Genetic and Expression Aberrations of E3 Ubiquitin Ligases in Human Breast Cancer

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Abstract
Recent studies revealed that E3 ubiquitin ligases play important roles in breast carcinogenesis. Clinical research studies have found that (epi)-genetic (deletion, amplification, mutation, and promoter methylation) and expression aberration of E3s are frequent in human breast cancer. Furthermore, many studies have suggested that many E3s are either oncogenes or tumor suppressor genes in breast cancer. In this review, we provide a comprehensive summary of E3s, which have genetic and/or expression aberration in breast cancer. Most cancer-related E3s regulate the cell cycle, p53, transcription, DNA repair, cell signaling, or apoptosis. An understanding of the oncogenic potential of the E3s may facilitate identifying and developing individual E3s as diagnosis markers and drug targets in breast cancer. (Mol Cancer Res 2006;4(10):695–707)

Introduction
Breast Cancer
Breast cancer is the second leading cause of cancer death in American women. The American Cancer Society estimates that there will be about 212,920 new cases of breast cancer, and that about 41,430 women will die of this disease in the United States in 2006 (1). Hormone therapy and chemotherapy have been shown to improve survival of patients with breast cancer; however, the serious side effects of these treatments inspire the development of targeted therapy. It is important to understand the mechanism(s) involved in breast cell transformation and to identify novel molecular targets for use in prevention, diagnosis, and therapy.

Genetic Alterations of Breast Cancer
Many genetic studies of breast cancer show that tumor development and progression involves the accumulation of various genetic defects, including amplification and concomitant overexpression of certain oncogenes and mutation or loss of various tumor suppressor genes. Accumulated evidence suggests that amplification and overexpression of Her-2/ErbB-2 (17q12), cyclin D1 (11q13), and Myc (8q24) contributes to a subset of breast cancer. Simultaneously, germ line mutations of BRCA1 and BRCA2 play important roles in a subset of familial and sporadic breast cancer.

However, breast cancer is heterogeneous, and many other genetic alterations have also been detected. For example, gene amplification at 17q23 (2) and 20q11-13 (3) and chromosome loss at 8p22 (4), 11q23 (5), and 16q22 (6) are frequently detected in breast cancer by fluorescent in situ hybridization, comparative genomic hybridization, and loss of heterozygosity studies. The implicated target genes of these genetic alterations have not been firmly revealed, although a number of interesting candidate genes may contribute to progression of breast cancer. In addition to chromosomal instability and gene mutation, epigenetic alteration by abnormal promoter methylation seems to be another approach to inactivate some tumor suppressor genes in breast cancer (7).

There are an overwhelming number of abnormalities that have been identified in breast cancer in molecules involved in protein degradation through the ubiquitin proteasome system (UPS). The field of protein ubiquitination and degradation has experienced explosive growth in the past decade. The UPS regulates an enormous range of biological processes, including the cell cycle, apoptosis, transcription, protein trafficking, signaling, DNA replication and repair, and angiogenesis (see refs. 8, 9 for reviews). Consistently, defects in the UPS have already been directly implicated in many diseases, including breast cancer (10-13). Well-known examples are the deregulation of the ubiquitin ligases BRCA1, Mdm2, and Skp2 (see below in detail).

UPS
Protein homeostasis in mammalian cells is tightly controlled by protein synthesis and degradation. Rapid protein turnover is a typical feature for many important proteins that regulate the cell cycle, apoptosis, and transcription, such as p53, p27kip1, and Myc. Selective degradation of these proteins through the
UPS plays an essential role in normal cell growth and differentiation, whereas abnormal accumulation or hyperactive degradation of these regulatory proteins are associated with carcinogenesis.

Proteins degraded through the proteasome are often first tagged by a K48 polyubiquitin chain (below in detail), through the action of three classes of enzymes: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Although the human genome encodes a unique E1 and limited numbers of E2s (~50), there are hundreds of different E3s in human cells. Ubiquitin is a small conserved protein with 76 amino acids. E1 binds to and activates ubiquitin and then passes the activated ubiquitin to an E2. The E2s have only limited substrate specificity. Typically, an E2 transfers the ubiquitin to a substrate and conjugates the ubiquitin moieties to the NH2 groups of the substrate’s Lys through interaction with an E3. The E3 is a scaffold protein that recognizes specific substrates.

Attachment of a single ubiquitin to a single lysine of a substrate protein is called monoubiquitination. Most membrane receptors, such as epidermal growth factor receptor and platelet-derived growth factor receptor, with monoubiquitin modification undergo endocytosis and are degraded in lysosome (reviewed in refs. 14, 15). Additionally, monoubiquitin modification for transcription factors, such as Smad4 (16) and p53 (17), can also serve as a signal for transcription activation or trafficking, respectively. Moreover, monoubiquitination of FANCD2 participates in DNA repair (reviewed in ref. 18).

Following monoubiquitination, the second ubiquitin molecule can be conjugated to previous one through an isopeptide bond between Gly76 of a new ubiquitin molecule and the ε-NH2 groups of one of the seven lysines (K6, K11, K27, K29, K33, K48, and K63) of the previous conjugated ubiquitin. After several rounds of catalysis by the E2/E3 complex, a polyubiquitin chain will be conjugated to a substrate protein. The predominant polyubiquitin chains in cells are through the K48 and K63 branching points. As mentioned earlier, proteins tagged with a K48 polyubiquitin chain are able to be rapidly degraded by a large cytosolic protease complex named the 26S proteasome. On the other hand, proteins with a K63 polyubiquitin chain will not be targeted for degradation but rather have been shown to play important role in nuclear factor-κB signaling transduction (reviewed in ref. 19).

Protein ubiquitination and degradation are usually regulated in a temporal and spatial manner. In most cases, the substrate must be modified first, through mechanisms such as phosphorylation, polyhydroxylation, acetylation, glycosylation, or cleavage before the destruction domain is recognized by E3s (20). Alternatively, the E3 itself could be activated by posttranslational modification. For example, the anaphase-promoting complex/cyclosome (APC/C) is phosphorylated and activated when cells enter mitosis. Activity of AIP4/ITCH is dramatically activated by c-Jun NH2-terminal kinase–mediated phosphorylation and negatively regulated by Src kinase Fyn-mediated tyrosine phosphorylation (21, 22). By ubiquitin-mediated proteolysis, cells quickly regulate the cell cycle, transcription, migration, and apoptosis in response to internal and external signaling.

As several extensive reviews of ubiquitination in breast cancer are available (12, 13), here, we provide a comprehensive review on the role of E3s in breast cancer. We focus on recent advances concerning aberrant expression and genetic alterations of E3s in breast cancer and also common mechanisms of E3 action in breast cancer. Finally, we discuss the challenge of identifying and developing E3s as drug targets and diagnosis markers in breast cancer.

### E3 Ligase Subfamilies

E3s carry out the key rate-limiting step in ubiquitin-mediated proteolysis. In mammalian cells, abundant ubiquitin ligases guarantee specific substrate recognition. Researchers have now identified >500 E3s (Table 1). Many of these E3s contain the homologous to E6-associated protein (E6-AP) COOH terminus (HECT) domain or the really interesting new gene (RING) finger domain. Although all HECT domain and some RING finger E3s can function independently, some RING finger proteins form E3 complexes with Cullins and several other proteins, such as F-box; brc a brac, tramtrack, and broad complex (BTB)-box; or suppressor of cell signaling (SOCS)-box proteins. Recently, two RING-like domains, the U-box and the plant homeo domain (PHD), have also been shown to have E3 activity. Here, we outline the basic features of each E3 ligase subfamily.

#### HECT Domain E3s

The number of proteins containing HECT domain in the human genome is estimated to be about 82 according to the Pfam database. The actual number that has been characterized from humans is around 30 (23). The HECT domain containing proteins can form thiol intermediates with ubiquitin and function independently as E3s (Fig. 1). Several HECT domain E3s, such as human papilloma virus gene product E6-AP, Smurf2, WWP1, and ARF-BP1, have been implicated in human breast cancer. For example, E6-AP is an E3 enzyme that targets the p53 protein (24). The catalytic cysteine near the COOH terminus of E6-AP can transfer ubiquitin from E2 to p53, leading to the ubiquitin-dependent degradation of the p53.

#### Table 1. Classification and Estimated Numbers of Human E3s

<table>
<thead>
<tr>
<th>E3s</th>
<th>Subfamily</th>
<th>No. proteins</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HECT</td>
<td>~30-82</td>
<td>(23)</td>
<td></td>
</tr>
<tr>
<td>RING finger</td>
<td>~350-510</td>
<td>(23)</td>
<td></td>
</tr>
<tr>
<td>U-box</td>
<td>6-16</td>
<td>(27)</td>
<td></td>
</tr>
<tr>
<td>PHD domain</td>
<td>211</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cullin-based</td>
<td>Cullins</td>
<td>~5-24</td>
<td>(202)</td>
</tr>
<tr>
<td>E3s and APC/C</td>
<td>F-box</td>
<td>~70</td>
<td>(33, 203)</td>
</tr>
<tr>
<td></td>
<td>SOCS</td>
<td>~40-72</td>
<td>(204)</td>
</tr>
<tr>
<td></td>
<td>BTB</td>
<td>~183-273</td>
<td>(38)</td>
</tr>
<tr>
<td></td>
<td>Skp1, ElonginC, DDB1/2, APC/C, etc</td>
<td>&gt;15</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** The estimated numbers from the cited literature and the Pfam data base are shown.
RING Finger E3s

There are >350 potential RING finger proteins; hence, this class represents the largest subfamily of ubiquitin ligases. Consistently, many RING finger E3s, including Mdm2, BRCA1, BARD1, and estrogen-inducible RING finger protein (EFP), have been suggested to play important roles in mammary carcinogenesis. The RING finger motif contains an octet of cysteine and histidine residues that constitute a zinc binding domain. It has become clear that many RING finger containing proteins function as E3 enzymes. Some RING finger proteins can function alone (Fig. 1), such as Mdm2 and Parkin, whereas others form complexes with other proteins to function as E3s (Fig. 1). For example, Roc1 is associated with E2 and Cullin1 (Cul1) in the Skp1, Cul1, F-box (SCF) complexes.

Additionally, two types of RING finger–like domains, such as U-box and PHD, also have E3 activity. The predicted three-dimensional structure of the U-box is similar to that of the RING finger except there are no metal-chelating residues in the dimensional structure of the U-box is similar to that of the RING finger. Similar to Skp1 in the SCF complex and Elongin C-SOCS, or BTB proteins. Right, examples for each type of E3. S, substrate of an E3.

F-Box Proteins

The F-box is an ~40-amino-acid motif, originally identified in cyclin F (34). F-box–containing proteins mediate substrate recognition for SCF complexes. F-box proteins bind to Skp1, which, in turn, binds Cul1. In addition to interaction with Skp1 through the F-box motif, F-box proteins recruit substrates through distinct protein-protein interaction domains, such as WD40 repeats and leucine-rich repeats. There are >70 F-box proteins in the human genome; however, few have been characterized in detail (33). The most well studied F-box proteins in breast cancer are Skp2, β-TrCP, and Fbw7.

SOCS Proteins

Proteins with the SOCS-box motif, similar to F-box proteins, can bridge the specific substrates in ECS-type E3s. Besides interacting with Cul2/5, SOCS proteins interact specific substrates through either WD40 repeats, ankyrin repeats, SPRY domains, or tubby domains. More than 40 members from nine families have been identified as SOCS proteins, the most well studied being von Hippel-Lindau. In the case of von Hippel-Lindau, a major substrate is HIF1-α. It has been well documented that von Hippel-Lindau is frequently mutated in renal carcinoma. It has been reported that deficiency of SOCS1 leads to accelerated mammary gland development (35), and methylation of SOCS1 contributes to breast tumorigenesis (36). Recently, SOCS1 was shown to inhibit tumor necrosis factor-α signaling through targeting apoptosis signal-regulating kinase 1 for ubiquitin-mediated degradation (37).

BTB-Box Proteins

Similar to Skp1 in the SCF complex and ElonginC in the ECS complex, BTB proteins function as subunits of Cul3-based E3s. Although Cul1 and Cul2 ligases each use a two-protein module in substrate recognition and culin binding, Cul-3 ligases use single BTB proteins. BTB proteins possess a protein-protein interaction domain so that they can function as substrate adaptors like F-box proteins and SOCS proteins.
Human BTB proteins typically recruit substrates through MATH, Kelch, or Zn-Finger domains. Although there are >183 BTB proteins in the human genome (38), at present, only a few of them have been shown to connect to the UPS. Furthermore, their in vivo substrates remain to be identified.

**APC/C E3**

The APC/C comprises ~12 subunits. Similar to SCF E3s, it contains a cullin domain protein APC-2 and a RING protein APC-11. The substrate recognition is mediated by two different activators: Cdc20 and Cdh1. APC/C is temporally regulated during the cell cycle (39). Cdc20 is up-regulated in late G2 and

**Table 2. Oncogenic E3 Ligases in Human Breast Cancer**

<table>
<thead>
<tr>
<th>E3</th>
<th>Type</th>
<th>Gene locus</th>
<th>Alteration in breast cancer</th>
<th>Major targets</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF-BP1</td>
<td>HECT</td>
<td>Xp11.22</td>
<td>Overexpression</td>
<td>P53, McI</td>
<td>Promoting cell growth and anti-apoptosis</td>
</tr>
<tr>
<td>WWP1</td>
<td>HECT</td>
<td>8q21</td>
<td>Amplification and overexpression</td>
<td>Smad2, TjR1, KLF2, KLF5</td>
<td>Transcription and signal transduction</td>
</tr>
<tr>
<td>Mdm2</td>
<td>RING</td>
<td>12q14.3-q15</td>
<td>Amplification, mutation, overexpression, and aberrant RNA splicing</td>
<td>P53</td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>RNF11</td>
<td>RING</td>
<td>1p31-32</td>
<td>Overexpression</td>
<td>AMSH</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>EFP</td>
<td>RING</td>
<td>17q23.1</td>
<td>Overexpression</td>
<td>14-3-3-σ</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>XIAP</td>
<td>RING</td>
<td>Xq25</td>
<td>Overexpression</td>
<td>Smac, Caspases</td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>BCA2</td>
<td>RING</td>
<td>1q21.1</td>
<td>Amplification and overexpression</td>
<td>P27, p21, FoxO1</td>
<td>Cell cycle, transcription</td>
</tr>
<tr>
<td>Skp2</td>
<td>F-box</td>
<td>5p13</td>
<td>Amplification and overexpression</td>
<td>InB, β-catenin, CDC25A, DDB2, Jun</td>
<td>Cell cycle, transcription, DNA repair and anti-apoptosis</td>
</tr>
<tr>
<td>Cul4A</td>
<td>Culin</td>
<td>13q34</td>
<td>Amplification and overexpression</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** For references, please see E3s as Oncogenes in Breast Cancer.
mediates the degradation of securins and cyclins to induce entry into and progression through mitosis, whereas Cdh1 levels increase during anaphase and control mitotic exit through the degradation of cyclin B and other substrates. Thus, inhibition of the APC/C complex is believed to be a good strategy for an anticancer therapy because it will block cell cycle progression.

**E3s as Oncogenes in Breast Cancer**

Many E3s could be oncogenes or tumor suppressor genes because frequent deregulation of E3s has been shown in human carcinogenesis. Some E3s have established roles in cell cycle and apoptosis, such as the Mdm2 and the SCF^Skp2 complex. More recently discovered E3s, such as ARF-BP1, WWP1, and breast cancer–associated gene 2 (BCA2), may be similarly important in breast tumorigenesis. These E3s are overexpressed in human breast cancer, and their inhibition leads to growth arrest or apoptosis. The well-studied oncogenic E3s in breast cancer are summarized in Table 2 and discussed in detail.

**ARF-BP1/HUWE1**

The protein ARF-BP1 is a HECT domain E3 that targets the p53 protein for ubiquitin-mediated proteolysis (40-42). ARF-BP1 was shown to be a critical mediator of both the p53-independent and p53-dependent tumor suppressor functions of ARF (42). The gene encoding ARF-BP1 is located at Xp11, and gains on chromosomes Xp11-13 have been detected in breast cancer (43). In agreement with this, ARF-BP1 has been shown to be overexpressed in colorectal and breast cancer.

**WWP1**

WWP1 is located at 8q21, a region frequently amplified in human breast cancer. Indeed, copy number gain and overexpression is frequently detected in human prostate and breast cancer (44, 45). WWP1 is also a HECT domain–containing E3. Growing evidence suggests that WWP1 negatively regulates the transforming growth factor-β (TGF-β) tumor suppressor pathway by mediating the ubiquitination and degradation of Smad2 (46), Smad4 (47), and TGF-β receptor 1 (48). Besides inhibiting the above components of the TGF-β pathway, WWP1 has recently been shown to function as an E3 for two transcription factors: KLF2 (49) and KLF5 (44). KLF2 and KLF5 have been shown to be frequently down regulated in ovarian and breast tumors, respectively (50, 51). Both transcription factors were shown to induce apoptosis through regulating transcription of their target genes (52, 53). WWP1 may also play a role in other biological processes, including regulation of epithelial sodium channels, viral budding, and receptor trafficking (54). Many studies have found that WWP1 directly binds to specific targets through its WW domains, which can interact with a proline-rich motif in the target proteins (44, 46). Alternatively, WWP1 targets Smad4 and TGF-β receptor 1 through adaptor proteins Smad2 (47) and Smad7, respectively (48).

**Mdm2**

Gene amplification, mutation, overexpression, and aberrant splicing have been identified in 19 tumor types, including breast (55-58). It has been shown that expression of aberrantly spliced Mdm2 mRNA in breast carcinoma was associated with a shortened overall patient survival (59). Although Mdm2 has been characterized as a RING finger E3 for the tumor suppressor p53 (60), Mdm2 also has transforming potential independent on the p53 (61, 62). Inhibiting the interaction of Mdm2 with p53 has therefore been a focus in drug discovery (63, 64). Recently, several potent and selective small-molecule antagonists of Mdm2 have been identified (65-67).

**EFP**

The EFP stimulates proliferation of breast cancer through facilitating ubiquitin-mediated destruction of a tumor suppressor 14-3-3ζ (68). Recently, EFP was shown to function as an E3 for ISGylation of 14-3-3ζ in response to IFNs (69). EFP may regulate a switch from estrogen-dependent to estrogen-independent growth of breast tumors because EFP overexpressed estrogen-dependent MCF-7 breast cancer cells gained the ability to form tumors in nude mice in the absence of estrogen (68). Inhibition of EFP expression by antisense oligonucleotides reduced tumor growth (68). The protein expression of EFP is significantly correlated with poor prognosis of breast cancer patients (70). Therefore, EFP may be a new valuable biomarker and molecular target for breast cancer (71).

**X-Linked Inhibitor of Apoptosis Protein/BIRC4**

Several inhibitor of apoptosis proteins (IAP) possess RING finger domains that are able to bind E2s, ultimately promoting ubiquitination of target proteins. In addition to directly binding and inhibiting caspases, IAPs may cause ubiquitination and subsequent proteolysis of caspases and other apoptotic regulators. For example, X-linked IAP (XIAP) targets caspase-3, caspase-7, and caspase-9 for degradation and may also enhance its own activity by mediating ubiquitination of its antagonists, including second mitochondria-derived activator of caspases (Smac; refs. 72-75). Overexpression of XIAP may cause the resistance to apoptosis induced by cytotoxic drugs in breast cancer. It has been shown that inhibition of XIAP by RNA interference suppresses MCF-7 xenograft tumor growth and sensitizes cells to etoposide and doxorubicin (76, 77). Thus, the role of IAP proteins in ubiquitination is an emerging field with important implications for resistance to apoptosis in tumor cell chemotherapy.

**RING Finger Protein 11**

Both mRNA and protein of RING finger protein 11 (RFN11) are highly expressed in breast cancer cells (78), although its role in breast tumorigenesis remains to be elucidated. RFN11 was found to cooperate with Smurf2 to degrade an enzyme AMSH, a positive regulator of both TGF-β and epidermal growth factor receptor signal pathways (79). RFN11 interacts with multiple HECT-domain E3s and Cul1 (80). Like the role of Skp1/2 in SCF E3, RFN11 may function as an adaptor to bridge some substrates to HECT domain E3s for ubiquitination because a large number of interacting partners (~80) have been revealed (80).
BRA2/ZNF364

BRA2 is another E3 with a RING domain. The BRA2 gene is located at 1q21.1, a region frequently amplified in breast cancer. Using immunohistochemistry and tissue microarray, Burger et al. showed that BRA2 protein was overexpressed in invasive breast cancer but correlated with positive estrogen receptor, negative lymph node metastasis, and increased survival (81). Inhibition of BRA2 by small interfering RNA suppressed T-47D and MCF7 cell proliferation (81). The targets of BRA2 E3 have not been identified.

Skp2

The Skp2 gene is located at 5p13, a region found to be amplified in 11% of breast cancer cell lines in a comparative genomic hybridization study (82). Skp2 has been reported to be overexpressed in a subset of breast carcinomas (estrogen receptor and Her-2 negative), and Skp2 expression inversely correlates with p27KIP1 levels in a variety of human tumors, including breast carcinomas (83, 84). Thus, Skp2 may be a potential specific biomarker and therapeutic target in a subset of aggressive breast carcinomas. Skp2 is an extensively studied F-box protein required for the ubiquitin-mediated degradation of the p27KIP1 (85), p21CIP1 (86), p130 (87), and FoxO1 (88) by SCF E3. Interestingly, the ubiquitination of p27KIP1 by SCFKIP2 requires an accessory protein Csk1 (89). Recent structural studies show that Csk1 binds to the phosphorylated Thr187 side chain of p27KIP1 (90). Expression of Csk1 is associated with decreased tumor differentiation and poor disease-free and overall survival outcome in breast cancer (91).

β-TrCP1/BTRC1

β-TrCP1 is another F-box protein but differs from Skp2 in that it harbors a β-transducin repeat domain. Overexpression of β-TrCP1 in mouse mammary gland epithelium promotes nuclear factor-κB activity and epithelial cell proliferation (92). Thirty-eight percent of transgenic mice develop tumors, including mammary, ovarian, and uterine carcinomas (92). Consistently, targeting of β-TrCP1 either by RNA interference or by forced expression of a dominant-negative β-TrCP1 mutant suppresses cell growth and sensitizes human breast cancer cells to the antiproliferative effects of anticancer drugs (93). β-TrCP1 mediates ubiquitination and degradation of β-catenin (94), IκB (95), CDC25A (96), Smad4 (97, 98), and Emi1 (99).

Cul-4A

The Cul-4A gene is located at 13q34 and is frequently amplified and overexpressed in breast cancer (100). Recently, Cul4A protein was shown to participate in the Mdm2-mediated proteolysis of p53 (101) and DET1-regulated c-Jun degradation (102). Additionally, Cul-4A expression is critical for early embryonic development because the homozygous deletion or heterozygous deletion of Cul-4A is lethal in a knock-out mouse model (103).

In addition to the E3s described above, many other E3s have also been shown to be oncogenic proteins in breast cancer, such as E6-AP (24), COP1 (104), and Pirh2 (105). All of these E3s target p53 for ubiquitin-mediated degradation.

E3s as Tumor Suppressor Genes in Breast Cancer

In contrast to oncogenic E3s, many other E3s, including BRCA1 and Fbw7, have been shown to be tumor suppressors in breast cancer. Frequently inactivating mutations or down-regulated expression of these E3s has been detected in breast cancer. Several recently discovered E3s, such as CHFR, SIAH1, and CHIP, may play a significant role in regulating breast tumorigenesis. Besides mutation and gene copy loss, epigenetic alteration (i.e., promoter methylation) also contributes to inactivation of these tumor suppressors. The E3s with tumor suppressor function in breast cancer were summarized in Table 3 and discussed below in detail.

BRCA1-BARD1

BRCA1 is a well-known tumor suppressor gene and is mutationally inactivated in familial forms of breast and ovarian cancer. BRCA1 protein contains a RING finger domain in its NH2 terminus. This RING finger domain has been shown to autoubiquitinate and monoubiquitinate histone H2A in vitro (106). BRCA1 interacts with another RING finger protein, tumor suppressor BARD1, which is also mutated albeit with low frequency in breast cancer (107, 108). BARD1 has BRCA1-independent and p53-dependent proapoptotic activity (109). BARD1 mRNA is lower in invasive breast cancers compared with normal breast tissues (110). Interestingly, BARD1 expression is highly up-regulated in the cytoplasm of most breast cancer cells and correlates with poor clinical outcome (111). The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase, and the mutations in the RING finger domain of BRCA1 found in familial breast cancer abolish the E3 activity (112, 113). The activity of BRCA1-BARD1 was shown to be

Table 3. E3 Ligases as Tumor Suppressors in Human Breast Cancer

<table>
<thead>
<tr>
<th>E3</th>
<th>Type</th>
<th>Locus</th>
<th>Alteration in breast cancer</th>
<th>Major targets</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>RING</td>
<td>17q21</td>
<td>Mutation</td>
<td>H2A, Rb1, γ-tubulin</td>
<td>DNA repair, genomic stability</td>
</tr>
<tr>
<td>BARD1</td>
<td>RING</td>
<td>2p24-q35</td>
<td>Mutation, expression aberration</td>
<td>H2A, Rb1, γ-tubulin</td>
<td>DNA repair, apoptosis</td>
</tr>
<tr>
<td>SIAH1</td>
<td>RING</td>
<td>16q22-23</td>
<td>Loss of expression</td>
<td>β-Catenin, ORF-1, CHIP, Kid, Numb</td>
<td>Cell cycle, transcription, and apoptosis</td>
</tr>
<tr>
<td>Parkin</td>
<td>RING</td>
<td>4q25</td>
<td>Deletion and loss of expression</td>
<td>cSp22, p38/JTV1, Pael-R</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>CHFR</td>
<td>RING</td>
<td>12q24.33</td>
<td>Loss of expression by promoter methylation</td>
<td>Pkl1, Aurora A</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Cul5</td>
<td>Culin</td>
<td>1q22-23</td>
<td>Loss of expression</td>
<td>Cyclin E, Myc, Notch</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Fbw7</td>
<td>F-box</td>
<td>4q21</td>
<td>Mutation and loss of expression</td>
<td>ErbB2, estrogen receptor α, AR, Smads</td>
<td>Signaling</td>
</tr>
<tr>
<td>CHIP</td>
<td>U-box</td>
<td>16q13.3</td>
<td>Loss of expression</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: For references, please see E3s as Tumor Suppressor Genes in Breast Cancer.
down-regulated by CDK2 (114) and enhanced by BRCC36 whose mRNA expression is elevated in breast tumors (115). BRCA1-BARD1 E3 was shown to ubiquitinate γ-tubulin, which regulates centrosome number and genome stability (116). Following that, the largest subunit of RNA polymerase II (Rpb1) was shown to be ubiquitinated by this E3 complex (117).

**SIAH1**

The **SIAH1** gene was first identified as a candidate tumor suppressor gene at 16q12.1 in human hepatocellular carcinomas because of frequent loss of heterozygosity and expression down-regulation (118). Overexpression of SIAH1 in MCF7 cells suppresses cell growth by altering the mitotic process (119, 120). SIAH1 is a RING finger E3 targeting β-catenin for ubiquitin-mediated degradation in response to activation of p53 (121). The ubiquitin ligase complex contains SIAH1, SIAH1-interacting protein, Skp1, and the F-box protein Ebi (122).

Importantly, this degradation pathway is different from degradation of β-catenin by SCFβ-catenin. The latter requires the β-catenin phosphorylation by glycogen synthase kinase 3β (GSK3β). It is well documented that β-catenin promotes cell cycle progression and inhibits cell cycle arrest. Because **SIAH1** is a p53-induced gene (123), SIAH1-mediated β-catenin degradation may contribute to p53-dependent cell cycle arrest and apoptosis. In addition to β-catenin, SIAH1 has been reported to promote degradation of several substrate proteins, such as transcriptional coactivator OBF-1 (124), transcriptional coexressor CIP (125), kinesin-like DNA-binding protein Kid (120), and cell fate regulator Numb (126).

**CHFR**

Epigenetic inactivation of CHFR by aberrant promoter methylation was found in several types of carcinomas, including breast (127, 128). In a knock-out mouse model, CHFR was suggested to be a tumor suppressor because mice deficient in its expression spontaneously develop a variety of epithelial tumors in major organs, including lung, liver, gastrointestinal tract, and reproduction system (129). The **CHFR** gene also encodes a RING finger domain containing E3, which was shown to play an important role in mitosis through targeting key mitotic proteins Plk and Aurora A for ubiquitin-mediated proteolysis (129, 130).

**Parkin**

Although mutations of the **Parkin** gene are the most common cause of hereditary parkinsonism, Parkin has also been suggested as a candidate tumor suppressor gene on chromosome 6q25-q27 in human breast cancer, ovarian cancer, and lung cancer (131, 132). The expression of the **Parkin** gene has been shown to be frequently down-regulated or absent in breast tumor cells (131). Although no mutations or in vitro changes in cell proliferation or cell cycle were observed, forced expression of Parkin was shown to suppress tumorigenicity in nude mice (132). Parkin is a RING-type E3 involved in the ubiquitination pathway for misfolded proteins (133). Although αSp22, Pael receptor (Pael-R), and p38/JTV1 were identified as the Parkin substrates in brain cells (134-136), the targeted substrate in breast cancer cells remains unclear.

**Fbw7/hCDC4**

Human F-box protein Fbw7/hCDC4 has been reported to promote ubiquitin-mediated degradation of several oncoproteins involved in the control of cell division and growth, such as cyclin E (137), c-Myc (138), c-Jun (139), and Notch (140). Mice lacking Fbw7 die at about day 10 with multiple abnormalities (140, 141). Although cyclin E expression is frequently dysregulated in breast cancer (142), a truncated mutant form of Fbw7, which can not bind to phosphorylated cyclin E target, has been found in one breast cancer cell line (143). However, the frequency of Fbw7 mutation in breast cancer has not been studied. Proteasomal degradation of cyclin E via the UPS is distinct from elastase, and calpain-mediated NH2-terminal proteolysis of cyclin E that generates low molecular weight forms of cyclin E, which are frequently detected in breast tumor tissue (144-146).

**CUL-5**

CUL-5 is located at 11q22-23, which is frequently deleted in breast cancer (147). A statistically significant decrease in CUL-5 expression versus the matched normal tissue was detected in 82% (41 of 50) of the breast cancers (148). Overexpressing Cul-5 significantly attenuated cellular proliferation of the T-47D breast cancer cell line (149).

**CHIP/STUB1**

The chaperone-dependent U-box E3 CHIP mediates a degradative pathway for ErbB2/Neu (28). ErbB2 overexpression is frequent in breast cancer and associated with poor prognosis. In addition to ErbB2, CHIP also promotes estrogen receptor α and glucocorticoid receptor degradation and attenuates receptor-mediated gene transcription (29, 150). However, CHIP negatively controls the sensitivity of TGF-β signaling by targeting Smads for ubiquitin-mediated degradation (151, 152). Interestingly, CHIP was found to be associated with Parkin and enhances Parkin E3 activity (153). The **CHIP** gene is located at 16p13.3, which is frequently deleted in papillary carcinomas of the breast (154).

**Functional Mechanisms of E3s in Breast Cancer**

Every E3 specifically targets one or several substrate proteins for ubiquitination. However, if an E3 plays an important role in breast tumorigenesis, the targets are usually involved in the cell cycle, p53 actions, transcription, DNA repair, signaling transduction, or apoptosis (Fig. 2). All these cellular processes are known to be critical in breast cancer initiation and progression.

**Cell Cycle**

The cell cycle plays a central role in regulating cell proliferation and tumorigenesis. It is well documented that alterations of cell cycle regulators occur in breast cancer, such as overexpression of cyclin E (155) and down-regulation of the p27Kip1 (156). Because rapid protein turnover by ubiquitin-mediated proteolysis is the most important mechanism of controlling protein abundance for cell cycle regulators, the frequent genetic and expression alterations of their E3s contribute to breast tumorigenesis. Typically, cullin-based
E3s, including SCF complexes, function through regulating the cell cycle. For example, F-box proteins Fbw7 and Skp2 target cyclin E and p27KIP1 for degradation, respectively. In addition, APC/C facilitates proteolysis of cyclin B, securin, and several other proteins to regulate mitosis exit.

\[ p53 \]

\[ p53 \] is a key tumor suppressor targeted by multiple E3s, including Mdm2 (60), ARF-BP1 (40-42), E6-AP (24), Pirh2 (105), p300 (157), Topo1 (158), and COP1 (159). Interestingly, p53 itself induces expression of Mdm2 and COP1 to negatively control p53 activity. Besides Mdm2 and ARF-BP1, COP1, a RING finger-containing E3, is also significantly overexpressed in control p53 activity. Besides Mdm2 and ARF-BP1, COP1, a RING finger-containing E3, is also significantly overexpressed in breast cancer (104). Additionally, Pirh2 protein expression is up-regulated in major human lung neoplasms (160). It would be interesting to know if Pirh2 is also overexpressed in human breast cancer.

\[ Transcription \]

Protein instability is a typical feature of many transcription factors, such as HIF1α (161), Myc (162), β-catenin (163), c-Jun (164), and KLF5 (165). Each of these transcription factors regulates expression of a large number of target genes, and alterations of these transcription factors are frequently involved in tumorigenesis. Besides proteolysis, accumulating evidence suggests that ubiquitination of transcription factors can enhance their transcriptional activation function (8). Therefore, it is not surprising that E3s of these transcription factors, such as von Hippel-Lindau, Fbw7, SIAH1, and WW1P1, play important roles in tumorigenesis through controlling the abundance and activity of transcription factors.

\[ DNA Repair \]

DNA damage is an important cause of gene mutation and carcinogenesis. Many proteins participating in DNA repair are regulated by the ubiquitin-proteasome pathway. For example, UV-induced p21 degradation is essential for DNA repair (166). Growing results indicate that BRCA1-BARD1 E3 is required for double-strand break repair after exposure to ionizing radiation (167). Additionally, a BRCA1-dependent zinc finger transcriptional repressor ZBRK1 is degraded through the UPS upon DNA damage (168). Interestingly, altered expression of the ZBRK1 gene has been frequently found in human breast carcinomas (169). No E3 has been identified for ZBRK1 ubiquitination upon DNA damage, although RNF11 has been shown to be a ZBRK1-associated protein (80). Recently, DNA damage–induced degradation of Cdt1 (170, 171) and DDB2 (172), two key proteins in DNA repair mechanisms, has been shown to link to Cul4A and DDB1. Therefore, compromise of DNA damage could be an important deregulating mechanism for BRCA1 and Cul4A in breast cancer.

\[ Growth Factor Signaling \]

Many growth factor signal receptors, such as epidermal growth factor factor receptor, estrogen receptor, and TGF-β receptors, are frequently altered in breast cancer. For example, ErbB-2 is frequently amplified and overexpressed in breast tumors, and this alteration is associated with poor prognosis. In addition to CHIP, which promotes ligand-independent degradation of growth factor receptors, c-Cbl is another E3 that can negatively regulate the epidermal growth factor receptor by monoubiquitinating them for endocytosis upon ligand stimulation (173, 174). It has been reported that degradation of ErbB-2 by Herceptin (a humanized ErbB-2 antibody) involves the recruitment of c-Cbl to ErbB-2 (174). The Cbl gene is located at 11q23.3, a region frequently lost in breast cancer (175). Mutation of Cbl is oncogenic in many types of cancer (176). Additionally, containing TGF-β receptors by WW1P1/Smurfs is implicated in the loss of sensitivity of tumorogenic cells to TGF-β–induced growth inhibition (48, 177, 178).

\[ Apoptosis \]

Inhibition and resistance to apoptosis is one of the major obstacles in cancer therapy. Apoptotic proteins have been identified as substrates of E3s and are frequently altered in breast cancer. In addition to p53 and the IAP family described above, Bcl-2 and Bcl2 family proteins are frequently deregulated by E3s in human cancer (9). It is well known that degradation of IκB by the SCFεB-AP complex is a critical step for the activation of nuclear factor-κB and up-regulated expression of antiapoptotic genes. Additionally, the prosapoptotic proteins Bax, Bid, and Bim are degraded through the UPS, which is believed to be a survival mechanism in human cancer cells, although their E3 has not been revealed thus far (179, 180). Up-regulated expression of Bim following detachment of normal breast epithelial cells from the extracellular matrix has been shown to be required for detachment-induced apoptosis (181). Recently, an antiapoptotic Bcl-2 family member, Mcl-1, was shown to be an ARF-BP1 E3 substrate (182). Therefore, alteration of apoptosis by E3s is an important mechanism for breast carcinogenesis.

\[ Future Directions \]

In conclusion, ubiquitin-mediated protein degradation plays an important role in many cancer-related cellular processes. E3s play critical roles because they control the substrate specificity. Accumulating evidence suggests that genetic and expression alteration of E3s contributes to breast carcinogenesis. In >500 human E3s, a number of E3s have been characterized as either oncogenes or tumor suppressor genes in breast cancer. Cell cycle, p53, transcription, signaling transduction, DNA repair, and apoptosis are major targets of E3s in breast cancer development. Several directions listed below should be important to develop E3s as diagnostic and therapeutic targets in the future.

\[ Identification of Novel Breast Cancer Related E3s as Biomarkers and Drug Targets \]

Although there are >500 E3s in the human genome, to date, only a limited number of E3s have been examined for their specific targets, genetic changes, and expression pattern in a large number of human breast tumors. High-throughput screening using E3 cDNA and small interfering RNA libraries have been developed to identify the cell cycle–related E3s. However, systematic screening of the E3s with genetic and expression alteration in human tumors has not been reported. With development of microarray technology, a cDNA chip with
all E3s should be a useful tool to identify more cancer-related E3s. Tissue microarray is another powerful tool to detect expression of a candidate E3 or its substrates simultaneously in large numbers of tumors by immunohistochemical staining of a single microscope slide (183). An E3 with genetic and expression alteration in breast cancer could be developed as a biomarker for breast cancer diagnosis. Emerging technologies, such as array-based comparative genomic hybridization, cDNA microarray, tissue microarray, and RNA interference, will provide a better validation of many breast cancer–related E3s. It is anticipated that more breast cancer–related E3s will be identified in the future.

Identification of Specific Substrates for E3s

Each E3 targets a small number of proteins for proteolysis. The target proteins for most E3s remain unclear. The identification of all substrates for all E3s is still a big challenge. Several strategies, including bioinformatics, in vitro expressing cloning, fusion protein, RNA interference, and arrays of synthetic phosphopeptides, certainly facilitate the identification of E3 substrates (184-186). A high-throughput screening to identify substrates for the yeast ubiquitin ligase Rsp5 has been reported recently (187). Because the E3s interact with their substrate proteins, maps of the interactome network generated from yeast (188), Drosophila (189), Caenorhabditis elegans (190), and human proteome (191, 192) by high-throughput yeast two-hybrid assays and newly developed mass spectrometric analysis technology will also facilitate the identification of the specific substrates for E3s.

Development of Specific Inhibitors for E3s

Approval of the general proteasome inhibitor Velcade by the Food and Drug Administration for the treatment of multiple myeloma suggests the promise of targeting the UPS in anticancer therapy. However, Velcade showed limited clinical activity against metastatic breast cancer (193) and many side effects (194). Specific inhibitors of E3s should be highly specific drugs with few side effects because of the specificity of target recognition. The oncogenic E3s that target tumor suppressors could be potential targets for developing small-molecule inhibitors. In contrast, the E3s with tumor suppressor function could also be promising targets for small-molecule activators. Several common approaches used for high-throughput screening for ubiquitin ligase inhibitors have been described recently (195). Moreover, high-throughput screening inhibitors for Mdm2-p53 (196), Skp2-Csk1 (197), β-Trcp1-Ie-B (198), and APC/C (199) have been reported recently. Several companies are actively pursuing the inhibitors for E3s, such as Mdm2, Skp2, and Smurf (200). Furthermore, two inhibitors of Mdm2 actually were reported to have substantial p53-dependent antitumor effect in vivo (65, 66). More detail of drug discovery in the UPS was discussed in a recent review (201). Although E3s are believed to be ideal drug targets, targeting E3s is still in its infancy because no inhibitors have reached the clinic yet. The challenge remains to discover small molecular drugs that selectively inhibit the E3s. Emerging technologies, such as bioinformatics, high-throughput screening, structure-based drug design, and virtual library screening, will enhance the future E3-based drug discovery.

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References


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