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Award Number: DAMD17-02-1-0426

TITLE: HOXB7: An Oncogenic Gene in Breast Cancer

PRINCIPAL INVESTIGATOR: Ethel Rubin, Ph.D.
Saraswati Sukumar, Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
School of Medicine
Baltimore, MD 21231

REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-05-2005	Annual Summary	15 Apr 2004 – 14 Mar 2005

4. TITLE AND SUBTITLE

HOXB7: An Oncogenic Gene in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0426

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Ethel Rubin, Ph.D.
Saraswati Sukumar, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
School of Medicine
Baltimore, MD 21231

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Homeobox genes control anterioposterior body axis patterning during development. Although expressed primarily in developing embryos, a growing body of evidence shows that homeobox gene re-expression in adult tissues is associated with tumorigenesis. Earlier work has shown that introduction of HOXB7, one of the members of this gene family, into non-expressing breast cancer cells induced expression of a number of pro-angiogenic factors and formed tumors upon xenograft into nude mice. Since a growing body of evidence showed that HOXB7 may be involved in ovarian, skin and breast cancerm, we wanted to investigate its expression levels in breast carcinomas and evaluate its oncogenic potential in this study.
To characterize the role of HOXB7 in breast cancer, a number of HOXB7-interacting proteins in breast cancer cells were identified and binding to HOXB7 was confirmed *in vivo*. Interestingly, all of the HOXB7-associated proteins identified have well characterized roles in the non-homologous end joining (NHEJ) pathway for DNA double strand break (DSB) repair as well as a larger role in maintenance of genomic integrity. Cell survival experiments demonstrated that breast cancer cells stably transfected with HOXB7 survive better after induction of DNA DSB with fewer chromosomal abnormalities following exposure to gamma radiation. HOXB7 overexpression in primary tumors occurs due to amplification of the gene. HOXB7 amplification correlates with poor prognosis. Thus it is possible that HOXB7 is a novel marker for identifying aggressive tumors.

15. SUBJECT TERMS
Breast cancer, HOX genes, DNA amplification, marker

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	47	

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Introduction

Like all HOX genes, HOXB7 has a role in vertebrate patterning during development (reviewed in 1, 2). In addition, several lines of evidence point to the importance of HOXB7 in myeloid hematopoiesis (3), with expression found in many hematopoietic cell lineages (4-8). Nevertheless, there is increasing evidence of a role for HOXB7 in breast cancer. When transduced into breast cancer cells, HOXB7 expression has been shown to promote the growth of xenografts of SKBR3 breast cancer cells in nude mice, with concomitant upregulation of both mitogens and pro-angiogenic factors (9-11). In addition, Hyman et al recently showed that HOXB7 overexpression in human breast cancer may be attributed to gene amplification and is correlated with poor patient prognosis (12). Despite increasing evidence of the involvement of HOXB7 in cancers of the breast, ovary (13) and skin (9), the relevance of this gene to breast cancer, and its functional role in tumorigenesis is poorly understood.

Our work in the last 3 years on HOXB7 and its interaction with DNA doubles strand repair proteins DNAPKcs, Ku-70 and Ku-80 and the PARP are described in the attached manuscript that has been submitted to EMBO journal for publication. In the last year we refined these studies by showing that this interaction is not only prevalent in cells transfected with HOXB7 but also in breast cancer cells with endogenous high level expression of HOXB7, and that this overexpression renders these cells better able to perform double strand break repair.

2005 Annual Summary

Body

Task 1: Investigate the transforming potential of HOXB7 *in vitro* (months 1-12)**- Completed**

- Stably expressed HOXB7 mutant constructs in MCF10A normal mammary epithelial cells
- Showed that only the wild type HOXB7-expressing cell line, but none of the mutants (above) formed colonies in soft agar assays.
- Showed requirement for PARP activity specific to HOXB7-dependent transformation *in vitro*

Task 2: Identify HOXB7 target genes and interacting proteins (months 12-36)

- Prepared RNA for oligonucleotide microarrays.
- Further characterized the interaction between HOXB7 and Ku/DNA-PK and PARP *in vivo* in co-immunoprecipitation experiments
- Discovered that two previously uncharacterized post-translational modifications of HOXB7 block its DNA-binding functions
- Identified effects of HOXB7 on cell survival of MCF10A normal epithelial cells following exposure to radiation
- Attenuated HOXB7-mediated transformation by PARP inhibition
- **Months 24-36.** Repeated all the above experiments in cells with endogenous overexpression of HOX B7
- Showed that- MDAMB 468 cell extracts perform DSB efficiently, an effect that is abrogated by transfecting the cells with an antisense construct to HOXB7
- MDAMB468 and MDAMB 435 breast cancer cells have a greater ability to resist the deleterious effects of gamma radiation. siRNA to HOXB7 abrogates this protection as shown by colony formation assays.
-

Task 3: Develop HOXB7 as a detection marker for diagnosis (months 1-36)

- Determined HOXB7 mRNA levels in 41 mammary carcinoma samples compared to normal controls.
- Characterized HOXB7 antibodies from a commercial source (ongoing).
- Characterized HOXB7 antibodies for immunohistochemistry without success.
- Showed by Q-RT-PCR that breast cancer metastasis also overexpress HOXB7.
- By Q-PCR showed that overexpression is likely due to gene amplification of HOXB7
- Array analysis of a group of 89 tumors with followup after Tamoxifen monotherapy showed that HOXB7 is overexpressed, but only borderline significance p=0.6 was achieved.
- FISH analysis of a 500 patient cohort with 10 years of followup after Tamoxifen monotherapy is ongoing to ask whether HOXB7 amplification will correlate with recurrence.

Task 1 of the statement of work investigates the transforming potential of HOXB7 *in vitro* by soft agar growth assay. Completion of this task has been reported in 2004.

Task 2 of the statement of work aims to identify HOXB7 target genes and interacting proteins. This task has been completed and was reported in detail in the 2004 report. Results are detailed in attached manuscript. The results are summarized below.

Homeobox genes encode transcription factors which function in body axis patterning in the developing embryo. Recent evidence suggests that maintenance of specific HOX expression

patterns is necessary for regulating homeostasis of adult tissues as well. Since all HOX genes contain a highly conserved homeodomain, through which DNA binding is achieved, specificity of DNA binding and transactivation are thought to be determined through protein-protein interactions. In this study, we identified four HOXB7 binding proteins by GST pull down/affinity chromatography and confirmed the interactions *in vivo* by co-immunoprecipitation. Interestingly, all four HOXB7 binding proteins identified in this assay shared functions as genomic caretakers, and included PARP and members of the DNA-PK holoenzyme responsible for DNA double strand break (DSB) repair by the non-homologous end joining (NHEJ) pathway. Exogenous and endogenous expression of HOXB7 enhanced NHEJ and DNA repair functions *in vitro* and *in vivo*, which were reversed by silencing HOXB7. This is the first mechanistic study providing definitive evidence for involvement of HOX protein in DNA repair.

Task 3 of the statement of work is to develop HOXB7 as a detection marker for diagnosis.

1) Initial attempts to detect either circulating antibodies to HOXB7 or HOXB7 protein in patient serum failed as discussed in the annual report of year one. It is possible that levels were too low to detect by ELISA methods employed. Nevertheless, we are characterizing a number of HOXB7 antibodies being developed by a commercial source (Zymed, Inc., San Francisco, CA) which we hope to use to screen breast cancer tissue arrays for presence of HOXB7 in tissue samples. With the new antibodies, we were able to detect HOXB7 by Western analysis and by immunofluorescence but not by IHC. We have now generated antibodies in chicken since the antigenicity of the human peptide may not be high in rabbits or mice since HOX proteins are so well conserved through evolution. These are still undergoing characterization.

2) To determine if HOXB7 mRNA levels were upregulated in breast cancer patients, quantitative, real-time PCR was employed. By comparison to levels of HOXB7 in normal samples (12 mammoplasty, 8 normal enriched epithelial organoid), mRNA for this gene was overexpressed an average of 4-fold in 39 of 41 mammary carcinomas, indicating that it may be of use as a diagnostic marker of the disease. We have since repeated this analysis with an additional set of 10 metastatic tissues from lymph nodes, ovary and liver. These tissues also displayed high levels of HOXB7.

3) A microarray analysis of purified epithelial cells revealed that compared to normal breast epithelial cells, HOXB7 was overexpressed 4-fold higher levels in primary metastatic breast carcinoma cells, and 12 –fold higher levels in bone metastasis.

4) Array CGH analysis of 146 breast cancers revealed that HOXB7 was amplified in breast cancer- 25% at log2ratio of >0.3 and 5% at >0.7. Independently, Hyman et al had published that HOXB7, as analyzed by FISH was systematically amplified in breast cancer cell lines, and in 10% of 363 primary breast cancers by FISH to a tissue microarray and was associated with poor prognosis ($p=<0.001$). Our efforts in the next few months will be focused on developing a FISH analysis to test amplification of HOXB7 of archival breast cancers.

Key Research Accomplishments

1) For the first time identified four HOXB7 interacting partners and submitted paper to EMBO journal.
2) Demonstrated an oncogenic role for HOXB7
3) Showed that HOXB7 is amplified in breast cancer, and has potential to be detected by FISH analysis.

List of reportable outcomes

2. **A role for homeodomain protein, HOXB7, in DNA repair** Ethel Rubin[1], Xinyan Wu[1], Tao Zhu, Hexin Chen, Annaka Lorincz, Raj K. Pandita, Girdhar G. Sharma, Judith Gasson, Tej K. Pandita and Saraswati Sukumar *(submitted to EMBO, October, 2005)*

3. Based on prelimnary data generated in this grant, the PI, Dr, Sukumar successfully competed for a DOD Center of Excellence award that has been initiated since Nov 2004.

Conclusions

This study examined the role of HOXB7 in breast cancer from a biochemical and cellular viewpoint. We have found that HOXB7 is a substrate for DNA-PK and PARP, two enzymes with which it interacts, and have determined that post-translational modification by these enzymes blocks DNA binding functions of HOXB7. While the role of PARP in tumorigenesis is not clear-cut and requires more study, it appears from our studies that HOXB7 and PARP function in the same pathway in breast cancer.

We have also functionally analyzed different regions of HOXB7 with respect to interaction with PARP and anchorage independent growth. Taken together, our results suggest that protein-protein interactions and post-translational modification of HOXB7 are crucial for modulating its biological activities and that mammary cells overexpressing this protein have oncogenic potential and survival advantages over non-expressing cells.

HOXB7 mRNA levels were upregulated in breast cancers as compared to normal tissues as shown by quantitative, real-time PCR. Metastatic tissues from lymph nodes, ovary and liver also displayed high levels of HOXB7. A microarray analysis of purified epithelial cells revealed that compared to normal breast epithelial cells, HOXB7 was overexpressed 4-fold higher levels in primary metastatic breast carcinoma cells, and 12 –fold higher levels in bone metastasis. Overexpression could be attributed to gene amplification. Array CGH analysis of 146 breast cancers revealed that HOXB7 was amplified in breast cancer- 25% at log2ratio of >0.3 and 5% at >0.7. Independently, Hyman et al had published that HOXB7, as analyzed by FISH was systematically amplified in breast cancer cell lines, and in 10% of 363 primary breast cancers by FISH to a tissue microarray and was associated with poor prognosis (p=<0.001). Our efforts in the next few months will be focused on developing a FISH analysis to test amplification of HOXB7 of archival breast cancers.

References

1. Veraksa A, Del Campo M, McGinnis W. **2000**. Developmental patterning genes and their conserved functions: from model organisms to humans. *Mol Genet Metab.* **69** (2): 85-100.

2. Capecchi MR. **1997**. Hox genes and mammalian development. *Cold Spring Harb. Symp. Quant. Biol.* **62**:273-281.

3. Yaron, Y., J. K. McAdara, M. Lynch, E. Hughes and J. C. Gasson. **2001**. Identification of novel functional regions important for the activity of HOXB7 in mammalian cells. *J. Immun.* **166**: 5058-5067.

4. Lawrence H.J., Sauvageau G., Humphries R.K., Largman C. **1996.** The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* **14** (3):281-291.

5. Fuller J.F., McAdara J., Yaron Y., Sakaguchi M., Fraser J.K., Gasson J.C. **1999.** Characterization of HOX gene expression during myelopoiesis: role of HOX A5 in lineage commitment and maturation *Blood* **93**(10):3391-400.

6. Lill M.C., Fuller J.F., Herzig R., Crooks G.M., Gasson J.C. **1995.** The role of the homeobox gene, HOX B7, in human myelomonocytic differentiation. *Blood* 85(3):692-697.

7. Care A, Testa U, Bassani A, Tritarelli E, Montesoro E, Samoggia P, Cianetti L, Peschle C. **1994.** Coordinate expression and proliferative role of HOXB genes in activated adult T lymphocytes. *Mol Cell Biol.* **14**(7):4872-4877.

8. Deguchi Y, Kehrl JH. **1991.** Selective expression of two homeobox genes in CD34-positive cells from human bone marrow. *Blood.* **78**(2):323-328.

9. Care A., A. Silvani, E. Meccia, G. Mattia, A. Stoppacciaro, G. Parmiani, C. Peschle and M. P. Colombo. 1996. HOXB7 constitutively activates basic fibroblast growth factor in melanomas. *Mol. Cell. Biol.* **16**: 4842-4851.

10. Care, A., A. Silvani, E. Meccia, G. Mattia, C. Peschle and M. P. Colombo. 1998. Transduction of the Skbr3 breast carcinoma cell line with the HOXB7 gene induces bFGF expression, increases cell proliferation and reduces growth factor dependence. *Oncogene* **16**: 3285-3289.

11. Care, A., F. Felicetti, E. Meccia, L. Bottero, M. Parenza, A. Stoppacciaro, C. Peschle and M. P. Colombo. 2001. HOXB7: a key factor in tumor-associated angiogenic switch. *Cancer Res.* **61**: 6532-6539.

12. Hyman, E., P. Kauraniemi, S. Hautaniemi, M. Wolf, S. Mousses, E. Rozenblum, M. Ringner, G. Sauter, O. Monni, A. Elkahloun, O.-P. Kallioniemi and A. Kallioniemi. 2002. Impact of DNA amplification on gene expression patterns in breast cancer. *Cancer Res.* **62**: 6240-6245.

13. Naora H, Yang YQ, Montz FJ, Seidman JD, Kurman RJ, Roden RB. **2001.** A serologically identified tumor antigen encoded by a homeobox gene promotes growth of ovarian epithelial cells. *Proc Natl Acad Sci U S A.* **98**(7):4060-4065.

46,884 with spaces

words: 7,034

A role for homeodomain protein, HOXB7, in DNA repair

Ethel Rubin[1], Xinyan Wu[1], Tao Zhu[1], Hexin Chen[1], Annaka Lorincz[1], Raj K. Pandita[2], Girdhar G. Sharma[2], Judith Gasson[3], Tej K. Pandita[2] and Saraswati Sukumar[1]

[1]Breast Cancer Program, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD

[2]Department of Radiation Oncology, Washington University School of Medicine, St. Louis, MO

[3]Department of Biological Chemistry, University of California, Los Angeles, CA

Address correspondence to:

Saraswati Sukumar, PhD

Johns Hopkins University School of Medicine

1650 Orleans Street, Rm 410

Baltimore, MD, 21231-1000

Email: saras@jhmi.edu

Phone: 410-614-2479

FAX: 410-614-4073

Key words: HOXB7/Ku70/ Ku80/ PARP/ NHEJ

Classification: Genome Stability and Dynamics

Running title: HOXB7 promotes DNA repair

Abstract

Homeobox genes encode transcription factors which function in body axis patterning in the developing embryo. Recent evidence suggests that maintenance of specific HOX expression patterns is necessary for regulating homeostasis of adult tissues as well. Since all HOX genes contain a highly conserved homeodomain, through which DNA binding is achieved, specificity of DNA binding and transactivation are thought to be determined through protein-protein interactions. In this study, we identified four HOXB7 binding proteins by GST pull down/affinity chromatography and confirmed the interactions *in vivo* by co-immunoprecipitation. Interestingly, all four HOXB7 binding proteins identified in this assay shared functions as genomic caretakers, and included PARP and members of the DNA-PK holoenzyme responsible for DNA double strand break (DSB) repair by the non-homologous end joining (NHEJ) pathway. Exogenous and endogenous expression of HOXB7 enhanced NHEJ and DNA repair functions *in vitro* and *in vivo*, which were reversed by silencing HOXB7. This is the first mechanistic study providing definitive evidence for involvement of HOX protein in DNA repair.

Introduction

HOX genes encode transcription factors that are characterized by a highly conserved tri-helical homeodomain that binds to specific DNA sequences. A total of 51 HOX genes have been identified that are organized into five paralogous clusters, HOX-A to –D on autosomal chromosomes, and a newly discovered cluster on the X chromosome, RHOX (McLean et al, 2005).

The functions of homeodomain-containing proteins are diverse and include roles as both classical regulators of transcription and novel roles outside of transcriptional regulation. HOX genes are functionally important in anterioposterior patterning during embryogenesis, maintenance and homeostasis in adult tissue, cell-cell interactions, and cell-ECM interactions (reviewed by Chen and Sukumar, 2003). Examples of novel roles for homeodomain containing proteins include the role of human proline-rich homeodomain protein, PRH (known as Hex in studies on hematopoiesis), which interacts with eIF4E to inhibit its mRNA nuclear-cytoplasmic transport function (Topisirovic et al., 2003). Given that many HOX genes are similar in sequence, a fundamental question arises of how each HOX protein achieves functional specificity. One hypothesis is that functional specificity is attained by physical interaction with various co-factors.

There is evidence for physical interaction between several HOX proteins and components of the non-homologous end joining pathway (NHEJ) suggesting a possible novel role for HOX proteins in DNA repair. Some of the major protein components of NHEJ are the Ku antigen (Ku 70/80), DNA-dependent protein kinase (DNA-PK), and poly (ADP)

ribose polymerase (PARP), which catalyzes the transfer of polymers of ADP-ribose from NAD+ onto protein targets (de Murcia et al., 1983). The Ku antigen binds to and recruits DNA-PK to sites of DNA strand breaks, where DNA-PK is activated to participate in DNA repair. In a separate interaction, the Ku antigen interacts with PARP to form a complex and bind to matrix attachment DNA sequences (Galande et al., 1999; Shigematsu et al., 1999). PARP is also activated by DNA strand breaks and play important roles in base excision repair, regulation of p53 response and other mechanisms (Herceg et al, 2001). HOXC4 and HOXD4, along with homeodomain-containing proteins Octamer transcription factor-1 and -2 and Dlx2, interacts with the C-terminus of the Ku antigen causing their recruitment to broken DNA ends and their phosphorylation by DNA-PK (Schild-Poulter et al., 2001). However, the functional significance of this interaction has not been determined.

Here, we provide evidence for novel non-transcriptional functions for HOXB7 mediated through physical interactions between HOXB7 and Ku70, Ku 80, the catalytic subunit of DNA-PK (DNA-PK_{cs}), and PARP. We chose to focus our study on HOXB7 since it is overexpressed in different tumor types (reviewed in Chen and Sukumar, 2003) and since NHEJ is commonly considered an error-prone mechanism that is mutagenic (Allen et al., 2003; Lieber et al., 2004). Our work suggests that the interaction between HOXB7 and the Ku antigens is functionally significant since HOXB7 expression enhances NHEJ and DNA damage repair in mammalian cells.

Results

HOXB7 interacts in vitro and in vivo with PARP and NHEJ proteins

To identify proteins in breast epithelial cells that associate with HOXB7, SKBR3 (Figure 1A, lanes 2 and 4) and MCF10A (Figure 1A, lane 5) cell extracts were fractionated by affinity chromatography on GST-HOXB7-Sepharose. Analysis of the proteins in column eluates by silver and coomassie staining after SDS-PAGE revealed the presence of 4 polypeptides of approximate sizes of 70, 85, 110, and >250 kD which did not bind to the GST, or to the unrelated GST-fusion protein, GST-PRL3 columns used as controls (Figure 1A). Similar results were obtained for fractionation of MCF-12A and MDA-MB-231 cell extracts (data not shown) on GST-HOXB7-Sepharose. Direct sequencing from PVDF membranes yielded results for only the 85 kD band, which identified Ku 80 from an N-terminal 17-amino acid sequence (VRSGNKAAVVLCMDVGF). For the 110 and 70 kD protein bands, peptide mass fingerprints were obtained by MALDI-TOF and compared against those in public databases. Both ProFound and MS-FIT public database searches for the peptide mass maps obtained from the 110 and 70 kD protein bands identified PARP and Ku 70, respectively. Protein identities were confirmed by immunoblotting with antibodies against PARP, Ku 80 and Ku 70 (Figure 1B). Since Ku 70/80 are known binding subunits of the DNA-dependent protein kinase (DNA-PK), the high molecular weight band appearing at the top margin of the gel (>250 kD) was predicted and confirmed as the catalytic subunit of DNA-PK (DNA-PK_{cs}) by immunoblot analysis (Figure 1B, top panel).

The interaction between HOXB7 and PARP, Ku 70, Ku 80 and DNA-PK_{cs} was then analyzed *in vivo* by co-immunoprecipitation. A HOXB7-YFP fusion protein was stably introduced into the HOXB7-null breast cancer cell line, SKBR; fluorescence microscopy confirmed that HOXB7-YFP localized solely to the nucleus (data not shown). Immunoprecipitation with GFP antibodies (which also recognize the YFP variant) showed that PARP, Ku 70, and Ku 80 associated with HOXB7 *in vivo* (Figure 2A, lane 4). Complementary immunoprecipitations using Ku 80 (Figure 2B lanes 4-6) or DNA-PK_{cs} (Figure 2B, lanes 7-9) antibodies confirmed the presence of HOXB7-YFP in their complexes (Figure 2B, lanes 4 and 7) following transient transfection of this construct into SKBR3 cells. Identical results were obtained when FLAG-HOXB7 (FB7pcDNA3) was transiently expressed in SKBR3 cells (see Figure 3A lane 2). Complex formation was not affected by DNA damage by UV or gamma-irradiation (data not shown). The interaction between HOXB7 and Ku80 or PARP was also confirmed by co-immunoprecipitation analysis using protein extracts of breast cancer cells, MDA-MB-435 (Figure 2C), which express high levels of endogenous HOXB7 (data not shown). This interaction was equally strong in the presence or absence of DNase I (Figure 2C).

The common DNA binding properties of these proteins raise the possibility that the interactions observed above are linked to DNA binding rather than direct protein-protein interaction. To test this possibility we performed the following experiments. To remove contaminating DNA prior to immunoprecipitation cell extracts were treated with DNAse I (Figures 2C and 3A) or fractionated on the weak anionic exchanger, DEAE-Sepharose (Figure 3B). In each case, cell extracts were normalized by protein concentration prior to

co-immunoprecipitation with HOXB7 (figure 2C) or FLAG (Figure 3) antibodies. Treatment with DNAse I had no effect on the interactions (Figure 2C and 3A, compare lane 3 with lane 2). Fractionation on DEAE-Sepharose did not eliminate the binding altogether (Figure 3B, lane 4). However, the presence of DNA did appear to stimulate the interactions between HOXB7 and PARP and Ku proteins to some extent (Figure 3B, lane 3). Finally, we investigated whether HOXB7 interacts non-specifically with two other DNA–binding proteins, BRCA-1 and E2F1; no such binding was observed (data not shown). These results suggest that the interaction between HOXB7 with PARP and Ku are likely to be specific. The interaction may not depend on the presence of DNA, but is possibly enhanced in its presence.

Since complexes formed by interactions between Ku 70, Ku 80 and DNA-PK_{cs} are well established, as is the interaction between Ku 80 and PARP (Galande and Kohwi-Shigematsu, 1999; Shigematsu et al., 1999), we investigated the nature of these complexes and the order of their formation. To examine the possibility that the interaction between HOXB7 and PARP was direct and not through PARP's association with Ku 80, FLAG-HOXB7 was transiently expressed in the Ku 80-mutant Chinese hamster ovary (CHO) cell line, XRV15B or its wild type control cell line, V79-4 (Zdzienicka et al., 1988) and cell extracts were subjected to immunoprecipitation and western blot analysis. Our results showed that PARP associated with HOXB7 even in the presence of mutant Ku (Figure 4A, Lane 4). Further experiments introducing FLAG-HOXB7 into the CHO cell line that has undetectable Ku 80 protein expression (Figure 4B), showed that PARP bound to HOXB7 in the absence of Ku proteins, but Ku

monomers did not. These results suggest that Ku 70/Ku 80 heterodimer formation may be a prerequisite for HOXB7 binding. Thus, these data support the premise that HOXB7 binds to PARP, and not through the Ku proteins.

Next, we analyzed the functional regions of HOXB7 involved in the interaction with PARP. We constructed a number of HOXB7 mutants in which distinct regions of HOXB7 were removed, or obtained deletion and mutant constructs described in (Yaron et al., 2001). These included mutations in: 1) the PBX1-binding region [FB7WM (W129F, M130I)], which has been shown to be required for high affinity DNA binding and transactivation; 2) deletion of the glutamic acid-rich carboxyl terminal tail (FB7 ΔGLU), or 3) deletion of helix 3 of the homeodomain (FB7 Δh3, deletion of I183 to W192). In addition, mutants of the FLAG-tagged HOXB7 expression construct were tested which had deletions of amino acids 1-14 [FB7Δ1-14)], or amino acids 38-79 [FB7 Δ38-79)] or 81-120 [FB7 Δ81-120)]. Transient transfection of these constructs into CHO cells together with PARP showed that, with the exception of FB7 Δh3 (Figure 5A, lane 4) which showed reduced PARP binding, there was no effect of any of these deletions/mutations in HOXB7. Immunoprecipitation of PARP with *in vitro* transcribed and translated HOXB7 deletion mutant proteins, FB7-D1 and FB7-D2 confirmed that HOXB7 utilizes its homeodomain for interaction with PARP (Figure 5B, lane 3).

HOXB7 stimulates DNA repair in vitro and in vivo

Based on our findings that HOXB7 associates with members of the DNA-PK holoenzyme, we investigated whether introduction of HOXB7 into a HOXB7-null cell

line, SKBR3, would affect DNA repair kinetics. First, we tested the DNA repair activities of HOXB7-containing nuclear extracts *in vitro* by plasmid end-joining assays (Sharma et al., 2003). This analysis revealed that expression of HOXB7-YFP stimulated the end-joining activity almost 2.5-fold over vector-transfected controls (Figure 6A and B). These results were verified by knockdown of endogenous HOXB7 expression in MDA MB-468 cells using antisense constructs. Transient transfection of these plasmids into MDA-MB-468 cells could suppress the expression of HOXB7 as shown by Western blot analysis (Figure 6C) and reduce NHEJ activity (Figure 6D, lane 5). These results suggest a role for HOXB7 in stimulating DNA repair *in vitro*.

To determine whether overexpression of HOXB7 helps to protect cells following ionizing radiation exposure, several tests were performed. G1 (Figure 7A) and G2-type (Figure 7B) chromosomal aberrations were examined in metaphase spreads from subcultured SKBR3 cells at various time points post-irradiation. In these analyses, the frequency of residual damage was lowest in HOXB7-YFP expressing cells, indicative of enhanced DNA DSB repair. These cells possessed an intact G2/M checkpoint (data not shown), though their elevated mitotic index (Figure 7C) appears to be indicative of enhanced recovery and repair of DNA damage. Next, we measured cell survival of HOXB7-YFP/SKBR3 or control cells following treatment with ionizing radiation. HOXB7-YFP expressing cells exhibited a 1.5-2.5 fold enhanced survival rate at low doses of radiation compared to vector-transfected control or parental cells (Figure 7D). To extend these observations, clonogenic survival assay (Collis et al., 2003) was performed on MCF10A breast epithelial cells stably transfected with FLAG-HOXB7. Upon exposure to low-dose

gamma radiation MCF10A-HOXB7 cells, like SKBR3 cells, had about a 2-fold enhanced survival advantage over the vector-transfected controls (Figure 7E). Conversely, knocking down endogenous HOXB7 expression level in either MCF-7 or MDA-MB-468 cells by specific siRNA abrogates cell survival (Figure 7F). Taken together, these results suggest first, that HOXB7 functions to enhance DNA repair in mammalian cells, and second, that HOXB7-expressing cells have a survival advantage over non-expressing cells in response to DNA damage.

Discussion

Since all HOX proteins bind to similar, heavily represented consensus DNA sequences, we hypothesized that protein-protein interactions may play a role in DNA binding specificity and target discrimination. Identification of specific HOX protein-protein interactions might shed a light on the functional specificity of HOX proteins. We therefore set out to identify HOXB7 binding proteins. Although our original purpose was to identify HOXB7 binding partners which regulate transciptional specificity of HOXB7, it led us to unveil a more intriguing aspect of HOXB7 and other homeobox proteins: DNA repair and genomic stability. Our results show that HOXB7 binds to Ku 70, Ku 80, DNA-PK_{cs} and PARP, proteins involved in DNA DSB repair and genomic stability. This is the first report demonstrating HOXB7 protein interaction with PARP and with the members of the DNA-PK holoenzyme, and a role for a HOX protein in DNA DSB repair. Our results show that, in addition to its function as a transcriptional regulator, HOXB7 functions in DNA DSB repair. While our data suggests that this is most likely through its association with Ku and PARP, our results do not rule out transcriptional regulation of

DNA repair genes as a possible mechanism. For example, when the *Pem* homeodomain-containing gene was expressed in murine Sertoli cells, it appeared to increase the number of DNA SS and DS breaks in the neighboring cells by regulating the expression of genes which affect DNA repair or chromatin remodeling (Wayne et al., 2002).

Most of the efforts in characterizing functions of homeobox proteins have focused on their role in transcriptional regulation. Nevertheless, in recent years, a small number of studies suggest that homeodomain-containing proteins may also play roles outside of transcriptional regulation, or have homeodomain-independent functions. The human proline-rich homeodomain protein, PRH (known as Hex in hematopoietic studies), interacts with eIF4E and inhibits its mRNA nuclear-cytoplasmic transport function (Topisirovic et al., 2003). In addition, a variant of the CSX1 (CSX1b) protein lacking the homeodomain, retained function (Shiojima et al., 1996), and a splice variant of *Meis2* (Meis2e) lacking a complete homeodomain possessed some regulatory function (Yang et al., 2000). Studies in *Drosophila* have shown that the *fushi tarazu* protein has homeodomain-independent functions (Hyduk and Percival-Smith, 1996). Thus, novel functions of homeobox proteins, and those independent of their homeodomains, are beginning to be described. In the case of HOXB7, a truncated HOXB7 protein lacking the homeodomain failed to bind PARP *in vitro* (Figure 5B), suggesting that the homeodomain region of HOXB7 is required for this interaction.

Since the HOXB7-binding proteins identified in this study have functions in DNA repair, and many of the known Ku and PARP binding proteins have DNA repair and genomic

stability functions in common (Pleschke et al., 2000), we investigated if expression of HOXB7 would exhibit effects on these cellular pathways as well. Towards this end, the response of HOXB7-expressing cells to the effects of DNA DSB induced by gamma irradiation was explored. While these cells did not exhibit defects in cell cycle checkpoint control following DNA damage (data not shown), other changes were noted. Most notably, cell survival following gamma irradiation was enhanced in two different HOXB7-transfected cell lines. *In vitro* experiments also indicated enhanced end-joined product formation (Figure 6A, D). When the chromosomal damage and cell survival following gamma irradiation was measured (Figures 7 A-E), we found that somewhat less residual damage was apparent in those cells expressing HOXB7, an effect that was reversed by HOXB7 silencing. These results indicate that cells expressing HOXB7 have enhanced survival and DNA repair rates compared to non-expressing controls. Unlike yeast, DNA DSB repair by NHEJ predominates in mammalian cells in all phases of the cell cycle except at G2/M, where the more conservative homologous recombination plays an important role (Rothkamm et al., 2003). Since the NHEJ pathway for DNA DSB repair is error-prone compared to that of homologous recombination (Collis et al., 2005), HOXB7-expressing cells which survive gamma irradiation and have enhanced NHEJ activity may harbor more potentially deleterious mutations, leading to a decrease in genomic stability. Their greater endurance to irradiation may also allow them to accumulate further mutations that initiate tumorigenesis. This line of reasoning may resolve the apparent paradox that on the one hand, HOXB7 is overexpressed in many tumor types including breast cancer, and on the other hand, it is involved in DNA repair

system. Determination of the genomic integrity and chromosomal stability of these cells is therefore warranted.

Interactions similar to the ones reported in this study, with Ku and PARP, were recently shown for Werner's syndrome protein (WRN) (Li et al., 2004). While WRN was not poly(ADP-ribosyl)ated by PARP, the Ku proteins were thus modified, resulting in a decrease in their affinity for DNA and their ability to regulate WRN exonuclease activity. WRN binds to many other proteins involved in DNA replication and repair, including Rad 52 (Baynton et al., 2003), which we have also found associated with HOXB7 immunocomplexes (our unpublished observations). It is plausible that many other DNA repair-associated proteins form complexes with Ku and PARP, and that this type of complex formation may represent a hallmark of a subset of proteins involved in the same pathway regulating genomic stability. The evidence shown here, indicating roles for HOXB7 in enhanced cell survival and DNA repair rates after irradiation, suggests that HOXB7 joins other important proteins in its involvement in DNA repair and maintenance of genomic stability. Taken together, it appears that HOXB7 may play a novel role in DNA repair through forming complexes with Ku and PARP.

Materials and Methods

Cell culture, plasmids, transfections and antibodies SKBR3 cells were cultured in McCoy's 5A medium containing 15% FBS. CHO cells were cultured in DMEM/F-12 medium containing 10% FBS. V79-4 and XRV15B cells, a kind gift of Dr. Les Hanakahi, were cultured in DMEM containing 10% FBS. MCF10A cells were cultured as described in (Zhang et al., 2003). All plasmids were sequenced to verify fidelity. Cells were selected in 800 μg/mL G418-containing medium and stable clones were analyzed for expression of the fusion protein by western blot and fluorescence microscopy. Expression vectors for Flag-tagged HOXB7 and mutants thereof are described in (Yaron et al., 2001). All transfections were performed using Genejammer (Stratagene) with the exception of transfection in MCF10A cells which was performed using Effectene (Qiagen) according to manufacturer's suggestions. His-tagged Ku70 and Ku80 expression plasmids were provided by Dr. Kathrin Muegge (NCI-Frederick) and PARP-pCR3.1 was provided by Drs. Jason Ha and Sol Snyder, Johns Hopkins. SDS-PAGE and western blots were performed as described (Rubin et al., 2001). The following monoclonal antibodies were used for protein detection by immunoblot: Anti-FLAG M2 (Sigma), PARP (clone C-2-10, Zymed), DNA-PK_{cs} (clone G-4, Santa Cruz Biotechnology), Ku 70 (clone 2C3.11, Novus Biologicals), Ku 86 (clone B-1, Santa Cruz Biotechnology), Living Colors A.v. (clone JL-8, Clontech) for detection of YFP and HOXB7-YFP, and GST goat polyclonal antibody (Pharmacia).

GST-HOXB7 affinity chromatography and identification of GST-HOXB7 binding proteins GST-HOXB7 was expressed as described in (Yaron et al., 2001). The GST and GST-PRL3 expression plasmids were provided by Dr. Bert Vogelstein. Quantitation of GST or GST fusion proteins was performed by silver staining SDS-PAGE gels using BSA as a standard. Soluble fusion proteins, used as controls on protein gels, were eluted from the Sepharose beads with 25 mM glutathione (Sigma)/PBS pH 8.0. For affinity chromatography, 5 μg (50-100 μL) of GST-HOXB7-Sepharose or control fusion protein was mixed for 2.5 hours at 4°C with 5 mg of cell protein extracts (SKBR3, MCF10A, MCF-12A, MDA-MB-231) prepared by scraping cells in 500 μL EBC lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40) supplemented with complete protease inhibitor cocktail (Roche). All cell extracts were pre-cleared by prior incubation for 1 hr with 5 μg of GST-Sepharose. The beads were washed five times with 1 mL of EBC cell lysis buffer and eluted in 25 mM glutathione/PBS pH 8.0. Elutes were divided into two aliquots for protein staining or western blot following SDS-PAGE. Protein identities were determined by one of two methods: direct sequencing from Coomassie-blue stained PVDF membranes or peptide mass fingerprinting from tryptic peptides of Coomassie-blue stained bands on the gel, both performed at the Stanford PAN facility (Palo Alto, CA). All protein identifications were confirmed by immunoblot with corresponding antibodies.

Co-immunoprecipitation For co-immunoprecipitation of HOXB7-binding proteins from SKBR3 cells expressing HOXB7-YFP or Flag-tagged HOXB7, 1-2 mg of cell protein extracts prepared as described above, were pre-cleared as described (Rubin et al., 2001),

and subjected to immunoprecipitation for 2.5 hours at 4^0C with the following antibodies: full length A.v. polyclonal antibodies (Clontech) for immunoprecipitation of HOXB7-YFP , or anti-FLAG polyclonal antibodies (Sigma) for precipitation of FLAG-HOXB7 complexes according to manufacturer's suggestions. Complementary co-immunoprecipitation of HOXB7-YFP with its binding proteins was performed with polyclonal antibodies to DNA-PK_{cs} and Ku 80 (clones H-163 and C-20, respectively, Santa Cruz Biotechnology) as described (Rubin et al., 2001). To verify the interaction between endogenous HOXB7 with Ku70, Ku80 and PARP, 1-2 mg MDA-MB-435 whole cell lysates (with or without DNase 1 treatment) was subjected to immunoprecipitation with HOXB7 rabbit polyclonal antibodies (Zymed) and the immune complexes were loaded onto 4-12% NuPAGE gel (Invitrogen) and blotted with the Ku80 (C-20, Santa Cruz Biotechnology) and PARP (Zymed) monoclonal antibodies. In vitro binding experiments were performed by immunoprecipitating PARP with HOXB7 and its C-terminal deletions, B7-D1 and B7-D2 from *in vitro* TNT-generated according to the manufacturer's instructions (Promega).

DNA repair and cell survival assays Plasmid end-joining assays were performed essentially as described in (Sharma et al., 2003). Briefly, nuclear extracts of HOXB7-YFP or vector-transfected SKBR3 cells were prepared with NE-PER Reagent (Pierce). 2 µl (4 µg) of nuclear extracts were mixed with 0.25 µg of linearized pCDNA3 digested with EcoRV for 1 hr at 25^0C in a buffer containing 20 mM Hepes-KOH pH 7.5, 10 mM $MgCl_2$ and 80 mM KCl. The reaction was stopped by incubation at 37°C with addition of 2 µL of 5% SDS, 2uL of 0.5M EDTA and 1 µL of 10 mg/mL proteinase K. Half of each

reaction was resolved by electrophoresis on agarose gels. UV detection and densitometric quantitation was performed using EagleEye Software. Similar results were also obtained in experiments performed on BamH1-digested plasmid (data not shown). All experiments were performed in duplicate and repeated twice. Relative NHEJ activity was obtained by calculating mean densitometric units of all the end joined products on the gel.

The HOXB7 antisense plasmid: This plasmid, used for NHEJ assays transfected to generate MDA-MB-468-antisense cells was constructed as follows: HOXB7 (ΔB7-AS) was PCR-amplified using primers- 5'ATGAGTTCATTGTATTATGCGAATG-3', and 5'GCGGTCAGTTCCTGAGCTTC-3' using the HOXB7-YFP plasmid as the template. Inserts in pcDNA3.1/His-topo (Invitrogen) in the antisense orientation were selected.

HOXB7-V5 Retrovirus: The HOXB7-V5-RTV DNA was co-transfected with pCL-Ampho (IMGENEX) into HEK-293 cells following the instructions of the manufacturer. The virus supernatant was used to infect MDA-MB-468 cells, and GFP-positive infected cells were FACS-sorted 7-days after infection.

Expression of antisense HOXB7: 468-RV-B7V5 cells seeded in 30 mm wells were transfected with 2 μg of ΔHOXB7-AS (ΔB7-AS); 468-RV (vector control) and 468-RV-B7V5 cells were transfected with 2 μg of pcDNA3.1-V5/His vector plasmid. Cells were harvested 36 hours later, and 2 X 10^6 cells were used to prepare nuclear extracts for the NHEJ assay as described above.

Cell survival following gamma-irradiation, measurements of mitotic indexes as well as determination of G1 and G2-type chromosomal aberrations after DNA damage: These experiments were performed as described in (Dhar et al., 2000).

Clonogenic survival assays: Briefly, cells were plated at appropriate density to give rise to at least 100 colonies in 100 mm dishes following irradiation as described in (Collis et al., 2005). Cells were plated in triplicate, observed for 10-14 days and the experiment was repeated three times.

Small Interfering RNA expression construct and Transfection: The siRNA sequences used targeting human *HOXB7* are 5'-ATATCCAGCCTCAAGTTCG-3' and 5'-ACTTCTTGTGCGTTTGCTT-3'. Oligonucleotides encoding siRNAs (Invitrogen) were annealed and ligated into *pSilencer*-U6 vector (Genscript). Two HOXB7 siRNA expression plasmids were mixed 1:1 for transfection. 1 μg of siRNA/control/ per well was transfected into six-well plates by use of Effectene (Qiagen) for 24 hours.

Acknowledgements

The authors would like to acknowledge B. Vogelstein, K. Muegge, and S. Snyder and L. Hanakahi for generously sharing reagents, L. Hanakahi and M. J. Fackler for helpful discussions and Dr. A. R. Rein for critical review of the manuscript. This work was supported by DAMD-17-02-1-0426 Department of Defense Breast Cancer Research Program postdoctoral fellowship grant to E. R. and the NIH SPORE grant P50CA88843 and DOD COE BCO30054 to S. S. Much thanks to D. A. R. for continued support.

Figure legends

Figure 1: Analysis of HOXB7 interaction with PARP and DNA-PK *in vitro* .

A. Affinity chromatography. GST-HOXB7 interacting proteins from SKBR3 (left panel-silver stained gel) or MCF10A (right panel-coomassie stained PVDF membrane) cells. Lane 1 indicates proteins binding to GST alone used as a control, in comparison with GST-HOXB7 in Lane 2, which bound 4 polypeptides of molecular masses 75, 85, 110 and >250 kD. Lane 3 shows proteins which bound to GST alone during the preclearing step. Positions of GST (lane 1) and GST-HOXB7 (lanes 2, 4, 5) are indicated by asterisks. Mw indicates protein molecular ladder. Lanes 6 and 7 show proteins bound to an unrelated control (GST-PRL3) and GST, respectively.

B. Immunoblot confirmation of HOXB7-interacting proteins. Proteins which bound to GST-HOXB7 or control matrices were eluted with 50 mM glutathione, separated by SDS-PAGE and transferred to nitrocellulose. Western blots using antibodies to the catalytic subunit of DNA-PK (DNA-PK_{cs}), PARP, Ku 80 and Ku 70 showed that these proteins specifically bound to GST-HOXB7 (lane 3) but not GST alone (lane 2) or GST-PRL3 (lane 4) used as controls. 100 μg of total SKBR3 cell extracts (2% of input) was loaded in lane 1 as positive controls for proteins detected by immunoblot.

Figure 2: Interaction of HOXB7 with PARP and DNA-PK *in vivo*

A. Co-immunoprecipitation of PARP, Ku 80 and Ku 70 with HOXB7-YFP in SKBR3 cells. SKBR3 cells were stably transfected with HOXB7-YFP (lanes 1, 4 and 7) or YFP alone as a vector control (lanes 2, 5 and 8) prior to immunoprecipitation with

GFP antibodies and subsequent western blot of precipitated proteins. SKBR3 parental cells, which do not express detectable HOXB7, were used as controls as well (lanes 3, 6 and 9). Lanes 1-3 show protein levels in 100 μg of total cell extracts (5% of input), while lanes 4-6 are proteins that precipitated with HOXB7-YFP or controls that did not express HOXB7. Normal rabbit serum was used as a control for specificity (lanes 7-9). While YFP is expressed at an excess over the HOXB7-YFP fusion protein (compare levels in lane 1 with lane 2), PARP, Ku 80 and Ku 70 specifically interacted only with HOXB7-YFP (lane 4).

B. Co-immunoprecipitation of HOXB7-YFP with DNA-PK$_{cs}$ and Ku 80 in SKBR3 cells. Complementary immunoprecipitations to those in **A.** were performed using SKBR3 cells transiently transfected with HOXB7-YFP (lanes 1, 4 and 7), YFP (lane 2, 5 and 8) or SKBR3 parental cells which do not express HOXB7 (lanes 3, 6 and 9). Levels of HOXB7-YFP (lane 1) in 100 μg of total cell extracts (lanes 1-3) were more similar to YFP (lane 2) upon transient transfection than in stable transfectants (**A**). Antibodies to Ku 80 (lanes 4-6) and DNA-PK$_{cs}$ (lane 7-9) co-immunoprecipitated HOXB7-YFP alone (lanes 4 and 7). Normal rabbit serum (NRS) (lanes 10-12) was used as a control.

C. Co-immunoprecipitation of endogenous HOXB7 in MDA-MB-468 cells with Ku80 and PARP. Immunoprecipitation was carried with either HOXB7 polyclonal antibody (lanes 1, 2) or IgG (lanes 3) from lysates with or without treatment with DNase I, and precipitates were analyzed by western blot with polyclonal antibody to either Ku80 or PARP.

Figure 3: DNA is not required for the interaction.

A. Co-immunoprecipitation of FLAG-HOXB7 with interacting proteins in the absence of DNA in SKBR3 cells. SKBR3 cells were transiently transfected with a FLAG-HOXB7 construct prior to immunoprecipitation with FLAG antibodies and detection by immunoblot (lane 2). 2000 units of molecular-biology grade DNase I was added during lysis and immunoprecipitation in lane 3. Lane 1 shows protein levels in 100 μg of cell extract (5% of input).

B. Co-immunoprecipitation following fractionation on DEAE-Sepharose. Protein extracts of SKBR3 cells expressing Flag-tagged HOXB7 were either fractionated on DEAE-Sepharose to remove DNA (lanes 2 and 4) or left untreated (lane 1 and 3) prior to co-immunoprecipitation with anti-FLAG antibodies (lanes 3 and 4) and immunoblot to detect HOXB7-interacting proteins PARP (top), Ku 80 (middle) or Ku 70 (bottom). Lanes 1 and 2 are 100 μg (10% of input) of protein extracts.

Figure 4. PARP binds to HOXB7 in the absence of Ku.

A. Co-immunoprecipitation of HOXB7 and PARP from XRV15B cells. Wild type V79-4 cells (wt) (lanes 1, 3, 5 and 7) or Ku-negative XRV15B cells (mu) (lanes 2, 4, 6 and 8) transfected with Flag-tagged HOXB7 (lanes 1-4) or vector alone (lanes 5-8) were subjected to immunoprecipitation with FLAG antibodies (lanes 3, 4, 7 and 8) to determine if PARP would interact with HOXB7 in the absence of Ku proteins. Lanes 1, 2, 5 and 6 are 100 □g of cell extracts (10% of input) used for the experiment.

B. Co-immunoprecipitation of HOXB7 with interacting proteins in CHO cells. FLAG-HOXB7 was transiently expressed in the CHO cell line with or without combinations of PARP, Ku 70 and Ku 80, prior to co-immunoprecipitation with FLAG antibodies, SDS-PAGE and immunoblot with the antibodies indicated on the right. The following transfections were performed: pCDNA3 (lanes 1, 11) and PARP (lanes 10, 20) as controls; FB7-pCDNA3 (lanes 2, 12) alone or together with Ku 70 (lanes 3, 13), Ku 80 (lanes 4, 14), PARP (lanes 5, 15) or in combination with Ku 70 + Ku 80 (lanes 6, 16), Ku 70 + PARP (lanes 7, 17), Ku 80 + PARP (lanes 8, 18) or all 3 (Ku 70+ Ku 80 + PARP, lanes 9 and 19). The Ku 80 and Ku 70 expression plasmids carried a histidine-tag but were detected on immunoblots with monoclonal antibodies to Ku 80 and Ku 70. 100 μg of total protein lysates (5% of input) are shown in lanes 1-10. Anti-FLAG immunoprecipitations are shown in lanes 11-20.

Figure 5: Homeodomain of HOXB7 protein is important for its interaction with PARP.

A. Wild-type Flag-tagged HOXB7 or constructs in which select regions were deleted or mutated, were transiently transfected into CHO cells together with a PARP expression construct (PARP-pCR3.1, where indicated, empty plasmid was used as control) to determine if a specific region of HOXB7 mediated its interaction with PARP. Co-immunoprecipitation with FLAG antibodies (top panel) was performed followed by immunoblot with PARP antibodies. The lower panel shows protein expression of all transfected plasmids. Structure of FLAG-HOXB7 showing point mutations and deletions

is shown below (Yaron et al., 2001). F: Flag tagged HOXB7, P: pentapeptide, H: homeodomain, h: helix 3 of the homeodomain.

B. Full length HOXB7 or deletion constructs B7-D1 or B7-D2 were transcribed and translated *in vitro* in the presence of ^{35}S-methionine prior to mixing with *in vitro* transcribed and translated PARP. Immunoprecipitation was performed with PARP monoclonal antibodies (lanes 1, 3 and 5). Complexes were resolved by SDS-PAGE and subjected to autoradiography for 24 hours. Lanes 2, 4 and 6 are input (15%) from the TNT reactions. Deletions D1 and D2 of the HOXB7 protein are shown in figure below.

Figure 6: HOXB7 stimulates DNA repair *in vitro* and *in vivo*

A. Plasmid end-joining assay Nuclear extracts of SKBR3 cells expressing HOXB7-YFP (lane 3) or YFP alone (lane 4) were mixed with 0.25 ug of blunt-digested pCDNA3.0 in a plasmid end-joining reaction. Products were resolved on 0.7% ethidium-bromide stained agarose gels Lane 1: DNA ladder, lane 2: undigested pCDNA3.0, lanes 3 and 4: HOXB7-YFP/SKBR3 and YFP/SKBR3 nuclear extracts, respectively, added to end-joining reaction; lane 5: control reaction containing nuclear extraction buffer with no protein. lane 6: control reaction containing protein omitting DS DNA substrate.

B: Band intensities for HOXB7-YFP and YFP control were quantitated on Eagleye software and shown as a bar graph.

C and D: Blocking of NHEJ by antisense HOXB7

C. Different amounts of partial HOXB7 antisense (ΔB7-AS) plasmid were transiently transfected into MDA-MB-468 cells previously infected with HOXB7-V5 retrovirus.

Cell lysates were western blotted with anti-V5 monoclonal antibody to determine transfection parameters for effective HOXB7 suppression.

D. Nuclear extracts of MDA-MB-468 empty vector infected cells (lane 3, 468-RV), infected with HOXB7-V5 retrovirus (lane 4, 468-RV-B7V5), or stably expressing HOXB7-V5 transfected with partial antisense (lane 5 ΔB7-AS) plasmids were mixed with blunt-digested pcDNA3.0 in plasmid end-joining assays (as in Figure 6A) to determine the effects of HOXB7 repression on *in- vitro* DNA repair. The data shown is representative of 3 experiments that showed similar results.

Figure 7: Altered response of HOXB7- expressing SKBR3 and MCF10A cells to radiation

A. G1 type chromosomal aberrations after radiation treatment Cells in plateau phase were irradiated with 3 Gy, incubated for 24 hours post-irradiation, subcultured, and metaphases were collected. G1-type aberrations were examined at metaphase. All categories of asymmetric chromosome aberrations were scored: dicentrics, centric rings, interstitial deletions/acentric rings, and terminal deletions. The frequency of aberrations following ionizing radiation treatment was calculated in HOXB7-YFP expressing SKBR3 cells and compared to vector-transfected and parental controls.

B. G2 type chromosomal aberrations after radiation treatment Cells in exponential phase growth were irradiated with 1 Gy gamma radiation. Metaphases were harvested 45 and 90 minutes following irradiation and examined for chromatid breaks and gaps. Fifty metaphases were scored for each post-irradiation time point. Results for HOXB7-YFP

expressing SKBR3 cells were compared to those of vector-transfected (YFP/SKBR3) and parental (SKBR3) controls.

C. Mitotic index after radiation treatment Cells in exponential phase were irradiated with increasing doses of gamma radiation and then examined for the frequency of mitotic cells. Results for HOXB7-YFP expressing SKBR3 cells (solid squares) were compared to those of YFP vector-transfected (solid triangles) and parental controls (open squares) to determine if DNA repair activities were enhanced in cells where HOXB7 was expressed.

D. Cell survival after radiation treatment Dose response curves are shown for HOXB7-YFP stably-transfected SKBR3 cells (solid squares) in comparison to control YFP-expressing (solid triangles) and parental cells (open squares) treated with ionizing radiation while growing exponentially.

E. Clonogenicity of MCF10A-HOXB7 cells after radiation treatment.

Clonogenic survival assays of MCF10A cells stably transfected with FLAG-HOXB7 (squares) or vector alone (triangles) were performed. Percent survival was calculated compared to mock-irradiated (0 Gy) controls.

F. Clonogenicity of MCF-7 and MDA-MB-468 cells transfected with HOXB7 specific siRNA after radiation treatment.

Clonogenic survival assays of MCF-7 or MDA-MB-468 cells stably transfected with either scrambled sequence siRNA or HOXB7-specific siRNA were performed. Percent survival was calculated on day 14 for MCF-7 and day 10 for MDA-MB-468 and compared to mock-irradiated (0 Gy) controls.

REFERENCES

Allen C, Miller CA, Nickoloff JA (2003) The mutagenic potential of a single mutagenic double-strand break in a mammalian chromosome is not influenced by transcription. *DNA Repair* **2**: 1147-1156.

Baynton K, Otterlei M, Bjoras M, von Kobbe C, Bohr VA and Seeberg E (2003) WRN interacts physically and functionally with the recombination mediator protein RAD52. *J Biol Chem* **278**: 36476-36486.

Bernstein C, Bernstein H, Payne CM and Garewal H (2002) DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. *Mutat Res* **511**: 145-178.

Bostrom K, Tintut Y, Kao SC, Stanford WP and Demer LL (2000) HOXB7 overexpression promotes differentiation of C3H10T1/2 cells to smooth muscle cells. *J Cell Biochem* **78**: 210-221.

Care A, Felicetti F, Meccia E, Bottero L, Parenza M, Stoppacciaro A, Peschle C and Colombo MP (2001) HOXB7: a key factor for tumor-associated angiogenic switch. *Cancer Res* **61**: 6532-6539.

Care A, Silvani A, Meccia E, Mattia G, Peschle C and Colombo MP (1998) Transduction of the SkBr3 breast carcinoma cell line with the HOXB7 gene induces bFGF expression, increases cell proliferation and reduces growth factor dependence. *Oncogene* **16**: 3285-3289.

Care A, Silvani A, Meccia E, Mattia G, Stoppacciaro A, Parmiani G, Peschle C and Colombo MP (1996) HOXB7 constitutively activates basic fibroblast growth factor in melanomas. *Mol Cell Biol* **16**: 4842-4851.

Chen F, Greer J and Capecchi MR (1998) Analysis of Hoxa7/Hoxb7 mutants suggests periodicity in the generation of the different sets of vertebrae. *Mech Dev* **77**: 49-57.

Chen H and Sukumar S (2003) Role of homeobox genes in normal mammary gland development and breast tumorigenesis. *J Mammary Gland Biol Neoplasia* **8**: 159-175.

Collis SJ, DeWeese TL, Jeggo PA and Parker AR (2005) The life and death of DNA-PK. *Oncogene* **24**: 949-961.

Collis SJ, Swartz MJ, Nelson WG and DeWeese TL (2003) Enhanced radiation and chemotherapy-mediated cell killing of human cancer cells by small inhibitory RNA silencing of DNA repair factors. *Cancer Res* **63**: 1550-1554.

de Murcia G, Jongstra-Bilen J, Ittel ME, Mandel P and Delain E (1983) Poly(ADP-ribose) polymerase auto-modification and interaction with DNA: electron microscopic visualization. *EMBO J* **2**: 543-548.

Dhar S, Squire JA, Hande MP, Wellinger RJ and Pandita TK (2000) Inactivation of 14-3-3sigma influences telomere behavior and ionizing radiation-induced chromosomal instability. *Mol Cell Biol* **20**: 7764-7772.

Galande S and Kohwi-Shigematsu T (1999) Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences. *J Biol Chem* **274**: 20521-20528.

Hayashi S and Scott MP (1990) What determines the specificity of action of Drosophila homeodomain proteins? *Cell* **63**: 883-894.

Herceg Z and Wang ZQ (2001) Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat Res* **477**: 97-110.

Hyduk D and Percival-Smith A (1996) Genetic characterization of the homeodomain-independent activity of the Drosophila fushi tarazu gene product. *Genetics* **142**: 81-492.

Krumlauf R (1994) Hox genes in vertebrate development. *Cell* **78**:191-201.

Lees-Miller SP and Meek K (2003) Repair of DNA double strand breaks by non-homologous end joining. *Biochimie* **85**:1161-1173.

Li B, avarro S, Kasahara N and Comai L (2004) Identification and biochemical characterization of a Werner's syndrome protein complex with Ku70/80 and poly(ADP-ribose) polymerase-1. *J Biol Chem* **279**:13659-13667.

Lieber MR, Ma Y, Pannicke U, Schwarz K (2003) Mechanism and regulation of human non-homologous DNA end-joining *Nat Rev Mol Cell Biol* **4**:12-720.

Maclean JA, 2nd, Chen MA, Wayne CM, Bruce SR, Rao M, Meistrich ML, Macleod C and Wilkinson MF (2005) Rhox: a new homeobox gene cluster. *Cell* **120**: 369-382.

Makiyama K, Hamada J, Takada M, Murakawa K, Takahashi Y, Tada M, Tamoto E, Shindo G, Matsunaga A, Teramoto K, Komuro K, Kondo S, Katoh H, Koike T and Moriuchi T (2005) Aberrant expression of HOX genes in human invasive breast carcinoma. *Oncol Rep* **13**: 673-679.

Naora H, Yang YQ, Montz FJ, Seidman JD, Kurman RJ and Roden RB (2001) A serologically identified tumor antigen encoded by a homeobox gene promotes growth of ovarian epithelial cells. *Proc Natl Acad Sci U S A* **98**: 4060-4065.

Pellerin I, Schnabel C, Catron KM and Abate C (1994) Hox proteins have different affinities for a consensus DNA site that correlate with the positions of their genes on the hox cluster. *Mol Cell Biol* **14**: 4532-4545.

Pleschke JM, Kleczkowska HE, Strohm M and Althaus FR (2000) Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *J Biol Chem* **275**: 40974-40980.

Rothkamm K, Kruger I, Thompson LH and Lobrich M. (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol Cell Biol* **23**: 5706-5715.

Rubin E, Mittnacht S, Villa-Moruzzi E and Ludlow JW (2001) Site-specific and temporally-regulated retinoblastoma protein dephosphorylation by protein phosphatase type 1. *Oncogene* **20**: 3776-3785.

Schild-Poulter C, Pope L, Giffin W, Kochan JC, Ngsee JK, Traykova-Andonova M and Hache RJ (2001) The binding of Ku antigen to homeodomain proteins promotes their phosphorylation by DNA-dependent protein kinase. *J Biol Chem* **276**: 16848-16856.

Sharkey M, Graba Y and Scott MP (1997) Hox genes in evolution: protein surfaces and paralog groups. *Trends Genet* **13**: 145-151.

Sharma GG, Gupta A, Wang H, Scherthan H, Dhar S, Gandhi V, Iliakis G, Shay JW, Young CS and Pandita TK (2003) hTERT associates with human telomeres and enhances genomic stability and DNA repair. *Oncogene* **22**: 131-146.

Shigematsu M, Watanabe H and Sugihara H (1999) Proliferation and differentiation of unilocular fat cells in the bone marrow. *Cell Struct Funct* **24**: 89-100.

Shiojima I, Komuro I, Mizuno T, Aikawa R, Akazawa H, Oka T, Yamazaki T and Yazaki Y (1996) Molecular cloning and characterization of human cardiac homeobox gene CSX1. *Circ Res* **79**: 920-929.

Topisirovic I, Culjkovic B, Cohen N, Perez JM, Skrabanek L and Borden KL (2003) The proline-rich homeodomain protein, PRH, is a tissue-specific inhibitor of eIF4E-dependent cyclin D1 mRNA transport and growth. *EMBO J* **22**: 689-703.

Wayne CM, Sutton K and Wilkinson MF (2002) Expression of the pem homeobox gene in Sertoli cells increases the frequency of adjacent germ cells with deoxyribonucleic acid strand breaks. *Endocrinology* **143**: 4875-4885.

Yaneva M, Kowalewski T and Lieber MR (1997) Interaction of DNA-dependent protein kinase with DNA and with Ku: biochemical and atomic-force microscopy studies. *EMBO J* **16**: 5098-5112.

Yang Y, Hwang CK, D'Souza UM, Lee SH, Junn E. and Mouradian MM (2000) Three-amino acid extension loop homeodomain proteins Meis2 and TGIF differentially regulate transcription. *J Biol Chem* **275**: 20734-20741.

Yaron Y, McAdara JK, Lynch M, Hughes E. and Gasson JC (2001) Identification of novel functional regions important for the activity of HOXB7 in mammalian cells. *J Immunol* **166**: 5058-5067.

Zdzienicka MZ, Tran Q, van der Schans GP and Simons JW (1988) Characterization of an X-ray-hypersensitive mutant of V79 Chinese hamster cells. *Mutat Res* **194**: 239-249.

Figure 1

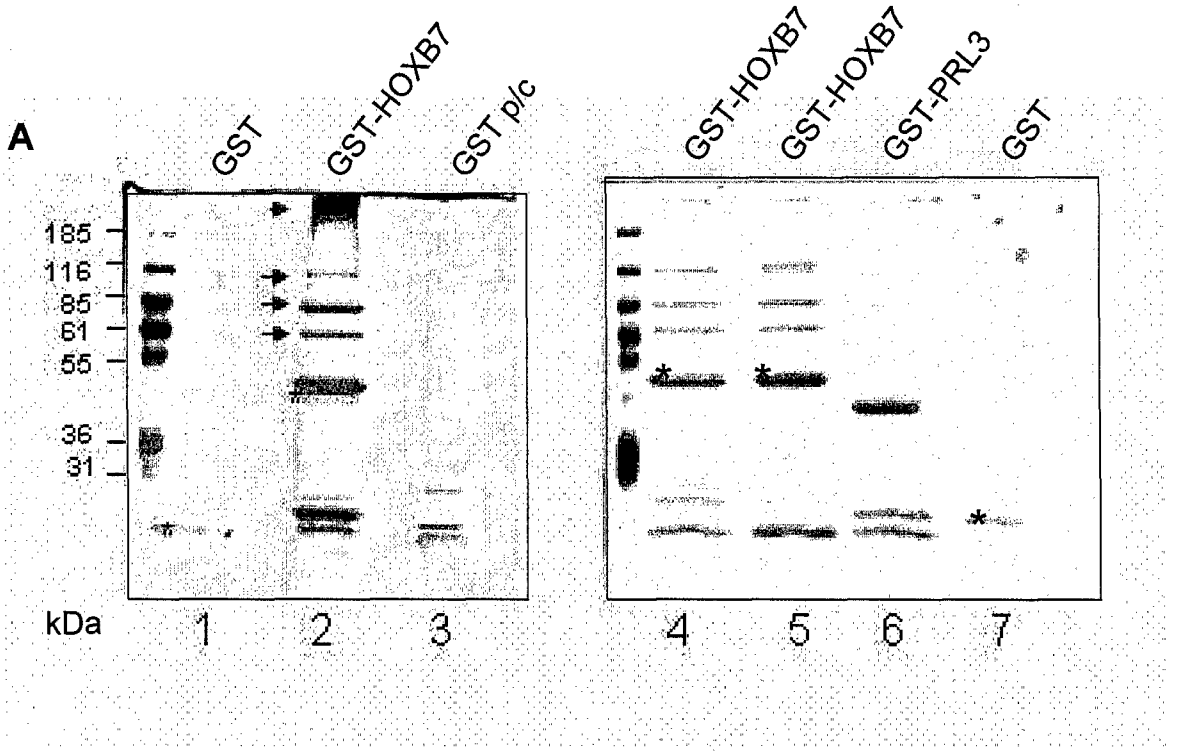

A
GST
GST-HOXB7
GST p/c
GST-HOXB7
GST-HOXB7
GST-PRL3
GST
185
116
85
61
55
36
31
kDa
1
2
3
4
5
6
7
B
input
GST
GST-HOXB7
GST-PRL3
250
185
DNA-PKcs
116
PARP
85
Ku 80
61
Ku 70
kDa
1
2
3
4

Figure 2

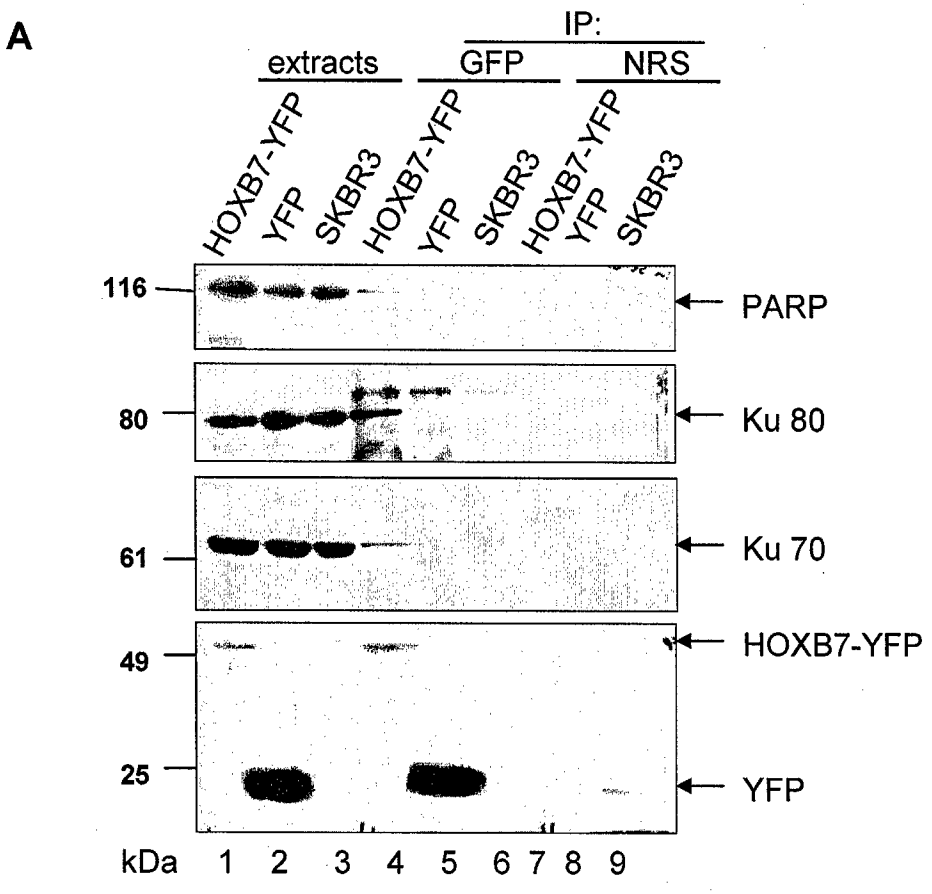
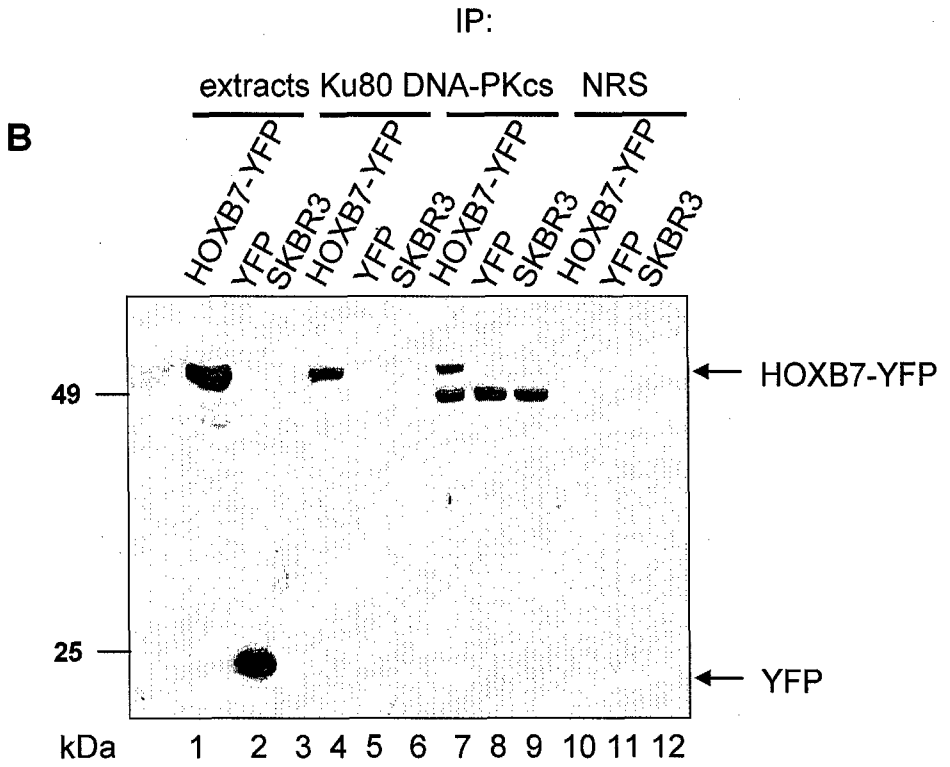

A
IP:
extracts
GFP
NRS
HOXB7-YFP
YFP
SKBR3
HOXB7-YFP
YFP
SKBR3
HOXB7-YFP
YFP
SKBR3
116
80
61
49
25
PARP
Ku 80
Ku 70
HOXB7-YFP
YFP
kDa 1 2 3 4 5 6 7 8 9

Figure 2


B
IP:
extracts
Ku80
DNA-PKcs
NRS
HOXB7-YFP
YFP
SKBR3
49
25
HOXB7-YFP
YFP
kDa
1 2 3 4 5 6 7 8 9 10 11 12

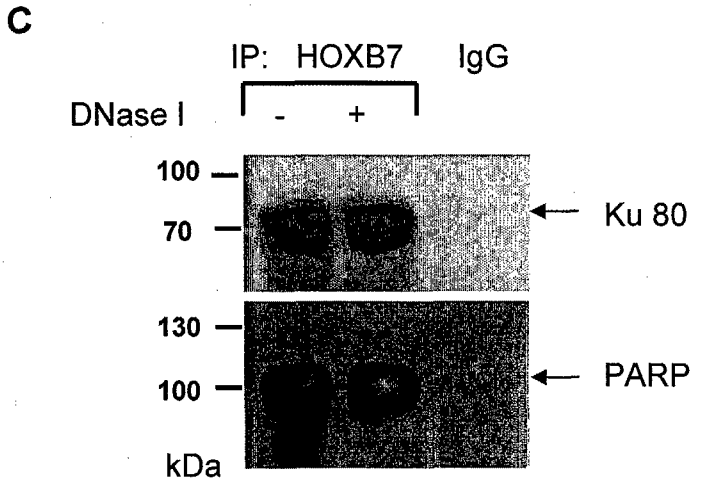

C
IP: HOXB7
IgG
DNase I
-
+
100
70
Ku 80
130
100
PARP
kDa

Figure 3

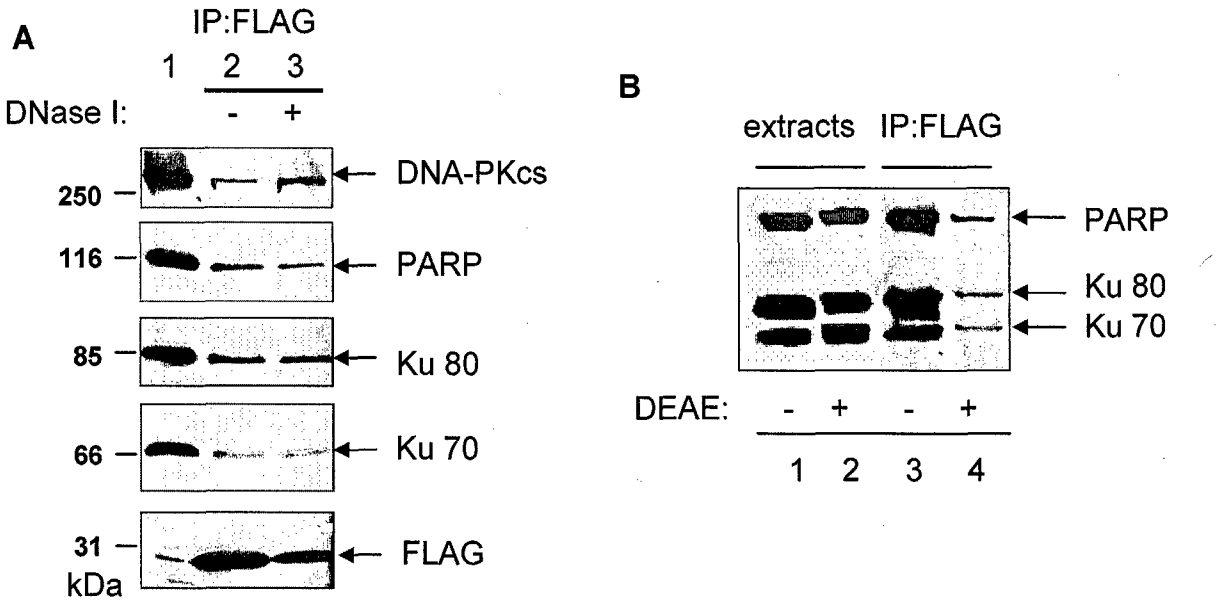

A
IP:FLAG
1 2 3
DNase I: - +
250
116
85
66
31
kDa
DNA-PKcs
PARP
Ku 80
Ku 70
FLAG
B
extracts IP:FLAG
PARP
Ku 80
Ku 70
DEAE: - + - +
1 2 3 4

Figure 4

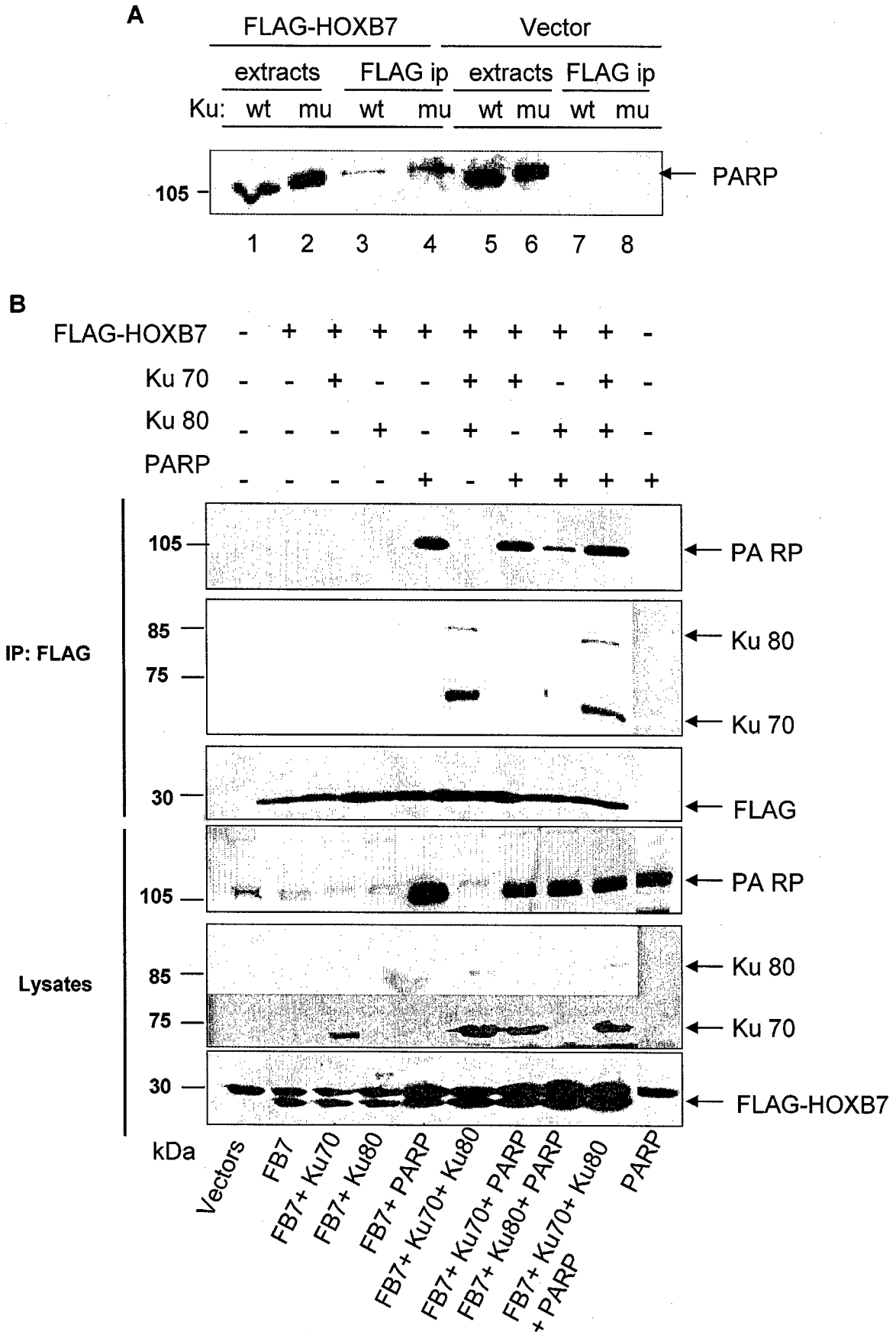

A
FLAG-HOXB7
Vector
extracts
FLAG ip
extracts
FLAG ip
Ku: wt mu wt mu wt mu wt mu
105
PARP
1 2 3 4 5 6 7 8
B
FLAG-HOXB7 - + + + + + + + + -
Ku 70 - - + - - + + - + -
Ku 80 - - - + - + - + + -
PARP - - - - + - + + + +
IP: FLAG
105
PA RP
85
75
Ku 80
Ku 70
30
FLAG
Lysates
105
PA RP
85
75
Ku 80
Ku 70
30
FLAG-HOXB7
kDa
Vectors
FB7
FB7+ Ku70
FB7+ Ku80
FB7+ PARP
FB7+ Ku70+ Ku80
FB7+ Ku70+ PARP
FB7+ Ku80+ PARP
FB7+ Ku70+ Ku80 + PARP
PARP

Figure 5

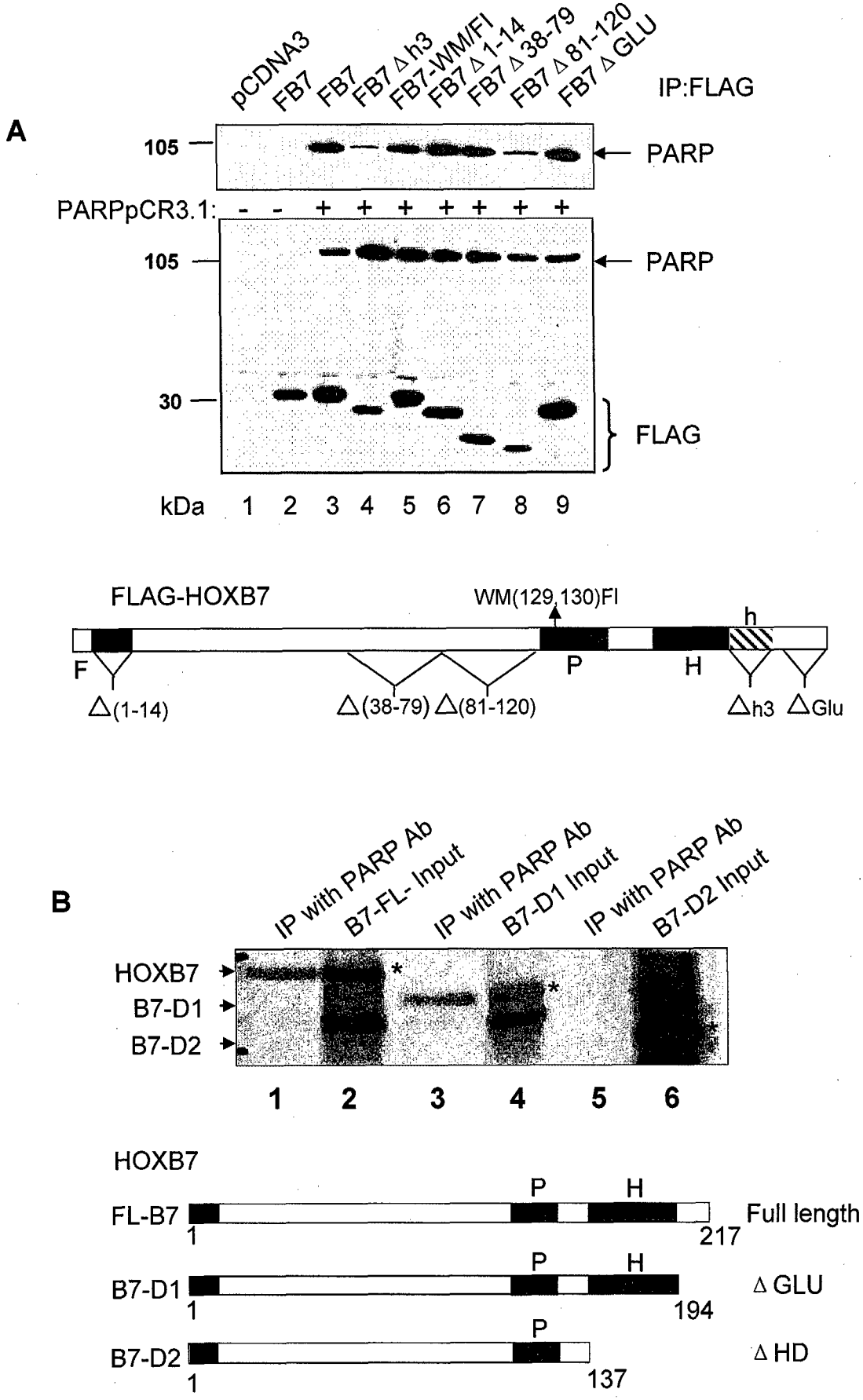

A
pCDNA3
FB7
FB7
FB7 Δh3
FB7-WM/FI
FB7 Δ1-14
FB7 Δ38-79
FB7 Δ81-120
FB7 ΔGLU
IP:FLAG
105
PARP
PARPpCR3.1: - - + + + + + + +
105
PARP
30
FLAG
kDa 1 2 3 4 5 6 7 8 9
FLAG-HOXB7
WM(129,130)FI
h
F
P
H
Δ(1-14)
Δ(38-79) Δ(81-120)
Δh3
ΔGlu
B
IP with PARP Ab
B7-FL- Input
IP with PARP Ab
B7-D1 Input
IP with PARP Ab
B7-D2 Input
HOXB7
B7-D1
B7-D2
1 2 3 4 5 6
HOXB7
FL-B7
P
H
1
217
Full length
B7-D1
P
H
1
194
Δ GLU
B7-D2
P
1
137
Δ HD

Figure 6

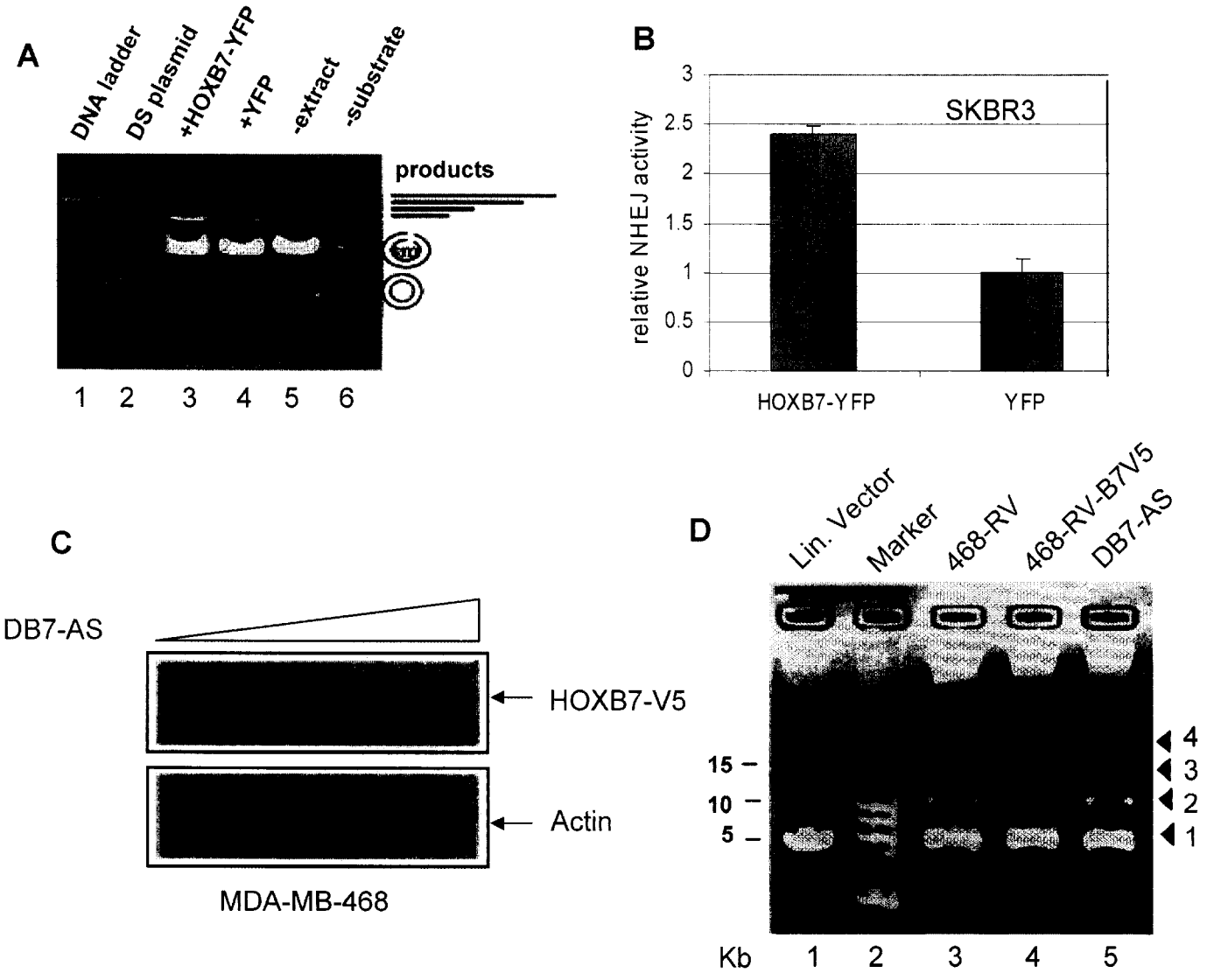
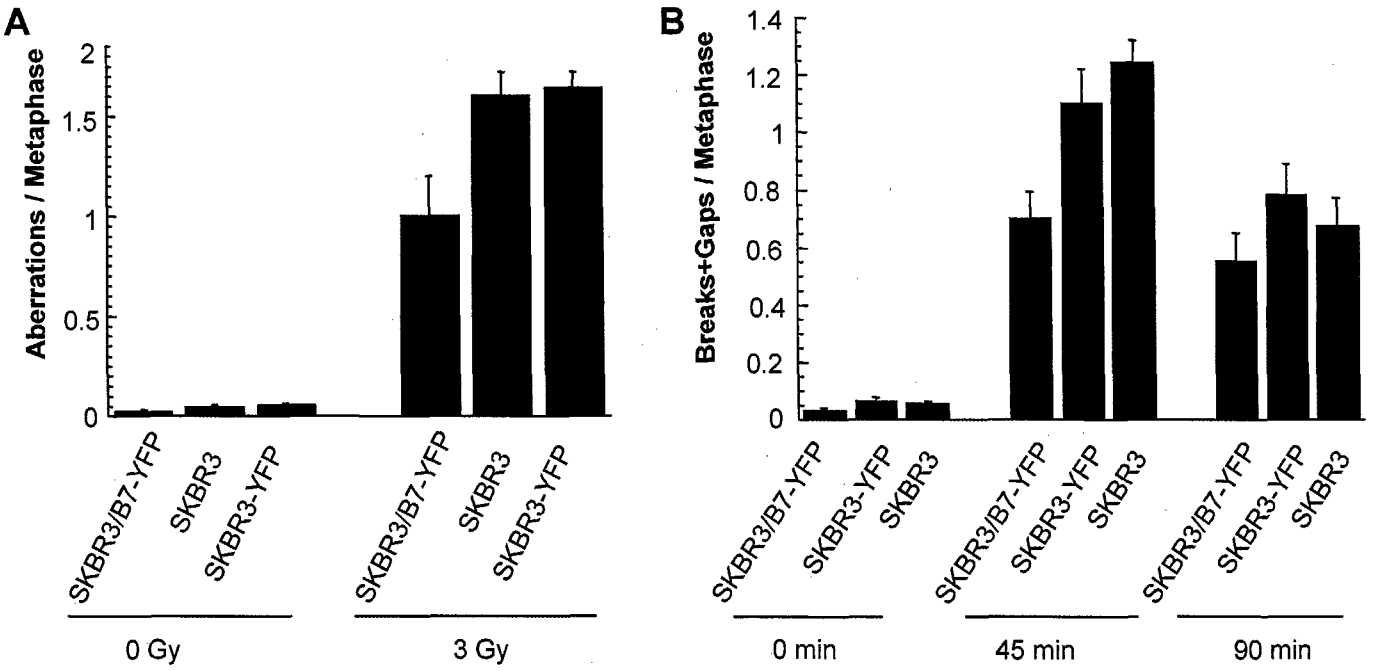
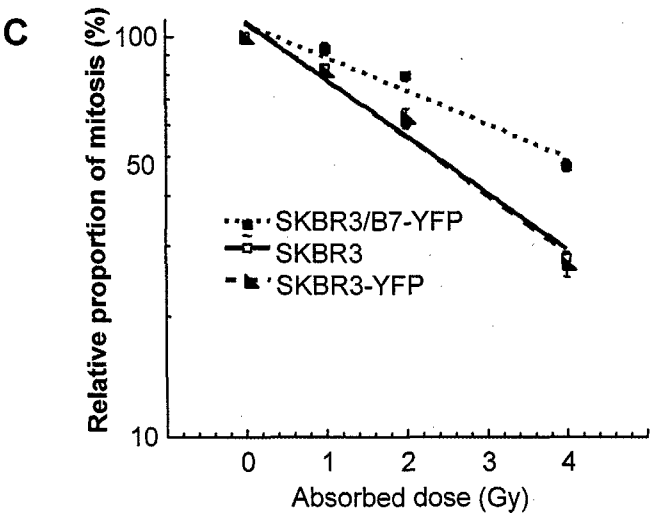
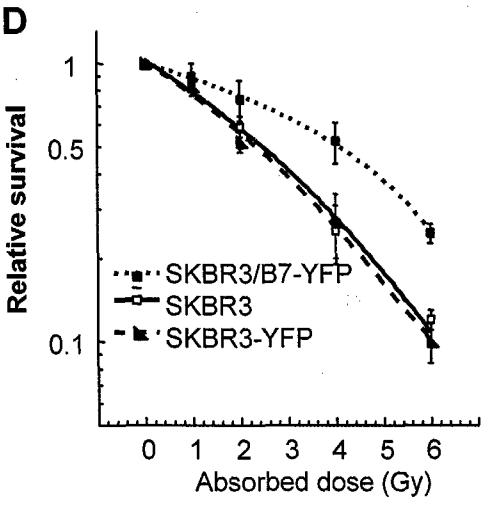
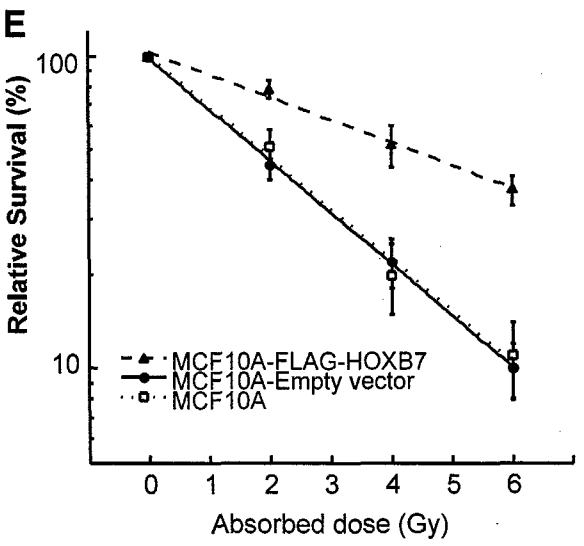

A
DNA ladder
DS plasmid
+HOXB7-YFP
+YFP
-extract
-substrate
products
1 2 3 4 5 6
B
SKBR3
relative NHEJ activity
3
2.5
2
1.5
1
0.5
0
HOXB7-YFP
YFP
C
DB7-AS
HOXB7-V5
Actin
MDA-MB-468
D
Lin. Vector
Marker
468-RV
468-RV-B7V5
DB7-AS
15
10
5
4
3
2
1
Kb 1 2 3 4 5

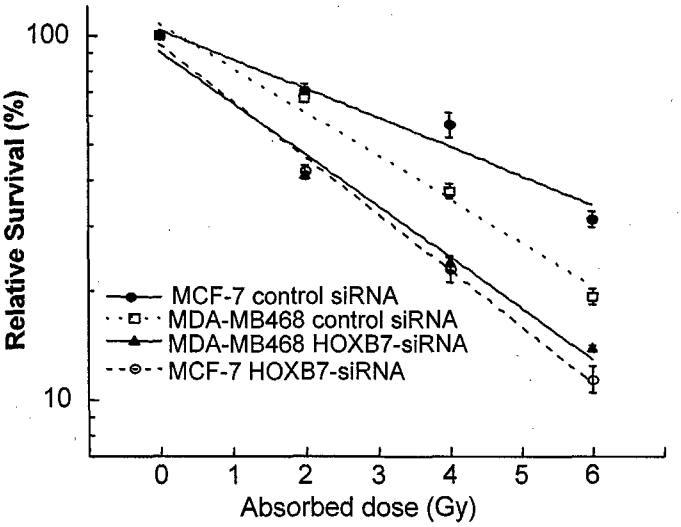
Figure 7


A
Aberrations / Metaphase
2
1.5
1
0.5
0
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
0 Gy
3 Gy
B
Breaks+Gaps / Metaphase
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SKBR3/B7-YFP
SKBR3-YFP
SKBR3
SKBR3/B7-YFP
SKBR3-YFP
SKBR3
SKBR3/B7-YFP
SKBR3-YFP
SKBR3
0 min
45 min
90 min


C
Relative proportion of mitosis (%)
100
50
10
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
0
1
2
3
4
Absorbed dose (Gy)


D
Relative survival
1
0.5
0.1
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
0
1
2
3
4
5
6
Absorbed dose (Gy)


E
Relative Survival (%)
100
10
MCF10A-FLAG-HOXB7
MCF10A-Empty vector
MCF10A
0
1
2
3
4
5
6
Absorbed dose (Gy)


F
Relative Survival (%)
100
10
MCF-7 control siRNA
MDA-MB468 control siRNA
MDA-MB468 HOXB7-siRNA
MCF-7 HOXB7-siRNA
0
1
2
3
4
5
6
Absorbed dose (Gy)