Novel strategies to enforce an epithelial phenotype in mesenchymal cells

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Precis: A novel functional assay for E-cadherin expression was used in a genetic screen to identify candidate therapeutic targets to block or reverse EMT as a generalized strategy for treatment of metastatic solid tumors.

Key Words: E-cadherin, epithelial-to-mesenchymal transition, metastasis, Listeria monocytogenes, RNA interference, ZEB1, FLASH
ABSTRACT

E-cadherin downregulation in cancer cells is associated with epithelial-to-mesenchymal transition (EMT) and metastatic prowess, but the underlying mechanisms are incompletely characterized. In this study, we probed E-cadherin expression at the plasma membrane as a functional assay to identify genes involved in E-cadherin downregulation. The assay was based on the E-cadherin-dependent invasion properties of the intracellular pathogen *Listeria monocytogenes*. On the basis of a functional readout, automated microscopy and computer-assisted image analysis were used to screen siRNAs targeting 7,000 human genes. The validity of the screen was supported by its definition of several known regulators of E-cadherin expression, including ZEB1, HDAC1 and MMP14. We identified three new regulators (FLASH, CASP7 and PCGF1), the silencing of which was sufficient to restore high levels of E-cadherin transcription. Additionally, we identified two new regulators (FBXL5 and CAV2), the silencing of which was sufficient to increase E-cadherin expression at a post-transcriptional level. FLASH silencing regulated the expression of E-cadherin and other ZEB1-dependent genes, through post-transcriptional regulation of ZEB1, but it also regulated the expression of numerous ZEB1-independent genes with functions predicted to contribute to a restoration of the epithelial phenotype. Finally, we also report the identification of siRNA duplexes that potently restored the epithelial phenotype by mimicking the activity of known and putative microRNAs. Our findings suggest new ways to enforce epithelial phenotypes as a general strategy to treat cancer by blocking invasive and metastatic phenotypes associated with EMT.
INTRODUCTION

E-cadherin (CDH1) is a major component of cell-cell junctions in epithelial cells (1). The extracellular domains of E-cadherin connect neighboring cells through $\text{Ca}^{2+}$-dependent homotypic interactions, whereas the cytoplasmic domain interacts with components of the adherens junctions, including p120, $\gamma$-catenin/plakoglobin and the protooncogene $\beta$-catenin (2, 3). As an adherens junction component, E-cadherin act as a tumor suppressor not only by contributing to epithelium integrity, thereby preventing metastasis, but also by sequestering $\beta$-catenin at the plasma membrane, thereby controlling the mitogenic activity of $\beta$-catenin/TCF signaling pathway. E-cadherin expression is tightly regulated at the transcriptional level by repressors (4-6) and at the post-transcriptional level by phosphorylation, ubiquitination and proteolysis (7, 8). Various E-cadherin repressors such as the E-box binding factors Snail, Slug, ZEB1 and ZEB2 and the basic helix-loop-helix (bHLH) factor Twist were implicated in E-cadherin regulation and epithelial-to-mesenchymal transition during normal development and cancer progression (6). Recently, microRNAs of the miR-200 family were uncovered as modulators of E-cadherin expression, through regulation of ZEB1 and ZEB2 expression (9, 10). The loss of E-cadherin expression has been extensively documented in cancer metastasis (11, 12). Frequently, metastatic tumor cells display decreased E-cadherin expression (13, 14) and re-expression of E-cadherin in invasive tumor cell lines reduces their invasive behavior (15, 16). Systematic investigations on the regulation of E-cadherin expression have been difficult in the past due to the absence of functional assays amenable to powerful genetic approaches. Here we developed an innovative assay using the E-
cadherin-mediated invasive properties of *Listeria monocytogenes* (17), as readout of E-cadherin expression at the plasma membrane. We used the approach in combination with the RNAi methodology and report the identification of novel regulators of E-cadherin expression that may represent new therapeutic targets. In addition, we also identified potent siRNA duplexes, potentially acting through the miRNA pathway, which enforced an epithelial phenotype in mesenchymal cells and may therefore serve as therapeutic molecules.
MATERIALS AND METHODS

Bacterial and eukaryotic cell growth conditions

*Listeria monocytogenes* strain 10403S (18) was grown overnight in BHI (Difco) at 30°C without agitation prior to infection. HeLa229 cells and MDA-MB 231 (ATCC #CCL-2.1 and # HTB-26) were obtained from the ATCC. Cells were authenticated by the ATCC by analysis of Short Tandem Repeat loci using the Promega PowerPlex Systems and were passaged in our laboratory for fewer than 6 months after resuscitation. Cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Gibco) at 37°C in a 5% CO₂ incubator. HT 29 cells (ATCC #HTB-38) were grown in McCoy (Invitrogen) supplemented with 10% FBS (Gibco) at 37°C in a 5% CO₂ incubator.

RNAi screen

The Dharmacon library (ThermoFisher) covering the human Druggable genome (Thermo Scientific) was aliquotted in black clear-bottom 384-well plates (Corning, 3712) by dispensing 10 µl of the corresponding siRNA pools (200nM). Reverse RNAi transfection and *Listeria monocytogenes* infection was performed as previously described (19). For a detailed description please see “Supplemental Materials and Methods”.

High-throughput imaging and computer-assisted image analysis

384-well plates were imaged using a TE 2000 microscope (Nikon) equipped with an Orca ER Digital CCD Camera (Hamamatsu), motorized stage (Prior), motorized filter wheels
(Sutter Instrument, Inc.) and a 10X objective (Nikon) mounted on a piezo focus drive system (Physik Instrumente). Image acquisition and analysis were conducted using the MetaMorph 7.1 software (Molecular Devices, Inc.).

Validation procedure
Cells were transfected by reverse transfection with Dharmafect1 and individual siRNA (D1, D2, D3 and D4, 50 nM final) or a pool of the four silencing reagents (12.5 nM each, 50nM total) and incubated for 72hrs in a 96-well plate format. For real-time PCR analysis, total RNA and first-strand cDNA synthesis was performed using the TaqMan Gene Expression Cells-to-Ct Kit (Applied Biosystems), as recommended by the manufacturer.

DNA constructs
HeLa229 cells were transiently transfected with either wild-type pME18S-FLASH-GFP or the siRNA-resistant form of FLASH. The resistant siRNA form of FLASH was constructed by silent mutagenesis at the siRNA D4-binding sequence site.

Immunofluorescence
For immunofluorescence, cells grown on coverslips were fixed and permeabilized in methanol (at −20°C) for 5 min and stained (at the dilutions shown) for anti-E-cadherin (1:1,000). The secondary antibody used was anti-mouse Alexa Fluor 594 (1:1,000) (Molecular Probes).
Colony formation assay

Mock-transfected or siRNA-transfected HeLa229 cells (1x10^3) were suspended in 1ml 0.5% agarose in DMEM supplemented with 10% FBS. The cell suspension was layered over 1ml of medium containing 0.8% agarose in 6-well plates. Plates were incubated for 21 days and stained with crystal violet. Colonies were counted using the Metamorph Imaging software from images of the plates captured with a digital camera (see Supplementary Fig. 4).

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation (ChIP) assay was performed with the protocol described by Upstate (Millipore) with optimizations for DNA shearing. Briefly, 5 x 10^6 cells per assay were cross-linked with 1% formaldehyde at room temperature for 10 minutes. Cells were resuspended in the SDS lysis buffer for 15 minutes on ice. The lysate was sonicated 10 times, 10 seconds on ice. After centrifuging at 13,000 rpm at 4°C for 10 minutes, the supernatant was precleared for 1h with salmon sperm DNA and Protein-A Sepharose before ZEB-1 antibody (Santa Cruz Biotechnology) or control rabbit IgG was added overnight at 4°C. Immune complexes were collected with Protein-A Sepharose (Amersham, GE Healthcare). Quantitative real-time PCR was performed using SYBR-Green with primers previously described for E-cadherin and β-globin (20, 21). The enrichment of E-cadherin promoter is relative to the β-globin gene as control.
**Gene expression profile analysis**

RNA samples were prepared from HeLa229 cells three days after mock and siRNA transfection. Sample preparation and hybridization to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix) were performed at the Yale University W.M. Keck facility, using an Affymetrix GeneChip Instrument System according to the manufacturer’s recommendations. The images were processed with Affymetrix Microarray Suite version 5.0, scaled to a target intensity of 500. For generating Log2 expression values the raw data was processed and normalized using “affy” package in Bioconductor 2.9 (22). For more information please see “Supplemental Materials and Methods”.

The microarray data are available on the GEO website ([http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57660](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57660)) under the accessions numbers GSM1386221, GSM1386222 and GSM1386223.
RESULTS

A functional approach for assessing the level of E-cadherin expression

In a survey for E-cadherin expression in epithelial cells, we found that HT-29 and HeLa229 cells expressed high and low levels of E-cadherin, respectively (Fig. 1A). Although standard assays such as immuno-fluorescence procedures allowed for the detection of high level of E-cadherin expression in HT-29 cells, similar procedures failed to detect low level of E-cadherin expression in HeLa229 cells (Fig. 1B). To develop a functional assay that reports on differential levels of E-cadherin expression in low expressing cells, we used the invasive property of the Gram-positive bacterium *Listeria monocytogenes*. *L. monocytogenes* invasion in epithelial cells relies on the interaction of the bacterial factor InlA with its host cell receptor, E-cadherin (23). Consequently, the proportion of cells successfully infected by *L. monocytogenes* depends on the levels of E-cadherin expression in these cells. Accordingly, RNAi-mediated silencing of E-cadherin (CDH1) in HeLa229 cell decreased the level of E-cadherin expression (Fig. 1C, bottom panel, CDH1 KD), which resulted in a decrease in the proportion of cells infected with GFP-expressing *L. monocytogenes* (Fig. 1D, Mock, 14 % +/- 0.9 vs. CDH1 KD, 2 % +/- 0.5). Conversely, silencing ZEB1, a negative regulator of E-cadherin expression, increased the level of E-cadherin expression in HeLa229 cells (Fig. 1C, bottom panel, ZEB1 KD), which increased the proportion of infected cells (Fig. 1D, ZEB1 KD 64 % +/- 2.7 vs. Mock 14% +/- 0.9). We validated these results by using the antibiotic protection assay, a standard approach for measuring the efficiency of *L. monocytogenes* invasion in mammalian cells (Supplementary Fig. S1). Thus, the combination of the invasive
properties of *L. monocytogenes*, quantitative imaging procedures and the RNAi methodology offers an unprecedented functional approach to investigate the regulatory network that controls E-cadherin expression.

**Identification of novel regulators of E-cadherin expression**

To identify novel regulators of E-cadherin expression, we used the functional approach presented in Fig. 1 and screened the Dharmacon siRNA library covering the Druggable Human Genome (ThermoFisher). This library harbors pools of four independent siRNA duplexes for a given gene, targeting a total of 7,000 genes. We identified 52 pools whose transfection led to increased infection levels that reproducibly deviated from the mean by at least 2 Standard Deviation (SD) units in three independent experiments. The main challenge in RNAi studies is associated with the unintended silencing of genes displaying limited sequence homology with the targeted gene, a phenomenon referred to as the “off-target effect” (24). We have previously shown that validation of RNAi screens requires the use of independent silencing reagents in order to establish a functional relationship between the silencing of the targeted gene and the observed phenotype (25). To validate the identified candidate genes, we re-tested individually the four siRNA duplexes that constituted the pools used in the primary screen. We identified 78 duplexes that conferred an increase in *L. monocytogenes* invasion. These duplexes corresponded to 4 pools displaying the phenotype for three or four of the four siRNA duplexes, 19 pools displaying the phenotype for two of the duplexes tested and 26 pools displaying the phenotype for only one of the four duplexes (Supplementary Table 1). In only 3 cases did the pools, but not the individual duplexes, displayed a phenotype. All the results obtained
by microscopy procedures were also validated using the antibiotic protection assay, a standard procedure for measuring the efficiency of *L. monocytogenes* infection in mammalian cells (see Materials and Methods and Supplementary Table 1, Plating). We next determined the silencing efficiency conferred by all siRNA duplexes investigated (Supplementary Table 1, RT PCR gene). Among the 52 pools analyzed, the 4 pools displaying the phenotype for at least three of the four duplexes tested and 4 pools displaying the phenotype for two of the duplexes tested, showed a very good correlation between silencing efficiency and *L. monocytogenes* invasion. These results therefore validated CASP8AP2 (FLASH) (Fig. 2A), FBXL5 (Fig. 2B), HDAC1 (Fig. 2C), PCGF1 (Fig. 2D), Caspase 7 (Fig. 2E), Caveolin 2 (Fig. 2F), MMP14 (Fig. 2G) and ZEB1 (Fig. 2H) as *bona fide* regulators of E-cadherin expression in HeLa229 cells. Thus, our screen led to the identification of known regulators of E-cadherin such as ZEB1 (5, 6, 9, 12), HDAC1 (26, 27) and MMP14 (7), as well as factors playing a previously unappreciated role in E-cadherin regulation, including FLASH, FBXL5, CASP7, PCGF1 and CAV2. We also replicated our *L. monocytogenes* invasion assay in the MDA-MB 231 cell line and obtained similar results for the *bona fide* regulators identified in HeLa229 cells (Supplementary Table 2).

**Transcriptional and post-transcriptional regulators of E-cadherin expression**

We further tested whether the identified regulators controlled E-cadherin expression at the transcriptional or post-transcriptional level. To this end, we tested whether increased E-cadherin protein levels correlated with increased mRNA levels. Depletion of FLASH resulted in a significant increase in E-cadherin mRNA (Supplementary Fig. S2A, D1-D4)
and total protein levels (Supplementary Fig. S2B, WCL, D1-D4). We obtained similar results for ZEB1, HDAC1, Caspase 7 (CASP7) and polycomb group (PcG) protein PCGF1 (Supplementary Fig. S3). We also performed surface biotinylation experiments in order to quantify the level of E-cadherin at the plasma membrane. Biotinylated proteins were collected by streptavidin beads pull-down, and subsequent E-cadherin blotting showed an increase in surface E-cadherin in FLASH-depleted cells (Supplementary Fig. S2B, IP:streptavidin). Altogether these results not only confirmed the previously reported role of ZEB1 and HDAC1 in E-cadherin regulation (5, 6, 9, 26, 27), but also uncovered previously unappreciated roles for FLASH, PCGF1 and CASP7 as transcriptional regulators of E-cadherin expression. 

As opposed to the situation observed for the depletion of the transcriptional regulators, depletion of FBXL5 did not alter significantly the levels of E-cadherin mRNA (Supplementary Fig. S2C). However, we observed a significant increase in E-cadherin protein level (Supplementary Fig. S2D, WCL, D1-D3). Furthermore, plasma membrane E-cadherin biotinylation and streptavidin recovery showed that FBXL5 depletion resulted in increased E-cadherin expression at the plasma membrane (Supplementary Fig. S2D, IP:streptavidin). These results suggested that FBXL5 affected E-cadherin expression at the post-transcriptional level. We obtained similar results for MMP14 and Caveolin 2 (CAV2) (Supplementary Fig. S3). These experiments not only confirmed the established role for MMP14 in E-cadherin post-translational modification (7), but also suggested a previously unappreciated role for FBXL5 and CAV2 in the post-transcriptional regulation of E-cadherin expression.
Depletion of the newly identified E-cadherin regulators affects the anchorage-independent growth of HeLa229 cells

We next studied the impact of silencing the expression of the identified candidate genes on the ability of HeLa229 cells to proliferate independently of both external and internal regulatory cues in a soft agar assay. We silenced the expression of the identified candidate with two independent siRNA duplexes (Fig. 3A). We found that the increase in E-cadherin expression in cells depleted for CASP8AP2 (FLASH), FBXL5, HDAC1, PCGF1, Caspase 7, Caveolin 2, MMP14 and ZEB1 resulted in a significant suppression of anchorage-independent cell growth in soft agar (Fig. 3B). These results suggest that the increase in E-cadherin expression resulting from the silencing of the identified candidate genes reduces the proliferating capacity of Hela229 cells and support the notion that these newly identified E-cadherin regulators constitute potential targets for therapeutic intervention.

Full validation of FLASH as a regulator of E-cadherin expression

We next conducted a full validation of the specific involvement of FLASH in E-cadherin expression. To this end, we first confirmed that siRNA treatment with the four duplexes targeting FLASH expression led to FLASH depletion at the mRNA as well as the protein level (Fig. 4A). We also ruled out a potential off-target activity for siRNA duplex D4 by taking advantage of the C911 mismatch approach in which bases 9 through 11 of the siRNA duplexes were replaced by their complement (28). We found that transfection of HeLa229 and MDA-MB 231 with the C911 version of siRNA duplex D4 did not result in the striking increase in E-Cadherin expression observed in cells transfected with
siRNA duplex D4 (Fig. 4B, HeLa229 and MDA-MB 231), suggesting that the increased in E-cadherin expression observed in cells transfected with siRNA duplex D4 was specifically due to FLASH depletion. Finally, to unambiguously establish the specificity of the observed phenotype, we conducted a “genetic rescue” experiment by designing a FLASH cDNA construct resistant to treatment with siRNA duplex D4 (referred to as FLASH(m) cDNA construct). We showed that, in contrast to FLASH-GFP, FLASH(m)-GFP was not depleted in cells transfected with siRNA duplex D4 (Supplementary Fig. S5A and S5B). We analyzed the levels of E-cadherin expression in Mock-transfected (Fig. 4C, top panel) and FLASH-depleted HeLa229 cells (Fig. 4C, bottom panel) transiently transfected with the FLASH(m)-GFP construct. E-cadherin expression was very low in Mock-transfected cells that did, or did not, express FLASH(m)-GFP (Fig. 4C, Mock). As expected, transfection with siRNA duplex D4 resulted in a striking increase in E-cadherin expression in cells that did not express FLASH(m)-GFP (Fig. 4C, compare Mock vs. FLASH D4, E-cadherin, arrowheads). Statistical significance of these visual differences was determined on a cell-by-cell basis by computer-assisted image analysis (Fig. 4C, Mock vs. FLASH D4). By contrast, the observed increase in E-cadherin expression was severely attenuated in cells expressing FLASH(m)-GFP (Fig. 4C, compare Mock vs. FLASH D4, FLASH(m) and E-cadherin, arrows; and Fig. 4D, FLASH D4 vs. FLASH D4 (FLASH positive)). These results suggested that the expression of FLASH(m)-GFP effectively rescued the increase in E-cadherin expression mediated by depletion of endogenous FLASH. Altogether these results unambiguously demonstrate that FLASH plays a specific role in E-cadherin expression.
FLASH regulates ZEB1 expression

As previously shown (5, 6, 9) and as illustrated in this study (Fig. 2H and Supplementary Fig. S2), the transcriptional repressor ZEB1 acts as negative regulator of E-cadherin expression. We tested whether the depletion of FLASH, PCGF1 and CASP7 may lead to an increase in E-cadherin transcription by modulating ZEB1 expression. We found that FLASH depletion, but not CASP7 or PCGF depletion, led to a decrease in ZEB1 protein expression (Fig. 5A, FLASH KD). We next determined whether FLASH depletion affected ZEB1 expression at the transcriptional or post-transcriptional level. We used 4 different siRNA duplexes to silence FLASH in HeLa229 cells and quantitative real-time PCR revealed that ZEB1 mRNA levels were not affected in the FLASH-depleted cells (Fig. 5B), but the ZEB1 protein levels were decreased by all FLASH-targeting siRNA duplexes in HeLa229 cell line (Fig. 5C) and similarly in the MDA-MB 231 cell line (Fig. 5D). Thus, FLASH regulates ZEB1 expression at the post-transcriptional level.

We next carried out co-immuno-precipitation experiments with cytosolic and nuclear extracts of HeLa229 cells to determine whether FLASH and ZEB1 may form a complex. As expected, ZEB1 and FLASH were largely identified in the nuclear fraction of HeLa229 cells (Fig. 5E, Lysate). Using an anti-FLASH antibody, we showed co-immuno-precipitation of FLASH and ZEB1 with extracts from mock-treated cells, but not with extracts from FLASH-depleted cells (Fig 5E, IP, Mock vs. FLASH KD). As expected, the converse experiments using an anti-ZEB1 antibody also showed co-immuno-precipitation of ZEB1 and FLASH (Fig. 5F). Thus, endogenous FLASH and ZEB1 form a complex in the nucleus. We next analyzed whether FLASH depletion modified the occupancy of the E-cadherin promoter by ZEB1. To this end, we carried out
chromatin immuno-precipitation assay with an anti-ZEB1 antibody, as previously described (20, 29). Quantitative PCR of the E-cadherin promoter region revealed that FLASH depletion led to a 2.5 decrease in the occupancy of the E-cadherin promoter by ZEB1 (Fig. 5G). Altogether, these results are in agreement with the notion that FLASH modulates E-cadherin expression by regulating ZEB1 protein levels, potentially through formation of a complex, which impacts the occupancy of the E-cadherin promoter and therefore the level of E-cadherin expression.

**Gene expression profiles in ZEB1- and FLASH-depleted cells**

ZEB1 acts as a regulator of the mesenchymal phenotype through its role as a transcriptional repressor of E-cadherin expression (5, 6). To determine whether ZEB1 may modulate the expression of additional genes, we compared the gene expression profiles of mock-treated and ZEB1-depleted cells. In addition to E-cadherin (CDH1), we found that ZEB1 negatively regulated the expression of EPCAM, JAM-A (F11R), DIRAS3 and MARVELD3 (Fig. 6A and Supplementary Table 3). We also compared the gene expression profiles of mock-treated and FLASH-depleted cells. As expected, we found that FLASH depletion increased the expression of the ZEB1-regulated genes, including CDH1 and EPCAM (Fig. 6A and Supplementary Table 3). In addition, we found that FLASH-depletion led to a > 4-fold increase in the transcription of more than 100 genes (Fig. 6B and Supplementary Table 4). We observed enrichment in histone transcripts, as previously described in SW480 cells (30). In addition, gene ontology analysis (Fig. 6C) revealed enrichment in regulators of apoptosis, cell adhesion molecules and tumor suppressors, including DHRS2, DKK1 and TFPI-2 (Fig. 6B). We confirmed
these microarray data by real time PCR (Fig. 6D). These results not only establish the role of FLASH in the regulation of ZEB1-dependent genes, but also uncover a role for FLASH in the negative regulation of ZEB1-independent genes previously associated with tumor suppression in mesenchymal cells.

**siRNA duplexes that mimic microRNAs**

In addition to the pools that passed our validation procedures for gene specificity, we identified 26 pools for which only one out of the 4 tested siRNA duplexes conferred the observed phenotype. For instance, when targeting ZNF281 gene expression, siRNA duplexes D2 and D3 displayed silencing efficiency similar to siRNA duplex D1 (Fig. 7A, mRNA level, D2 and D3), but did not result in an increase in *L. monocytogenes* infection (Fig. 7A, Infection, D2 and D3) or E-cadherin mRNA (Fig. 7B, D2 and D3). This is the signature of the so-called “off-target effect” whereby, in addition to their silencing activity through the siRNA pathway, siRNA duplexes may also affect gene expression through the microRNA pathway (24). Interestingly, sequence analysis revealed that 7 of the 26 duplexes displaying an off-target effect harbored a seed region identical to the seed region of the miR-200 family members (Supplementary Fig. S6, top panel). miR-200 regulates E-cadherin expression by down-modulating the expression of the E-cadherin transcriptional repressors, ZEB1 and ZEB2 (9, 10). Therefore, the identified siRNA duplexes plausibly regulated E-cadherin expression by mimicking the activity of miR-200 family members. Although the seed region of ZNF281_D1 did not match the seed region of any known human microRNA, it displayed a perfect match with the seed region of *C. elegans* miR-70 (Supplementary Fig. S6, bottom panel), suggesting that
ZNF281_D1 may regulate E-cadherin expression by mimicking the activity of a yet uncovered human homolog of *C. elegans* miR-70. We refer to those siRNA duplexes that potentially mimic the activity of endogenous microRNAs as miR-mimics.

**Effects of miR-mimics on the epithelial phenotype**

We explored the mechanisms supporting the activity of miR-mimics PON3_D2 and ZNF281_D1. Transfection with miR-mimics led to a strong increase in *L. monocytogenes* infection (Fig. 7A, Infection, D1) paralleled by high levels of E-cadherin mRNA (Fig. 7B, D1), total E-cadherin protein (Fig. 7C, WCL, D1) and an increased availability of E-cadherin protein at the plasma membrane (Fig. 7C, IP:streptavidin, D1). Strong E-cadherin expression was still detected 21 days after a single transfection with 50nM siRNA (Supplementary Fig. S6B) and the lowest effective dose tested that induced E-cadherin expression was 5nM (Supplementary Fig. S6C). Increased E-cadherin expression (Supplementary Fig. S7A) was paralleled by the decreased expression of its transcriptional repressors, including ZEB1, ZEB2 and SNAIL (Supplementary Fig. S7B, S7C and S7D). To examine the ability of ZNF281_D1 to enforce an epithelial phenotype, we also examined occludin expression, a critical component of tight-junctions involved in the establishment and maintenance of cell polarity (31). In mock-treated Hela229 cells, occludin expression was not detectable (Fig. 7D, Mock). However, occludin expression was restored in miR-mimic-transfected cells (Fig. 7D, PON3_D2 and ZNF281_D1). In addition to the transcriptional up-regulation of epithelial markers, such as E-cadherin (Supplementary Fig. S7A) and occludin (Supplementary Fig. S7E), we also observed repression of mesenchymal markers, such as vimentin (Supplementary Fig. S7F). Finally,
we showed that the enforcement of the epithelial phenotype observed in miR-mimic-transfected cells resulted in suppression of anchorage-independent cell growth in soft agar (Fig. 7E, PON3_D2 vs. Mock and ZNF281_D1 vs. Mock). Altogether, these results indicated that miR-mimics enforced an epithelial phenotype in mesenchymal cells.
DISCUSSION

Our genetic investigations led to the identification of novel regulators of E-cadherin expression as well as potent siRNA duplexes that enforce an epithelial phenotype in mesenchymal cells. Not only were E-cadherin levels restored in HeLa229 cells depleted for these regulators but the anchorage-independent growth, a hallmark of cell transformation, was significantly reduced. Below we discuss the implications of our findings in the context of our understanding of the mechanisms supporting E-cadherin expression and the design of novel strategies for enforcing an epithelial phenotype in mesenchymal cells.

Post-transcriptional regulation of E-cadherin expression

We identified FBXL5 and CAV2 as post-transcriptional regulators of E-cadherin expression. The F-box protein FBXL5 is a component of the ubiquitin protein ligase complex SCF (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination and subsequent proteolysis (32). FBXL5 was previously involved in polyubiquitination and degradation of p150Glued which plays an important role in dynein-dynactin complex (33). In agreement with our results showing that E-cadherin accumulated at the plasma membrane in FBXL5-depleted cells, we speculate that FBXL5 regulates E-cadherin degradation and accumulation at the plasma membrane. In addition to FBXL5, we also identified CAV2 as a post-transcriptional regulator of E-cadherin expression. This result is in agreement with previous reports showing that CAV1 and CAV2 expression is associated with highly aggressive breast tumors (34). In addition, an
inverse correlation between CAV2 and E-cadherin expression was found in invasive breast cancer (34). Our results indicate that CAV2 depletion leads to an accumulation of E-cadherin at the plasma membrane, suggesting that CAV2 regulates E-cadherin endocytosis.

**Transcriptional regulation of E-cadherin expression**

We identified CASP7 and PCGF1 as regulators whose depletion led to an increase in E-cadherin transcription. Given its cytoplasmic localization, it is likely that CASP7 plays an indirect role in E-cadherin transcription. Interestingly, a recent report revealed that CASP3-induced cleavage of δ-catenin generates a fragment that localizes to the nucleus and binds to a zinc finger transcriptional factor (ZIFCAT), potentially modulating its function (35). We speculate that CASP7 might exhibit a similar role and process unknown factor(s) that modulate E-cadherin transcription in the nucleus. In addition to CASP7, we identified the Polycomb repressive complex (PRC) component PCGF1 as a regulator of E-cadherin transcription. PRC components are divided into two sub-complexes (PRC1 and PRC2) which play an important role in embryonic development and carcinogenesis through gene silencing (36, 37). A role for PRC2 in E-cadherin repression has been formerly described (38). BMI1, a component of the PRC1 group, has been recently shown to repress E-cadherin expression (39). Since it is a member of the PRC1 group (40), we suggest that PCGF1, potentially in interaction with BMI1, is a component of a complex that represses E-cadherin transcription.
A role for FLASH in ZEB1-regulated E-cadherin transcription

In addition to CASP7 and PCGF1, we identified FLASH as a transcriptional regulator of E-cadherin expression. The role of FLASH in transcriptional regulation has been formerly described and recent studies support its emerging role as a co-factor in specific transcription processes (41, 42). For instance, FLASH was found to act as a co-factor of c-Myb and enhance the expression of c-Myb-dependent genes (43). As our results suggest that FLASH interacts with ZEB1 in the nucleus and FLASH depletion decreases the E-cadherin promoter occupancy by ZEB1, we speculate that FLASH may be a component of the ZEB1-containing repressor complex. Alternatively and since FLASH depletion impacted the protein level of ZEB1, we cannot exclude the possibility that FLASH regulates the stability or the nuclear localization of ZEB1 through unknown mechanisms.

A role for FLASH in the regulation of the mesenchymal phenotype

To further understand the role of ZEB1 and FLASH in the regulation of gene expression in mesenchymal cells, we conducted gene expression profile analyses. In addition to E-cadherin, our results revealed novel genes involved in tumorigenesis whose expression is subject to ZEB1-dependent repression, including EPCAM, JAM-A, DIRAS3 and MARVELD3. Previous studies detailed the controversial biological role of EPCAM in carcinogenesis. As an adhesion molecule, EPCAM mediates homophilic adhesion interactions, which in turn might prevent metastasis (44, 45). On the other hand, EPCAM abrogates E-cadherin-mediated cell-cell adhesion thereby promoting metastasis (46).
findings are consistent with data showing a negative correlation between EPCAM and ZEB1 in several cancer cell lines (47, 48). The roles of JAM-A, DIRAS3 and MARVELD3 as potential tumor suppressors and promoters of epithelial phenotype have been previously described (49-51). To the best of our knowledge, this is the first report on the negative correlation between ZEB1 and JAM-A, DIRAS3 and MARVELD3. These results reveal that in addition to E-cadherin, ZEB1 regulates the expression of genes whose products may contribute to the epithelial phenotype.

In agreement with our findings showing that FLASH regulates ZEB1 expression, our gene expression profile analyses showed that FLASH regulates the expression of ZEB1-dependent genes. In addition, we uncovered that FLASH regulates the expression of numerous ZEB1-independent genes. These genes not only include histone genes, as previously reported (30, 42), but also numerous genes whose products may contribute to the epithelial phenotype, including DHRS2, DKK1 and TFPI-2. Previous studies showed that mitochondrial Hep27 (DHRS2) is a c-Myb target gene that inhibits Mdm2 and stabilizes p53 (52). Also, a role for DKK1 as a tumor suppressor by Wnt-signaling inhibition was documented (53). Tissue factor pathway inhibitor-2 (TFPI-2) is a matrix-associated serine protease inhibitor, which has been previously described as a tumor suppressor gene in several types of cancer (54, 55). Our results therefore support the notion that FLASH represses the expression of numerous genes that support the epithelial phenotype and therefore is a critical determinant of the mesenchymal phenotype.

**Novel strategies for enforcing an epithelial phenotype**
Our genetic investigation led to the identification of several novel regulators of E-cadherin expression that may constitute potential targets in the context of therapeutic intervention aiming at preventing the epithelial-to-mesenchymal transition. In particular, our results revealed that FLASH is a major enforcer of the mesenchymal phenotype whose inhibition effectively restores the expression of numerous genes that support the epithelial phenotype. Similarly, our results revealed that siRNA duplexes such as miR-mimics, not only restored very high levels of E-cadherin expression, but also enforced a mesenchymal-to-epithelial transition in mesenchymal cells. In addition to miR-mimic-70 (ZNF281_D1) and miR-mimic-200 (PON3_D2), our genetic screen uncovered other siRNA duplexes displaying very potent properties with respect to E-cadherin expression (Supplementary Table 1). Although, the seed-region of these siRNA did not match the seed-region of any known human miRNAs, these siRNA duplexes may mimic the activity of yet undiscovered, endogenous microRNAs. We note, however, that we cannot exclude the possibility that these siRNA molecules may exert their effects on E-cadherin expression independently of the siRNA and miRNA pathways, through uncovered mechanism(s). Also, we acknowledge that the targets we identified may be cell-specific and various cancer cell lines with low expression of E-cadherin may require depletion of other regulators in order to restore high levels of E-cadherin. Nonetheless, these molecules should be considered as a novel class of compounds that display remarkably potent activities on E-cadherin expression and the enforcement of the epithelial phenotype.
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FIGURE LEGENDS

**Fig. 1: Functional assay for E-cadherin expression**

(A) E-cadherin expression in epithelial cell lines. E-cadherin expression in HeLa229 and HT-29 cells was detected by Western blot. Actin was used as loading control. (B) Immuno-fluorescence was performed for E-cadherin expression in HeLa229 and HT-29 cells grown to confluency for three days. (C) Effect of ZEB1 depletion on E-cadherin expression. HeLa229 cells were transfected with a pool of siRNA duplexes targeting E-cadherin (CDH1) or its transcriptional repressor ZEB1 or both (CDH1/ZEB1). ZEB1 and E-cadherin protein levels were determined by Western blot analysis. (D) Quantification of *L. monocytogenes* infection by computer-assisted image analysis. Cells (Nuclei-DAPI) infected with GFP-expressing *L. monocytogenes* (*Listeria*-FITC) were determined by computer-assisted image analysis. The nuclei of infected and non-infected cells are represented in green and red, respectively (Segmentation).

**Fig. 2: Correlation between silencing efficiency and *L. monocytogenes* infection**

HeLa229 cells were transfected with four different duplexes (D1-D4) targeting the corresponding genes. (A-H, Infection) *L. monocytogenes* infection was quantified by computer-assisted image analysis. (A-H, mRNA level) Silencing efficiency was determined by Q-PCR. The graphs represent the average of three independent experiments. The significance of the differences was confirmed by Student’s t-test for infection quantification (**, p<0.0025) and silencing efficiency (††, p<0.0025).
**Fig. 3: Depletion of E-cadherin regulators affects the anchorage-independent growth of HeLa cells**

(A) Anchorage-independent growth of HeLa229 cells transfected with two independent siRNA duplexes targeting ZEB1, FLASH, CASP7, HDAC1, PCGF1, CAV2, FBXL5 and MMP14 in soft agar assay. (B) Colony counts were conducted by computer-assisted analysis of images as shown in (A). The graphs represent the average of three independent experiments. The significance of the differences was confirmed by Student’s t-test (**, p<0.0025).

**Fig. 4: Validation of FLASH as a specific regulator of E-cadherin expression**

(A) FLASH protein levels in FLASH-depleted and mock-treated cells (A, top panels) and actin loading controls (A, lower panels) were assessed by Western blot analysis. Four independent siRNA duplexes were used to deplete FLASH in HeLa229 cells. (B) E-cadherin protein levels and actin loading controls were assessed by Western blot analysis in HeLa229 or MDA-MB 231 transfected with FLASH D4 duplex (D4) and FLASH D4-C911 control duplex (D4(C911)). (C) Immunofluorescence revealed restoration of E-cadherin expression in FLASH-depleted (FLASH D4) HeLa229 cells. Cells expressing an siRNA-resistant version of FLASH (FLASH(m)) display low levels of E-cadherin (bottom panels, arrow). (D) E-cadherin expression in individual cells was quantified by computer-assisted analysis of images as shown in (C). The significance of the differences was confirmed by Student’s t-test (**, p<0.0025).
Fig. 5: FLASH controls ZEB1 expression

(A) HeLa229 cells were transfected with a pool of duplexes targeting CASP7, FLASH or PCGF1. ZEB1 protein level in siRNA- and mock- transfected cells was determined by Western blot analysis (A, top panels) and the expression normalized to actin loading control (A, lower panels). (B) Relative mRNA level of ZEB1 in FLASH-depleted cells as determined by Q-PCR. (C) ZEB1 protein levels in FLASH-depleted and mock-treated cells were determined by Western blot analysis (C, top panels) and the expression normalized to actin loading control (C, lower panels). Four different siRNA duplexes were used to deplete FLASH in HeLa229 cells. (D) MDA-MB 231 cells were transfected with four different siRNA duplexes targeting FLASH. E-cadherin (D, top panel) and ZEB1 (D, middle panel) proteins expression was detected by Western blot analysis. ZEB1 expression was normalized to actin loading control (D, lower panel). (E) FLASH interacts with ZEB1. Immuno-precipitation was performed with cytosolic and nuclear fraction from mock-treated or FLASH-depleted cells (FLASH KD). Immuno-blotting with anti-ZEB1 antibody reveals a ZEB1-FLASH complex formation in the nucleus (E, top panels, IB: ZEB1) in the presence, but not in the absence of FLASH (E, middle panel, IB: FLASH). α-Tubulin, a cytosolic marker, was largely detected in the cytosolic fractions (E, lower panel, IB: Tubulin). (F) ZEB1 interacts with FLASH. Immuno-precipitation was conducted as in (E) but with an anti-ZEB1 antibody and immunoblotting was conducted with and anti-FLASH antibody. (G) ChIP assay. ZEB1 binding to the E-cadherin promoter was analyzed by ChIP assay carried out with anti-ZEB1 antibody in mock-treated and FLASH-depleted (FLASH KD) cells. Q-PCR was
performed for E-cadherin promoter using SYBR-green and Ct values were normalized to β-globin.

**Fig. 6: Gene expression profile in ZEB1- and FLASH-depleted cells**

(A) Heat map representation of gene expression levels showing at least a 2-fold increase in ZEB1-depleted cells and the corresponding expression in FLASH-depleted cells. Representative genes with a role in tumor suppression are indicated.  
(B) Heat map representation of gene expression levels showing at least a 4-fold increase in FLASH-depleted cells and the corresponding expression in ZEB1-depleted cells.  
(C) The enriched categories for FLASH-regulated genes were determined using the gene ontology software from [www.pantherdb.org](http://www.pantherdb.org).  
(D) Q-PCR validation of the microarray results for CDH1, DHRS2, DKK1, TFPI2 and HIST1H2BD.

**Fig. 7: miRNA-like duplexes restore E-cadherin expression and suppress anchorage-independent cell growth**

*L. monocytogenes* infection efficiency in HeLa229 cells transfected with siRNA duplexes targeting ZNF281 (A, Infection) and gene knock-down efficiency (A, mRNA level) were quantified as described in Fig. 1 and 2. The mRNA level (B, CDH1 mRNA fold increase) and cell-surface E-cadherin expression (C, IP:streptavidin) was analyzed as described in Supplementary Fig. S2.  
(D) Expression of E-cadherin and occludin was determined by immuno-blotting using anti-E-cadherin and anti-occludin antibodies in HeLa229 mock-transfected or miR-mimic-200 (PON3_D2) and miR-mimic-70 (ZNF281_D1) on day one and day three during a six-day period.  
(E) Anchorage-independent growth of cells
transfected with miR-mimic-200 and miR-mimic-70 was determined by soft agar colony formation assay and quantified using the MetaMorph Imaging software.
Figure 1

A

HeLa229
HT-29

IB: E-cadherin (short exp)
IB: E-cadherin (long exp)
IB: Actin

B

HeLa229
HT-29

E-cadherin
Nuclei-DAPI
Listeria -FITC
Segmentation

CDH1 KD  ZEB1 KD  CDH1/ZEB1 KD  Mock

IB: E-cadherin (short exp)
IB: ZEB1
IB: E-cadherin
IB: NS

C

D

IB: E-cadherin
IB: ZEB1
IB: NS

CDH1 KD
ZEB1 KD
CDH1/ZEB1 KD
Mock

14% +/- 0.9
2% +/- 0.5
64% +/- 2.7

Research.
Figure 2

(A) siRNA FLASH

(B) siRNA FBXL5

(C) siRNA HDAC1

(D) siRNA PCGF1

(E) siRNA CASP7

(F) siRNA CAV2

(G) siRNA MMP14

(H) siRNA ZEB1

** Infection

†† mRNA
Figure 3

**Mock**

**ZEB1 D1**

**ZEB1 D2**

**FLASH D3**

**FLASH D4**

**CASP7 D2**

**CASP7 D3**

**HDAC1 D1**

**HDAC1 D4**

**PCGF1 D2**

**PCGF1 D3**

**CAV2 D1**

**CAV2 D4**

**FBXL5 D1**

**FBXL5 D2**

**MMP14 D2**

**MMP14 D3**

Anchorage-independent growth

| Sample       | Colonies/well |
|--------------|---------------|
| **Mock**     | **500**       |
| **ZEB1 D1**  | **200**       |
| **ZEB1 D2**  | **100**       |
| **FLASH D3** | **600**       |
| **FLASH D4** | **400**       |
| **CASP7 D2** | **300**       |
| **CASP7 D3** | **200**       |
| **HDAC1 D1** | **100**       |
| **HDAC1 D4** | **500**       |
| **PCGF1 D2** | **400**       |
| **PCGF1 D3** | **300**       |
| **CAV2 D1**  | **200**       |
| **CAV2 D4**  | **100**       |
| **FBXL5 D1** | **600**       |
| **FBXL5 D2** | **500**       |
| **MMP14 D2** | **400**       |
| **MMP14 D3** | **300**       |

* **p < 0.01"
A

siRNA FLASH
IB: FLASH
IB: Actin

B

D4 (C911) D4 Mock
IB: E-cadherin
IB: Actin

HeLa 229 MDA-MB 231

C

Mock FLASH D4
Merged FLASH(m) E-cad DAPI

D

E-cadherin expression

** **

Mock (FLASH pos) FLASH D4 (FLASH pos)
Relative intensity

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Figure 5
Figure 6

**A**

Heatmap showing Log2 expression levels for **FLASH KD**, **Mock**, and **ZEB1 KD** conditions for genes **DIRAS3**, **MARVELD3**, **CDH1**, and **EPCAM**.

**B**

Heatmap showing Log2 expression levels for **FLASH KD**, **Mock**, and **ZEB1 KD** conditions for genes **HIST1H2BD**, **DHRS2**, **DKK1**, and **TFPI2**.

**C**

Gene Ontology Categories graphic highlighting categories such as **Cell Adhesion**, **Cell Communication**, **Cell Cycle**, **Cell Motility**, **Cell Proliferation**, **Cellular Component Organization**, **Developmental Process**, **Immunological System Process**, **Metabolic Process**, and **Transport**.

**D**

Bar graphs showing gene expression fold increase for **CDH1**, **DHRS2**, **DKK1**, **TFPI2**, and **HIST1H2BD** in **Mock** and **FLASH KD** conditions.

**Legend**

- **Mock**
- **FLASH KD**
- **ZEB1 KD**

**Note**

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited. 

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