Ixr1 Is Required for the Expression of the Ribonucleotide Reductase Rnr1 and Maintenance of dNTP Pools

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Abstract
The Saccharomyces cerevisiae Dun1 protein kinase is a downstream target of the conserved Mec1-Rad53 checkpoint pathway. Dun1 regulates dNTP pools during an unperturbed cell cycle and after DNA damage by modulating the activity of ribonucleotide reductase (RNR) by multiple mechanisms, including phosphorylation of RNR inhibitors Sml1 and Dif1. Dun1 also activates DNA-damage-inducible genes by inhibiting the Crt1 transcriptional repressor. Among the genes repressed by Crt1 are three out of four RNR genes: RNR2, RNR3, and RNR4. The fourth RNR gene, RNR1, is also DNA damage-inducible, but is not controlled by Crt1. It has been shown that the deletion of DUN1 is synthetic lethal with the deletion of IXR1, encoding an HMG-box-containing DNA binding protein, but the reason for this lethality is not known. Here we demonstrate that the dun1 ixr1 synthetic lethality is caused by an inadequate RNR activity. The deletion of IXR1 results in decreased dNTP levels due to a reduced RNR1 expression. The ixr1 single mutants compensate for the reduced Rnr1 levels by the Mec1-Rad53-Dun1-Crt1-dependent elevation of Rnr3 and Rnr4 levels and downregulation of Sml1 levels, explaining why DUN1 is indispensable in ixr1 mutants. The dun1 ixr1 synthetic lethality is rescued by an artificial elevation of the dNTP pools. We show that Ixr1 is phosphorylated at several residues and that Ser366, a residue important for the interaction of HMG boxes with DNA, is required for Ixr1 phosphorylation. Ixr1 interacts with DNA at multiple loci, including the RNR1 promoter. Ixr1 levels are decreased in Rad53-deficient cells, which are known to have excessive histone levels. A reduction of the histone gene dosage in the rad53 mutant restores Ixr1 levels. Our results demonstrate that Ixr1, but not Dun1, is required for the proper RNR1 expression both during an unperturbed cell cycle and after DNA damage.

Introduction
Cells experiencing DNA damage or replication blocks activate stress response pathways, or checkpoints, that arrest the cell cycle and facilitate DNA repair. In budding yeast, the key checkpoint protein kinases are Mec1 (homolog of human ATR) and Rad53 (homolog of CHK2 and functional homolog of CHK1 in human), reviewed in [1,2]. In human cells, ATR and CHK2 are upstream protein kinases are Mec1 (homolog of human ATR) and Rad53 and facilitate DNA repair. In budding yeast, the key checkpoint pathway. Dun1-Crt1–dependent elevation of Rnr3 and Rnr4 levels and downregulation of Sml1 levels, explaining why DUN1 is indispensable in ixr1 mutants. The dun1 ixr1 synthetic lethality is rescued by an artificial elevation of the dNTP pools. We show that Ixr1 is phosphorylated at several residues and that Ser366, a residue important for the interaction of HMG boxes with DNA, is required for Ixr1 phosphorylation. Ixr1 interacts with DNA at multiple loci, including the RNR1 promoter. Ixr1 levels are decreased in Rad53-deficient cells, which are known to have excessive histone levels. A reduction of the histone gene dosage in the rad53 mutant restores Ixr1 levels. Our results demonstrate that Ixr1, but not Dun1, is required for the proper RNR1 expression both during an unperturbed cell cycle and after DNA damage.

The Saccharomyces cerevisiae Dun1 protein kinase is a downstream target of the conserved Mec1-Rad53 checkpoint pathway. Dun1 regulates dNTP pools during an unperturbed cell cycle and after DNA damage by modulating the activity of ribonucleotide reductase (RNR) by multiple mechanisms, including phosphorylation of RNR inhibitors Sml1 and Dif1. Dun1 also activates DNA-damage-inducible genes by inhibiting the Crt1 transcriptional repressor. Among the genes repressed by Crt1 are three out of four RNR genes: RNR2, RNR3, and RNR4. The fourth RNR gene, RNR1, is also DNA damage-inducible, but is not controlled by Crt1. It has been shown that the deletion of DUN1 is synthetic lethal with the deletion of IXR1, encoding an HMG-box-containing DNA binding protein, but the reason for this lethality is not known. Here we demonstrate that the dun1 ixr1 synthetic lethality is caused by an inadequate RNR activity. The deletion of IXR1 results in decreased dNTP levels due to a reduced RNR1 expression. The ixr1 single mutants compensate for the reduced Rnr1 levels by the Mec1-Rad53-Dun1-Crt1-dependent elevation of Rnr3 and Rnr4 levels and downregulation of Sml1 levels, explaining why DUN1 is indispensable in ixr1 mutants. The dun1 ixr1 synthetic lethality is rescued by an artificial elevation of the dNTP pools. We show that Ixr1 is phosphorylated at several residues and that Ser366, a residue important for the interaction of HMG boxes with DNA, is required for Ixr1 phosphorylation. Ixr1 interacts with DNA at multiple loci, including the RNR1 promoter. Ixr1 levels are decreased in Rad53-deficient cells, which are known to have excessive histone levels. A reduction of the histone gene dosage in the rad53 mutant restores Ixr1 levels. Our results demonstrate that Ixr1, but not Dun1, is required for the proper RNR1 expression both during an unperturbed cell cycle and after DNA damage.

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Author Summary

Dun1 is a non-essential protein kinase important for the maintenance of genome stability in budding yeast. Earlier studies found that simultaneous deletion of DUN1 and IXR1 results in lethality, but the reason for this so-called synthetic lethality is not clear. Ixr1 is implicated in DNA repair based on its ability to bind to DNA modified by the anticancer drug cisplatin. Here, we investigated the mechanism behind the ixr1 dun1 synthetic lethality. We demonstrate that yeast strains lacking Ixr1 have decreased amounts of dNTPs, the building blocks of DNA. This is because Ixr1 is required for the normal expression of Rnr1, one of the essential subunits of the enzyme ribonucleotide reductase (RNR), which catalyzes the rate-limiting step in the production of all four dNTPs. Cells lacking Ixr1 compensate the decreased expression of Rnr1 by the increased expression of other RNR genes and degradation of RNR inhibitors. These compensatory processes require Dun1. Hence, cells lacking both Dun1 and Ixr1 have dNTP pools that are too low for survival. Our work identifies a new important player in the synthesis of the building blocks of DNA.

The lethality of mec1 and rad53 mutants can be rescued either by deletion of SML1 (suppressor of mec1 lethality) [5], CRT1 [17], DIF1 [15,16], or by overexpression of RNR1 or RNR3 [6], all resulting in increased RNR activity. In contrast, the deletion of DUN1 is not lethal and does not cause any obvious proliferation defects except for a slightly prolonged S phase, defects in mitochondrial propagation and decreased dNTP levels [3,14,19]. It is therefore possible that another pathway exists downstream of Mec1 and Rad53, in contrast to Dun1, is involved in a plethora of important chromosomal transactions, reviewed in [1].

Others have performed large-scale analyses of synthetic genetic interactions, in which the DUN1 gene was one of the baits. In two of such screens, DUN1 mutants were found to be synthetic lethal with a gene encoding the intrastrand cross-link recognition protein (Ixr1), but the reason for this synthetic interaction remains unknown [20,21]. Ixr1 is a high mobility group (HMG) transcription factor first identified by its ability to bind DNA modified by the anticancer drug cisplatin (cis-diaminedichloroplatinum(HI)) [22]. Very little is known about the cellular function of Ixr1, other than its ability to bind cisplatin-modified DNA [18,23].

Here we demonstrate that Ixr1 is required for the maintenance of the Rnr1 levels. In the absence of Ixr1, Rnr1 levels are decreased and become even lower after DNA damage, instead of increasing as in the wild-type. This observation explains the sensitivity of ixr1 mutants to hydroxyurea (HU), an inhibitor of RNR. In contrast, the levels of Rnr3 and Rnr4 in ixr1 mutants are increased due to the activation of the Mec1-Rad53-Dun1-Crt1 pathway, and increase even further after DNA damage and replication blocks, similar to wild-type. We show that deletion of SML1 or overexpression of RNR1 or RNR3 elevates dNTP pools and rescues the ixr1 dun1 synthetic lethality. The requirement for RNR activation in ixr1 via Dun1 explains why Dun1 is indispensable in ixr1 mutants.

Results
dun1Δ is synthetic lethal with ixr1-S366F

Earlier screens for synthetic genetic interactions between dun1Δ and other genes used a collection of yeast strains with null alleles in all nonessential genes. To facilitate identification of synthetic genetic interactions of DUN1 with essential genes we performed a colour-based synthetic lethal screen using a dun1Δ strain as described in the Materials and Methods. Briefly, the ade2 ade3 yeast strains are white unless a plasmid with ADE3 is present, conferring red color. Three mutations resulting in synthetic lethality with dun1Δ (first designated as mut1, mut2, and mut3) were isolated based on the inability of the ade2 ade3 dun1Δ mut strains to lose the pK503 plasmid carrying DUN1 and ADE3. MUT1 was identified as RAD53, MUT2 as WHI3, and MUT3 as IXR1. Sequencing of the rad53Δ mutant allele identified a single point mutation changing His 622 to Tyr. The H622 residue of Rad53 was identified before as crucial for the interaction of Rad53 with Rad9 [25], but the synthetic lethality of rad53Δ-H622Y was not known. Sequencing of whi3Δ identified a single point mutation changing Gln in position 481 to a stop codon. Consistent with this observation, deletion of WHI3 showed a synthetic growth defect with dun1Δ in a large-scale analysis [20]. Finally, sequencing of the ixr1 Δ mutant allele identified a single point mutation that changed Ser 366 to Phe in the first of the protein’s two HMG boxes (Figure 2A). This highly conserved serine residue (Figure 2B) forms a hydrogen bond with the DNA minor groove, a conserved feature of HMG proteins [26].

Deletion of SML1 or elevated expression of RNR genes rescues dun1Δ ixr1 synthetic lethality

A well-established role of Dun1 is to increase RNR activity by targeting Sm1l and Dif1 for degradation and by transcriptional activation of RNR2, RNR3 and RNR4. Therefore, we asked whether deletion of SML1 or elevated expression of RNR genes rescues the lethality of dun1Δ ixr1. The dun1Δ ixr1-S366F [pK503] strain, originally identified in the screen, was crossed with a dun1Δ sml1Δ strain. Both strains are ade2 ade3 mutants. The unstable pK503 plasmid was lost in dun1Δ sml1Δ ixr1-S366F colonies, based on their white color, but not in dun1Δ sml1Δ ixr1-S366F colonies (Figure 2C). Next, we crossed ixr1Δ sml1Δ with dun1Δ. Tetrad analysis confirmed that dun1Δ ixr1Δ sml1Δ spores were viable while dun1Δ ixr1Δ were unable to germinate (Figure 2D). An additional copy of RNR1 rescues the dun1Δ ixr1Δ synthetic lethality, as demonstrated by sporulation and tetrad analysis of the ixr1Δ/IXR1 dun1Δ/DUN1 diploid strain transformed with a centromeric plasmid pBJ6 containing RNR1 under the control of the native promoter (Figure 2E). Finally, after transformation with plasmids overexpressing RNR1 (Figure 2F) or RNR3 (Figure 2H) or with the centromeric pBJ6 plasmid containing an additional copy of RNR1 (Figure 2G), we also observed the loss of the pK503 plasmid from the dun1Δ ixr1Δ-S366F strain, leading to the sectoring phenotype.

Rnr3 and Rnr4 levels are increased in ixr1 mutants, while Sm1l levels are decreased

The requirement of Dun1 for ixr1 viability suggests that the Mec1-Rad53-Dun1 pathway is activated in ixr1 strains. A highly sensitive readout of this pathway’s activation is induction of Crt1-controlled RNR2, RNR3, and RNR4. Indeed, Rnr3 and Rnr4 levels were higher in ixr1Δ-S366F and ixr1Δ strains compared to wild-type
Dun1 downstream of Rad53. A Dun1 viiae type, leading to a shift of the Rad53 band (Figure 3D). Resulted in hyperphosphorylation of Rad53 both in ixr1 that the Mec1-Rad53-Dun1 pathway was not compromised in RAD53 Rad53 dependent. Deletion of did not observe a mobility shift of the Rad53 band in the Mec1-Rad53-Dun1 pathway was activated (Figure 3C). We damage was identical in the elevation of Rnr2, Rnr3 and Rnr4 in response to DNA Rad53-Dun1 pathway activation is low (Figure S1A). Importantly, polyclonal anti-Rnr3 antibodies, also indicating that the Mec1-Rad53-Dun1 pathway in ixr1 (Figure 3A, lanes 1–3). Rnr2 levels were not significantly changed in ixr1 compared to wild-type (Figure 3B). Activation of the Mecl-Rad53-Dun1 pathway in ixr1 strains was not maximal; increase in Rnr3-HA levels in the undamaged continuous; Rnr1 levels remained lower in ixr1 activation of the Mec1-Rad53-Dun1 pathway in ixr1 is not only due to a decreased dNTP production, but also due to some other defects.

Levels of Rnr1 in ixr1 decrease after DNA damage

The paradoxical finding that dNTP pools decreased despite the activation of the Mec1-Rad53-Dun1 pathway in ixr1 strains indicates a deficiency in another component(s) of the RNR machinery. Analysis of Rnr1 steady state levels demonstrated moderately decreased levels in ixr1 and in ixr1-sml1D compared to wild-type (~64%, and ~62% respectively) (Figure 5A, 5B, and 5C (0 min lanes)). As expected, incubation of wild-type cells in the presence of 4-NQO or HU for two hours led to an increase in Rnr1 levels (~39% and 57%, respectively) (Figure 5A and 5B). Interestingly, the same treatment of ixr1-S366F and ixr1 led to a further reduction of Rnr1 to ~19% and ~37%, respectively, after 4-NQO treatment, and to ~56% and ~46%, respectively, after the HU treatment (Figure 5A and 5B). This reduction was continuous; Rnr1 levels remained lower in ixr1 throughout a 12-hour incubation with 4-NQO (Figure 5C). Based on flowcytometric analysis, the ixr1 strain had a slightly greater proportion of cells in S phase compared to wild-type both before and during 4-NQO treatment (Figure 5C), although the overall proliferation rate was similar between ixr1 and wild-type (Figure 5D).

The decreased Rnr1 levels caused by ixr1 provide an explanation for the synthetic lethality displayed by the ixr1-dun1A double mutant strain; the dun1A strains are defective in relieving the inhibition of RNR imposed by Sml1, Dif1, and Crt1.

Deletion of IXR1 negatively affects RNR1 transcription

Using a β-galactosidase assay we demonstrated that the decreased Rnr1 levels in ixr1 are due to a lower RNR1 promoter activity, which indicates that Ixr1 directly or indirectly regulates RNR1 transcription (Figure 6A). Deletion of IXR1 also caused a concomitant increase in RNR3 and RNR4 promoter activities (Figure 6A), in agreement with the observed increase in Rnr3 and Rnr4 protein levels (Figure 3A). To gain further insight into the mechanism of RNR1 regulation by Ixr1, we performed chromatin immunoprecipitation (ChIP) experiments followed by qPCR using Ixr1-9xMyc fusion protein and 9E10 antisera. We analyzed binding of Ixr1 to the RNR1 promoter (pRNR1) region. In addition, we analyzed the DSF2 promoter (pDSF2) region earlier identified as an Ixr1-interacting locus [27] and the actin (ACT1)
Figure 2. **dun1 ixr1 synthetic lethality is rescued by increased RNR activity.** (A) Schematic representation of the Ixr1 protein. HMG boxes are shown in blue and polyQ regions in black. (B) Alignment of homologous HMG proteins close to the conserved S366. (C) Tetrad analysis demonstrating that deletion of SML1 rescues the dun1Δ ixr1-S366F synthetic lethality. The dun1Δ ixr1-S366F strain (TOY544) carries a DUN1-containing plasmid, pK503, which confers a red color; this plasmid is lost in ixr1-S366F dun1Δ sml1Δ colonies (TOY544 x TOY588). (D) Tetrad analysis demonstrating that deletion of SML1 rescues the dun1Δ ixr1-S366F synthetic lethality (TOY604 x TOY527). (E) Tetrad analysis demonstrating that a low-copy RNR1 vector rescues the ixr1-S366F dun1Δ synthetic lethality. The diploid strain ixr1/IXR1 dun1/DUN1 (TOY527 x TOY603) was transformed with pRS316 and pB6 (pRS316-RNR1), sporulated and tetrad analysis was performed. (F) Overexpression of RNR1 rescues the ixr1-S366F dun1Δ synthetic lethality. The pK503 plasmid, containing the DUN1 gene and conferring red color, is lost on YP-Gal medium in the ixr1-S366F dun1Δ strain transformed with pESC-URA-pGAL1-RNR1 plasmid (left panel, sectoring phenotype), but not when transformed with pESC-URA-pGAL1 (right panel). (G) Low-copy RNR1 vector rescues the ixr1-S366F dun1Δ synthetic lethality. The pK503 plasmid, containing the DUN1 gene and conferring red color, is lost in the ixr1-S366F dun1Δ strain transformed with the pB6 plasmid (left panel, sectoring phenotype), but not when transformed with pRS316 (right panel). (H) Overexpression of RNR3 rescues the ixr1-S366F dun1Δ synthetic lethality. The pK503 plasmid, containing the DUN1 gene and conferring red color, is lost in the ixr1-S366F dun1Δ strain transformed with pBAD79 plasmid (left panel, sectoring phenotype), but not when transformed with pRS414 (right panel).

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open reading frame, a commonly used negative control. As another control, we used an untagged congenic IXR1 strain. Ixr1 interacted with all three loci (Figure 6B): relative to the input DNA we recovered 0.46%, 0.35% and 0.79% of pRNR1, pDSF2 and ACT1 loci, respectively, in the IXR1-9Myc strain. The interaction of Ixr1 with the RNR1 promoter did not change after the treatment of cells with 4-NQO (0.46% and 0.48%, respectively). In the untagged strain, DNA recovery was at background levels as judged by the ChIP samples where 9E10 antiserum was omitted. The precipitation of the ACT1 ORF locus indicates that Ixr1 binds to many loci in the genome.

Elevation of Rnr1 levels in response to DNA damage depends on Rad53, but not Dun1

The DNA-damage-inducible genes become damage uninducible in dun1 mutants, because Dun1 is required to relieve the inhibition imposed by the transcriptional inhibitor Crt1. Indeed, induction of Rnr4 in response to 4-NQO is less pronounced in the dun1Δ strain compared to wt (Figure 7A). The RNR1 promoter, however, does not contain Crt1 sites and the expression of RNR1 is not affected by the CRT1 deletion [17,18]. In Figure 7A, we demonstrate that the elevation of Rnr1 levels in response to DNA damage does not depend on DUN1, but does depend on Rad53 and Mec1. All checkpoint mutant strains in this experiment contained sml1Δ, because mec1Δ and rad53Δ are inviable otherwise. As a control, we demonstrate that the deletion of SML1 by itself has little effect on Rnr1 and Rnr4 levels in the wild type and ixr1Δ strains treated by 4-NQO (Figure S1C).

Ixr1 levels depend on histone dosage

Because Mec1, Rad53 and Dun1 are protein kinases, it was possible that they directly phosphorylate Ixr1 and modulate its
changes in Ixr1 mobility in of Ixr1 to that of Ixr1-S366F. However, we did not observe important for Ixr1 phosphorylation, as Ixr1-S366F separated by likely phosphorylated at several residues (Figure 7B). Serine 366 is S366F proteins and found that Ixr1 is a phosphoprotein most function. We analyzed the phosphorylation status of Ixr1 and Ixr1-IXR1 leads to decreased dNTP levels. A) dNTP levels are decreased in ixr1Δ (TOY736), increased in ixr1Δ sml1Δ (TOY714) compared to wild-type (W1588-4C), but lower than in sml1Δ (U952-3B). Values shown are the average from two independent experiments with the minimum and maximum values represented as error bars. (B) dNTP levels increase in ixr1Δ (TOY736, black triangles) during the treatment with 0.2 mg/L 4-NQO, but less than in a wild-type strain (W1588-4C, open circles). Values shown are the average from two independent experiments with the minimum and maximum values represented as error bars. (C) ixr1Δ (TOY736) has a higher frequency of petite formation than a wild-type strain (W1588-4C). (D) ixr1Δ (TOY736) is more sensitive to HU than a wild-type strain (W1588-4C).

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Discussion

The Dun1 kinase is a downstream target of the Mec1-Rad53 checkpoint, which monitors the genome integrity. In S. cerevisiae, the Mec1-Rad53-Dun1 pathway also regulates the RNR activity both during the normal cell cycle and after DNA damage [4]. RNRs are instrumental in controlling dNTP balance and concentration [30]. Deletion of Dun1 is synthetic lethal with the deletion of many genes involved in DNA replication and DNA repair [20,21]. Synthetic lethality of dun1 with a number of other genes remains unexplained. Here, we demonstrate that Ixr1, deletion of which is synthetic lethal with dun1, is required for the normal expression of the RNR1 gene and maintenance of the dNTP pools.

In the absence of DNA damage, the deletion of Ixr1 leads to a moderate decrease of Rnr1 and dNTP levels. This decrease is partially compensated by the activation of the Mec1-Rad53-Dun1 pathway. We base this conclusion on the following observations. First, Rnr3 and Rnr4, whose levels are controlled by the Mec1-Rad53-Dun1 pathway, are upregulated in ixr1Δ, and Rad53 is required for this upregulation. Second, RNR inhibitor Smr1, whose levels are also controlled by the Mec1-Rad53-Dun1 pathway, is downregulated in ixr1Δ. Third, DUN1 is indispensable in ixr1Δ, but ixr1 Δ dun1Δ synthetic lethality is rescued by elevated dNTP levels. We note that elevation of dNTP levels in ixr1Δ caused by the SML1 deletion does not eliminate the checkpoint activation (Figure 3E), indicating that deletion of Ixr1 leads to replication stress not only because of the decreased dNTP levels expression but also because of other effects. It is conceivable that in addition to RNR1 some other genes involved in the DNA biosynthesis are regulated by Ixr1. The reported synthetic lethality of ixr1Δ with the origin recognition complex mutant ori2-1 [31] and synthetic sickness with the thymidylate kinase mutant cdk8-2 [32] indicate the importance of Ixr1 for the processes involved in DNA replication.

RNR expression increases in response to DNA damage in most organisms. In E. coli, unnA and unnB (encoding the large and the small RNR subunits, respectively) are among the most highly induced genes following UV exposure (induced ~20- and ~7-fold, respectively) [33,34]. In mammalian cells, DNA damage induces the p53R2 protein, an alternative small RNR subunit, about 4-fold in a p53-dependent manner [35–37]. Similarly, the Drosohila large RNR subunit, RnL1, is induced by ionizing radiation in wild-type, but not p53-deficient strains [38]. In the yeast Schizosaccharomyces pombe, RNR genes are among the most robustly induced genes following DNA damage [39]. All four S. cerevisiae RNR genes are activated by DNA damage and replication blocks [8,10,12]. The pathway involved in the activation of RNR2, RNR3 and RNR4 is well understood and requires the Mec1-Rad53-Dun1 kinase cascade, which targets Ctr1, transcriptional inhibitor of DNA-damage-inducible genes (Figure 1A). Here we demonstrate that elevation of Rnr1 in response to DNA damage requires Mec1 and Rad53, but not Dun1 (Figure 7A). Earlier, it has been shown that RNR1 expression does not depend on Ctr1 [17,18]. Thus, the downstream Dun1-Ctr1 part of the Mec1-Rad53-Dun1-Ctr1 pathway, which is known to control the DNA-damage-inducible genes in yeast, is not involved in the regulation of Rnr1. Instead, the elevation of Rnr1 levels in response to DNA damage requires Ixr1 (Figure 7E).

In addition to Ctr1, transcription of RNR2, RNR3 and RNR4 genes is also controlled by Rox1 and Mot3, the DNA binding proteins that repress the hypoxic genes by recruiting the Snf6/Tup1 general repression complex. Again, in contrast to RNR2, RNR3, and RNR4 genes, no Rox1 or Mot3 sites are present in the
RNR1 promoter [18]. Transcription of RNR1 is controlled by MBF, a dimeric transcription factor composed of Swi6 and Mbp1 [40–42]. Interestingly, Swi6 is directly phosphorylated by Rad53 in response to DNA damage [43]. It will be interesting to investigate whether Ixr1 is important for MBF-dependent regulation of the RNR1 promoter. Earlier, Ixr1 was implicated in controlling the levels of the hypoxic gene COX5b [24]. Currently, we do not know whether Ixr1 is involved in the activation of RNR1 expression in response to oxygen deprivation.

In contrast to many other HMG-box proteins, Ixr1 is rather large (68 kDa) and contains several polyglutamine repeats, which are often involved in protein-protein interactions and are present in many transcription factors. The HMG box is a conserved domain of 80 amino acids, binding to the minor groove of DNA. Proteins containing two or more HMG boxes usually recognize structural features of DNA without sequence specificity, while proteins containing one HMG box can recognize DNA in a sequence specific manner. In S. cerevisiae, there are two proteins...
containing two HMG boxes (Ixr1 and Abf2) and five proteins containing one HMG box (Nhp6A, Nhp6B, Nhp10, Hmo1 and Rox1). The closest homolog of Ixr1, Abf2, binds to many loci in the mitochondrial genome [44]. Yet, the HMG-box proteins with two or more HMG boxes can bind to specific loci in the genome. For example, human transcription factor UBF, which has 6 HMG boxes and belongs to the sequence-nonspecific class of HMG-box proteins, binds specifically to rDNA or to heterologous UBF-binding sequences from Xenopus integrated into ectopic sites on human chromosomes [45]. Our ChIP analysis of Ixr1 identified the RNR1 promoter as a binding locus. However, Ixr1 bound equally well at two other tested loci, the DSF2 promoter and the ACT1 open reading frame. Still, it is possible that, in the context of the RNR1 promoter, Ixr1 together with other proteins directly regulates RNR1 gene expression.

Interestingly, the mutation in the Ixr1 S366 residue that is important for interaction of HMG boxes with DNA results in the phosphorylation of Ixr1. This S366 residue is required for the phosphorylation of several amino acid residues in Ixr1. Currently we do not know the phosphorylation status of S366. It is possible that S366 itself is not phosphorylated, but its interaction with the DNA or other proteins is required for the phosphorylation of other residues in Ixr1. Multiple phosphorylation of Ixr1 causes an increase in the apparent molecular weight of the protein: Ixr1 separates by SDS-PAGE as a ~95 kDa protein (not as predicted 60 kDa). We demonstrate that neither Mec1, nor Rad53, nor Dun1 are responsible for the phosphorylation of Ixr1, as its mobility is not affected in the respective mutants. The region of the HMG domain around the Ser366 residue has been shown to affect DNA binding specificity. All sequence-specific HMG proteins have a serine (e.g., Ser10 in the D. melanogaster HMG-D box co-crystallized with DNA) [26]. To our knowledge, crystal structures analyzing the interaction of HMG boxes and DNA were solved with the non-phosphorylated proteins. It would be interesting to investigate whether S366 is phosphorylated in Ixr1, whether the corresponding serine residues are phosphorylated in other HMG proteins in other species, and whether Ser366 phosphorylation affects DNA binding and/or makes binding of the HMG box sequence specific.

Although the mobility of Ixr1 is not changed, its levels are significantly reduced in the rad53Δ strain (Figure 7C). rad53 mutant strains are known to have increased histone levels due to a defect in histone degradation [28]. Increased histone levels presumably lead to decreased Ixr1 levels, because we show that decreasing histone dosage in the rad53Δ strain restores Ixr1 levels (Figure 7D). There are at least two possibilities explaining this interplay between Ixr1 and histone levels. Because Ixr1 contains two HMG boxes and therefore binds DNA presumably without sequence specificity, it might compete with histones for DNA binding. Increased histones in rad53Δ might displace Ixr1 from the Ixr1 promoter, where it was shown to bind and regulate its own expression [46]. Alternative, but not exclusive possibility is that Ixr1 displaced by histones from DNA undergoes degradation.

In summary, we identify Ixr1 as a novel factor involved in regulation of dNTP pools and RNR1, a gene that, in contrast to all other known DNA-damage inducible genes, is not controlled by Dun1 and Crt1.

Materials and Methods

Yeast strains and primers

All yeast strains used in this study are congenic to W1588-4C [5]. Table 1 gives only the allele(s) that differ from the W1588-4C genotype. Table S1 lists primers used for strain construction. DUN1 was deleted using the KanMX4 cassette PCR-amplified with primers F_Dun1 and R_Dun1 from the dun1Δ::KanMX4 Y03798 strain (Euroscarf). The CY12625 ade3::HIS5 strain [47] was crossed with W1588-4C to select ade2 ade3 clones (TOY502). The resulting strain was crossed with dun1Δ::KanMX4 to create the strain used for the synthetic lethality screen (TOY527). The TOY836 (IXR1-9MYC) strain was generated by amplifying and introducing the 9MYC-TRP1 cassette from the Z1580 strain [48] into W1588-4C.

Plasmids

To overexpress Rnr1 or Rnr3, the previously described pESC-pGAL1-RNR1 or pBAD79 plasmids were used [6,49]. To express RNR1 under its own promoter from a low-copy centromeric vector, the pBR6 (pRS316-RNR1) plasmid was used (gift of Anders Byström, Umeå University). To construct pK303 (urn3A::LEU2, ADE3, DUN1), the DUN1 gene including the promoter region was PCR-amplified using primers Dun1_F and Dun1_rev. The PCR product was cloned into the SalI site of p2013 [50], and the URA3 gene in the

Figure 6. Ixr1 regulates RNR1 promoter activity. (A) β-Galactosidase assay of RNR1, RNR3, and RNR4 promoter activity in wild-type (W1588-4C) and ixr1Δ (TOY736) strains. Promoters of the analyzed genes were fused with the lacZ reporter gene and the respective plasmids were transformed in the wild type and ixr1Δ strains. β-Gal units were quantified as described in Materials and Methods. (B) Analysis of DNA associated with Ixr1 was performed by chromatin immunoprecipitation (ChIP) followed by qPCR using locus-specific primers for the RNR1 promoter (pRNR1), the DSF2 promoter (pDSF2) and the ACT1 gene. ChIP was performed with anti-Myc antiserum 9E10 or mock-antiserum. For pRNR1, ChIP was performed both with and without addition of 4-NQO (0.2 mg/L). Y axis represents the amount of precipitated DNA relative to the input DNA. Values shown are the average from two independent experiments with the minimum and maximum values represented.
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Materials and Methods

Yeast strains and primers

All yeast strains used in this study are congenic to W1588-4C [5]. Table 1 gives only the allele(s) that differ from the W1588-4C genotype. Table S1 lists primers used for strain construction. DUN1 was deleted using the KanMX4 cassette PCR-amplified with primers F_Dun1 and R_Dun1 from the dun1Δ::KanMX4 Y03798 strain (Euroscarf). The CY12625 ade3::HIS5 strain [47] was crossed with W1588-4C to select ade2 ade3 clones (TOY502). The resulting strain was crossed with dun1Δ::KanMX4 to create the strain used for the synthetic lethality screen (TOY527). The TOY836 (IXR1-9MYC) strain was generated by amplifying and introducing the 9MYC-TRP1 cassette from the Z1580 strain [48] into W1588-4C.

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resulting plasmid was then replaced by LEU2. To construct pK521 (TRP1, DUN1) a Sall/Sall fragment of DUN1 from pK503 was cloned into the Sall site of pRS414 [51]. To construct plasmids for the β-Galactosidase assay, the RNR1, RNR3 and RNR4 promoters were PCR amplified from the W1588-4C genomic DNA using primers pR1-F, pR1-R, pR3-F, pR3-R, pR4-F and pR4-R. The RNR3 promoter was cloned in the BamHI site of pJO20, and the RNR1 and RNR4 promoters were cloned in the BamHI site of pJO21 [52], resulting in plasmids pK505, pK504 and pK506, respectively. β-Galactosidase levels were assayed as described [52].

**Synthetic lethal screen**

To identify mutations synthetic lethal with dun1Δ, we used a color-based synthetic lethal screen [53]. The TOY541 strain carrying pK503 was grown in selective medium to ~2×10^7 cells/ml. Cells were spun down, resuspended in water, plated onto YPD plates at 1500 cells/plate, and UV-mutagenized with a dose of 150 J/m^2, resulting in 30% survival. Plates were placed in the dark and incubated for 3 days at 30°C. Non-sectoring red colonies were re-streaked twice on YPD, and those retaining the red color were selected for further analysis. Candidate mutants were transformed with pK521 to exclude mutants synthetically lethal with the plasmid-borne ADE3 or LEU2 genes. Transformants were grown on –Trp medium, and strains with a sectoring phenotype were selected. The candidate mutants were crossed with TOY566 to test recessiveness/dominance, and tetrad analysis was performed to select mutations with monogenic inheritance. Selected strains were mated in all possible combinations to establish complemen-

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**Figure 7. Elevation of Rnr1 levels in response to DNA damage requires MEC1, RAD53, and IXR1, but not DUN1.** (A) Western blot analysis of Rnr1 and Rnr4 levels. The following strains were incubated with 0.2 mg/L 4-NQO for 2 hours: wt (W1588-4C), ixr1Δ (TOY736), dun1Δ sml1Δ (TOY728), rad53Δ sml1Δ (TOY782), mec1Δ sml1Δ (TOY711), dun1Δ rad53Δ sml1Δ (TOY786), and dun1Δ mec1Δ sml1Δ (TOY774). Rnr1 and Rnr4 levels were quantified in relative units (RU, levels of Rnr1 or Rnr4 divided by the levels of tubulin in corresponding sample) as described in Materials and Methods. (B) Western blot analysis of β-phosphatase treated extracts from ixr1-Ha (TOY655) and ixr1-S366F-Ha (TOY650) strains. (C) Western blot analysis of ixr1 levels in wt (W1588-4C), dun1Δ sml1Δ (TOY728), rad53Δ sml1Δ (TOY782), mec1Δ sml1Δ (TOY711), rad53Δ dun1Δ sml1Δ (TOY786) and mec1Δ dun1Δ sml1Δ (TOY774). (D) Western blot analysis of ixr1 levels in wild-type (W1588-4C), hht2-hhf2Δ (TOY806), hht2-hhf2Δ sml1Δ (TOY821), rad53Δ sml1Δ (TOY782) and hht2-hhf2Δ rad53Δ sml1Δ (TOY819). (D) Western blot analysis of Ixr1 levels in wild-type (W1588-4C), mec1Δ (TOY806), hht2-hhf2Δ sml1Δ (TOY821), rad53Δ sml1Δ (TOY782) and mec1Δ dun1Δ sml1Δ (TOY774). (E) Western blot analysis of Ixr1 levels in wild-type (W1588-4C), hht2-hhf2Δ (TOY806), hht2-hhf2Δ sml1Δ (TOY821), rad53Δ sml1Δ (TOY782) and hht2-hhf2Δ rad53Δ sml1Δ (TOY819). Ixr1 levels were quantified in relative units (RU, levels of Ixr1 divided by the levels of tubulin in corresponding sample) as described in Materials and Methods. (E) Mec1-Rad53-Dun1-dependent regulation of S. cerevisiae ribonucleotide reductase. Expression of RNR1 depends on Mec1, Rad53 and Ixr1, but does not depend on Dun1 or Crt1.

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tation groups. In total, we isolated 4 mutants falling into three complementation groups and identified them as one ixr1, one rad53 and two whi3 mutants as outlined below.

One strain from each complementation group was transformed with a pRS314-based yeast genomic DNA library [54], and transformants were selected on –Trp plates. Clones that showed a sectoring phenotype were re-streaked onto –Trp plates, and plasmids were isolated from these clones and partially sequenced using T3 and T7 standard primers. The obtained sequences were subjected to BLAST homology searches using the S. cerevisiae genome database, and genomic regions were retrieved. One of the regions contained the genome database, and genomic regions were retrieved. One of the regions contained the genome database, and genomic regions were retrieved. One of the regions contained the genome database, and genomic regions were retrieved.

| Strain     | Genotype                        | Reference |
|------------|---------------------------------|-----------|
| W1588-4C   | MATa ade2-Ican1-100 his3-11.15 leu2-3,112 trp1-1ura3-1 RADS+ | [5]       |
| AC447-2A   | MATa ade2-1 can1-100 his3-11.15 leu2-3,112 trp1-1 ursa-1 rad5 RNR3-3HA::KanMX6 | [49]      |
| U952-3B    | MATa sml1Δ::His53              | [5]       |
| CY1263     | MATa ade3::HSG                 | [47]      |
| CUY995     | MATs metl-D1::His3 ade2-101 lue2-3,112 ursa-3-S2 | [11]      |
| TOY502     | MATa ade3::HSG                 | This study|
| TOY510     | MATa dun1Δ::KanMX6             | This study|
| TOY527     | MATa ade3::HSG dun1Δ::KanMX6   | This study|
| TOY541     | MATa ade3::HSG ixr1-S366F::TRP1 | This study|
| TOY544     | MATa ade3::HSG dun1Δ::KanMX6 ixr1-S633F [pK503] | This study|
| TOY566     | MATa ade3::HSG dun1Δ::URA3     | This study|
| TOY588     | MATa ade3::HSG dun1Δ::KanMX6 sm1lΔ::His5 | This study|
| TOY598     | MATs ade3::HSG dun1Δ::KanMX6 ixr1-S366F::TRP1 [pK503] | This study|
| TOY603     | MATa ade3::HSG ixr1Δ::TRP1     | This study|
| TOY604     | MATa ade3::HSG ixr1Δ::TRP1 sm1lΔ::His5 | This study|
| TOY619     | MATa ade3::HSG ixr1Δ::TRP1 RNR3-Ha::KanMX6 | This study|
| TOY621     | MATa ade3::HSG ixr1Δ::TRP1 RNR3-Ha::KanMX6 | This study|
| TOY650     | MATa ade3::HSG ixr1Δ::TRP1 RNR3-Ha::KanMX6 | This study|
| TOY655     | MATa ade3::HSG IXR1-Ha::KanMX6 | This study|
| TOY711     | MATa mec1Δ::TRP1 sm1lΔ::His5 | This study|
| TOY714     | MATa ixr1Δ::TRP1 Δ::URA3 sm1lΔ::His5 | This study|
| TOY728     | MATa ixr1Δ::TRP1 sm1lΔ::His5 | This study|
| TOY732     | MATa ixr1Δ::URA3 RNR3-Ha::KanMX6 | This study|
| TOY734     | MATa ixr1Δ::URA3 RNR3-Ha::KanMX6 | This study|
| TOY736     | MATa ixr1Δ::URA3 RNR3-Ha::KanMX6 | This study|
| TOY772     | MATa ixr1Δ::URA3 dun1Δ::ura3A::LEU2 sm1lΔ::His5 RNR3-Ha::KanMX6 | This study|
| TOY774     | MATa dun1Δ::ura3A::LEU2 mec1Δ::TRP1 sm1lΔ::His53 RNR3-Ha::KanMX6 | This study|
| TOY778     | MATa ixr1Δ::tra1A::URA3 sm1lΔ::His3 RNR3-Ha::KanMX6 | This study|
| TOY781     | MATa ixr1Δ::tra1A::URA3 rad53Δ::HphMX4 sm1lΔ::His3 RNR3-Ha::KanMX6 | This study|
| TOY782     | MATa rad53Δ::HphMX4 sm1lΔ::His3 RNR3-Ha::KanMX6 | This study|
| TOY786     | MATa rad53Δ::HphMX4 sm1lΔ::His3 RNR3-Ha::KanMX6 | This study|
| TOY806     | MATa hht2::Hht2Δ::KanMX6 | This study|
| TOY819     | MATa rad53Δ::HphMX4 hht2::Hht2Δ::KanMX6 sm1lΔ::His5 | This study|
| TOY821     | MATa hht2::Hht2Δ::KanMX6 sm1lΔ::His5 | This study|
| TOY836     | MATa IXR1-9Mye::TRP1           | This study|

Table 1. Yeast strains used in this study.

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Western blotting and antibodies

Protein samples for Western blotting were prepared as described [55]. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Protran BA 85, Whatman, USA) using the Minigel System (C.B.S. Scientific Co., USA).

Ixr1 and Maintenance of dNTP Pools
Rabbit polyclonal anti-Rnr1 (AS09 576), anti-Rnr2 (AS09 575), anti-Rnr3 (AS09 574), and anti-Sml1 (AS10 847) antibodies were produced by Agrisera, Sweden (peptides used for immunization are listed in Table S2). For the detection of Ixr1 we used rabbit polyclonal antibodies produced by Agrisera, Sweden (Table S2). For the detection of the HA-tag, mouse monoclonal 12CA5 antibodies were used (1:5000). For the detection of both Rnr4 and α-tubulin [56], we used YL1/2 rat monoclonal antibodies (Sigma) at 1:2500. These antibodies recognize C-termini of α-tubulin and small RNR subunits from different species. The absence of the Rnr4 band on a Western blot with an extract from an mR4A strain (CUY995, [11]) confirmed that YL1/2 antibodies specifically recognize yeast Rnr4 (Figure S1B). For the detection of Rad53, we used γ-C-19 goat polyclonal antibodies at 1:2000 (Santa Cruz Biotechnology, USA).

Quantification of protein levels was performed using ImageJ software [http://rsweb.nih.gov/ij]. Protein levels were calculated as relative units (RU, levels of the particular protein divided by the levels of tubulin in corresponding sample). To quantify Rnr1 levels three independent clones were analyzed on the same membrane.

Chromatin immunoprecipitation and quantitative PCR
Chromatin immunoprecipitation followed by qPCR was performed as previously described [Barsoum et al., 2010]. DNA damaging agent 4-NQO was added to the cells to final concentration 0.25 mg/L at OD ~0.5 and cells were grown 2 hours to OD ~1.2–1.5. To amplify RNR1 promoter, DFS2 promoter and ACT1 open reading frame ChiP_pRNR1, ChiP_pDSF2 and ChiP_ACT1 primers were used (Table S1).

Treatment with λ Phosphatase
9 x 10^7 cells were collected, vortexed with glass beads in 10% w/v trichloroacetic acid and spun down 10 min in microcentrifuge in cold room. Pellet was re-suspended in 150 μL of λ-Phosphatase buffer, pH was adjusted to 7.5 with basic 1 M Tris and 15 mM 10 Complete Protease Inhibitor Cocktail (Roche Applied Biosytems) was added to the samples. 60 μL of 10× PhosStop Phosphatase Inhibitor Cocktail (Roche Applied Biosytems) or 6 μL of λ-Phosphatase (New England Biolabs) was added to the respective samples and all samples were incubated 1 hour at 30°C. Then, samples were boiled 10 min with Laemmli buffer and analyzed by SDS PAGE followed by the Western blotting.

Measurement of dNTP levels
NTP and dNTP extraction and quantification were performed as previously described [7]. Nucleotides were analyzed by HPLC on a Partisphere SAX-5 HPLC column (4.6 mm x 125 mm, Whatman International Ltd.) using a UV-2075 Plus detector (Jasco, Tokyo, Japan).

Analysis of HU tolerance and measurement of the frequency of petite formation
Mid-log phase cells were collected, sonicated, and plated at appropriate dilutions. For spot assays, 2 μL of 10-fold serial dilutions were spotted onto YPD plates or YPD plates containing 200 mM HU. Cells were grown at 30°C for 3 days. Measurement of the frequency of petite formation was done as described before [5].

Supporting Information
Figure S1 (A) Western blot analysis of Rnr2, Rnr3-HA (detected with anti-HA or with rabbit polyclonal anti-Rnr3 antibodies), and Rnr4 in the wild-type (wt) (AC447-2A) and in ixr1Δ (TOY621) strains before and after 2 hours treatment with 0.2 mg/L 4-NQO. (B) Specificity of the YL1/2 antibodies used for the detection of Rnr4. Wild-type (W1588-4C), ixr1Δ (TOY736), and mR4A (CUY995) strains were analyzed before and after treatment with 0.2 mg/L 4-NQO. The Rnr4 band is absent in the mR4A strain. Instead, a band of higher molecular weight appears in the position corresponding to Rnr2. (C) Western blot analysis of Rnr1 and Rnr4 levels in the wild-type (wt) (W1588-4C), ixr1Δ (TOY736), sml1Δ (U952-3B), and ixr1Δ sml1Δ (TOY778) strains after 2 hours treatment with 0.2 mg/L 4-NQO. Rnr1 and Rnr4 levels were quantified as described in Materials and Methods. RU, relative units.

Table S1 Primers used in this study. All insertions were confirmed with PCR, each strain was back-crossed with W1588-4C.

Table S2 Antibodies used in this study.

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Author Contributions
Conceived and designed the experiments: OT EB SUA˚ AC. Performed the experiments: OT EB. Analyzed the data: OT EB SUA˚ AC. Wrote the paper: OT AC.
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