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Over-expression of the BRMS1 family member SUDS3 does not suppress metastasis of human cancer cells

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Abstract

BRMS1 and SUDS3 are related members of SIN3-HDAC chromatin remodeling complexes. We hypothesized that they might have overlapping functions and that SUDS3 over-expression could compensate for BRMS1 deficiency. SUDS3 expression was ubiquitous in seven breast cell lines, regardless of metastatic potential. SUDS3 over-expression in BRMS1-non-expressing metastatic cells did not suppress metastasis, motility, osteopontin secretion nor EGF receptor expression, phenotypes associated with BRMS1-mediated metastasis suppression. This study demonstrates functional differences for BRMS1 family members and highlights how the composition of SIN3-HDAC (BRMS1/SUDS3) complexes uniquely affects protein expression and biological behaviors.

Keywords

SUDS3; BRMS1; metastasis suppression; motility

1. Introduction

Breast Cancer Metastasis Suppressor 1 (BRMS1) is a functionally validated metastasis suppressor, defined by blockage of metastasis without preventing orthotopic tumor growth, in both human and murine breast cancer and melanoma cells lines, as well as ovarian cancer cells (reviewed in [1]). The mechanism(s) by which BRMS1 suppresses metastasis are complex and varied, but appear to be dependent upon transcriptional regulation through interaction with SWI-independent 3 (SIN3)-histone deacetylase (HDAC) chromatin remodelling complexes [2-4].

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BRMS1 re-expression in metastatic cancerous cells restores homotypic and heterotypic gap junctional intercellular communication [5;6], increases the sensitivity of cells to anoikis [7; 8], and significantly decreases motility [9]. BRMS1 also alters transcription and expression of multiple genes [10-12]; in particular BRMS1 decreases expression of several tumor promoting and metastasis activating genes including epidermal growth factor receptor (EGFR; [13]), osteopontin (OPN; [14;15]), urokinase-type plasminogen activator [16], and fascin [17].

Suppressor of Defective Silencing 3 (SUDS3, formerly SDS3) was first identified in yeast when mutation restored silencing at the HMR locus [18]. Co-immunoprecipitation studies showed it to be an integral part of orthologous SIN3-HDAC chromatin remodeling complexes in yeast, mouse, and human cells [19-22]. Moreover Sds3, the yeast ortholog of SUDS3, promoted Sin3 complex integrity and was essential for histone deacetylase activity [19]. Mammalian studies demonstrated that SUDS3 is essential for embryonic development [23] and implicated a role for SUDS3 in cancer [24].

In addition to being involved in many of the same chromatin remodeling complexes [3;4; 25-27], BRMS1 shares 23% identity and 49% similarity [3] with the entirety of the SUDS3 protein. Amino acids 69-110 of BRMS1 and 63-104 of SUDS3 were originally designated as the Sds3 domain; but recently, the domain was redefined to include amino acids 52-223 of BRMS1 and 58-229 of SUDS3 [28]. The Sds3 domain is common to all BRMS1 family members including BRMS1, SUDS3, BRMS1-like (p40), as well as the yeast protein Dep1 [28]. Based on sequence homology, we hypothesized that these two proteins might share overlapping functions. Of the phenotypes examined, SUDS3 over-expression in BRMS1 non-expressing cells did not mimic BRMS1 re-expression. Therefore, we conclude that, despite being related, the two proteins are functionally distinct.

2. Materials and Methods

2.1. Cell lines and cell culture

MDA-MB-231 and MDA-MB-435 are human estrogen receptor- and progesterone receptor-negative cell lines derived from metastatic infiltrating ductal breast carcinomas [29;30]. While there is some controversy as to the origin of MDA-MB-435 [31;32], the findings presented here are not dependent upon cellular origin. MDA-MB-436 and MDA-MB-468 cells are human mammary adenocarcinoma cells. These cells were cultured in a mixture (1:1, v/v) of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) with 5% fetal bovine serum, 2mM L-glutamine (Invitrogen, Carlsbad, CA), and 0.02 mM non-essential amino acids (Mediatech, Herndon, VA) without antibiotics or antimycotics. Cells were grown on 100-mm tissue culture dishes (Corning, Corning, NY) at 37°C with 5% CO_2 in a humidified atmosphere. Cultures were passaged upon reaching 80-90% confluency using a solution of 0.05% Trypsin/ 2 mM EDTA (Invitrogen) and were confirmed negative for *Mycoplasma spp*. infection using a PCR-based test (TaKaRa, Shiga, Japan).

MCF7 cells are tumorigenic human mammary cells. These cells were cultured in minimal essential medium (MEM) with L-glutamine and Earle's salts supplemented with 10% fetal bovine serum (Invitrogen), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (Mediatech), and 10 mg/mL insulin (Sigma-Aldrich, St. Louis, MO).

MCF10 and derived cell lines model cancer progression and originated from benign fibrocystic breast tissue [33-37]. They include MCF10A (immortalized but nontumorigenic epithelial cells), MCF10AT (mutant ras-expressing, premalignant, mildly tumorigenic epithelial cells), and MCF10CA1a.1 and MCF10CA1d.1α (form invasive orthotopic tumors that metastasize to lung and regional lymph nodes). These cell lines were cultured as described above with the substitution of 5% horse serum for fetal bovine serum. MCF10A and MCF10AT growth

medium was supplemented with 10 ng/mL EGF, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin (Sigma-Aldrich).

Stably transfected cells were selected using 500 μg/mL active G418 (Mediatech) and maintained in 100 μg/mL G418.

2.2. Constructs and transfection

pcDNA3.1-V5/His-SUDS3, pcDNA3.1-V5/His, and pcDNA3 (Invitrogen) plasmids were transfected into MDA-MB-435 and MDA-MB-231 cells using Lipofectamine 2000 (Invitrogen). Vector controls were kept as a mixed population while SUDS3 transfectants were single cell cloned and screened for expression of SUDS3 by immunoblotting.

2.3. Antibodies and immunoblots

A polyclonal rabbit anti-SUDS3 antibody was previously described [22]. The antibody was generated against a peptide corresponding to amino acids 83-328 of the SUDS3 protein. Other antibodies were purchased as indicated: mouse-anti-β-actin (A2228; Sigma-Aldrich), rabbit-anti-OPN (WH0006696M1; Sigma Aldrich), mouse-anti-V5 (R962-25; Invitrogen), anti-EGFR (2232; Cell Signaling Technology, Danvers, MA), mouse-mab-anti-GAPDH (ab9482; Abcam, Cambridge, MA), anti-mouse secondary antibody conjugated to horseradish peroxidase (NXA931; Amersham-Pharmacia, Biotech, Buckinghamshire, UK) and anti-rabbit secondary antibody conjugated to horseradish peroxidase (NA934; Amersham-Pharmacia).

Cells were grown to 80-90% confluence and then lysed in either RIPA Buffer (Millipore, Billerica, MA) or a 0.1% Triton X-100 lysis buffer as previously described [7]. Both lysis buffers were supplemented with 1 μL/mL protease inhibitor cocktail (P8340; Sigma-Aldrich). To evaluate OPN secretion, media were collected from cells that had been washed three times with ice-cold PBS and serum starved for 12 -24 hours in 5 mL of media/10-cm dish. Equal protein loading was determined by using the BCA assay for whole cell lysates (Pierce, Rockford, IL) or by cell count for media loading.

2.4. In vitro growth, wound healing, and motility assay

Cells at 80–90% confluence were detached and seeded at a density of 50,000 cells per well in a 6-well tissue culture dish (Corning) in triplicate. The growth of cells was monitored for 14-16 days. Cell number and viability were determined using a hemacytometer.

Cells at 80–90% confluence were detached and seeded in triplicate at a density of 100,000 cells per well in a 6-well tissue culture dish. A scratch was made in the shape of an octothorpe (#) using 10 μL pipette tips. Cells were kept in serum-free media to minimize effects of proliferation. Phase contrast photomicrographs were taken using a Nikon Eclipse inverted microscope (Nikon, Chiyoda-ku, Tokyo) equipped with the QICAM Mono capture device (Media Cybernetics, Inc., Bethesda, MD). Four images were taken, one from each intersection, at 0 and 8 hr for MDA-MB-231 cell lines. Initial and final distances were measured using QCapture Pro software (Media Cybernetics, Inc., Bethesda, MD). Parent and vector control cell lines and SUDS3-transfected clones were compared using Dunn's comparison test. Calculations were performed using SigmaStat statistical analysis software (SPSS Inc., Chicago, IL). Statistical significance was defined as a probability $p \leq 0.05$.

2.5. Experimental metastasis assay

Experimental metastasis assays were performed as previously described [38]. Ten mice per experimental group were initially injected with 5×10^5 cells. Animals were maintained under the guidelines of the National Institutes of Health and the University of Alabama at Birmingham. All protocols were approved by the Institutional Animal Care and Use

Committee. Food and water were provided *ad libitum*. ANOVA calculations were performed using SigmaStat statistical analysis software (SPSS Inc., Chicago, IL). Statistical significance was defined as a probability $p \leq 0.05$.

3. Results

3.1. SUDS3 expression does not correlate with metastasis

Previous studies have shown that BRMS1 re-expression in metastatic cells blocks metastasis without blocking orthotopic tumor growth [4;7;39]. Immunohistochemical analysis showed that BRMS1 expression was inversely correlated with prognosis and metastasis in a subset of human breast cancers [40]. Based primarily upon relatedness of BRMS1 and SUDS3, we hypothesized that SUDS3 shared BRMS1 metastasis suppressor as well as other functions. Levels of SUDS3 were measured in multiple human breast cell lines using a polyclonal antibody generated specifically against SUDS3. SUDS3 (45 kDa) was present in all of the cell lines examined, regardless of their tumorigenicity or metastatic potential (Fig. 1A). Although all lanes were loaded with equal amounts of whole cell lysate protein, probing with antibodies directed against housekeeping proteins (i.e., GAPDH; β-actin, α-tubulin) exhibited variability among cell lines, suggesting that we have not yet identified a consistent loading control. Nonetheless, it is apparent that no gross trend in expression levels was observed with tumor progression.

3.2. Ectopic expression of SUDS3 in MDA-MB-231 and MDA-MB-435 cells does not affect proliferation

To examine whether ectopic of SUDS3 affected *in vitro* cell growth or phenotypes associated with BRMS1 metastasis suppression, stable MDA-MB-231 and -435 breast cancer cell lines were generated to ectopically express a SUDS3-V5/His fusion protein. Several clones were isolated and SUDS3 expression was evaluated by immunoblot. Endogenous SUDS3 (45 kDa) and SUDS3-V5 (~ 50 kDa) were detected with anti-SUDS3 (Fig. 1B & 1C). Since all of the clones were derived from the same parental population, endogenous SUDS3 was used as a loading control to assess ectopic SUDS3 expression. The identity of the 50 kDa band was verified using an anti-V5 antibody (data not shown). Ectopic SUDS3 expression did not affect *in vitro* growth rates or saturation densities (Fig. 1D & 1E). Similarly, no gross differences in morphology were observed (data not shown). Several clones of each cell line were selected to represent varying levels of ectopic SUDS3 expression in MDA-MB-435 (clones 5, 10, 17, and 25) and -231 (clones 1, 5, and 22) cells.

3.3. Ectopic expression of SUDS3 does not affect motility of MDA-MB-231 cells

Motility is required for tumor cell invasion and metastasis. 231^{BRMS1} cells showed a modest, but significant inhibition (~60%) of motility as measured using an *in vitro* wound healing assay [41]. To determine whether ectopic expression of SUDS3 affected *in vitro* motility in MDA-MB-231 cells, a similar *in vitro* wounding/motility assay was performed. SUDS3 did not alter motility compared to parental or vector controls (Fig. 2A).

3.4. Ectopic expression of SUDS3 does not consistently suppress metastasis of MDA-MB-231 cells

To examine whether over-expression of SUDS3 could suppress metastasis, representative clones of 231^{SUDS3} were injected into the lateral tail vein of athymic mice. Formation of macroscopic lung metastases was assessed as described previously [4;7;42]. 231^{SUDS3} clones 5 and 22 produced an average of 37 and 76 lung colonies/lung compared to 70-109 lung colonies in parental and vector controls (Fig. 2B & 2C). The size of the lung colonies were

approximately equal for all of the cells. The equivalence of clone 22 to parental and vector cells demonstrates that, in this model, over-expression of SUDS3 does not suppress metastasis.

3.5. Ectopic expression of SUDS3 does not reduce OPN or EGFR

OPN is a secreted glycoprotein that can bind cell surface receptors to promote cell adhesion and migration. High expression of OPN generally correlates with aggressive tumor cell behavior and poor prognosis [43]. There exist various forms of endogenous osteopontin due to differential RNA splicing, protein modification, and susceptibility to proteases [44;45]. Ectopic expression of BRMS1 in MDA-MB-435 cells decreased OPN mRNA and protein by 90- 95% [4;14;15]. OPN down-regulation is crucial to BRMS1 metastasis suppression since restoration of OPN in 435^{BRMS1} cells resulted in increased incidence of spontaneous metastasis to lymph nodes and lungs [15].

To assess whether OPN was similarly affected by SUDS3, 435^{SUDS3} cell-conditioned media and whole cell lysate was collected from serum-starved cells. Immunoblots with anti-OPN identified a single band at 50 kDa in the whole cell lysate and two bands between 40 and 70 kDa in conditioned media as previously described [15]. Ectopic expression of SUDS3 did not decrease either intracellular or secreted levels of OPN (Fig. 3A & 3B).

The EGF receptor tyrosine kinase activates a variety of signaling transduction pathways that affect cell proliferation, differentiation, adhesion, migration, and apoptosis. EGFR is expressed in patients with breast cancer and activated EGFR is often associated with poor patient survival in invasive breast cancer [46]. BRMS1 significantly decreased EGFR in MDA-MB-231 and -435 cells by ~50 to 100%, respectively [13]. However, ectopic over-expression of SUDS3 did not change EGFR when comparing parental, vector, and SUDS3 over-expressing MDA-MB-231 and -435cells (Fig. 3C).

4. Discussion

Functional and transcriptional compensation among protein families is a well-characterized phenomenon, as clearly demonstrated by the Rb family of proteins for example [47]. The studies reported here were undertaken, in part, because of the strong homology between SUDS3 and BRMS1 and the involvement of both proteins in SIN3-HDAC chromatin remodeling complexes. These facts suggested that perhaps SUDS3 and BRMS1 share some functional redundancy and that SUDS3 could compensate for BRMS1 deficiency. Not only did SUDS3 not suppress metastasis like BRMS1, but their apparent functions and/or regulation of chromatin remodeling-based transcriptional regulation differed as well.

We chose to isolate clones from the transfected MDA-MB-231 population in order to address phenotypic changes related to tumor heterogeneity and dose-dependency. That SUDS3 transfectant clones were not universally low (compared to parental MDA-MB-231) for metastatic ability or motility allows the conclusion that SUDS3 is not a metastasis suppressor. Differences among clones are most likely attributable to genetic instability and tumor cell heterogeneity. Taken together, these experiments clearly show that BRMS1 and SUDS3, while sharing the Sds3 domain and participating in similar (and sometimes even the same) SIN3-HDAC complexes, have distinct functions in the breast cancer cell lines examined.

Among the many proteins that comprise the SIN3-HDAC core complexes, there are several that have high sequence similarity for which both overlapping and distinct functions have already been, and are continually being, discovered. These proteins include SIN3A/B, HDAC1/2, ARID4A/B, and Rbbp4/7 [48;49]. Similarly, although BRMS1 and SUDS3 did not share overlapping functions for all of the phenotypes examined, there may be other shared characteristics. Nonetheless, our findings emphasize BRMS1 and SUDS3 distinctness and

further emphasize that the diversity and mix-and-match nature of chromatin remodeling machinery remains relatively ill-defined. Further defining the binding partners and functions of the myriad proteins in the larger SIN3-HDAC complexes is essential.

While SUDS3 is necessary for normal somatic cell survival [23], there have been no reports to date on levels of endogenous SUDS3 in breast cell lines. SUDS3 is expressed in every breast cell line examined. SUDS3 expression did not appear to change to compensate for loss of BRMS1 expression in the MDA-MB-231 and -435 cell lines. Not shown here, we frequently observed a second SUDS3 band in MCF10-derived cells (data not shown). Thus, modified variants may exist within these cells, however, additional experimentation is required in order to identify and characterize this band.

A possible explanation for the apparent differences between SUDS3 and BRMS1 relates to protein-protein interactions. A part of the originally defined Sds3 domain has been shown in separate studies to be necessary for the homodimerization of murine SUDS [21]. SUDS3 directly interacts with BRMS1 as shown in yeast two-hybrid genetic screens [3]. Studies are currently underway to determine whether the Sds3 domain is responsible for direct binding of BRMS1 to SUDS3 or whether BRMS1-SUDS3 interactions occur at other portions of each molecule. Although the data presented here do not support our original hypothesis - that SUDS3 is a metastasis suppressor - the collective findings begin to address the role(s) that the Sds3 domain plays in these individual proteins and in the formation/regulation of the SIN3-HDAC complexes.

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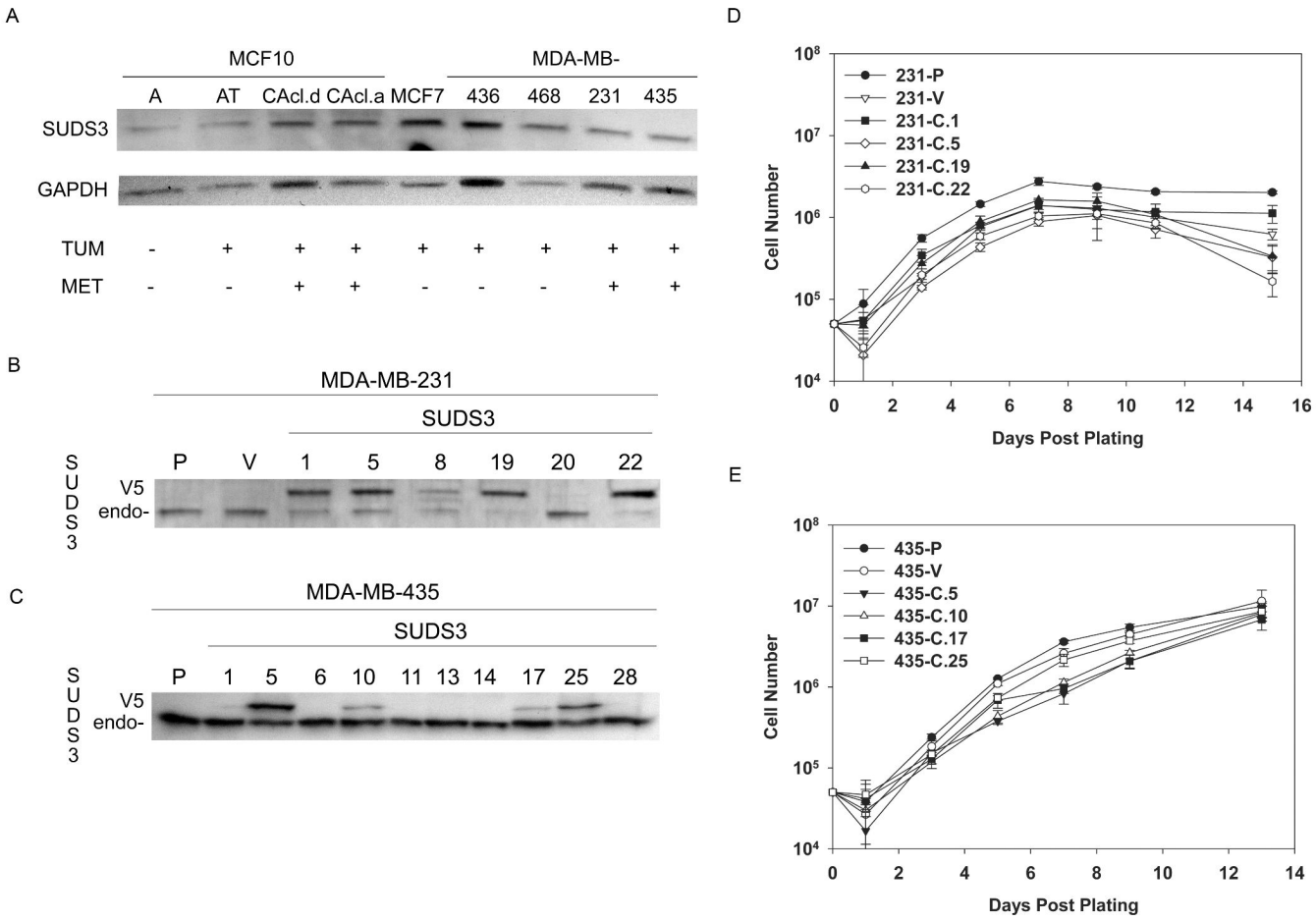
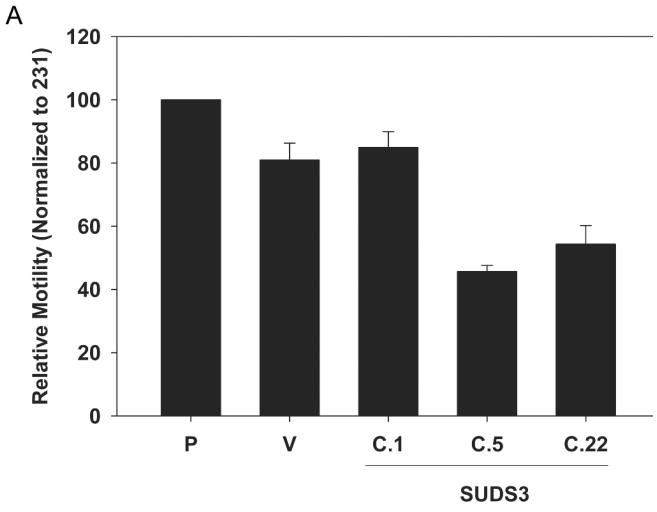
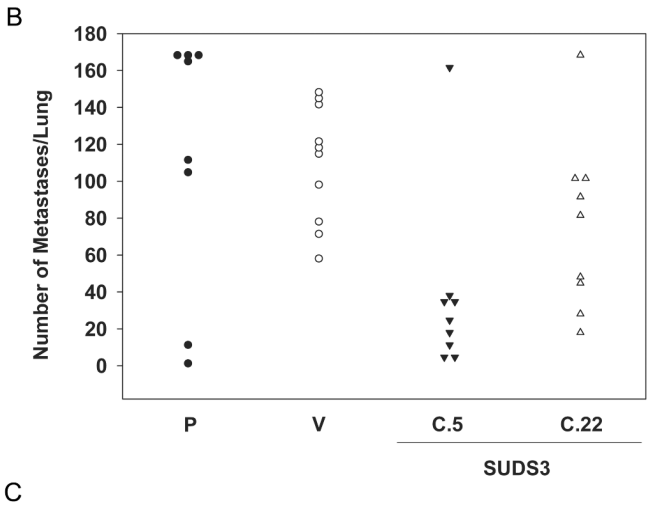


Fig. 1.

SUDS3 expression does not correlate with tumor progression or metastatic potential (**A**). A panel of human breast cell lines were probed with anti-SUDS3 antiserum. Endogenous SUDS3 was ubiquitously present. Levels varied from experiment to experiment, but did not correlate with metastatic potential. Blots were re-probed with GAPDH to verify equal loading. MDA-MB-231 (**B**) and -435 (**C**) cells were stably transfected to express a SUDS3-V5 fusion protein. Western blots with SUDS3 specific antiserum shows an endogenous (endo-) 45 kDa band in all clones. A second band at 50 kDa corresponding to the fusion protein (V5) was verified with anti-V5 antibody (data not shown). Ectopic over-expression of SUDS3 did not affect the *in vitro* growth of MDA-MB-231 cells (**D**) or -435 (**E**) cells when compared to parental and vector controls.





C

	Lung Metastases		
Cell Line	Incidence	Mean ±SEM	p<0.05
MDA-MB-231	8/8	70 ± 24	
231^{vector}	10/10	109 ± 31	
231^{SUDS3}-C.5	9/9	37 ± 48	**
231^{SUDS3}-C.22	9/9	76 ± 47	

Fig. 2.

SUDS3 does not significantly nor consistently suppress motility or metastatic behavior of MDA-MB-231 breast carcinoma cells. (**A**) Motility was measured using an *in vitro* scratch/wound healing assay. Confluent MDA-MB-231 monolayers were scratched and distances from edge to edge were measured at 0 and 8 hr. Relative motility is normalized to parental MDA-MB-231 (P) cells. Vector control cells (V) and three selected 231^{SUDS3} clones are shown. Data are cumulative for 3 independent experiments with replicate wells. (**B**) Lung colonization of MDA-MB-231 (P), vector control (V), and 231^{SUDS3} transfectants (C.5 and C.22) was measured following i.v. of 5×10^5 cells into athymic mice. Each symbol represents the number of surface lung metastases (maximum counted = 170) per mouse. (**C**) Summary statistics of

experimental metastasis for data represented in panel B. One-way analysis of variance with Dunn's post-test was used to determine differences among groups (** = $p<0.05$).

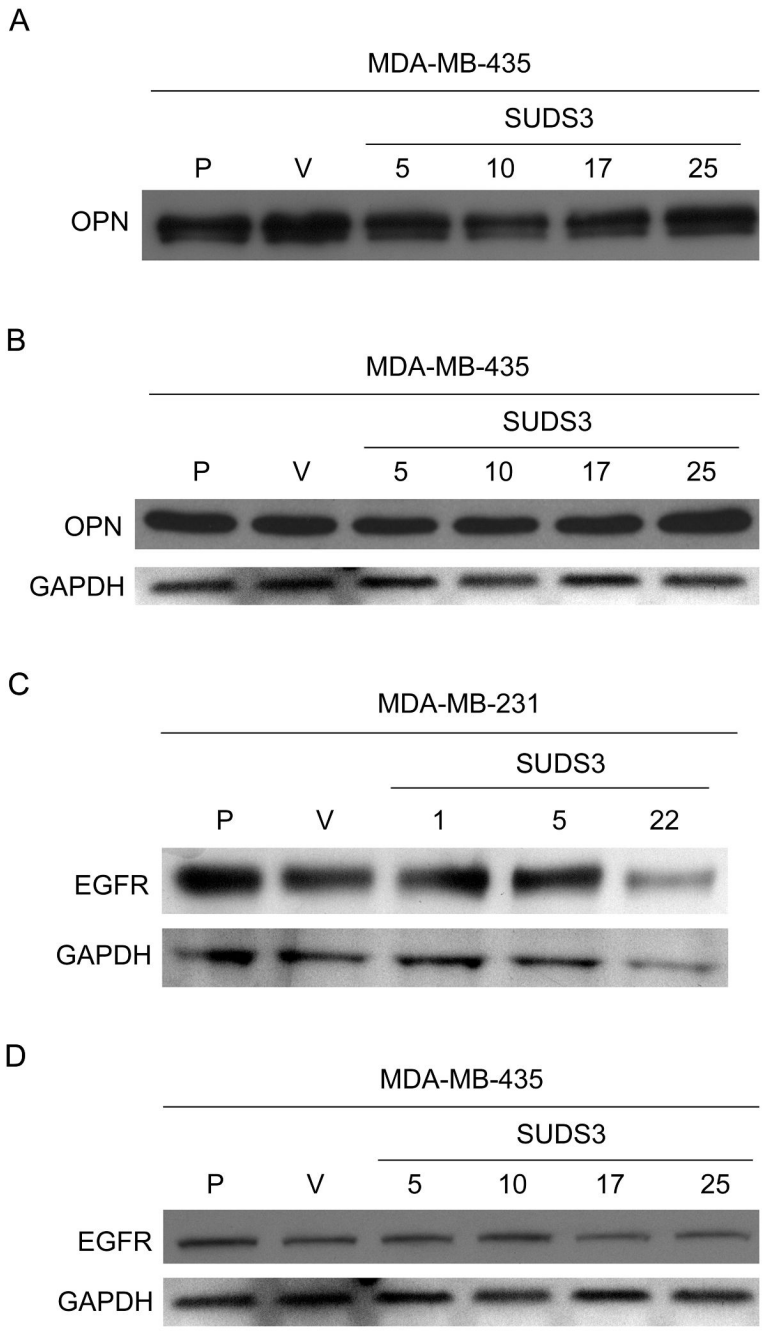


Fig. 3.

SUDS3 does not affect levels of secreted (**A**) or intracellular (**B**) OPN or EGFR in MDA-MB-231 (**C**) or -435 (**A, B, D**) cells. GAPDH was evaluated as a control for equal loading (**B**, **C**, **D**), while secreted OPN measurements were normalized by cell number (**A**).