NQO1 prevents radiation-induced aneuploidy by interacting with Aurora-A

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Aneuploidy is the most common characteristic of human cancer cells. It also causes genomic instability, which is involved in the initiation of cancer development. Various lines of evidence indicate that nicotinamide adenine dinucleotide(P)H quinone oxidoreductase 1 (NQO1) plays an important role in cancer prevention, but the molecular mechanisms underlying this effect have not yet been fully elucidated. Here, we report that ionizing radiation (IR) induces substantial aneuploidy and centrosome amplification in NQO1-deficient cancer cells, suggesting that NQO1 plays a crucial role in preventing aneuploidy. NQO1 deficiency markedly increased the protein stability of Aurora-A in irradiated cancer cells. Small interfering RNA targeting Aurora-A effectively attenuated IR-induced centrosome amplification concerned with aneuploidy in NQO1-deficient cancer cells. Furthermore, we found that NQO1 specifically binds to Aurora-A via competing with the microtubule-binding protein, TPX2 (targeting protein for Xklp2), and contributes to the degradation of Aurora-A. Our results collectively demonstrate that NQO1 plays a key role in suppressing IR-induced centrosome amplification and aneuploidy through a direct interaction with Aurora-A.

Introduction

Aneuploidy is closely associated with aberrant chromosome numbers, abnormal chromosome structures and genomic instability, which is a hallmark of most human cancers and a known contributor to carcinogenesis (1). However, how aneuploidy leads to genomic instability has not been well understood. For normal mitotic progression, the entire genome must be duplicated and one copy of each chromosome must be distributed to each daughter cell (2). Aneuploidy can result from defective chromosome segregation during mitosis (1,3). To prevent this, cells generally activate the mitotic checkpoint (also known as the spindle assembly checkpoint) during mitosis (3). When the mitotic checkpoint is activated, the separation of duplicated chromosomes is delayed until each chromosome has been correctly attached to the mitotic spindle; this supports accurate chromosome segregation leading to genetically identical progeny (3,4). Therefore, defects in the mitotic checkpoint lead to the development of aneuploidy. Aneuploidy can also occur via other routes, including premature loss of sister chromatid cohesion, hyperstabilized kinetochore–microtubule interactions and centrosome amplification (5). Ionizing radiation (IR) has been known to cause high frequency of genomic instability due to aneuploidy (6,7).

In particular, centrosome amplification is known to contribute to the generation of aneuploidy via the formation of multiple mitotic spindle poles during mitosis (8). As a major cellular organelle, the centrosome plays a fundamental role in regulating the migration of chromosomes to the daughter cells and organizing the mitotic spindle during mitosis (2). Normally, cells in the G2 phase of the cell cycle have a single centrosome containing a pair of orthogonally placed centrioles that duplicates synchronously as cells progress to S phase, thereby forming two new centrosomes. The number of centrosomes must be rigidly regulated in a cell to ensure accurate chromosome segregation during mitosis (9). Inhibition of centrosome duplication induces the formation of incomplete mitotic spindles that lack cell polarity, whereas centrosome amplification can cause the formation of aberrant mitotic spindles and the subsequent migration of chromosomes to numerous poles (10). Many human cancers are known to have abnormal numbers of centrosomes, leading to genomic instability (11–13). Centrosome amplification can be induced by dysfunctions of the DNA repair system, checkpoint proteins and telomerase, as well as through the effects of viral onecogens (14–16). IR and a variety of DNA-damaging agents are also known to induce centrosome amplification in both normal and cancerous cell lines (17–20). Although the underlying mechanism through which IR induces centrosome amplification has not yet been fully elucidated, some reports indicate that a Chk1-dependent process is involved in IR-induced centrosome amplification (18,21).

The activation of Aurora-A has been implicated in the entry and progression of cells through mitosis, which is a strictly regulated dynamic process (22). Aurora-A plays a key role in inducing centrosome maturation, duplication and cell cycle movement (22). The absence of Aurora-A has been shown to cause mitotic spindle assembly defects and misalignment of chromosomes, which can lead to embryonic lethality (23), whereas the upregulation of Aurora-A promotes centrosome amplification, aneuploidy and carcinogenesis (24). Because the overexpression of Aurora-A transforms normal cells and induces tumors in nude mice, it is considered to be an oncogene (24,25). Consistent with this, Aurora-A has been shown to be overexpressed in many types of cancer (26).

Nicotinamide adenine dinucleotide(P)H quinone oxidoreductase 1 (NQO1, DT-diaphorase) protects cells from various cytotoxic quinines and oxidative stress by catalyzing the reduction and detoxification of quinone substrates (27). It has also been shown to protect animals from diverse chemical carcinogens (28,29) and stabilizes the tumor suppressor p53 by preventing its proteosomal degradation (30). However, although NQO1 may be considered as a cancer preventive enzyme, its precise role in cancer prevention has not yet been clarified.

In this study, we investigated the relationship between NQO1 and IR-induced aneuploidy. We show for the first time that NQO1 significantly contributes to suppressing aneuploidy and centrosome amplification in irradiated human cancer cells and negatively regulates the protein stability of Aurora-A. Furthermore, we present evidence that NQO1 specifically binds to Aurora-A by competing with TPX2 (targeting protein for Xklp2), a binding partner of Aurora-A, and subsequently participates in the degradation of Aurora-A. These findings indicate a novel role for NQO1 in preventing centrosome amplification via the negative regulation of Aurora-A in irradiated human cancer cells.

Materials and methods

Reagents

Antibodies against γ-tubulin, cyclin E, cyclin B1 and His6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Aurora-A, BubR1 and Mad2 were purchased from BD Science (Franklin Lakes, NJ). An anti-phospho-Aurora-A (Thr288) antibody was purchased from Cell Signalling Technology (Beverly, MA). Antibodies against NQO1 and ubiquitin were purchased from Invitrogen (Carlsbad, CA). Antibodies targeting TPX2 and securin were purchased from Novus Biologicals (Littleton, CO). The antibody against pericentrin was purchased from Abcam (Cambridge, MA). The antibody against phospho-histone H3 was purchased from Millipore.
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Corporation (Billericia, MA). Anti-β-actin, anti-rabbit IgG and anti-mouse IgG were purchased from Sigma–Aldrich Co. (St Louis, MO). Nocodazole, thymidine, MG132 and cycloheximide were purchased from Sigma–Aldrich Co. 4′,6-diamidino-2-phenylindole was purchased from Molecular Probes (Eugene, OR).

Cell culture

Parental NQO1−/−MDA-MB-231 human breast cancer cells (NQO1-deficient) and NQO1+/−MDA-MB-231 human breast cancer cells (stably transfected with an expression vector for NQO1) were obtained from Dr David Boothman (University of Texas Southwestern Medical Center, Dallas, TX). Parental RKO human colorectal cancer cells carrying wild-type p53 were also used. The MDA-MB-231 and RKO cells were cultured, respectively, in RPMI 1640 medium and Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) bovine calf serum (all from Gibco BRL, Grand Island, NY), penicillin (50 units/ml) and streptomycin (50 μg/ml), in a 37°C incubator under 95% air and 5% CO₂ (both vol/vol).

Establishment of inducible NQO1 short hairpin RNA-containing cell lines

The appropriate double-stranded oligonucleotide (5′-TCGAGGCGATGATCAGATATCCTGTTGATCAAGAGATCAAGGATCTACGGTTTT TTTACCGGTA-3′ and its complement) was cloned into the pSIN32-LTR-shRNA vector (Clontech Laboratories, Mountain View, CA). RKO cells were transfected with the plasmid or control empty vector, using Lipofectamine 2000 (Gibco Invitrogen Corporation, Carlsbad, CA) in accordance with the manufacturer’s recommendations. Inducible NQO1 shRNA-containing stable clones were selected using 1 mg/ml of G418 for 7 days. Stable clones were isolated and treated with 1 μg/ml of doxycycline (a tetracycline analog; Sigma–Aldrich Co.) for 24 h, and endogenous NQO1 knockdown was determined by western blot analysis using an anti-NQO1 antibody.

Small interfering RNA transfection

RNA interference-mediated small interfering RNAs (siRNAs) was achieved using double-stranded RNA molecules. The siRNA against Aurora-A (5′-AA CCUCUUCGAUAACAGCAG-3′) was purchased from Bioneer Corporation (Daejeon, Korea). Stealth™ RNAi (Gibco Invitrogen Corporation) was used as a control. Cells were grown to 30% confluency on 60 mm dishes and transfected with the siRNA duplexes (100 nM), using Lipofectamine 2000 (Gibco Invitrogen Corporation) in accordance with the manufacturer’s instructions. Assays were performed 24 h after transfection.

Plasmids

To generate pCDNA3.1-myc-his6-NQO1, the complementary DNA of NQO1 was obtained from RKO cells using reverse transcription–PCR, annealed and ligated into KpnI- and XbaI-digested vector (pCDNA3.1- myc-his6). The primer sequences used were as follows: 5′-GGGGTACCATGGTGGCGGACGAC GAGCACA3′ (forward) and 5′-CCGTCGATTCCTTCA GCTGGTATGCTGG TTGTC3′ (reverse).

Irradiation

Cells were exposed to γ-rays with a 137Cs irradiation source (Model 68; L.J. Shepherd and Associates, Glenwood, CA) at a dose rate of 200–300 cGy/min.

Synchronization and cell cycle analysis

Cells were synchronized at the G1/S boundary with the use of the double-thymidine block method. Cells were first treated with 2 mM of thymidine for 12 h, incubated with fresh regular medium without thymidine for 12 h and then re-treated with thymidine for another 12 h. The cells were finally released from the arrest by replacing the thymidine solution with fresh regular medium. The cells were irradiated with 10 Gy after finally replacing the thymidine solution with fresh regular medium. For mitotic synchronization, cells were treated with 100 nM of nocodazole for 22 h and then released from the blocking by incubating with fresh regular medium. The cells were harvested at various times after they were released from the synchronization, fixed in cold 70% (vol/vol) ethanol, washed with cold phosphate-buffered saline (PBS) and then stained with 40 μg/ml propidium iodide in the presence of 50 μg/ml ribonucleoside A for 30 min at room temperature. Cellular DNA (10,000 cells per sample) was analyzed by flow cytometry (Becton Dickinson, Mountain View, CA).

Western blotting and immunoprecipitation

Cells treated with lysis buffer [40 mM Tris–HCl (pH 8.0), 120 mM NaCl and 0.1% (vol/vol) NP40] were supplemented with protease inhibitors and centrifuged for 15 min at 12,000g. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% (wt/vol) non-fat dry milk in Tris-buffered saline and then incubated for 1 h with primary antibodies at room temperature. Specific reaction bands were detected using peroxidase-conjugated secondary antibodies, and proteins were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Immunoprecipitation was performed by incubating 200 μg of lysate with antibodies for 90 min and then adding 25 μl of protein G magnetic beads (New England Biolabs, Ipswich, MA). After 90 min of incubation at 4°C, the immune complexes were recovered by centrifugation, washed twice with lysis buffer and resuspended in 30 μl of sodium dodecyl sulfate–loading buffer. The samples were then resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to western blot analysis.

Confocal microscopy

Cells grown on coverslips were washed twice in ice-cold PBS, fixed in ice-cold methanol, blocked with 2% (wt/vol) bovine serum albumin in PBS containing 0.2% (vol/vol) Triton X-100 and then incubated at room temperature for 1 h with primary antibodies against γ-tubulin, pericentrin, Aurora-A and NQO1. The cells were then washed three times with blocking solution and incubated for 1 h with a fluorescein isothiocyanate-conjugated secondary antibody (Molecular Probes). Nuclei were then stained with 4′,6-diamidino-2-phenylindole (Molecular Probes) for 10 min. After further washes with PBS, the coverslips were mounted onto microscope slides using the ProLong Antifade mounting reagent (Molecular Probes) and were viewed using a confocal laser scanning microscope (Nikon TE-2000E, Tokyo, Japan).

Statistical analysis

The optical densities of all western blots were quantified using the Image J software (NIH, Bethesda, MD) and normalized to β-actin. Comparisons among groups were analyzed using the Student’s t-test (SPSS version 17.0; SPSS Corporation, Chicago, IL). P values < 0.05 were considered significant.

Results

NQO1 is required to suppress IR-induced centrosome amplification

We investigated the role of NQO1 in IR-induced alternations of cell cycle progression in NQO1−/−MDA-MB-231 cells (deficient in NQO1) and NQO1+/−MDA-MB-231 cells (possessing abundant NQO1) irradiated with 10 Gy. As shown in Figure 1A, 10 Gy of irradiation triggered G1/M arrest in both NQO1−/− and NQO1+/−MDA-MB-231 cells at 24 h, but the degree of G1/M arrest in NQO1−/−MDA-MB-231 cells was greater than that in NQO1+/−MDA-MB-231 cells. The accumulation of cells with ≥4N DNA content (i.e. polyploid/aneuploid cells) in NQO1+/−MDA-MB-231 cells was markedly greater than that seen in NQO1−/−MDA-MB-231 cells at 48 h after irradiation, suggesting that NQO1 may prevent IR-induced aneuploidy (Figure 1A). This possibility was further studied using human colon cancer RKO cells expressing NQO1 shRNA. We synchronized cells at G1/S boundary using double-thymidine blocking, released the cells from the arrest and then promptly irradiated with 10 Gy. In sh-control cells, 10 Gy of irradiation induces only G1/M arrest. In shNQO1 cells, however, 10 Gy of irradiation triggered both G1/M arrest and an increase in the aneuploid cell population. These results indicate that NQO1 plays a key role in preventing IR-induced aneuploidy.

Because previous reports demonstrated that centrosome amplification is a major cause of aneuploidy (11–16), we assessed centrosomes in irradiated NQO1−/−MDA-MB-231 and NQO1+/−MDA-MB-231 cells by using anti-γ-tubulin and anti-pericentrin antibodies (two centrosomal markers) for confocal microscopy. As shown in Figure 1C, γ-tubulin and pericentrin localized to the centrosomes in both NQO1−/− and NQO1−/−MDA-MB-231 cells, and 10 Gy of irradiation increased the numbers of centrosomes in NQO1−/−MDA-MB-231 cells but not in NQO1+/−MDA-MB-231 cells (Figure 1C and D). Essentially, similar phenomena were observed in RKO cells expressing NQO1 shRNA. As shown in Figure 1E and F, shRNA-mediated NQO1 knockdown greatly increased the numbers of cells with ≥3 centrosomes following irradiation with 10 Gy. These results clearly indicate that NQO1 plays a key role in preventing IR-induced aneuploidy and centrosome amplification.

Aurora-A contributes to centrosome amplification and generation of aneuploidy in irradiated NQO1-deficient cancer cells

Because cyclin E has been implicated in the regulation of centrosome duplication during normal cell cycle progression (31) and its overexpression is closely related to centrosome amplification (32), we assessed the involvement of NQO1 in the expression of cyclin E in 10 Gy irradiated cells. We first used western blot analysis to...
measure the protein levels of cyclin E in the presence or absence of NQO1 following irradiation. As shown in Figure 2A, the expression of cyclin E increased at 1 h and remained elevated for 6 h after 10 Gy of irradiation in both NQO1−MDA-MB-231 and NQO1+MDA-MB-231 cells. Similar results were obtained in RKO cells (Figure 2A), suggesting that NQO1 does not affect the radiation-induced expression of cyclin E. Previously, Aurora-A was shown to control centriole duplication, centrosome maturation and cytokinesis (22,24), and overexpression of Aurora-A was shown to cause centrosome amplification and genomic instability (22,24). Therefore, we investigated whether NQO1 is involved in Aurora-A expression in irradiated MDA-MB-231 or RKO cells. About 10 Gy of irradiation increased both the activity and protein levels of Aurora-A markedly in NQO1−MDA-MB-231 cells and slightly in NQO1+MDA-MB-231 cells (Figure 2B). Similar results were obtained in RKO cells (Figure 2B). To further elucidate whether Aurora-A was associated with IR-induced centrosome amplification in NQO1-deficient human cancer cells, we pretreated NQO1-deficient human cancer cells with siRNAs against Aurora-A. As shown in Figure 3A and B, siRNA-mediated Aurora-A knockdown efficiently inhibited IR-induced centrosome amplification in NQO1−MDA-MB-231 cells. We also observed similar results in RKO cells expressing shNQO1 (Figure 3C and D).
Role of NQO1 in preventing aneuploidy

Fig. 1. NQO1 deficiency induces aneuploidy in irradiated cancer cells. (A) NQO1⁻ or NQO1⁺-MDA-MB-231 cells were irradiated with 10 Gy. After incubation for 0, 6, 12, 24 and 48 h, the cells were analyzed by flow cytometry for DNA content. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (B) Parental or shNQO1 RKO cells were synchronized at the G₁/S boundary by double-thymidine blocking, irradiated with 10 Gy, harvested at various length of time after they were released from the synchronization and then analyzed by flow cytometry for DNA content. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (C) Representative confocal images show centrosomes and nuclei at 48 h after 10 Gy of irradiation in NQO1⁻ or NQO1⁺-MDA-MB-231 cells. Centrosomes are demonstrated by overlaps of γ-tubulin (green) and pericentrin (red), resulting in a yellow color. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (D) A total of 200 cells were counted for each group at 24 and 48 h after irradiation in three independent experiments. The means ± SE are shown for NQO1⁻ or NQO1⁺-MDA-MB-231 cells with abnormal centrosomes in each group (*P < 0.05). (E) Representative confocal images show centrosomes and nuclei at 48 h after 10 Gy of irradiation in parental or shNQO1 RKO cells. Centrosomes are demonstrated by overlaps of γ-tubulin (green) and pericentrin (red), resulting in a yellow color. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (F) A total of 200 cells were counted for each group at 24 and 48 h after 10 Gy of irradiation in three independent experiments. The means ± SE are shown for parental or shNQO1 RKO cells with abnormal centrosomes in each group (*P < 0.05).
Fig. 2. NQO1 deficiency increases the protein expression of Aurora-A in irradiated cancer cells. (A and B) Cells were irradiated with 10 Gy. After incubation for the indicated times, cell lysates were subjected to western blot analysis using anti-cyclin E, anti-NQO1, anti-phospho-Aurora-A (Thr288), anti-Aurora-A and anti-β-actin antibodies. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment.
These results clearly indicate that Aurora-A plays key roles in IR-induced centrosome amplification and aneuploidy in NQO1-deficient human cancer cells.

**NQO1 deficiency leads to aneuploidy during mitotic progression**

To determine whether NQO1 affects mitotic cell cycle progression, we treated parental and NQO1-deficient RKO cells with thymidine or nocodazole to synchronize the cells at the G₁/S and G₂/M boundaries, respectively. Following release from thymidine- or nocodazole-induced cell cycle arrest, the cells were harvested at various times and their cell cycle distributions were analyzed by flow cytometry. As shown in Figure 4A, in NQO1-deficient RKO cells, aneuploidy initially increased after the cells were released from G₁/S arrest, but then began to decrease at 6h. When NQO1-deficient cells were released from G₂/M arrest, aneuploidy initially increased, but then began to decrease at 1h (Figure 4B). No significant increase in aneuploidy was observed in parental cells following their release from G₁/S or G₂/M arrest. Western blot analysis was used to assess the expression levels of various proteins involved in mitosis, as shown in Figure 4C. Although no significant change was seen in the protein levels of BubR1, cyclin B1 or Mad2 in both cell types following their release from G₂/M arrest, the levels of phospho-histone 3 (a mitotic marker protein) and Aurora-A were much higher in NQO1-deficient RKO cells than in parental cells, suggesting that NQO1 deficiency caused an aberrant mitotic delay (Figure 4C). The protein expression of securin slightly increased in both parental and NQO1-deficient RKO cells (Figure 4C). These results collectively indicate that NQO1 deficiency leads to aneuploidy via defective mitotic progression.
To determine whether NQO1 changes the protein stability of Aurora-A during mitotic progression, parental and NQO1-deficient RKO cells were arrested in prometaphase by nocodazole treatment, released and then treated with cycloheximide to block protein translation for various lengths of time. Thereafter, the levels of Aurora-A were assessed. As shown in Figure 4D and E, after release from G2/M arrest, Aurora-A was stabilized more extensively in NQO1-deficient RKO cells than in parental cells. These results indicate that Aurora-A is rapidly degraded during mitotic progression in the presence of NQO1 and this degradation is inhibited by NQO1 deficiency.

Ubiquitination is an essential modification for the 26 S proteasome-mediated recognition and degradation of cellular proteins. Prior to its cell cycle-dependent degradation, Aurora-A is poly-ubiquitinated by the anaphase-promoting complex/cyclosome (33).
To examine the mechanism underlying the increase in Aurora-A stability in NQO1-deficient cells, we determined whether NQO1 plays a role in the ubiquitination of Aurora-A during mitotic progression. Figure 4F shows that the poly-ubiquitination of Aurora-A was evident in parental RKO cells. However, in NQO1-deficient RKO cells released from G₂/M arrest, the poly-ubiquitination of Aurora-A was greatly abolished during mitotic progression, compared with that in parental cells (Figure 4F). These results collectively indicate that NQO1 may suppress the generation of aneuploidy by negatively regulating Aurora-A.

NQO1 competes with TPX2 for binding to Aurora-A

A recent report showed that TPX2 interacts with Aurora-A and plays a key role in protecting it from degradation, suggesting that TPX2 may be involved in regulating the stability of Aurora-A during mitosis (34). As NQO1 negatively regulates the protein stability of Aurora-A (Figure 4), we speculated that NQO1 may directly bind to Aurora-A to suppress its stability. Therefore, we treated parental and NQO1-deficient RKO cells with nocodazole to synchronize the cells at the G₂/M boundary, released the cells from the arrest, harvested them at different time points and used
coimmunoprecipitation assay to analyze the ability of TPX2 or NQO1 to bind Aurora-A. As shown in Figure 5A, the interaction between Aurora-A and TPX2 was greater and remained for 4h in NQO1-deficient cells than in parental RKO cells. Interestingly, we found that NQO1 interacted with Aurora-A during mitosis in parental cells (Figure 5A). We subsequently confirmed the interaction between Aurora-A and NQO1 in NQO1-expressing cell lines during mitosis, using confocal microscopy. Figure 5B clearly shows that NQO1 co-localized with Aurora-A in the mitotic spindle poles during prometaphase.

To better assess the formation of mitotic spindle poles and the subcellular distribution of TPX2 and Aurora-A during mitosis in the presence or absence of NQO1, we stained NQO1− or NQO1+ MDA-MB-231 cells in prometaphase with anti-TPX2 and anti-Aurora-A antibodies. In NQO1− MDA-MB-231 cells, we observed abnormal formation of spindle poles (where both TPX and Aurora-A intensely co-localized) and defective spindle pole separation (Figure 5C). However, in NQO1+ MDA-MB-231 cells, two mitotic spindle poles were normally formed and both TPX and Aurora-A were mainly co-localized in two clearly separated spindle poles (Figure 5C). These results indicate that NQO1 competes with TPX2 for binding to Aurora-A, which may contribute to the correct formation and separation of mitotic spindle poles.

To further confirm the interaction between NQO1 and Aurora-A, we transfected NQO1− MDA-MB-231 cells with increasing doses of a plasmid vector encoding His6-tagged NQO1 and performed coimmunoprecipitation assays using an anti-His6 antibody followed by western blot analysis using an anti-Aurora-A antibody. Our results indicated that the interaction between NQO1 and Aurora-A increased with increasing doses of plasmid expressing His6-tagged NQO1 (Figure 5D). Western blot analysis using an anti-His6 antibody after coimmunoprecipitation using an anti-Aurora-A antibody yielded similar results (Figure 5D). We also found that Aurora-A in lysates from RKO cells overexpressing NQO1 readily binds to the purified His6-tagged NQO1 proteins (Supplementary Data 1, available at Carcinogenesis Online). In contrast, as shown in Figure 5D, the interaction between Aurora-A and TPX2 effectively decreased with increasing doses of a plasmid expressing His6-tagged NQO1. These results collectively indicate that NQO1 can directly bind to Aurora-A and competes with TPX2 for this binding.
Role of NQO1 in preventing aneuploidy

NQO1 deficiency increases the interaction between TPX2 and Aurora-A in irradiated cancer cells

We next investigated whether 10 Gy of irradiation increases the interaction between NQO1 and Aurora-A in MDA-MB-231 and RKO cells. As shown in Figure 6A, NQO1 specifically interacted with Aurora-A in irradiated NQO1−/−MDA-MB-231 cells but not in irradiated NQO1+/−MDA-MB-231 cells, suggesting the binding specificity of NQO1 to Aurora-A. Furthermore, IR increased the interaction between TPX2 and Aurora-A markedly in NQO1+/−MDA-MB-231 cells but only slightly in NQO1−/−MDA-MB-231 cells, as shown by communoprecipitation experiments using anti-TPX2 and anti-Aurora-A antibodies (Figure 6A). NQO1 failed to interact with TPX2 in both irradiated and non-irradiated cells (Figure 6A). We further confirmed this effect using parental and NQO1-deficient RKO cells. As shown in Figure 6B, NQO1 deficiency effectively suppressed the interaction between NQO1 and Aurora-A but markedly increased the interaction between TPX2 and Aurora-A in irradiated RKO cells, suggesting that NQO1 deficiency contributes to high-affinity binding between TPX2 and Aurora-A. Moreover, irradiation did not affect the lack of binding between NQO1 and TPX2 (Figure 6B). These results collectively indicate that NQO1 deficiency greatly enhances the interaction between TPX2 and Aurora-A, which may be involved in IR-induced centrosome amplification and aneuploidy. Thus, NQO1 may protect against aneuploidy.

Fig. 4. NQO1 deficiency contributes to the generation of aneuploidy during mitotic progression. (A) Parental or shNQO1 RKO cells were synchronized at the G1/S boundary by double-thymidine blocking, harvested at various length of time after they were released from the synchronization and then analyzed by flow cytometry for DNA content. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (B) Parental or shNQO1 RKO cells were synchronized at the G1/M boundary by nocodazole, harvested at various length of time after they were released from the synchronization and then analyzed by flow cytometry for DNA content. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (C) Parental or shNQO1 RKO cells were synchronized at mitotic phase by nocodazole (100nM) and then harvested at various length of time after they were released from the synchronization. Cell lysates were subjected to western blot analysis using anti-phospho-histone H3, anti-Aurora-A, anti-BubR1, anti-cyclin B1, anti-Mad2, anti-securn, anti-NQO1 and anti-β-actin antibodies. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (D) Parental or shNQO1 RKO cells were synchronized at mitotic phase by nocodazole (100nM) and then harvested at various length of time after they were released from the synchronization in the presence of 10 μg/ml of cycloheximide. Cell lysates were subjected to western blot analysis using anti-Aurora-A and anti-β-actin antibodies. (E) The relative density value of each band is shown in the graph (*P < 0.05). Experiments were conducted in triplicate, and the data shown are representative of a typical experiment. (F) Parental or shNQO1 RKO cells were synchronized at mitotic phase by nocodazole (100nM) and then harvested at various length of time after they were released from the synchronization in the presence of 5 μM of MG132. Cell lysates were immunoprecipitated with an anti-ubiquitin antibody and then subjected to western blot analysis using an anti-Aurora-A antibody. Inputs 1 and 2 indicate the total extracted proteins from RKO (sh cont) and RKO (sh NQO1) cells harvested 24h after 10 Gy of irradiation, respectively. Experiments were conducted in triplicate, and the data shown are representative of a typical experiment.
Discussion

It has been reported that IR, a well-known carcinogen, causes aneuploidy and subsequent genomic instability (6,7,35), which may be intimately involved in radiation-induced cancer development (1). Despite several previous investigations (6,7,35), the cellular and molecular mechanisms underlying IR-induced aneuploidy are not yet well understood. NQO1 is a major enzyme for the bioreduction of toxic quinones in cells and plays a preventive role against chemical-induced carcinogenesis (27–30). Interestingly, NQO1 has also been demonstrated to enhance the effectiveness of certain anticancer drugs (e.g. β-lapachone and RH1), and polymorphisms in NQO1 have been associated with cancer risk (36–39). The aim of this study was to elucidate the potential role of NQO1 in IR-induced aneuploidy. We observed that NQO1 prevents IR-induced aneuploidy and centrosome amplification, and further found that NQO1 directly interacts with Aurora-A and decreases its protein stability, thereby preventing centrosome amplification and aneuploidy.

In this study, we observed that IR induces aneuploidy preferentially in NQO1-deficient cells versus NQO1-expressing cells, suggesting that NQO1 plays a preventive role against IR-induced aneuploidy (Figure 1A). Centrosome amplification is an important event in the generation of aneuploidy (8,10–16). MDA-MB-231 cells have been previously reported to exhibit centrosome amplification, microtubule nucleation and defects in cytokinesis (40). We found that irradiation increased centrosome amplification in parental NQO1−/−-MDA-MB-231 cells (Figure 1C–F). Several lines of evidence have demonstrated that NQO1 positively regulates the

[Diagram of Immunoprecipitation (IP) and Western Blotting (WB) showing interactions between TPX2, Aurora-A, and NQO1]
stability of p53, a well-known tumor suppressor protein, by interfering with its 20S proteasome-mediated degradation (30), and p53 mutation is thought to critically contribute to the generation of aneuploidy associated with abnormal amplification of centrosomes in tumors (32). Therefore, it is plausible that NQO1 suppresses aneuploidy by stabilizing p53 in irradiated cells. Because MDA-MB-231 cells carry a mutant form of p53 (41), we further elucidated the possible role of NQO1 in the suppression of aneuploidy using human colon cancer RKO cells, which express wild-type p53. Consistent with our observations in MDA-MB-231 cells, IR induced aneuploidy and centrosome amplification in NQO1-deficient RKO cells, but not in parental cells (Figure 1B, E and F), suggesting that NQO1 deficiency is responsible, at least in part, for IR-induced aneuploidy regardless of the cell’s p53 status. Notably, IR-induced aneuploidy in the NQO1-deficient RKO cells lasted for 6 h and then decreased (Figure 1B). Previous studies showed that tetraploid cancer cells die or undergo senescence via p53-dependent pathways in the G1 phase of the subsequent cell cycle (42–45). Because NQO1-deficient RKO cells also carry wild-type p53, we speculate that the aneuploid cells were eliminated through p53-dependent pathways upon re-entry to G1 phase.

As cyclin E and Aurora-A are known to increase the frequency of centrosome amplification (24,32), we assessed the effect of NQO1 on the expression of cyclin E and Aurora-A in irradiated MDA-MB-231 and RKO cells. In both cases, NQO1 deficiency significantly increased the protein expression and activity levels of Aurora-A, but
not the expression of cyclin E expression (Figure 2). Thus, Aurora-A, but not the cyclin E, may be critically important for IR-induced centrosome amplification in NQO1-deficient cells. The overexpression of Aurora-A has been implicated in overriding cell cycle checkpoints, inducing aneuploidy and promoting cell transformation (24–26). In particular, Aurora-A has been demonstrated to play a key role in centrosome amplification (24), as well as the maturation and migration of the centrosome to organize a functional mitotic spindle during cell cycle progression (22). Consistent with these reports, siRNA-mediated knockdown of Aurora-A effectively attenuated IR-induced centrosome amplification in NQO1-deficient cells (Figure 3). These observations strongly indicate that Aurora-A contributes to the generation of aneuploidy in irradiated NQO1-deficient cells.

Aurora-A is elevated at the G2/M transition to regulate centrosome separation and is thereafter decreased by anaphase-promoting complex/cyclosome-Cdh1-dependent proteasome-mediated proteolysis during normal mitotic progression (33). Several reports previously suggested that overexpression of Aurora-A can override the mitotic checkpoint, leading to aneuploidy (24–26). Consistent with these findings, we observed that NQO1 deficiency leads to aneuploidy during normal cell cycle progression and found that this aneuploidy was closely correlated with the increased stability and abundance of Aurora-A during mitotic progression (Figure 4). These results suggest that NQO1 is a negative regulator of Aurora-A. Similar to our observations in irradiated NQO1-deficient RKO cells (Figure 1B), aneuploidy subsequently decreased at 6 and 1 h, after release from thymidine (G2/S arrest) and nocodazole (G2/M arrest), respectively, in non-irradiated NQO1-deficient RKO cells (Figure 4A). As NQO1-deficient RKO cells carry wild-type p53, we speculate that the aneuploid cells were eliminated via p53-dependent pathways upon re-entry to G1 phase.

The protein stability and activity of Aurora-A are regulated by a number of factors (22,46). Among them, the microtubule-binding protein, TPX2 plays a conspicuous role in the regulation of Aurora-A (22,46). TPX2 directly binds to C-terminus of Aurora-A and triggers a conformational change of it that modifies the position of a key residue (Thr 288) responsible for the autophosphorylation of Aurora-A (46). This conformational change of it that modifies the position of a key residue (Thr 288) responsible for the autophosphorylation of Aurora-A (46). This conformational change renders Aurora-A inaccessible to protein phosphatase 1, which is known to activate Aurora-A (46). In addition to stabilizing Aurora-A, TPX2 also promotes its translocation to the mitotic spindle (46). Recently, however, it has been suggested that the TPX2-induced stabilization of Aurora-A is independent of the phosphorylation status of Aurora-A or the ability of TPX2 to target it to the mitotic spindle (34). Furthermore, TPX2 is reportedly required for centrosome integrity and contributes to the nucleation of microtubule arising from both centrosomes and chromosomes (47). Consistent with these reports, we found that the interaction of TPX2 with Aurora-A during normal mitotic progression was markedly stronger in NQO1-deficient cells than that in parental cells, suggesting that NQO1 deficiency contributes to the TPX2-mediated stabilization of the Aurora-A protein (Figure 5A). Interestingly, we observed that NQO1 specifically binds to Aurora-A in NQO1-expressing cells (Figure 5A) and co-localizes with Aurora-A in the mitotic spindle poles of NQO1-expressing mitotic cells (Figure 5B). Consistent with our results, a recent paper also showed that NQO1 localizes to mitotic spindles in human lung cancer cells (48). Furthermore, NQO1 deficiency disrupted normal spindle pole formation (Figure 5C). Taken together, these findings indicate that NQO1 participates in the maintenance of mitotic spindle poles during normal cell cycle progression. However, additional studies are warranted to further clarify the role of NQO1 in mitotic spindle poles. The addition of an NQO1-expressing plasmid dose-dependently increased the interaction of
Aurora-A with NQO1, whereas it substantially decreased the interaction of Aurora-A with TPX2 (Figure 5). Moreover, NQO1 deficiency markedly increased the binding of TPX2 to Aurora-A in irradiated MDA-MB-231 and RKO cells (Figure 6). These findings collectively suggest that NQO1 binds to Aurora-A in competition with TPX2. In light of previous reports that the N-terminus of TPX2 could directly interact with the C-terminal catalytic domain of Aurora-A (49), we speculate that the TPX2-binding motif of Aurora-A is the target site for NQO1. TPX2 is overexpressed in many types of cancer; this overexpression positively correlates with tumor grade and stage and negatively correlates with survival rate (47). These previous findings suggest that TPX2 has oncogenic potential. We may thus conclude that NQO1 plays a critical role in preventing aneuploidy and safeguarding normal mitotic progression by competing with TPX2. Future studies of the mechanisms underlying the reciprocal regulation of Aurora-A stability by NQO1 and TPX2 in irradiated cells may shed further light on the molecular mechanisms responsible for radiation-induced carcinogenesis.

In summary, we herein report for the first time that NQO1 prevents centrosome amplification and aneuploidy in irradiated cancer cells. Furthermore, we present evidence that NQO1 competes with TPX2 for binding to Aurora-A and inhibits excessive increase of Aurora-A, thereby suppressing the generation of aneuploidy in irradiated cells (Figure 6C).

**Supplementary material**

Supplementary Data 1 can be found at http://carcin.oxfordjournals.org/

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**References**

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