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SHORT COMMUNICATION

Ha-*Ras* transformation of MCF10A cells leads to repression of Singleminded-2s through NOTCH and C/EBPβ

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We have previously shown that Singleminded-2s (SIM2s), a member of the basic helix-loop-helix Per-Arnt-Sim (bHLH/PAS) family of transcription factors, is downregulated in breast cancer samples and has tumor suppressor activity. However, the mechanism by which SIM2s is repressed in breast cancer cells has not been determined. In this study, we show that transformation of MCF10A cells by Harvey-*Ras* (Ha-*Ras*) induces CCAAT/enhance binding protein β (C/EBPβ) and activates the NOTCH signaling pathway to block SIM2s gene expression. NOTCH-mediated repression acts through a C-repeat binding factor 1 (CBF1)-independent mechanism, as introduction of CBF1 had no effect on SIM2s expression. Consistent with C/ebpβ-dependent inhibition of SIM2s, *C*/ebpβ$^{-/-}$ mouse mammary glands express high levels of SIM2s and reestablishment of C/ebpβ isoforms decreased SIM2s mRNA levels in C/ebpβ immortalized mammary epithelial cell lines. These studies illustrate a novel pathway of tumor suppressor gene silencing in Ha-*Ras*-transformed breast epithelial cells and identify SIM2s as a target of C/EBPβ and NOTCH signaling.
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Introduction

Singleminded-2 (SIM2) is a member of the basic helix-loop-helix Per-Arnt-Sim (bHLH/PAS) family of transcription factors (Kewley *et al*., 2004). SIM2 is one of two mammalian orthologs of the *Drosophila* singleminded (sim) gene, which regulates central midline development (Crews *et al*., 1992). Previous studies have demonstrated that SIM2 is differentially expressed in prostate, colon and pancreatic cancers (Deyoung *et al*., 2002; Aleman *et al*., 2005; Halvorsen *et al*., 2007). We have shown that a short splice variant of SIM2, SIM2s, is expressed in human breast epithelial cells and is downregulated in primary human breast cancer samples (Kwak *et al*, 2007). Reestablishment of SIM2s in highly invasive breast cancer cells significantly inhibited growth and motility, suggesting that SIM2s is a breast tumor suppressor gene. Indeed, loss of SIM2s causes aberrant mammary ductal development with features suggestive of malignant transformation including increased proliferation, changes in polarity, downregulation of E-cadherin and invasion into the surrounding stroma (Laffin *et al*., 2008). Furthermore, short-hairpin RNA (shRNA)-mediated reduction of SIM2s (SIM2*i*) in MCF7 human breast cells promotes an epithelial-mesenchymal transition (EMT) and increases tumorigenic potential. These results suggest that SIM2s is required for normal mammary ductal development and inhibition of SIM2s is permissive for acquisition of an invasive mesenchymal phenotype; however, the mechanism by which SIM2s is suppressed during breast cancer progression has not been determined.

The *Ras* proto-oncogene is an important effector molecule that is essential for growth-dependent induction of numerous growth factors and cytokines. Although mutations in *Ras* are rare in breast cancers (Khleif *et al*., 1999), *Ras* is inappropriately activated in 50% of breast tumors and positively correlates with early neoplasia and poor prognosis (von Lintig *et al*., 2000). *Ras* activation is also associated with ERBB2-overexpressing breast tumors, which represent 30% of breast cancer cases (Slamon *et al*., 1989). Harvey-*Ras* (Ha-*Ras*)-induced tumors are characterized by activation of mitogen-activated protein kinase signaling and are cyclin D1 dependent (Yu *et al*., 2001; Dunn *et al*., 2005). In addition, by activating oncogenic pathways, Ha-*Ras* overexpression, driven by the mouse mammary tumor virus promoter, induces mammary adenocarcinomas (Sinn *et al*., 1987; Nielsen *et al*., 1991). Two Ha-*Ras* targets associated with breast cancer are the NOTCH signaling pathway and the CCAAT/enhance binding protein β (C/EBPβ) transcription factor (Nakajima *et al*., 1993; Weijzen *et al*., 2002). Increased NOTCH signaling has been demonstrated in a variety of human breast carcinomas (Stylianou *et al*., 2006), whereas C/EBPβ has been identified as an important mediator of *Ras*-induced tumorigenesis in a mouse skin

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carcinogenesis model known to cause Ha-*Ras* mutations (Zhu *et al*., 2002; Shuman *et al*., 2004; Shim *et al*., 2005). In the studies presented here, we show that Ha-*Ras*-mediated transformation of MCF10A normal breast epithelial cells leads to a decrease in SIM2s expression resulting from C/EBPβ induction and NOTCH signaling.

Results and discussion

Ha-Ras *transformation of MCF10A decreases SIM2s gene expression*

To define the mechanisms by which SIM2s is silenced in breast cancer, normal immortalized breast MCF10A cells were stably transduced with control vector or Ha-*Ras* expression constructs. Ha-*Ras*-MCF10A cells displayed hallmarks of transformation and EMT including a significant increase in invasion, anchorage-independent growth (data not shown), focus formation (Figure 1a) and downregulation of E-cadherin protein levels (Figure 1c) as compared to control cells. Evaluation of SIM2s expression in Ha-*Ras*-MCF10A cells, using real-time PCR and western blot analysis, showed a significant decrease in SIM2s mRNA (Figure 1a) and protein levels (Figure 1b), respectively. These results suggest that SIM2s is downregulated during Ha-*Ras*-dependent transformation and pathways downstream of Ha-*Ras* suppress SIM2s gene expression.

Reduction of SIM2s in MCF10A cells by shRNA induces an invasive EMT-like phenotype

To determine if downregulation of SIM2s contributes to Ha-*Ras*-mediated transformation of MCF10A cells, we stably silenced SIM2s in MCF10A cells using a previously confirmed SIM2s-specific lentivirus shRNA construct (Laffin *et al*., 2008) (Figures 2a and b). When grown in three-dimensional cultures, control MCF10A cells formed regular, spherical acini, whereas a majority of SIM2*i* MCF10A acini were misshapen with cells budding into the surrounding matrix (Figure 2c). Loss of SIM2s also correlated with significantly increased *in vitro* invasive potential (Figure 2d). Furthermore, western blots showed that SIM2*i*-MCF10A cells had decreased E-cadherin and increased vimentin expression (Figure 2e). Together, these results show that reduction of SIM2s in MCF10A cells to levels observed in

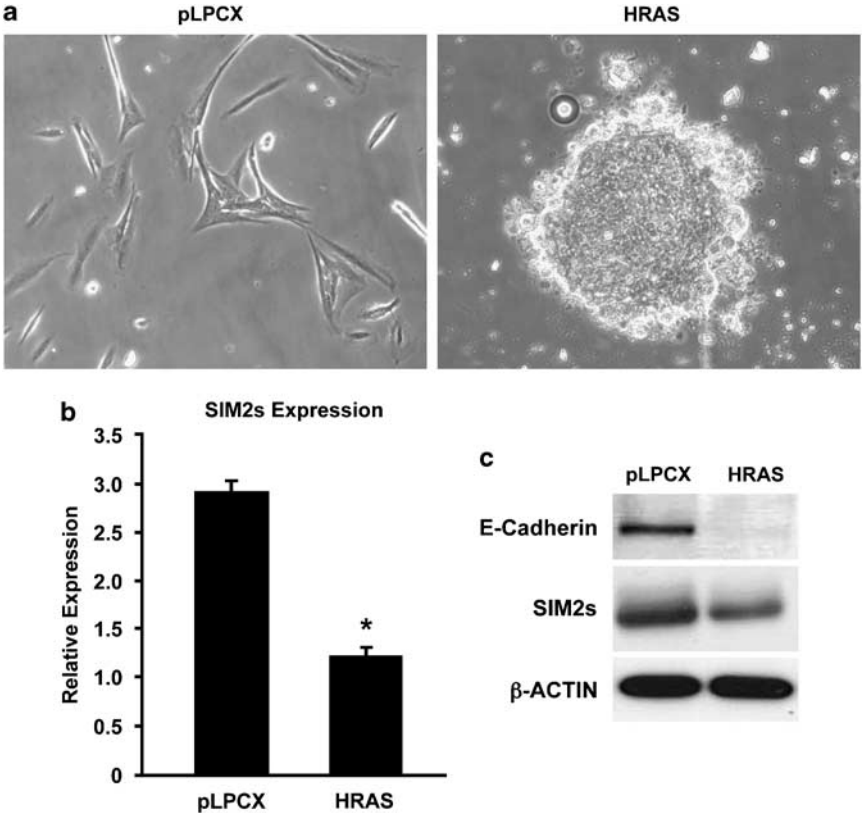


Figure 1 Downregulation of Singleminded-2s (SIM2s) expression in HRAS-transformed MCF10A cells. (**a**) Altered morphology of Harvey-*Ras* (Ha-*Ras*)-transformed MCF10A cells. Cells were transduced with either empty vector (pLPCX, left panel) or a vector containing the Ha-*Ras* coding sequence (HRAS, right panel). Differential contrast imaging of cells following 2 days of selection in medium containing 1 μg/ml puromycin, cells, showed that Ha-*Ras*-transduced cells lost their normal ovoid shape and displayed abnormal cellular organization. Ha-*Ras* construct was provided by Brian and Alana Welm and subcloned into *Eco*RI sites in pLPCX (Clontech). (**b**, **c**) SIM2s and E-cadherin expression is decreased in Ha-*Ras*-overexpressing MCF10A cells. Total RNA and protein were isolated from control (pLPCX) and Ha-*Ras*-overexpressing cells (HRAS) and analysed for SIM2s expression by quantitative real-time RT-PCR and western analysis (Chemicon) as previously described (Laffin *et al*., 2008). The results show that SIM2s and E-cadherin expression are significantly reduced in Ha-*Ras*-transduced MCF10A cells in comparison to control cells (mean ± s.e. for three replicates; $*P = 0.0002$).

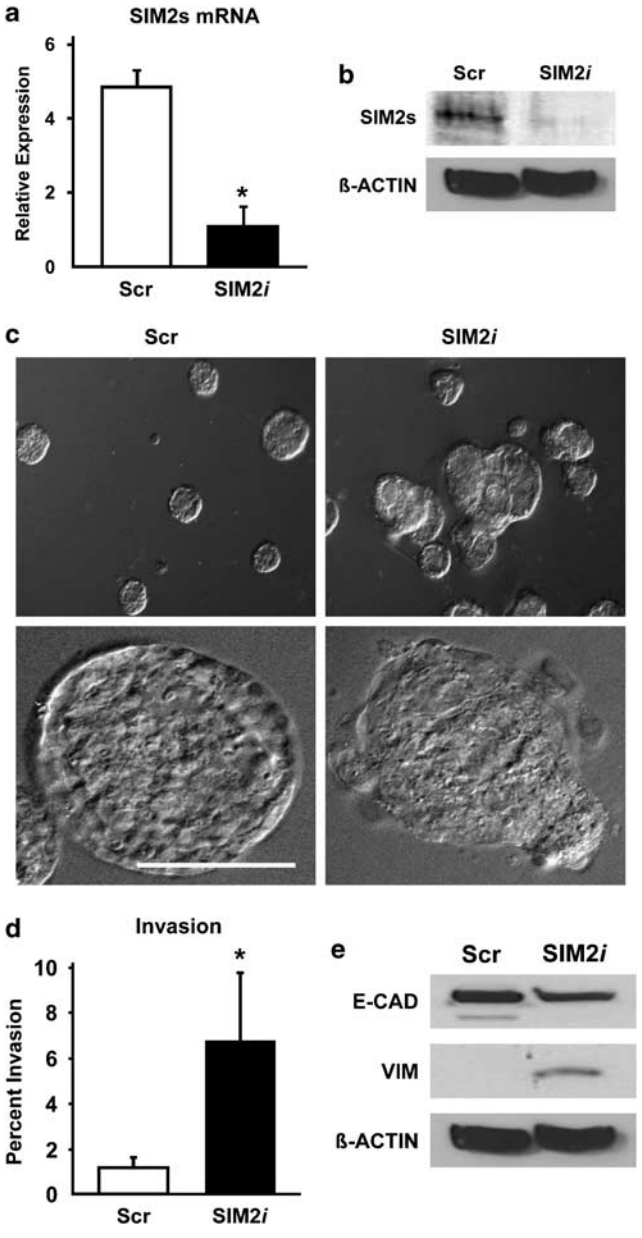


Figure 2 Knockdown of Singleminded-2s (SIM2s) in MCF10A mammary epithelial cells by short-hairpin RNA (shRNA) disrupts three-dimensional acinar morphogenesis and induces a partial epithelial-mesenchymal transition (EMT). (**a**, **b**) Inhibition of SIM2s expression in MCF10A cells by shRNA. MCF10A cells transduced with a confirmed SIM2-specific construct (SIM2s-sequence 3116; SIM2*i*) showed a significant reduction in SIM2s mRNA and protein as compared to a nonspecific control shRNA retroviral construct (Scr) (Laffin *et al*., 2008). (**c**) Low- and high-power differential interference contrast images of MCF10A acini grown on matrigel demonstrate that acinar morphogenesis is disrupted in SIM2*i* MCF10A cells as compared to Scr control. (**d**) SIM2*i* MCF10A cells display an increased ability to invade through a matrigel matrix using transwell invasion assays (Kwak *et al*., 2007). (**e**) Loss of SIM2s induces a partial EMT. Western blot analysis of Scr and SIM2*i* cells found that E-cadherin expression is reduced and vimentin expression is increased in SIM2*i* MCF10A cells as compared to Scr controls and is consistent with an EMT-like phenotype (Laffin *et al*., 2008). Data are represented as mean ± s.e.m., asterisk (*) indicates $P<0.05$; Bar = 100 μM.

Ha-*Ras*-transformed MCF10A cells induces an invasive EMT-like phenotype.

Activation of NOTCH in Ha-Ras-*MCF10A cells represses SIM2s through a CBF1-independent mechanism*

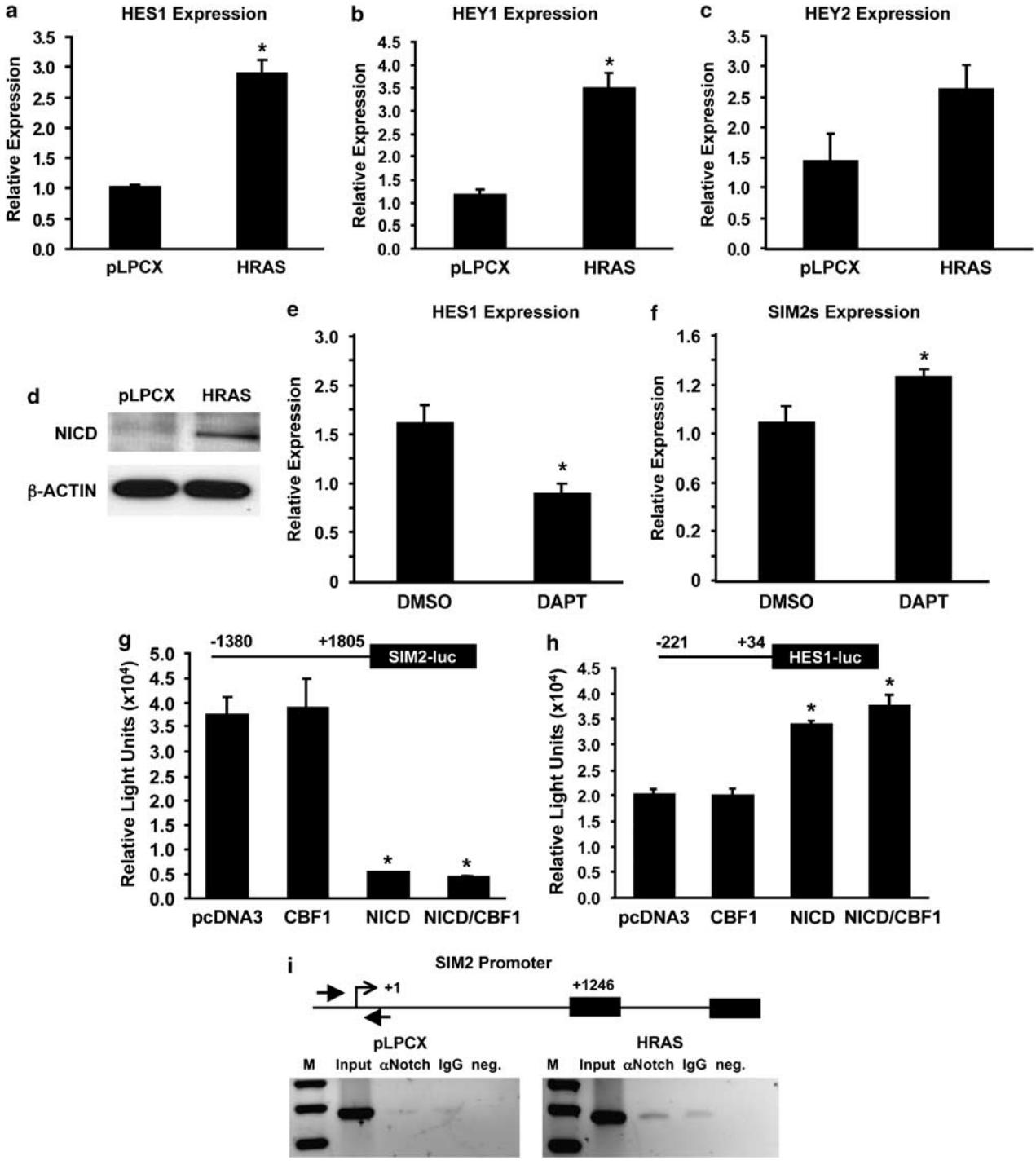
Increased NOTCH signaling has been demonstrated in a variety of human breast carcinomas (Stylianou *et al*., 2006). Studies have shown that Ha-*Ras* activates NOTCH, which is required to maintain the transformed phenotype in human cells (Weijzen *et al*., 2002). Activation of NOTCH signaling is an important mediator of *Ras*-induced tumorigenesis in the mouse mammary gland and is also implicated in stem cell maintenance (Weijzen *et al*., 2002; Kiaris *et al*., 2004; Sansone *et al*., 2007). Indeed, in Ha-*Ras*-MCF10A cells we observed a significant increase in the expression of known NOTCH target genes HES1, HEY1 and HEY2 by real-time PCR (Figures 3a–c) and detection of the NOTCH intracellular domain (NICD) by western blot analysis (Figure 3d), indicating that the NOTCH pathway is active in these cells. Furthermore, treatment with the γ-secretase inhibitor, DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester), significantly blocked NOTCH-induced expression of HES1 and suppression of SIM2s gene expression (Figures 3e and f), implying that Ha-*Ras*-activated NOTCH signaling targets SIM2s.

Previous studies have shown that the canonical NOTCH signaling pathway acts through C-repeat binding factor 1 (CBF1) to govern normal developmental processes (Bray, 2006). In addition, CBF1-independent mechanisms of NOTCH signaling that contribute to cell fate determination have also been identified (Martinez Arias *et al*., 2002). To evaluate the function of NOTCH signaling in regulation of SIM2s, a SIM2 promoter-luciferase reporter construct was cotransfected in the presence or absence of NICD or CBF1 expression vectors. NICD significantly repressed SIM2 promoter activity ($P<0.0004$); however, CBF1 had no effect on SIM2 promoter activity, suggesting a CBF1-independent mechanism of SIM2s repression by the NOTCH signaling pathway (Figure 3g). NICD function was confirmed using an HES1 promoter-controlled reporter. As expected, reporter activity was increased in cells cotransfected with either NICD or NICD plus CBF1, but not in CBF1 cells alone (Jarriault *et al*., 1995; Figure 3h). To determine whether SIM2 was a direct target of NICD, chromatin immunoprecipitation (ChIP) assays were employed to evaluate the region near the transcriptional start site of the SIM2 promoter (Figure 3i). The results show increased binding of NICD to the endogenous SIM2 promoter in Ha-*Ras*-over-expressing cells, suggesting that activation of NOTCH and release of the NICD by Ha-*Ras* contributes to inhibition of SIM2s expression.

C/EBPβ-dependent regulation of SIM2s in vitro *and* in vivo

C/EBPβ is a member of the basic leucine zipper family of transcription factors, which has an important

function in mammary development and has recently been shown to be a critical mediator of *Ras*-induced tumorigenesis (Seagroves *et al*., 1998; Zhu *et al*., 2002; Grimm and Rosen, 2003). The C/EBPβ gene encodes a single 1.4 kb mRNA that, through alternative translation start sites, leads to production of three isoforms: LAP1, LAP2 and the dominant negative isoform, LIP. Both LAP isoforms contain the DNA binding and dimerization domains, whereas LIP is missing the transactivation domain and antagonizes LAP-mediated gene transcription by competing for LAP binding sites or by forming LIP/LAP heterodimers (Descombes and Schibler, 1991). The ratio of LAP to LIP has been proposed to have an important function in breast cancer (Zahnow, 2002; Grimm and Rosen, 2003). A low LIP/LAP ratio, indicating an increase in LIP expression, is associated with higher histological grading (Zahnow *et al*., 1997; Milde-Langosch *et al*., 2003) and has recently been shown to point toward a loss of transforming growth factor-β-cytostatic responses (Gomis *et al*., 2006). Indeed, overexpression of LIP in mouse mammary glands causes an increase in prolifera-



tion resulting in hyperplasias and invasive carcinoma (Zahnow *et al*., 2001). Although C/EBPβ is associated with breast cancer, its specific function in tumorigenesis is not well understood.

To determine the effect of Ha-*Ras* transformation on C/EBPβ, we used western blot analysis to evaluate levels of LAP1, LAP2 and LIP in Ha-*Ras*-MCF10A cells. These data revealed similar levels of LAP1 and LAP2; however, there was a significant increase in the truncated dominant negative isoform (LIP) in the Ha-*Ras*-MCF10A cells (Figure 4a). This is consistent with recent studies showing Ha-*Ras*-dependent induction of EGFR, which induces LIP (Martinez-Lacaci *et al*., 2000; Baldwin *et al*., 2004). To confirm if the increase in LIP alone alters SIM2s, MCF10A cells were stably transduced with control (pBABE), LAP1, LAP2 or LIP vectors. Western blot analysis revealed that LAP1 and LAP2 did not affect SIM2s levels, whereas overexpression of LIP leads to a reduction in SIM2s protein (Figure 4b).

C/ebpβ$^{-/-}$ mice are viable, but display immune system defects, abnormal brown adipose tissue function, skin irregularities and female infertility (Grimm and Rosen, 2003). In addition, mammary glands from *C*/ebpβ$^{-/-}$ mice have impaired ductal outgrowth and differentiation due to a disruption in cell fate determination (Seagroves *et al*., 1998). We have previously shown that SIM2s is expressed in luminal mammary epithelial cells and is required for proper ductal morphogenesis (Laffin *et al*., 2008). Using real-time PCR, we found that SIM2s is significantly increased in *C*/ebpβ$^{-/-}$ mammary glands when compared to wild-type mice (Figure 4c). The increase in SIM2s was confirmed at the protein level by immunohistochemistry and was found uniformly in the luminal epithelial cells of C/ebpβ null glands, compared to the more punctate expression pattern seen in wild-type glands (Figure 4d). To define further the relationship between C/ebpβ and SIM2s, we used an immortalized cell line established from mammary glands of C/ebpβ null virgin females. When compared to control cells, SIM2s expression was significantly increased in this *in vitro* model, consistent with the *in vivo* data. Furthermore, reestablishing each of the three C/ebpβ isoforms by lentiviral transduction (Figure 4f) led to a reduction in SIM2s expression (Figure 4g). Together, these results show that SIM2s is differentially regulated by C/EBPβ under both transforming and normal developmental conditions.

In summary, we have identified a pathway connecting Ha-*Ras* activation to SIM2s silencing through NOTCH and C/EBPβ. Ha-*Ras* has been shown to promote cleavage of NOTCH, releasing the active NICD (Weijzen *et al*., 2002), and to increase the LIP/LAP ratio (Nakajima *et al*., 1993). In addition, we also found that activation of NOTCH and C/EBPβ by Ha-*Ras* independently regulate SIM2s expression. We observed no changes in the LIP/LAP isoforms in Ha-*Ras*-MCF10A cells following treatment with a γ-secretase inhibitor (Supplementary Figure 1a), which we had previously shown to block RAS-mediated activation of HES1 and partially alleviated SIM2s suppression (Figures 3e and f), nor upregulation of NOTCH or NOTCH target genes HES1 and HEY1 in LIP-transduced MCF10A cells (Supplementary Figure 1b and c). We do not suggest that these are the only mediators of SIM2s repression in breast cancer cells, because *Ras* signaling affects many other pathways including phosphatidylinositol 3-kinase. Also, it will be important to evaluate SIM2s expression in other epigenetic and oncogene-driven models of breast cancer, such as MYC and WNT. Because NOTCH and C/EBPβ are differentially expressed in differing tumor types, these results may also explain the oncogenic function SIM2s has in pancreatic and prostate cancers (Sundaram, 2005; Gomis *et al*., 2006). It is of interest that two proteins involved in cell fate determination independently regulate SIM2s (Liu *et al*., 2005), suggesting that SIM2s has a function in mammary gland cell fate, possibly directing cells to differentiate. Future studies using gain- and loss-of-function mouse mammary gland models will elucidate the function of SIM2s in this process. We believe that downregulation of SIM2s expression is a requirement for breast cancer progres-

Figure 3 Activation of NOTCH in Harvey-*Ras* (Ha-*Ras*)-MCF10A cells. (**a**, **b**, **c**) Expression of NOTCH target genes in control and Ha-*Ras*-transduced MCF10A cells. Analysis of gene expression by real-time RT-PCR found that NOTCH target genes HES1, HEY1 and HEY2 are significantly higher in Ha-*Ras*-transduced cells compared to control MCF10A cells (*$P<0.005$). (**d**) Western blot analysis of Ha-*Ras*-MCF10A cells using a NOTCH intracellular domain (NICD)-specific antibody (Cell Signaling Technology) shows a significant increase in the active form of NOTCH. Together, these results suggest that the NOTCH pathway is activated by overexpression of RAS in MCF10A cells. (**e**, **f**) Treatment of Ha-*Ras*-MCF10A cells with 1 μM of the γ-secretase inhibitor, DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) (Calbiochem), significantly blocked NOTCH-induced expression of HES1 and suppression of SIM2 gene expression (*$P<0.05$). (**g**, **h**) Effect of NICD and C-repeat binding factor 1 (CBF1) on HES1 and SIM2 promoter activity. Promoter reporter assays using either SIM2 or HES1 promoter constructs were (diagrammed above) cotransfected with vector control, NICD, CBF1 or both in HEK293T cells as previously described (Metz *et al*., 2006). The results show that HES1 promoter activity was significantly increased by NICD and NICD/CBF1 cotransfection compared to vector control, whereas SIM2 luciferase activity was suppressed by the NICD, independent of CBF1 (*$P\leqslant0.001$). Data are expressed as the mean ± s.e. for three wells per condition. The SIM2 promoter was amplified in two pieces with primers 5-ATCTGGGTAATCCCTTTCAAGCC-3 and 5-CCTGAGCTCCGAGCAACC-3, and 5-GTGGACAGCGGAGGTGCT-3 and 5-CCAAACCAAACCAGAATGC-3. Each piece was cloned onto pCR2.1 TOPO. The former fragment was subcloned onto pGL2 Basic into *Kpn*I and *Xho*I restriction sites. The latter fragment was then subcloned into *Sac*I and *Xho*I restriction sites to obtain the full-length SIM2 promoter on pGL2 Basic. (**i**) Binding of NOTCH to the SIM2 promoter. Chromatin immunoprecipitation (ChIP) analysis of the SIM2 promoter (Laffin *et al*., 2008) using a NOTCH1 antibody (Upstate) showed increased binding of NOTCH to the SIM2 promoter in MCF10A-HRAS cells as compared to control (pLPCX) cells (SIM2 primers FP: 5-GCCCACCCTGTGACCCTG-3, RP: 5-AAGTGACCCTTCTGCCCTTTC-3).

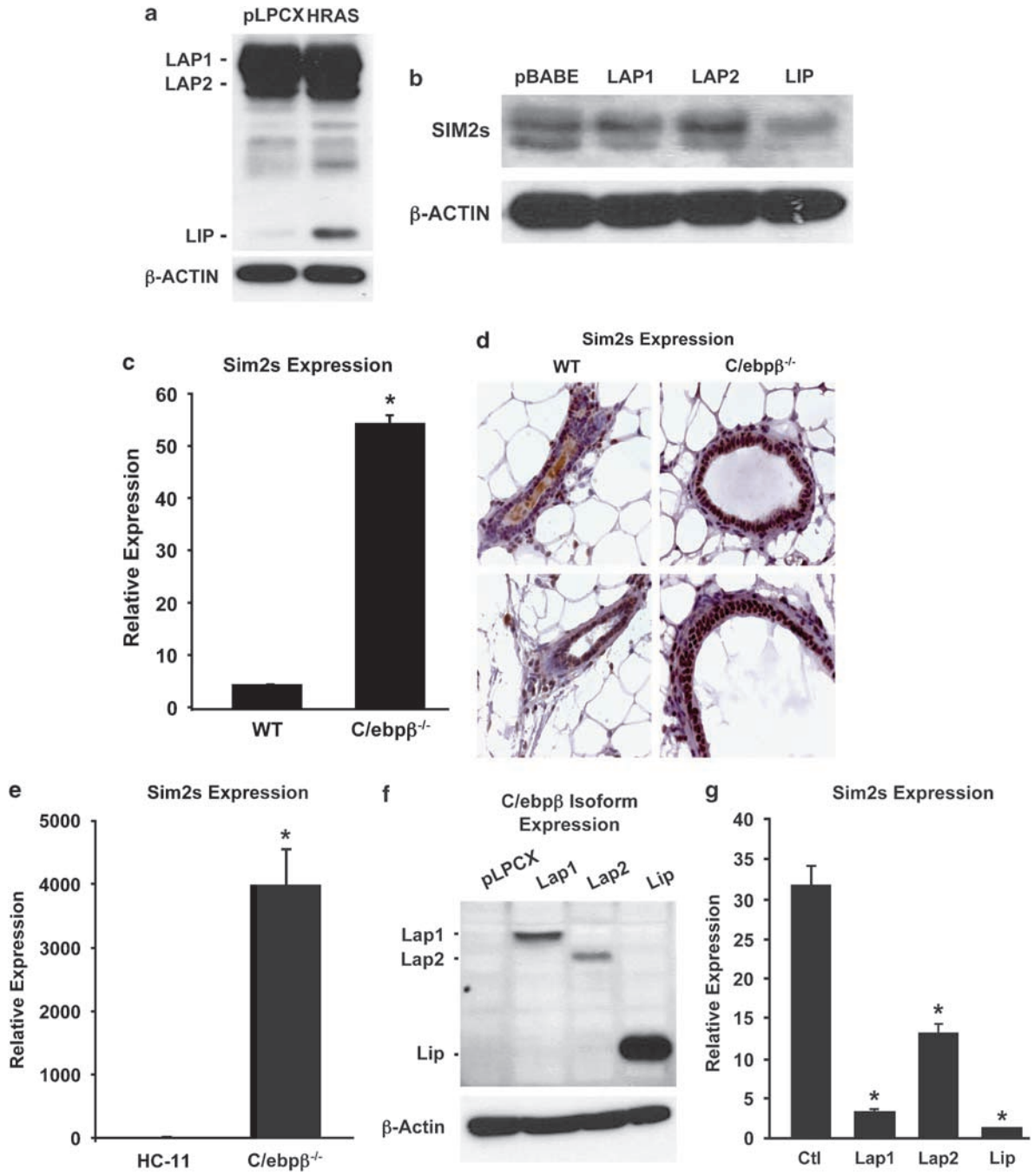


Figure 4 Ha-*Ras* and developmental regulation of SIM2s by C/EBPβ. (**a**) C/EBPβ isoform expression in Ha-*Ras*-MCF10A and control (pLPCX) MCF10A cells. Western blot analysis of Ha-*Ras*-MCF10A and control cell shows an Ha-*Ras*-dependent increase in C/EBPβ-LIP isoform expression (C/EBPβ antibody; Santa Cruz). (**b**) C/EBPβ-LIP-mediated suppression of SIM2s expression in MCF10A cells. Western blot for SIM2s (Chemicon) in MCF10A cells stably transduced with pBABE (vector control) or the indicated C/EBPβ isoforms shows a significant reduction of SIM2s protein levels in the LIP-transduced cells. Vectors (pBABEpuro or pEFIRESpuro backbones) containing human and mouse C/EBPβ isoforms were graciously provided by C Zahnow. (**c**, **d**) SIM2s expression in wild-type and *C*/ebpβ$^{-/-}$ mammary glands. Elevated SIM2s gene expression and protein levels in *C*/ebpβ$^{-/-}$ mammary glands as determined by real-time RT-PCR and immunohistochemistry, respectively (Laffin *et al*., 2008). (**e**) SIM2s expression in a *C*/ebpβ$^{-/-}$ mouse mammary epithelial cell line. Similar to the *C*/ebpβ$^{-/-}$ mammary glands, SIM2s levels are increased in mouse mammary epithelial cells derived from *C*/ebpβ$^{-/-}$ mammary glands (Lamb *et al*., 2003) by real-time RT-PCR as compared to normal HC11 cells (*$P \leqslant 0.001$). (**f**, **g**) Reestablishment of mouse C/ebpβ isoforms in the *C*/ebpβ$^{-/-}$ cells as determined by western blot analysis correlates with a decrease in SIM2s mRNA levels (*$P \leqslant 0.001$). Data are expressed as the mean ± s.e. for three plates per condition.

sion, and that elucidating pathways involved in SIM2s silencing will aid in the development of targeted therapies for treatment of this disease.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)