Supplementary Information - Additional Methods

Chromatin immunoprecipitation (ChIP) assay. Components of buffers used in all ChIP assays are as follows: SDS buffer (50 mM Tris-HCl at pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors including freshly prepared PMSF), IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 16.7 mM Tris-HCl at pH 8.1, 167 mM NaCl), low salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl at pH 8.1, 2 mM EDTA, and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl at pH 8.1, 2 mM EDTA, and 0.5 M NaCl), IP buffer#3 (1% Nonidet P-40, 1% sodium deoxycholate, 100 mM Tris-HCl at pH 8.1, and 500 mM LiCl), TE buffer (10 mM Tris-HCl at pH 8.1 and 1 mM EDTA), and IP elution buffer (1% SDS and 50 mM sodium bicarbonate)

Quantitative real-time PCR analysis. To assess mRNA expression, 100 ng aliquots of cDNA prepared from total RNA samples were used in PCR reactions containing TaqMan Universal master mix (Applied Biosystems, Foster City, CA), 30 nM ROX (Stratagene, La Jolla, CA) and premixed Taqman probes and primers (Applied Biosystems) in a final volume of 25 µL. Following activation of the AmpliTaq Gold for 10 min at 95°C, 45 cycles (15 s at 95°C and 1 min at 60°C) were performed using the Mx3000p real-time PCR system (Stratagene). Fluorescence intensity was monitored in real-time, and cycle threshold cycles (CTs) were calculated based on dRn fluorescence with an adaptive baseline using software supplied with the MX3000p. Standard curves were generated based on 2-fold serial dilutions of cDNA from MCF7 and RKO cells, and these data were used to calculate PCR amplification efficiencies. Comparative quantitation was performed by comparing the CTs obtained from amplification of a given target gene to that observed for 18S rRNA or actin as a normalizer, and relative mRNA

abundance was calculated using the -ΔΔCT method (Stratagene). *BRCA1*, *DEC1*, and *NBR2* probes were FAM-labeled and endogenous control genes were VIC-labeled, and assays were performed in triplicate or quadruplicate. To detect *VEGF* expression, PCR amplifications were performed in the presence of SYBR Green I dye (Stratagene) with primers specific to the *VEGF* gene and to *Actin* for normalization.

Quantitative PCR analysis of ChIP assays was performed using 5 μL of ChIP DNA (representing $1/50^{th}$ of immunoprecipitate or 1% of input DNA) in a reaction mixture containing: 200 nM primers (shown below), 200 μM dNTPs, 10 mM Tris-HCl at pH 8.5, 50 mM KCl, 2 mM $MgCl_2$, 0.5 μL Advantage II Taq polymerase (BD Pharmingen, Franklin Lakes, NJ), 5 M Betaine (Calbiochem, San Diego, CA), 0.167x SYBR Green I and 30 nM ROX dye (Stratagene). Accumulation of fluorescent products was monitored by real-time PCR, and each PCR reaction generated only the expected specific amplicon, as shown by the melting-temperature profiles (dissociation curves) of final products. PCR assays were run in triplicate, and threshold cycles (CTs) were calculated as described earlier. Standard curves were generated based on 2-fold serial dilutions of input DNA which were used to calculate amplification efficiencies. Amplification products were also analyzed by agarose gel electrophoresis, and selected amplicons (e.g. *BRCA1* promoter fragments) were isolated by gel excision, purified using a QIAquick gel extraction kit (Qiagen) and sequenced to confirm specific amplification of target sequences (data not shown). Calculations of relative promoter occupancy were based on previously described equations (1) and an example is shown in Fig. S3c. The consistency of calculated promoter occupancies is shown in Fig. S3e for a representative ChIP assay across several independent replicates and experiments.

ChIP assay antibodies. Antibodies specific for the following proteins were used in the ChIP assays: E2F1 (clone KH20&KH95, Cat. no. 05-379, Upstate Biotech), E2F4 (clone C-20, Cat. no. sc-8366x, Santa Cruz), E2F6 (clone E-20, Cat. no. sc-8366x, Santa Cruz; and clone TFE61, Cat no. 39509, Active Motif, Carlsbad, CA), p130 (clone C-20, Cat. no. sc-317, Santa Cruz), p107 (clone C-18, Cat. no. sc-318, Santa Cruz), Rb (clone C-15, Cat. no. sc-50), HDAC1 (clone H-51, Cat. no. sc-7872, Santa Cruz; and clone 2E10, Cat. no. 05-614, Upstate), c-Myc (clone N-262, Cat. no. sc-764, Santa Cruz).

ChIP assay primers. The following primers were used for amplification by PCR in the ChIP assays with a 60^{o}C annealing temperature: *BRCA1p* F1/R1 (upstream region, schematic shown in Fig. S3a), (F) 5'-CCACACTTCCCCGCAAGCAGAGG-3' and (R) 5'-TGTTCTGAGGGACCGAGTGGGCG-3'; *BRCA1p* F2/R2 (proximal region) (F) 5'-GATTGGGACCTCTTCTTACG-3' and (R) 5'-TACCCAGAGCAGAGGGTGAA-3'; *BRCA1p* F3/R3 (upstream) (F) 5'TCACTTGAAATGGCAAAATAAAATCATGGATGA-3' and (R) 5'-GGTTCAAGTGATTCTCCTGCCTCAGCC-3'; *DHFR* 3'-UTR F/R (E2F negative control)(F) 5'-CTGATGTCCAGGAGGAGAAAGG-3' and (R)5'-AGCCCGACAATGTCAAGGACTG-3'; pBRC-FF F/R (luciferase construct, see Fig. 4a) (F) 5'-TTCTTCCTCTTCCGTCTCTTTCCTT-3' and (R) 5'-TCCAGCGGTTCCATCTTCCA-3'. *BRCA1p* F2/R2 and *DHFR* 3'-UTR F/R have been described previously (1).

Transient and sequential ChIP assays. To assess binding of endogenous E2F4 to the *BRCA1* promoter luciferase vectors, 1-3 μg of pBRC-FF plasmid (WT or mutants) was transiently transfected in RKO or MCF7 cells followed by a 24-48h incubation in normoxia. Cells were then harvested and ChIP assays were performed as described earlier. Only 1x10^{6} cells were used for each immunoprecipitation in these assays, as the target sequence is present in

higher copy number. In addition, antibody-nucleoprotein complex mixtures were washed 8-9 times in the buffers described above to further reduce background amplification in the subsequent PCR analyses. Detection of pBRC-FF (promoter) occupancy was performed using a forward primer specific to the *BRCA1* promoter and a reverse primer specific to the *firefly* luciferase ORF. The specific locations of these primers are shown in Fig. 4a for reference. Endogenous proximal *BRCA1* promoter and *DHFR* 3'-UTR occupancy was analyzed using the primers described above (which were also used in the conventional ChIP assays) and these data served as internal positive and external negative controls, respectively, for each sample. Amplification products were analyzed by agarose gel electrophoresis followed by calculation of relative pBRC-FF promoter occupancy essentially as described earlier using qPCR. Importantly, these calculations included subtraction of the background signal obtained from the no antibody control, as well as normalization to endogenous proximal *BRCA1* promoter occupancy for each sample in the following equation: [(pBRC-FF relative promoter occupancy)-(No Ab relative promoter occupancy)]/(endogenous *BRCA1* promoter occupancy). While similar results were obtained without these modifications, and clear differences were observed by agarose gel analysis (e.g., Fig. 4b), the background correction and normalization permitted more consistent comparisons across independent replicates (data not shown). Additionally, luciferase activities were measured in parallel plates for each pBRC-FF plasmid transfection as described below.

In sequential ChIP analyses, conventional ChIP assays were first performed as described above, except that nucleoproteins complexes were eluted by incubation in 10 mM DTT at 30^{o}C for 30 min. Duplicates from each antibody immunoprecipitation were then pooled to enhance the signal obtained in the second immunoprecipitation. 1/5th volume (40 uL) was reserved for subsequent analysis of the first immunoprecipitation, and the remaining solution was then diluted

in 3 mLs of IP dilution buffer. The diluted immunoprecipitates were pre-cleared and assayed exactly as described for the first immunoprecipitation, except that the purified DNA fragments (both input samples and immunoprecipitates) were only diluted 1/2 in dH_20.

Additional antibodies. For western blot analyses, the antibodies described in the ChIP assay antibody section above were used, in addition to the following: E2F1 (clone C-20, Santa Cruz), E2F4 (clone RK-13, Santa Cruz), BRCA1 (clone Ab-1, Calbiochem), MSH6 (clone 44, BD Biosciences), and tubulin (clone B-512, Sigma, St. Louis, MO).

Luciferase reporter gene assays. For luciferase reporter gene analyses, fragments from the *BRCA1/NBR2* intergenic were selected using the UCSC genome browser (UCSC, Santa Cruz, CA), isolated by genomic PCR and subsequently sub-cloned into the pGL3-basic vector (Promega, Madison, WI). Primers used to generate these amplicons are available upon request. Bidirectional promoters were constructed as follows: fragments containing the *renilla* luciferase ORF and the proximal *BRCA1* promoter region were obtained from a *renilla* pRL-SV40 control vector (Promega) and pBRC-FF, respectively (with a restriction digestion strategy resulting in compatible ends), and these fragments were then sub-cloned into the pGL3-basic vector in the orientation shown in Fig. 1e. Mutagenesis of pBRC-FF was performed using the Stratagene Quick Change mutagenesis kit (Stratagene) according to the manufacturer's instructions and supplemented with One-Shot Competent cells (Invitrogen) to increase transformation efficiency.

For transfections, $1\text{-}5x10^5$ RKO or MCF7 cells were seeded into 6- or 12-well culture plates and transfected with 1 μg of each reporter construct using the Fugene 6 reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's specifications. At the indicated times, *firefly* luciferase activity was measured using a Luciferase Reporter Assay

System kit (Promega) according to the manufacturer's instructions. Luciferase activity was normalized to total protein, which was assayed in triplicate as described earlier.

NHEJ assays. Functional changes in non-homologous end joining (NHEJ) in hypoxia were assessed using a plasmid end-joining assay involving the transient transfection of a linearized pGL3 plasmid (Promega) to assess NHEJ activity in hypoxia (schematic shown in Fig. 5c). Restriction endonuclease digestion of pGL3 with *Hind*III or *Nco*I results in cleavage at the linker region between the promoter and luciferase coding sequence or at the ATG start codon, respectively. Following transfection into cells, any end-joining activity resulting in re-ligation of the linearized plasmid is detected as luciferase activity. Due to the location of the *Hind*III site in the vector, end-joining activity which results in small insertions or deletions at this site will not affect luciferase expression, while restoration of luciferase activity following digestion with *Nco*I only results when precise end-joining occurs. In this manner, the assay permits the study of both overall and precise end-joining activity. The validation and use of this protocol to assess NHEJ activity has been described previously (2). Experimentally, 25 μg of pGL3 vector was digested either with *Hin*dIII, *Nco*I, or no enzyme overnight at 37^{o}C and purified by phenol-chloroform extraction followed by ethanol precipitation. To confirm complete digestion of plasmid, DH5α cells were transformed with 1 μg of linearized or uncut purified vector and plated on LB-ampicillin plates in triplicate. The amount of undigested vector after treatment with *Hin*dIII or *Nco*I was estimated based on the number of bacterial colonies obtained following transformation with each linearized vector compared to the number obtained following transformation with uncut vector (and thus is referred to as background). Background values were calculated to be 1.5% and 1.0% in the plasmids linearized with *Hin*dIII and *Nco*I, respectively. RKO cells were transiently transfected with 1 μg of either linearized or uncut vector 6h prior to normoxic or

hypoxic exposure (48h), immediately followed by measurement of luciferase activity as described earlier. Luciferase activity in the cleaved samples was normalized to the activity obtained following transfection with undigested plasmid and thus is expressed as a percentage.

Supplementary Information – Supplemental Figure Legends

Fig. S1. (a) Repression of *BRCA1* expression by hypoxia persists during the post-hypoxic, reoxygenation phase**.** Northern blotting was performed to assess *BRCA1* mRNA expression in MCF7 cells in response to hypoxia (H, 0.01% O_2, 48h) and following reoxygenation (R) at the indicate time-points. As a control to show that *BRCA1* expression does not change over the same time period under normoxia, *BRCA1* mRNA expression in normoxic cells (N) grown in parallel is shown for each time-point. *VEGF* expression is shown for comparison to verify that physiologically relevant states of hypoxia and reoxygenation were obtained in the treated cells, and *18S* rRNA expression is presented to confirm equal sample loading. Similar results were obtained using A549 cells (data not shown). **(b)** Hypoxia-induced repression of *BRCA1* expression is not dependent on cell cycle profile. qPCR analysis of *BRCA1* mRNA abundance in G_1- and S-phase populations of normoxic and hypoxic A549 cells isolated by FACS and normalized to *18S* rRNA expression. Unsorted cells were processed in parallel as a control (Total). Error bars are based on standard errors calculated from triplicate assays. **(c)** Western blot analysis to analyze total amounts of the indicated E2F proteins in RKO, MCF7 and A549 cells in normoxia and hypoxia. Dashed lines indicate separate lanes from the same gel, and tubulin protein levels served as a loading control. **(d)** Elucidation of the role of E2F sites in the regulation of *BRCA1* promoter activity by E2F1. The dominant negative Dp1 expression vector,

Dp1(Δ103-126), was cotransfected with pBRC-FF WT or mutant promoter constructs and assayed for changes in luciferase activity in RKO cells. Results are expressed as the fold change in normalized luciferase activity following transfection with the Dp1 mutant transcription factor (TF) compared to that obtained with an empty vector (pCDNA; thus TF/pCDNA). The E2F reporter vector (3XE2F) is induced by endogenous activating E2Fs, and thus serves as a positive control to confirm repression of luciferase activity by exogenous dominant negative Dp1 expression. Error bars are based on standard errors calculated from three pairs of TF/pCDNA replicates.

Fig. S2. (a) Northern blot analysis to assess the effect of exogenous wild-type or mutant HIF-1α overexpression (48h) on *BRCA1* mRNA levels in HeLa and A549 cells. *VEGF* expression was measured to confirm induction of an endogenous HIF target by the exogenously expressed HIF proteins. **(b)** Western blot analysis to confirm HIF-1α WT and ODD mutant protein expression following transfection with the corresponding cDNA expression vectors or with an empty pCDNA vector (Con) in HeLa cells. **(c)** Co-transfection of mutant HIF-1α expression vectors (ODD and Pro) or an empty vector (pCDNA) with the HIF-inducible luciferase report vector, 5X-HRE, to confirm activity of the expressed mutant proteins in RKO cells. **(d)** qPCR analysis of *BRCA1* mRNA abundance in HeLa cells following exogenous overexpression of HIF-1α proline mutant protein (Pro), normalized to *Actin* expression. *DEC1* expression was also measured to confirm induction of another endogenous HIF target by the exogenously expressed HIF protein. Error bars are based on standard errors calculated from triplicate assays. **(e)** Northern blot analyses were performed to determine the expression of *BRCA1* in log-phase 786-0 cells expressing a wild-type (WT) or mutant (Mut) *VHL* gene. The expression of *VEGF* is

shown for comparison to verify the constitutively hypoxic phenotype of the *VHL* mutant cells, and *18S* rRNA expression is also presented to confirm equal sample loading.

Fig. S3. (a) Schematic of primer locations in the *BRCA1* promoter region used in the ChIP analyses. Nucleotide positions are shown relative to the first exon of the *BRCA1* gene, and the approximate locations of the CCAAT and E2F sites are shown for reference. *BRCA1* F1/R1 and *BRCA1* F3/R3 primers were designed to amplify regions upstream and downstream from exon 1, respectively, that are sufficiently far from the proximal *BRCA1* promoter region (which is amplified using *BRCA1* F2/R2 primers) such that occupancy by E2Fs at the proximal promoter should not be detected when these primer pairs are used. The F1/R1 and F3/R3 primers pairs thus served as controls both for the efficiency of chromatin sonication and for the specificity of E2F binding at the proximal *BRCA1* promoter region. **(b)** ChIP assays were performed with PCR primers specific for the regions of the *BRCA1* promoter as described in (a). Detectable *BRCA1* promoter occupancy by E2F1 was only observed using primers specific to the proximal promoter region (F2/R2). Dashed lines indicate separate lanes from the same agarose gel. **(c)** Overview of relative promoter occupancy calculations using real-time qPCR with primers specific to the *BRCA1* proximal promoter region. PCR amplification curves from a representative ChIP assay using normoxic and hypoxic samples with an antibody specific to p130 are shown as an example. Cycle thresholds using the MX3000p instrument software were calculated based on a dRn fluorescence threshold set to the linear range of all reactions (performed in triplicate) as indicated by the solid line intersecting the ordinate axis. Relative promoter occupancy (%), in which the input signal was set as 100% binding, was calculated by comparing the CTs between input (representing 1% of total lysate) and immunoprecipitation (IP) samples using the equation shown

in the upper left corner of the graph. Standard curves were generated based on 2-fold serial dilutions of input DNA (data not shown) which were used to calculate PCR amplification efficiency (Eff). The amplification efficiency of PCR using the *BRCA1* F2/R2 primer pair was calculated to be 94%. Using these formulas with this representative pair of ChIP (N/H) samples, a 4-fold increase in relative *BRCA1* promoter occupancy by p130 was observed in hypoxia compared to normoxia, while minimal signal was observed in the no antibody control sample. **(d)** Relative *BRCA1* promoter occupancies (%) for each factor in normoxic and hypoxic MCF7 cells as calculated in (c). Error bars are based on standard errors calculated from ChIP replicates (number, n, shown below each factor) performed over at least 3 independent experiments. Binding by E2F6, Rb, HDAC1 and c-MYC ChIP is shown only in duplicate for each factor because agarose gel analysis consistently revealed undetectable binding above background using several different antibodies of varying isotypes, and thus a limited number of representative samples were analyzed by qPCR. Importantly, these antibodies also served as isotype controls, since the isotypes matched those used in the analysis of *BRCA1* promoter occupancy by E2Fs and pocket proteins. Additionally, no amplification products were detected above background following PCR with primers specific to the 3'-UTR of the *DHFR* gene for any of the E2Fs or pocket proteins (data not shown), which served as another negative control. **(e)** The consistency of calculated relative *BRCA1* promoter occupancies was confirmed across several independent replicates (A and B) and experiments (1-3) for E2F4 in normoxia. Error bars are based on standard errors calculated from triplicate qPCR analyses and dashed lines indicate the boundaries of the 95% confidence interval (CI) calculated from a single sample t-test. Similar results were observed using E2F1, p130 and p107 antibodies both in normoxia and hypoxia (data not shown).

1. Oberley MJ, Inman DR, Farnham PJ. E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9. J Biol Chem 2003 Oct 24;278(43):42466-76.
2. Zhong Q, Chen CF, Chen PL, Lee WH. BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks. J Biol Chem 2002 Aug 9;277(32):28641-7.