Induction of Lumen Formation in a Three-dimensional Model of Mammary Morphogenesis by Transcriptional Regulator ID4

ROLE OF CaMK2D IN THE EPIGENETIC REGULATION OF ID4 GENE EXPRESSION*

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Concomitant loss of lumen formation and cell adhesion protein CEACAM1 is a hallmark feature of breast cancer. In a three-dimensional culture model, transfection of CEACAM1 into MCF7 breast cells can restore lumen formation by an unknown mechanism. ID4, a transcriptional regulator lacking a DNA binding domain, is highly up-regulated in CEACAM1-transfected MCF7 cells, and when down-regulated with RNAi, abrogates lumen formation. Conversely, when MCF7 cells, which fail to form lumena in a three-dimensional culture, are transfected with ID4, lumen formation is restored, demonstrating that ID4 may substitute for CEACAM1. After showing the ID4 promoter is hypermethylated in MCF7 cells but hypomethylated in MCF/CEACAM1 cells, ID4 expression was induced in MCF7 cells by agents affecting chromatin remodeling and methylation. Mechanistically, CaMK2D was up-regulated in CEACAM1-transfected cells, effecting phosphorylation of HDAC4 and its sequestration in the cytoplasm by the adaptor protein 14-3-3. CaMK2D also phosphorylates CEACAM1 on its cytoplasmic domain and mutation of these phosphorylation sites abrogates lumen formation. Thus, CEACAM1 is able to maintain the active transcription of ID4 by an epigenetic mechanism involving HDAC4 and CaMK2D, and the same kinase enables lumen formation by CEACAM1. Because ID4 can replace CEACAM1 in parental MCF7 cells, it must act downstream from CEACAM1 by inhibiting the activity of other transcription factors that would otherwise prevent lumen formation. This overall mechanism may be operative in other cancers, such as colon and prostate, where the down-regulation of CEACAM1 is observed.

Lumen formation, a central feature of mammary morphogenesis, is lost during mammary tumorigenesis, starting with filling in the lumen with cancer cells in ductal carcinoma in situ (1) and becoming more evident in invasive solid tumors (2). Identification of the molecules that change during this process and the underlying mechanisms causing these changes are major goals of breast cancer research. Among the many molecules identified so far, CEACAM1 stands out as a luminal expressed protein that is rapidly down-regulated in both ductal carcinoma in situ (3) and invasive breast cancer (2). Not surprisingly, luminal expression of CEACAM1 occurs throughout ductal tissues such as the liver, digestive and urogenital tract, and prostate, and its loss upon malignant transformation is one of the earliest events observed (4). To study its role in lumen formation, we originally placed MCF10F, a cell line that expresses CEACAM1, in a three-dimensional culture system pioneered by Bissell and co-workers (5), and observed lumen formation with CEACAM1 at the luminal surface (3). When its expression was blocked by antisense, anti-CEACAM1 antibody, or peptides derived from the N terminus of CEACAM1, lumen formation was abrogated, demonstrating that its expression played a central role in the complex process of lumenogenesis (3). As a next step, we demonstrated that MCF7 breast cancer cells that fail to express CEACAM1 or form a lumen in the three-dimensional culture, regain this property when transfected with CEACAM1 (6). Although these experiments demonstrated a role for CEACAM1 in lumenogenesis, they did not reveal the underlying mechanism.

As a next step in identifying factors downstream from CEACAM1 in lumenogenesis, we identified key residues in the short cytoplasmic domain isoform (CEACAM1-SF), the predominant isoform in breast that comprised a short stretch of 12 amino acids (7). Because CEACAM1 can be expressed as multiple isoforms, depending on its tissue of origin, we refer to the cytoplasmic isoform expressed exclusively in the mammary gland as the short form, or CEACAM1-SF (referring only to the cytoplasmic domain). Once we demonstrated that a key threonine residue (Thr-457) and a back-up serine (Ser-459) were phosphorylated during lumen formation, and null mutations in these two residues blocked lumen formation (7), we performed gene chip experiments to determine which genes changed between MCF7 cells transfected with wild type CEACAM1-SF (5W cells) and those transfected with the double alanine null mutants (DA cells). Not surprisingly, over 300 genes were either up- or down-regulated (8), indicating that lumenogenesis is a complex process. Nonetheless, we asked if transfection of parental MCF7 with any of the up-regulated genes could substitute for the action of CEACAM1, demonstrating that they were directly involved in the lumenogenesis pathway downstream of CEACAM1.

Among the many genes identified, ID4, a transcriptional regulator, stood out as a candidate that could affect the transcription of a large number of genes by an inhibitory mechanism.
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ID4\(^2\), known as inhibitor of differentiation 4 (9), is a member of a gene family including ID1–4, all of which resemble basic helix-loop-helix (bHLH) transcription factors, but lack a DNA binding domain, thus suggesting their activity is based on a dominant-negative effect where they form inactivating heterodimers with functional bHLH transcription factors (9). Strikingly, \(ID1–3\) have ascribed oncogenic properties, although \(ID4\) is a putative tumor suppressor gene, due to inhibition of bHLH transcription factors, and perhaps due to its formation of heterodimers with \(ID1–3\) (10). Moreover, \(ID4\) has been shown to play an important role in breast, prostate, and colon (9) cancers, all tissues that show high CEACAM1 expression in normal to malignant tissue. On the other hand, \(ID4\) is expressed in a subset of mammary basal cells as a marker of lumen formation, a critical pathway in maintaining the normal differentiated state of mammary epithelial cells. The fact that \(ID4\) is both necessary and sufficient for lumen formation in the three-dimensional model for lumenogenesis, that is, when parental MCF7 cells that express low levels of \(ID4\) are transfected with \(ID4\), they fail to form a lumen, and when MCF7 cells transfected with CEACAM1 that express high levels of \(ID4\) are transfected with RNAi to \(ID4\), they fail to form a lumen. The \(ID4\) gene that is frequently silenced by hypermethylation (13, 14) is indeed hypermethylated in parental MCF7 cells, suggesting that this silencing affects lumen formation, a critical pathway in maintaining the normal differentiated state of mammary epithelial cells. The fact that introduction of CEACAM1 into MCF7 cells that have lost the ability to express CEACAM1, restores \(ID4\) expression suggests a direct link between CEACAM1 expression and relief of the \(ID4\) gene silencing of \(ID4\). In the studies that follow, we demonstrate that CEACAM1 can affect chromatin remodeling through cytoplasmic sequestration of HDAC4, a histone deacetylase, which in the nucleus, can maintain chromatin in a condensed state, effecting gene silencing. In our accompanying article (50), we show that a micro-RNA, miR-342, plays a role in the regulation of both \(ID4\) and DNMT1 expression levels, further implicating \(ID4\) gene activity as a major player in maintaining the differentiated state of mammary epithelial cells.

Results

\(\text{ID4 Expression Is Up-regulated by CEACAM1-SF and Is Both Necessary and Essential for Lumen Formation—}\)As previously reported, CEACAM1-SF restored lumen formation to MCF7 cells grown in a three-dimensional culture, whereas a functionally null, phosphorylation site mutant in the cytoplasmic domain of CEACAM1-SF failed to form lumena (7). A gene chip analysis comparing gene expression of CEACAM1-wild type (SW) transfected MCF7 cells and the functionally null, double alanine mutant (DA) MCF7 cells, indicated among other genes, that the \(ID4\) gene was up-regulated by a log2 of 6.7-fold (8). \(ID4\) was selected for further analysis because it was the only transcription regulator identified in the top 100 genes up-regulated in this analysis. We hypothesized that CEACAM1-SF must play a role in the regulation of \(ID4\), and that in turn, \(ID4\) must play a role in regulating genes critical for lumen formation. To test these hypotheses, we first validated its up-regulation by qPCR analysis of RNA isolated from MCF7 cells that were transfected with empty vector (V), CEACAM1-SF wild type cells (SW), and the phosphorylation site, double alanine mutant cells (DA) grown for 0, 2, 4, and 6 days in a three-dimensional culture. At each time point, \(ID4\) expression was 100-fold higher in the presence of the wild type CEACAM1 compared with the vector and DA-transfected cells (Fig. 1A).

We next tested the hypothesis that \(ID4\) up-regulation played a role in reversing the malignant status of MCF7 cells. The first experiment examined its role in CEACAM1-SF transfected MCF7 (SW) cells using RNAi oligos to inhibit \(ID4\) expression. At day 4 in a three-dimensional culture, ID4 expression was reduced to 75, 50, or 25% by the three different \(ID4\) RNAi oligos, when compared with the controls (Fig. 1B). The second experiment examined the role of \(ID4\) in the parental MCF7 cells lacking \(ID4\). When these cells were transfected with \(ID4\), high level expression of \(ID4\) was achieved (Fig. 1C). The corresponding protein levels of \(ID4\) in the SW cells treated with RNAi for \(ID4\) or the parental MCF7 cells transfected with \(ID4\) are shown in Fig. 1D. Transfer of these cells to a three-dimensional culture and examination for lumen formation revealed inhibition of lumen formation in parental MCF7 cells transfected with \(ID4\) (Fig. 1, E and F). Representative fields of acini are shown for the untreated SW cells and those treated with \(ID4\) RNAi (Fig. 1, left panel) and for parental MCF7 cells transfected with \(ID4\) (Fig. 1, right panel). When percentages of SW acini that possessed the central lumen were counted for over 300 acini, we observed 88% for the untreated, 77% for the scrambled RNAi control, and significantly lower for all three \(ID4\) RNAi oligos (Fig. 1F, left panel). Similarly, when percentages of parental MCF7 acini with central lumen were counted, we observed 5% for vector-transfected controls and 85% for \(ID4\)-transfected cells (Fig. 1F, right panel). These data indicate that the action of CEACAM1 on lumenogenesis is inhibited by blocking \(ID4\) expression in SW cells, and that overexpression of \(ID4\) alone in parental MCF7 cells was sufficient to mimic the action of CEACAM1-SF in reverting malignant MCF7 cells to a normal phenotype. Considering these lines of evidence, we conclude that \(ID4\) is both necessary and sufficient for lumen formation in this model system.

\(\text{ID4 Gene Hyper-methylation and Silencing Can Be Reversed by Re-introduction of CEACAM1-SF into MCF7 Cells—}\)Based on its down-regulation in many types of cancers, \(ID4\) has been proposed to play a role as a tumor suppressor gene (9). In this

\(^2\)The abbreviations used are: ID4, inhibitor of differentiation 4; bHLH, basic helix-loop-helix; miRNA, micro-RNA; DA, double alanine; DAC, 5-aza-2’-deoxycytidine; TSA, trichostatin A; IP, immunoprecipitation; qPCR, quantitative PCR; HDAC, histone deacetylase; DNMT, DNA methyltransferase; MSP, methylation-specific PCR.
regard, whereas normal breast cells express both CEACAM1 and ID4, breast cancer cells fail to express either. Thus, it was important to determine why the apparent re-introduction of CEACAM1 into a breast cancer cell line like MCF7 led to ID4 up-regulation. In addition, this knowledge may shed light on understanding how ID4 is down-regulated during tumorigenesis. Because ID4 gene silencing by hyper-methylation has been widely reported (13), it was likely that the same situation exists in MCF7 cells. To test this idea, parental MCF7 cells were treated with a combination of 5-aza-2′-deoxycytidine (DAC), a DNMT inhibitor, and trichostatin A (TSA), a histone deacetylase inhibitor to effect global chromatin re-modeling and demethylation. MCF7 cells transfected with empty vector (V) or CEACAM1-SF wild type (SW) cells were treated with DAC for 3 days and TSA on day 3 before RNA was harvested for qPCR analysis for ID4 expression. Using the ratio of V levels over SW levels, ID4 expression increased from 20% for the untreated to 70% for the DAC/TSA-treated cells (Fig. 2A). This striking increase suggested that the promoter of ID4 in MCF7 cells was functionally hypermethylated and that the gene could be reactivated by the combined treatment of DAC and TSA. When MSP analysis was performed on the ID4 promoter of MCF7 cells transfected with vector control (V) or ID4 plasmid. Equal protein amounts (50 μg) were loaded onto each lane, the gels were scanned and the lanes compared with a maximum signal as 1.00. Actin is shown as a loading control only. E, left, a representative view of SW cells ± treatment with RNAi to ID4 grown in a three-dimensional culture for 4 days. E, right, vector or ID4-transfected MCF7 cells grown in a three-dimensional culture. Insets show a magnified view of representative acini. F, quantitation of lumen formation for SW cells treated with RNAi or vector control parental cells compared with MCF7 cells transfected with ID4 (*, p < 0.05, ***, p < 0.001).

FIGURE 1. Role of ID4 in lumen formation. A, MCF7 cells transfected with empty vector (V), CEACAM1-SF wild type (SW), or CEACAM1 phosphorylation site mutant (DA) cells were grown in a three-dimensional culture for 0–6 days, and ID4 expression was analyzed by qPCR. B, SW cells were treated with 3 RNAi oligos to ID4, as well as no RNAi (none) or Lipofectamine (Lipo), or a low GC RNAi control (Ctrl) and the expression levels of ID4 were measured by qPCR. C, parental MCF7 cells were transfected with empty vector (V) or ID4 and the expression levels of ID4 measured by qPCR. D, left, immunoblot analysis of ID4 levels in SW cells untransfected (UT), Lipofectamine controls (Lipo), or transfected with different RNAi oligos to ID4. Right, immunoblot analysis of ID4 levels in parental MCF7 cells transfected with vector control (V) or ID4 plasmid. Equal protein amounts (50 μg) were loaded onto each lane, the gels were scanned and the lanes compared with a maximum signal as 1.00. Actin is shown as a loading control only. E, left, a representative view of SW cells ± treatment with RNAi to ID4 grown in a three-dimensional culture for 4 days. E, right, vector or ID4-transfected MCF7 cells grown in a three-dimensional culture. Insets show a magnified view of representative acini. F, quantitation of lumen formation for SW cells treated with RNAi or vector control parental cells compared with MCF7 cells transfected with ID4 (*, p < 0.05, ***, p < 0.001).
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Using reduced representation bisulfite sequence analysis, we found hypermethylation of the ID4 promoter in vector control MCF7 cells and hypomethylation in CEACAM1-transfected MCF7 cells (Fig. 3). Two CpG-rich regions in the promoter were identified, with hypomethylation at −655 to −565 from the transcription start site in the CEACAM1-transfected MCF7 cells. This site may be associated with a new regulatory region versus the transcription start site in the CEACAM1-transfected cells. B, right, normalized methylation levels. C, ID4 expression levels of MCF7 cells freshly transfected with CEACAM1 (SW) at 3 and 7 days compared with controls.

When we examined our gene chip data for changes in chromatin remodeling genes, most of those identified, including DMPA1, HDAC2, HDAC3, HDAC8, and HAT1, fell below the level of significant change, suggesting that changes in their expression per se were not responsible for the observed re-activation of the ID4 gene. However, immunoblot analysis of HDACs 1, 2, 4, 6, and 8 showed a dramatic decrease in the cytoplasmic expression for HDAC4 in SW compared with vector-transfected cells (Fig. 4A). Nevertheless, this analysis did not produce an obvious change in the nuclear levels of HDAC4. To further examine this issue, chromatin immunoprecipitation (ChIP) analysis on the ID4 promoter was performed for HDAC4 and −6. The results indicate less binding of HDAC4 and −6 to the ID4 promoter of CEACAM1 (SW) compared with vector-transfected cells (Fig. 4B). Thus, these results support the idea that less HDAC4 or -6 binding at the ID4 promoter could lead to less de-acetylation at this locus, hence less methylation. Although both HDAC4 and HDAC6 were candidates for further analysis, our attention turned to HDAC4, because it is a reported substrate for CaMK2D (16). Since we have previously shown that CaMK2D is up-regulated during CEACAM1-SF-mediated lumen formation and plays an essential role in the phosphorylation of the critical Thr-457 in the cytoplasmic domain of CEACAM1-SF (17), we hypothesized that it may play a dual role in the action of CEACAM1-SF by also causing it to phosphorylate HDAC4. It has been reported that upon phosphorylation, HDAC4 can be sequestered in the cytoplasm or exported from the nucleus by the adaptor 14-3-3 (18, 19), possibly resulting in less DNMT1 recruited to a given promoter. When we examined the possibility that more HDAC4 phosphorylation occurs in CEACAM1-SF-transfected MCF7 SW compared with V cells, we found that this is indeed the case (Fig. 4C). When HDAC4 was knocked down by RNAi, protein levels of ID4 in SW cells were relatively unaffected (Fig. 4D, left). Conversely, ID4 proteins levels were undetectable in parental MCF7 cells even after knockdown of HDAC4 by RNAi (Fig. 4D, right). Thus, the absolute levels of HDAC4 protein were not responsible for the regulation of ID4, instead the degree of phosphorylation was likely the key step in regulation.

Next, we asked if phosphorylated HDAC4 was sequestered by 14-3-3 in the cytoplasm and found that the two formed a complex as shown by a 14-3-3 immunoprecipitation experiment, where the complex was observed only in SW cells at day 4 in a three-dimensional culture (Fig. 5A). In addition, because methylation of ID4 was likely caused by the activity of DNMT1, the methylase responsible for the maintenance of CpG methylation (20), a DNMT1 ChIP analysis was performed, confirming that there was less binding to the ID4 promoter in CEACAM1 (35%) compared with the vector-transfected MCF7 cells (85%; Fig. 5B). In addition, levels of H3K27Me3 at the ID4 promoter of SW versus vector control cells were reduced and H3Ac were increased, indicating that the ID4 promoter was in a more accessible state (Fig. 5B). Based on these analyses, we conclude that the phosphorylation of HDAC4 plays a key role in the gene re-activation of ID4 in SW cells.

CaMK2D Is Crucial in Modulating the Epigenetic Action of HDAC4 on ID4—The above findings suggested the possibility that CaMK2D was the kinase responsible for phosphorylation...
of HDAC4 leading to chromatin remodeling at the ID4 promoter. In our previous study on the CaMK2D phosphorylation of CEACAM1-SF (17), we had blocked CaMK2 expression with RNAi inhibitors, and noted in unpublished data3 that ID4 expression decreased in SW cells, a perplexing result that can now be explained in view of its role in HDAC4 phosphorylation. As a follow up to that observation, we show here that overexpression of CaMK2D in parental MCF7 cells resulted in a 2-fold enhanced expression of ID4 (Fig. 6A) and inhibition of CaMK2D expression in SW cells decreases ID4 expression at least 100-fold lower compared with controls (Fig. 6B). When the occupancy of HDAC4 at the promoter of ID4 in SW cells was examined by ChIP analysis before and after knockdown of CaMK2D by RNAi, we found elevated levels of HDAC4 after treatment with CaMK2D RNAi (Fig. 6C) demonstrating that HDAC4 occupancy of the ID4 promoter correlated inversely with the expression of CaMK2D. As a further proof that CaMK2D plays a key role in the epigenetic control of ID4 expression, we treated SW cells with CaMK2D RNAi oligos or a CaMK2 kinase inhibitor in a three-dimensional culture. When total cell lysates from 4-day cultures were analyzed for CaMK2D expression, HDAC4 phosphorylation, and ID4 expression, the data clearly showed a large reduction in CaMK2D expression for two of the three RNAi oligos, a significant loss of phosphorylation of HDAC4, and the expected silencing of ID4 expression for the two oligos that successfully inhibited CaMK2D expression (Fig. 6D). In addition, KN93, a known inhibitor of CaMK2 (21), confirmed these results by blocking the in-cell activity of the kinase leading to impaired phosphorylation of HDAC4, and ultimately, to the silencing of ID4.

Discussion

Although CEACAM1 down-regulation is an early event in tumorigenesis for a number of cancers including breast cancer (22–24), the mechanism of down-regulation and subsequent effects are unclear. However, the observation that CEACAM1 expression is luminal in most normal tissues and correlates with lumen formation in a three-dimensional model of lumenogenesis, suggests its expression is coordinated with lumen formation, a phenotype lost in hyperplasia, ductal carcinoma in situ, and invasive tumors (25). MCF7 cells derived from a breast tumor that fail to express CEACAM1 or form lumena in a three-dimensional culture can be reverted to a more normal phenotype whether grown in vitro in a three-dimensional culture (6) or in humanized mammary fat pads in a

3 T. Nguyen and J. E. Shively, unpublished data.
murine model of lumenogenesis (26). Thus, restoration of CEACAM1 expression is able to trigger a normal tissue pathway in a breast cancer cell line in both in vitro (7) and in vivo models (26). In addition, mutation of two residues (Thr-457 and Ser-459) to alanine (double alanine or DA mutant) in the cytoplasmic domain of the short isoform abrogated its lumen formation function (7). Among the various up-regulated genes that were differentially expressed between the short isoform, wild type CEACAM1-transfected MCF7 cells (SW) and the double mutant, nonlumen forming CEACAM1-transfected cells (DA), ID4 was identified for further study due to its postulated role as a tumor suppressor in several cancers, including breast cancer (9). When ID4 was overexpressed in parental MCF7 cells, lumen formation was observed, whereas when ID4 expression was inhibited with RNAi in SW cells, lumen formation was inhibited, thus, confirming ID4 as a viable target for further study in lumenogenesis.

Because ID4 has the general structural motif of bHLH transcription factors, but lacks a DNA binding domain, it has been hypothesized that it functions by forming inactive heterodimers with bHLH transcription factors (9). Although several targets of ID4 have been identified (9), including ID1–3 (10), the question arose in our study, how does CEACAM1 up-regulate ID4? The first clue to a likely mechanism involves regulation of the ID4 promoter by hypermethylation, as reported for prostate cancer (13), a cancer in which CEACAM1 is also down-regulated (27, 28). When we examined the methylation status of the ID4 promoter in parental MCF7 cells, it was highly methylated, whereas hypomethylation was observed in CEACAM1-transfected SW cells. Thus, the next question was how can CEACAM1 change the methylation status of a critical gene?

To address this question we examined the role of HDACs, enzymes that de-acetylate histones at critical promoters destined for inactivation by methylation. Treatment of parental MCF7 cells sequentially with a DNMT inhibitor, followed by a HDAC inhibitor, led to both hypomethylation of the ID4 promoter and expression of ID4. However, because this approach...
has global effects and bypasses the natural mechanism of chromatin remodeling, it was important to determine the status of HDACs in MCF7 cells before and after transfection with CEACAM1. In other studies it has been shown that phosphorylation of HDACs and retention in the cytoplasm by binding to the adaptor protein 14-3-3 is able to effect specific gene activation via chromatin remodeling (19). When this analysis was performed on MCF7 cells before and after transfection with CEACAM1, cytoplasmic levels of HDAC4 and HDAC6 appeared to change the most, including the levels of phospho-HDAC4. Furthermore, immunoprecipitation (IP) of 14-3-3 revealed the presence of phospho-HDAC4 in the IPs of CEACAM1 transfected, but not parental MCF7 cells. Accordingly, the presence of DNMT1 at the ID4 promoter by ChIP analysis was significantly higher for parental versus CEACAM1-transfected cells. In addition, a histone methylation mark (H3K27Me3) for closed chromatin was reduced, whereas an acetylated histone mark (H3Ac) for open chromatin was increased at the promoter of ID4 in SW versus vector control cells. Thus it appears that CEACAM1 can effect chromatin remodeling at the ID4 promoter by retention of phospho-HDAC4 in the cytoplasm.

At this point, we were interested in identifying the critical kinase that phosphorylates HDAC4 for which several literature candidates were possibilities, including CaMK2 (19). Although many CaMK2 genes are widely expressed in a tissue-specific manner (29), the CaMK2D gene immediately caught our attention, because we had previously shown that CEACAM1-transfected cells specifically expressed CaMK2D during lumen formation (17) and that CaMK2D was associated with induction of apoptosis in ischemic cardiomyocytes (30). Notably, apoptosis is a central feature of lumen formation (6). Importantly, we had shown that the critical Thr-457 residue in the cytoplasmic tail of CEACAM1 was specifically phosphorylated by CaMK2D, and this phosphorylation was required for lumen formation (17). Thus, it was straightforward to inhibit this enzyme by either the drug KN93 or RNAi, both of which reduced both phospho-HDAC4 and ID4 protein levels in CEACAM1-transfected cells.

Based on these studies, we can outline a general mechanism for participation of CEACAM1 in the epigenetic activation of ID4 expression involving cross-phosphorylation of CEACAM1 and HDAC4 by CaMK2D, followed by sequestration of phospho-HDAC4 in the cytoplasm by 14-3-3 (Fig. 7). Subsequent steps likely include repression of DNMT1 by ID4, maintaining hypomethylation at the ID4 promoter. Because phosphorylated CEACAM1 recruits actin (31), and CaMK2D actively bundles actin (32), we speculate that changes in the cytoskeleton are responsible for binding 14-3-3 in the cytoplasm and maintenance of the low activity of HDAC4 at the ID4 promoter. In the case of malignant transformation, down-regulation of CEACAM1 would reverse this epigenetic remodeling, resulting in down-regulation of CaMK2D, ultimately leading to the silencing of ID4, and subsequent phenotypic changes characteristic of cancer including lack of lumen formation. In the cardiac model of hypertrophy, the retention of phospho-HDAC4 in the cytoplasm by 14-3-3 is well established (19), supporting this aspect of our model.

Although many of the players in the mechanism have been identified, several steps require further investigation. First, how does CEACAM1 induce CaMK2D? Unfortunately, regulation of the CaMK2D gene is hardly studied compared with its effects on the regulation of other genes such as Mef2 or p53 by CaMK2D in cardiac myopathy (33, 34). Studies in the brain...
suggest NFκB may regulate or be regulated by CaMK2D (35, 36). Second, can CEACAM1 directly interact with 14-3-3 explaining the sequestration of phosphor-HDAC4 in the cytoplasm? In earlier unpublished studies, we found that such an interaction may occur. Indeed, this may be a general phenomenon because 14-3-3 can also export phosphorylated β-catenin from the nucleus (37), and CEACAM1 has been shown by us to interact with β-catenin (38, 39). Thus, CEACAM, together with 14-3-3 may sequester several nuclear signaling molecules in the cytoplasm. Furthermore, 14-3-3 may also be regulated by phosphorylation with kinases such as PKC (40), involving a link to our studies on PKC and apoptosis induced by CEACAM1 (8). However, in this study we did not observe CEACAM1 in the 14-3-3 IPs (data not shown), suggesting further work is necessary. Third, the transcription factor targets of ID4 were not identified in this study. Because ID2 plays an important role in mammary gland development (41, 42), but may be oncogenic when not switched off (9), its regulation by ID4 is a real possibility. Along this line, Sharma et al. (10) have recently shown that ID4 can switch off ID1–3 inhibition by forming heterodimers, thus switching on their targets. Thus, it may be necessary to identify their targets, rather than the targets of ID4. In their study, the focus was on action of the bHLH protein E47 on the promoter of CDKN1 p21 in prostate cells. Although we have no evidence that E47 and CDKN1 p21 are critical regulators of lumen formation, it is likely that other targets of ID2 exist and need to be identified. Furthermore, ID4 may have direct targets including likely mammary gland candidates BRCA1 (43) and Hes1 (44). Specifically, ID4 was identified as a negative regulator of the BRCA1 promoter (43), suggesting an important role in BRCA1-related breast cancer. In the case of osteoblast differentiation, ID4 has been shown to release Hes1 from Hes1-Hey2 complexes (44), a potentially exciting observation, because Hes1 has been shown to negatively regulate MCF7 (45) and T47D (46) proliferation stimulated by estrogen and E2F-1. In addition, these investigators have shown that the growth inhibitory effects of all-trans-retinoic acid on breast cells is mediated by Hes1 up-regulation (8), and we find that Hes1 expression is up-regulated in either CEACAM1-transfected or all-trans-retinoic acid-treated MCF7 cells (46). Thus, the sequestration of Hes1 by Hey2 complexes may be relieved by ID4, allowing Hes1 to inhibit proliferation of MCF7 cells, a necessary step in their differentiation to lumen forming cells. There is also evidence that the expression of CEACAM1 and ID1 are inversely related in going from normal to malignant transformation in the breast (47), possibly implicating ID4 in the regulation of both ID1 and ID2. Finally, the oncogenic role of ID4 in triple negative breast cancer (11) and promotion of angiogenesis in glioblastoma argues that expression of ID4 is not always beneficial. Because ID4 functions as an inhibitor of transcription factors, it may promote cancer by inhibiting normal genes, or inhibit cancer by inhibiting oncogenes. Thus, the identification of ID4 targets in each cancer will remain a crucial first step in predicting its role.

We conclude that forced expression of CEACAM1 in breast cancer cell line MCF7 restores lumenogenesis by induction of CaMK2D, a kinase that coordinately

FIGURE 7. Proposed sequence of events in CEACAM1-mediated epigenetic activation of the ID4 promoter. MCF7 cells without CEACAM1 (left) have a silenced ID4 promoter maintained by HDAC4 and methyl-CpG (MeCpG). Transfection with CEACAM1 (middle) recruits CaM (17) to CaMK2D, which in turn, is regulated by autophosphorylation (48). Activated CaMK2D travels to the nucleus to phosphorylate HDAC4 (16), whereas at the same time, it triggers actin polymerization and bundling at the site of its activation in the cytoplasm (32). 14-3-3 binds phospho-HDAC4 (pHDAC4) and exports it to the cytoplasm (right) where it binds to the actin cytoskeleton (49). The ID4 promoter histones are acetylated by histone acetyltransferase (HAT), allowing an open chromatin structure accessible to demethylases and transcription machinery (not shown).
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phosphorylates CEACAM1 and HDAC4, which in turn, relieves the epigenetic silencing of ID4, and that this expression is sufficient to restore lumen formation.

 Experimental Procedures

Materials—MCF7 (ATCC HTB-22) cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/ampicillin. Anti-HDAC1, -2, -3, -4, -6 and pHDAC4 antibodies were from Cell Signaling Technology, Inc. (Danvers, MA). Anti-β-actin and 14-3-3 antibodies were from GeneTex, Inc. (Irvine, CA). Anti-CaMK2 antibody was from BD Biosciences. Anti-DNMT1 antibody was from Epigentek Group Inc. (Farmingdale, NY). Anti-lamin A/C was from Cell Signaling. Anti-H3ac and anti-H3K27me3 were from Active Motif. DAC and TSA were from Sigma. CaMKII-specific inhibitor KN-93 and inactive analog KN-92 were from Calbiochem, pCMV6 plasmids for CaMK2D and ID4 were from OriGene Technologies, Inc. (Rockville, MD). Lipofectamine RNAiMAX transfecting reagent, OptiMEM I Reduced Serum Medium, and Stealth RNAi siRNAs for CaMK2D, HDAC4, and ID4 were from Invitrogen Corp.

Cell Treatments—Cells (2 × 10^6 cells/well) in a 6-well plate were treated with 0.1 μM DAC on days 1–3, then treated with 300 nM TSA on day 3. Cells were harvested on days 3 or 7 for RNA and DNA extraction. Untreated cells were incubated without the addition of DAC or TSA and fresh medium was supplied on days 1, 2, and 3. Bisulfite conversion and MSP analysis were performed using the EZ DNA Methylation Lightning kit (Zymo Research, Orange, CA) according to the manufacturer’s specifications. For MSP, 1 μl of modified DNA was amplified using MSP primers that specifically recognize the unmethylated (forward, 5′-GTTAGTTGGATTTTGTGGTT-TTTAGTAT-3′ and reverse, 5′-AAGCTTATTTTAAACCA-CCATAACCCCA-3′) or methylated (forward, 5′-TAGTGCG-GATTTTTCTGTTTTTATC-3′ and reverse, 5′-CTA-TATTTTAAACCGTGACCGCCCGC-3′) ID4 promoter sequence after bisulfite conversion. Reaction products (15 μl) were visualized after electrophoresis in 2.0% agarose gel containing SYBR Safe DNA gel stain (Invitrogen). In addition, reduced representation bisulfite sequencing on the V and SW regions of each total RNA, 20 μl of cDNA from the CFX-96 Real-time System (Bio-Rad). Normalization was taken into account for differences in DNA input.

Quantitative RT-PCR—Total RNAs were extracted using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s specifications. From 1 μg of each total RNA, 20 μl of cDNA was synthesized using the Omni-Script RT kit (Qiagen). Amplification was performed using the CFX-96 Real-time System (Bio-Rad). Briefly, we amplified 1 μl of cDNA from the reverse transcription reaction with 20 pmol of each primer in a total volume of 25 μl using the IQ Supermixes (Bio-Rad) and the following conditions: initial denaturation step at 94 °C for 3 min, followed by 40 cycles of 94 °C for 10 s and 55 °C for 30 s. The fluorescence was measured at the end of the annealing step at 55 °C. Subsequently, a melting curve was recorded between 55 and 95 °C every 0.5 °C with a hold every 1 s. Samples were done in triplicate, and the values shown were normalized to their own GAPDH readings for quantitative purpose.
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Immunoblot Analysis—Total cell lysates and nuclear and cytoplasmic extracts were prepared using the M-PER™ Mammalian Protein Extraction and the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher), respectively, per the manufacturer’s specifications. For total lysates, protein concentrations were determined using the Pierce Coomassie Plus Assay kit and 50 μg of protein from each sample were resolved by SDS-gel electrophoresis and Western blot analysis with appropriate antibodies. Detection was carried out using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

Author Contributions—T. N. and J. E. S. designed the study; T. N. performed the experiments and analyzed the data; J. E. S. wrote the manuscript.

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References

1. Pradeep, C. R., Köstler, W. J., Lauriola, M., Granit, R. Z., Zhang, F., Jacob-Hirsch, I., Rechavi, G., Nair, H. B., Hennessey, B. T., Gonzalez-Angulo, A. M., Tekmal, R. R., Ben-Porath, I., Mills, G. B., Domany, E., and Yarden, Y. (2012) Modeling ductal carcinoma in situ: a HER2-Notch3 collaboration enables luminal filling. Oncogene 31, 907–917

2. Kirshner, J., Hardy, J., Wilczynski, S., and Shively, J. E. (2004) Cell-cell adhesion molecule CEACAM1 is expressed in normal breast and milk and associates with β1 integrin in a 3D model of morphogenesis. J. Mol. Histol. 35, 287–299

3. Huang, J., Hardy, J., Sun, Y., and Shively, J. E. (2007) Mutation analysis of the short cytoplasmic domain of the CEACAM1–4S, a cell-cell adhesion molecule, mediates apoptosis and reverts mammary carcinoma cells to a normal morphogenic phenotype in a 3D culture. Proc. Natl. Acad. Sci. U.S.A. 100, 521–526

4. Chen, C. J., Kirshner, J., Sherman, M. A., Hu, W., Nguyen, T., and Shively, J. E. (2007) Mutation analysis of the short cytoplasmic domain of the cell-cell adhesion molecule CEACAM1 identifies residues that orchestrate actin binding and lumen formation. J. Biol. Chem. 282, 5749–5760

5. Chen, C. J., Sun, Z. Y., and Shively, J. E. (2010) Role of calpain-9 and PKC-δ in the apoptotic mechanism of lumen formation in CEACAM1 transfected breast epithelial cells. Exp. Cell Res. 316, 638–648

6. Patel, D., Morton, D. J., Carey, J., Havrda, M. C., and Chaudhary, J. (2015) Inhibitor of differentiation 4 (ID4): from development to cancer. Biochem. Biophys. Acta 1855, 92–103

7. Sharma, P., Chinaranagari, S., and Chaudhary, J. (2015) Inhibitor of differentiation 4 (ID4) acts as an inhibitor of ID-1, -2 and -3 and promotes basic helix loop helix (bHLH) E47 DNA binding and transcriptional activity. Biochem. Biophys. Res. Commun. 4198, 424–428

8. Kuzontkoski, P. M., Mulligan-Kehoe, M. J., Harris, B. T., and Israel, M. A. (2010) Inhibitor of DNA binding-4 promotes angiogenesis and growth of glioblastoma multiforme by elevating matrix GLA levels. Oncogene 29, 3793–3802

9. Chen, C. J., Kirshner, J., Sherman, M. A., Hu, W., Nguyen, T., and Shively, J. E. (2007) Mutation analysis of the short cytoplasmic domain of the CEACAM1–4S, a cell-cell adhesion molecule, mediates apoptosis and reverts mammary carcinoma cells to a normal morphogenic phenotype in a 3D culture. Proc. Natl. Acad. Sci. U.S.A. 100, 521–526

10. Schumann, D., Chen, C.-J., Kaplan, B., and Shively, J. E. (2001) Carcinoembryonic antigen cell adhesion molecule 1 directly associates with cytoskeletal proteins actin and tropomyosin. J. Biol. Chem. 276, 47421–47433
Roles of ID4, CaMK2D, and CEACAM1 in Lumen Formation

Li, J., and Tang, X. D. (2011) The 8A isoform of calmodulin kinase II mediates pathological cardiac hypertrophy by interfering with the HDAC4-MEF2 signaling pathway. Biochem. Biophys. Res. Commun. 409, 125–130

Toko, H., Takahashi, H., Kayama, Y., Oka, T., Minamino, T., Okada, S., Morimoto, S., Zhan, D. Y., Terasaki, F., Anderson, M. E., Inoue, M., Yao, A., Nagai, R., Kitaura, Y., Sasaguri, T., and Komuro, I. (2010) Ca2+/calmodulin-dependent kinase IIδ causes heart failure by accumulation of p53 in dilated cardiomyopathy. Circulation 122, 891–899

Federman, N., Zalcman, G., de la Fuente, V., Fustiñana, M. S., and Rojas, C. (2014) Epigenetic mechanisms and memory strength: a comparative study. J. Physiol. Paris 108, 278–285

Culver, C., Sundqvist, A., Mudie, S., Melvin, A., Xirodimas, D., and Rocha, S. (2010) Mechanism of hypoxia-induced NF-κB. Mol. Cell Biol. 30, 4901–4921

Killoran, R. C., Fan, J., Yang, D., Shilton, B. H., and Choy, W. Y. (2015) Structural analysis of the 14–3–3ζ/Chibby interaction involved in Wnt/β-catenin signaling. PLoS ONE 10, e0123934

Li, Y., and Shively, J. E. (2013) CEACAM1 regulates Fas-mediated apoptosis in Jurkat T-cells via its interaction with β-catenin. Exp. Cell Res. 319, 1061–1072

Jin, L., Li, Y., Chen, C. J., Sherman, M. A., Le, K., and Shively, J. E. (2008) Direct interaction of tumor suppressor CEACAM1 with β-catenin: identification of key residues in the long cytoplasmic domain. Exp. Biol. Med. (Maywood) 233, 849–859

Woodcock, J. M., Coolen, C., Goodwin, K. L., Baek, D. J., Bittman, R., Samuel, M. S., Pitson, S. M., and Lopez, A. F. (2015) Destabilisation of dimeric 14–3–3 proteins as a novel approach to anti-cancer therapeutics. Oncotarget 6, 14522–14536

Kim, N. S., Kim, H. J., Koo, B. K., Kwon, M. C., Kim, Y. W., Cho, Y., Yokota, Y., Penninger, J. M., and Kong, Y. (2006) Receptor activator of NF-κB ligand regulates the proliferation of mammary epithelial cells via Id2. Mol. Cell Biol. 26, 1002–1013

Ithana, Y., Singh, J., Sumida, T., Coppe, J. P., Parrivello, S., Bennington, J. L., and Desprez, P. Y. (2003) Role of Id-2 in the maintenance of a differentiated and noninvasive phenotype in breast cancer cells. Cancer Res. 63, 7098–7105

Beger, C., Pierce, L. N., Kruger, M., Marcusson, E. G., Robbins, J. M., Welch, P., Welch, P. J., Welte, K., King, M. R., Barber, J. R., and Wong-Staal, F. (2001) Identification of Id4 as a regulator of BRCA1 expression by using a ribosome-library-based inverse genomics approach. Proc. Natl. Acad. Sci. U.S.A. 98, 130–135

Tokozawa, Y., Yagi, K., Yamashita, Y., Nakachi, Y., Nkaido, I., Bono, H., Ninomiya, Y., Kanesaka-Tatsuka, Y., Akita, M., Motegi, H., Wakana, S., Noda, T., Sablitzky, F., Ariai, S., Kurokawa, R., et al. (2010) Id4, a new candidate gene for senile osteoporosis, acts as a molecular switch promoting osteoblast differentiation. PLoS Genet. 6, e1001019

Mueller, P., Kietz, S., Gustafsson, J. A., and Strom, A. (2002) The anti-estrogenic effect of all-trans-retinoic acid on the breast cancer cell line MCF-7 is dependent on HES-1 expression. J. Biol. Chem. 277, 28376–28379

Hartman, J., Müller, P., Foster, J. S., Wimalasena, J., Gustafsson, J. A., and Ström, A. (2004) HES-1 inhibits 17β-estradiol and heregulin-β1-mediated upregulation of E2F-1. Oncogene 23, 8826–8833

Liu, Q., Yang, Y. M., Zhang, Q. H., Zhang, T. G., Zhou, Q., and Zhou, C. I. (2011) Inhibitor of differentiation is overexpressed with progression of benign to malignant lesions and related with carcinoembryonic antigen-related cell adhesion molecule 1 distribution in mammary glands. Ann. Diagn. Pathol. 15, 30–36

Tzortzopoulos, A., Best, S. L., Kalamida, D., and Török, K. (2004) Ca2+/calmodulin-dependent activation and inactivation mechanisms of αCaMKII and phospho-Thr286-αCaMKII. Biochemistry 43, 6270–6280

Zhang, T., Kohlihaas, M., Backs, J., Mishra, S., Phillips, W., Dybkova, N., Chang, S., Ling, H., Bers, D. M., Maiers, L. S., Olson, E. N., and Brown, J. H. (2007) CaMKIIδ isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. J. Biol. Chem. 282, 35078–35087

Weng, C., Nguyen, T., and Shively, J. E. (2016) miRNA-342 regulates CEACAM1-induced lumen formation in a three-dimensional model of mammary gland morphogenesis. J. Biol. Chem. 291, 16777–16786