Deletion of the Proline-Rich Region of the Murine Metastasis Susceptibility Gene Brd4 Promotes Epithelial-to-Mesenchymal Transition- and Stem Cell-Like Conversion

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Abstract

The bromodomain-containing chromatin-modifying factor BRD4 is an inherited susceptibility gene for breast cancer progression and metastasis, but its functionality in these settings has yet to be explored. Here we show that deletion of either of the BRD4 bromodomains had modest effects on the metastatic suppression ability of BRD4. In contrast, expression of the natural short isoform of BRD4 that truncates the protein after the SEED domain restored progression and metastatic capacity. Unexpectedly, deletion of the proline-rich region induced mesenchymal-like conversion and acquisition of cancer stem cell-like properties, which are mediated by the carboxy-terminal P-TEFb binding domain. Deletion of this proline-rich region also induced a gene expression signature that predicted poor outcome in human breast cancer data sets and that overlapped G3 grade human breast tumors. Thus our findings suggest that BRD4 may be altering the predisposition of tumors to undergo conversion to a more de-differentiated or primitive state during metastatic progression. Cancer Res; 71(8); 3121–31. ©2011 AACR.

Introduction

Metastasis is an extremely complex process that remains a major problem in the management of cancer. For breast cancer, the median survival of patients with metastatic cancer is 2–4 years, compared to an approximate survival rate of 80% for women with nonmetastatic tumors (1). The ability to control the spread or effectively treat patients with or at risk of metastatic disease would therefore significantly improve overall outcome of the disease.

Part of the complexity that complicates our understanding of tumor progression is likely due to the highly genetically heterogeneous nature of human populations. Previously, our laboratory showed that the genetic context on which a tumor arises significantly impacts the ability of that tumor to form pulmonary metastases (2). Subsequently, using a systems genetics approach we have identified a number of genes associated with mammary tumor metastatic progression in the mouse and validated their role in human breast cancer gene expression through analysis of publicly available breast cancer data sets (3–6).

One of these genes is the bromodomain containing gene Brd4. Ectopic expression of Brd4 in mice was found to repress primary tumor growth and metastatic capacity. Brd4 activation also induced a gene signature that predicted good outcome in human breast cancer data sets (5). BRD4 has 2 bromodomains within the N-terminus, an ET domain, a serine-rich SEED domain and a proline-rich C-terminal region. BRD4 interacts with acetylated chromatin via its 2 bromodomains (7) and also binds the metastasis modifier SIPA1 through its 2 bromodomains (7) and also binds the metastasis modifier SIPA1 through bromodomain II (8).

Brd4 is expressed normally as 2 alternatively spliced variants. Both isoforms retain the amino half of the long isoform encoded in the carboxy half of the longer isoform is essential for the tumor- and the metastasis-suppressive properties of Brd4. Unexpectedly, the extreme C-terminal domain containing the P-TEFb binding region (9) was found to act as a metastasis enhancer and to induce both epithelial-to-mesenchymal transition (EMT)- and cancer stem cell-like properties without the activation of the canonical pathways associated with these processes. These data suggest that the metastasis susceptibility function mediated by Brd4 may be
the result of a complex series of opposing positive and negative factors that modify the ability of tumor cells to revert to a more de-differentiated state, possibly by a novel mechanism. If true, this implies that inherited metastatic susceptibility may be due in part to polymorphisms lowering the barriers preventing tumor cells from reverting to more primitive transcriptional states.

Materials and Methods

Cell culture and transfection

The Mvt-1 cell line was obtained as a gift from Lalage Wakefield (NCI) in 2003. The cell line was validated as mouse by microsatellite-based PCR and in vivo passage in FVB/NJ mice. The most recent validation by in vivo passage was performed in November 2010. The ΔI, ΔII, and ΔC vectors (8, 10) were cotransfected along with pSuperRetro.Puro (Oligoengine), as a selectable marker for transfectants, using the FuGene 6 transfection reagent (Roche) to develop stable cell lines and clones generated by limiting dilution. Cells expressing Brd4 or β-Galactosidase (β-Gal) were previously described (5).

Lentiviral gene transfer

The SF and STer lentiviral vectors encode the FLAG-Brd4 short isoform gene and the first 699 amino acids (aa) of BRD4, respectively, driven by the mouse Pol2 promoter. The lentiviral particles were produced by transfection of HEK293T cells with 1 μg of SF vector, 750 ng psPAX2 packaging plasmid, and 250 ng pMD2.G envelope plasmid using the FuGene 6 transfection reagent. Forty-eight hours later, the virus-containing media was harvested and filtered through a 0.45 μm filter. Viral particles were then used to infect the Mvt-1 cells. STer viral particles were produced by the Viral Technology Group in NCI, Frederick.

Animal studies

Orthotopic injection was performed by injecting 6-week-old FVB/NJ female mice, into the forth mammary fat pad, with 1 × 10⁶ cells/animal. Primary tumor weight was compared 25–26 days postinjection. Lungs were harvested, sectioned, H&E stained, and metastasis nodules counted.

Tail vein injections were performed by injecting FVB/NJ female mice with 2.5 × 10⁷ cells/animal and lungs processed as described earlier.

All experiments were performed in compliance with the National Cancer Institute’s Animal Care and Use Committee guidelines.

In vitro invasion 3-dimensional assay

The invasiveness of the cells was assayed by culturing the cells in a 3-dimensional (3D) culture as described (5).

Total RNA isolation

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) as described (5). Samples containing high-quality total RNA with A260/A280 ratios between 1.8 and 2.1 were used for further analysis.

Microarray and survival analysis

Microarray expression analysis was performed at LMT, NCI Frederick. Microarray analysis of Brd4 and β-Gal clones was previously described (5). Normalization was performed using Partek Genomics Suite software. For survival analysis BRB array tool software was used.

Quantitative real-time PCR

cDNA was synthesized from RNA isolated from cells using the iScript cDNA synthesis kit (BioRad) per the manufacturer’s protocol. Quantitative real-time PCR was performed using an ABI PRISM 7900HT Sequence Detection System as described (5).

F-actin staining

Cells were seeded in 2-well plates (Nunc) at a density of 1 × 10⁶ cells/well. Twenty-four hours later, cells were fixed with paraformaldehyde-containing sucrose and Triton X-100, then refixed with paraformaldehyde-containing sucrose. Staining for F-actin was carried out by overnight incubation with Alexa-Fluor 488 Phalloidin (Molecular Probes). Cells were subsequently washed and mounted with VECTASHIELD (Vector Laboratories) and examined with Zeiss LSM 510 confocal microscope.

Tumorsphere formation assay

Single cells were seeded onto Ultra-low attachment 24-well plates (Corning) at a density of 3,000 cells/well in serum-free growth media. Pictures were taken 3 and 6 days after seeding using a light microscope.

Flow cytometry

Cells were trypsinized to a single cell suspension, washed with PBS and 1 × 10⁶ cells were resuspended in 100 μL stain buffer (FBS; BD Biosciences). Cells were stained for the pre-conjugated antibodies CD24a-PE and CD49f-FITC or matched isotope controls (BD Biosciences). Cells were then washed and resuspended in PBS and analyzed by FACS (LSR II Fortessa, BD Biosciences). The data were evaluated using FlowJo software.

Results

Brd4 proline-rich C-terminal domain mediates primary tumor growth suppression

To investigate which part of Brd4 is essential for its tumor suppressive property, stable clones of the highly metastatic mouse mammary tumor cell line Mvt-1 (11) expressing full length Brd4 (5), the short isoform (SF), or mutants deleted for the bromodomains (ΔI, ΔII; ref. 8), or for the SEED and proline-rich domains (ΔC; ref. 10) were established (Fig. 1A). These stable cell lines were orthotopically injected into FVB/NJ mice and 25 to 26 days later primary tumor weight was quantified.

Consistent with our previous findings Brd4-ectopic expression reduced primary tumor growth compared to controls [Fig. 1B(a, b)]. Tumorigenicity of ΔII-expressing cells, lacking the binding domain for SIPA1, was also significantly impaired [Fig. 1B(a)], suggesting that the interaction with SIPA1 was not
necessary for suppression of the primary tumor by Brd4. Primary tumor growth was partially restored by deletion of bromodomain I [ΔI; Fig. IB(a)]. In contrast, ΔC and SF clones generated tumors comparable in size to controls [Fig. IB(b, c)], suggesting that primary tumor growth suppression was mediated by the proline-rich domain between the SEED and P-TEFb binding domains. To further confirm this, a lentiviral construct containing the first 699 aa of ΔC but missing the last 83 aa P-TEFb binding domain (STer) was generated (Fig. IA).

Ectopic expression of the ΔC mutant results in an EMT-like phenotype

Unexpectedly, ΔC-ectopic expression profoundly changed cell morphology to a mesenchymal-like phenotype and induced a loss of contact inhibition compared to Brd4 or β-Gal cells that have an epithelial-like appearance (Fig. 2A and Supplementary Fig. S1A). In contrast, expression of SF (Fig. 2A and Supplementary Fig. S1A) or ΔI did not affect cell morphology (Fig. 2A). Expression of ΔII resulted in a more epithelial-like appearance (Fig. 2A) with reduced proliferation in vitro compared to the other cell lines, however this reduction in growth was not statistically significant [Supplementary Fig. S1B(a)]. However, ΔC-, SF-, and

Figure 1. Tumor suppressive property of Brd4 is mediated by the proline-rich domain. A, modular structure of BRD4 and BRD4 deletion mutants. In vivo/in vitro characteristics demonstrated throughout the study are summarized. EMT-like is represented here by cellular morphology. B, a-d, primary tumor weight of FVB/NJ female mice injected with 1 × 10⁵ cells expressing ΔI, ΔII, Brd4 and β-Gal (a), ΔC, Brd4 and β-Gal (b), SF and controls (c), STer and controls (d) 25–26 days after orthotopic injection into the mammary fat pad. Means ± SD, n = 5 for each clone were used. Mann–Whitney test was used to calculate the P value.

Brd4 Mediates EMT- and Stem Cell-Like Conversion

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ΔI-ectopic expression did not change cell proliferation in vitro compared to Brd4 or β-Gal cells [Supplementary Fig. S1B(a, b)].

The ability of the cells to grow in 3D cultures was also determined by performing 3D in vitro invasion assay. Consistent with previous data (5), 3D basement membrane extract cultures showed that Brd4 clones had reduced invasion properties (Fig. 2B). The invasiveness of ΔII clone was comparable to that of Brd4, whereas ΔI and SF clones were also able to proliferate and invade the basement membrane extract compared to β-Gal (Fig. 2B). ΔC clones in contrast were highly invasive and had an increased ability to grow in a 3D culture compared to the other cell lines (Fig. 2B).

Both cell spreading and cell migration are highly dependent on the dynamics of the actin cytoskeleton. Staining the cells with phalloidin showed an organized actin cytoskeleton in only SF, ΔI, ΔII, Brd4, and β-Gal cells (Fig. 2C). A dramatic change in actin organization was observed upon ΔC-ectopic expression, which is characterized by the presence of cortical actin but loss of stress fibers (Fig. 2C), suggesting that while the ΔC-expressing cells had undergone some mesenchymal-like changes, complete EMT was not achieved.
Domain analysis of the deletion mutants suggested that although the proline-rich domain mediates primary tumor growth, the EMT-like phenotype was most likely due to either the loss of the SEED domain or the retention of the 83 aa P-TEFb binding C-terminus. To address this question, STer-expressing cells were further investigated. ST-ectopic expression (Supplementary Fig. S1A) did not change cellular morphology or proliferation and invasion in a 3D culture and did not affect the actin cytoskeleton organization (Fig. 2A–C), compared to controls. These data suggest that the EMT-like changes appear to be mediated by the P-TEFb binding region and not by the SEED or the proline-rich domains.

\( \Delta C \) suppresses metastasis in spontaneous but not experimental metastasis assays

We have previously shown that full length \( \text{Brd4} \) profoundly reduces lung metastasis when subcutaneously implanted into mice (5). To investigate the domains essential for metastasis suppression, pulmonary metastatic lesions from the orthotopic tumors were counted. \( \Delta I \)- and \( \Delta II \)-expressing cells significantly suppressed metastatic burden [Fig. 3A(a)]. \( \Delta I I \) cells also showed reduced metastasis [Fig. 3A(a)], suggesting that the interaction with SIPA1 was not necessary for suppression. SF only partially inhibited metastatic capacity but was still significantly reduced compared to controls [Fig. 3A(b)] whereas STer cells were comparable to controls [Fig. 3A(c)]. Despite the ability of \( \Delta C \) clones to form large primary tumors, these cells formed very few lung metastases compared to \( \beta \)-Gal [Fig. 3A(d)] or \( \text{Brd4} \) cells [Fig. 3A(d)].

To determine whether the \( \Delta C \) metastatic defect was due to inhibition of lung colonization or occurred at an earlier step in the metastatic cascade, experimental metastasis assays were performed. \( \text{Brd4} \) expression reduced the number of the metastatic lesions compared to \( \beta \)-Gal controls [Fig. 3B(a)] whereas SF expression enhanced pulmonary metastasis [Fig. 3B(b)]. STer cells also formed lung metastatic lesions after tail vein injection; however, there was no difference compared to control mice [Fig. 3B(c)]. Unexpectedly, mice injected with \( \Delta C \) cells had a profoundly enhanced number of pulmonary lesions compared to mice injected with cells expressing SF, STer, \( \text{Brd4} \), or \( \beta \)-Gal [Fig. 3B(a)], indicating that the spontaneous metastatic defect in \( \Delta C \) cells was not due to an inability to grow in the secondary site. Furthermore, the in vivo invasive ability of \( \Delta C \) cells was tested to determine whether the

![Figure 3](image-url)

Figure 3. \( \Delta C \) suppresses metastasis from an orthotopic site but not when directly administered into the circulation. A, a–d, quantification of pulmonary metastasis nodules from mice injected orthotopically with clones expressing \( \Delta I \), \( \Delta II \), \( \text{Brd4} \), and \( \beta \)-Gal (a), SF and controls (b), STer and controls (c), and \( \Delta C \), \( \text{Brd4} \), and \( \beta \)-Gal (d) after sectioning and H&E staining of the lungs. B, a–c, quantification of pulmonary metastasis nodules from tail vein injection of mice with 1 clone of \( \Delta C \), \( \text{Brd4} \), or \( \beta \)-Gal (a), 2 clones of SF-expressing cells and control Mvt-1 cells (b), 2 clones of STer, or controls (c) after sectioning and H&E staining. Means ± SD, \( n = 5 \) for each clone were used. Mann–Whitney test was used to calculate the \( P \) value.
inability of ΔC to form spontaneous pulmonary metastases was due to an invasive defect. Histological analysis of the tumors and surrounding tissues showed that both ΔC and β-Gal clones invaded the surrounding tissues (Supplementary Fig. S2B, left) and grew within regional lymph nodes partially replacing lymph node parenchyma (Supplementary Fig. S2B, right), suggesting that the inhibition of ΔC metastasis to the lungs is not due to the inhibition of local invasion.

ΔC and SF induce distinct transcriptional signatures compared to Brd4

Previous studies have suggested that the short isoform opposes the function of the long isoform by competing for the target acetylated histones (12, 13). To assess the effect of SF expression on cellular transcription, Affymetrix-based gene expression analysis was performed. Principal component analysis (PCA) of the microarray data showed that ΔC and SF clones cluster closely to each other, whereas Brd4 clones were more distantly related (Fig. 4A). Hierarchical clustering analysis of the expression profiles revealed that the ΔC and SF expression signatures highly correlated and in general opposed the Brd4 expression profile (Fig. 4B). STer, which has the SEED domain absent, clustered with control cells (Fig. 4A and B). Taken together, these data are consistent with the possibility that SF negatively regulates Brd4 activity and that the SEED domain significantly contributes to SF suppressive activity. In addition, comparison of STer with ΔC suggests that the P-TEFb binding domain also contributed additional functions resulting in a SF-like transcriptional signature.

ΔC and SF induce signatures of poor survival in human breast cancer data sets

Ectopic expression of Brd4 induces a gene signature associated with good prognosis (5). The in vivo results for ΔC and SF would predict that signatures associated with these genes should be predictive of poor outcome. To test this, a human gene expression signature was generated by mapping the most significantly differentially regulated genes obtained by comparing ΔC or SF versus the control cells \( (P < 10^{-9}) \) from the mouse array data to human Affymetrix annotations. The ΔC or SF signatures were then used to cluster patients in publicly available human breast cancer data sets into 2 groups using unsupervised hierarchal clustering and Kaplan–Meier survival analysis performed. ΔC-ectopic expression induced a gene signature that predicted poor outcome in 4 independent human breast cancer data sets (Fig. 5A). Ectopic expression of SF also induced a gene signature that predicted poor outcome in 3 of the 4 data sets, and trending toward significance \( (P = 0.06) \) in the 4th data set (GSE3494; Fig. 5B). Furthermore, the ΔC and SF signatures were found to overlap a signature that defines poorly differentiated and highly malignant G3 grade breast cancers (14; Table 1 and Supplementary Table S1), consistent with the in vitro data, suggesting that ΔC is inducing a less differentiated phenotype in the Mvt-1 cells.

ΔC-ectopic expression modulates the expression of a subset of EMT markers

EMT has been shown to be associated with cancer progression and metastasis (15–18). Given the fact that ΔC-expressing
Figure 5. ΔC and SF microarray expression signatures predict poor outcome in human breast cancer data sets. A and B, Kaplan–Meier curves of 4 independent human breast cancer microarray data sets performed in Affymetrix GeneChips for ΔC-expressing cells or controls (A) and SF-expressing cells or controls (B). The endpoint for the GSE2034 and GSE4922 data sets differ in that disease-free survival was measured. Log-rank test was used to calculate the P value.
### Table 1. Ectopic expression of ΔC in the highly metastatic mouse mammary tumor cell line Mvt-1 up-regulates the expression of genes up-regulated in G3-grade tumors

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*aFold difference between Mvt-1/ΔC and Mvt-1 control cell lines.

Cells have a mesenchymal-like phenotype, the microarray gene expression data of ΔC and β-Gal cells were examined for expression of EMT markers. ΔC-ectopic expression was found to modulate the expression of some previously described EMT markers (Supplementary Table S2). To confirm this, the expression of 6 EMT markers was validated in ΔC and β-Gal cells using real-time PCR (Supplementary Table S3). Fbn1, Vim, and Foxc2 were up-regulated, and Ocn was down-regulated, by ΔC-ectopic expression consistent with an EMT phenotype. However, expression of Snai1 and Twist1 was down-regulated by ΔC-ectopic expression, opposite to what is usually seen by a standard EMT, suggesting that the ΔC cells had not fully undergone EMT.

**ΔC-expressing cells form tumorspheres in nonadherent conditions and regulate a subset of stem cell markers**

Human breast tumors have been found to contain cells that have the ability to undergo anchorage-independent growth in vitro and form spheres (19), akin to behaviors characterizing normal mammary stem cells (20). The EMT process has been shown to modulate the expression of some previously described EMT markers (Supplementary Table S4). Quantitative real-time PCR validated this finding demonstrating that ΔC cells up-regulated Abcb1a and Abcb1b 120- and 180-fold, respectively (Fig. 6B). In contrast, SF cells up-regulated these genes ~2-fold compared to controls (Supplementary Fig. S3B). Furthermore, the expression of Cdc24a stem cell marker was highly expressed in the ΔC-expressing clone compared to Brd4 and β-Gal clones both on the protein and mRNA levels (Fig. 6C). These results are consistent with the acquisition of cancer stem cell-like properties by ectopic expression of ΔC.

**Discussion**

The metastatic cascade is a complex multifactor process that involves both tumor autonomous as well as nonautonomous events. Recent studies have provided an increasing understanding of many of the mechanisms associated with somatic and acquired phenotypes involved in metastatic progression. In contrast, little is known about the molecular mechanisms involved in inherited predisposition to metastasis. This study was therefore designed to gain a better understanding of one of the most significant metastasis susceptibility genes, Brd4, that was identified in our previously described genetic screens (5, 6).

To do so we have taken advantage of a naturally occurring alternative isoform of Brd4 as well as a series of preexisting well-characterized Brd4 deletion mutants (8, 10). Brd4 has 2 alternatively spliced isoforms that differ in the 3’-UTR and the coding region. The shorter isoform retains both chromatin-binding bromodomains but lacks the C-terminal proline-rich and P-TEFb binding domains (9), suggesting that the short
isoform might compete with the longer isoform for binding to acetylated histones (12) and thus interfering with the long isoform function. This is consistent with our in vivo experiments that showed that Brd4 short isoform-ectopic expression does not affect primary tumor growth but enhances metastatic colonization. Furthermore, transcriptional analysis of the short isoform showed that it induced a gene signature indicative of poor survival in breast cancer data sets, consistent with the possibility that the shorter isoform inhibits the longer isoform and that metastatic susceptibility might in part be encoded by a ratio between the 2 isoforms. Further investigations, however, will be required to validate this possibility.

This possibility is further suggested by examination of the alternative 3′-UTRs. Computational predictions suggest that the 3′-UTR of BRD4 short isoform is a potential target of multiple microRNAs including some previously shown to be associated with aggressive breast cancer, such as the mir-200 and the let7 families (22–25). Loss of these miRNAs during tumor progression would result in an increase in transcription of the shorter isoform, consistent with its role in a more aggressive disease. In addition, in rare midline carcinomas BRD4 short isoform is frequently fused to the NUT (nuclear protein in testis) oncogene via an intronic translocation (26–29). Midline carcinomas are highly aggressive tumors with poor prognosis, consistent with the possibility that competitive inhibition of the longer Brd4 isoform would increase the ability of tumors to progress to metastatic disease.

These data also suggest that the C-terminal half of the full length isoform mediates the ability to suppress progression and metastasis. To gain further understanding of the mechanism, deletion analysis was performed. Deletion of bromodomain I had a least suppressive effect on tumor growth. Deletion of bromodomain II however resulted in further suppression of tumor growth and the conversion to a more epithelial morphology. The bromodomain II deletion, in addition to eliminating the chromatin binding domain,
also deletes the binding domain for SIPA1 (8). Previous studies demonstrated that increased levels of SIPA1 were associated with greater malignancy (30) and relocation of BRD4 from the nucleus to the cytoplasm (8). Our results are consistent with the possibility that interaction between BRD4 and SIPA1 contributes to tumor progression, although the mechanism by how this occurs is currently not fully understood.

In contrast, the opposite phenotype was observed during the analysis of the BRD4 C-terminal half. The C-terminus of the long isoform contains only a single defined domain, the P-TEFb binding domain at the extreme C-terminus (9), and regions of high serine and proline content of unknown function. Unexpectedly, introduction of a deletion mutant (ΔC; ref. 10) that deletes the serine-rich SEED and the proline-rich regions but retains the carboxy-terminal 83 aa, including the P-TEFb binding domain, induced significant morphological and physiological changes reminiscent of EMT-like and cancer stem cell-like properties. In contrast, the STer mutant that deletes the entire C-terminus before the SEED domain was not able to induce these phenotypes, suggesting that the 83 aa P-TEFb binding region is the domain responsible for the EMT-like and the stem cell-like conversion.

The EMT process has been implicated as an important intermediate in tumor progression (31, 32) and thought to be required during the later steps of the metastatic cascade (33, 34). Recently it has also been linked to the ability of self-renewal (21). Current thinking suggests that disseminated cancer cells may need to acquire self-renewal properties to achieve for- mation of macroscopic metastases (21). The ΔC data implies that one of the mechanisms by which BRD4 suppresses tumor growth and metastasis is by preventing de-differentiation to a stem cell-like state, mediated by the proline-rich domains. Consistent with this, microarray expression analysis of ΔC-like Brd4 short isoform, converted the Brd4 gene signature from being a predictor of good outcome to being a predictor of poor outcome. It is important to mention here that the cell phenotypes induced by ΔC are not the conventional EMT or stem cell transitions because ΔC cells do not fully exhibit the EMT and the cancer stem cell properties. The expression of genes, thought to be critical for EMT, was not up-regulated in ΔC-expressing cells as normally seen (e.g., Twist, Snail). Furthermore, the stem cell markers Nanog, Oct4, and Sox2 were not detected in ΔC cells, as measured by microarray analysis and quantitative PCR (data not shown). These data suggest that although ΔC-expressing cells have undergone the morpho- logical transformation and some of the molecular changes associated with EMT and stemness, either these transformations are incomplete or the ΔC transformation is a phenocopy of EMT and stemness induced by an alternative mechanism. Further investigations will be required to resolve this. As a result, we have opted to describe the ΔC transformation as EMT-like and stem cell-like until these discrepancies can be resolved.

Experimental metastasis assays further support the role of both Brd4 short isoform and ΔC as metastatic modifiers. ΔC significantly increased the metastatic capacity when intro-
References

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