BP1 Upregulates Twist, a Trigger of the Epithelial to Mesenchymal Transition (EMT), Which May Lead to Metastasis in Breast Cancer

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Abstract

BP1 Upregulates Twist, a Trigger of the Epithelial to Mesenchymal Transition (EMT), Which May Lead to Metastasis in Breast Cancer

In previous research, BP1 appears to be associated with metastasis, shown by immunostaining on 46 samples of inflammatory breast cancer (as well as matched lymph nodes in 9 metastatic cases); all cases were BP1 positive. Now we suggest that BP1 induces breast cancer cells to undergo the epithelial to mesenchymal like transition (EMT). EMT, a process in which cancer cells lose their epithelial features and gain mesenchymal markers, enables tumor cells to become more invasive and migratory, and can lead to metastasis. BP1 upregulate Twist, a basic helix-loop-helix (bHLH) transcription factor which triggers EMT. Twist is well known for initiation of EMT in various cancers. We found that BP1 can upregulate Twist expression in two breast cancer cell lines, MCF-7 and HS578T, and induce EMT, resulting in increased migratory ability. On the other hand, exogenous BP1 can also increase Twist expression and migration ability of cancer cell lines, indicating that BP1 might be able to induce EMT in both a paracrine and endogenous manner.
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List of Abbreviations:

Ab: antibody

bHLH: basic helix loop helix

BP1: Beta Protein 1

BSA: Bovine serum albumin

CDH1: Cadherin 1

ChIP: Chromosomal Immunoprecipitation

DLX: Distal-less

DMSO: Dimethyl sulfoxide

EGF: Epidermal growth factor

EMT: Epithelial to mesenchymal transition

ER: Estrogen receptor

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

GSK3: Glycogen synthase kinase 3

HIF1α: Hypoxia inducing factor 1 α

HMLE: Human mammary epithelial cells

HOXB7: Homeobox gene B7

MCC: Mitomycin C
MET: Mesenchymal to epithelial transition

MTT: 3-(4,5-dimehtylthiazol-2-yl)- 2,3-diphenyltetrazolium bromide

NF-KB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

RealTimePCR: Quantitative RealTime polymerase chain reaction

rpBP1: Recombinant protein BP1

SDS: Sodium dodecyl sulfate

Ser: Serine

TBST: Tris buffered saline and Tween 20

TGFβ: Tumor growth factor β

VEGF: Vascular endothelial growth factor

P/S: Penicillin/Streptomycin
Introduction

1. Epithelial to Mesenchymal Transition (EMT)

1.1 What is EMT?

The epithelial to mesenchymal transition (EMT) plays a very crucial role in formation of the body plan and it can promote tumor progression as well. During EMT, epithelial cells shed their differentiated characteristics which include cell-cell adhesion and planar and apical-basal polarity, and acquire mesenchymal features which lead to motility, invasiveness and resistance to apoptosis. EMT is crucial for both normal embryogenesis and carcinogenesis, and is characterized by disarrangements of apicobasal polarity, lack of basal laminal integrity, cell shape plasticity and dramatic remodeling of the cytoskeleton (for review see Polyak et al., 2009). We can observe this phenomenon as morphology changes in which a cubicle-like cell shape changes into an elongated, spindle-like shape on single cell layers (for review see Thiery J. et al, 2006). Cells also lose the molecular markers of epithelial cells and gain the molecular characteristics of mesenchymal cells (for review see Thiery et al., 2006).

Epithelial cells and mesenchymal cells represent two main cell types in mammals
with distinct characteristics (Fig 1). Epithelial cells have the following characteristics:

(i) cohesive interactions with each other, resulting cell layers;

(ii) three membrane domains: apical, lateral and basal;

(iii) tight junctions between apical and lateral domains;

(iv) apicobasal polarized with distribution of cytoskeleton components; Epithelial cell membranes are organized into at least two discrete regions: apical and basal

/lateral surface. Each surface has different membrane protein components and features (Lodish et al. 2004).

(v) Lack of mobility of individual epithelial cells.

On the other hand, mesenchymal cells feature:

(i) loose interaction between cells so no cell layer is formed;

(ii) no clear apical and lateral domain;

(iii) no apicobasal polarization of cytoskeleton;

(iv) motile cells.

For reviews see Christiansen et al., 2006, Polyak et al., 2009 and Thiery et al., 2009.
Figure 1 Differences between epithelial and mesenchymal cells (from Christiansen et al., 2006). There are morphological and molecular difference between typical epithelial cells from highly migratory mesenchymal cells.
However, the initiation of the same EMT signal transduction pathway can lead to disparate outcomes in different cell lines. Not all cell lines which had undergone EMT will acquire the entire characteristics of mesenchymal cells. Only a few cell lines undergo a complete transition to mesenchymal cells (for review see Christiansen et al., 2006). As reviewed by Christiansen et al, there are several reports describing a partial or incomplete EMT phenotype of advanced carcinomas displaying some mesenchymal features, but with retention of well-differentiated epithelial cell characteristics (Christiansen et al., 2006). For example, hypoxia was reported to induce different EMT phenotypes in different breast cancer cell lines, demonstrating incomplete EMT (Lundgren et al., 2009).

1.2 The EMT in normal development

The EMT is a highly conserved and fundamental process that governs morphogenesis in multicellular organisms. This process plays crucial roles in formation of the body plan and in the differentiation of multiple tissues and organs. In embryonic development, EMT occurs during gastrulation and facilitates the formation of the three layered embryonic structure. During gastrulation, endodermal epithelial cells in the marginal zone (red in the Figure 2) acquire mesenchymal features, appear at the primitive streak (green in Figure 2) and migrate to form the mesoderm. Mesenchymal
cells acquire higher migratory ability in the extracellular environment and settle in areas involved in organ development, which involves interactions between epithelial and mesenchymal cells. Without EMT, there would be no proper gastrulation (for review see Thiery J. et al., 2002, Thiery J. et al., 2009).

After gastrulation in vertebrates, neural crest cells undergo EMT within the dorsal neural epithelium and individual cells migrate to give rise to different derivatives, which including craniofacial structures (Sauka-Spengler T, et al. 2008).
Figure 2 EMT occurs at the formation of the gastrula (from Thiery J., 2002).

During gastrulation, endodermal epithelial cells (red) acquire a mesenchymal phenotype, appear at the primitive streak and migrate to form the mesoderm.
Mesenchymal cells can also form epithelial organs through the mesenchymal to epithelial transition (MET). The cell may undergo several rounds of EMT and MET to differentiate into final specialized cell types and organ structures. EMT is regulated in a system with plasticity in development (Fig. 3). There are multiple signal transduction pathways regulating EMT in development, such as Wnt, TGF-β, EGF, FGF and Nodal. The existence of regulatory loops among different EMT inducers explains why the absence of one player may be compensated for by another (for reviews see Thiery J. et al, 2002; Thiery, J. et al, 2009).
Figure 3. Pathways which regulate EMT and gastrulation (from Thiery J. et al, 2009). Pathways regulating EMT and gastrulation are highly conserved throughout evolution and are also involved in carcinogenesis. Shown here are the pathways for Sea urchin (A), Dorsophila(B) and Amniotes(C).
1.3 EMT in carcinogenesis

EMT is considered to play an important role promoting tumor metastasis (Fig. 4).

During progression toward metastatic competence, carcinoma cells acquire a mesenchymal gene-expression pattern and properties, and enhanced invasiveness. This transition allows the tumor cells to metastasize and establish secondary tumors at distant sites. In tissue culture, this transition is accompanied by partial or complete EMT (Thiery et al., 2006, Christiansen et al., 2006). It has been demonstrated that EMT, which is observed in cultured tumors, leads to increased metastatic potential in vivo (Yang et al., 2004). EMT was also reported to have a relationship with cancer stem cells: human mammary epithelial cells (HMLEs) which have undergone EMT acquire cancer stem cell marker expression, further indicating the role of EMT in promoting metastasis (Mani et al., 2008).
Figure 4. EMT/incomplete EMT positively correlates with the malignant phenotype (from Christiansen et al., 2006). During the progression of invasive and metastatic carcinoma, epithelial cells may lose their intercellular cohesion via incomplete EMT or fully convert to a mesenchymal phenotype, which all lead to increased metastatic ability.
Tumor metastasis consists of several distinct steps. In the first step, epithelial cells lose cell-cell adhesion and gain motility which leads to invasion of adjacent tissue and is associated with EMT (Yang et al., 2004). The second step is to penetrate through the endothelium cells of blood and lymphatic vessels to enter the systemic circulation. Only some cells can survive during circulation and extravasate through the endothelium at distal sites. Finally, minute growth proliferates into malignant secondary growth in the new host environment and acquires MET, recovering the epithelial phenotype (reviewed in Christiansen et al., 2006 and Cheng et al., 2008).

Therefore, EMT is considered an important step in metastasis (Fig. 5). But due to remarkable inherent heterogeneity and plasticity, tumors are a highly diverse population of cells that display a remarkable range of phenotypes. Many of the defining characteristics of EMT do not present in all invasive or metastatic tumor cells and a complete transition to a mesenchymal phenotype is not required for invasion and metastasis (Christiansen et al., 2006).

Interestingly, the signal transduction pathway which regulates tumour progression is also the same in regulating developmental EMT (Thiery et al., 2006). Therefore, EMT in carcinogenesis can be regarded as reactivation of developmental programs in adults (Thiery et al., 2009).
Figure 5. EMT contributes to the intravasation stage of metastasis (Thiery et al., 2002). EMT participates in the intravasation stage of metastasis. It can increase the migratory ability of tumor cells, allowing them to penetrate through the endothelial cells of blood vessels and lymphatic vessels to enter the systemic circulation.
1.4 Experimental approaches to monitor EMT.

We can experimentally monitor EMT by observing the morphological change of cells and displaying of epithelial and mesenchymal cell markers.

As the result of EMT, epithelial cells transform to elongated, more fibroblastic cells with a spindle-like shape since mesenchymal cells lack an apical basolateral polarity (Thiery et al., 2006, Christiansen et al., 2006). Instead, these cells possess an elongated morphology with front-back asymmetry that facilitates motility and locomotion (Christiansen et al., 2006). This elongated morphology with filapodial extensions are enriched in MMPs, which digest cellular basal membrane and promote invasion (Seiki, 2003).

Using molecular markers, we usually can detect this process by monitoring the shift in expression from a decrease or loss of epithelial marker expression and a gain or increase of mesenchymal markers expression. The widely used epithelial markers include such protein as E-cadherin, claudin 4, claudin 7, β-catenin and claudin (Thiery et al., 2006). They contribute to adheren junctions formation and maintain apical-basolateral polarity. Mesenchymal markers include, for example, vimentin, N-cadherin, fibronectin, smooth muscle actin, γ-actin, β-filamin and talin.
Overexpression of these proteins can facilitate pseudopod formation and cytoskeleton remodeling (Christiansen et al., 2006).

E-cadherin, a transmembrane glycoprotein encoded by the CDH-1 gene, mediates calcium-dependent intercellular adhesion (Lodish, 2004), is involved in stable cell-cell contact and development of adherens junctions (Fig. 6), which is an important feature of epithelial cells (Thiery et al., 2002). In epithelial cells, the E-cadherin molecules cluster into small junctional complexes and promote the formation of desmosomes (Thiery et al., 2002). The extracellular domain of E-cadherin mediates Ca-dependent homotypic interactions with E-cadherin molecules on adjacent cells. On the other hand, the intracellular domain binds to cytosolic β-catenin, which is linked to the actin cytoskeleton (Christiansen et al., 2006). Losing expression of E-cadherin disrupts cell-cell contacts, induces the mesenchymal phenotype and promotes invasion (Zheng et al., 2005). In most cases, downregulation of E-cadherin during carcinoma progression occurs by an epigenetic mechanism, such as repressor binding and promoter hypermethylation (Thiery et al., 2002). It was found that EMT of breast cancer cell lines is induced by hypermethylation instead of mutation of CDH1 gene (Lambaerts et al., 2006). On the other hand, it was reported that E-cadherin is downregulated by lysosomal degradation (Palacios et al., 2005). Several repressors, such as
Snail, Slug and Sip1 can repress the expression of E-cadherin by binding to E-box regulatory binding sites (Christiansen, et al., 2006).

Another well known epithelial marker is β-catenin (Fig. 6). It connects the intracellular region of E-Cadherin with the cytoskeleton, therefore maintaining the E-cadherin-mediated cell–cell interaction (Lodish, 2004; Thiery J. et al., 2002). Through β-catenin, E-cadherin indirectly links to actin and the microtubule cytoskeleton, which is essential for cell-cell adhesion (Schmalhofer et al., 2009). On the other hand, β-catenin also serves as a transcription factor which induces the expression of several EMT-inducing transcription factors (Polyak et al., 2009). During EMT, β-catenin leaves the cell membrane and translocates to the nucleus as a consequence of losing E-cadherin expression (Schmalhofer et al., 2009). Nuclear β-catenin induces a gene expression pattern favoring tumor invasion and leading to stabilization of the mesenchymal phenotype of epithelial tumor cells (Schmalhofer et al., 2009). β-catenin downregulation was also reported in some Twist induced EMT (Yang et al., 2004; Cheng et al., 2007).
Figure 6. E-cadherin mediated cell-cell junctions (Lodish et al., 2004). E-cadherin mediates calcium dependent intercellular adhesions which provides stable cell-cell interactions. β-catenin links E-cadherin with the cytoskeleton, maintaining E-cadherin mediated cell-cell interactions.
Vimentin is a mesenchymal marker which is one of the most widely expressed mammalian intermediate filament proteins. It is a type 3 intermediate filament normally expressed in all mesenchymal cells and tissues (Hay et al., 1989). Its exact mechanisms of function are not fully understood. It is also expressed in migratory epithelial cells and associated with the ability of tumor cell invasion or migration. Upregulation of vimentin predicts metastatic progression and poor patient prognosis (Domagala et al., 1994). Vimentin filaments coaligned with microtentacles promote metastatic spread (Whipple et al., 2008).

2. Twist promotes EMT and induces metastasis

2.1 Basic characteristics of TWIST and its role in development

Twist is a member of highly conserved family of basic helix-loop-helix (bHLH) transcription factors and is involved in the specification and differentiation of mesenchymal tissue. The bHLH is characterized by a stretch of basic amino acid adjacent to two amphipathic α-helices separated by an interhelical loop. Its structure is composed of a basic DNA binding domain that targets the consensus 5’-CANNTG-3, which known as the E-box sequence (for review see Puisieux, 2006). There are two TWIST gene in vertebrates, Twist-1 (Twist) and Twist-2 (Dermo-1). The two Twist protein sequences are more than 90% identical but Twist 2 lacks a glycine-rich region.
that is present in the Twist-1 N-terminal region (Li et al., 1995). The functions of Twist are regulated through heterodimerization or homodimerization. Twist-Twist homodimers share distinct even antagonistic properties toward the Twist-E heterodimer (Castanon et al., 2001).

Twist was initially found as a gene crucial for development for all mesoderm derived organs (Thisse et al., 1987). Twist is important in its regulatory role in neurogenesis, osteogenesis, and myogenesis in vertebrates (reviewed in Cheng et al., 2008). Twist plays a very important role in gastrulation and is evolutionary conserved even in sea urchins and flies (for review see Thiery et al., 2009). A loss of Twist function can cause severe consequences. In mice, Twist maintains limb bud, head mesenchyme and somite morphogenesis. Mice that have lost Twist expression can still undergo normal gastrulation, but would have defects in the closure of the cephalic neural tube, deficient cranial mesoderm, malformed branchial arches and facial primordium, and retarded limb buds, which together lead to death (Bourgeois et al., 1998). In humans, mutation of the Twist 1 gene causes Saethre-Chotzen syndrome (SCS). This disease is characterized by limb abnormalities, facial dysmorphism and premature fusion of cranial sutures and is transmitted hereditarily as an autosomal dominant trait (El Ghouzzi et al., 1997). Therefore, Twist plays a role in mesoderm differentiation of
vertebrates in which mesoderm formation is controlled by Snail (reviewed in Ansiauau S, 2010).

2.2 TWIST is an oncogene

Twist is overexpressed in large set of tumors (Ansiauau et al., 2010; Puisieux et al., 2006) and its expression positively correlates with increased invasiveness of cancer (Cheng et al., 2007). Overexpression of Twist leads to antiapoptosis, drug resistance, angiogenesis, EMT and metastasis (reviewed in Cheng et al., 2008). Twist overexpression can induce EMT and therefore promote metastasis in vivo (Yang, 2004). Twist 1 silencing was shown to prevent lung metastasis in mice which suggests that Twist might be essential for metastasis. Its potential to promote metastasis was through inducing EMT (Yang et al., 2008).

Twist expression was shown to be negatively correlated with E-cadherin expression and positively correlated with aggressiveness in different breast cancer cell lines (Vesuna et al., 2008). Twist can regulate EMT indirectly through other genes, e.g., Twist can activate Snail to promote EMT and metastasis (Smit et al., 2009). Overexpression of Twist induces chromosomal instability in breast cancer (Mironchik et al., 2005). Twist upregulates AKT2 expression in breast cancer cell lines (Cheng
GZ et al, 2007) so it may regulate EMT through the PI3K/AKT pathway (Larue et al., 2005) Interestingly, the knockdown effect of Twist can be rescued by reexpression of AKT2 showing that AKT2 is essential for Twist induction of EMT (Cheng et al., 2007). In some cell lines, Twist expression alone can only induce partial EMT. But with expression of both Twist and H-RasV12 or ErbB2 can induce complete EMT (Ansieau et al, 2008).

Twist is regulated by several pathways (Fig 7). It is a direct target of NF-κB and regulated by a negative feedback mechanism (Sosic et al., 2003). Twist might be a stress response gene which HIF-1α can directly regulate, inducing metastasis through EMT (Yang et al., 2008). The Wnt pathway might regulate Twist function; Wnt 1 was found to upregulate Twist expression in mouse cell lines (Howe et al., 2003). The Twist promoter was also found to be responsive to β-catenin, c-jun and Ets factors of the PEA 3 family, which are downstream of the Wnt pathway (Howe et al., 2003).
Figure 7. Schematic representation of the regulation of Twist expression (Ansiuau et al., 2010). Regulation of Twist can occur by stress, signaling factors/cytokines, transcription factors and miRNA.
In addition to inducing EMT and metastasis, Twist also inhibits apoptosis. It was shown that Twist can prevent Myc-dependent cell death. On the other hand, it can also indirectly affect p53 through the ARF/MDM2/p53 pathway (Maestro R, 1999). It was also shown that it can cooperates with Ras and disrupts both Rb and the p53 pathway (Ansieau S, 2008). Twist can also triggers a stem cell like phenotype in differentiated mammary epithelial cells (Mani et al., 2008).

Twist was also shown to induce angiogenesis in mice and thrombin-induced angiogenesis (Mironchik et al., 2005; Hu et al., 2008). On the other hand, it contributed to an anti-Paclitaxel effect (Cheng et al., 2007).

3. BP1 and breast cancer progression

3.1 BP1, a DXL family transcription factor.

Beta Protein 1 (BP1), an isoform of homeobox gene, DLX4, was indentified and cloned in our lab (Chase et al., 2002). Although this gene is named “DLX4 variant1” in the NCBI database, it is not DLX7 (another DLX4 variant 1). BP1 and DLX7 have distinct functions and the molecular weight is different, DLX7 is 24kDa and BP1 is 32kDa (Fu et al., 2001). It was initially found as a repressor of β-globin gene in erythroid cells (Chase et al., 2002) and frequently upregulated in 47% of adult and
81% of pediatric acute leukemias (Haga et al., 2000).

### 3.2 Association of BP1 with breast cancer

BP1 is located on chromosome 17q21-22, which is known to be amplified in breast cancer (Fu et al., 2001). BP1 was found to be correlated with estrogen receptor status in breast cancer. It was shown by an analysis of 46 invasive ductal breast tumors; 100% of ER negative tumors were BP1 positive (for mRNA) compared with 73% of ER positive tumors which were BP1 positive (Fu et al., 2003). On the other hand, BP1 expression also correlated with breast cancer progression and invasion: BP1 stained positively in 21%, 46% and 81% of hyperplastic, in situ and invasive lesions (Man et al., 2005). Recent findings on inflammatory breast cancer indicate that BP1 may promote breast tumor progression, invasion and metastasis (Man et al., 2009). BP1 mRNA expression was also found to be significant higher in the tumors with lymph node metastasis (Yu et al., 2008). However, these findings were all based on pathological staining showing BP1 “associated” with the progression and invasion of breast cancer but does not indicate the mechanism of BP1 involvement in breast cancer progression, invasion and metastasis. In general, Homeobox genes are found to be involved in tumorigenesis and development, and HOXB7 was found to induce EMT in epithelial cell lines (Wu et al., 2006).
We hypothesized that BP1 might promotes cancer metastasis and invasiveness by the epithelial to mesenchymal like transition. Homeobox genes are the master regulators of development, initially identified in Drosophila. They are essential for body plan and developmental patterning. On the other hand, they also play a role in cancer oncogenesis (reviewed in Shah et al., 2010). HOXB7 was overexpressed in breast cancer cell lines and promoted EMT and invasion in vivo (Wu et al., 2006). DLX4 is known to play an important role in controlling the development of tissues dependent on epithelial–mesenchymal cell interactions for morphogenesis in humans (Quinn et al., 1997; Quinn et al., 1998). In rodents, members of Dlx family are expressed in those structures that require epithelial-mesenchymal cell interactions for development, including the branchial arch, bone, ear, whisker and tooth, (as reviewed as Quinn et al., 1998 and Panganiban et al., 2002). It was also found that DLX4 is required for control of epithelial-mesenchymal cell interactions in endometrium (Quinn et al., 1998).

A former lab member, Jinguen Rheey, performed a PCR microarray which showed that BP1 upregulated Twist expression in MCF-7 O2 cells. These data were verified using RealTime PCR (unpublished data, Fig. 8). Here, we demonstrated that BP1 can upregulate Twist expression and induce breast cancer cell lines to undergo EMT and
increase migratory ability. Importantly, it might be possible to induce EMT in surrounding cancer cells due to the paracrine effects of BP1. Therefore, I hypothesized that BP1 might promote tumor invasion and metastasis by EMT, which is mediated by Twist. BP1 was also found to be secreted by breast cancer cell lines and has a potent paracrine activity (unpublished data).
Figure 8. Twist was shown to be upregulated in BP1 overexpressing cells by RealTime PCR (from Rheey). The following genes were assayed: C-fos, Met, MMP9 and Twist, comparing between BP1 overexpressing cells (O1) and empty vector cells (V1). Twist mRNA level increased for 5 folds in O1 cells.
4. Specific aims

This study aims to determine whether:

I. BP1 upregulates Twist.

II. BP1 induces EMT in breast cancer cell lines.

III. BP1 promotes migratory ability in breast cancer cell lines.

IV. Secreted BP1 can promote EMT.
Materials and Methods

1. Maintenance of mammalian cell lines.

The cell lines used in this project are listed in Table 1. Cells were maintained in 5% CO₂ at 37°C. They were passaged every 72 hrs, washed with PBS, then trypsinized. The MCF-7 O1 cell line contains a BP1 vector. It was made in our lab and overexpresses BP1 compared to the MCF-7 parental cell line and MCF-7 cell lines with an empty vector (MCF7 V1). HS578T cells were also used. HS578T O3 and O7 cells, contain a BP1 vector and therefore overexpress BP1, while the V2 cells contain an empty vector, serving as a negative control. The HS578T derivatives cells are from Dr. Sidney W Fu.

The RPMI 1640 media is from Invitrogen (11875-093). The DMEM media is from Invitrogen (12430-104). The Fetal Bovine Serum (FBS) is from Atlanta Biologicals (S11050H). The Penn/Strep is from Gibco (15140). The trypsin/EDTA solution is also from Gibco (0.025%, R-001-100). The cells were cultured in T25/T75 flasks (NUNC™, cat. 136196 and cat. 178905).
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media</th>
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<tbody>
<tr>
<td>1 MCF-7</td>
<td>RPMI 1640 containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (P/S).</td>
</tr>
<tr>
<td>2 MCF-7 V1</td>
<td>RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin (P/S) and 500μg/ml G418.</td>
</tr>
<tr>
<td>3 MCF-7 O1</td>
<td>RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin (P/S) and 500μg/ml G418.</td>
</tr>
<tr>
<td>4 T47D</td>
<td>RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin (P/S).</td>
</tr>
<tr>
<td>5 HS578T V2</td>
<td>DMEM media (glucose, glutamate, sodium pyruvate) supplemented with 10% FBS, 1% penicillin/streptomycin (P/S) and 500μg/ml G418.</td>
</tr>
<tr>
<td>6 HS578T O3</td>
<td>DMEM media (glucose, glutamate, sodium pyruvate) supplemented with 10% FBS, 1% penicillin/streptomycin (P/S) and 500μg/ml G418.</td>
</tr>
<tr>
<td>7 HS578T O7</td>
<td>DMEM media (glucose, glutamate, sodium pyruvate) supplemented with 10% FBS, 1%</td>
</tr>
<tr>
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<td>Cell Line</td>
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<td>MDAMB 231</td>
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penicillin/streptomycin (P/S) and 500μg/ml G418.
2. RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA extraction was performed using the Qiagen RNaseasy kit (Qiagen, cat. 74104). Cells were seeded in a 6 well plates at 150,000 to 300,000 cells per well (Corning, COSTAR 3516). Then, the cells were washed with PBS to remove cell debris, and RLT lysis buffer (from the kit) was added. Cells were scraped with RLT buffer into Eppendorf tubes. Samples were homogenized by passing them through a syringe and needle (20 gauge, BD 305178) 10 times. This was mixed with 350 μl of 70% ethanol, and then transferred to an RNaseasy spin column (from the kit). Next it was centrifuged for 15 seconds at 2100g, then 350μl of Buffer RW1 (from kit) was added to wash the column membrane. Next, to remove the genomic DNA contamination, the membrane was treated with 10μl of DNase 1 and 70μl Buffer RDD (both from Qiagen RNase-Free DNase Set, cat. 79254) and incubating for 15 minutes at room temperature. Then 350μl Buffer RW1 was used to wash the membrane again, followed by two washes using 500μl of Buffer RPE. The membrane was dried by centrifuging at 12,000 rpm for 1 minute. Last, 20μl of RNase free H2O (From Qiagen RNaseasy kit) was added to elute the RNA. The RNA was stored at -80°C.

RNA was quantified with the NanoDrop RD1000 instrument. It was diluted to 1μg/μl using RNase/DNase free H2O (Invitrogen 10977) and used for reverse transcription
for cDNA synthesis using SuperScript III First-Strand cDNA synthesis kit (Invitrogen, 18080-051).

Quantitative Real Time PCR of the cDNA sample was performed using an ABI Prism 7000 system (from Applied Biosystem). SYBR green master mix (Biorad, 1725850 or SA Bioscience, PA-012) was used to prepare each 25μl reaction mix. For each reaction, 1ng of cDNA and 20pmol of each primer was added. Each reaction was performed in triplicate. Nontemplate controls were used to test for DNA contamination. Samples were run as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 seconds followed by 60°C for 1 min. Next, a dissociation curve was made by an additional 60°C for 30 seconds. Primers are listed in Table2.
Table 2. RealTime PCR primers and sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S (F)</td>
<td>GCC GCT AGA GGT GAA ATT CTT G</td>
</tr>
<tr>
<td>18S (R)</td>
<td>CAT TCT TGG CAA ATG CTT TCG</td>
</tr>
<tr>
<td>BP1 (F)</td>
<td>CCT CCC CCA ATT TGT CCT ACT C</td>
</tr>
<tr>
<td>BP1 (R)</td>
<td>GGT TGC TGG CAG GAC AGG TA</td>
</tr>
<tr>
<td>Twist (F)</td>
<td>GGA CAA GCT GAG CAA GAT TCA GA</td>
</tr>
<tr>
<td>Twist (R)</td>
<td>TCT GGA GGA CCT GGT AGA GGA A</td>
</tr>
<tr>
<td>Vimentin (F)</td>
<td>GGA AGA GAA CTT TGC CGT TGA A</td>
</tr>
<tr>
<td>Vimentin (R)</td>
<td>GTG ACG AGC CAT TTC CTC CTT</td>
</tr>
<tr>
<td>Fibronectin (F)</td>
<td>GGA AGA GAA CTT TGC CGT TGA A</td>
</tr>
<tr>
<td>Fibronectin (R)</td>
<td>GTG ACG AGC CAT TTC CTC CTT</td>
</tr>
<tr>
<td>E-cadherin (F)</td>
<td>GAA CGC ATT GCC ACA TAC AC</td>
</tr>
<tr>
<td>E-cadherin (R)</td>
<td>ATT CGG GCT TGT TGT CAT TC</td>
</tr>
</tbody>
</table>
3. **Protein extraction, quantification and Western blotting analysis.**

Protein was extracted from cells and probed by Western blotting to test the expression of genes at the protein level. The protein was extracted using two different methods based on the suggestions from the company supplying the antibody. One method used RIPA lysis buffer and the other used SDS lysis buffer. The procedures were as follows:

(a) This procedure optimized the recognition of antibodies from BD Bioscience.  
All other samples were probed using method (b). SDS buffer was composed of 1% SDS (Quality Biological, Cat. 351-066-101), 1mM Na$_2$VO$_4$ (Sigma, cat. S6508), 10mM Tris. This formula was recommended by the BD Bioscience website. Cells were seeded in 6 well plates at 100,000 to 300,000 cells per well depending on the cell type. Then 350 $\mu$l of SDS lysis buffer was added to the plate, followed by a washing with PBS. Cells were then scraped from the plate and transferred to Eppendorf tubes, boiled for 5 minutes using a heat block, and cooled to room temperature. 1x protease inhibitor (Roche, 04683124001) was added. Then, the mixture was sonicated for 10 pulses using the lowest output followed by centrifugation for 30 minutes at 13,000 rpm at 4°C. Last, the
supernatant was collected and 35μl glycerol was added to each tube. The sample was stored at -80°C with. Protein concentration was determined using the BCA protein assay kit (Pierce, 23227) with a BSA standard (Pierce, cat. 23209) that was diluted in SDS lysis buffer, centrifugated for 30 minutes at 13,000 rpm at 4°C to precipitate the excess SDS.

(b) Cells were seeded in 6 well plates, at 100,000 to 300,000 cells per well depending on the cell type. Cells were trypsinized, followed by washing with cold PBS (without Ca/Mg, Quality Biological, cat. 114-057-101). Then the cells were lysed in 100 to 200μl of RIPA lysis buffer with freshly added 1x protease inhibitor (Roche, 04683124001). The RIPA lysis buffer is composed of 50mM Tris-HCl pH 7.5, 2mM EDTA (BioWhittaker, cat. 16-004Y), 100 mM NaCl (BioWhittaker, cat. 16-008Y) and 1% Igepal CA-630 (Sigma Aldrich, replacing the NP40), and was stored at 4°C. The cell lysates were kept on ice for 10 minutes for lysis, followed by sonication for 10 pulses (lowest output). Then the samples were centrifuged for 30 minutes at 130,000 rpm. The supernatant was collected and glycerol added to reach 10% glycerol in samples. The samples were stored at -80°C. Sample concentrations were determined using the BCA protein assay kit (Pierce, 23227) with the BSA standard (Pierce, cat. 23209) diluted in RIPA.
Western blotting was used to determine the protein expression level of genes.

Protein samples were loaded on a 10% Biorad Precast gel (Cat.465-1033). The samples were prepared using 20μl of total protein (protein diluted to desired amount with RIPA) and 10μl of 3X SDS loading buffer (Table 3), then heated at 100°C for 10 min. After heating, the samples were allowed to cool to room temperature and then loaded on the gel. The proteins were electrophoresed for about 1 hour at 30mA per membrane. When the electrophoresis was completed, samples were transferred to a nitrocellulose membrane (Biorad, cat.162-0117) at 100V for 1hr or 80v for 2 hours. After the transfer was completed, the membrane was incubated in blocking buffer (Table 4) for 2.5 to 3 hrs at room temperature on a shaking platform. After the blocking, the blocking buffer was removed and the blot was treated with primary antibody solution and incubated overnight at 4°C. The next day, the membrane was washed 3 times, followed with secondary antibody treatment and incubated for 1.5 to 2.5 hrs. The blots were then washed 3 times for 10 min each. Then 1 ml of each reagent from Pierce West Dura Kit was added for exposure. The blots were covered with foil and incubated for 5 min. Then, the blots were developed on Kodak 2000 Image Station.

For calculation of relative protein amounts and controlling equal amounts of proteins
loaded, the blots were also probed for β-actin after stripping. The membrane was stripped with 10ml of stripping buffer (Table 3) and incubated at 45°C for 10 min. Then, the blot was washed for at least 4 times by PBST to remove the β-mercaptoethanol (FisherBiotech, cat. BP176-100). After that, the blot was slightly blocked for another 20 minutes with 10ml of blocking buffer. After the blocking, the blot was probed with β-actin using the same protocol as described in the last paragraph.

Table 3 lists the components for all the running/transfer/stripping/washing buffers. Table 4 describes the wash/blocking/antibody buffers and their conditions for every protein.
Table 3. Buffers for Western blotting analysis.

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running Buffer (10X)</td>
<td>30.3g Tris, 144g Glycine, 50mL 20% SDS buffer, Total vol. 1L</td>
</tr>
<tr>
<td>Transfer Buffer(10x)</td>
<td>30.3g Tris, 144g Glycine, Total vol. 1L</td>
</tr>
<tr>
<td>TNE (20X)</td>
<td>200mM Tris/HCl, 50mM EDTA, 1M NaCl, total vol. 500ml (pH 7.4)</td>
</tr>
<tr>
<td>TNET (1X)</td>
<td>500µl of Tween-20 in 500ml of 1X TNE (pH 7.4)</td>
</tr>
<tr>
<td>TBS (1X)</td>
<td>2.7mM KCl, 138mM NaCl, 20mM Tris, pH7.4, total vol 1L (pH 7.4)</td>
</tr>
<tr>
<td>TBS/T (1X)</td>
<td>500µl of Tween-20 in 500ml of 1X TBS (pH 7.4)</td>
</tr>
<tr>
<td>Loading buffer</td>
<td>0.1538M Tris-HCl, 6.15% SDS, 23% glycerol and 23% of bromophenol blue</td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>1% Tris, 0.7% of β-mercaptoethanol, 2% SDS</td>
</tr>
</tbody>
</table>

The 1M Tris is obtained from BioWhittaker (cat. 16-015Y), the Tris Base from Fisher Scientific (cat. BP152-1) and the Glycine from Fisher Scientific (cat. BP381-1).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Wash buffer</th>
<th>Blocking buffer</th>
<th>primary AB</th>
<th>secondary AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP1</td>
<td>TNET</td>
<td>20ml of TNET + 1g milk</td>
<td>Bethyl Rabbit</td>
<td>Pierce anti-rabbit (cat. 1858415)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:5000 dilution in TNET</td>
<td>1:15,000 in 15ml TNET</td>
</tr>
<tr>
<td>Twist</td>
<td>TBST</td>
<td>20ml of TBST + 1g milk</td>
<td>Cell Signaling Rabbit (cat. 4119)</td>
<td>Cell Signaling anti Rabbit 1:2000 in TBST+5%BSA</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>TBST</td>
<td>20ml of TBST + 1g milk</td>
<td>Cell Signaling Rabbit (cat. 406)</td>
<td>Cell Signaling anti Rabbit 1:2000 in TBST+5%BSA</td>
</tr>
<tr>
<td>β-catenin</td>
<td>TBST</td>
<td>20ml of TBST</td>
<td>Cell Signaling</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Protein</td>
<td>Buffer Components</td>
<td>Wash Buffer Components</td>
<td>Antibody</td>
<td>Dilution</td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
<td>------------------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Vimentin</td>
<td>10Mm Tris, +100Mm NaCl+0.1% Tween20</td>
<td>+ 1g milk</td>
<td>Rabbit (cat. 9582)</td>
<td>anti Rabbit 1:2000 in TBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20ml wash buffer + 1 g milk</td>
<td>BD Pharmingen, Mouse (cat. 550513)</td>
<td>ECL antimouse (cat. NA931VS) 1:15000 in PBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1000 in 10ml wash buffer + 0.5g BSA</td>
<td>Or Thermo anti Mouse (cat. 32430) 1:2500 in PBST</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>PBST</td>
<td>10ml of PBST+0.5g milk</td>
<td>Sigma mouse monoclonal AC15 (cat. A5441)</td>
<td>ECL antimouse (cat. NA931VS) 1:15000 in PBST</td>
</tr>
</tbody>
</table>
|          |                  | 1:10,000 PBST | }
| Fibronectin | 10Mm Tris +100Mm NaCl+0.1% Tween20 | 20ml wash buffer + 1 g milk | BD Pharmingen, Mouse (cat. 610077) 1:1000 in 10ml wash buffer + 0.5g BSA | Or Thermo anti Mouse (cat. 32430) 1:2500 in PBST | ECL antimouse (cat. NA931VS) 1:15000 in PBST |
4. Chromosomal Immunoprecipitation (ChIP)

ChIP was performed with the Millipore kit (cat. DAM1692681). Cells were seeded in 10cm dishes with 500,000 cells per dish. When cells reached 80% confluence, they were fixed in 165μl of formaldehyde (cat. F8775) for 10 minutes. Excess formaldehyde was washed off with 6ml of cold PBS / 1 x protease inhibitor. The dishes were stored in -80°C.

The PBS/1x protease inhibitor was prepared by adding 1 tablet of protease inhibitor (Roche, 04683124001) to 100ml of PBS (without Ca/Mg).

On Day2, 200μl of SDS lysis buffer (from the Millipore kit) was added to the fixed plate, which was then put on ice for 5 min to lyse the cells. The cells were scraped and transferred into Eppendorf tubes for 10 minutes on ice for further lysis. The samples were sonicated for 20 pulses using the lowest output and the ratio: 40%, followed by centrifugation for 15,000 rpm for 15 minutes at 4°C. The supernatant, which contained DNA, was transferred to another Eppendorf tube and the pellet was discarded. The Nanodrop instrument was used to quantify the concentration of DNA. DNA samples were diluted to 434.28 μg with the dilution buffer (from the kit). Each sample was aliquoted into 3 tubes: test group (containing anti-BP1 antibody), negative
control group (without any antibody) and the input control (Salmon Sperm DNA, from the kit). The samples were allowed to shake at 4°C overnight.

On Day 3, 40µl of agarose beads were added to BP1 Ab test tubes and the mixture was shaken for 2 hrs at 4°C. 40µl of Salmon Sperm DNA was added to the positive control group and the mixture was shaken at 4°C for 1 hr. Then, the samples were centrifuged for 10 min at 3000 rpm at 4°C. The beads pulled down the immuno-precipitated DNA-protein complex. Then, the supernatant was transferred to new tubes which were stored at -80°C (the supernatant contains nonspecific DNA + Target DNA-protein complex unbound to Ab). Next, 1ml of high salt immune complex wash buffer was added to the pellet and the mixture was rotated for 10 minutes at 4°C followed by centrifugation for 3 min at 3000 rpm for 4°C. After removal of the supernatant, 1ml of low salt immune complex wash buffer was added and rotated for 10 minutes in the cold room, followed by centrifugation for 3 min at 3000 rpm at 4°C. After discarding the supernatant, 1ml of LiCl immune complex wash buffer was added to the tube and rotated for 10 minutes at 4°C followed by centrifugation for 3 min at 3000 rpm at 4°C. After discarding the supernatant, TE immune complex wash buffer was used to wash the samples twice. Elution buffer was added to each sample to elute the DNA by incubating the mixture at 65 °C overnight.
Elution buffer was freshly prepared and composed of 1ml 1M NaHCO₃ + 50µl 20% SDS + 9ml DNase/RNase free H₂O.

On Day 4, 10µl of 5M EDTA, 20µl of 1M Tris-HCl and 2µl of 10mg/ml Proteinase K were added to the combined eluates and incubated for 1hr at 45°C. Then 500µl (equal volume) of phenol/chloroform/isoamyl alcohol was added and samples were centrifuged at 8000 rpm for 1 minute at 4°C to separate the phases. The aqueous layer was transferred to another tube and twice the volume of cold ethanol (at 4°C) was added to it. After that, 3M sodium acetate was added to reach 0.3M in the mixture, followed by incubation at -80°C for 1 hr and centrifugation at 15,000 rpm for 15 minutes at 4°C. Then, after removal of the supernatant, 1ml of 70% EtOH was added to the pellet and vortexed, centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the samples were heated at 65°C for 10 minutes to remove all the EtOH. Finally, 20µl TE buffer was added, followed by heating at 60°C for 10 minutes. The DNA was stored at -20°C.

The software, Genomic Expression 1.10, was used to search for the BP1 consensus binding site in the upstream region of the Twist promoter to verify a possible binding site. The following sequence was used as the consensus DNA binding site: 5’
WTCWATATG 3’ (W=A/T). Primers were designed for ChIP using the software Primer3. The primer sequences used were:

Forward: 5’ TCA TGC TCT TCA CTC TTT GTG A 3’
Reverse: 5’ TGG AAT TTT CTC TTT TCT GCT G 3’

PCR was performed to amplify this region using the DNA harvested by ChIP. The procedure for PCR was as follows: 340ng of DNA sample was used with the MasterMix prepared as in Table 5. MasterMix and DNA were combined to 25μl final reaction volume in a PCR tube stripe. The PCR reaction was as follows: 5min 95°C, 40 cycles (30 sec 95°C→30sec 60°C→30 sec 72°C) and 5min 72°C for the final extension. Then the PCR product was electrophoresed on a 20% agarose gel. After the electrophoresis was finished, the gel image was developed on Kodak Machine 3000mm Image Station.
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free H2O</td>
<td>77.5μl</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>12.5μl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10μl</td>
</tr>
<tr>
<td>Primer</td>
<td>10μl (forward/reverse: 5μl each, final concentration 10uM)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1μl</td>
</tr>
</tbody>
</table>

( S Prime Lot # 136240576)
5. **Confocal staining**

On the first day, 250,000 cells were seeded in each well of a 6 well plate which contained a coverslip; cells attach to the coverslip. On the second day, the media was aspirated and PBS (without Ca/Mg) was used to wash the cells 3 times. Then, 1ml of 4% Paraformaldehyde/PBS was added to each well to fix the cells on the coverslip. The plate was covered with foil, kept in dark and incubated for 2 hrs. Then, 2ml PBS was added to rinse the sample for 20 min to remove the paraformaldehyde. PBS was poured off and 1 ml of 0.2% Triton X-100/PBS was added to the well and incubated for 15 min at room temperature to permeabilize the cells. Then, each well was blocked with 1ml of blocking buffer (Table 6) for 3hrs at room temperature. After blocking was finished, the blocking buffer was poured off and 1ml of antibody solution was added to each well and incubated for 12hrs at 37°C.

On the next day, the primary antibody solution was poured off and 1ml of PBS/Triton was added to each well to rinse the cells for 5 minutes. This was done three times to remove the excess primary antibody. Then 1ml of the second blocking buffer was added to each well and incubated for 10 minutes at room temperature. Next, secondary antibody solution was added to each well and incubated for 2hrs covered in foil.
Table 6. Buffers for confocal microscopy.

<table>
<thead>
<tr>
<th>BUFFER</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4% Paraformaldehyde/PBS</td>
<td>0.2g Paraformaldehyde + 4.8ml of 1X PBS (without Ca/Mg)</td>
</tr>
<tr>
<td>PBS/TRITON (0.2% Triton X-100/PBS)</td>
<td>60 $\mu$l of Triton X-100 + 30ml of 1X PBS</td>
</tr>
<tr>
<td>Blocking buffer (10% Normal Goat Serum in PBS/Triton)</td>
<td>for each sample $\rightarrow$ 100 $\mu$l of Normal Goat Serum (Sigma-Aldrich, Cat. G9023) + 900 $\mu$l PBS/TRITON (must be freshly made during fixation). One well of sample need 4 ml of Blocking buffer.</td>
</tr>
<tr>
<td>Secondary blocking buffer</td>
<td>0.3ml of blocking buffer + 0.7 ml PBS/Triton</td>
</tr>
<tr>
<td>primary antibody solution</td>
<td>1ml blocking buffer + 1:100 antibody. (Must be freshly made during blocking)</td>
</tr>
<tr>
<td>secondary antibody solution</td>
<td>1ml blocking buffer + 1:500 antibody. (Must be freshly made just before the secondary antibody treatment started)</td>
</tr>
</tbody>
</table>
The paraformaldehyde is from Sigma-Aldrich (cat. P6148). The Fluoromount G is from Southern Biotech (cat. 0100-01). The secondary antibody was Invitrogen Alexa Fluor 488, anti-Rabbit (cat. A11008). Table 6 shows the composition of the buffers used in this procedure. Table 7 describes the primary antibody used in confocal staining.
Table 7. E-cadherin antibodies for confocal staining.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Primary antibodies</th>
<th>Secondary antibodies</th>
</tr>
</thead>
</table>
6. rpBP1 treatment of cells

Recombinant pBP1 (rpBP1) was used to simulate the effect of secreted and internalized BP1 protein on neighboring cells. The rpBP1 was prepared by Anna Yakovleva in our lab. Cells were seeded in 6 well plates at 300,000 cells per well. 24hrs after seeding, the medium (containing FBS/PS, G418 for the cells containing the G418 selection marker) was changed to fresh medium containing rpBP1 at different concentrations.

7. Wound healing migration assay

Cells were seeded in 6 well plates and the plates were marked with lines by sharpies to indicate the location where the scratch was made. Each well was seeded at 300,000 to 500,000 cells per well. Cells were grown to 90% confluence. Then, 20μl tips (VWR: cat#87001-694) or cell scrapers (Corning 3008) were used to make scratches on the cells to create the “wound”. After the wound was induced, 1 μg/ml of Mitomycin C (MCC, from Sigma Aldrich cat. M2003) was used to inhibit cellular proliferation. The wound closure was visualized using a Nikon Inverted Phase Contrast Microscope. The pictures were taken at 6 hrs, 12 hrs, 24 hrs and 48hrs. The size of the wound was calculated using NCBI ImageJ software.

Cell proliferation was measured using the MTT assay (3-(4,5-dimehtylthiazol-2-yl)-2,3-diphenyltetrazolium bromide conversion assay). The cells were seeded in 96 well plates with 2000 cells per well. On the second day, the media was changed to the desired experimental media, this was Day 0. Proliferation was measured on Day 0, Day 3 and Day 5. The cells were incubated with 5mg/ml MTT for 4 hrs and formazan was released by DMSO from living cells; absorbance was read at 570mm.
Results

1. pBP1 upregulates Twist expression and binds to the upstream DNA region of the Twist gene in vivo.

In order to verify the microarray results that showed BP1 upregulates Twist expression (Berg, unpublished data), a series of experiments were performed. We used two different cell lines to study the relationship of BP1 and Twist, MCF7 and HS578T. These two cell lines originally have low expression of BP1 (Fu et al., 2003). MCF7 is an estrogen receptor positive, less aggressive cell line with nearly no Twist expression (Cheng et al., 2007; Vesuna et al., 2008, Mironchik et al., 2005) and has an epithelial phenotype. MCF7 breast cancer cell lines that were transfected with a BP1 containing plasmid and stably express BP1 (MCF7 O1) were compared with empty vector containing cells (MCF7 V1). HS578T is an estrogen receptor negative breast cancer cell line with Twist expression and has a more mesenchymal like phenotype (Vesuna F, 2008). Its BP1 overexpressing derivatives stably overexpress BP1 mRNA and protein. The cell lines O3/O7 are the BP1 overexpressing cell lines and the V2 is empty vector containing cell line (Song et al., 2009).

Real time PCR confirmed the microarray data, which revealed a significant change in Twist mRNA levels resulting from BP1 overexpression. The MCF7 O1 Twist mRNA level is 9 fold higher compared with Twist mRNA in MCF7 V1 cells, confirming the
qPCR data (Fig. 9). This experiment was further extended to the protein level.

Western blotting was performed to analyze Twist protein expression, which increased significantly in both MCF7 and HS578T BP1 overexpressing cell lines (Fig. 10). Although the HS578T parental cell line expressed Twist, BP1 overexpressing cells still showed a 2 fold increase in Twist expression compared with the empty vector.

A BP1 consensus binding site (5’WTCWATATG3’, W=A/T) was found upstream of the Twist gene (Fig. 11). Chromosome Immunoprecipitation (ChIP) was performed to verify whether BP1 actually binds to this site. The result shows that MCF7 O1 has a significantly stronger signal than MCF7 V1 cells. MCF7 cells have low BP1 expression, and a low signal (Fig. 12). In conclusion, BP1 appears to upregulate Twist expression by directly binding to its upstream region.
Figure 9. High BP1 expression increases TWIST mRNA levels in MCF7 O1 cells.

Relative expression levels of Twist mRNA in BP1 overexpressor (O1) and empty vector (V1). The MCF7 O1 Twist RNA level is 10 fold higher than in MCF7 V1 cells. RealTime PCR was performed as described in Material and Method. The numbering on the left side indicates the mRNA fold-changes compare to V1B.
Figure 10. The TWIST protein level increases in both MCF7 and HS578T BP1 overexpressing cells. Western blot analysis of Twist expression in MCF7 and HS578T cells BP1 overexpressing BP1. The BP1 overexpressing cells show a reproducible increase in Twist expression. MCF7 O1 and HS578T O3/O7 are BP1 overexpressing cell lines and MCF7 V1/V2 and HS578T V2 are controls. Numbering on the bottom of the graph indicates the relative fold increase of signal compare to the V2 cells. The Western blot was performed as described in Material and Method.

Figure 11. A BP1 consensus binding site on the upstream of Twist gene.
Figure 12. BP1 binds upstream of the Twist gene. ChIP assay was performed as described in Material and methods to verify a BP1 binding site upstream of the Twist gene. Here we found that MCF7 O1 (BP1 overexpressing) cells have a significantly stronger signal than MCF7 V1 (empty vector) cells. The signal is the expected PCR product amplifying from the BP1 consensus binding site at the upstream of Twist gene.
2. BP1, a novel factor that induces an incomplete EMT phenotype in breast cancer cell lines.

MCF7 O1 and HS578T O3/O7 cells were used as test group, while MCF7 V1 and HS578T V2 were empty vector controls. Cell morphology was observed under a phase contrast microscope (10x magnification). The morphological change induced by BP1 is not dramatic in MCF7 cells; MCF7 V1/O1 share nearly the same cobblestone like structure. But HS578T O3 and O7 exhibits a more elongated morphology than the V2 cells, which have a more round shape (Fig. 13).

The expression of epithelial and mesenchymal markers was also probed to verify whether BP1 promotes a more mesenchymal phenotype or decreased epithelial phenotype. E-cadherin was probed at both the RNA and protein level. The E-cadherin mRNA level did not change in MCF7 cells comparing MCF7 O1 and V1 (Fig. 14), but the protein was downregulated two fold in MCF7O1 (Fig. 15). Confocal microscopy further confirmed that E-cadherin was downregulated in MCF7 BP1 overexpressing cells. The samples were fixed and then stained for E-cadherin (Fig. 16). β-catenin, another epithelial marker, demonstrated a decrease in BP1 overexpressing cells of MCF7 and HS578T cells (Fig. 17).
Figure 13. The cell morphology of MCF7 and HS578T derived cell lines. The morphology of MCF7 O1 cells does not show a significant alteration. HS578T O3/O7 cells show a more elongated, spindle like shape compared with V2 cells. The cells were monitored using Nikon Eclipse TE300 phase contrast microscope and under 10x magnification.

Figure 14. E-cadherin mRNA levels in MCF7 derived cell lines. E-cadherin mRNA levels do not have a significant change between BP1 overexpressing cells (O1) and empty vector cells (V1). The numbering on the left side of the legend indicates the relative RNA fold compared to the V1 cells. Same as the figure legend 10.
Figure 15. E-cadherin protein levels in MCF7 and HS578T derived cell lines.

E-cadherin protein levels decreased in MCF7 O1. HS578T cells have no E-cadherin expression. The numbering on the bottom indicates the relative fold decrease of signal compared to the V1 cells. Western blot described as the same in the figure legend 10.

Figure 16. E-cadherin protein localization in vivo. MCF7 V1 and O1 were fixed and stained for E-cadherin and observed by confocal microscopy as described in Materials and Methods. The E-cadherin level was reduced dramatically in BP1 overexpressing cells.
Vimentin, a mesenchymal marker, is shown to be slightly increased in MCF7 O1 compared to MCF7 V1 cells. But in HS578T cells, which already have a mesenchymal phenotype, there is no significant change of vimentin expression in BP1 overexpressing cells compared with empty vector (Fig. 18). Fibronectin, another mesenchymal marker protein, was also shown to be increased in HS578T O7 cells compared to HS578T V2 cells. However, fibronectin is not expressed in either MCF7 V1 or O1 cells (Fig 19).

In conclusion, BP1 can induce an incomplete EMT in MCF7 and HS578T cells under normal growth conditions. It inhibited cell-cell interaction by downregulating E-cadherin and β-catenin expression. Therefore BP1 can push cancer cells toward a more malignant phenotype.
Figure 17. β-catenin protein levels in MCF7 and HS578T derived cell lines. The β-catenin expression levels decreased by 40 to 50% in BP1 overexpressing cell lines compare to empty vector controls. Western blot was performed as described in Material and Methods. The numbering on the bottom indicates the relative fold decrease of signal which MCF7 O1 cells compare to the V1 and HS578T O3/O7 compare to the V2 cells.

Figure 18. Vimentin protein levels in MCF7 and HS578T derivative cell lines.

Vimentin protein levels increased in MCF7 O1 compared with V1 cells. There was no significant difference between V2 and O3/O7 cells. Western blot performed as described in Material and Methods.
Fibronectin protein levels increased in HS578T O7 compared with V2 cells.

Fibronectin was not expressed in either MCF7 V1 or O1 cells. Western blot was performed as described in Material and Methods. The numbering on the bottom indicates the relative folds of signal which the O7 cells compare to the V2 cells.
3. BP1 promotes migratory ability in breast cancer cell lines.

A wound healing assay was conducted to test the ability of BP1 to promote breast cancer cell motility. Two approaches were used: adding exogenous rpBP1 and comparing overexpressor (O1 and O7) and empty vector (V1 and V2) cell lines. The scratch test was done in T47D breast cancer cells line in media supplemented with different concentrations of recombinant BP1 protein (rpBP1), 0, 1ng/ml, 2ng/ml.

The closure rate of the wound was measured at 0hr, 24hrs and 48hrs after the wound was induced. T47D cells grown in 1ng/ml rpBP1 supplemented media have a 1.5 fold increase in wound closure rate compared to the cells grown without rpBP1 (Fig. 20).

The migration assay was also performed in MCF7 and HS578T derived cells to determine whether the overexpressing cells would have a higher migratory ability compared to the empty vector. Overexpressing BP1 seems to increase the migratory ability of HS578T (Fig.11B) but not MCF7 cells (Fig.11C). These results correspond to our previous data showing that BP1 induces incomplete EMT in MCF7 cells.
Figure 20. rpBP1 can increase cancer cell migratory ability. A wound healing assay was performed with T47D cells grown in media supplemented with 0ng/ml, 1ng/ml and 2ng/ml rpBP1. Phase contrast microscopic observations were recorded at 0, 24 and 48hrs at same condition described in the figure legend 13. The closure speed was calculated and compared.
**Figure 21. BP1 expression can increase HS578T cell migratory ability.** The wound was totally filled and recorded at 0 and 24 hrs under same conditions as described in the figure legend 13. The wound closed after 24hrs in O7 cells but not in V2 cells, demonstrating that HS578T O7 (overexpressing BP1) has significantly higher migratory ability than V2.
MCF7 V1B

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MCF7 O1

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**Figure 22. BP1 expression does not induce significant changes in MCF7 cell migration.**

This wound healing assay was performed with MCF7 V1 and O1 cells; the time frames selected were 0hr, 6hr and 12hrs, the same conditions as described in the figure legend 13.
4. Secreted BP1 protein can promote EMT.

Exogenous BP1, secreted into microenvironment, can promote Twist mRNA expression (unpublished data, J Rheey and P. Berg). MCF7 cells were grown in media supplemented with 1ng/ml rpBP1. Cells were passaged in media containing rpBP1 for 3 passages and the protein was harvested. A Western blot probing for Twist was analysed. In the presence of 1ng/ml of rpBP1, Twist expression upregulated about 1.5 fold (Fig. 23).

5. Recombinant BP1 proteins can stimulate cell proliferation.

MCF7 cells were grown for 5 days in unsupplemented and rpBP1 supplemented complete media (containing 10% FBS and 1% PS). By day 3, the viability of the cells grown in 1 μg/ml and 5 μg/ml rpBP1 supplemented media was 1.7 to 2.2 fold higher for cells grown in the unsupplemented media. By day 5, the cells grown in 100ng/ml, 500ng/ml, 1 μg/ml and 5 μg/ml of rpBP1 supplemented media showed viability 3 to 4 fold higher than the cells grown in unsupplemented media (Fig. 24). This indicated that exogenous BP1 can increase cell proliferation.
Figure 23. Twist expression is upregulated in the presence of 1ng/ml rpBP1.

Western blotting was performed and showed Twist protein levels upregulated by 1.47 fold in MCF7 cells cultured in rpBP1 supplemented media. The numbering on the bottom indicates the relative fold increase of which signal compared to the V1 cells. Western blot was performed as the same as figure legend 10.
Figure 24. The mitogenic effect of rpBP1. MTT assay showing that rpBP1 can promote MCF7 cell proliferation. Cells were grown in media supplemented with 100ng/ml, 500ng/ml, 1ug/ml or 5ug/ml rpBP1. The numbering on the left side indicated the fold increase of MTT absorbance at 570nm as compared to the cells grown in the control with no rpBP1.
Discussion

From past research, we know that BP1 is associated with breast cancer aggressiveness (Man et al., 2005), estrogen receptor negativity (Fu et al., 2003) and probably metastasis (Man et al., 2009; Yu et al., 2008). However, knowledge of the mechanism of BP1 action is very limited, especially its downstream targets and how it affects cancer cells. The purpose of this study was to seek mechanisms by which BP1 may promote cancer invasiveness and metastasis.

We found that BP1 binds upstream of Twist gene, which may lead us to know more about regulation of Twist expression in metastasis and development, since homeotic proteins also play important roles in development. Few upstream regulators of Twist have been identified, only TNF induced NF-KB pathway (Sosic et al., 2003), Wnt-1 (Howe et al., 2003) and HIF-1α (Yang et al., 2008). It will be important to determine whether these upstream regulators interact with each other. It is highly possible that this BP1/Twist axis also plays a role in development. Since BP1 is directing EMT through Twist, one interesting question is what is the upstream regulator or environmental factor which controls this BP1/Twist axis? Since Twist is a direct target of HIF1-α (Yang et al., 2008), hypoxia may be a possible upstream regulator of the BP1/Twist axis. It was found that the HIF-1α/Twist axis upregulates VEGF expression
(Yang et al., 2008), which is consistent with the finding that BP1 directly upregulates VEGF expression (Song et al., 2009). On the other hand, a HIF-1α consensus binding site was found upstream of the BP1 promoter region (unpublished data). Therefore, we hypothesize that HIF-1α might upregulate BP1 and Twist, therefore promoting metastasis.

We tested the effect of BP1 on the expression of various epithelial and mesenchymal markers. We found that BP1 alone might not be sufficient to induce epithelial cancer cells to gain the full mesenchymal phenotype. Therefore, BP1 is more likely to promote cancer metastasis by incomplete or partial EMT. On the other, complete EMT is not required for metastasis (reviewed in Christiansen, 2006); there is a low frequency of complete EMT in invasive human breast cancer (Trimboli et al., 2008). The reason why BP1 induces incomplete EMT instead of complete EMT might be explained if it only upregulates Twist but not Snail 1. It was reported that during development and carcinoma progression, Snail 1 is expressed at the onset of the transition while Twist is subsequently induced to maintain the migratory mesenchymal state (Peinado et al., 2007; also reviewed in Foubert et al., 2010). On the other hand, Twist seems to regulate different pathways than Snail, and Twist expression cannot compensate for a Snail knockdown (Yang MH, 2008). Therefore,
this might explain why BP1 can only promote partial EMT. So our next step in this project is to verify whether BP1 regulates Snail expression.

EMT might provide tumor cells with the ability to adapt to physiologically relevant stresses such as low oxygen or nutrient levels (Trimboli et al., 2008). It was also consistent with our finding that BP1 expression is upregulated by serum starvation (unpublished). The possibility of starvation controlling the BP1/Twist axis and inducing EMT is under investigation.

Our results also show that BP1 downregulates the E-cadherin protein level significantly in MCF7 cells (Fig. 15/D) but the mRNA level did not change appreciably (Fig. 17). This indicates that BP1 might downregulate E-cadherin expression by a posttranslational mechanism, although the mechanism is unclear. For example, it was reported that E-cadherin can be internalized and shuttled to lysomes for degradation during EMT, which is mediated by E-cadherin ubiquitination (Palacios, et al., 2005).

Our results also indicate that BP1 increases breast cancer cell migration. It is consistent with the past finding that BP1 overexpression in HS578T cells increased
invasiveness, which was shown by a matrigel chamber assay (Fu et al., 2009). This is also consistent with our previous microarray finding showing BP1 can upregulate MMPs which play a very important role in tumor invasion (as reviewed in Seiki, 2003). Our finding that a BP1/Twist axis promotes EMT explains the mechanism for our previous finding that BP1 increases breast cancer cell migration.

Our data showing that BP1 can upregulate Twist expression and promote EMT is also consistent with the previous finding that BP1 can inhibit apoptosis (Stevenson et al., 2007). EMT participates in resistance to apoptosis, which is relevant to tumor progression. TGF-β exposure induces hepatocytes and breast tumor cells to undergo EMT and become resistant to apoptosis (Gal et al., 2008; Valdés et al., 2002). Whether TGF-β activates BP1 will be studied in the future. Twist-AKT2 signaling was also found to promote survival of breast cancer cells by resistance to Paclitaxel (Cheng et al., 2007). On the other hand, Twist was also reported to inhibit apoptosis through modulation of the ARF/MDM2/p53 pathway (Maestro et al., 1999). This also could explain why BP1 is associated with breast cancer metastasis. Metastasis is considered an inefficient process since thousands of cancer cells migrate into the blood circulation but only a few can survive and make their way to secondary organs and colonize them (DiMeo et al., 2009). Therefore, BP1 might increase metastasis by
inhibiting apoptosis.

Our results also indicate the connection between BP1, EMT and estrogen receptor (ER) negativity. BP1 is associated with ER negative breast cancer (Fu et al., 2003). ER signaling can regulate E-cadherin expression and therefore influence EMT. Knocking down ER increases Slug expression, which is a transcription factor trigger of EMT in cell lines (Ye et al., 2010). ER was found to indirectly activate MTA3 (metastasis associated 1 family, member 3), which can repress Snail1, and Snail 1 is known to induce EMT (Fujita et al., 2003). Snail also represses ER expression and increases TGF-β signaling (Dhasarathy et al., 2007). On the other hand, ER signaling can downregulate Slug, thereby inhibiting EMT in breast cancer (Ye et al., 2010). BP1 was also found to downregulate ER expression (unpublished data). Therefore, we can further link the BP1/Twist axis with EMT and ER. BP1 is associated with ER negativity and ER negativity is associated with increased EMT, while ER is positivity associated with decreased EMT.

BP1 can promote cell migration and Twist expression in a paracrine manner, shown by growing cells in the presence of rpBP1 (Fig 4 and 5). This indicates that BP1 could potentially promote surrounding cells to undergo EMT and affect the
microenviroment. Twist is known to be under the control of the microenviroment for induction of EMT (reviewed in Foubout et al., 2010). Moreover, we found BP1 expression is upregulated by serum starvation (unpublished data). Therefore we hypothesize that BP1 might play an important role in tumor microenviroment regulation of tumor progression and metastasis.

To further verify the mechanism of the BP1/Twist axis in promoting EMT, a series of experiments are planned. RNA interference experiment of BP1 would be included to see whether BP1 knockout in mesenchymal breast cancer cells, e.g., MDA-MB-231, revert its mesenchymal phenotype to become more epithelial. The next question we would like to ask is which pathway the BP1/Twist axis regulates in the induction of EMT. BP1 expression was found to upregulate AKT phosphorylation in MCF7 breast cancer cells in our lab (unpublished data). Activated AKT can mediate the NFKB/Snail pathway, which induces EMT (Julien et al., 2007). AKT phosphorylation on Ser 473 can increase Snail protein and therefore promote EMT. Snail1 is marked for ubiquitination after phosphorylation by activated GSK-3 and phosphorylated AKT (Ser 473) can inhibit GSK-3 activity therefore increasing the Snail1 protein half-life (Rowe et al., 2009). Therefore, the BP1/Twist axis association with the PI3K/AKT pathway is another target for investigation.
This research indicated the role BP1 might play in cancer metastasis. Since BP1 induces cancer metastasis through upregulation of Twist expression and EMT, both essential steps in embryonic development, it could also indicate the role that BP1 might play in normal development.
References


