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Two fission yeast high mobility group box proteins in the maintenance of genomic integrity following doxorubicin insult

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ABSTRACT

Drug resistance is a challenge in chemotherapy, and, to date, there has been little resolution as to how it is induced. We previously isolated a host of doxorubicin resistance (DXR) genes in fission yeast and here we investigate the regulation of this resistance through two high mobility group (HMG) motif-containing DXR proteins, Nht1 and Hap2. The concurrent deletion of *nht1* and *hap2* did not confer cumulative sensitivity to doxorubicin, indicating that these factors cooperate closely in similar epistatic groups. We show that doxorubicin treatment resulted in the subcellular reorganization of Rhp54, a homologous recombination-dependent DNA damage repair protein. The disruption of either *nht1* or *hap2* attenuated Rhp54-foci formation, suggesting that these factors modulate the repair of doxorubicin-induced DNA lesions via the recruitment of homologous recombination machinery. Epistatic analyses further confirmed that Nht1 and Hap2 act in similar functional groups with complexes related to DSB repair but act synergistically with factors that regulate transcription and chromosome segregation. Overall, this work shows the molecular crosstalk coordinated by HMG proteins in conferring doxorubicin resistance in fission yeast.

1. Introduction

Doxorubicin is one of the most useful chemotherapeutic agents employed against leukemia, sarcoma, lymphoma and tumors of the breast, stomach, and gastrointestinal tract (Carvalho et al., 2009). It acts as a DNA intercalator to inhibit the re-ligation of transiently cleaved double stranded breaks (DSB) in DNA, which are created by topoisomerase II (TOPII); this results in overwhelming DSB accumulation and, consequently, apoptosis (Pommier et al., 2010; Tacar et al., 2013). Doxorubicin has also been linked to the disruption of mitochondrial oxidative phosphorylation and the generation of reactive oxygen species (Carvalho et al., 2009).

The clinical utility of doxorubicin is hampered by the rapid development of resistance in tumor cells, and the molecular mechanism(s) of such resistance remains unclear (Carvalho et al., 2009; Tacar et al., 2013). To shed light on this issue, we previously undertook a screen in *Schizosaccharomyces pombe* (fission yeast) to discover genes that confer resistance by preserving robust cell viability in the presence of doxorubicin (Tay et al., 2013). We found that many of these doxorubicin resistance (DXR) genes were integrated in a genetic network that regulates doxorubicin responsiveness and is, at least in part, conserved in human cells (Tay et al., 2014).

Among our list of DXR genes, we identified a prominent group of chromatin factors (Tay et al., 2013). Yet, it is largely unknown how these nuclear proteins regulate doxorubicin sensitivity in fission yeast. Intriguingly, a pair of these unknown factors—Nht1/Hap1 and Hap2—contain the highly conserved high mobility group (HMG) box motif. Previous work has shown that Nht1 co-purifies with the chromatin remodeling complex Ino80; albeit, the function mediated by this interaction has not been defined (Hogan et al., 2010). Recently, a HMG box1 (HMGB1) factor was reported to regulate doxorubicin resistance in human osteosarcoma cells (Huang et al., 2012). Given the paucity of evidence surrounding these two genes, we therefore proceeded to clarify the function of Nht1/Hap1 and Hap2 in safeguarding the viability of fission yeast cells following their exposure to doxorubicin.

Our analysis revealed that these HMG box factors form a unique cluster with several DXR genes in the context of the entire DXR gene

Abbreviations: DXR, doxorubicin resistance; HMG, high mobility group; WT, wild-type; HR, homologous recombination.

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network (Tay et al., 2013). In addition, we showed that doxorubicin treatment caused an increase in the formation of DNA repair foci harboring Rhp54, a homologous recombination (HR) factor required against DNA-damaging agents, in wild-type cells, and that the loss of either Nht1 or Hap2 reduced Rhp54 foci formation in the presence of doxorubicin. Therefore, our findings suggest that these HMG box factors may function to coordinate DNA damage response in the presence of doxorubicin (Khasanov et al., 1999; Tay et al., 2013). Further epistatic analyses confirmed these findings, and implicated histone acetyltransferase and chromatin remodeling in this DNA damage response. Taken together, our observations have uncovered a role for HMG box-containing proteins in regulating genomic integrity through the recruitment of a DNA repair factor to counteract doxorubicin cytotoxicity.

2. Materials and methods

2.1. Yeast culture and manipulation

Single fission yeast null mutants hosting specific gene deletions were constructed by insertion of a G418-resistant gene cassette, as previously reported (Tay et al., 2013). Double mutants were constructed by crossing the single mutants using tetrad dissection, and disruption of the appropriate gene was confirmed by PCR with gene-specific primers (Tay et al., 2013). YEA media was used for all experiments and contained 3% D-glucose, 0.5% yeast extract and 75 mg/L adenine. Viability assay (Tay et al., 2014) and pulse-field gel electrophoresis (PFGE) (Akamatsu et al., 2008) were performed as previously described.

2.2. Drug hypersensitivity test

Isogenic WT, single and double mutant cells were ten-fold serially diluted. Aliquots (2.3 μl) of each diluted cell suspension were then transferred to YEA media containing different concentrations of doxorubicin. A mutant strain was deemed to show hypersensitivity towards the drug if growth on the drug-containing plates was less than that of WT cells after normalizing for growth on plates in the absence of the drug. If a double mutant showed higher hypersensitivity relative to each of the single mutants, then the two genes were deemed to function synergistically; in contrast, a similar degree of hypersensitivity between the double and single mutants indicated that the genes most probably acted in a similar functional group (Dixon et al., 2009).

2.3. Antibodies

Green fluorescent protein (GFP) was detected with a mouse monoclonal antibody (11814460001, Roche, Indianapolis, IN, USA). Cdc2 was detected with a rabbit polyclonal antibody directed against the PSTAIRE peptide sequence (sc-53, Santa Cruz, Dallas, TX, USA);

2.4. Microscopy

Rhp54-GFP signals were studied on methanol-fixed samples using an Eclipse Ti inverted microscope (Nikon, Tokyo, Japan). Experiments were repeated three times, and the means and standard deviations were calculated using standard formulae in Microsoft Excel 2013 (Redmond, WA).

3. Results

3.1. Nht1 and Hap2 act in a similar functional group to confer doxorubicin resistance

To ascertain how cells respond to doxorubicin at the molecular level, we recently screened for genes that confer resistance against the drug in fission yeast *Schizosaccharomyces pombe*. Among the 91 genes that were identified, two genes – SPAC10F6.08c and SPCC16C4.20c – were found to code for proteins that contain a high mobility group (HMG) box domain (Reeves, 2010; Tay et al., 2013). One of these, SPAC10F6.08c, which encodes Nht1, was recently affinity purified with the fission yeast Ino80 chromatin remodeling complex; albeit, the functional significance of this interaction was not investigated (Hogan et al., 2010). Given that these two HMG box-containing proteins counteract adriamycin toxicity in *S. pombe*, we henceforth refer to them as Nht1/Hap1 (SPAC10F6.08c) and Hap2 (SPCC16C4.20c); Nht1 will be used to be consistent with published literature (Tay et al., 2013).

To understand how these HMG proteins confer doxorubicin resistance, we first determined if there was a genetic relationship between the two proteins by comparing the hypersensitivity of single *Δnht1* and *Δhap2* deletion mutants with that of the double null mutant, *Δnht1Δhap2*. If Nht1 and Hap2 worked in parallel, we would expect to see a cumulative growth defect in the cells in the presence of doxorubicin; if no additive defect is observed, then it would be assumed that these two genes act in a similar functional group (Dixon et al., 2009; Tay et al., 2013). We serially diluted these mutants and spotted the cultures on doxorubicin-incorporated media. Wild-type (WT) and *Δrav1* – a null mutant of the gene encoding a regulator of the vacuolar-ATPase proton pump assembly (Dawson et al., 2008) – were used as controls. Consistent with previous observations (Tay et al., 2013), *Δnht1* showed a weak hypersensitivity to doxorubicin at higher drug concentrations, whereas *Δhap2* exhibited a stronger hypersensitivity to the drug (10-fold at 360 μg/ml and 100-fold at 240–360 μg/ml doxorubicin relative to non-treated control). The *Δnht1Δhap2* double mutant showed no cumulative hypersensitivity towards doxorubicin as compared with *Δhap2*, indicating that Nht1 and Hap2 may act in a similar functional group to regulate a response to doxorubicin (Fig. 1A). This relationship was consistent in cell viability assays for the aforementioned strains over a shorter period of 4–8 h in the presence of 75 μg/ml doxorubicin (Figs. 1B, S1).

3.2. Rhp54 foci formation in the presence of doxorubicin is regulated by Nht1 and Hap2

Next, we explored the mechanism by which Nht1 and Hap2 conferred doxorubicin resistance. Even though the nature of doxorubicin-induced DNA adducts has not been clarified in fission yeast, others have uncovered indicators of DSB in mammalian cells following doxorubicin treatment (Capranico et al., 1989; Kujjo et al., 2010; Verma et al, 2010). Considering that Nht1 interacts with the Ino80 complex, which mediates DSB response (Morrison et al., 2004), we therefore sought to assess the appearance of DSB damage following drug treatment using two methods: (1) pulse field gel electrophoresis (PFGE), to identify the presence of a faster migrating DNA species, which is indicative of DNA fragmentation (Akamatsu et al., 2008); and (2) microscopy, to examine any change in the localization of the DSB repair protein, Rhp54 (Yu et al., 2013).

The fission yeast *rad50S* strain lacks the ability to repair DNA by HR during meiotic prophase of the cell cycle, and this appears as a smear of broken DNA, as previously reported (Young et al., 2002). In our experiment, however, we did not find any lower molecular weight smear for doxorubicin-treated WT, *Δnht1*, *Δhap2*, or *Δnht1Δhap2* cells that resembled what was observed in *rad50S* (Fig. 2). For the experiment, PFGE was performed at a lower concentration of doxorubicin (20 μg/ml), as higher concentrations (50 μg/ml) prohibited proper resolution of chromosomal DNA in the gel (data not shown).

Rhp54 is an HR protein reported to accumulate in DNA repair foci in the presence of DSB (Yu et al., 2013). In our previous work, Rhp54 was also isolated as a DXR factor (Tay et al., 2013). Interestingly, we detected a significant upregulation (2-fold) in Rhp54-GFP foci upon exposure to doxorubicin in WT cells as compared with untreated WT cells, consistent with the formation of DNA lesions that require Rhp54 for repair. Although there was an increase in the proportion of cells showing one