Published in final edited form as:

J Cell Physiol. 2011 December ; 226(12): 3355–3367. doi:10.1002/jcp.22695.

Ubc9 Mediates Nuclear Localization and Growth Suppression of BRCA1 and BRCA1a Proteins

YUNLONG QIN¹, JINGYAO XU¹, KARTIK AYSOLA¹, NURJAHAN BEGUM¹, VAISHALI REDDY¹, YULI CHAI¹, WILLIAM E. GRIZZLE², EDWARD E. PARTRIDGE³, E. SHYAM P. REDDY¹, and VEENA N. RAO^{1,*}

¹Cancer Biology Program, Department of OB/GYN, Morehouse School of Medicine, Georgia Cancer Center for Excellence, Grady Health System, Atlanta, Georgia

²Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama

³Division of Gynecological Oncology, Department of Obstetrics & Gynecology, University of Alabama at Birmingham, Birmingham, Alabama

Abstract

BRCA1 gene mutations are responsible for hereditary breast and ovarian cancers. In sporadic breast tumors, BRCA1 dysfunction or aberrant subcellular localization is thought to be common. BRCA1 is a nuclear–cytoplasm shuttling protein and the reason for cytoplasmic localization of BRCA1 in young breast cancer patients is not yet known. We have previously reported BRCA1 proteins unlike K109R and cancer-predisposing mutant C61G to bind Ubc9 and modulate ER- α turnover. In the present study, we have examined the consequences of altered Ubc9 binding and knockdown on the subcellular localization and growth inhibitory function of BRCA1 proteins. Our results using live imaging of YFP, GFP, RFP-tagged BRCA1, BRCA1a and BRCA1b proteins show enhanced cytoplasmic localization of K109 R and C61G mutant BRCA1 proteins in normal and cancer cells. Furthermore, down-regulation of Ubc9 in MCF-7 cells using Ubc9 siRNA resulted in enhanced cytoplasmic localization of BRCA1 protein and exclusive cytoplasmic retention of BRCA1a and BRCA1b proteins. These mutant BRCA1 proteins were transforming and impaired in their capacity to inhibit growth of MCF-7 and CAL51 breast cancer cells. Interestingly, cytoplasmic BRCA1a mutants showed more clonogenicity in soft agar and higher levels of expression of Ubc9 than parental MCF7 cells. This is the first report demonstrating the physiological link between cytoplasmic mislocalization of mutant BRCA1 proteins, loss of ER- α repression, loss of ubiquitin ligase activity and loss of growth suppression of BRCA1 proteins. Thus, binding of BRCA1 proteins to nuclear chaperone Ubc9 provides a novel mechanism for nuclear import and control of tumor growth.

Mutations in the BRCA1 gene are associated with hereditary breast cancers (Miki et al., 1994; Rowell et al., 1994). In sporadic breast cancers, BRCA1 mutations are rare, but reduced expression or aberrant subcellular localization of BRCA1 is common among young African-American women with triple negative breast cancers (TNBC) (Bogdani et al., 2002; Rakha et al., 2008). Several BRCA1 isoforms are found in different tissues, but their functional significance is currently not understood (Lu et al., 1996; Wang et al., 1997; Wilson et al., 1997; Orban and Olah, 2001; Maniccia et al., 2009). We have previously described two major splice variants of BRCA1, namely BRCA1a/p110 and BRCA1b/p100,

which are expressed at reduced levels in tumors compared to normal mammary epithelial cells (Shao et al., 1996; Thangaraju et al., 2000; Orban and Olah, 2001; Maniccia et al., 2009). Other groups have named BRCA1a as BRCA1-delta 11b (Wilson et al., 1997), BRCA1s (Lu et al., 1996), BRCA1 delta11q (Orban and Olah, 2001), and BRCA1b as BRCA1s-9, 10 (Lu et al., 1996), BRCA1 11q delta 9, 10 (Orban and Olah, 2001). BRCA1a and BRCA1b differ from BRCA1 in having a deletion of a majority of exon 11 sequences (within amino acids 263–1,365). BRCA1b has an additional deletion of exon 9 and 10 sequences (Wang et al., 1997). BRCA1 proteins induce apoptosis (Shao et al., 1996; Thangaraju et al., 2000) and function as tumor suppressors of ER- α positive, TNBC, ovarian and prostate cancers, but their mechanism of tumor suppression is not known (Yuli et al., 2007). BRCA1 is a predominantly nuclear phosphoprotein (Coene et al., 2005), which has several functional domains, an N-terminal RING finger domain which interacts with several proteins and two BRCA1 C-terminal domains. We have shown BRCA1, BRCA1a, and BRCA1b proteins to be localized in the mitochondria and their nuclear–cytoplasmic shuttling to be a regulated process (Wang et al., 1997; Coene et al., 2005; Maniccia et al., 2009). BRCA1 nuclear import is regulated by the action of nuclear localization signal (NLS) and nuclear export signals (NES) located in the RING domain which mediates nuclear export via association with BARD1 (Rosen et al., 2006). The BRCA1 delta 11 isoform which lacks NLS also enters the nucleus via the RING-domain mediated BARD1 import pathway (Fabbro et al., 2002). BARD1 by binding to BRCA1 masks its NES resulting in nuclear localization of BRCA1 proteins (Fabbro et al., 2002). The RING domain of BRCA1 in complex with BARD1, mediates an E3 ubiquitin ligase activity on ER- α in vitro (Hashizume et al., 2001; Eakin et al., 2007). Recent findings using an ubiquitin ligase-deficient BRCA1 I26A mutant suggested that the ubiquitin ligase activity is dispensable for both genomic stability as well as homology-directed repair of double-strand DNA breaks, but is required for repression of ER- α activity (Reid et al., 2008; Ma et al., 2010).

Post-translational modification of transcriptional factors is important for regulated gene expression (Hay, 2005). Small ubiquitin-like modifier (SUMO) modification of proteins has effects on the stability, localization, protein–protein interactions, and transcriptional regulation (reviewed by Kim et al., 2002; Seeler and Dejean, 2003; Johnson, 2004). The SUMO modification pathway was shown to be involved in BRCA1 response to DNA damage and transcriptional repression (Park et al., 2008; Morris et al., 2009). We have previously reported the amino-terminal domain of BRCA1, BRCA1a, and BRCA1b proteins to bind SUMO-E2-conjugating enzyme Ubc9 and regulate ER- α activity by promoting its degradation in vivo (Xu et al., 2009). These studies suggested a potential cross-talk between the SUMO and ubiquitin pathways similar to the ubiquitin ligase RNF4 by highlighting the biochemical function of BRCA1 as a putative SUMO-1 and Ubc9-dependent E3 ubiquitin ligase for ER- α SUMO conjugates (Sun et al., 2007; Uzunova et al., 2007). Mutation in the Ubc9 binding site as well as BRCA1 RING domain cancer-predisposing mutation (C61G) disrupted the ability to modulate Ubc9-mediated SUMO-dependent/independent estrogen-induced ER- α transcriptional activity in breast cancer cells (Xu et al., 2009) but did not disrupt SUMO-1 binding (Morris et al., 2009) nor auto ubiquitination activity of BRCA1 (Xu et al., 2009). Ubc9 plays an important role in both cancer progression and resistance to chemotherapy (Mo et al., 2004; Dunnebie et al., 2009; Ronen et al., 2009; Driscoll et al., 2010; Moschos et al., 2010). In fact Ubc9 was found to act as both a positive and negative regulator of proliferation and transformation of HMGA1 proteins (Li et al., 2007). Here we have further investigated these findings and have studied the effect of altered Ubc9 binding and knockdown on the subcellular localization and growth inhibitory function of BRCA1 proteins in breast cancer cells.

Materials and Methods

Expression constructs

Full-length BRCA1a, BRCA1b, BRCA1a Mut #1, BRCA1a Mut #4, AS-Ubc9 (antisense), Ubc9, BRCA1 Mut #1, BRCA1 Mut #4 were cloned 3' of the RFP or YFP or GFP in p-EGFP (Maniccia et al., 2009) or pCDNA3 (Xu et al., 2009) or pFLAG vectors. Point mutations were generated as described previously (Xu et al., 2009).

Cell culture and stable cell lines

MCF-7, COS-1, MCF10A, LNCap, PC-3, NIH-3T3, HCC70, MDAMB231, and T47D cells were obtained from American Type Culture Collection (Rockville, MD). CAL-51 cell line was obtained from J. Gioanne and Jean Louis Fischel and cultivated as described previously (Yuli et al., 2007). Stable MCF-7 pCDNA3 BRCA1a, BRCA1, BRCA1a Mut#1, BRCA1a Mut#4, BRCA1 Mut#1, BRCA1 Mut#4, and MCF-7 EGFP-AS Ubc9 expressing clones were generated as described previously (Yuli et al., 2007).

Subcellular fractionation and Western blot analysis

Cos-1 cells were transfected with pRFP-BRCA1a or its respective mutants and subjected to nuclear and cytoplasmic fractionation as described previously (Maniccia et al., 2009). The extracts were separated on 4–20% SDS-PAGE gradient gel and processed for Western blotting using BRCA1 Ab1, HSP90, Lamin A + C antibodies as previously described (Maniccia et al., 2009). The OncoPair INSTA-Blot cat: No: 1MB-130e IMGENEX (San Diego, CA) which is ready to use PVDF membrane containing denatured protein lysates from ductal carcinomas and matched adjacent tissues obtained from seven patient donors was probed with Ubc9 (H-81, Santa Cruz Biotechnology (Santa Cruz, CA)) antibody as described by the manufacturer (IMGENEX).

Antibodies and reagents

The antibodies used in this study were MS110 ascites (Ab1, EMD (Gibbstown NJ)), Ubc9 N-15, beta-Actin C4, ER- α D12, and D20 antibodies (Santa Cruz Biotechnology).

Immunofluorescence microscopy and live imaging

To analyze the subcellular localization MCF-7, T47D, COS-1, MCF10A, LNCap, PC-3, NIH-3T3, and HCC70, cells were seeded into six-well plates a day before transfection with pCDNA3, pCMVtag2, RFP, GFP or YFP-tagged BRCA1, BRCA1a and their respective mutant plasmids. The DNA was stained with Hoechst dye and after 24 h the cells were visualized under a fluorescent microscope (Olympus (Center Valley, PA), 20 $\times$ lens). Similar experiments were performed in the presence of control or Ubc9 siRNA.

Growth suppression studies

The growth suppression assay using MCF-7 and CAL51 were done as previously described (Yuli et al., 2007).

Luciferase assays

T47D cells were plated at a concentration of 2×10^5 cells per six-well plates, starved for 72 h, followed by transfection with control or Ubc9 siRNA by using HiPerFect Transfection Reagent from Qiagen (Valencia, CA). Twenty-four hours later cells were transfected with pS2-ERE-luc and pCMVTag2C or pCMVTag2C BRCA1a by using Lipofectamine 2000 kit from Invitrogen (Carlsbad, CA). Twenty-four hours after transfection, luciferase activity

was analyzed as previously described (Xu et al., 2009). The data presented are mean $\pm$ s.d. of three independent experiments performed in triplicate.

Soft agar assays

Soft agar assay was carried out as previously described (Yuli et al., 2007). Each soft agar assay was performed in triplicate.

siRNA transfection

MCF-7, COS-1, MCF10A, LNCaP, PC-3, NIH-OVCAR-3, HCC70, MDAMB23, and T47D cells were seeded into culture dishes. Ubc9 SMART pool siRNA (50 nM) or 50 nM control RISC-free siRNA (Dharmacon, Lafayette, CO) was diluted in serum-free medium. HiPerFect transfection reagent (Qiagen) was added to the diluted siRNA and mixed by vortexing. It was allowed to sit at room temperature for 5–10 min then added drop wise to the cells and incubated in normal growth medium for 24 h. For experiments to show that Ubc9 is required for growth suppression exerted by BRCA1 proteins stable MCF-7pCDNA3 and MCF-7 BRCA1a cells were seeded in six-well plates and transfected with Ubc9 siRNA (100 nM) or 100 nM control siRNA as described above. Growth of the cells was measured 48 h after transfection using the CellTiter-Glo Luminescent cell viability assay as described by the manufacturer Promega (Madison, WI). Similarly after 24 h of siRNA transfection, cells were starved in serum-free medium for 48 h and treated with E2 (100 nM) for an additional 24 h. The cells were then visualized under fluorescence microscope (Olympus, 4 $\times$ lens).

Results

Ubc9 is required for nuclear import of BRCA1 and BRCA1a proteins

As mentioned earlier BRCA1 is a nuclear–cytoplasm shuttling protein and many cancer-associated mutations have altered subcellular localization of BRCA1 protein (Bogdani et al., 2002; Rakha et al., 2008). This is especially true in some cases of sporadic breast cancers among younger women where the majority of BRCA1 protein is found in the cytoplasm (Bogdani et al., 2002). BRCA1 nuclear import is regulated by the combined action of NLS (Fabbro et al., 2002) and interaction with the RING domain binding protein, BARD1, which masks the NES (Fabbro et al., 2002). BRCA1a and BRCA1b proteins both lack the two NLS, but are still imported to the nucleus suggesting that they may use an alternate mechanism for nuclear import. Since our previous results showed lack of binding of BRCA1/BRCA1a Mut #1 (K 109 to R) and disease-associated Mut #4 C61G to Ubc9, we wanted to study the impact of these mutations on the subcellular localization of BRCA1 and BRCA1a proteins. We initially transfected COS-1 cells with pCDNA3 BRCA1, BRCA1 Mut #1, BRCA1 Mut #4, pCMV Tag2 BRCA1a, BRCA1a Mut#1, BRCA1a Mut#4 and studied the subcellular localization of BRCA1 and BRCA1a proteins by immunofluorescence using BRCA1 antibody. BRCA1 was localized both in the nucleus as well as cytoplasm unlike the mutant BRCA1 proteins where the BRCA1 immunofluorescence in the cytoplasm increased from 4.4% to 12% and 22%, respectively (Fig. 1a). Similarly, BRCA1a was also found to be localized both in the nucleus as well as cytoplasm, but BRCA1a Mut#1 and Mut#4 were found exclusively in the cytoplasm (Fig. 1b). We have further confirmed these results by examining the subcellular localization using RFP-epitope tagged BRCA1a, BRCA1a Mut #1, and BRCA1a Mut #4 after transient transfection into COS-1 (Fig. 1c). We observed exclusive cytoplasmic localization of both BRCA1a Mut #1 and Mut #4 unlike BRCA1a which was both in the nucleus as well as cytoplasm of COS-1 cells (Fig. 1). We have confirmed these results after subcellular fractionation followed by Western blot analysis in COS-1 cells using BRCA1 antibody (Fig. 1c). It should be noted that BRCA1a Mut#1 and Mut#4 proteins are more stable compared

to BRCA1a (Rao et al. unpublished work). These results suggest that Ubc9 may be required for nuclear import of BRCA1 and more so for BRCA1a proteins that lack NLS. Previous work from Dr. Henderson's group suggested that both NLS and RING domain sequences are necessary for nuclear localization (Fabbro et al., 2002) of BRCA1 proteins. To demonstrate that the BRCA1 RING domain is required for Ubc9-mediated nuclear import, we tested Ubc9 for its effect on nuclear localization of BRCA1-NLS mutants. In transfected COS-1 cells approximately 53% of BRCA1-NLS mutant was found in the cytoplasm (Fig. 1d). Co-transfection with Ubc9 enhanced nuclear localization of BRCA1-NLS mutant and 11% was found in the cytoplasm (Fig. 1d). These results indicate that Ubc9 similar to BARD1 functions as a new chaperone promoting the nuclear entry of BRCA1 and its splice variants. We have further confirmed these results using normal mammary epithelial cells MCF10A (Fig. 2a), breast cancer cells MCF-7 (Fig. 2b), prostate cancer cells LNCaP (Fig. 2c) and PC-3 (Fig. 2d), ovarian cancer cells NIHOCAR3 (Fig. 2e) and TNBC cells obtained from an African-American women with breast cancer HCC-70 (Fig. 2f).

Ubc9 knockdown causes cytoplasmic localization of BRCA1 proteins

If Ubc9 is required for nuclear localization of BRCA1 and BRCA1a proteins, then knockdown of endogenous Ubc9 in MCF-7 breast cancer cells should result in cytoplasmic localization of BRCA1 proteins. We observed cytoplasmic localization of full-length RFP BRCA1a, YFP-BRCA1, GFP-BRCA1b, and endogenous BRCA1 proteins in MCF-7 cells that has been treated with siRNA to Ubc9 by live imaging and immunofluorescence analysis using BRCA1 antibody (Fig. 3a–d). These results further confirm the requirement of Ubc9 in the nuclear localization of BRCA1, BRCA1a, and BRCA1b proteins. MCF-7 breast cancer cell line was used in this experiment since it has lost one of the BRCA1 alleles, expresses low levels of both BRCA1 and BARD1 (Fabbro et al., 2002) and so it is an ideal cell line for this study. To determine whether Ubc9 influences BRCA1 localization, we modified MCF-7 cells with stable transfection of human antisense Ubc9 (MCF-7-EGFP AS-Ubc9). Compared to MCF-7 cells, these MCF-7-EGFPAS-Ubc9 cells expressed decreased levels of endogenous Ubc9 (Fig. 3e). As shown above, when expressed in MCF-7-EGFPAS-Ubc9 cells, YFP-BRCA1, RFP-BRCA1a, and GFP-BRCA1b accumulated in the cytoplasm, suggesting that BRCA1, BRCA1a, and BRCA1b need Ubc9 to enter the nucleus (Fig. 3e). These results once again demonstrated a role for Ubc9 in the nuclear import of BRCA1 proteins.

Ubc9 knockdown impairs BRCA1a inhibition of ER- α activity

BRCA1, BRCA1a, and BRCA1b are known to repress E2-induced ER- α activity, and their respective Mut #1 and Mut #4, which do not bind Ubc9, are impaired in their capacity to inhibit ER- α activity in breast cancer cells (Xu et al., 2009). Ubc9 also functions as an ER- α co-activator independent of its SUMO E2-conjugating activity. Recently knockdown of endogenous BRCA1 was found to alter the association of ER- α co-regulators (Wen et al., 2009). To study whether the inhibition of E2-induced ER- α activity by BRCA1a is mediated through interaction with Ubc9, we have knockdown the endogenous Ubc9 in T47D breast cancer cells using siRNA to Ubc9, which resulted in significant loss of BRCA1a repression of ER- α activity, suggesting the requirement of Ubc9 in BRCA1-mediated repression of ER- α transcription (Fig. 3f) in breast cancer cells.

Altered Ubc9 binding promotes cell proliferation of BRCA1 and BRCA1a mutants

BRCA1 and BRCA1a proteins suppress the growth of human ER- α positive, TNBC, and ovarian cancer cells (Holt et al., 1996; Aprelikova et al., 1999; Tait et al., 2000; Randrianarison et al., 2001; Marot et al., 2006; Yuli et al., 2007). To study the consequence of altered Ubc9 binding on the growth suppressor function of BRCA1 proteins, we subjected BRCA1, BRCA1a, and their corresponding Mut#1, cancer-predisposing Mut#4 C61G to

colony suppression assays using MCF-7 an ER- α positive, and CAL51 a TNBC cells as described previously (Yuli et al., 2007). BRCA1 Mut#1, BRCA1a Mut#1, BRCA1 Mut#4, and BRCA1a Mut#4 lost their capacity to suppress growth of human breast cancer cells (Fig. 4a,b). These results are consistent with the notion that a direct interaction of BRCA1 proteins with Ubc9 is critical for inhibiting the growth of ER- α positive and TNBC cells.

Cytoplasmic BRCA1 and BRCA1a mutant proteins are transforming

To examine the functional consequences of altered Ubc9 binding and altered subcellular localization of BRCA1 proteins, we generated MCF-7 cells that stably express BRCA1, BRCA1a, BRCA1 Mut#1, BRCA1 Mut#4, BRCA1a Mut#1, and BRCA1a Mut#4 proteins. These stable cell lines expressed higher levels of BRCA1 proteins by Western blot analysis (Fig. 5a). The MCF-7 BRCA1a cells, as observed previously (Yuli et al., 2007) were slow growing compared to MCF-7 pCDNA3 cells. BRCA1a Mut#1 and BRCA1a Mut#4 clones proliferated faster than MCF-7 pCDNA3 cells (data not shown). These results once again suggest that lack of Ubc9 binding has altered the growth property of these cells. The MCF-7 BRCA1 Mut#1, MCF-7 BRCA1 Mut#4, MCF-7 BRCA1a Mut#1, and MCF-7 BRCA1a Mut#4 cell lines were highly clonogenic and formed anchorage-independent colonies in soft agar unlike MCF-7 BRCA1 and MCF-7 BRCA1a cells indicating considerable loss of in vitro tumor suppressor function (Fig. 5b,c). It is worth noting that there is a significant difference in the clonogenicity of BRCA1a mutants and BRCA1 mutants. BRCA1a mutants were three to five times more transforming when compared to pCDNA3 MCF-7 cells. On the other hand, BRCA1 mutants were less clonogenic than pCDNA3 MCF-7 cells. Thus, lack of Ubc9 interaction resulted in an increase in transforming potential and an increase in average colony size (Fig. 5). As mentioned earlier BRCA1a is totally dependent on Ubc9 for nuclear import unlike BRCA1 where some of it can enter the nucleus via its NLS and this can explain the functional differences between the two isoforms.

Cytoplasmic BRCA1a mutants promote survival

BRCA1 and BRCA1a proteins induce apoptosis of breast cancer cells on serum starvation (Shao et al., 1996; Thangaraju et al., 2000) and inhibit the growth of breast cancer cells in the presence of E2. Since mutant BRCA1a cells were more transforming when compared to mutant BRCA1 clones, we examined the cell proliferation of MCF-7 BRCA1a stable cell lines grown in serum-free media for 48 h followed by treatment with E2. BRCA1a Mut#1 and Mut#4 cells were hypersensitive to E2 compared to MCF-7 BRCA1a clones which ceased to proliferate both in serum restricted as well as E2-induced conditions (Fig. 6a). These findings and the fact that BRCA1a mutant cells exhibited an increased ability to form colonies in soft agar suggests for the first time that non-nuclear BRCA1a promotes growth and survival of breast cancer cells.

Knockdown of Ubc9 alters growth suppression by BRCA1a protein

We showed that BRCA1 and BRCA1a Mut#1 and Mut#4 proteins that do not bind Ubc9 lost the ability to suppress growth of tumor cells compared to wild-type BRCA1 and BRCA1a proteins. We have attributed this effect to the lack of Ubc9 binding by these mutants. Since knockdown of Ubc9 expression resulted in cytoplasmic localization of BRCA1 proteins, it is logical for us to test whether knockdown of Ubc9 can reverse suppression of growth by BRCA1 proteins. We down-regulated Ubc9 expression by using siRNA to Ubc9 in MCF-7BRCA1a stable cells and studied their growth rate compared to control siRNA transfected cells. The results indicated that Ubc9 siRNA-treated MCF-7BRCA1a cells showed 3.4-fold increase in proliferation compared with cells treated with control siRNA (Fig. 6b, compare third and fourth lanes). A comparable reduction in Ubc9 levels in MCF-7pCDNA3 cells had only 1.2-fold increase in growth (first and second lanes). Similarly, MCF-7 BRCA1a cells that were transfected with Ubc9 siRNA proliferated faster

under serum depleted conditions and in presence of E2 compared to control siRNA transfected MCF-7 BRCA1a cells (Fig. 6c). From these experiments we can conclude a clear link between Ubc9-BRCA1 interaction and requirement of Ubc9 in BRCA1-mediated growth suppression of breast cancer cells.

Ubc9 is elevated in BRCA1a mutants, breast cancer cell lines, and breast tumor tissues

Ubc9 is the only E2 enzyme essential for protein sumoylation and is a multifunctional protein, which is over-expressed in several cancers like colon, prostate, breast, lung, ovarian, and melanomas (Mo et al., 2004; Dunnebie et al., 2009; Ronen et al., 2009; Driscoll et al., 2010; Moschos et al., 2010). Ubc9 functions as a co-activator of several nuclear receptors (Kobayashi et al., 2004; Chang et al., 2007). Ubc9 has also been shown to bind to HMGA1 proteins and integrate both positive and negative signals for proliferation and transformation (Li et al., 2007). Recently Ubc9 was found to promote cell invasion and metastasis of breast cancer cells (Zhu et al., 2010) implicating a role in tumorigenesis. Ubc9 levels have also been shown to correlate with drug resistance in ovarian cancer cells (Mo et al., 2004). Since BRCA1 mutant proteins do not bind Ubc9 and are transforming, we wanted to test if the inability to bind Ubc9 could result in aberrant Ubc9 expression as observed previously in tumors. We found Ubc9 protein to be expressed at elevated levels in BRCA1a Mut#1 and BRCA1a Mut#4 clones compared to BRCA1a cells (Fig. 7). We studied Ubc9 expression in MCF10A, a normal mammary epithelial and several other ER-positive, ER-negative, triple negative luminal-like, Her-2 like, and basal-like breast cancer cells (T47D, MCF-7, SKBR-3, MDAMB231, MDA-MB-468, CAL-51, and HCC70). We observed over-expression of Ubc9 in basal-like, Her-2 like, and luminal-like breast cancer cells compared to normal mammary epithelial cells (Fig. 7a). Comparison of Ubc9 expression levels by Western blot in seven ductal carcinomas and adjacent normal mammary tissues revealed significantly higher levels of Ubc9 in tumors (Fig. 7b). This is in agreement with our results with breast cancer cell lines and previous report that documented higher levels in several cancers compared with their normal tissue counterparts (Moschos et al., 2010). Since Ubc9 functions as a co-activator of ER- α (Sentis et al., 2005), we examined the expression of ER- α in each of the stable BRCA1a cell lines. ER- α was expressed at significantly higher levels in the mutant cell lines compared to BRCA1a and pCDNA3 transfected MCF-7 cells (Fig. 7a). In fact ER- α level was down-regulated in MCF-7 BRCA1a cells. These results are consistent with the model that a direct interaction of BRCA1 with Ubc9 is critical for growth/tumor suppression by BRCA1 proteins and lack of binding results in deregulated Ubc9 and ER- α levels causing transformation (Fig. 8a,b).

Discussion

Identifying the functional implications of loss of nuclear BRCA1 localization in young African-American Women with aggressive breast tumors is critical to understanding how BRCA1 dysfunction results in TNBC. BRCA1 is a tumor suppressor that undergoes active nuclear import and export, which can provide a regulatory function (Thompson, 2010). As somatic mutations in BRCA1 are rarely found in sporadic breast cancers, the nuclear-cytoplasm shuttling of BRCA1 may offer an important mechanism for regulating its function. The mistargeting of tumor suppressors like BRCA1 can have severe consequences on the normal function of these proteins. Nuclear transport of proteins can be altered either by mutations or through post-translational modifications (include phosphorylation, ubiquitylation, glycosylation, and sumoylation) within or proximal to nuclear import/export signals which could cause a conformational change, thus preventing the binding of proteins like Ubc9 resulting in non-nuclear distribution of BRCA1 proteins.

In this study we are demonstrating for the first time that both BRCA1/BRCA1a K109R and disease-associated C61G mutants, which fail to bind Ubc9, fail to repress ER- α activity, lack

E3 ubiquitin ligase activity (Xu et al., 2009) and lack growth inhibitory function are localized in the cytoplasm of breast, ovarian, prostate, and normal mammary epithelial cells (Table 1). Knockdown of endogenous Ubc9 using siRNA resulted in enhanced cytoplasmic retention of BRCA1, BRCA1a, and BRCA1b proteins. Co-expression of Ubc9 induced nuclear accumulation of NLS-deficient forms of BRCA1 proteins. These results suggest, for the first time, the existence of a new import pathway involving Ubc9, which transports BRCA1 proteins to the nucleus via a piggyback mechanism (Fig. 9) similar to BARD1 (Fabbro et al., 2002). BRCA1 is known to enter the nucleus by binding to importin alpha, which subsequently binds importin beta as well as through interaction with BARD1 (Thompson, 2010). Ubc9 does not bind importin alpha nor beta proteins (Mingot et al., 2001). Recently, importin 13 was identified as a novel importin beta-related transport receptor that mediates nuclear import of Ubc9 via RanGTPase system (Mingot et al., 2001). We can propose the existence of a new nuclear import pathway for BRCA1 proteins involving the Ubc9: importin 13 and BRCA1a/1b proteins may use only this mechanism for nuclear transport (Fig. 9). We have generated stable MCF-7 cell lines expressing mutant BRCA1, BRCA1a proteins in the cytoplasm and found BRCA1a mutants to be hypersensitive to estrogen and transforming unlike BRCA1a. We found a clear difference between the colony forming ability of mutant BRCA1 and BRCA1a stable clones. BRCA1a mutant cell lines were highly clonogenic indicating that the enhanced cytoplasmic accumulation of BRCA1a mutants was sufficient to change the growth properties of these cells. Knockdown of endogenous Ubc9 in these MCF-7BRCA1a cells by using Ubc9 siRNA resulted in reversal of suppression of growth by BRCA1a. These findings indicate a role for Ubc9 in BRCA1-mediated growth/tumor suppression. These findings indicate a role for Ubc9 in BRCA1 mediated growth/tumor suppression. Our results suggest that the apoptosis inducing function of BRCA1 proteins may require nuclear localization which agrees with our previous observations where serum depletion resulted in nuclear transport and apoptosis of BRCA1 proteins (Wang et al., 1997). We have observed elevated levels of expression of Ubc9 in these mutant clones as well as several ER- α positive luminal-like, Her-2-like, and TN basal-like breast cancer cells compared to normal mammary epithelial cells. Infact, it made no difference whether the breast cancer cells were luminal-like, Her-2-like, basal-like obtained from African American or White women with breast cancer unlike what was observed previously using TMA of breast tumor samples (Moschos et al., 2010). We have observed high levels of expression of Ubc9 in breast tumors compared to normal mammary tissues. These findings agree with others regarding over-expression of Ubc9 in several tumors such as lung adenocarcinoma, ovarian carcinoma, melanoma, and breast cancer cells where it promotes invasion and metastasis (Zhu et al., 2010). Together, these results support the notion that Ubc9 can function as a dual regulator of transformation and tumor suppression as shown for HMGA1b family of proteins (Li et al., 2007).

These results suggest a molecular interplay between BRCA1 and Ubc9, which maintains the balance of two opposing effects: tumor suppression (inhibition of ER- α) or tumorigenesis (activating ER- α). BRCA1 dysfunction can tilt this delicate balance resulting in ER- α positive or ER- α negative breast cancer (Fig. 8a,b). BRCA1 thus functions as a master switch, which by turning off or on Ubc9 binding regulates ER- α transcription and cell growth. This model explains how BRCA1 can both activate and repress ER- α transcription in hormone responsive tissues like breast and ovaries. Post-translational modification of BRCA1 proteins could prevent the binding of BRCA1 to Ubc9 resulting in breast cancers. We can speculate that the nuclear-cytoplasm shuttling of BRCA1 protein may provide an important mechanism for regulating its tumor suppression function in sporadic breast cancers where somatic mutations in BRCA1 are rarely found. Finally, we have presented simple in vitro functional assays where lack of Ubc9 binding can predict predisposition to breast cancer (Table 1). The question whether sumoylation of BRCA1 by Ubc9 is involved in the nuclear import of BRCA1 proteins and whether DNA repair activity is required for

BRCA1 growth/tumor suppressor function is currently under investigation. Future efforts will be directed towards developing targeted drugs that could facilitate the interaction of Ubc9 with BRCA1 proteins to combat BRCA1-associated breast cancers.

Acknowledgments

We are grateful to Dr. Erdos (National Human Genome Research Institute, Bethesda, MD, USA), Dr. Henderson (West Mead Institute for Cancer Research, Australia), Professor Parker (London, UK), Professor Hay and Dr. Jaffrey (University of Dundee, UK) for generously providing BRCA1, YFP-BRCA1, pSG5-ER- α , ERE-TK-luciferase, pCDNA3 Ubc9 constructs. We thank all the members of Rao and Reddy labs for their help. We thank Ms Printupp for administrative help. We thank Mr. Abramson, research media services and RCMI core facilities at Morehouse School of Medicine, for their help. V.N.R. dedicates this article to her mother, Rohini N. Rao. This work was supported in part by Georgia Cancer Coalition Distinguished Cancer Scholar award, NIH-NCRR-RCMI grant G-12-RR003034 and 5P20RR11104, U54 CA118638 to V.N.R. Georgia Cancer Coalition Distinguished Cancer Scholar award to E.S.P.R. V.N.R.'s lab was also supported in part by funds from ING Foundation.

Literature Cited

- Aprelikova ON, Fang BS, Meissner EG, Cotter S, Campbell M, Kuthiala A, Bessho M, Jensen RA, Liu ET. BRCA1-associated growth arrest is RB-dependent. *Proc Natl Acad Sci USA*. 1999; 96:11866–11871. [PubMed: 10518542]
- Bogdani M, Teugels E, De Greve J, Bourgain C, Neyns B, Pipeleers-Marichal M. Loss of nuclear BRCA1 localization in breast carcinoma is age dependent. *Virchows Arch*. 2002; 440:274–279. [PubMed: 11889597]
- Chang YL, Huang CJ, Chan JY, Liu PY, Chang HP, Huang SM. Regulation of nuclear receptor and coactivator functions by the carboxyl terminus of ubiquitin-conjugating enzyme 9. *Int J Biochem Cell Biol*. 2007; 39:1035–1046. [PubMed: 17336575]
- Coene ED, Hollinshead MS, Waeytens AA, Schelfhout VR, Eechaute WP, Shaw MK, Van Oostveldt PM, Vaux DJ. Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria. *Mol Biol Cell*. 2005; 16:997–1010. [PubMed: 15591126]
- Driscoll JJ, Pelluru D, Lefkimmatis K, Fulciniti M, Prabhala RH, Greipp PR, Barlogie B, Tai YT, Anderson KC, Shaughnessy JD Jr, Annunziata CM, Munshi NC. The sumoylation pathway is dysregulated in multiple myeloma and is associated with adverse patient outcome. *Blood*. 2010; 115:2827–2834. [PubMed: 19965618]
- Dunnebie T, Bermejo JL, Haas S, Fischer HP, Pierl CB, Justenhoven C, Brauch H, Baisch C, Gilbert M, Harth V, Spickenheuer A, Rabstein S, Pesch B, Bruning T, Ko YD, Hamann U. Common variants in the UBC9 gene encoding the SUMO-conjugating enzyme are associated with breast tumor grade. *Int J Cancer*. 2009; 125:596–602. [PubMed: 19358266]
- Eakin CM, Maccoss MJ, Finney GL, Kleivit RE. Estrogen receptor alpha is a putative substrate for the BRCA1 ubiquitin ligase. *Proc Natl Acad Sci USA*. 2007; 104:5794–5799. [PubMed: 17392432]
- Fabbro M, Rodriguez JA, Baer R, Henderson BR. BARD1 induces BRCA1 intranuclear foci formation by increasing RING-dependent BRCA1 nuclear import and inhibiting BRCA1 nuclear export. *J Biol Chem*. 2002; 277:21315–21324. [PubMed: 11925436]
- Hashizume R, Fukuda M, Maeda I, Nishikawa H, Oyake D, Yabuki Y, Ogata H, Ohta T. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem*. 2001; 276:14537–14540. [PubMed: 11278247]
- Hay RT. SUMO: A history of modification. *Mol Cell*. 2005; 18:1–12. [PubMed: 15808504]
- Holt JT, Thompson ME, Szabo C, Robinson-Benion C, Arteaga CL, King MC, Jensen RA. Growth retardation and tumour inhibition by BRCA1. *Nat Genet*. 1996; 12:298–302. [PubMed: 8589721]
- Johnson ES. Protein modification by SUMO. *Annu Rev Biochem*. 2004; 73:355–382. [PubMed: 15189146]
- Kim KI, Baek SH, Chung CH. Versatile protein tag, SUMO: Its enzymology and biological function. *J Cell Physiol*. 2002; 191:257–268. [PubMed: 12012321]
- Kobayashi S, Shibata H, Yokota K, Suda N, Murai A, Kurihara I, Saito I, Saruta T. FHL2, UBC9, and PIAS1 are novel estrogen receptor alpha-interacting proteins. *Endocr Res*. 2004; 30:617–621. [PubMed: 15666801]

- Li Y, Lu J, Prochownik EV. Dual role for SUMO E2 conjugase Ubc9 in modulating the transforming and growth-promoting properties of the HMGA1b architectural transcription factor. *J Biol Chem*. 2007; 282:13363–13371. [PubMed: 17350957]
- Lu M, Conzen SD, Cole CN, Arrick BA. Characterization of functional messenger RNA splice variants of BRCA1 expressed in nonmalignant and tumor-derived breast cells. *Cancer Res*. 1996; 56:4578–4581. [PubMed: 8840964]
- Ma Y, Fan S, Hu C, Meng Q, Fuqua SA, Pestell RG, Tomita YA, Rosen EM. BRCA1 regulates acetylation and ubiquitination of estrogen receptor- α . *Mol Endocrinol*. 2010; 24:76–90. [PubMed: 19887647]
- Maniccia AW, Lewis C, Begum N, Xu J, Cui J, Chipitsyna G, Aysola K, Reddy V, Bhat G, Fujimura Y, Henderson B, Reddy ES, Rao VN. Mitochondrial localization, ELK-1 transcriptional regulation and growth inhibitory functions of BRCA1, BRCA1a, and BRCA1b proteins. *J Cell Physiol*. 2009; 219:634–641. [PubMed: 19170108]
- Marot D, Opolon P, Brailly-Tabard S, Elie N, Randrianarison V, Connault E, Foray N, Feunteun J, Perricaudet M. The tumor suppressor activity induced by adenovirus-mediated BRCA1 overexpression is not restricted to breast cancers. *Gene Ther*. 2006; 13:235–244. [PubMed: 16208422]
- Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M, Frye C, Hattier T, Phelps R, Haugen-Strano A, Katcher H, Yakumo K, Gholami Z, Shaffer D, Stone S, Bayer S, Wray C, Bogden R, Dayananth P, Ward J, Tonin P, Narod S, Bristow PK, Norris FH, Helvering L, Morrison P, Rostek P, Lai M, Barrett JC, Lewis C, Neuhausen S, Cannon-Albright L, Goldgar D, Wiseman R, Kamb A, Skolnick MH. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*. 1994; 266:66–71. [PubMed: 7545954]
- Mingot JM, Kostka S, Kraft R, Hartmann E, Gorlich D. Importin 13: A novel mediator of nuclear import and export. *EMBO J*. 2001; 20:3685–3694. [PubMed: 11447110]
- Mo YY, Yu Y, Ee PL, Beck WT. Overexpression of a dominant-negative mutant Ubc9 is associated with increased sensitivity to anticancer drugs. *Cancer Res*. 2004; 64:2793–2798. [PubMed: 15087395]
- Morris JR, Boutell C, Keppler M, Densham R, Weekes D, Alamshah A, Butler L, Galanty Y, Pangon L, Kiuchi T, Ng T, Solomon E. The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature*. 2009; 462:886–890. [PubMed: 20016594]
- Moschos SJ, Jukic DM, Athanassiou C, Bhargava R, Dacic S, Wang X, Kuan SF, Fayewicz SL, Galambos C, Acquafondata M, Dhir R, Becker D. Expression analysis of Ubc9, the single small ubiquitin-like modifier (SUMO) E2 conjugating enzyme, in normal and malignant tissues. *Hum Pathol*. 2010; 41:1286–1298. [PubMed: 20561671]
- Orban TI, Olah E. Expression profiles of BRCA1 splice variants in asynchronous and in G1/S synchronized tumor cell lines. *Biochem Biophys Res Commun*. 2001; 280:32–38. [PubMed: 11162473]
- Park MA, Seok YJ, Jeong G, Lee JS. SUMO1 negatively regulates BRCA1-mediated transcription, via modulation of promoter occupancy. *Nucleic Acids Res*. 2008; 36:263–283. [PubMed: 18025037]
- Rakha EA, El-Sheikh SE, Kandil MA, El-Sayed ME, Green AR, Ellis IO. Expression of BRCA1 protein in breast cancer and its prognostic significance. *Hum Pathol*. 2008; 39:857–865. [PubMed: 18400253]
- Randrianarison V, Marot D, Foray N, Cabannes J, Meret V, Connault E, Vitrat N, Opolon P, Perricaudet M, Feunteun J. BRCA1 carries tumor suppressor activity distinct from that of p53 and p21. *Cancer Gene Ther*. 2001; 8:759–770. [PubMed: 11687899]
- Reid LJ, Shakra R, Modi AP, Lokshin M, Cheng JT, Jasin M, Baer R, Ludwig T. E3 ligase activity of BRCA1 is not essential for mammalian cell viability or homology-directed repair of double-strand DNA breaks. *Proc Natl Acad Sci USA*. 2008; 105:20876–20881. [PubMed: 19088202]
- Ronen O, Malone JP, Kay P, Bivens C, Hall K, Paruchuri LP, Mo YY, Robbins KT, Ran S. Expression of a novel marker, Ubc9, in squamous cell carcinoma of the head and neck. *Head Neck*. 2009; 31:845–855. [PubMed: 19309722]

- Rosen EM, Fan S, Ma Y. BRCA1 regulation of transcription. *Cancer Lett.* 2006; 236:175–185. [PubMed: 15975711]
- Rowell S, Newman B, Boyd J, King MC. Inherited predisposition to breast and ovarian cancer. *Am J Hum Genet.* 1994; 55:861–865. [PubMed: 7977346]
- Seeler JS, Dejean A. Nuclear and uncLEAR functions of SUMO. *Nat Rev Mol Cell Biol.* 2003; 4:690–699. [PubMed: 14506472]
- Sentis S, Le Romancer M, Bianchin C, Rostan MC, Corbo L. Sumoylation of the estrogen receptor alpha hinge region regulates its transcriptional activity. *Mol Endocrinol.* 2005; 19:2671–2684. [PubMed: 15961505]
- Shao N, Chai YL, Shyam E, Reddy P, Rao VN. Induction of apoptosis by the tumor suppressor protein BRCA1. *Oncogene.* 1996; 13:1–7. [PubMed: 8700535]
- Sun H, Leversson JD, Hunter T. Conserved function of RNF4 family proteins in eukaryotes: Targeting a ubiquitin ligase to SUMOylated proteins. *EMBO J.* 2007; 26:4102–4112. [PubMed: 17762864]
- Tait DL, Obermiller PS, Holt JT. Preclinical studies of a new generation retroviral vector for ovarian cancer BRCA1 gene therapy. *Gynecol Oncol.* 2000; 79:471–476. [PubMed: 11104622]
- Thangaraju M, Kaufmann SH, Couch FJ. BRCA1 facilitates stress-induced apoptosis in breast and ovarian cancer cell lines. *J Biol Chem.* 2000; 275:33487–33496. [PubMed: 10938285]
- Thompson ME. BRCA1 16 years later: Nuclear import and export processes. *FEBS J.* 2010; 277:3072–3078. [PubMed: 20608972]
- Uzunova K, Gottsche K, Miteva M, Weisshaar SR, Glanemann C, Schnellhardt M, Niessen M, Scheel H, Hofmann K, Johnson ES, Praefcke GJ, Dohmen RJ. Ubiquitin-dependent proteolytic control of SUMO conjugates. *J Biol Chem.* 2007; 282:34167–34175. [PubMed: 17728242]
- Wang H, Shao N, Ding QM, Cui J, Reddy ES, Rao VN. BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases. *Oncogene.* 1997; 15:143–157. [PubMed: 9244350]
- Wen J, Li R, Lu Y, Shupnik MA. Decreased BRCA1 confers tamoxifen resistance in breast cancer cells by altering estrogen receptor-coregulator interactions. *Oncogene.* 2009; 28:575–586. [PubMed: 18997820]
- Wilson CA, Payton MN, Elliott GS, Buaas FW, Cajulis EE, Grosshans D, Ramos L, Reese DM, Slamon DJ, Calzone FJ. Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-delta11b. *Oncogene.* 1997; 14:1–16. [PubMed: 9010228]
- Xu J, Watkins T, Reddy A, Reddy ES, Rao VN. A novel mechanism whereby BRCA1/1a/1b fine tunes the dynamic complex interplay between SUMO-dependent/independent activities of Ubc9 on E2-induced ERalpha activation/repression and degradation in breast cancer cells. *Int J Oncol.* 2009; 34:939–949. [PubMed: 19287951]
- Yuli C, Shao N, Rao R, Aysola P, Reddy V, Oprea-Ilie G, Lee L, Okoli J, Partridge E, Reddy ES, Rao VN. BRCA1a has antitumor activity in TN breast, ovarian and prostate cancers. *Oncogene.* 2007; 26:6031–6037. [PubMed: 17384678]
- Zhu S, Sachdeva M, Wu F, Lu Z, Mo YY. Ubc9 promotes breast cell invasion and metastasis in a sumoylation-independent manner. *Oncogene.* 2010; 29:1763–1772. [PubMed: 20023705]

Fig. 1.

Ubc9 is required for nuclear localization of BRCA1 and BRCA1a proteins. The subcellular localization and fractionation of BRCA1, BRCA1a and its mutants in various cancer cells by immunofluorescence, live imaging and Western blot analysis. a–d: COS-1 cells. COS-1 cells were seeded into six-well plates and transfected with pCDNA3 BRCA1 or pCDNA3 BRCA1 Mut#1 or BRCA1 Mut#4 or YFP-BRCA1 (yellow fluorescent protein) NLS mu with and without Ubc9 or pCMVTag BRCA1a, pCMVTag BRCA1a Mut#1 or pCMVTag BRCA1a Mut#4 or pRFP-BRCA1a or its mutants using Lipofectamine 2000 (Invitrogen). The nuclei were visualized with DNA staining dye Hoechst 24 h after transfection. The live images of cells were taken by fluorescent microscope (20 $\times$) (Olympus). Graphs show the proportion of transfected COS-1 cells showing predominantly nuclear (N), nuclear/cytoplasmic (NC), cytoplasmic (C) BRCA1 (wild-type or mutant). Bars represent the mean $\pm$ SE of at least two experiments. BRCA1 transfected cells were fixed and probed with Ab-1 antibody followed by Alexa Fluor 488-labeled secondary antibody staining. The nuclei were visualized with DAPI staining. Western blot analysis after subcellular fractionation of BRCA1a transfected COS-1 cells is shown. Cells were transfected with pRFP-BRCA1a or its mutants and harvested 48 h after transfection and nuclear and cytoplasmic extracts were prepared as described (Maniccia et al., 2009). Blots were probed with Ab-1, cytoplasmic marker HSP 90 α/β and nuclear envelope marker Lamin A + C.

Fig. 2.

Ubc9 is required for nuclear localization of BRCA1a proteins. The subcellular localization of BRCA1a and its mutants in various normal and cancer cells by live imaging was carried out as described previously (Xu et al., 2009). a: MCF10A, (b) MCF-7, (c) LNCap, (d) PC-3, (e) NIH OVCA93, (f) HCC-70. Cells were seeded into six-well plates and transfected with pRFP-BRCA1a or its mutants using Lipofectamine 2000 (Invitrogen). The live images of cells were taken by fluorescent microscope (20 $\times$) (Olympus). The nuclei were visualized with DAPI staining.

Fig. 3.

Effect of endogenous Ubc9 knockdown on E2-induced ER- α repression and subcellular localization of BRCA1, BRCA1a, and BRCA1b proteins in breast cancer cells. Live imaging (a) or immunofluorescence using BRCA1 Ab1 antibody on MCF-7 cells transfected with control or Ubc9 siRNA (b) and RFP-BRCA1a (a), or YFP-BRCA1 (c), or GFP-BRCA1b (d). The nuclei were visualized with DAPI or blue fluorescent Hoechst dye staining using a fluorescent microscope (20 $\times$ or 100 $\times$). Western blot analysis of Ubc9 expression in siRNA-treated MCF-7 cells. The control and Ubc9 siRNA-treated MCF-7 cells were lysed and probed with Ubc9 antibody. β -Actin was used as an internal control. The stoichiometry of Ubc9 protein levels is shown. The signal of Ubc9 protein band was quantified using software MultiGauge and the values were used to plot efficiency of Ubc9 knockdown in MCF-7 cells. Live image of MCF-7 EGFP-AS Ubc9 stable cells transfected with RFP-BRCA1 a, EGFPBRCA1b and YFP-BRCA1 plasmids (e). T47D cells were transfected with control and Ubc9 siRNA in the presence of pSV2 ERE-luc, pCMVtag

BRCA1a, treated with E2 and assayed for luciferase activity as described previously (Xu et al., 2009) (f).

Fig. 4.

Altered Ubc9 binding results in loss of growth suppression by BRCA1 and BRCA1a in ER- α positive and TNBC cells. MCF-7 or CAL51 cells were transfected with pCDNA3 or pCDNA3-BRCA1 (a), or pCDNA3-BRCA1a (b) and selected with G418 and the colonies were stained with crystal violet and counted as described (Yuli et al., 2007). The number of colonies obtained by pCDNA3 was considered as 100%. Each experiment was repeated three times and the bars shown represent s.d. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcp>]

Fig. 5. MCF-7 BRCA1/BRCA1a Mut#1, Mut#4 stable clones are transforming and promote survival. Expression of wild-type BRCA1a and mutant BRCA1a protein levels in BRCA1a, BRCA1a Mut#1, and BRCA1a Mut#4 transfected MCF-7 stable clones (a). BRCA1a Mut#1 and Mut#4 (b), BRCA1 Mut#1 and Mut#4 (c), cell lines are clonogenic in soft agar compared to BRCA1/BRCA1a clones. Soft agar assays were performed as previously described (Yuli et al., 2007). [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcp>]

Fig. 6. BRCA1a Mut#1 and Mut#4 clones are hypersensitive to E2 and proliferative unlike BRCA1a clones which were growth arrested both in serum depleted as well as in presence of E2 (a). Knockdown of endogenous Ubc9 in MCF-7BRCA1a cells resulted in increased cell proliferation. MCF-7pCDNA3 and MCF-7BRCA1a cells were transfected with Ubc9 or control siRNA. After 48 h the growth was measured using CellTiter-Glo kit. Each assay was performed in triplicate and the bars shown represent s.d. (b). Ubc9 is required for E2-induced growth suppression exerted by BRCA1a in breast cancer cells. The experiment was performed as in (b) except that 24 h after transfection with siRNAs the cells were starved in serum depleted medium for 48 h. The cells were then treated with E2 for 24 h and visualized under a fluorescence microscope (Olympus, 4 $\times$ lens) (c).

Fig. 7.

Ubc9 is expressed at high levels in several breast cancer cells lines and tissues, stable MCF-7BRCA1a Mut#1, Mut#4 clones compared to normal mammary epithelial cells and tissues. Expression of Ubc9 and ER- α in T47D, MCF-7, MCF10A, MDAMB231, CAL51, HCC-70, SKBR-3, MDAMB468, MCF-7BRCA1a, and mutant clones by Western blot analysis. Protein concentrations were normalized using β -actin (a). Ubc9 protein levels were screened in seven patient sets of ductal carcinomas/normal adjacent tissue lysates using INSTA-blot Breast Tissue OncoPair from INGENEX. Approximately 14 μ g/lane of protein was loaded (b).

Fig. 8. Model to explain how BRCA1 functions as a growth/tumor suppressor by regulating the SUMO-dependent/independent activity of Ubc9 and any tilt in this delicate balance results in ER- α positive and ER- α negative breast cancers (a). Working hypothetical model showing how BRCA1, by binding to Ubc9 regulates ER- α activity resulting in tumor suppression. Mutant BRCA1 is unable to interact with Ubc9 resulting in deregulated levels of UBC9, which activates ER- α resulting in cancer (b).

Fig. 9.

Model showing how Ubc9 serves as a new import pathway to shuttle BRCA1 and BRCA1a proteins to the nucleus. BRCA1 can enter the nucleus in an import in α/β receptor pathway and/or Ubc9 pathway. BRCA1a/1b proteins can enter the nucleus by binding to Ubc9.

TABLE 1

Summary of functional differences between wild-type and mutant BRCA1/BRCA1a proteins

	WT BRCA1/1a	(K109R) Mut #1	(C61G) Mut #4	Refs.
UBC9 binding	+	–	–	Xu et al. (2009)
ER- α binding (337–379)	+	–	–	Xu et al. (2009), Rosen et al. (2006)
Repression of ER- α activity	+	–	–	Xu et al. (2009), Rosen et al. (2006)
UBC9 C93S binding	+	–	–	Xu et al. (2009)
Relieve E2-induced ER- α repression by UBC9	+	–	–	Xu et al. (2009)
Repression of E2-induced ER- α activity by UBC9 C93S	+	–	–	Xu et al. (2009)
E3 ubiquitin ligase activity	+	–	–	Xu et al. (2009), Hashizume et al. (2001)
Growth suppression	+	–	–	This study
Subcellular localization	Nucleus + cytoplasm	Enhanced/exclusively cytoplasm	Enhanced/exclusively cytoplasm	This study
Transforming/growth promoting	–	+	+	This study