The Hippo Transducer TAZ Confers Cancer Stem Cell-Related Traits on Breast Cancer Cells

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SUMMARY

Cancer stem cells (CSCs) are proposed to drive tumor initiation and progression. Yet, our understanding of the cellular and molecular mechanisms that underlie CSC properties is limited. Here we show that the activity of TAZ, a transducer of the Hippo pathway, is required to sustain self-renewal and tumor-initiation capacities in breast CSCs. TAZ protein levels and activity are elevated in prospective CSCs and in poorly differentiated human tumors and have prognostic value. Gain of TAZ endows self-renewal capacity to non-CSCs. In epithelial cells, TAZ forms a complex with the cell-polarity determinant Scribble, and loss of Scribble—or induction of the epithelial-mesenchymal transition (EMT)—disrupts the inhibitory association of TAZ with the core Hippo kinases MST and LATS. This study links the CSC concept to the Hippo pathway in breast cancer and reveals a mechanistic basis of the control of Hippo kinases by cell polarity.

INTRODUCTION

Human tumors disproportionally arise in tissues and organs that undergo constant remodeling and regeneration. As these processes are normally maintained by stem cells, this has led to the attractive hypothesis that tumor initiation and progression are also driven by cancer stem cells (CSCs), here defined as the fraction of tumor cells specifically endowed with self-renewal and tumor-seeding potential and ability to spawn non-CSC progeny (Visvader and Lindeman, 2008). The definition of CSCs remains largely operational, i.e., based on functional assays that register self-renewal in vitro and tumor initiation in vivo (Gupta et al., 2009). In fact, we have only a scattered understanding of the molecular and cellular mechanisms that are responsible for the special attributes of CSCs.

In breast cancer, tumor cells can be physically separated in at least two distinct populations, one of which is enriched with CSCs (Visvader and Lindeman, 2008). Malignant progression is accompanied by an increased proportion of these CSCs within the tumor (Pece et al., 2010) and activation of the epithelial-to-mesenchymal transition (EMT) (Chaffer and Weinberg, 2011; Gupta et al., 2009). EMT is a complex transdifferentiation program that is instrumental for the acquisition of stemness by nontransformed and tumor cells (Mani et al., 2008; Thiery et al., 2009). Although EMT is rarely observed in vivo (Chaffer and Weinberg, 2011; Savagner, 2010), one of its central features, namely loss of cell polarity, is a fundamental histopathological trait of most human cancers (Thiery et al., 2009). The mechanistic relationship between EMT, loss of cell polarity, and CSC characteristics remains an open question in cancer biology. Here we report the identification of TAZ, a transcriptional effector of the Hippo signaling cascade (Pan, 2010), as molecular thread between these events.

RESULTS

TAZ/YAP Activity Correlates with High Histological Grade and Metastasis in Breast Cancer

We initiated this study with the aim to identify signaling pathways driving tumorigenesis and tumor heterogeneity in human breast cancer. Importantly, these traits have been traced to the CSC
subpopulation (Pece et al., 2010; Visvader and Lindeman, 2008).
Indeed, CSCs are enriched in high-grade breast cancers (i.e., poorly differentiated tumors classified as G3 by histopathological criteria) when compared to well-differentiated, low-grade (G1) tumors (Pece et al., 2010). We thus based our initial analysis on clinical data and reasoned that if a given signaling cascade is relevant for amplification/activity of CSCs, this pathway should be identifiable by the specific upregulation of its target genes (gene signature) in G3 versus G1 tumors (Figure 1A, left diagram). For this, we generated a compendium of data (metadataset) from seven independent breast cancer gene-expression datasets that contained information on histological grading. This cohort of 993 primary tumors was analyzed by the significance analysis of microarrays (SAM) algorithm to identify a list of genes differentially expressed in G3 versus G1 tumors. This identified 78 Affymetrix probesets specifically upregulated more than...
two times in G3 cancers and that showed 99.9% of statistical confidence for differential expression (Table S1 available online). We then used a computational enrichment analysis to test whether the G3-enriched gene set included a higher-than-randomly-expected representation (expected p value < 0.05) of gene signatures that denote the activity of individual signal transduction pathways (Figure 1A, left diagram). In particular, we screened for associations with signatures registering the activity of ERBB2, RAS, Notch, TGF-β, Wnt/β-catenin, Src, STAT3, NF-kB, and Hippo signaling pathways in human mammary cell lines (See Extended Experimental Procedures). Strikingly, the only signatures significantly over-represented in G3 breast cancers were those denoting the activity of the Hippo transducers TAZ and YAP (Figure 1A). The same result was obtained using an independent dataset from the Netherland Cancer Institute (NKI) (van de Vijver et al., 2002) that was not included in our metadata (Figure S1A). These findings indicate that tumors classified as poorly differentiated/high grade by histopathological criteria display elevated TAZ/YAP activity. As previously reported, G3 breast tumors are also characterized by expression of embryonic and normal mammary stem cell signatures (Ben-Porath et al., 2008; Pece et al., 2010) (Figures 1A and 1B), suggesting that TAZ/YAP activity correlates with stemness potential. To reinforce this link, we turned our attention on the most heterogeneous category of breast cancer, that is, G2 tumors, whose characteristics in terms of gene expression and clinical outcome vary from being similar to G1 to being closer to G3 (Ivshina et al., 2006). We stratified G2 patients from our metadata in two categories according to high or low expression levels of TAZ/YAP target genes and found that G2 tumors with high TAZ/YAP activity are associated with an enrichment of stem cell signatures (Figure 1B).

Because metastasis is intimately wired to tumor-initiating capacity of a primary tumor (Chaffer and Weinberg, 2011), we tested whether TAZ/YAP activity was associated to this event in human patients’ datasets. As shown by univariate Kaplan-Meier analyses, tumors with high expression of the TAZ/YAP signature displayed a significantly higher probability to develop metastasis and reduced survival compared to tumors characterized by low TAZ/YAP activity (Figures 1C and 1B). Taken together, the data support the notion that activation of TAZ or YAP specifically parallels tumor traits previously linked to their CSC content, such as high histological grade, expression of stem cell signatures, tumor heterogeneity, and metastasis.

**TAZ Is Required to Sustain Self-Renewal and Tumorigenic Potential of Breast Cancer Cells**

We next sought to determine whether TAZ/YAP activity plays a causal role in defining CSC traits. As a model of tumor progression, we used two isogenic derivatives of the human mammary MCF10A cell line: Ras-transformed MCF10A-T1k cells (hereafter MII) and their malignant derivative, MCF10A-Ca1a cells (also known as MIV), derived from in vivo spontaneous evolution of MII cells (Santer et al., 2001). Once injected orthotopically in the fat pad of immunocompromized mice, MII cells generate low-grade tumors, whereas MIV cells form high-grade tumors, resembling G3 tumors (Figure 2A).

To validate MII and MIV as a model of increased CSC content during breast cancer progression (Pece et al., 2010), we compared their self-renewal potential, as assayed by the capacity to form and propagate mammospheres in vitro and to give rise to tumors in vivo when transplanted in the mouse fat pad (Dontu et al., 2003; Visvader and Lindeman, 2008). Once cultured in suspension, MIV cells formed more primary and six times more secondary mammospheres than MII cells (Figure 2B). The sphere-forming capacity in subsequent serial passages remained stable for MIV cells but declined for MII cells (Figure 2B and data not shown). In order to measure tumorigenicity, we injected orthotopically 10⁵ and 10⁶ MII or MIV cells. Although both concentrations of MIV cells readily formed tumors in all injected mice, MII-derived tumors were detected only in one-third of mice injected with 10⁶ cells but not at the lower cell concentration (Figure S2A). Thus, MII and MIV cells are endowed with significantly different self-renewal and tumorigenic potential, recapitulating the differences in CSC representation previously reported for G1 versus G3 tumors (Pece et al., 2010).

We then asked whether the differences in these CSC-related properties could be ascribed to differential expression of TAZ and/or YAP between MII and MIV cells. Intriguingly, western blot analyses showed that TAZ levels are higher in MIV than in MII cells, whereas YAP is expressed similarly in both cell lines (Figure 2C). Moreover, levels of TAZ in MIV cells were comparable to those in MDA-MB-231 cells (Figure 2C), one of the most established model systems of aggressive breast cancer (Adorno et al., 2009).

To directly address the role of TAZ as a determinant of self-renewal, we knocked down endogenous TAZ in MIV cells by shRNA. TAZ knockdown strongly downregulated the expression of Survivin and CTGF, known TAZ/YAP target genes (Pan, 2010) (Figure 2D), but had no overt effects on cell viability and proliferation in 2D cultures (data not shown). Remarkably, however, MIV-shTAZ formed significantly less primary and secondary mammospheres than control MIV cells (MIV-shControl) (Figure 2E). We also noticed that the sizes of the mammospheres in control and TAZ-depleted populations were surprisingly similar (Figure 2F), suggesting that high levels of endogenous TAZ in MIV cells do not primarily influence cell proliferation during mammosphere growth. To confirm that these observations are not due to off-target effects of the RNAi approach, we coinfected MIV-shTAZ with an shRNA-insensitive mouse TAZ construct (MIV-shTAZ+mTAZ); this fully rescued TAZ-dependent gene responses and mammosphere-forming capacity (Figures S2B and S2C).

To assay whether TAZ regulates tumor-initiating potential of breast cancer cells, we compared MIV-shControl and MIV-shTAZ cells for capacity to seed tumors at limiting dilutions. Whereas shControl cells are able to form tumors in the majority of injected mice, even upon injection of only 20 cells, TAZ-depleted cells showed a dramatic decrease of tumor-seeding ability as up to 10⁴ cells were required to induce tumors in 100% of the animals (Figures 2G and S2D). Based on these data, it is possible to estimate that depletion of TAZ causes about a 20-fold reduction in the number of tumor-initiating cells (p value < 0.0001). Taken together, these in vitro and in vivo
results indicate that TAZ is required for self-renewal and tumor initiation of breast cancer cells.

**TAZ Endows Self-Renewal Capacity to Breast Cancer Cells**

We next sought to investigate the role of TAZ as a potential driving force for self-renewal in a pool of CSCs naturally arising within breast cancer cells. To test this, we used fluorescence-activated cell sorting (FACS) to sort MII cells according to expression of the cell-surface antigen markers CD44 and CD24; this approach has been successfully used to identify putative CSCs contained in primary breast tumors or mammary cell lines based on a CD44^{high}/CD24^{low} immunophenotypical profile, whereas the excluded cells represent a more differentiated tumor progeny of limited stemness potential (Al-Hajj et al., 2003). As shown in Figures 3A and 3B, FACS analysis readily revealed a CD44^{high}/CD24^{low} subpopulation that displayed higher levels of TAZ protein and transcriptional activity once compared to the remaining cell population (CD44^{low}/CD24^{low}). Moreover, the CD44^{high}/CD24^{low} cells display many CSC-like properties: (1) the CD44^{high}/CD24^{low} cells can regenerate the CD44^{low}/CD24^{low} population but not vice versa, suggesting a lineage hierarchy between these immunologically distinct populations (Figures S3A–S3C); (2) CD44^{high}/CD24^{low} cells are endowed with better long-term self-renewal capacity as measured by mammosphere assays (Figure 3C); (3) CD44^{high}/CD24^{low} cells include the so-called “side population (SP)” (Figure S3D), which is defined by its ability to exclude the Hoechst vital dye (Dontu et al., 2003). Collectively, these data provide evidence that cell populations endowed with CSC properties express higher levels of TAZ.

To establish whether TAZ is functionally relevant for the biological traits of the CD44^{high}/CD24^{low} population, we used three independent shRNAs to knock down endogenous TAZ in MII cells (Figure S3E). TAZ is required for the maintenance of the CD44^{high}/CD24^{low} antigen phenotype (Figure 3D) and for primary and secondary mammosphere formation in MII cells (Figure 3E).
These findings suggest that TAZ expression is important to maintain self-renewal of prospective CSCs. We next tested whether raising TAZ activity could establish self-renewal properties. To this end, we overexpressed in MII cells a constitutive active point mutant of TAZ, TAZ(S89A) (Pan, 2010), and confirmed that it induced the expression of TAZ target genes (Figure S4A). By flow cytometry, expression of TAZ(S89A) induced an en masse enlargement of the CD44<sup>high</sup>/CD24<sup>low</sup> population (Figures 4A and S4B), resembling MIV and MDA-MB-231 cells (Figure 4B). Functionally, MII-TAZ(S89A) formed more primary, secondary, and tertiary mammospheres than control cells (Figure 4C). Of note, this was not accompanied by increased proliferation, as the sizes of mammospheres were similar in control and TAZ(S89A)-expressing cells (Figure 4D).

To directly show that activated TAZ is sufficient to endow CSC-like properties to non-CSCs, MII cells were transduced with expression vectors encoding for a doxycycline-inducible TAZ(S89A) or enhanced green fluorescent protein (EGFP) as control. Transduced cells were FACSc-sorted into CD44<sup>high</sup>/CD24<sup>low</sup> and CD44<sup>low</sup>/CD24<sup>low</sup> populations, cultured in the presence or absence of doxycycline, and assayed for mammosphere formation (see diagram in Figure 4E). CD44<sup>high</sup>/CD24<sup>low</sup> cells increased their self-renewal potential after activation of TAZ expression (Figure 4F, compare lane 6 with 8). More remarkably, TAZ(S89A) promoted abundant secondary mammosphere formation in the CD44<sup>low</sup>/CD24<sup>low</sup> population, making it indistinguishable from the CD44<sup>high</sup>/CD24<sup>low</sup> cell population (Figure 4F, compare lanes 5, 7, and 8). This effect was specific, as a similar shift was not observed in doxycycline-treated MII-EGFP cells. These findings suggest that TAZ activity can confer attributes of self-renewal to the more differentiated progeny of prospective CSC populations.

In addition to self-renewal, another characteristic of CSCs is their capacity to resist chemotherapy (Dean et al., 2005). We noticed that TAZ overexpression in MII cells increased the activity of multidrug resistance (MDR) proteins, as revealed by the 4-fold increase of the SP (Figures 4G and S4C). Consistently, TAZ-expressing cells were more resistant than control cells to two widely used chemotherapeutic drugs, Doxorubicin and Paclitaxel (Figures 4H and S4D). The latter result is consistent with the recently reported dependency of MDA-MB-231 cells on TAZ for Taxol resistance (Lai et al., 2011).

**TAZ Promotes the Formation of High-Grade Tumors**

Data presented so far indicate that raised levels of TAZ are instrumental for several phenotypic traits so far associated with the behavior of prospective CSC populations in vitro. Because in vivo CSCs are defined operationally by their ability to seed new tumors at limiting dilutions (Visvader and Lindeman, 2008), we determined whether TAZ activation promotes the
Figure 4. TAZ Endows Self-Renewal Capacity to Breast Cancer Cells

(A) Representative FACS profiles with CD24 and CD44 markers of MII cells transduced with control (empty vector) or TAZ(S89A)-encoding retroviral vectors. See Figure S4B for quantifications.

(B) Representative FACS profiles of MIV and MDA-MB-231 cells with CD24 and CD44 markers. In contrast to parental MII cells, only the CD44(high)/CD24(low) cell population is discernible after FACS in these cell lines.

(C and D) Effects of stable TAZ activation on the mammosphere-forming abilities of MII cells. (C) Quantification of primary, secondary, and tertiary mammospheres formed by MII-empty vector or MII-TAZ(S89A) cells (mean + SEM of six experiments). (D) Representative images of primary mammospheres. Scale bars represent 200 μm.

(E and F) Forced expression of TAZ(S89A) enhances mammosphere formation in the CD44(low)/CD24(low) cell population. (E) Schematic representation of the experiments. Cells were transduced with vectors encoding for the reverse tetracycline-dependent transactivator (rTA) and for a doxycycline-inducible TAZ(S89A) or EGFP. Doxycycline was used at 0.5 μg/ml. (F) Quantification of secondary mammospheres formed by the eight cell groups shown in (E). Data are presented as in (C).

(G and H) TAZ confers partial drug resistance. (G) Quantification of the SP (Hoechst-low) in control or TAZ(S89A)-expressing MII cells. We used this assay because chemoresistance is linked to increased activity of MDR proteins that are also responsible for the Hoechst exclusion phenomena. To ensure MDR involvement, MII-TAZ(S89A) cells were pretreated with the MDR1 inhibitor Verapamil. Data are reported as mean + SEM. See also Figure S4C for FACS data. (H) Dose-response curves of MII-empty vector and MII-TAZ(S89A) cells treated with Paclitaxel. Bars denote the standard error (n = 4).

See also Figure S4.
formation of tumor-initiating cells. As shown in Figures 5A and S5A, palpable tumor masses developed in mice injected with \( >3 \times 10^5 \) control or MII-TAZ(S89A) cells, although at a considerably higher frequency in the TAZ-expressing cells; in contrast, only MII-TAZ(S89A) cells formed tumors when as few as \( 10^4 \) cells were injected. Interestingly, this increase in

Figure 5. TAZ Promotes the Formation of High-Grade Tumors and Is Overexpressed in G3 Primary Human Tumors

(A) Tumor-seeding ability of MII-empty vector and MII-TAZ(S89A) cells. The indicated number of cells were injected into the fat pads of RAG-/- mice. Results are shown as the fraction of mice developing palpable tumors 5 weeks after transplantation. The complete set of data is in Figure S5A.

(B and C) Representative hematoxylin and eosin staining of tumors emerging from fat-pad injection of MII-empty vector (B) and MII-TAZ(S89A) cells (C).

(D) Frequency of TAZ-positive G1 or G3 primary human breast cancers as judged by immunohistochemistry (IHC). Tumors were scored as positive when over 10% of cells displayed nuclear TAZ staining similar to or stronger than that of the cells of normal ducts included in the same section. 35/44 G3 tumors and only 1/26 G1 tumor were found positive for TAZ expression (p value < 0.0001).

(E and F) Representative IHC pictures for TAZ expression in G1 (E) or G3 (F) invasive human breast cancer samples. Nuclei are counterstained with hematoxylin. Black arrowheads in (E) point to the TAZ-positive cells of the normal mammary ductal tissue (NT) included in the same section with G1 tumor structures (G1). Inset in (E) shows (white arrowhead) very rare TAZ-positive cells that are seldom associated with G1 tumors. Further pictures of TAZ staining of G1 and G3 tumors are shown in Figures S5C and S5D.

(G) Frequency of amplification of the TAZ locus, WWTR1, in breast cancers from the EMC dataset stratified according to the TAZ/YAP signature.

(H) Box-and-whisker plots comparing TAZ mRNA expression between primary breast tumors from the EMC dataset with or without amplifications in the TAZ-encoding locus.

See also Figure S5.
Figure 6. TAZ Is Required downstream of EMT for Self-Renewal of Breast Cancer Cells

(A and B) The CD44high/CD24low population of MII cells display EMT features. (A) Immunofluorescence (IF) images for the epithelial marker E-Cadherin and the mesenchymal marker Vimentin in FACS-sorted CD44low/CD24low and CD44high/CD24low populations of MII cells. Nuclei are stained with Hoechst. (B) Western blot analysis for E-Cadherin and TAZ expression in the same cell populations described in (A).

(C) Representative IF pictures of E-Cadherin in the indicated MII cell derivatives.

(D) Quantification of primary and secondary mammospheres formed by the indicated cells (mean of six replica experiments + SEM).
tumor-initiation capacity was accompanied by increased size and histological grade of the tumors. Control MII cells formed small tubular carcinomas displaying no nuclear atypia, mostly resembling human G1 tumors (Figure 5B). Conversely, the MII-TAZ(S89A) cells formed invasive carcinomas that phenocopied several traits of human G3 breast cancers, such as lack of tubular structures, high nuclear pleomorphism, and prominent nucleoli (Figure 5C and data not shown). Thus, TAZ promotes tumorigenic potential and the acquisition of a more malignant and less differentiated phenotype.

**TAZ Protein Is Overexpressed in High-Grade Breast Cancers**

Because TAZ protein levels correlate with tumorigenic potential in our cellular model systems, we next used immunohistochemistry to examine TAZ expression in human primary tumor samples. We compared 26 G1 and 44 G3 invasive ductal carcinomas. In G1 tumors, TAZ expression was typically hardly detectable (Figures 5D, 5E, and S5B), with strong staining appearing only on rare individual cells (Figure 5E, inset). In contrast, the vast majority of G3 tumors (80%) contained a substantial fraction of cells displaying an intense TAZ nuclear staining (Figures 5D, 5F, and S5C). The frequency of TAZ-positive tumors in this analysis recapitulates the frequency of G3 tumors with high TAZ/YAP activity as assayed by TAZ/YAP target gene expression in patients’ datasets (82%, data not shown). Thus, G3 breast cancers are enriched with TAZ-expressing cells, a finding that links the increased CSC content of high-grade tumors (Pece et al., 2010) with the present functional characterization of TAZ as a promoter of CSC-like traits.

**The TAZ-Encoding Locus Is Amplified in a Fraction of Breast Cancers**

We next wondered about the mechanisms leading to increased TAZ expression in breast cancer. First, by analyzing the EMC dataset that contains combined clinical, gene-expression, and comparative genomic hybridization (CGH) data, we investigated whether the TAZ-encoding locus WWTR1 is amplified in primary tumors (Zhang et al., 2009). Notably, 27 out of 313 tumors exhibit evidence of amplification at the WWTR1 locus, the majority of which (22) belong to the group showing high TAZ/YAP activity (Figure 5G). An essential criterion for establishing whether an amplified gene might contribute to tumorigenesis is that its mRNA has to be overexpressed in the tumors where it is amplified. In agreement with this prediction, we found that TAZ mRNA levels are specifically increased in tumors with amplified WWTR1 (Figure 5H).

Although amplification provides the proof-of-principle for a biological selective mechanism leading to TAZ overexpression, the relatively low frequency of this event (14% of G3 tumors) contrasts with the previous immunohistochemical and bioinformatic analyses (Figures 1B and 5D–5F), indicating increased TAZ protein levels and activity in at least 80% of G3 tumors. This indicates that other mechanisms must be in place to elevate TAZ levels during tumor progression in the larger fraction of breast cancers.

**EMT Promotes Self-Renewal through TAZ**

The biological activity of TAZ in breast cancer cells presents intriguing analogies with the cellular traits acquired after an EMT (Thiery et al., 2009): both TAZ and EMT promote the formation of cell populations enriched with CSCs, as assayed by tumor-seeding ability, mammosphere formation, and expression of cell-surface markers (Mani et al., 2008 and this study). EMT and TAZ activity are linked: we found that cells displaying high levels of endogenous TAZ protein, such as the CD44high/CD24low fraction of MII or MIV and MDA-MB-231 cells, also display loss of junctional E-Cadherin (Figures 6A, 6B, and S6A), which is a hallmark of the EMT process (Thiery et al., 2009). Moreover, as shown previously—and repeated here—overexpression of TAZ can trigger EMT (Chan et al., 2008; Lei et al., 2008 (Figure 6C). We thus asked whether a cardinal feature of CSCs, i.e., self-renewal potential, is induced by TAZ because TAZ induces EMT or, rather, if TAZ is downstream of EMT and serves as a molecular mediator of its pro-stemness effects. For this, we overexpressed E-Cadherin in MII-TAZ(S89A) cells (Figure 6C), but this had no effect on the generation of primary and secondary mammospheres from these cells (Figure 6D). This suggested that TAZ promotes self-renewal of breast cancer cells independently of loss of junctional E-Cadherin.

Next, we tested whether TAZ is required for the EMT phenotype and for EMT-induced self-renewal. For this, we used MDA-MB-231 cells that displayed an overt mesenchymal phenotype and expressed high levels of endogenous TAZ (Figure 2C). As shown in Figures 6E–6G, loss of TAZ dramatically affected TAZ target gene expression as well as primary and secondary mammosphere formation from MDA-MB-231 cells but did not change their mesenchymal morphology or induce E-Cadherin expression.

To extend our study to nontransformed cells, we used immortalized human mammary epithelial cells (HMLEs) and induced EMT by ectopic overexpression of the transcription factor Twist (Mani et al., 2008) (Figure 6H). As previously reported, HMLE-Twist cells displayed a >5-fold increased mammosphere-forming capacity than empty vector control cells (Figure 6I) (see Mani et al., 2008). Interestingly, TAZ protein levels, but not mRNA levels, were also significantly upregulated in HMLE-Twist cells, leading to TAZ-dependent induction of
Figure 7. Scribble Delocalization Activates TAZ by Inhibiting the Hippo Pathway

(A) Western blot analysis for Scribble, TAZ, CTGF, and E-Cadherin expression in MII cells transfected with control or three independent anti-Scribble siRNAs. Scribble loss increases TAZ protein levels and activity without affecting E-Cadherin expression.

(B) Western blot for the indicated proteins in lysates of MII-empty vector and MII-Snail cells transfected with control or Scribble siRNA#3 (SCRIB siRNA).

(C) Confocal images showing localization of Scribble in control or Snail-expressing MII cells. For each cell line, panels show XY images (top) and the indicated (red line) Z stacks (bottom). White arrowheads point to lateral membrane domains. Nuclei are stained with propidium iodide (PI).

(D) Western blot analysis for Scribble, TAZ, CTGF, and Survivin expression in lysates of MII cells transduced with a retroviral vector coding for ScribP305L. Expression of ScribP305L causes an increase of total Scribble levels and the displacement of this protein from the lateral plasma membrane (see Figure S7E).

(E) Scheme of the proposed epistatic relationships between EMT, Scribble, TAZ, and self-renewal.

(F) Quantification of secondary mammospheres formed by control or ScribP305L-expressing MII cells. Data are mean of six replica experiments + SEM.

(G) CoIP/western blot analysis of MII cell lysates showing endogenous TAZ bound to Scribble, MST2, and LATS1. As a specificity control, immunoprecipitation was repeated with unrelated mouse IgG.
CTGF, Survivin, and PAI-1 (Figures 6H and S6B). We confirmed that TAZ can be induced by EMT also in MII cells ectopically expressing Snail (Figure S6C). Crucially, in HMLE-Twist cells, TAZ knockdown significantly reduced the Twist-induced mammosphere-forming ability (Figure 6I) but did not alter the Twist-induced mesenchymal phenotype (Figure 6H and data not shown).

We conclude from these experiments that levels of endogenous TAZ protein increase during EMT and that TAZ is a relevant mediator of the mammosphere-forming capacity promoted by EMT in both nontumorigenic and transformed epithelial cells.

Scribble Delocalization Activates TAZ

Although full EMT is rare in human tumors, most carcinomas show evidence of a partial EMT (Savagner, 2010). As such, it appears that cancer cells exploit only the onset of the EMT program without the need to reach a completely mesenchymal state. This partial EMT invariably entails loss of apicobasal polarity and destabilization of junctional complexes (Thiery et al., 2009). A key gatekeeper of epithelial polarity is Scribble, which establishes the basolateral domain of the cell membrane (Macara, 2004). Scribble is frequently disabled during mammary tumorigenesis and also serves as a tumor suppressor in Drosophila (Zhan et al., 2008; Humbert et al., 2008). Interestingly, in fly tissues, the overgrowth of scribble mutant clones is dependent on yorkie, the TAZ Drosophila ortholog (Grzeschik et al., 2010); yet, the molecular mechanisms of this genetic interaction remain unknown.

With this background in mind, we tested whether Scribble is a relevant inhibitor of TAZ in breast cancer cells. As shown in Figure 7A, Scribble knockdown in MII cells resulted in robust upregulation of TAZ protein levels but not mRNA levels and did not affect epithelial morphology (Figures S7A and S7B). Notably, Scribble regulates TAZ in epithelial cells but not after Snail-induced EMT (Figure 7B); this reinforces the notion that Scribble inactivation is part of the EMT program necessary for TAZ upregulation.

We next wondered what the mechanism is by which EMT inhibits Scribble. EMT did not affect Scribble protein levels (Figure S7C) but dramatically changed its subcellular localization: in MII and HMLE cells, Scribble is localized to the basolateral membrane, but after expression of Snail or Twist, it is excluded from this domain and becomes predominantly cytoplasmic (Figures 7C and S7D). This raised the possibility that the delocalization of Scribble could be sufficient for TAZ induction in breast cancer. To test this, MII cells were transduced with a mutant version of Scribble (ScribP305L) that is not localized to the cell membrane and even disrupts membrane recruitment of endogenous Scribble (Zhan et al., 2008) (Figure S7E). We found that ScribP305L induced TAZ protein levels and activity (Figure 7D). Conversely, overexpression of a membrane-tethered version of Scribble, but not of wild-type Scribble, strongly inhibited TAZ expression and activity in MII-Snail cells (Figures S7F and S7G).

The data thus suggest a scenario whereby EMT leads to Scribble delocalization from the cell membrane and, in so doing, relieves TAZ from Scribble inhibition to promote CSC-related traits (Figure 7E). In agreement with this scenario, expression of ScribP305L increased the self-renewal potency of MII cells, as assayed by second-generation mammosphere formation (Figure 7F). Moreover, the CD44high/CD24low subpopulation of MII cells—enriched with CSC-like cells—is also specifically characterized by loss of junctional Scribble, correlating with their elevated levels of endogenous TAZ (Figure S7H). This was also verified in MIV and MDA-MB-231 tumor cells (Figure S7I).

Scribble Regulates the Hippo Pathway in Breast Cancer Cells

TAZ is the downstream effector of the Hippo pathway. This signaling cascade is controlled by two evolutionarily conserved kinases, MST1/2 and LATS1/2. MST1/2 (the homologs of Drosophila Hippo) phosphorylate LATS1/2 that, in turn, phosphorylate and thereby inhibit TAZ and YAP. Loss of this negative control stabilizes TAZ and promotes its nuclear activity (Pan, 2010). However, the biochemical mechanisms by which the Hippo signal transduction pathway is regulated upstream of MST remain poorly understood. Our findings on TAZ inhibition by Scribble raised the attractive possibility that Scribble functions as an upstream regulator of Hippo signaling in breast cancer cells. Remarkably, in coimmunoprecipitation (coIP) assays of endogenous proteins, we found that TAZ forms a biochemical complex with MST, LATS, and Scribble itself in both MII and HMLE cells (Figures 7G and S7J).

We thus asked whether Scribble is required for the recruitment of MST and/or LATS to TAZ. Interestingly, the interaction between TAZ and LATS was only weakened in colP from Scribble-depleted cells; in contrast, Scribble was required for the recruitment of MST2 to the TAZ/LATS complex (Figure 7H, compare lanes 1 and 2; see also Figures S7J and S7K). Accordingly, in TAZ immunoprecipitates, MST-mediated phosphorylation of LATS on S909—and LATS-mediated phosphorylation of TAZ—were lost upon Scribble knockdown. Thus, Scribble is required for the assembly of a complex between TAZ, LATS, and MST and for MST-dependent activation of the LATS kinase. In line with the notion that EMT regulates this pathway, induction of EMT in MII and HMLE cells blocked TAZ association to Scribble and recapitulated the biochemical consequences of Scribble inactivation (Figure 7H, lane 3; see also Figures S7J and S7K).

(A) Western blot analysis of TAZ immunocomplexes purified from control, SCRIB siRNA#3-transfected, or Snail-MII cells. See Figure S7K for inputs of these CoIPs.
(B) Scribble depletion and EMT disable TAZ interaction with β-TrCP as verified by western blot analysis on TAZ immunocomplexes described in (G).
(C) Western blots for the indicated proteins in lysates from MII cells expressing wild-type mTAZ or TAZS89/306A, transfected with control (Control siRNA) or SCRIB siRNA#3 (SCRIB siRNA).
(D) A model depicting the proposed mechanism for TAZ regulation by Scribble and the Hippo pathway. See also Figure S7.
It is known that TAZ phosphorylation by LATS on S306 promotes its degradation through association to the β-TrCP E3 ubiquitin-ligase complex (Liu et al., 2010) (Figure 7L). We found that TAZ interacted with β-TrCP at the endogenous level in MLL cells (Figure 7I, lane 1). Crucially, Scribble knockdown or EMT induction impaired the formation of such a TAZ/β-TrCP complex (Figure 7I, lanes 2 and 3). Furthermore, LATS-mediated phosphorylation is essential for TAZ regulation by Scribble, as a mutant TAZ missing LATS phosphorylation sites (S89 and S306) is insensitive to loss of Scribble (Figure 7J). Thus, in epithelial cancer cells, Scribble is an upstream activator of the Hippo pathway leading to TAZ inhibition. Scribble inactivation upon EMT allows TAZ to escape this inhibition and accumulate in stem-like progenitors resulting in more aggressive tumors (see diagram in Figure 7K).

DISCUSSION

TAZ Is a Molecular Determinant of Biological Properties Associated with Breast CSCs

We found that TAZ protein levels and TAZ biological activities go hand-in-hand with some salient traits that have been associated with CSCs. The following evidence supports this conclusion: (1) the molecular imprints of TAZ/YAP activity are tightly linked with progression from well-differentiated to high-grade tumors, during which the CSC content has been shown to increase (Pece et al., 2010); indeed, low-grade G1 tumors have low numbers of TAZ-positive cells that greatly increase in G3 tumors; (2) TAZ protein levels are specifically enriched in prospective CSCs purified by cell sorting from genetically identical heterogeneous cell populations; (3) in human tumors, TAZ activity is associated with molecular signatures of stem cells; (4) TAZ activity is a clinically relevant tool to predict the proclivity to develop metastasis, itself another hallmark of CSC activity.

At the functional level, we show that endogenous TAZ is required for self-renewal and tumor-initiation properties in breast cancer cells, and that raising TAZ levels promotes these CSC-related traits. Increased TAZ activity is sufficient to convert otherwise benign experimental tumors into a more aggressive G3-like histopathological phenotype. Thus, TAZ expression levels and activity embody three characteristics that have traditionally been linked to CSCs in breast tumors, i.e., tumor heterogeneity, reduced differentiation, and tumor-seeding potential.

One unsettled issue in the CSC field is whether the potential of self-renewal is an exclusive property of CSCs, or whether plasticity exists in these hierarchical lineages, such that any cell, even a non-CSC, could acquire self-renewal potential under appropriate intrinsic or extrinsic cues (Chaffer and Weinberg, 2011; Gupta et al., 2009). Interestingly, it has been recently demonstrated that non-stem mammary epithelial cells could spontaneously revert to a stem-like state in a seemingly stochastic manner (Chaffer et al., 2011). The data presented here show that a molecular player could induce a similar type of transition: TAZ activation in non-CSCs could reactivate their self-renewal potential, suggesting that TAZ levels may regulate phenotypic plasticity in breast cancer.

TAZ Activity Is Promoted by EMT

Experimentally induced EMT instills stem-like properties to mammary epithelial cells. As such, EMT has been evoked as the culprit for tumor progression and tumor heterogeneity (Chaffer and Weinberg, 2011; Mani et al., 2008; Thiery et al., 2009). We found that TAZ is turned on by overexpression of the EMT-inducing transcription factors Twist or Snail and is required for self-renewal induced by Twist. Others have shown that TAZ itself can sustain EMT (Chan et al., 2008; Lei et al., 2008); however, we found that acquisition of mesenchymal traits can be uncoupled from TAZ-induced CSC-like properties. Indeed, attenuation of endogenous TAZ has no effect on Twist-induced mesenchymal transition of nontransformed mammary cells or on the intrinsic mesenchymal phenotypes of malignant cells. We thus envision TAZ-induced EMT as an embedded self-sustaining mechanism of TAZ activity. Importantly, neither stabilization of TAZ protein levels nor induction of self-renewal require the entire EMT program. In fact, only a specific segment of EMT, the loss or delocalization of the cell-polarity determinant Scribble, is necessary, with loss of Scribble increasing TAZ activity and CSC properties without inducing overt mesenchymalization. Conversely, adding back a membrane-tethered version of Scribble in post-EMT/Snail-expressing cells is sufficient to destabilize TAZ without modifying their mesenchymal phenotype (data not shown). These findings also offer a plausible explanation for a conundrum regarding the role of EMT in high-grade malignancies, as transition of epithelial cells toward a full mesenchymal cell fate is rare in cancers (Chaffer and Weinberg, 2011; Savagner, 2010). However, if we consider EMT “only” in terms of loss of apicobasal polarity that results in TAZ stabilization, then this conundrum would be resolved because disruption of cell polarity and tissue organization is a fundamental trait present in the majority of human tumors. Although intriguing, a more definitive demonstration of this possibility must await dedicated in vivo studies.

It is worth considering what other signals may drive TAZ activity in addition to EMT. For example, structural/physical properties of the tumor microenvironment are just as relevant as soluble cues in driving progression (Butcher et al., 2009). Indeed, we recently discovered a central role for TAZ/YAP in mechanotransduction (Dupont et al., 2011), raising the interesting possibility that TAZ may be locally activated at specific “biomechanical niches” within tumors.

A Mechanism that Links Cell Polarity to Hippo Signaling

TAZ and YAP are central mediators of the Hippo pathway, which regulates organ size from Drosophila to mammals (Pan, 2010). How this pathway responds to extracellular signals remains unclear. Further evidence points to cell adhesion and polarity proteins as likely candidates of Hippo pathway activation, but the underlying biochemical mechanisms remain an unsolved mystery. In the present study, we found that in nontransformed and tumorigenic mammary epithelia, the Hippo cascade is activated by Scribble at the cell-cell contact. Scribble cascade is activated by Scribble at the cell-cell contact. Scribble is an adaptor to assemble a protein complex with TAZ, LATS, and MST and is required for MST-dependent activation of LATS and ultimately TAZ phosphorylation (Figure 7K). Indeed, Scribble is frequently delocalized from the cell membranes in breast
cancer (Zhan et al., 2008), and we now show that mimicking this subcellular relocalization event is sufficient to allow TAZ to escape LATS inhibition.

In addition to the basolateral Scribble complex, the establishment of epithelial apicalbasal polarity is mediated by the apical Crumbs and Par/aPKC complexes. Apical and basolateral complexes mutually regulate each other, leading to the segregation of the apical and basolateral membranes (Humbert et al., 2008; Macara, 2004). We suggest that basolateral polarity controlled by the Scribble complex is the primary polarity signal that activates the Hippo cascade in mammary epithelia. This offers a remarkably simple mechanism by which cell polarity can control cell fates through the Hippo pathway, whereby the degree of Hippo activation is proportional to the extent of the cell basolateral domain. Although the generality of this concept must await validation in other contexts, genetic evidence in Drosophila is consistent with this idea: deregulation of Crumbs, gain of active forms of aPKC, or mutation in Scribble or other members of the Scribble complex all lead to a yorkie-dependent hyperproliferation (Grzeschik et al., 2010; Robinson et al., 2010).

Understanding tumor formation is ultimately directed to cure patients. Unfortunately, the option of targeting the core Hippo cascade for breast cancer therapies is presently frustrated by the fact that the known kinases are negative regulators of TAZ, as these would need to be activated to counteract tumor progression. However, the present study pinpoints extrinsic and intrinsic cellular determinants upstream of the core signaling cascade, which could be exploited to curb tumorigenic potential.

**EXPERIMENTAL PROCEDURES**

**Gene-Expression Analysis**

Gene-expression profiles from seven breast cancer datasets analyzed on Affymetrix HG-U133A arrays and annotated with histological tumor grade and clinical outcome were downloaded from NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). A list of these datasets is provided in Table S2. After reorganization, microarray data were merged in a compendium (metadataset) comprising 993 unique samples (see Extended Experimental Procedures). Expression values of the metadataset were generated from raw CEL files using RMA algorithm implemented by the affy R package (Irizarry et al., 2003). The type and content of clinical and pathological annotations of the metadataset samples were derived from the original cohorts.

Genes upregulated in histological G3 tumors were identified, comparing the expression profiles of G3 versus G1 samples with SAM algorithm coded in the samr package (Tusher et al., 2001).

Detailed statistical methods are provided in the Extended Experimental Procedures.

**Enrichment Analysis of Signaling Pathways and Stem Cell Signatures**

We used an over-representation analysis based on Fisher’s exact test to assess the statistical association between genes overexpressed in histological G3 tumors and gene signatures of signal transduction pathways. The significance of such association was evaluated, taking into account the number of genes in the two lists and the total number of genes in the microarray. Under the null hypothesis that the proportion of probesets overexpressed in G3 samples and present in the gene signature is smaller than or equal to the proportion of probesets that are not contained in the gene signature (one-sided test), we built a 2 x 2 contingency table for each gene signature recording the relation between the genes in the signature and the genes overexpressed in histological grade 3. Given m probesets in the G3-enriched gene set and n probesets in a predefined pathway signature, the probability of observing an overlap of k probesets for that gene signature, under the hypothesis that the m probesets were picked out randomly from the N total probesets of the microarray, is given by the hypergeometric distribution. For any given signature, the significance of the observed overlap k (p value) is computed as the sum of the probabilities for all possible contingency tables with an overlap greater than or equal to k. The null hypothesis is then rejected if the p value is smaller than a predetermined threshold a (e.g., a ≤ 0.05). Considering that multiple signatures are being tested, p values can be finally adjusted for false discovery rate (FDR) using, e.g., Benjamini-Hochberg correction.

The over-representation analysis has been conducted using the phyper function of the R stats package. The p value threshold has been set to 0.05 and p values adjusted using the p.adjust function of the R stats package (FDR < 5%).

**Mammosphere Assays**

Confluent monolayers of cells were trypsinized, counted, and plated as single-cell suspensions (1000 cells/cm²) on ultra-low attachment plates (Costar). Mammospheres were counted after 5 days; cells were thereafter dissociated to be reseeded for a second round of mammosphere formation. Mammosphere cultures of MII, HMLE, MDA-MB-231, and their derivatives were performed as described in Dondu et al. (2003); for MIV cells, culture conditions were as in Ponti et al. (2005). Statistical analyses were done with Prism software (GraphPad).

**Fluorescence-Activated Cell Sorting**

Cells were detached from plates with TrypLE (Invitrogen), resuspended (5 x 10⁶ cells/ml), incubated in running buffer (PBS 1X, BSA 0.5%, and EDTA 5 mM) with anti-human CD44 (clone G44-26, FITC-conjugated, BD Biosciences) and anti-human CD24 (clone ML5, PE-conjugated, BD Biosciences), and finally analyzed on a MoFlo XDP sorter (Beckman Coulter).

**Tumorigenesis Assays**

For xenograft tumor-seeding studies, the indicated numbers of M11 or MIV cells were suspended in 100 μl Matrigel (BD Biosciences) and injected in the fat pads of immunocompromized female mice. Tumor formation was assayed by palpation. After the indicated periods, mice were sacrificed and tumors were explanted for histological analyses as previously described (Adorno et al., 2009).

Numbers of tumor-initiating cells were estimated by using ELDA software (http://bioinf.wehi.edu.au/software/elda/).

**Western Blot and Immunoprecipitations**

Protein lysates were obtained from cells grown for 2 days at high density, after sonication in lysis buffer (20 mM HEPES [pH 7.8], 100 mM NaCl, 5% glycerol, 5 mM EDTA, 0.5% NP40, and protease and phosphatase inhibitors). The western blot procedure was carried out as described in Dupont et al. (2009). Primary antibodies are listed in the Extended Experimental Procedures.

Immunoprecipitations were carried out as in Adorno et al. (2009), with the following modifications: extracts were diluted to 20 mM HEPES (pH 7.8), 100 mM NaCl, 5% glycerol, 2.5 mM MgCl₂, 1% Triton X-100, 0.5% NP40 and incubated with protein A-Sepharose-bound anti-TAZ/YAP monoclonal antibody (Santa Cruz, sc-101199).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2011.09.048.

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