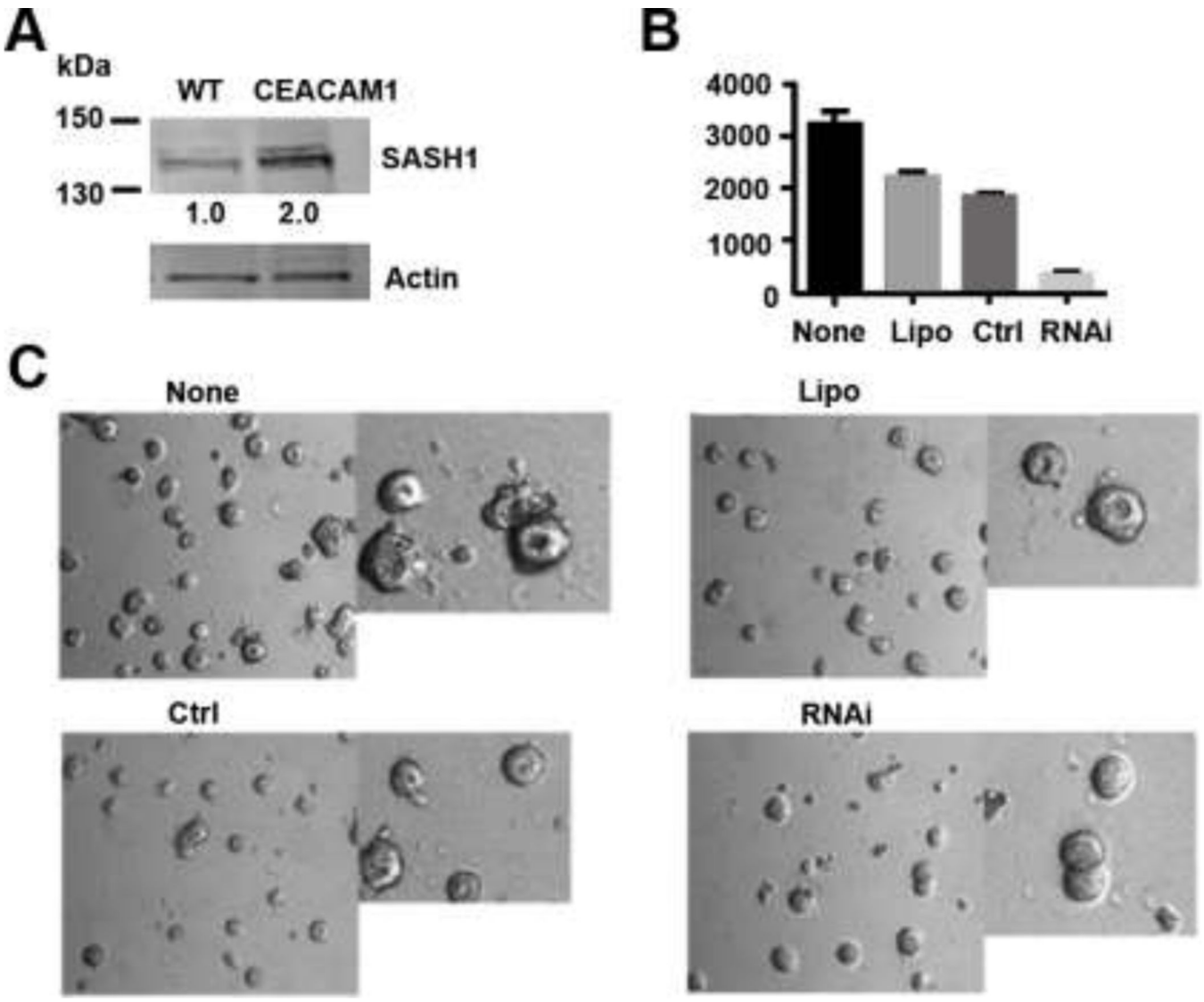

Figure 1: Sash1 is up-regulated by CEACAM1 expression and is critical for lumenogenesis.

(**A**) MCF7 parental cells (WT) and CEACAM1/MCF7 cells (CEACAM1) were grown in 3D culture for six days, proteins separated by SDS gel electrophoresis and analyzed by western blotting for SASH1 and β-actin. (**B**) mRNA expression of SASH1 in CEACAM1/MCF7 cells untreated, treated with lipofectamine (lipo), treated with a negative, scrambled oligo control (ctrl) or treated with SASH1 specific RNAi. mRNA levels were quantified by qRT-PCR in triplicate. (**C**) CEACAM1/MCF7 cells treated with controls or with SASH1 RNAi were grown on Matrigel for 6 days and lumen were examined at 50x by phase-contrast microscopy (n= 200, P= 0.001, for RNAi treated vs transfectamine control). Magnification 40x, inset 80x. Acini counted had the following dimensions: D= 40–60-μm, with lumen size D= 8–10 μm.

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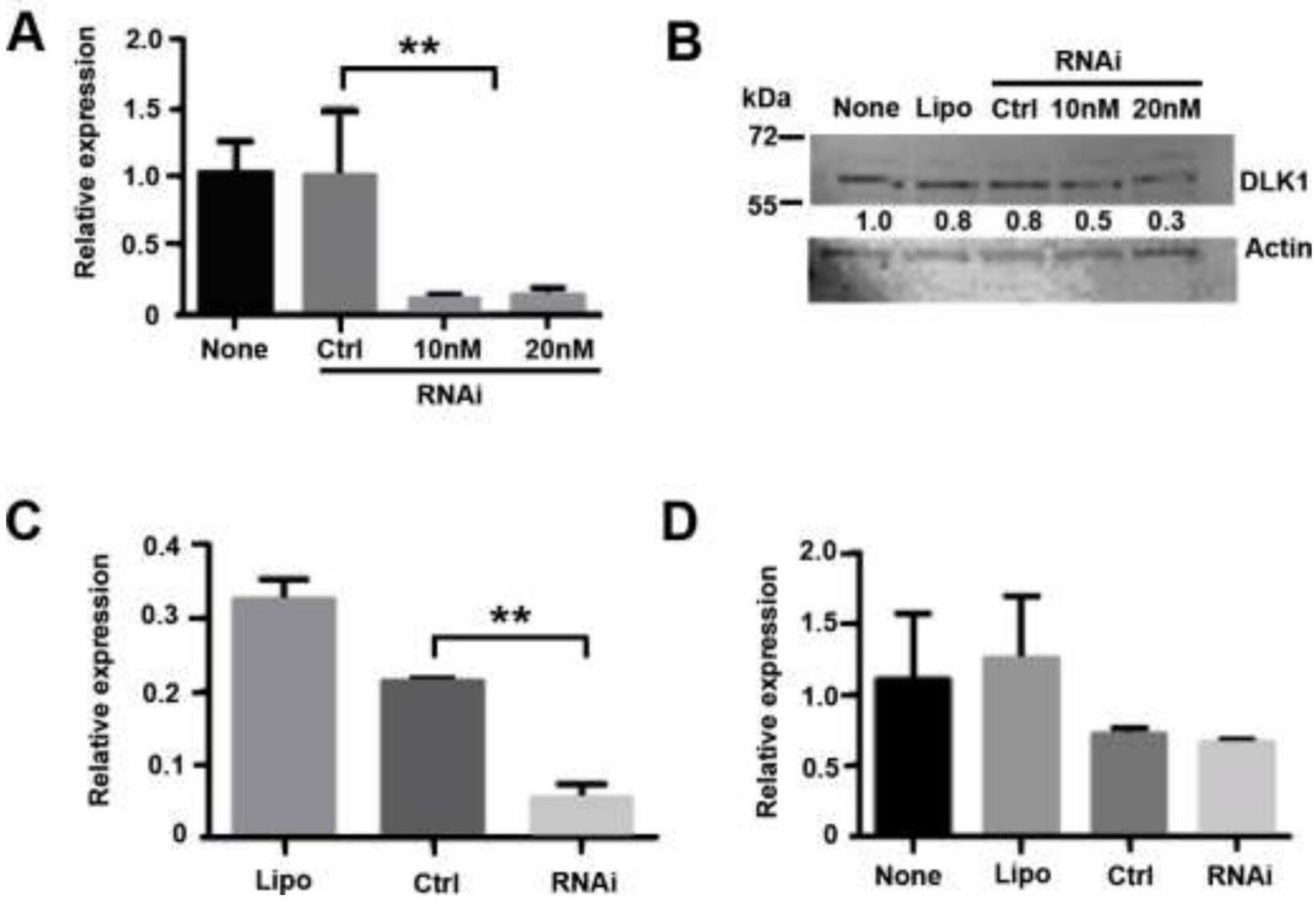

Figure 2: Inhibition of SASH1 expression by RNAi inhibits DLK1 expression.

(**A**) mRNA expression of DLK1 in CEACAM1/MCF7 cells untreated (None), treated with a negative, scrambled oligo control (Ctrl) or treated with SASH1 specific RNAi at 10 nM or 20 nM concentrations. mRNA levels were quantified by qRT-PCR in triplicate. (**B**) CEACAM1/MCF7 cells were untreated (None), treated with lipofectamine (Lipo), treated with a negative, scrambled oligo control (Ctrl), or treated with SASH1 RNAi for three days and proteins analyzed by SDS gel electrophoresis followed by western blotting for DLK1 and β-actin. Values normalized for equal protein loading are shown. (**C**) mRNA expression of DLK1 in CEACAM1/MCF7 cells treated with lipofectamine (Lipo), a negative, scrambled oligo control (Ctrl) or DLK1 RNAi for three days was quantified by qRT-PCR in triplicate. (**D**) mRNA expression of SASH1 in CEACAM1/MCF7 cells untreated (None), treated with lipofectamine (Lipo), treated with a negative, scrambled oligo control (Ctrl) or treated with DLK1 specific RNAi. mRNA levels were quantified by qRT-PCR in triplicate (**, $p<0.001$).

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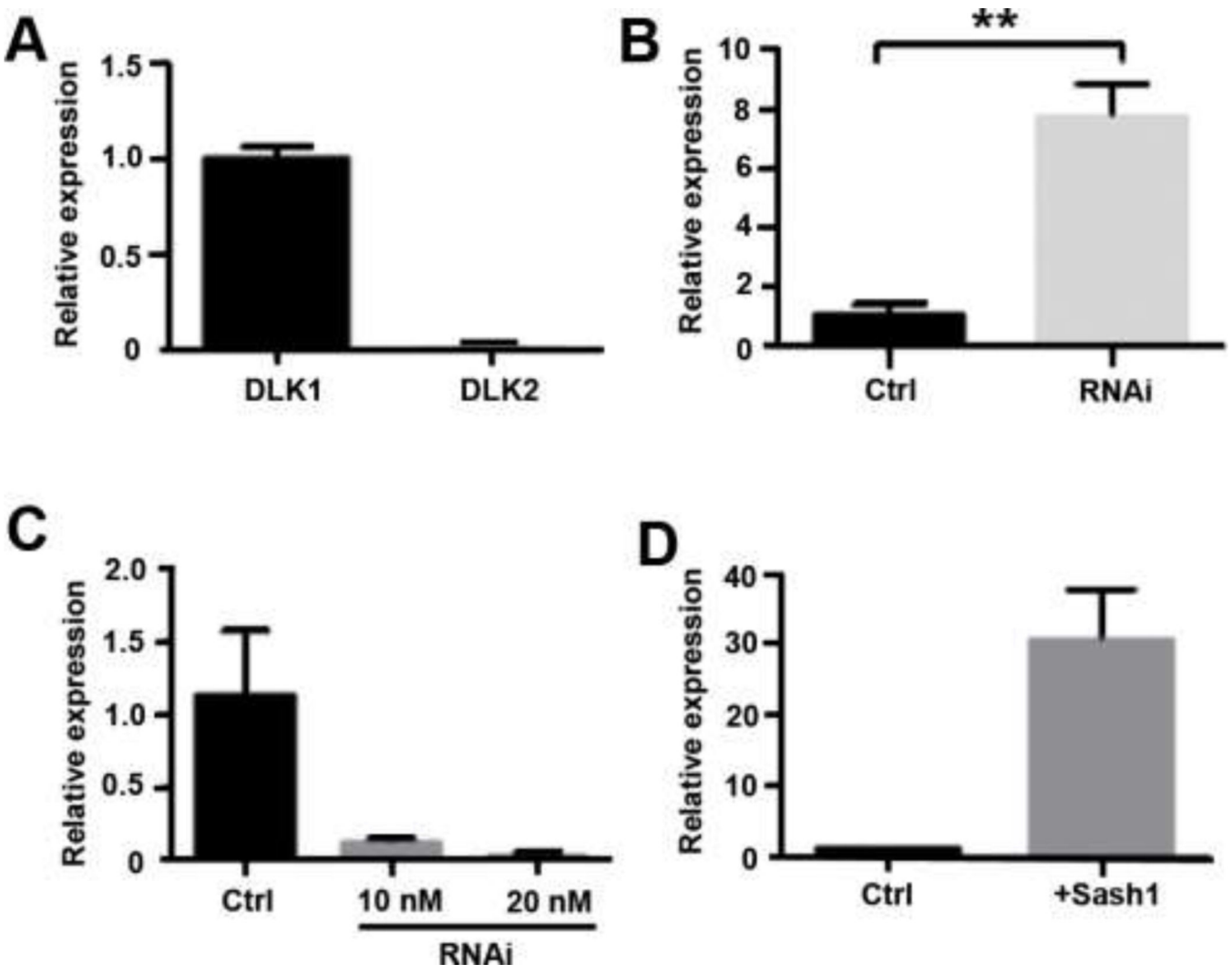

Figure 3: Inhibition of SASH1 by RNAi downregulates both DLK1 and DLK2.

(**A**) mRNA levels of DLK2 compared to DLK1 by qRT-PCR in triplicate in CEACAM1/MCF7 cells. (**B**) mRNA levels of DLK2 from untreated or DLK1 RNAi treated CEACAM1/MCF7 cells. (**C**) mRNA levels of DLK2 by qRT-PCR in triplicate from untreated or SASH1 RNAi treated CEACAM1/MCF7 cells at two different concentrations (10 nM and 20 nM) after growth in 3D culture for six days. (**D**) DLK2 mRNA expression by qRT-PCR in triplicate from CEACAM1/MCF7 cells over expressing SASH1-GFP compared to the non-transfected control.

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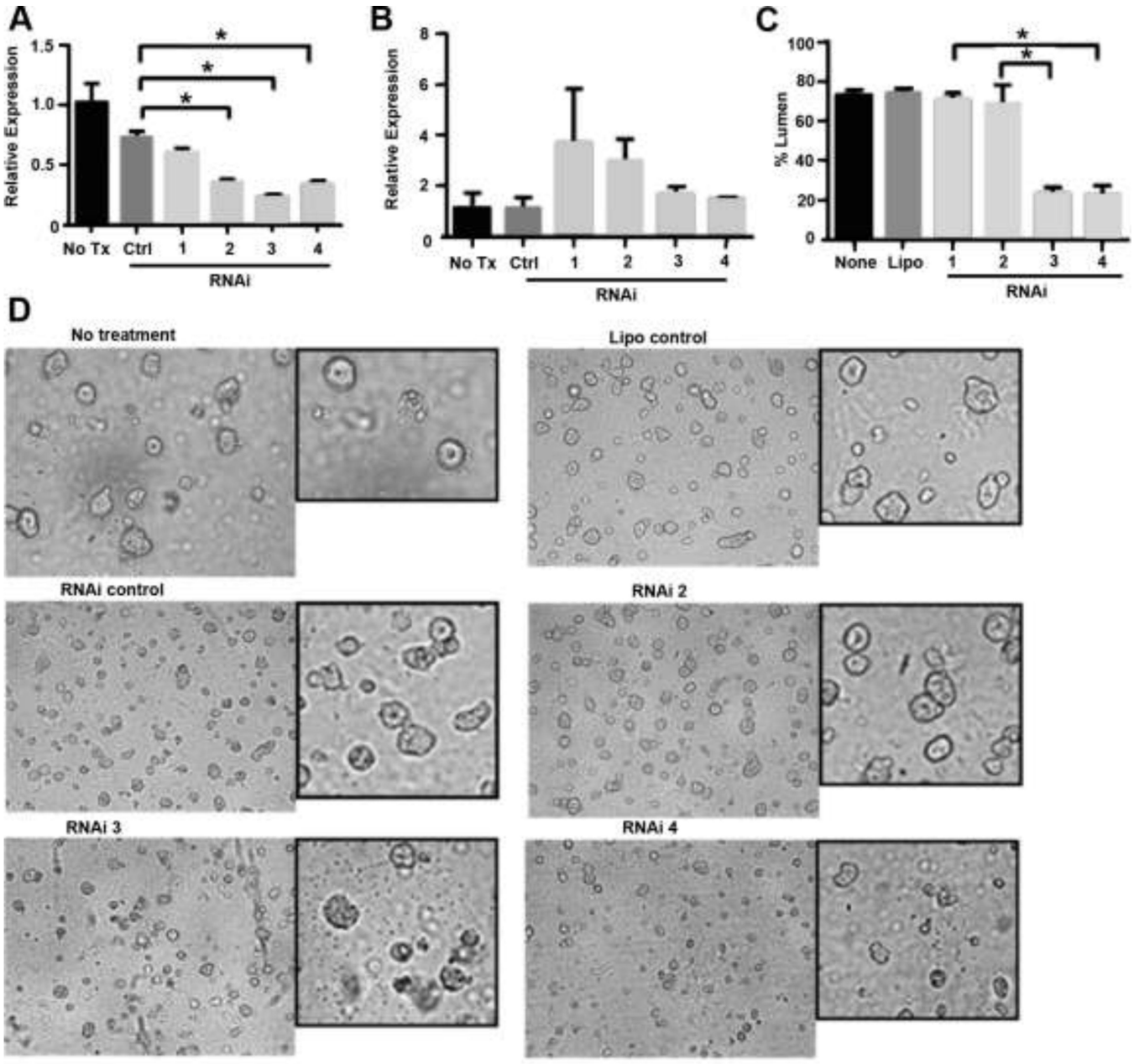

Figure 4: Inhibition of both DLK1 and DLK2 are required for inhibition of lumen formation.

(**A**) mRNA expression of DLK1 in CEACAM1/MCF7 cells untreated (No Tx), treated with control RNAi (Ctrl), or DLK1 RNAi 1 through 4 (1 and 2 from Origene; 3 and 4 from Ambion). (**B**) mRNA expression of DLK2 in CEACAM1/MCF7 cells untreated (No Tx), treated with control RNAi (Ctrl), or treated with DLK1 RNAs 1 through 4 as above. (**C**) Quantitation of lumen formation of CEACAM1/MCF7 cells treated with RNAi 1–4 and grown in 3D vs no treatment (None) or lipofectamine (Lipo). (**D**) Representative micrographs (low resolution and inset, high resolution) of lumen formation of CEACAM1/MCF7 cells untreated, treated with lipofectamine (Lipo control), treated control RNAi or treated with RNAi oligos 2–4 (n= 200, p= 0.001 for RNAi2 vs transfectamine control).

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Magnification 40x, inset 80x. Acini counted had the following dimensions: D= 40–60-μm, with lumen size D= 8–10 μm.

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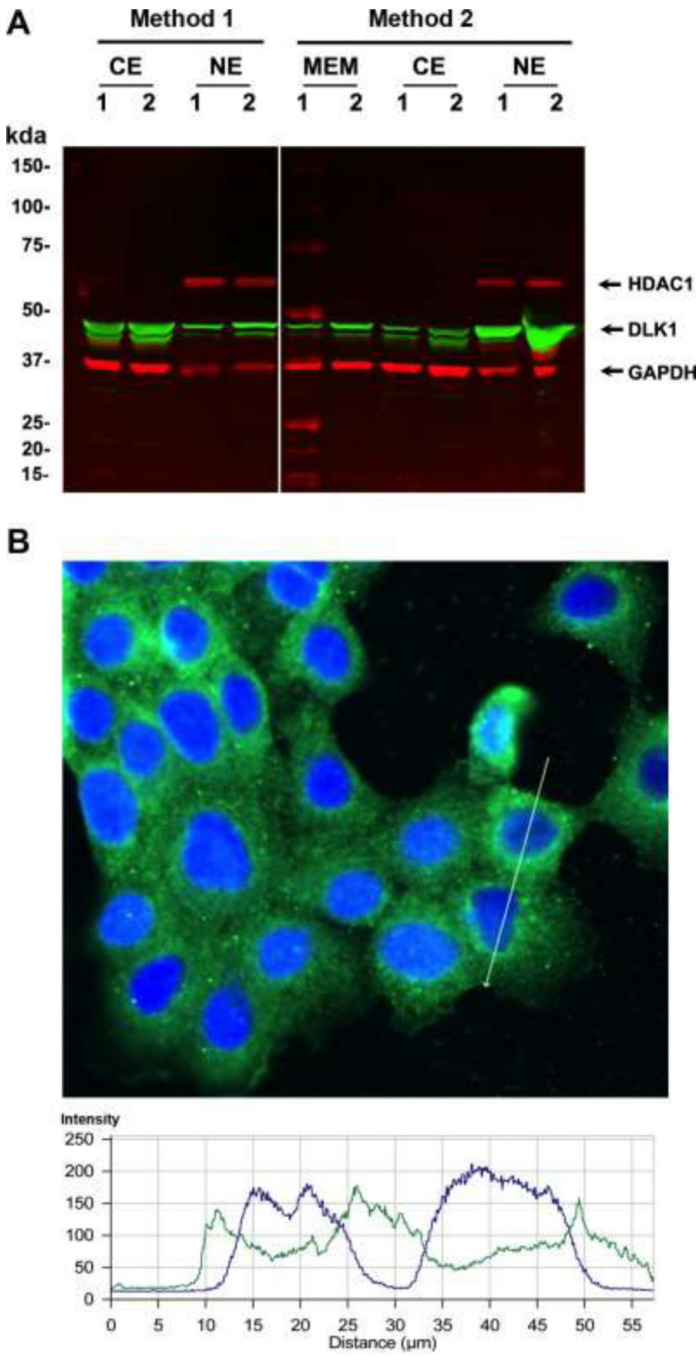

Figure 5: Subcellular location of DLK1 is affected by SASH1 expression.

(A) CEACAM1/MCF7 cells before (1) and after treatment (2) with SASH1 RNAi were fractionated by two methods and the proteins analyzed by western blotting for DLK1. In method 1 (left), DLK1 was found mainly in the cytosolic fraction (CE) with an increase in the nuclear fraction (NE). In method 2 (right), three subcellular fractions were analyzed, membrane (MEM), cytoplasm (CE) and nuclear (NE). In this method DLK1 was found mainly in the nuclear fraction with a further increase after treatment with SASH1 RNAi. Markers for the nuclear fraction were HDAC1 and GAPDH for the cytosolic fraction. The

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degree of contamination of the CE fraction as measured by GAPGH staining was substantial for method 2. (**B**) Confocal staining of CEACAM1/MCF7 cells with DLK1 (green) reveals substantial perinuclear staining (nuclei counter staied with DAPI, blue). The line drawn across two cells reveals the amount of green and blue staining shown in the histogram below.

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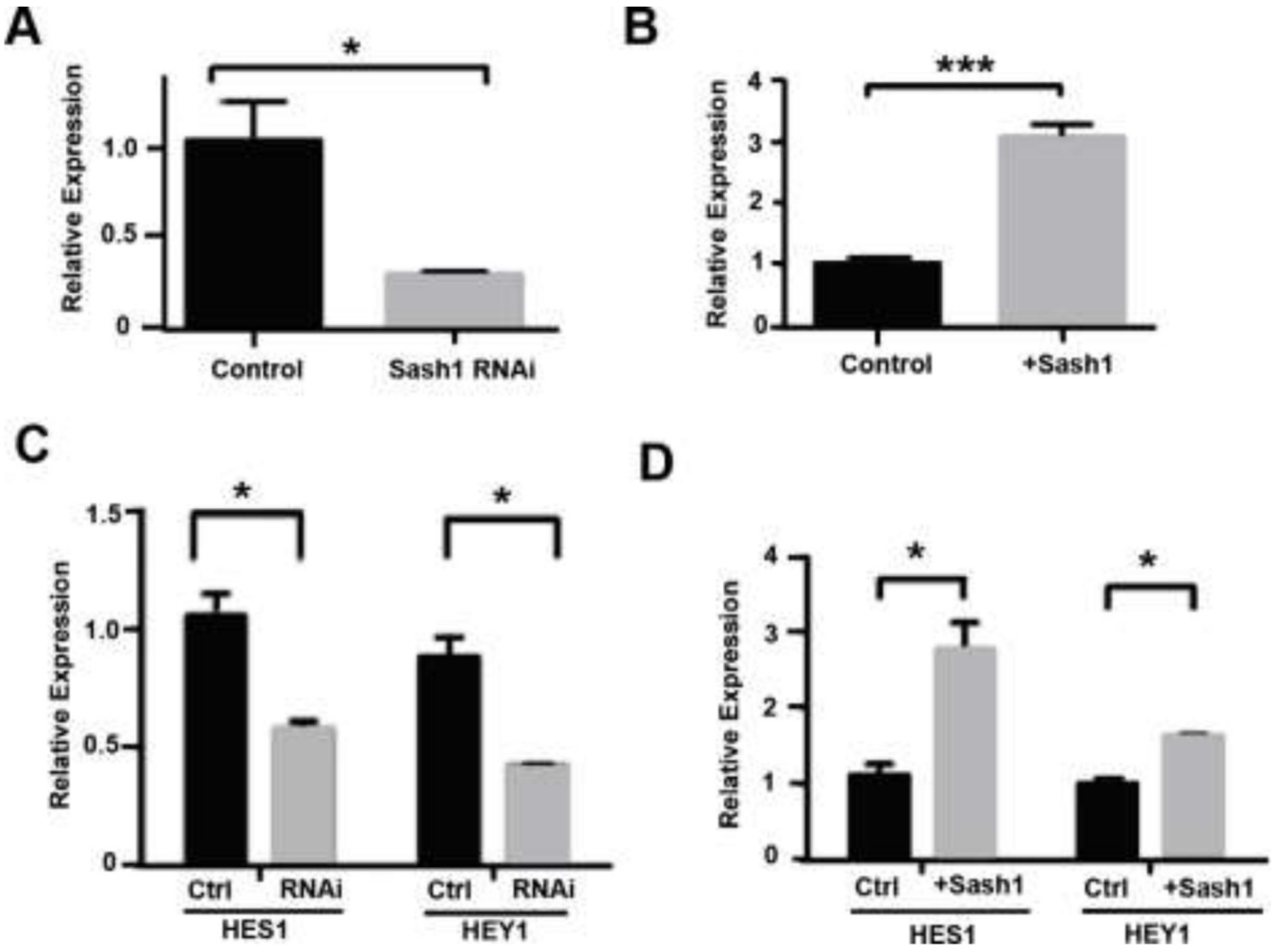

Figure 6. SASH1 expression affects NOTCH1, HES1 and HEY1 expression.

(**A**) NOTCH1 expression quantitated by qRT-PCR in triplicate from CEACAM1/MCF7 cells treated with control RNAi or SASH1 RNAi. (**B**) NOTCH1 expression quantitated by qRT-PCR in triplicate from CEACAM1/MCF7 cells transfected with vector control or SASH1-GFP plasmid. (**C**) HES1 and HEY1 expression quantitated by qRT-PCR in triplicate from CEACAM1/MCF7 cells treated with control RNAi or SASH1 RNAi. (**D**) HES1 and HEY1 expression quantitated by qRT-PCR in triplicate from CEACAM1/MCF7 cells transfected with vector or SASH1-GFP plasmid.

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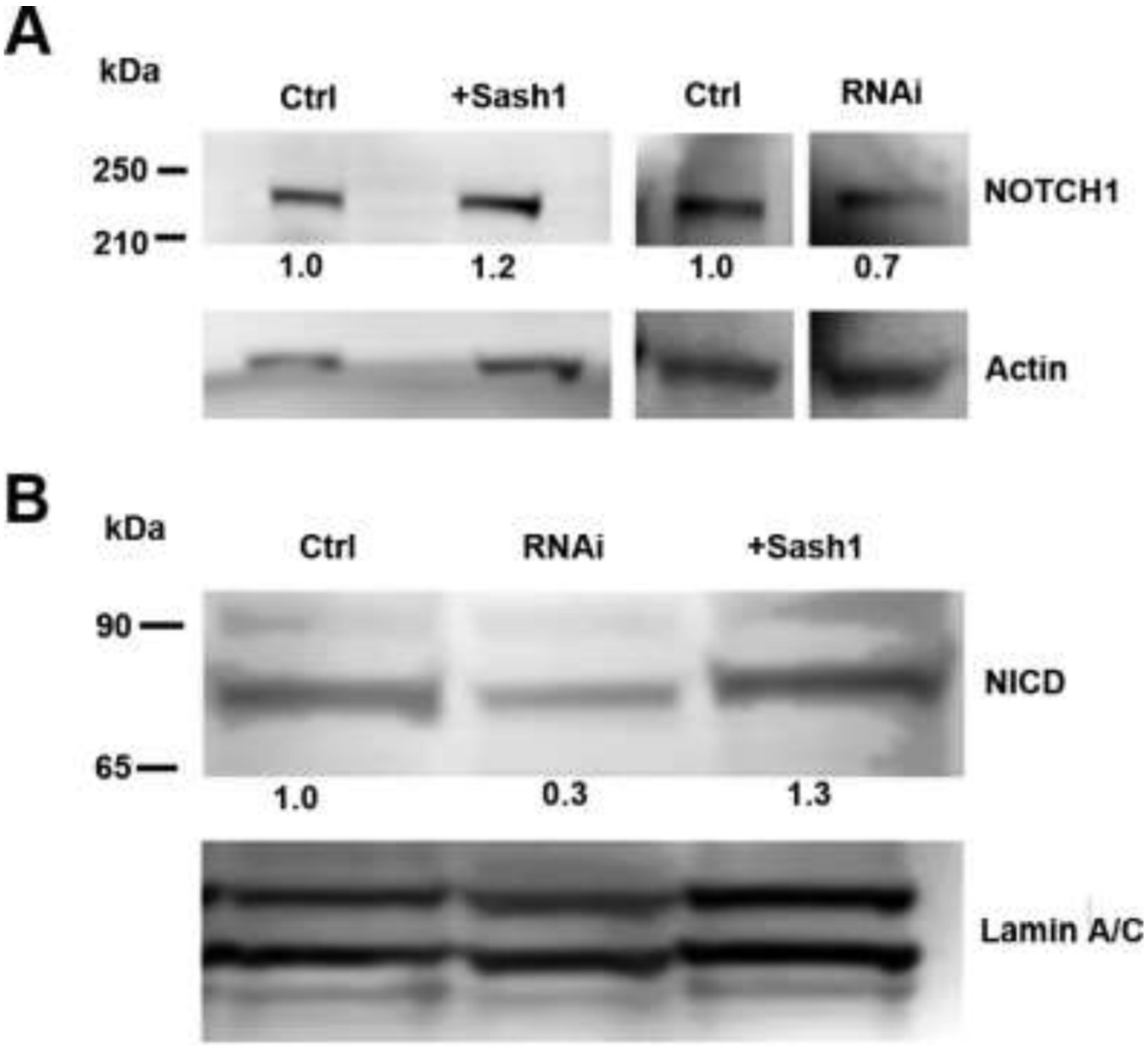

Figure 7. SASH1 expression affects protein levels of NOTCH1 and NICD.

(**A**) CEACAM1/MCF7 cells were transfected with vector or SASH1-GFP plasmid (left) or with RNAi control or SASH1 RNAi (right) and the protein levels of NOTCH1 measured by western blotting of total cell lysates (actin was run as a loading control; numbers refer to relative values for NOTCH1). (**B**). Same as in (**A**), except nuclear fractions were prepared and western blotted for NICD with a Lamin A/C control (numbers refer to relative amounts of NICD).

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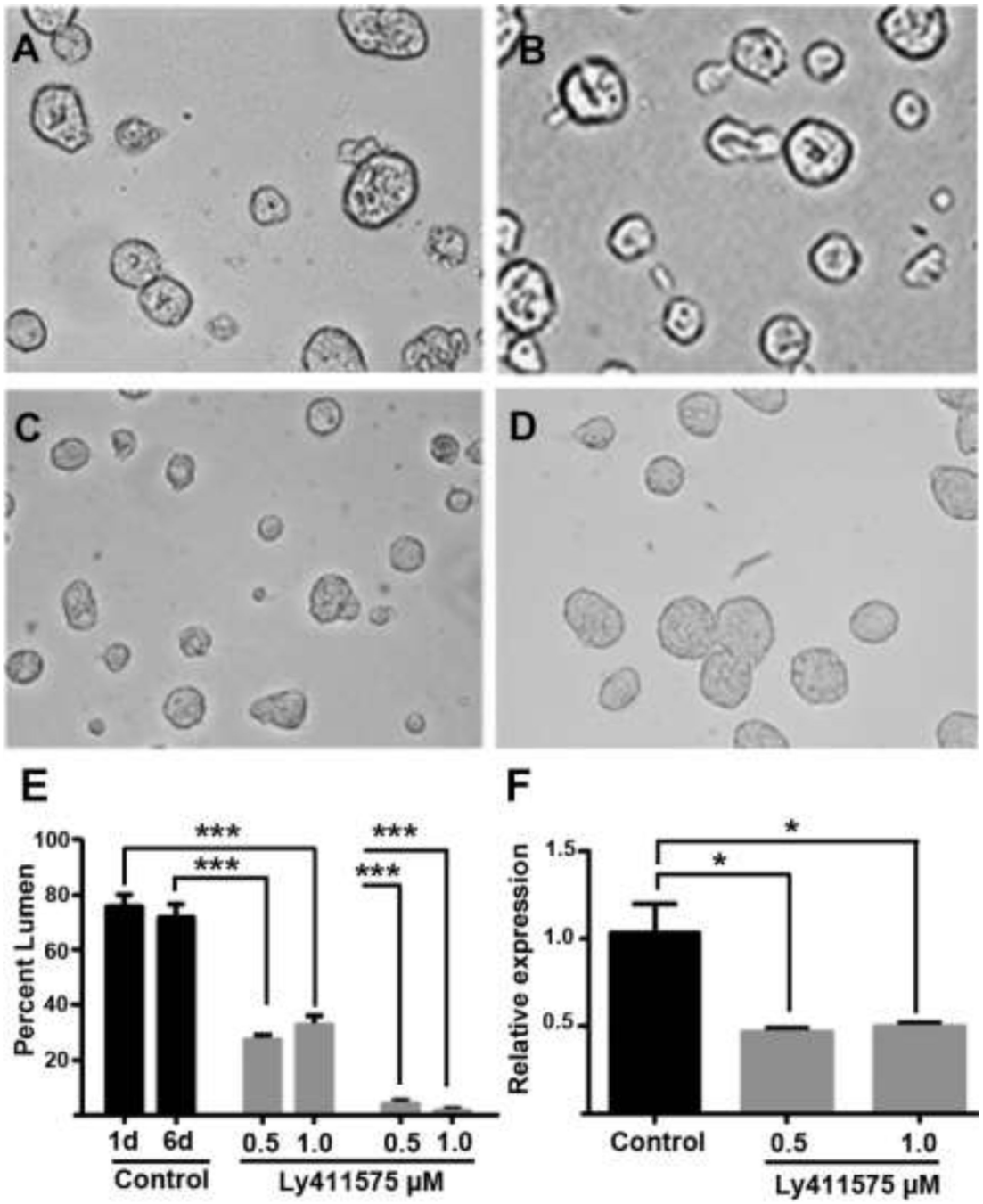

Figure 8. Treatment of CEACAM1/MCF7 cells with γ-Secretase inhibitor LY411575 inhibits lumen formation.

(**A, B**) CEACAM1/MCF7 cells were treated with 0.5 μM or 1.0 μM γ-secretase inhibitor LY411575 in 3D culture for 1d or 6d, respectively, and lumen formation scored by microscopic analysis at 6d (n= 200, p= 0.001 for LY411575 treated vs DMSO control). Acini counted had the following dimensions: D= 40–60-μm, with lumen size D= 8–10 μm. (**C, D**) same as in **A** and **B** except cells were treated with vehicle only (0.5% or

1.0% DMSO). (**E**). Quantitation of lumen from **A-D**. (**F**) RNA isolated from 6d acini and expression of NOTCH1 measured by qRT-PCR in triplicate. P values are * <.01, *** <0.001.

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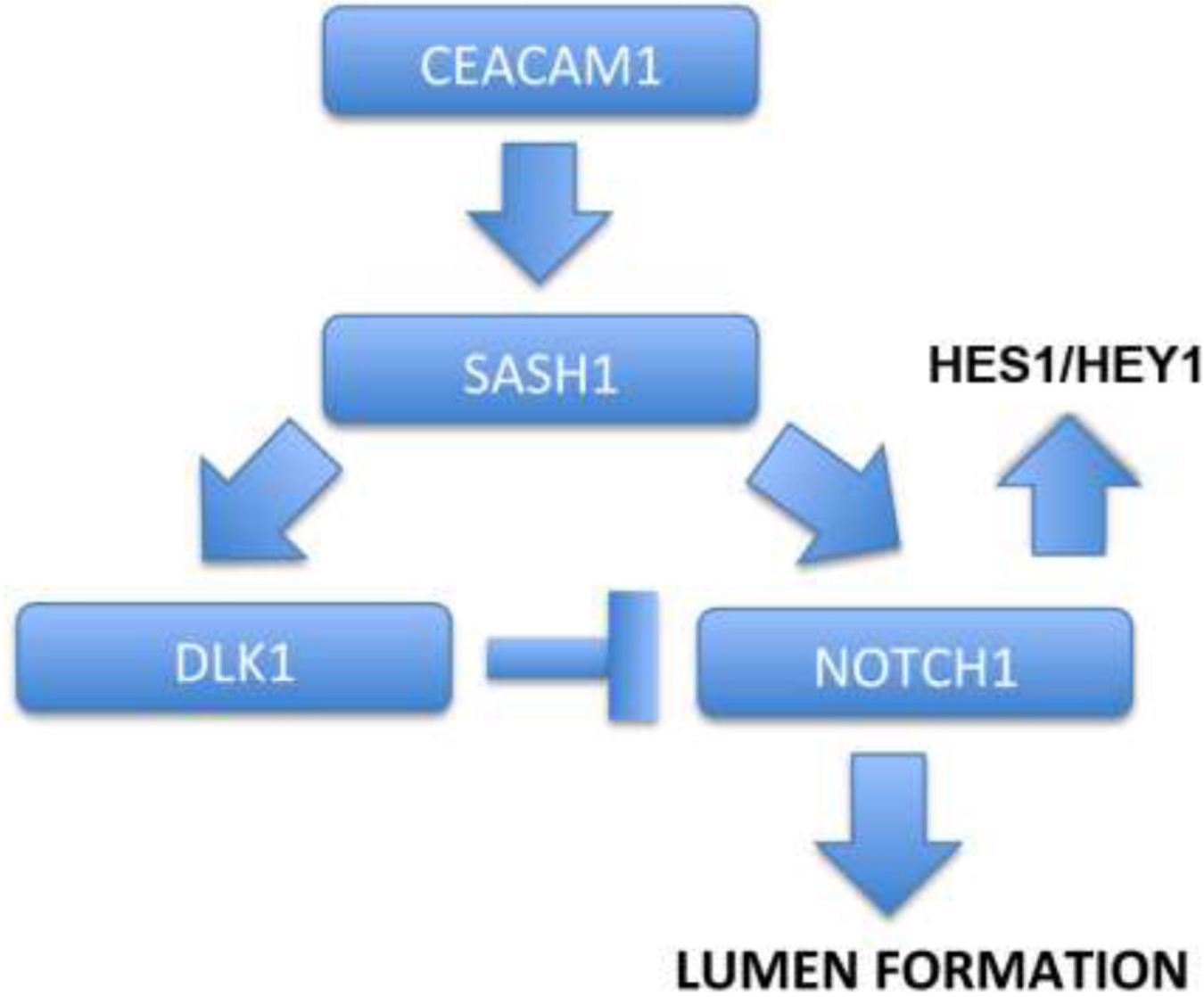

Figure 9. Expression of SASH1, regulated by CEACAM1, leads to branched regulation of DLK1 and NOTCH1.

Genetic analysis of lumen formation of CEACAM1/MCF7 cells reveals SASH1 as a major up-regulated gene that, in turn, up-regulates DLK1 and NOTCH1. Since DLK1 negatively regulates NOTCH1 and its genes (HES1 and HEY1), NOTCH1 signaling is likely under temporal control during lumenogenesis.

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Table 1.

Inhibition of lumen formation of CEACAM1/MCF7 cells grown in 3D culture and treated with RNAi or γ-secretase[1].

Treatment	Percent Lumen	P value
None	93	-
Transfectamine control	81	-
RNAi control	76	NS
SASH1 RNAi	13	<0.001
DLK1 RNAi1	70	NS
DLK1 RNAi2	23	<0.001
DLK1 RNAi3	23	<0.001
DMSO control	71	-
γ-secretase 1d	33	<0.001
γ-sectease 6d	2	<0.001

[1]Results are percent of lumen counted out of 200–300 acini. P values are calculated versus the transfectamine or DMSO controls (NS= not significant).

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Table 2:

Gene expression analysis of CEACAM1/MCF7 cells treated with SASH1 RNAi[1].

Gene	log2 ratio
SASH1	−2.257
DLK1	−2.215
IGFBP5	−1.88
SOCS2	−1.63
CDC42EP5	−1.476
SCIN	−1.175
VAV3	−0.448
ELF5	−0.349
RUNX2	1.502

[1]RNA was isolated from CEACAM1/MCF7 cells with or without treatment of SASH1 RNAi and subjected to microarray analysis. The ratio of the two (log2 ratio) of the top 9 genes down-regulated by the treatment are shown.