Selenium Protection from DNA Damage Involves a Ref1/p53/Brca1 Protein Complex

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Abstract. *Selenium, in the form of seleno-L-methionine (SeMet), induced Redox-factor-1 (Ref1) and p53 proteins in normal human and mouse fibroblasts. Ref1 and p53 are known to be associated with each other, resulting in enhanced sequence-specific DNA binding by p53 and transactivation of p53-regulated effector genes. SeMet preferentially induced the DNA repair branch of the p53 pathway, while apoptosis and cell cycle arrest were unaffected. Accordingly, pretreatment with SeMet protected normal fibroblasts from subsequent DNA damage. In the current study, Brca1 and Ref1 were shown to interact concurrently with p53 in targeting a SeMet-induced DNA repair response. Moreover, like p53 and Ref1, Brca1 was required for SeMet-mediated DNA damage protection, as brca1-/- mouse fibroblasts were not protected from UV-radiation by SeMet treatment. These findings indicate that besides p53 and Ref1, Brca1 is required for selenium protection from DNA damage. The data are consistent with selective induction of the DNA repair branch of the p53 pathway by SeMet.*

The tumor suppressor/transcription factor p53 interacts with a number of cellular proteins to coordinate its complex biological functions of apoptosis, cell cycle arrest and DNA repair. Specifically, other authors have shown that p53 interaction with Brca1 is required for the p53-mediated nucleotide excision (NER) DNA repair pathway, involving the p53-regulated effector genes *XPC, p48XPE* and *Gadd45a* (1, 2). One unanswered question is whether known p53-interacting proteins can interact concurrently with p53, as opposed to potentially exclusionary interactions. Both redox factor-1 (Ref1) and Brca1 are well-known to interact physically and functionally with p53. This is the first report demonstrating that they may do so concurrently, *i.e.* the p53 function(s) that Ref1 and Brca1 govern are compatible and not mutually exclusive.

Selenium, in the form of seleno-L-methionine (SeMet), promotes the reduction of key p53 cysteine residues 275 and/or 277, a reduction required for sequence-specific DNA binding of p53 to its target genes (3). The redox factor Ref1 is the mediator of selenium signaling to p53, since a Ref1 mutant carrying alanine substitution for cysteine 65 (the known redox center of Ref1) blocked p53 cysteine reduction in response to SeMet (4). Recently, Ref1 was shown to bind to p53, promote p53 tetramerization and enhance p53 sequence-specific DNA binding in its reduced state (5).

SeMet protected normal human or mouse fibroblasts from UV-radiation corresponding to a SeMet-inducible DNA repair response (4, 6). This repair response was p53-dependent as p53-null mouse embryo fibroblasts did not exhibit increased DNA repair and were not protected (4). Likewise, cells carrying dominant-negative Ref1 were UV-sensitive and did not elicit a DNA repair or protective response to SeMet (4). Importantly, the induction of DNA repair and DNA damage protection required a 15-h pretreatment with SeMet (4). Neither DNA repair nor protection was observed when SeMet was given concurrently with DNA damage (4, 6). The mechanism whereby SeMet induced DNA repair involves a non-genotoxic signal transduction pathway, since SeMet alone did not cause DNA damage (3, 4, 6). Rather, DNA repair proteins were elevated by SeMet pretreatment, which protected cells from subsequent DNA damage (4, 6, 7).

In determining the mechanism for SeMet-enhanced DNA repair, p53- and Ref1-containing protein complexes in SeMet-treated cells were probed under the exact conditions previously shown to induce NER and DNA damage protection (4, 6). One important p53-interacting protein, Brca1, was found to interact with p53 concurrently with Ref1, indicating that Ref1 could potentially regulate the

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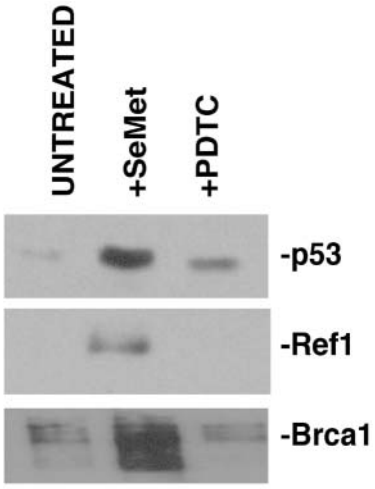

Figure 1. *Detection of Ref1 and Brca1 in p53-immune complexes of selenium-treated cells. Antibody to p53 D01/agarose conjugate was used to immunocapture p53, then p53 and p53 interacting proteins were detected by immunoblotting. Cellular Ref1 and Brca1 were pronounced in SeMet-treated cells compared to untreated cells and were decreased to absent in PDTC-treated cells. All detected proteins were endogenous to the RKO cells. The cellular treatment conditions (10 μM SeMet; 15 h) have been shown to elicit a DNA repair and protective response to subsequent DNA damage (4, 6). Pyrollidone dithiocarbamate (PDTC) blocked DNA repair and DNA damage protection in those same studies (4).*

activities of p53 and Brca1, *e.g.,* by the redox-factor-1 function. DNA repair genes regulated by transcription factors p53 and Brca1 may be sensitive to changes in cellular redox state *via* the presence of Ref1 in the complex.

Materials and Methods

Immunocapture of cellular and recombinant p53 using anti-p53 D01/agarose conjugate. Recombinant p53, Rad51 and Brca1 were purchased from Santa Cruz Biotech, Santa Cruz, CA, USA, as baculovirus-encoded proteins. Ref1 (APE/Ref1) was a bacterially-expressed histidine-tagged protein from Dr. Mark R. Kelley, Indiana University, USA. RKO cells carrying wild-type p53 were treated with SeMet (10 μM, 15 h). Untreated cells and cells treated with pyrrolidine dithiocarbamate (PDTC; Sigma, St. Louis, MO, USA) were used as controls. PDTC is known to oxidize p53 cysteine residues, an effect opposite to that of SeMet. The immune complexes were collected by overnight agitation with anti-p53 D01 agarose conjugate (Oncogene Research Products, San Diego, CA, USA). The immunoprecipitation lysis buffer consisted of 50 mM Tris/HCl pH 8, 150 mM NaCl, 1% Triton X-100 and protease inhibitors. The immune complexes were washed 4 times with lysis buffer, then boiled in SDS gel-loading buffer and subjected to electrophoresis and transfer. Proteins were detected with horseradish peroxidase conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce Inc., Rockford, IL, USA). The complexes were analyzed using mouse monoclonal antibodies for p53 (D01); APE/Ref1 (NB100-116A3, Novus Biologicals, Littleton, CO, USA), BRCA1 (Ab-1, Oncogene Research Products) and Rad51 (Ab1, NeoMarkers, Fremont, CA, USA) antibodies.

For the immunocapture of cellular p53, RKO human colon cancer cells wild-type for p53 or H1299 human lung cancer cells null for p53 were used. Endogenous wild-type p53 and bound Brca1 and Ref1 are depicted in Figure 1. A pcDNA3.1 plasmid encoding wild-type p53 was used to transfect H1299 cells by the FuGene method, together with pcDNA3.1 plasmids encoding the wild-type Ref1 or C65A Ref1 mutant. The cells were treated with 10 μM SeMet overnight and immunoprecipitation was conducted as reported in reference 4.

Chemical crosslinking of recombinant Ref1, p53 and Brca1. Recombinant proteins (1 μg) were allowed to interact at room temperature, were then treated with 5 mg/mL dimethylsuberimidate for 20 min at 37˚C, in a 20 μL volume of 20 mM HEPES pH7.9, 100 mM KCl, 1 mM DTT, followed immediately by SDS/polyacrylamide gel electrophoresis and immunoblotting on nitrocellulose (8). The monoclonal antibody to APE/Ref1 (NB100) was used to detect Ref1.

Sequence-specific DNA binding of p53 (4). The prototype p53-binding site is identical to that of the p53-regulated *Gadd45a* gene (9). We showed that SeMet treatment enhanced sequence-specific p53 binding to the prototype sequence (4). In the current study, the protein complexes were large, owing to the presence of the 200 kilodalton Brca1 protein in the complex, as well as to p53 tetramerization and formation of higher-order p53-containing complexes. Therefore, an agarose conjugate in which the prototype p53-binding site was covalently coupled to agarose beads (Santa Cruz Biotech) was used. Recombinant proteins were incubated with the beads in a 25-μl volume in 20 mM HEPES pH 7.8, 100 mM KCl, 1 mM EDTA, 1 mM DTT for 20 min at 37˚C, and were then washed 4 times in 1 mL of lysis buffer composed of 10 mM Tris pH 7.5, 150 mM NaCl and 1% Triton-X100. Bound proteins were dissociated from the beads by boiling in 3X SDS gel-loading buffer and were separated by electrophoresis. Bound proteins were detected by immunoblotting as above. The detection of Brca1 and Ref1 required p53, as neither was detected in controls lacking p53 (results not shown).

Cell survival assays. To evaluate the role of BRCA1 in the selenium protective response to DNA damage, brca1-/- MEFs (homozygous for the Δ exon 11 allele) were treated in the presence or absence of 10 μM SeMet for 15 h and were then exposed to UV radiation as indicated. Non-irradiated cells served as controls. The cell yield was determined after 7 days by a thiazolyl blue viability assay (Ref. 4; Figure 3).

Results

Both p53 and Ref1 proteins were elevated in cells treated overnight with 10-20 μM SeMet (4, 7), a relatively physiological and nontoxic concentration (10, 11). Both proteins are regulated by post-translational protein stabilization and p53 cysteine residues 275 and/or 277 were reduced in SeMet-treated cells (4). Although human p53 contains 12 cysteine residues, cysteines 275 and/or 277 reside in the DNA-binding domain and are known to be critical for sequence-specific binding of p53 to its downstream effector

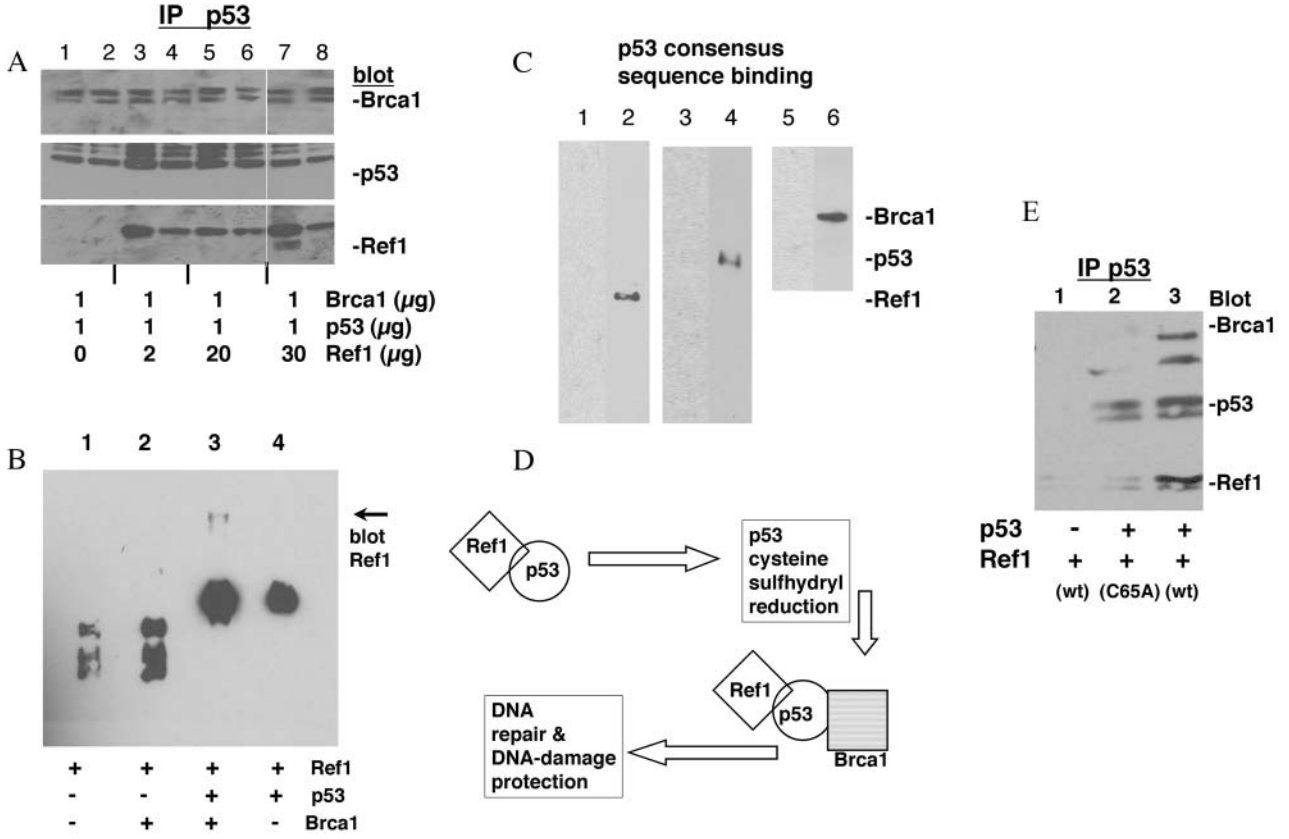

Figure 2. *Evidence of concurrent interaction of Ref1 and Brca1 with p53 using recombinant proteins. A) Excess Ref1 does not compete with Brca1 for p53 binding. Recombinant proteins were mixed as indicated and allowed to interact for 1 h at room temperature. Then, p53 was immunocaptured using antibody D01-agarose. After extensive washing in 1 mL of buffer containing 1% Triton-X100, the immunocaptured proteins were subjected to electrophoresis and immunoblotting. The amount of Ref1 added did not affect the binding of Brca1 to p53, suggesting that different p53 domains are involved in binding to Ref1 versus Brca1. Note that the amount of added Ref1 (20 μg, 30 μg) saturated the p53 binding sites. Duplicate lanes shown. B) Chemical crosslinking of putative ternary complex containing p53, Ref1 and Brca1. Recombinant proteins were allowed to interact as in A, were then crosslinked by addition of dimethylsuberimidate for 20 min at 37˚C and were subjected to electrophoresis and immunoblotting. Ref1 and Brca1 alone showed no evidence of interaction (lanes 1 and 2), however, p53 addition yielded slower-migrating species where all 3 proteins were present (lane 3). Immunodetection was carried out with a Ref1 antibody, hence the higher molecular weight band was not detected in controls lacking Ref1 (not shown). C) Immunocapture of p53 by sequence-specific DNA binding results in concurrent capture of Ref1 and Brca1 in complex with p53. Recombinant proteins were used as in A and B. The consensus p53 DNA-binding sequence conjugated to agarose was used to bind p53 and p53-interacting proteins. After extensive washing in buffer containing 1% Triton X-100, electrophoretically-resolved proteins were detected by their respective antibodies. The data suggest that Brca1 and Ref1 can interact concurrently with p53 and do not compete for p53 binding, consistent with the other experiments. Lanes 1, 2, blot Ref1, p53 absent in lane 1, p53 present in lane 2; lanes 3,4, blot p53, p53 absent in lane 3, p53 present in lane 4; lanes 5,6, blot Brca1, p53 absent in lane 5, p53 present in lane 6. D) Current model of DNA damage protection by selenium involving the Ref1/p53/Brca1 complex. Key p53 cysteine residues are sulfhydryl-reduced in selenium-treated cells, a response that requires Ref1/p53 interaction (4, 9). Brca1 can interact with p53 even in the presence of excess Ref1, suggesting a ternary complex. Brca1 cooperates with p53 in driving p53-mediated DNA repair and protective response to UV-radiation (2, 18, 26, 27). E) Binding of cellular Brca1 and Ref1 to p53 are linked. Cellular p53 was immunocaptured from transfected H1299 cells null for p53, then probed for the presence of Brca1 and Ref1 in the immune complexes; lane 1, Brca1 and Ref1 not detected in p53-minus control; lane 2, little Brca1 or Ref1 detected in presence of Ref1 C65A mutant, unable to reduce p53 cysteines; lane 3, clear Brca1 and Ref1 detection in presence of wild-type Ref1. The interacting Brca1 was endogenous to the cell line. The lower band detected by the Brca1 antibody may be a truncated Brca1 protein endogenous to the cell line, or a degradation product.*

gene sequences (3). Immunoprecipitation and immunoblot experiments of extracts of SeMet-treated cells were conducted. A p53-antibody/agarose conjugate was used to capture p53 and then the membranes were probed for the interacting proteins Ref1 and Brca1. Both Ref1 and Brca1 were detected in complex with p53 in SeMet-treated cells (Figure 1). As a control, pyrrolidine dithiocarbamate (PDTC), known to oxidize p53 cysteine residues (12), was used. PDTC completely blocked the interaction of p53 with Ref1 and Brca1 (Figure 1).

To ascertain that the protein interactions we observed were direct interactions, recombinant proteins of bacterial or baculovirus origin were used. The recombinant proteins were incubated together and the immune complexes were then captured by p53-antibody/agarose beads. Blots were probed for Brca1 and Ref1 proteins as in Figure 1. Brca1 was not displaced from p53, even in the presence of excess Ref1 (Figure 2A). As a control, Rad51, which strongly competed for interaction with p53, displacing both Ref1 and Brca1 (results not shown), was employed. Reciprocal experiments, in which recombinant protein complexes were formed and then probed for the presence of Ref1 (Figure 2B), were conducted. Rather than immunocapture, the chemical crosslinking agent dimethylsuccinimide was used to covalently link the interacting proteins (8). No evidence of crosslinking between Ref1 and Brca1 alone was found, in as much as the 2 proteins are not known to interact (Figure 2B). However, the presence of p53 caused a larger protein complex to be detected. The putative ternary complex containing p53, Brca1 and Ref1 was detected only in lanes where all 3 proteins were present (Figure 2B).

Most p53-regulated cellular functions depend on p53 interaction with downstream effector gene sequences. A consensus p53-binding site has been derived from the study of a number of p53-dependent gene elements (13). We used a consensus p53-binding double-strand oligonucleotide coupled to agarose to capture p53 and any p53-bound proteins (Figure 2C). Recombinant proteins were mixed as above, then immunocapture was conducted in an excess volume of a buffer containing 1% Triton X-100. The beads were washed extensively in the same buffer and the proteins were detected by immunoblotting. Both Brca1 and Ref1 were detected in the p53 complexes (Figure 2C). EMSA assays were conducted using the radiolabelled free double-strand p53-binding sequence as in (4). However, the high molecular weight of the putative ternary complex caused most of the protein to remain in the wells (results not shown). We chose the less complex assay shown in Figure 2C to demonstrate that Ref1 and Brca1 can concurrently interact with p53 and do not compete for p53 binding in the context of p53 sequence-specific DNA binding.

The data point to a model (Figure 2D) in which Ref1 and Brca1 bind cooperatively to p53. Although recombinant Brca1 binds p53, irrespective of the presence or absence of Ref1 (Figure 2A), cellular Brca1 bound to p53 in the presence of wild-type but not mutant Ref1 (Figure 2E). The C65A Ref1 mutant affects the redox center of Ref1 and cannot promote p53 cysteine 275/277 sulfhydryl reduction. Likewise, PDTC, a known oxidizer of p53 cysteines 275/277, blocked or decreased Brca1 and Ref1 interaction with p53 (Figure 1). Recombinant proteins were already in the cysteine-sulfhydryl reduced form, owing to the presence of DTT (Figure 2A).

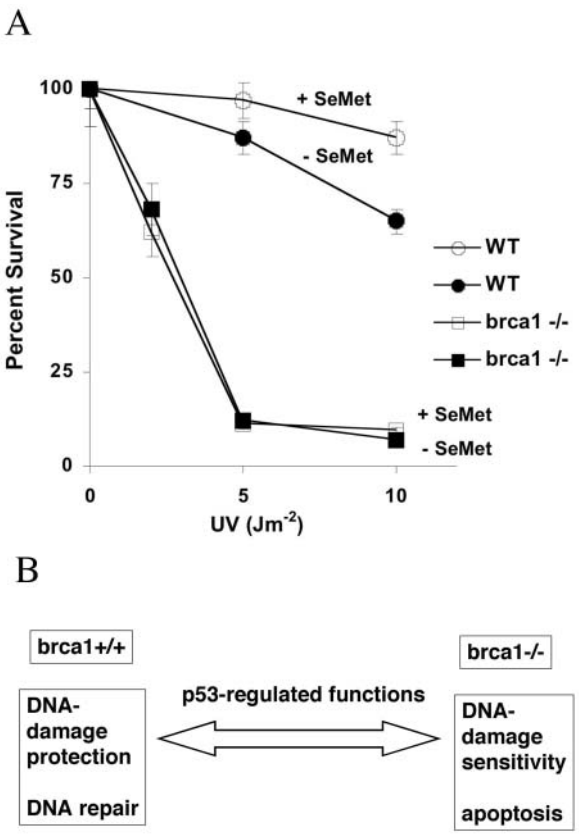

Figure 3. *Brca1 is required for selenium protection from DNA damage. A) Primary, low-passage, brca1-/- MEFs were treated with 10 μM SeMet for 15 h and were then exposed to UV radiation as indicated. The cell yield was determined after 7 days. SeMet did not protect brca1 -/- MEFs from UV-radiation. The mean±SD of 3 independent determinations is shown. B) Model depicting a role for Brca1 in directing p53 in DNA repair and DNA damage protection. In the absence of Brca1, p53-mediated apoptosis is favored over DNA damage protection, i.e. Brca1 is a key component of the p53-mediated protective response to UV-radiation (15, 18, 26, 27).*

The above findings suggest that, in addition to p53 and Ref1 (4), Brca1 might also be involved in DNA damage protection by SeMet. We used brca1-/- mouse embryo fibroblasts to test the hypothesis. Similar to our earlier findings using p53-/- mouse fibroblasts (4), SeMet did not protect brca1-/- fibroblasts from UV-radiation (Figure 3). Thus, the SeMet-enhanced DNA repair and protective responses observed in normal fibroblasts require not only p53 and Ref1, but also Brca1.

Discussion

The tumor suppressor p53 has multiple cellular functions that are regulated by protein-protein interactions. Quite a number of proteins are known to interact with p53 and to regulate p53 functions, leading to distinct cellular end-points

pertaining to cell survival, including apoptosis, DNA repair and cell cycle effects (14). It is extremely unlikely that the many p53-interacting proteins are able to bind p53 simultaneously, thus some interactions are probably mutually exclusive. Interactions that are mutually exclusive would presumably mediate opposing (*i.e.* mutually exclusive) effects on p53 function. Interactions that occur concurrently would be compatible in the regulation of p53 function toward a singular functional end-point, *i.e.* DNA repair (15).

We showed that p53-dependent DNA repair was the predominant pathway whereby selenium protected normal fibroblasts from DNA damage, and that the redox factor Ref1 was required to convey selenium signal transduction to p53 (4, 6). Specifically, Ref1 binds to p53 causing the reduction of sulfhydryl groups on key p53 cysteines 275 and/or 277 (4). Mutation of Ref1 cysteine 65 to alanine blocked p53 cysteine reduction in response to selenium, possibly involving the selenoenzyme thioredoxin reductase (3, 4, 16). Selenium enhanced the sequence-specific DNA binding and trans-activation of DNA repair genes by p53, with no evidence of cell cycle arrest or apoptosis in normal fibroblasts, at least in response to seleno-L-methionine (SeMet) at concentrations below 45 μM (4, 6, 17). The use of higher (non-physiological) SeMet concentrations or other chemical forms of selenium may lead to apoptosis or other functional consequences (7). Thus, SeMet, at relatively physiological nontoxic concentrations, selectively enhanced the DNA repair branch of the p53 pathway and protected mouse or human fibroblasts from DNA damage in the form of UV-radiation (4, 6). Both DNA repair and DNA damage protection were p53-dependent and both required a 15-h SeMet pretreatment prior to DNA damage (4).

We focused on the DNA repair branch of the p53 pathway. One p53-interacting protein known to regulate the DNA repair branch of the p53 pathway is Brca1 (2, 18). Therefore, the finding that Ref1 and Brca1 can interact concurrently with p53 supports the hypothesis, among the many alternative p53 functions, that the DNA repair branch of the p53 pathway was preferentially activated in selenium-treated cells (4, 6).

The data also suggested that cells lacking either p53, Ref1, or Brca1 would be defective in mounting a protective response to selenium treatment. We showed previously that selenium protection from DNA damage required p53 and Ref1 (4). Here, we showed that Brca1 is additionally required (Figure 3). Besides NER, Brca1 participates in other DNA repair pathways, about which less is known mechanistically (19). For example, Brca1 is required for Rad51 protein assembly at sites of double-strand break DNA damage (20, 21). Curiously, p53 also binds to Rad51 and p53-/- MEFs exhibited a phenotype of rad51 "stalling" at double-strand break sites (22, 23). Brca1 functions are complex and may involve DNA damage signaling *via* the poly-ADP-ribose polymerase (PARP) signaling system (24). It is likely that different domains of Brca1 are involved in DSB repair, as compared to its role in transcription of DNA repair genes (2), given that Rad51 and Brca1 interactions with p53 were mutually exclusive in the current study. Of course, the role of Brca1 in DSB repair may be a direct one, while its role in NER requires transcription of the NER genes *XPC, p48XPE* and *Gadd45a* (15, 17, 25). Moreover, NER occurs in the G1- and G2-phases of the cell cycle, while Rad51 functions mainly in the S-phase. More studies are needed for mechanism-driven approaches to cancer prevention and therapy, such as employing selenium in conjunction with DNA repair targets.

Acknowledgements

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