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Dual functions of the homeoprotein DLX4 in modulating responsiveness of tumor cells to topoisomerase II-targeting drugs

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Abstract

Topoisomerase II (TOP2)-targeting poisons such as anthracyclines and etoposide are commonly used for cancer chemotherapy and kill tumor cells by causing accumulation of DNA double-strand breaks (DSBs). Several lines of evidence indicate that overexpression of *TOP2A*, the gene encoding topoisomerase II α , increases sensitivity of tumor cells to TOP2 poisons but it is not clear why some *TOP2A*-overexpressing (*TOP2A*-High) tumors respond poorly to these drugs. In this study, we identified that *TOP2A* expression is induced by DLX4, a homeoprotein that is overexpressed in breast and ovarian cancers. Analysis of breast cancer datasets revealed that *TOP2A*-High cases that also highly expressed *DLX4* responded more poorly to anthracycline-based chemotherapy than *TOP2A*-High cases that expressed *DLX4* at low levels. Overexpression of *TOP2A* alone in tumor cells increased the level of DSBs induced by TOP2 poisons. In contrast, DLX4 reduced the level of TOP2 poison-induced DSBs irrespective of its induction of *TOP2A*. DLX4 did not stimulate homologous recombination-mediated repair of DSBs. However, DLX4 interacted with Ku proteins, stimulated DNA-dependent protein kinase activity, and increased erroneous end-joining repair of DSBs. Whereas DLX4 did not reduce levels of TOP2 poison-induced DSBs in Ku-deficient cells, DLX4 stimulated DSB repair and reduced the level of TOP2 poison-induced DSBs when Ku was reconstituted in these cells. Our findings indicate that DLX4 induces *TOP2A* expression, but reduces sensitivity of tumor cells to TOP2 poisons by stimulating Ku-dependent repair of DSBs. These opposing activities of DLX4 could explain why some *TOP2A*-overexpressing tumors are not highly sensitive to TOP2 poisons.

Keywords

chemosensitivity; topoisomerase II; DNA repair; homeobox genes

INTRODUCTION

DNA double-strand breaks (DSBs) are the most lethal form of DNA damage and are primarily repaired by two pathways. The homologous recombination (HR) pathway is a high fidelity repair mechanism that utilizes the sister chromatid as a template. The canonical non-homologous end-joining (NHEJ) pathway is initiated by the binding of Ku heterodimers to DNA ends which recruit the catalytic subunit of DNA-dependent protein kinase (DNA-PK) to form a complex that enables ligation of DNA ends with little or no homology (1,2). Cells

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that are defective in the HR or NHEJ pathways are highly sensitive to poisons that target topoisomerase II (TOP2) (3–6). TOP2 is an enzyme that induces transient DSBs to relieve torsional stress imposed on DNA during replication (7). Of the two mammalian TOP2 isoforms, TOP2 α is essential for cell growth (7). TOP2 poisons such as etoposide and anthracyclines (e.g. doxorubicin, epirubicin) are commonly used as anticancer drugs. TOP2 poisons trap the enzyme in a complex with DNA at a step after DSBs have occurred, thereby causing DSBs to accumulate to lethal levels (8). Experimental studies have demonstrated that overexpressing TOP2 α in cells increases TOP2 poison-induced cell death, whereas down-regulating TOP2 α decreases sensitivity (9–11). On the other hand, there have been conflicting studies regarding the clinical value of TOP2 α in predicting responsiveness to TOP2 poisons. The *TOP2A* gene that encodes TOP2 α maps to 17q21-22, a region that is amplified in ~12% of breast and ovarian cancers (12,13). Whereas several studies have reported that *TOP2A* amplification identifies cancer patients who respond favorably to anthracycline-based chemotherapy (13,14), others have found the *TOP2A* status has no predictive value (15–17). However, a molecular explanation for the discordance between these experimental and clinical studies has not been identified.

Homeobox genes constitute a gene super-family encoding transcription factors (often termed homeoproteins) that control developmental patterning (18–20). Increasing evidence indicates that aberrant expression of many homeobox genes in tumors deregulates cell proliferation and cell survival (19,20). For example, the homeobox gene *CDX2* inhibits cell proliferation and is down-regulated in many colon cancers (21,22). Overexpression of *SIX1* in ovarian cancers confers resistance to TRAIL-mediated apoptosis (23), whereas loss of *BARX2* in ovarian cancers is associated with cisplatin-resistance (24). However, only few *bona fide* transcriptional targets of homeoproteins have been identified, and the mechanisms of these regulatory factors in tumorigenesis remain poorly understood.

The *DLX4* gene (also reported as *DLX7*, *BPI*) is a member of the *DLX* family of homeobox genes that controls diverse developmental processes including skeletal patterning and neurogenesis (25). *DLX4* is absent from most normal adult tissues, but is frequently expressed in breast and ovarian cancers (26,27) and also in lung cancers and leukemias (28,29). We previously identified that *DLX4* inhibits gene responses that are central to the TGF- β cytosolic program (30), and also stimulates tumor angiogenesis by inducing expression of fibroblast growth factor-2 and vascular endothelial growth factor (27). Others have found that *DLX4* is more highly expressed in a doxorubicin-resistant subclone of MCF-7 breast cancer cells than in the parental cell line (26), raising the possibility that *DLX4* also modulates sensitivity to specific chemotherapeutic agents. In this study, we identified that *TOP2A* is a transcriptional target of *DLX4*, but that induction of *TOP2A* by *DLX4* is not associated with increased sensitivity to TOP2 poisons. *DLX4* was found to interact with Ku proteins, stimulate Ku-dependent repair of DSBs and reduce levels of DSBs induced by TOP2 poisons. These apparently opposing activities of *DLX4* could explain why some tumors have elevated *TOP2A* expression but are not sensitive to TOP2 poisons.

MATERIALS AND METHODS

Reagents

Sources of antibodies (Abs) were as follows: *DLX4* (Abcam, Abnova); TOP2 α (TopoGEN); Ku70, Ku80 (Thermo Fisher Scientific); DNA-PK catalytic subunit (DNA-PKcs) (Cell Signaling Technology); DNA ligase IV, XRCC4, FLAG, actin (Sigma-Aldrich); histone H2A (Millipore). Doxorubicin, etoposide, paclitaxel, 5-fluorouracil, cyclophosphamide, 5-bromo-4-chloro-3-indolyl- β -D-galacto-pyranoside (X-gal) and isopropyl-L-thio- β -D-galacto-pyranoside (IPTG) were purchased from Sigma-Aldrich.

Plasmids

All shRNAs and cDNAs encoding Ku80 and TOP2 α -GFP fusion protein were purchased from OriGene Technology. pUC19 plasmid was purchased from Invitrogen. *BRCA1* and I-*Sce* I plasmids (31) and full-length and truncated *DLX4* expression plasmids (30) have been previously described. pGL2 plasmids containing wild-type *TOP2A* promoter sequences were provided by William Beck (University of Illinois). The motif ATATAAAAG in the *TOP2A* promoter (nucleotides -127 to -119) was mutated to ACAGAGACG by using the QuikChange II Site-directed Mutagenesis Kit (Agilent Technologies).

Cell lines

Parental MDA-MB-468 and XR-V15B cells, authenticated by STR analysis, were purchased from American Type Culture Collection and passaged within 6 months of receipt. Parental TOV112D cells (provided by Ju-Seog Lee, MD Anderson Cancer Center) and U2OS cells were also tested by STR analysis. The stable +DLX4 MDA-MB-468 and U2OS DR-GFP reporter cell lines are described in our previous studies (30,31). Cell lines were cultured in the following media supplemented with 10% FBS and penicillin/streptomycin: MDA-MB-468 (RPMI 1640); U2OS (McCoy's 5A); XR-V15B (DMEM); TOV112D (1:1 mixture of MCDB 105 medium and Medium 199). Lipofectamine 2000 reagent (Invitrogen) was used for transfecting cell lines.

Cell viability and cell death assays

Cells were plated in 96-well plates (2,000 per well) and incubated for 2 d with drugs at concentrations ranging from 10^{-4} to 10^4 μ M. Cell viability was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche). IC₅₀ values were calculated from the average of dose-response curves obtained in 3 independent experiments. Cell death was measured by assaying mono- and oligo- nucleosomes in cell lysates by using Cell Death Detection ELISA (Roche).

Chromatin immunoprecipitation (IP) and luciferase reporter assays

Chromatin IP assays were performed by using the ChIP Assay kit (Upstate Biotechnology). Sheared chromatin was incubated with DLX4 Ab and DNA purified from precipitated protein-DNA complexes. A 375 bp fragment of the *TOP2A* promoter was amplified by using the following primers: forward, 5'-GAAGCTAAGGCTCCCATTC-3'; reverse, 5'-CGTCCAGAAGAACCAATCGT-3'. As a negative control, a 166 bp *GAPDH* fragment was amplified using the following primers: forward, 5'-TACTAGCGGTTTTACGGGCG-3'; reverse, 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'. Cells were co-transfected with pGL2 firefly luciferase reporter plasmids and with pRL-CMV Renilla luciferase reporter plasmid (Promega) for normalizing transfection efficiency. Luciferase activities were assayed using the Dual-luciferase reporter assay kit (Promega).

Comet assay

DSBs were measured by neutral comet assay using the Comet Assay kit (Trevigen). Comet images were captured by fluorescence microscopy. Tail moments (percentage of DNA in tail \times tail length) were quantified using CometScore software (www.autocomet.com). For each cell line, 3 independent assays were performed where comet tails were analyzed in a minimum of 50 randomly selected cells in each assay.

HR assay

Cells of the U2OS DR-GFP reporter cell line were transfected with I-*Sce* I plasmid to generate a DSB within the *SceGFP* reporter gene. At 1 d thereafter, cells were transfected

with empty vector, *DLX4* or *BRCA1* plasmids and cultured for 2 d. Repair of the DSB was assayed by PCR and flow cytometric analysis of GFP fluorescence intensity as previously described (31).

End-joining assay

Cells were suspended in lysis buffer (10 mM HEPES, pH 7.9, 0.5 M sucrose, 1 mM dithiothreitol (DTT), 5 mM MgCl₂, 0.2% Nonidet P-40) and centrifuged at 800×*g* for 5 min. Pellets were suspended in nuclear buffer (10 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1 mM DTT, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2% Nonidet P-40) and centrifuged at 12,000×*g* for 10 min. Supernatants were dialyzed at 4°C in dialysis buffer (20 mM HEPES, pH 7.9, 10% glycerol, 100 mM KCl, 1 mM DTT, 0.2 mM EDTA). *Eco* RI-digested pUC19 DNA (100 ng) was incubated at 20°C for 16 h with dialyzed nuclear extract (15 µg) in a reaction containing 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM ATP, 50 µM dNTPs, 1 mM DTT. *E. coli* strain DH5α (Invitrogen) was transformed with 10 ng of pUC19 DNA and plated on LB agar plates containing ampicillin, X-gal and IPTG. Numbers of blue and white colonies were counted on each plate. Plasmid DNA was isolated from individual colonies and sequenced.

IP and immunoblotting

Whole cell extracts were prepared by lysing cells in M-PER buffer (Pierce Biotechnology). Nuclear extracts were prepared as previously described (30). Where indicated, genomic DNA was removed by treatment with benzonase. For protein IP, extracts were pre-cleared with protein G agarose and 1 mg of extract was incubated with Abs indicated in the text. For DNA IP, nuclear extracts were pre-cleared with streptavidin agarose and incubated with biotinylated oligonucleotide duplexes as previously described (30). Immunoprecipitates were subjected to SDS-PAGE and immunoblotting using PVDF membrane (GE Healthcare).

DNA-PK assay

Nuclear extracts were prepared as for IP assays and incubated with DEAE Sepharose® Fast Flow (GE Healthcare) to remove genomic DNA. DEAE sepharose was removed by centrifugation and extracts dialyzed in buffer containing 20 mM Tris-HCl, pH 8.0, 10% glycerol, 100 mM NaCl, 1% Nonidet P-40, 2 mM EDTA. DNA-PK assays were performed by using the SignaTECT® DNA-PK Assay System (Promega).

Bioinformatic analysis

DLX4 and *TOP2A* transcript levels in tumors were analyzed in publicly available gene expression datasets as described in Supplementary Methods.

Statistical analysis

Statistical analysis was performed using STATISTICA6 software (StatSoft Inc.). Statistical significance of data was assessed by two-tailed Student's *t*-test unless noted otherwise. Data represent mean + s.d. Significance of differences in gene expression between groups of patients was assessed by Mann-Whitney *U*-test. Fisher exact test was used for evaluating differences in response rates between groups. *P* values of >0.05 were considered not significant.

RESULTS

DLX4 overexpression in tumor cells selectively decreases TOP2 poison-sensitivity

Increasing evidence indicates that various homeobox genes modulate responsiveness of cells to diverse anti-proliferative and apoptotic signals (23,24,30). We initially investigated the

possibility that DLX4 modulates the sensitivity of tumor cells to commonly used chemotherapeutic agents by using a MDA-MB-468 breast cancer cell line that we transduced with DLX4 in earlier studies (30). Expression of DLX4 significantly reduced sensitivity of MDA-MB-468 cells to doxorubicin ($P=0.005$), but did not alter sensitivity to paclitaxel (microtubule disruptor), 5-fluorouracil (pyrimidine analog) or cyclophosphamide (alkylating agent) (Supplementary Table I). Similar results were obtained when DLX4 was overexpressed in U2OS osteosarcoma cells (Supplementary Table I). These findings suggest that DLX4 overexpression selectively decreases TOP2 poison-sensitivity.

DLX4 induces *TOP2A* promoter activity and TOP2 α levels

Because down-regulation of TOP2 α has been reported to reduce sensitivity to TOP2 poisons (10,11), we evaluated whether DLX4 inhibits TOP2 α expression. Contrary to our expectation, enforced expression of DLX4 induced TOP2 α levels (Fig. 1A). Conversely, TOP2 α levels decreased when endogenous DLX4 was knocked-down by shRNAs that targeted two different sites in *DLX4* but were not affected by non-targeting shRNA (Fig. 1B). When breast cancer cases in two independent cohorts were stratified according to expression of *DLX4* in tumors, levels of *TOP2A* transcripts were found to be significantly higher in *DLX4*-High than in *DLX4*-Low tumors in each of these cohorts (the Cancer Genome Atlas (TCGA) dataset, $P=0.0001$; Van de Vijver dataset (32), $P=0.0012$; Fig. 1C). *TOP2A* transcript levels were also higher in *DLX4*-High than in *DLX4*-Low tumors in TCGA datasets of ovarian and lung cancers (ovarian cancers, $P=0.022$; lung cancers, $P=0.028$; Supplementary Fig. S1). Because homeoproteins are thought to function as transcription factors (18), we evaluated the effect of DLX4 on *TOP2A* promoter activity in luciferase reporter assays. DLX4 stimulated *TOP2A* promoter activity through a region located between positions -162 to -90 relative to the transcription start site ($P=0.006$; Fig. 1D). Binding of endogenous DLX4 to this region was detected by chromatin IP (Fig. 1E). An AT-rich motif located within the responsive region is homologous to the DLX4-binding site in the β -*globin* promoter that is the only well-characterized target of DLX4 (33). Mutation of the AT-rich motif significantly reduced the ability of DLX4 to stimulate *TOP2A* promoter activity ($P=0.004$; Fig. 1D). Together, our findings indicate that *TOP2A* is a transcriptional target of DLX4, but that DLX4 reduces TOP2 poison-sensitivity irrespective of its induction of *TOP2A*.

DLX4 is associated with reduced responsiveness to anthracycline-based chemotherapy

Several studies have published datasets of transcriptional profiles of breast cancers linked to outcomes following anthracycline chemotherapy in combination with 5-fluorouracil, cyclophosphamide and/or a taxane (34–37). Because DLX4 did not affect sensitivity of tumor cells to 5-fluorouracil, cyclophosphamide or paclitaxel (Supplementary Table I), we analyzed four independent breast cancer datasets to gain insight into the relationship between *DLX4* and *TOP2A* expression and anthracycline-sensitivity. Cases in each cohort were classified into groups according to levels of *DLX4* and *TOP2A* transcripts in tumors. Response rates of a given group were found to be consistent across all four cohorts (Table I). Because the sizes of several groups were too small to compare in an individual cohort, response rates were compared between groups of cases from the four cohorts combined. The response rate was higher in the *TOP2A*-High group than in the *TOP2A*-Low group ($P=0.00001$; Table I). However, the response rate was lower in the sub-group of *TOP2A*-High cases that were also *DLX4*-High than in the sub-group of *TOP2A*-High cases that were *DLX4*-Low ($P=0.043$; Table I). These findings raise the possibility that *TOP2A*-High tumors that also highly express *DLX4* respond to anthracyclines more poorly than *TOP2A*-High tumors that have low levels of *DLX4* expression.

DLX4 reduces DSBs and cell death induced by TOP2 poisons

To determine whether tumor cells that highly express both *TOP2A* and *DLX4* are less sensitive to TOP2 poisons than tumor cells that highly express *TOP2A* but not *DLX4*, we initially evaluated levels of doxorubicin-induced cell death in MDA-MB-468 cells in which TOP2 α alone was overexpressed (Supplementary Fig. S2A) and in which TOP2 α was induced by DLX4 (Fig. 1A). As compared to vector-control cells, cell death was increased in cells that overexpressed TOP2 α alone ($P=0.005$) but was reduced in cells that overexpressed DLX4 ($P=0.009$) (Fig. 2A). To confirm our findings, we evaluated cell death in TOV112D ovarian cancer cells in which TOP2 α was knocked-down by *TOP2A* shRNAs (Supplementary Fig. S2B) and in which TOP2 α was down-regulated by DLX4 knockdown (Fig. 1B). Doxorubicin-induced cell death was reduced when TOP2 α was knocked-down by *TOP2A* shRNAs ($P=0.02$) but was increased when DLX4 was knocked-down ($P=0.009$) (Fig. 2B). Similar results were obtained when TOV112D cells were treated with etoposide (Fig. 2B). As compared to vector-control MDA-MB-468 cells, higher levels of doxorubicin-induced DSBs were detected by neutral comet assays in MDA-MB-468 cells that overexpressed TOP2 α ($P=0.006$), whereas lower levels were detected in cells that overexpressed DLX4 ($P=0.02$) (Fig. 2C). Conversely, levels of DSBs were reduced in TOV112D cells when TOP2 α was knocked-down ($P=0.008$) but were increased when DLX4 was knocked-down ($P=0.009$) (Fig. 2D). These findings indicate that DLX4, notwithstanding its induction of TOP2 α , inhibits TOP2 poison-induced cell death by reducing accumulation of DSBs.

DLX4 does not stimulate HR repair but stimulates error-prone end-joining repair of DSBs

In mammalian cells, DSBs are primarily repaired by HR and by end-joining (1,2). To determine whether DLX4 stimulates HR-mediated repair of DSBs, we used a U2OS cell line that stably expresses a *DR-GFP* reporter cassette. Transient expression of the restriction enzyme *I-SceI* in this cell line induces a DSB within the *SceGFP* gene. Repair of the DSB by HR is facilitated by using the adjacent *iGFP* gene (encoding a truncated GFP) as a template and results in restoration of GFP expression by the *SceGFP* gene (Supplementary Fig. S3A). Restoration of GFP expression (measured as % of GFP+ cells) was compared when DLX4 and BRCA1, a well-characterized regulator of HR, were expressed in the reporter cell line (Supplementary Fig. S3B). Restoration of GFP expression was markedly increased by BRCA1 (from 5.3% to 17.1% GFP+ cells) but not by DLX4 (from 5.3% to 5.9% GFP+ cells) (Supplementary Fig. S3C). PCR amplification was performed to confirm increased repair of the *SceGFP* gene in cells when BRCA1 but not DLX4 was overexpressed (Supplementary Fig. S3D). These findings indicate that DLX4 does not stimulate HR-mediated repair of DSBs.

Whereas HR is a high fidelity repair mechanism, repair of DSBs by end-joining is error-prone (1,2). To investigate the effect of DLX4 on end-joining, nuclear extracts of vector-control U2OS cells and U2OS cells that expressed FLAG-tagged DLX4 (+DLX4) were assayed for the ability to rejoin an *EcoRI*-cut DSB within the *lacZ* gene of pUC19 plasmid DNA. Frequency and efficacy of plasmid repair were measured by the formation of blue colonies (correct repair) and white colonies (misrepair) following transformation of *E. coli* (Fig. 3A). As compared to vector-control U2OS extracts, extracts of +DLX4 U2OS cells exhibited an overall increase in repair activity, but this increase was due to a higher frequency of misrepair ($P=0.004$; Fig. 3B). This increase in misrepair was inhibited when extracts were depleted of FLAG-tagged DLX4 by using Ab to FLAG ($P=0.005$; Fig. 3C). To confirm that DLX4 stimulates erroneous repair, we sequenced pUC19 DNA isolated from individual white colonies. Representative examples of sequences surrounding the *EcoRI*-cut DSB and tracts of nucleotide microhomology identified around breakpoint junctions are shown in Supplementary Fig. S4A. As compared to assays using vector-control U2OS

extracts, the size of deletions surrounding the DSB were significantly larger in assays using +DLX4 U2OS extracts ($P=0.009$; Fig. 3D). To confirm our findings, we performed end-joining assays using extracts of TOV112D cells that were transfected with non-targeting and *DLX4*-specific shRNAs. Knockdown of endogenous *DLX4* reduced the frequency of misrepair ($P=0.018$, Fig. 3E), and also reduced the size of deletions surrounding the DSB ($P=0.007$; Fig. 3F; Supplementary Fig. S4B). These findings indicate that *DLX4* stimulates erroneous end-joining repair of DSBs.

DLX4 interacts with the NHEJ machinery and stimulates DNA-PK activity

To determine the mechanism by which *DLX4* stimulates end-joining repair, we initially evaluated the effect of *DLX4* on expression of components of the canonical NHEJ machinery. *DLX4* did not alter expression levels of Ku70 (also known as XRCC6), Ku80 (also known as XRCC5, Ku86), DNA-PKcs, XRCC4 or DNA ligase IV (Fig. 4A). *DLX4* also did not affect interactions between Ku70 and Ku80 (Fig. 4B). Furthermore, *DLX4* neither affected binding of Ku proteins to DNA ends nor the interaction of DNA-PKcs with Ku-DNA complexes (Fig. 4C). On the other hand, *DLX4* increased DNA-PK activity ($P=0.011$; Fig. 4D). Interaction of FLAG-tagged *DLX4* with DNA-PKcs was not detected in the absence of DNA and only weakly detected in the presence of DNA (Fig. 4E). However, interactions of FLAG-tagged *DLX4* with Ku70 and with Ku80 were strongly detected both in the absence and presence of DNA (Fig. 4E). Interactions of endogenous *DLX4* with these NHEJ proteins were confirmed in IP assays using extracts of TOV112D cells (Fig. 4F). We compared the abilities of full-length and truncated FLAG-tagged *DLX4* proteins to interact with Ku70 and Ku80 (Fig. 4G). The N-terminal transcriptional activation domain (TA) of *DLX4* did not interact with Ku70 or Ku80 (Fig. 4H). However, deletion of the DNA-binding homeodomain of *DLX4* (*DLX4-ΔHD*) abolished Ku-binding ability (Fig. 4H), indicating that *DLX4* interacts with Ku proteins through its homeodomain. As compared to vector-control U2OS cells, error-prone end-joining was significantly increased in U2OS cells that expressed full-length *DLX4* ($P=0.004$) but not in cells that expressed the *DLX4-ΔHD* form (Fig. 4I). These findings indicate that *DLX4* interacts with Ku proteins and stimulates DNA-PK activity, and that the ability of *DLX4* to stimulate end-joining repair of DSBs depends on its interaction with Ku proteins.

The ability of DLX4 to reduce levels of TOP2 poison-induced DSBs is Ku-dependent

In subsequent experiments, we investigated whether the ability of *DLX4* to reduce levels of TOP2 poison-induced DSBs is due to its stimulation of Ku-dependent NHEJ activity. We firstly evaluated the effect of *DLX4* on end-joining repair of DSBs in Ku-deficient cells. Cells of the Ku80-deficient cell line XR-V15B (38) were transfected with Ku80 and/or *DLX4* (Fig. 5A). Very little end-joining activity was detected in vector-control XR-V15B cells (Fig. 5B). Reconstitution of Ku80 in XR-V15B cells increased end-joining activity ($P=0.02$), but expression of *DLX4* alone had no stimulatory effect (Fig. 5B). In contrast, *DLX4* increased end-joining activity in XR-V15B cells that were reconstituted with Ku80 ($P=0.003$; Fig. 5B). Expression of *DLX4* alone in XR-V15B cells did not reduce the levels of DSBs induced by doxorubicin (Fig. 5C). However, significantly lower levels of doxorubicin-induced DSBs were detected when *DLX4* was expressed in XR-V15B cells that were reconstituted with Ku80 ($P=0.004$; Fig. 5C). Together, our findings support a model in which *DLX4* induces *TOP2A* expression, but reduces levels of TOP2 poison-induced DSBs by interacting with Ku proteins and stimulating Ku-dependent end-joining repair of DSBs.

DISCUSSION

TOP2A has been the most extensively studied gene for determining responsiveness to TOP2 poisons, but there have been conflicting studies regarding its clinical value as a predictive

marker (13–17). Reasons that have been proposed to explain this discordance include differences in techniques that evaluated the *TOP2A* status in tumors and the possible importance of co-amplification of *HER2* (17). In this study, we identified that *TOP2A* is a transcriptional target of DLX4, a homeoprotein that is overexpressed in various types of tumors including breast and ovarian cancers. Our study supports prior experimental evidence that overexpression of *TOP2A* alone enhances sensitivity of cells to TOP2 poisons, whereas down-regulating *TOP2A* increases resistance (9–11). However, notwithstanding its induction of *TOP2A*, DLX4 inhibited levels of TOP2 poison-induced DSBs and cell death. These apparently opposing activities of DLX4 might provide a molecular explanation as to why some tumors have elevated levels of *TOP2A* expression but respond poorly to TOP2 poisons.

The importance of NHEJ in repairing TOP2 poison-induced DNA damage has been supported by several studies. NHEJ-deficient cells are highly sensitive to TOP2 poisons (3,6) and inhibition of DNA-PK activity increases sensitivity to TOP2 poisons (5). Our studies demonstrated that DLX4 does not stimulate HR-mediated DSB repair, but interacts with Ku proteins and stimulates end-joining repair of DSBs in a Ku-dependent manner. DLX4 did not stimulate end-joining in the absence of its Ku-interacting domain. Whereas levels of end-joining activity and TOP2 poison-induced DSBs were not affected by DLX4 in Ku-deficient cells, DLX4 stimulated DSB repair and reduced the level of TOP2 poison-induced DSBs when Ku was reconstituted in these cells. These results support a model in which DLX4 inhibits accumulation of TOP2 poison-induced DSBs by stimulating the canonical NHEJ pathway but not alternate Ku-independent end-joining pathways (2). DNA-PKcs is activated when recruited to DNA-bound Ku complexes (1). Because DLX4 neither stimulated expression of NHEJ components, Ku70-Ku80 dimerization, the binding of Ku proteins to DNA nor the interaction of DNA-PKcs with Ku-DNA complexes, it is as yet unclear how DLX4 stimulates DNA-PK activity. Because DLX4 can bind Ku proteins in the absence of DNA, it is possible that this binding induces conformational changes in Ku-DNA complexes that enhance DNA-PK activity. This suggests that DLX4 stimulates DNA repair in a non-transcriptional capacity. There is evidence that some homeoproteins have non-transcriptional functions (39,40). However, the possibility that DLX4 might also regulate transcription of genes that control DNA-PK activity cannot at this present time be excluded.

Several other homeoproteins, such as CDX2 and HOXB7, have been reported to interact with Ku proteins (41,42). Interestingly, CDX2 inhibits DNA repair in intestinal cells (41). HOXB7 has been reported to stimulate DNA repair (42), but it is not known whether HOXB7 promotes correct or erroneous repair. An intriguing finding of our study is that DLX4 increases both the frequency and magnitude of erroneous end-joining. NHEJ depends on DNA ends being held in alignment and in proximity in order for end-processing and ligation to occur (1). Ku proteins not only serve as ‘scaffolding’ proteins to bring DNA ends together but also have lyase activity (43). DLX4 did not block ligation but caused larger deletions at breakpoint junctions. The interaction of DLX4 with Ku proteins might alter alignment of DNA ends and also end-processing. On the other hand, it is possible that binding of CDX2 to Ku proteins sterically blocks alignment or processing of DNA ends so that ligation does not occur. The opposite effects of DLX4 and CDX2 on end-joining might be due to different conformational changes in Ku-DNA complexes.

An implication of our finding that DLX4 increases end-joining infidelity is that DLX4 overexpression in tumor cells might promote genomic instability. The role of NHEJ in maintaining genomic stability is unclear. Deficiency in NHEJ components has been reported to increase chromosomal aberrations (44,45), but NHEJ has also been found to promote genomic rearrangements (46). We observed large deletions in end-joining assays using control TOV112D cells (Fig. 3F). This suggests that TOV112D cells have constitutively

overactive NHEJ and is consistent with findings that this cell line has numerous chromosomal aberrations (47). Several studies indicate that error-prone repair is increased in tumors. Leukemic cells exhibit higher NHEJ activity and misrepair frequency than normal hematopoietic cells (48,49). CDX2 inhibits end-joining in intestinal cells and is down-regulated in colon cancers (22,41). The susceptibility of *Cdx2*^{+/-} mice to intestinal cancer has been attributed in part to increased genomic instability (21). Conversely, DLX4 stimulates erroneous end-joining and is overexpressed in tumors. Because the precise functions of DLX4 in normal development are not known, it is not clear how its DNA repair activity is related to its developmental functions. One possibility is that the ability of DLX4 to stimulate repair of DSBs might have evolved as a protective mechanism against the potentially lethal effects of its induction of TOP2 α .

The *DLX4* and *TOP2A* genes are both located on 17q21-22 region, a region that is amplified in ~12% of breast and ovarian cancers (12,13). However, DLX4 levels are elevated in >50% of breast and ovarian cancers (26,27), indicating that DLX4 overexpression is not solely due to gene amplification. TOP2 α levels in breast cancers also do not correlate with *TOP2A* gene copy number (50). The frequency of co-amplification of *DLX4* and *TOP2A* is not known, but co-amplification might account for elevated expression of both genes in a subset of tumors. Our findings that DLX4 induces TOP2 α levels could explain at least in part why TOP2 α levels are elevated in tumors in which the *TOP2A* gene is not amplified. Deletion of *TOP2A* also occurs in 6–14% of breast cancers (13,14,16), and could explain why a subset of tumors that highly express *DLX4* have low *TOP2A* mRNA levels. TOP2 α levels are regulated by other mechanisms in addition to transcription (17). Further investigation of TOP2 α protein levels in tumors is therefore needed. Based on our findings, evaluation of DLX4 levels in tumors that have elevated TOP2 α protein levels but respond poorly to TOP2 poisons warrants further investigation.

In summary, our study identifies two opposing activities of DLX4 in inducing *TOP2A* expression and stimulating repair of DSBs that could explain why some *TOP2A*-overexpressing tumors respond poorly to TOP2 poisons. Our study also supports increasing evidence that homeoproteins confer survival advantages to tumor cells by diverse mechanisms. Further studies of DLX4 and other homeoproteins that control DNA repair and cell survival could provide important insights into predicting responsiveness of tumor cells to chemotherapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	antibody
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	DNA double-strand break
HR	homologous recombination
IP	immunoprecipitation
NHEJ	non-homologous end-joining
TOP2	topoisomerase II
TOP2α	topoisomerase II α

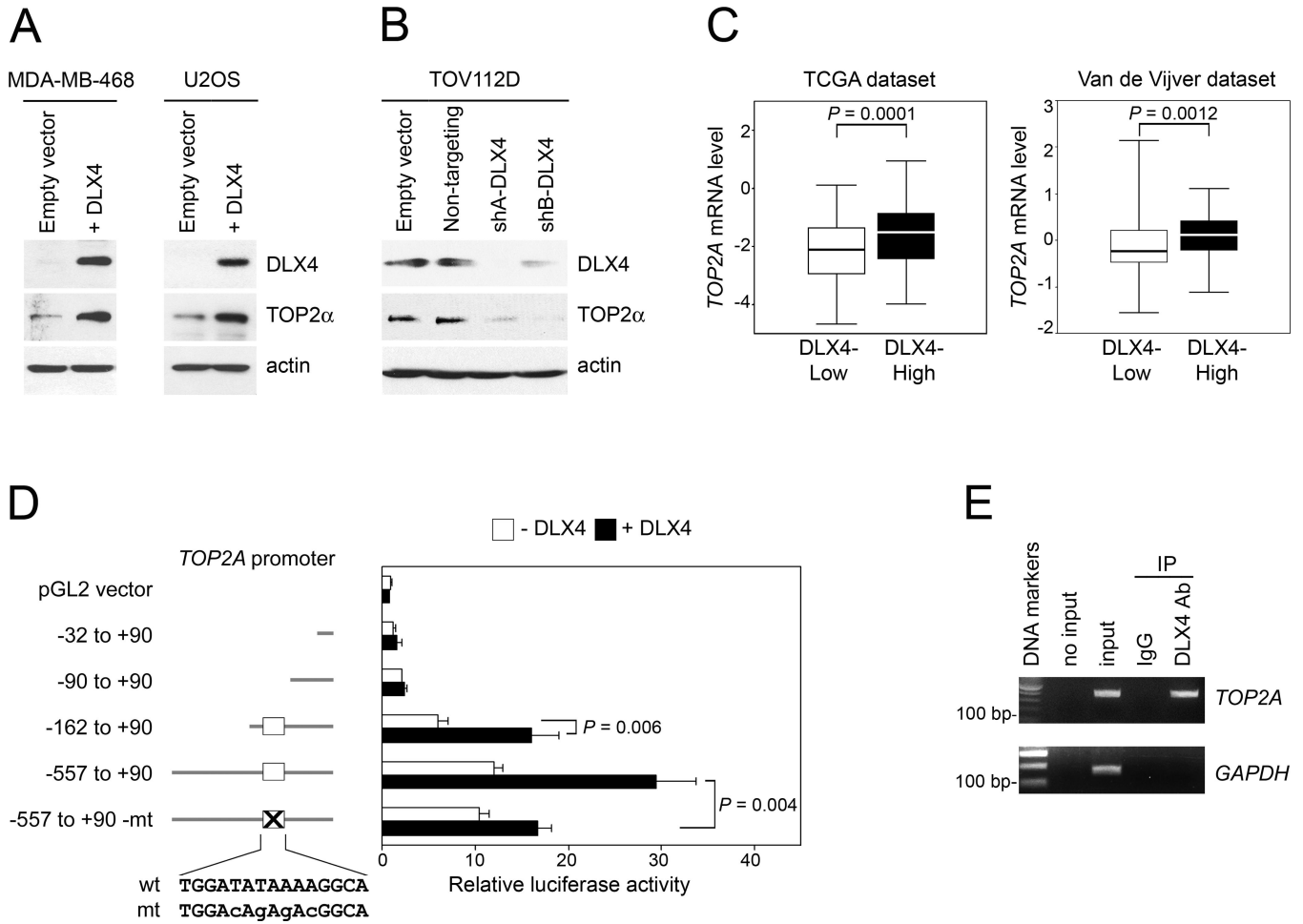


Fig. 1. DLX4 induces TOP2A promoter activity and TOP2α levels

[A,B] Western blot of DLX4 and TOP2α levels in [A] vector-control and *DLX4*-overexpressing MDA-MB-468 and U2OS cell lines, and [B] TOV112D cells transfected with empty vector, non-targeting shRNA and shRNAs targeting two different sites of *DLX4* (shA-DLX4, shB-DLX4). [C] Breast cancer cases from the TCGA Project (n=537) and study of Van de Vijver *et al* (32) (n=295) were stratified according to *DLX4* expression in tumors, where *DLX4* transcript levels in each dataset were defined as High (> upper quartile) and Low (< lower quartile). Significance of differences in *TOP2A* transcript levels (log2 scale) between upper and lower quartile sub-groups was evaluated by Mann-Whitney *U*-test. [D] U2OS cells that lacked DLX4 (white bar) or expressed DLX4 (black bar) were transfected with pGL2 luciferase reporter plasmids containing the indicated regions of the *TOP2A* promoter. Wild-type (capitals) and mutated (small case) sequences of the DLX4-binding site (white box) are indicated. Shown are average relative luciferase activities of 3 independent experiments. [E] Detection of binding of endogenous DLX4 to the *TOP2A* promoter in TOV112D cells by chromatin IP. Input corresponds to 1% of the chromatin solution before IP.

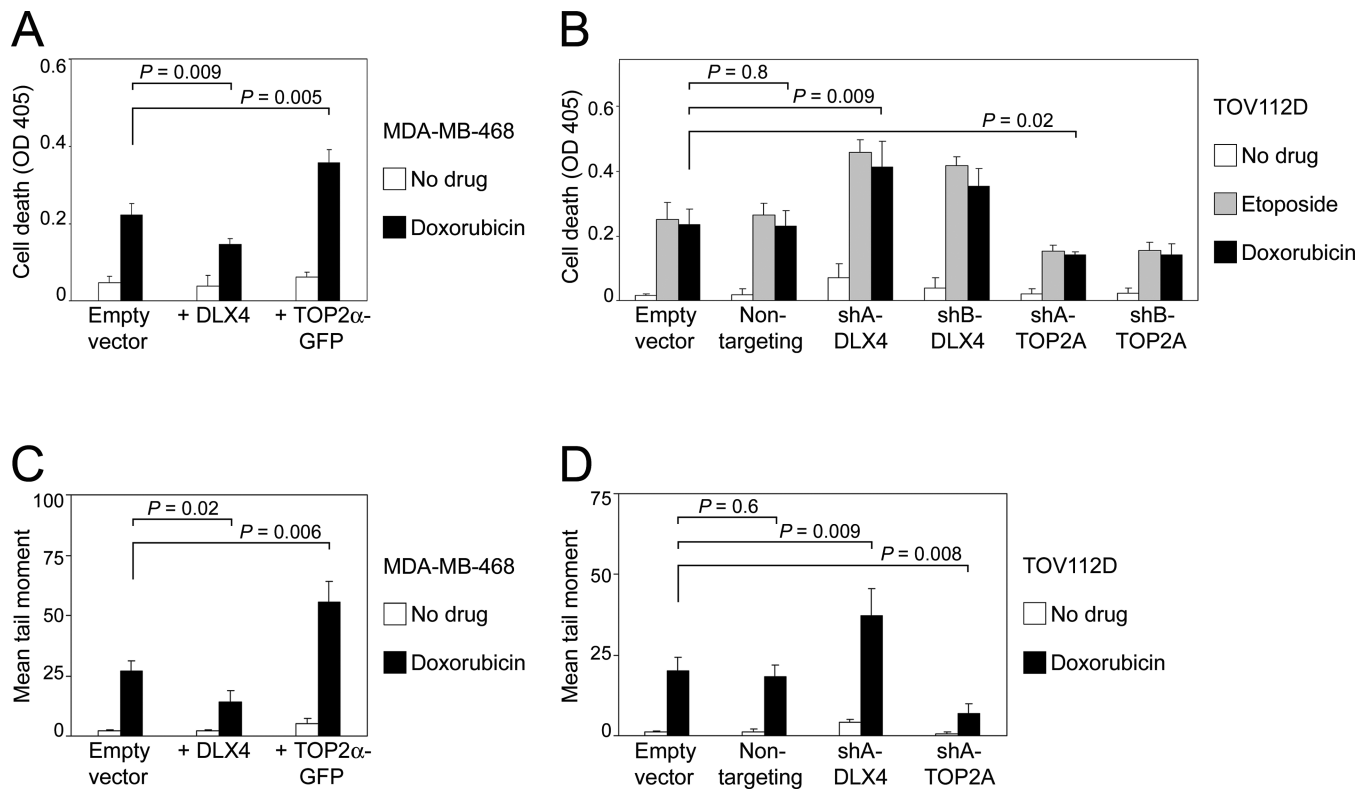


Fig. 2. DLX4 reduces TOP2 poison-induced DSBs and cell death

[A,B] Cell death was assayed by ELISA in tumor cells at 2 d after treatment with TOP2 poisons. Shown are average results of 3 independent experiments. [A] Vector-control, +DLX4 and +TOP2 α MDA-MB-468 cells were incubated without and with doxorubicin (100 nM). [B] TOV112D cells were transfected with empty vector, non-targeting shRNA, shRNAs targeting *DLX4* (shA-DLX4, shB-DLX4) and *TOP2A* (shA-TOP2A, shB-TOP2A). At 1 d thereafter, doxorubicin (200 nM) or etoposide (500 nM) was added to cells. [C,D] DSBs were assayed by neutral comet assay in [C] MDA-MB-468 and [D] TOV112D cells at 1 d after doxorubicin treatment. Shown are mean tail moments of 3 independent assays where 50 randomly selected cells were scored in each assay.

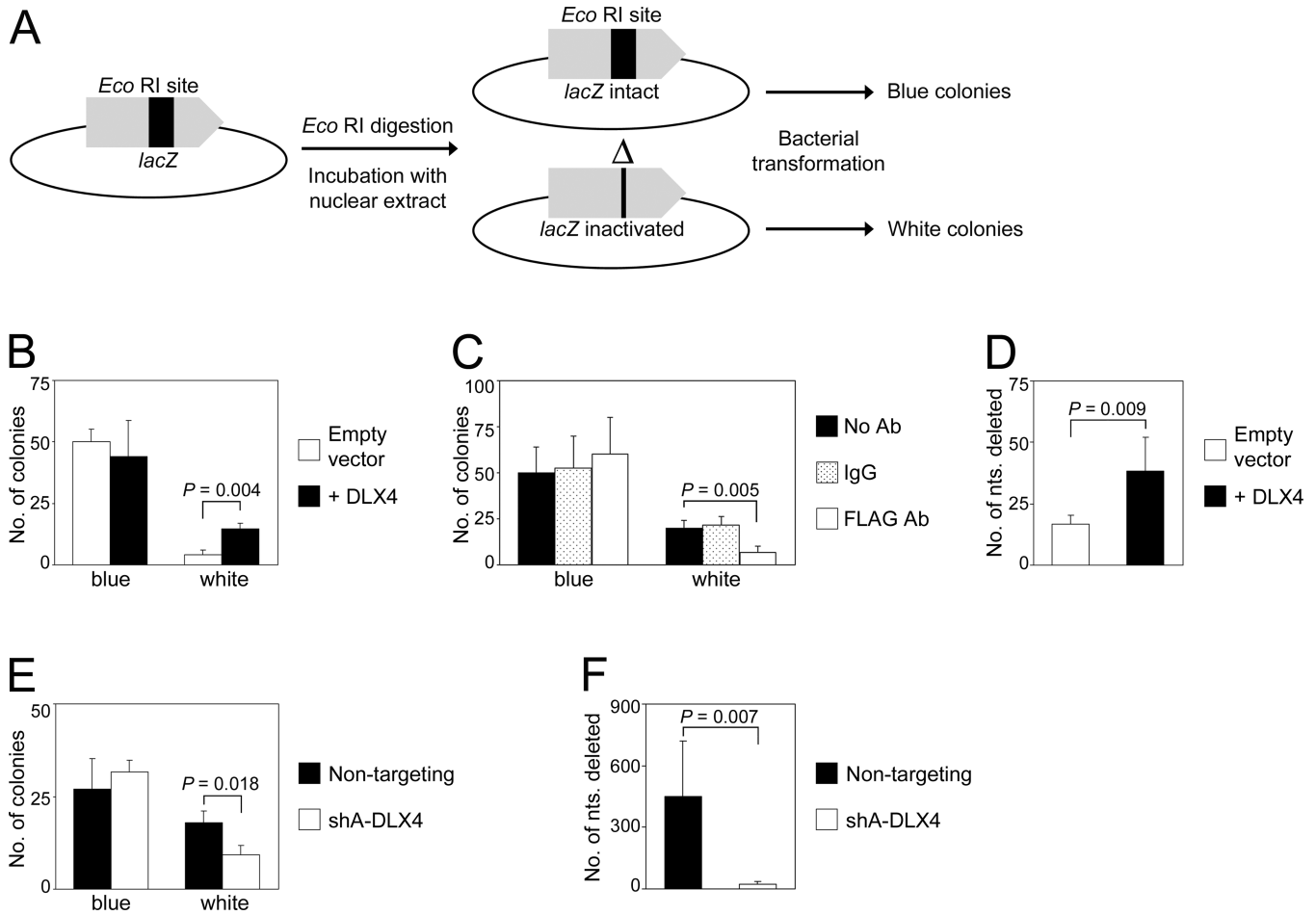


Fig. 3. DLX4 stimulates erroneous end-joining repair of DSBs

[A] Schematic diagram of the end-joining assay. Repair of the *Eco* RI-cut *lacZ* gene of pUC19 DNA incubated in nuclear extracts was assayed by formation of blue colonies (correct repair) and white colonies (misrepair) following transformation of *E. coli* and plating on plates containing X-gal and IPTG. **[B]** Colony formation in end-joining assays using nuclear extracts of vector-control U2OS cells and U2OS cells that expressed FLAG-tagged DLX4 (+DLX4). **[C]** Colony formation in end-joining assays using nuclear extracts of U2OS cells that expressed FLAG-tagged DLX4, where extracts were depleted using FLAG Ab, IgG or left untreated. **[D]** Numbers of nucleotides surrounding the *Eco* RI-cut DSB that were deleted in pUC19 DNA isolated from individual white colonies of end-joining assays using U2OS extracts. **[E]** Colony formation in end-joining assays using nuclear extracts of TOV112D cells transfected with non-targeting and *DLX4* shRNAs. **[F]** Numbers of deleted nucleotides in pUC19 DNA isolated from white colonies of end-joining assays using TOV112D extracts. Shown are average results of 3 independent experiments.

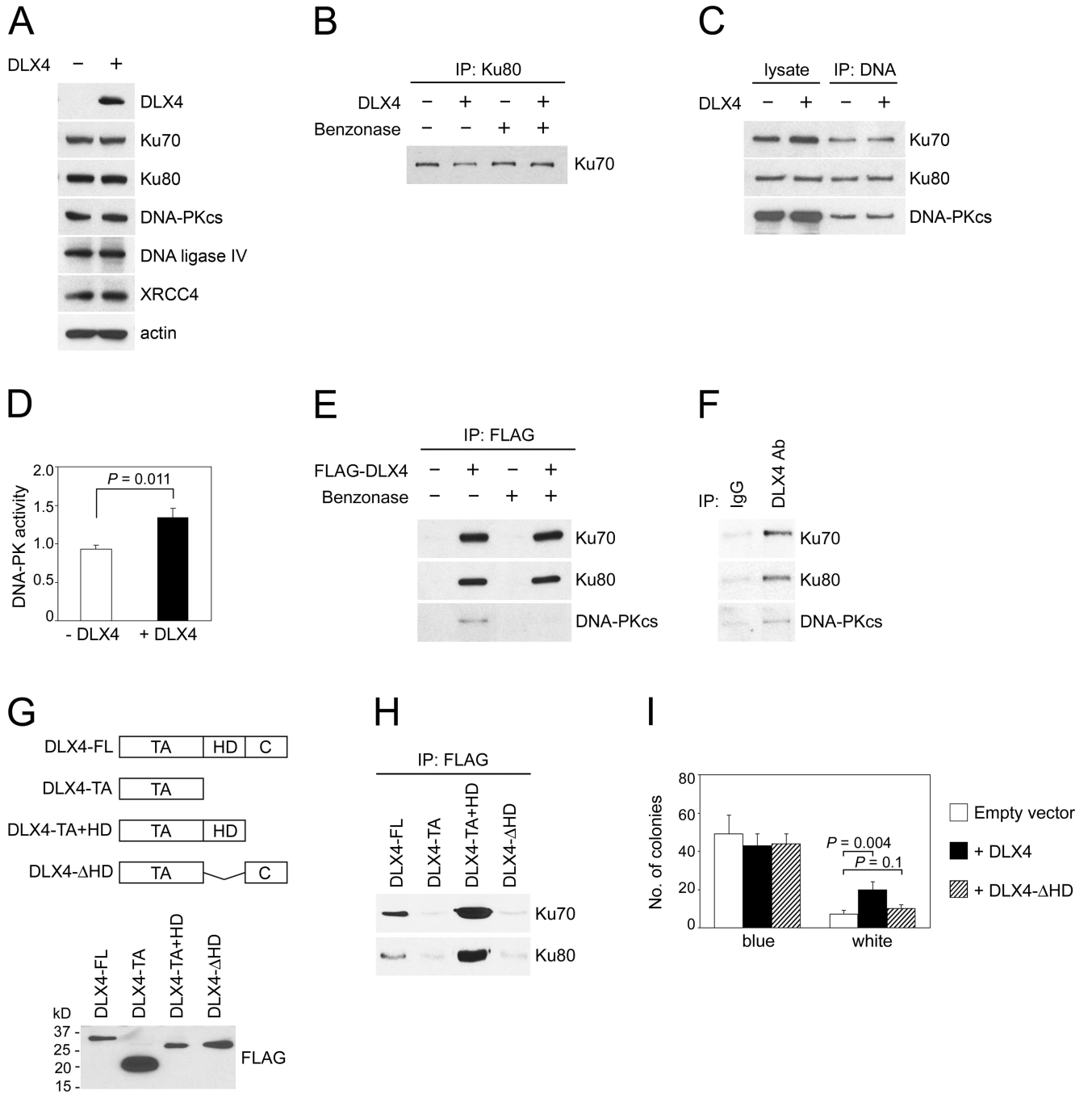


Fig. 4. DLX4 interacts with the NHEJ machinery and stimulates DNA-PK activity
[A] Western blot of levels of canonical NHEJ components in vector-control (-DLX4) and +DLX4 U2OS cells. **[B]** Interaction of Ku80 with Ku70 was assayed by IP in extracts treated with benzonase to remove DNA or left untreated. **[C]** Interactions of Ku proteins and DNA-PKcs with DNA ends were assayed by incubating biotinylated oligomer duplexes with U2OS nuclear extracts. Following DNA pull-down, DNA-associated proteins were analyzed by immuno-blotting. **[D]** DNA-PK activity in U2OS nuclear extracts (expressed as pmol ATP/minute/μg of protein). **[E]** Interactions of FLAG-tagged DLX4 with Ku70, Ku80 and DNA-PKcs were assayed in U2OS whole cell extracts (treated with benzonase or left

untreated) by IP using FLAG Ab and immunoblotting using Abs to NHEJ components. **[F]** Interactions of endogenous DLX4 with Ku proteins and DNA-PKcs were assayed by IP in TOV112D extracts (not treated with benzonase). **[G]** Western blot of FLAG-tagged proteins containing full-length DLX4 (FL), transactivation domain (TA), homeodomain (HD) and C-terminal tail (C) expressed in U2OS cells. **[H]** Interactions of FLAG-tagged DLX4 proteins with Ku proteins were detected by IP as in [E]. **[I]** Colony formation in end-joining assays using extracts of U2OS cells that were transfected with empty vector, full-length DLX4 and a truncated form lacking the Ku-interacting domain (DLX4- Δ HHD). Shown are average results of 3 independent experiments.

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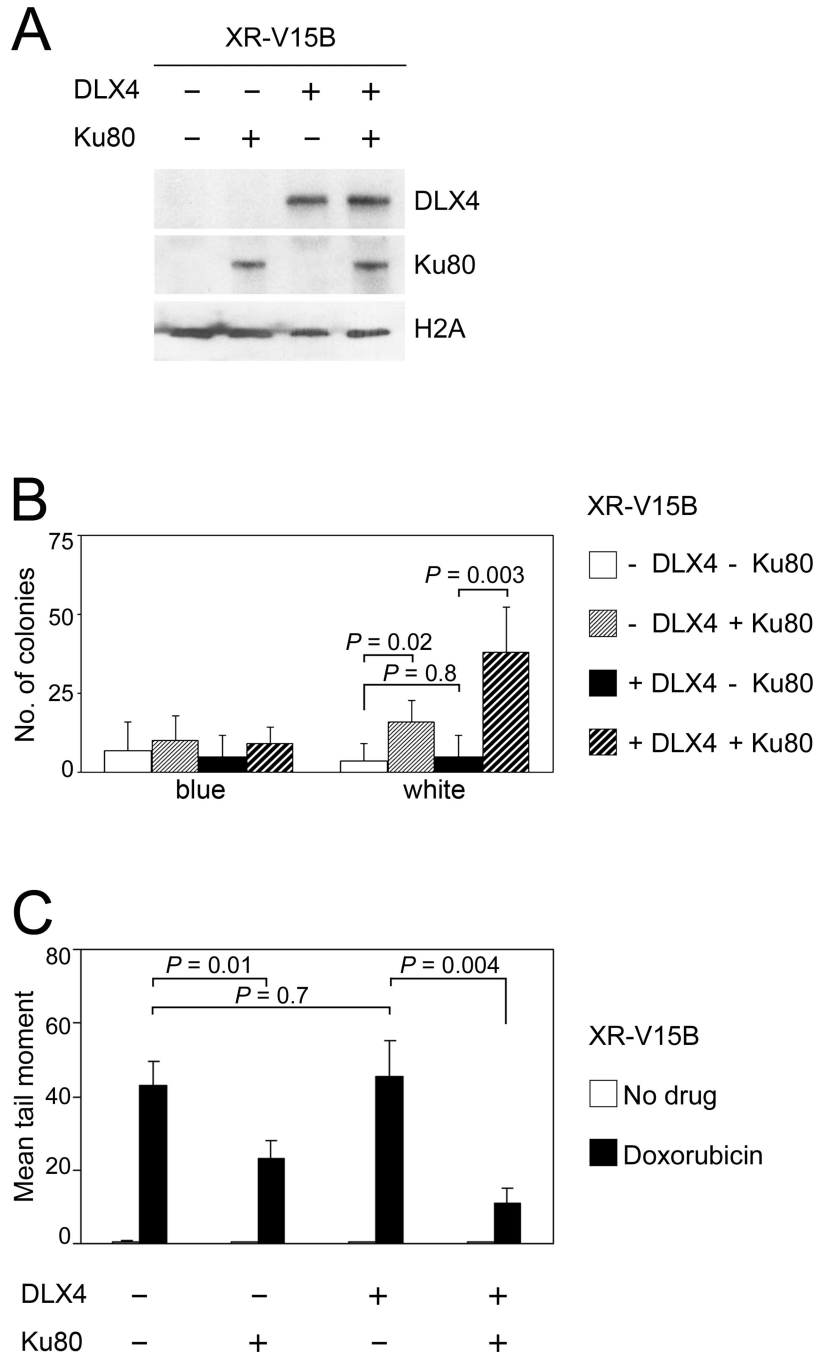


Fig. 5. DLX4 reduces levels of TOP2 poison-induced DSBs by stimulating Ku-dependent NHEJ activity

Cells of the Ku80-deficient cell line XR-V15B were transfected with Ku80 and/or DLX4. **[A]** Western blot of DLX4 and Ku80 levels in XR-V15B nuclear extracts. **[B]** Colony formation in end-joining assays using XR-V15B nuclear extracts. Shown are average results of 3 independent experiments. **[C]** DSBs were assayed in XR-V15B cells by neutral comet assays at 1 d after doxorubicin treatment (500 nM). Shown are mean tail moments of 3 independent assays where 50 randomly selected cells were scored in each assay.

Table 1

Response rates of breast cancer patients grouped by *TOP2A* and *DLX4* expression levels¹

	GSE25055	GSE22093	GSE20194	GSE4779	Combined	P-value ²
<i>TOP2A</i> -Low	4/76 (5.3%)	2/24 (8.3%)	5/69 (7.2%)	7/25 (28.0%)	18/194 (9.3%)	0.00001
<i>TOP2A</i> -High	26/76 (34.2%)	11/24 (45.8%)	19/69 (27.5%)	10/25 (40.0%)	66/194 (34.0%)	
<i>DLX4</i> -Low	15/76 (19.7%)	7/24 (29.2%)	17/69 (24.6%)	9/25 (36.0%)	48/194 (24.7%)	0.18
<i>DLX4</i> -High	13/76 (17.1%)	2/24 (8.3%)	12/69 (17.4%)	9/25 (36.0%)	36/194 (18.6%)	
<i>TOP2A</i> -High + <i>DLX4</i> -Low	6/13 (46.2%)	2/5 (40.0%)	6/13 (46.2%)	4/9 (44.4%)	18/40 (45.0%)	0.043
<i>TOP2A</i> -High + <i>DLX4</i> -High	5/22 (22.7%)	2/9 (22.2%)	3/13 (23.1%)	2/7 (28.6%)	12/51 (23.5%)	

¹Breast cancer cases from four independent datasets (refs. 34–37) were classified by levels of *TOP2A* and *DLX4* transcripts in tumors. Transcript levels of each gene were defined as High (> upper quartile) and Low (< lower quartile) in each cohort. Shown are numbers and proportions of patients, within a given group defined by transcript levels of *TOP2A* and/or *DLX4*, who had a pathologic complete response following anthracycline-based chemotherapy. Details of datasets and analysis are described in Supplementary Methods.

²Differences in response rates between the indicated groups of cases from the four cohorts combined were evaluated by two-tailed Fisher exact test.