A vertebrate gene, *ticrr*, is an essential checkpoint and replication regulator

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Eukaryotes have numerous checkpoint pathways to protect genome fidelity during normal cell division and in response to DNA damage. Through a screen for G2/M checkpoint regulators in zebrafish, we identified *ticrr* (for TopBP1-interacting, checkpoint, and replication regulator), a previously uncharacterized gene that is required to prevent mitotic entry after treatment with ionizing radiation. *Ticrr* deficiency is embryonic-lethal in the absence of exogenous DNA damage because it is essential for normal cell cycle progression. Specifically, the loss of *ticrr* impairs DNA replication and disrupts the S/M checkpoint, leading to premature mitotic entry and mitotic catastrophe. We show that the human TICRR ortholog associates with TopBP1, a known checkpoint protein and a core component of the DNA replication preinitiation complex (pre-IC), and that the TICRR–TopBP1 interaction is stable without chromatin and requires BRCT motifs essential for TopBP1's replication and checkpoint functions. Most importantly, we find that *ticrr* deficiency disrupts chromatin binding of pre-IC, but not prereplication complex, components. Taken together, our data show that TICRR acts in association with TopBP1 and plays an essential role in pre-IC formation. It remains to be determined whether Ticrr represents the vertebrate ortholog of the yeast pre-IC component Sld3, or a hitherto unknown metazoan replication and checkpoint regulator.

[*Keywords*: DNA replication; S/M checkpoint; G2/M checkpoint; pre-RC; pre-IC; TopBP1; SLD3]

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Eukaryotic cells possess numerous mechanisms to ensure the fidelity of the genome. In dividing cells, DNA replication is the primary potential source of errors. To ensure that DNA replication occurs at the appropriate cell cycle stage, and that the DNA is copied once and only once, this process is divided into two temporally distinct steps (Bell and Dutta 2002; Sclafani and Holzen 2007). During G1, the replicative helicase, the Mcm2–7 complex, is loaded onto origin DNA by the ORC (origin recognition complex), Cdc6, and Cdt1 proteins in a process called prereplication complex (pre-RC) formation. The loaded helicase is inactive, however, and is only activated upon entry into S phase during preinitiation complex (pre-IC) formation. This process requires CDK (cyclin-dependent kinase) and DDK (Dbf4-dependent kinase) activity, and involves recruitment of additional proteins to the Mcm2–7 complex, including TopBP1, Cdc45, and the GINS complex (Hashimoto and Takisawa 2003; Kubota et al. 2003; Aparicio et al. 2009). Once activated, the helicase and its associated proteins recruit the remaining DNA synthesis machinery leading to the formation of a pair of bidirectional replisomes.

In addition to the core replication machinery, multiple checkpoint pathways exist to protect cells from DNA damage arising from replication errors and/or genotoxins (Bartek et al. 2004; Harper and Elledge 2007). Two of these pathways function during DNA replication: The intra-S-phase checkpoint stabilizes existing replication forks while inhibiting firing of late origins, and the S/M checkpoint prevents the cell from prematurely entering mitosis before it has fully replicated the genome (Bartek et al. 2004). Abrogation of the latter pathway allows cells to enter mitosis with incompletely replicated chromosomes, leading to chromosome fragmentation and segregation defects and, often, mitotic catastrophe (Canman 2001). In G2, a checkpoint blocks mitotic entry when DNA is damaged. This pathway is rapidly activated when cells are exposed to ultraviolet (UV) or ionizing radiation (IR), which primarily cause bulky DNA adducts or dsDNA breaks, respectively. Mutations that prevent cells from appropriately responding to DNA damage cause human developmental disorders, cancer, and aging (Harper and Elledge 2007). Importantly, nearly all cancer cells have

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partially impaired checkpoints, and thus checkpoint pathway components have emerged as important targets for anti-cancer drugs.

Considerable attention has focused on identifying the proteins that contribute to these checkpoint pathways. This has established the PIKK kinases, ATM and ATR, as playing a central role in DNA damage response (Harrison and Haber 2006). In vertebrates, ATM responds primarily to double-stranded breaks, while ATR is more versatile, responding to a wide range of damage or replication stress (Brown and Baltimore 2003). ATR is a key player in the S/M checkpoint and is also required for the radiation-induced G2/M checkpoint (Nghiem et al. 2001). Once activated, ATR phosphorylates and activates the CHK1 kinase. Importantly, activated CHK1 phosphorylates and inhibits the CDC25 phosphatases, thereby preventing the activation of the Cyclin/CDK kinases and blocking cell cycle progression.

Numerous other proteins have been identified as sensors and/or mediators in checkpoint signaling pathways (Harrison and Haber 2006). Human TopBP1 and its orthologs Cut5 (in *Xenopus*), Mus101 (in *Drosophila*), Cut5/Rad4 (in *Saccharomyces pombe*), and Dpb11 (in *Saccharomyces cerevisiae*) are particularly intriguing because they have been shown to be critical not only for checkpoint response, but also DNA replication initiation in unperturbed cells (Garcia et al. 2005). Studies in numerous organisms establish TopBP1 as essential for the initiation of DNA replication. Consistent with these observations, TopBP1 associates with Cdc45, and the recruitment of TopBP1, Cdc45, and GINS that is required for the pre-RC-to-pre-IC transition appears to be interdependent (Van Hatten et al. 2002; Kubota et al. 2003; Takayama et al. 2003; Schmidt et al. 2008). Sld3, a protein that is additionally essential for pre-IC formation in yeast, also associates with Dpb11^{TopBP1}. However, to date, Sld3 orthologs have not been identified in higher eukaryotes. Importantly, TopBP1 has also been identified as being essential for both the intra-S and S/M checkpoints in numerous organisms (Garcia et al. 2005). Although it seemed plausible that these checkpoint functions are an indirect consequence of TopBP1's replication function, this is not the case: Studies with conditional and separation of function mutants show that TopBP1's checkpoint function can be clearly separated from its action in pre-IC formation (McFarlane et al. 1997; Saka et al. 1997; Hashimoto et al. 2006; Yan et al. 2006). Finally, TopBP1 is also required for activation of the G2/M checkpoint in response to DNA-damaging agents (Garcia et al. 2005). The widespread roles of TopBP1 are consistent with the broad spectrum of phenotypes resulting from TopBP1 deficiency. For example, mus101^{TopBP1} mutants display defects in chorion gene amplification, hypersensitivity to DNA-damaging agents, and mitotic chromosome instability (Yamamoto et al. 2000).

The predominant feature of the TopBP1 protein is that it contains multiple BRCT motif repeats (from four in yeast to eight in humans) (Garcia et al. 2005). These domains commonly mediate protein–protein interactions, and in some cases pairs of BRCT domains act as phosphopeptide-binding motifs. Structure–function studies have revealed a critical role for the N-terminal BRCT domains in TopBP1 function (Garcia et al. 2005). The BRCT domains in this part of the protein are conserved from yeast to humans. Accordingly, this N-terminal half is both necessary and sufficient for DNA replication (Hashimoto et al. 2006). Moreover, BRCT motifs I and II are required for binding to CDK-phosphorylated Sld3 and also for TopBP1's checkpoint function in vertebrates (Lee et al. 2007; Tanaka et al. 2007; Zegerman and Diffley 2007; Yan and Michael 2009). Additional BRCT domains in the C-terminal half of TopBP1 of higher eukaryotes are also involved in the response to DNA damage and replication stress. Specifically, BRCTs VII and VIII are important for ATR-dependent phosphorylation of Chk1 in response to replication stress in *Xenopus* extracts (Yan et al. 2006; Yan and Michael 2009), and BRCT domain V is required for TopBP1 to form nuclear foci in response to damage or stalled replication forks (Yamane et al. 2002).

In this study, we use zebrafish to conduct a screen for vertebrate DNA damage regulators. This screen identified a novel gene, *ticrr* (for TopBP1-interacting, checkpoint, and replication regulator), which is required for both the G2/M and S/M checkpoints and for normal DNA replication. This spectrum of defects is highly reminiscent of those arising in TopBP1 mutants. Accordingly, we show that TICRR binds to TopBP1 in vivo and is essential for pre-IC formation in a similar manner to TopBP1.

Results

An insertional mutation in zebrafish that abrogates IR-induced cell cycle arrest

Zebrafish is an excellent model in which to conduct genetic screens for vertebrate cell cycle and checkpoint regulators. This is due primarily to its small size and fecundity, but also because maternal mRNA stores allow embryos to survive to developmental stages at which defects in cell-essential genes can be assayed. In addition, through a pilot genetic screen, we validated our ability to identify novel cell cycle regulators using zebrafish (Sansam et al. 2006). In this prior study, we assayed mitotic index through whole-mount staining of zebrafish embryos for phosphorylated (Ser 10) histone H3 (pH3). Moreover, we established that the number of pH3-positive cells decreases rapidly when zebrafish embryos are exposed to 15 Gy IR, showing that the G2/M checkpoint is intact in these embryos.

Given this success, we now applied this screen to a large collection of zebrafish mutants that carry stable viral insertions within 335 different genes (Amsterdam et al. 2004). These lines are fully viable as heterozygotes, but the homozygous mutants display developmental defects 24–72 h post-fertilization (hpf) that are typically lethal. For our cell cycle screen, we intercrossed the heterozygous mutants, treated 50 or more of the resulting embryos at 32 hpf with IR, and assayed pH3 staining 1 h later (Fig. 1A). Lines were considered to have altered mitotic index if

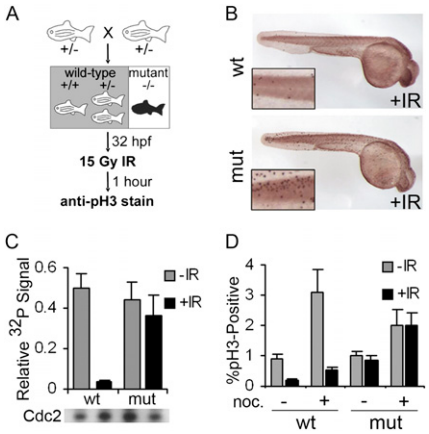

Figure 1. hi1573 zebrafish embryos show a MAI phenotype. (*A*) Scheme of checkpoint screen. (*B*) Whole-mount immunostaining of pH3 showed that IR treatment induces the appropriate block to mitotic entry in 36-hpf wild-type (wt) embryos, but the hi1573 homozygous mutant (mut) clutchmates retained a high mitotic index. (*C*) Cyclin B1–Cdc2 was immunoprecipitated from 40-hpf nonirradiated (−IR) and irradiated (+IR) pools of wild-type (+/+ and +/−) and mutant (−/−) hi1573 embryos. The total levels of Cdc2 were determined by Western blotting (representative experiment shown). Assessment of cyclin B1–Cdc2 kinase activity (mean ± SD; n = 3 biological replicates) showed that the hi1573 mutants retain high activity after IR treatment. (*D*) Nonirradiated and irradiated 36-hpf wild-type (+/+ and +/−) and hi1573 mutant (−/−) clutchmates were maintained in the absence or presence of nocodazole for 2 h. The percentage of pH3-positive cells was quantified by FACS (mean ± SD; n = 20,000 cells counted for each of three biological replicates).

at least one-quarter of the embryos showed altered pH3 staining relative to the rest of the clutch. PCR genotyping of the embryos was used to confirm that the phenotype was linked to the mutant insert. Using this approach, we found that hi1573 (Hopkins insertion line 1573) homozygotes showed a high mitotic index compared with their wild-type and heterozygous clutchmates after IR exposure (Fig. 1B). In contrast, the mitotic index without radiation in the hi1573 mutants was indistinguishable from that of the wild-type embryos (0.91% and 1% pH3-positive for wild-type and mutant embryos, respectively) (Fig. 1D). We refer to this phenotype as mitosis after irradiation (MAI).

A critical step in regulating entry into mitosis is the activation of the Cdc2 kinase by the Cdc25 phosphatases. In the presence of DNA damage, Cdc25 phosphatases are inhibited, thereby preventing Cdc2 kinase activation. We wished to determine whether the increased mitotic index in the hi1573 mutants after DNA damage was associated with high Cdc2 kinase activity. Thus, we performed an in vitro assay of Cyclin B1–Cdc2 complex immunoprecipitated from lysates of 40-hpf embryos identified as either mutant or wild type, based on the presence or absence of the characteristic hi1573 developmental phenotype. IR exposure of wild-type embryos resulted in a 14-fold decrease in the phosphorylation of a synthetic peptide substrate for Cdc2 (Fig. 1C). This is consistent with the decrease in mitotic index and the activation of the G2/M checkpoint in response to IR. In contrast, the activity of Cyclin B1–Cdc2 remained high after DNA damage in the hi1573 mutants (Fig. 1C), consistent with the persistence of mitotic cells.

We reasoned that the MAI phenotype and the high Cdc2 kinase activity could arise in two possible ways: The mutation could abrogate the checkpoint that prevents mitotic entry after DNA damage, or it could reflect a failure to efficiently exit mitosis. To distinguish between these two possibilities, we performed a nocodazole trapping experiment using 36-hpf embryos. For this assay, nocodazole was added immediately after exposure to IR, and then the embryos were incubated for 2 h before disaggregating and quantifying the percentage of pH3-positive cells with 4N DNA content by fluorescence-activated cell sorting (FACS). Nocodazole causes early to mid-mitotic arrest, so an accumulation of mitotic cells following IR and nocodazole treatment would indicate that the cells continue to enter mitosis. In the absence of irradiation, the level of 4N, pH3-positive cells was increased in the nocodazole-treated, versus the untreated, embryos for both the wild-type (3.4-fold) and hi1573 mutants (twofold) (Fig. 1D). IR treatment greatly reduced the level of mitotic cells in the wild-type embryos in both the absence and presence of nocodazole (Fig. 1D). This is consistent with the existence of a robust G2/M checkpoint. In contrast, the level of 4N, pH3-positive cells in the hi1573 mutants was completely unaffected by IR treatment. Indeed, we still observed the twofold increase in mitotic cells that results from nocodazole treatment. Taken together, our data show that the G2/M checkpoint is abrogated in the hi1573 mutants, and these cells continue to enter mitosis following DNA damage.

*The MAI phenotype is caused by insertions in a novel gene (*ticrr*)*

We mapped the hi1573 viral insertion just upstream of an uncharacterized ORF (*5730590G19-like*) on chromosome 25 (Fig. 2A). A second line in the collection, hi3202A, included a distinct mutant allele of *5730590G19-like* with a viral insertion in the first predicted coding exon (Fig. 2A). Notably, hi3202A homozygotes have a normal mitotic index in the absence of irradiation (data not shown), and display the MAI phenotype (Fig. 2B) just like the hi1573 mutants. The hi1573 and hi3202A homozygotes also have identical developmental phenotypes: At 36 hpf, these lines develop a dark head that is characteristic of widespread apoptosis in the CNS (Amsterdam et al. 2004). We confirmed that these lines are apoptotic, with increased acridine orange and anti-cleaved caspase-3 staining appearing at 26 hpf (data not shown). Typically, the retroviral insertions in the Hopkins mutant collection cause a decrease in mRNA expression that results in recessive, loss-of-function phenotypes. We performed quantitative RT–PCR to measure the expression of *5730590G19-like* at 36 hpf in wild-type and mutant embryos, and found that both the hi1573 and hi3202A insertions cause a strong reduction in mRNA expression of this gene (Fig. 2C,D). Taken together, these data indicate that disruption

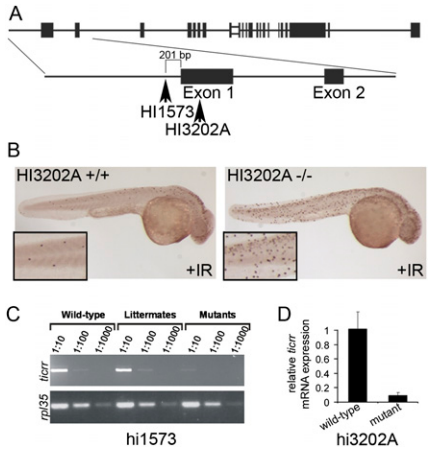

Figure 2. The checkpoint defect results from disruption of a novel gene, *ticrr*. (*A*) Schematic of the zebrafish *ticrr* gene denoting the position of viral insertions in the hi1573 and hi3202A mutant lines. (*B*) Analysis of pH3 showed a low mitotic index in wild-type embryos but not hi3202A mutant clutchmates 1 h after IR exposure, showing that hi3202A also has a checkpoint defect. (*C*,*D*) Quantification of total mRNA by RT–PCR showed that the levels of *ticrr* mRNA were greatly reduced in both the hi1573 (*C*) and hi3202A (*D*) homozygous mutants at 36 hpf.

of the novel gene *5730590G19-like* causes embryonic lethality and the MAI phenotype. In the following sections, we describe additional roles for this gene. In recognition of its broad spectrum of functions, we named this gene *ticrr*.

Disruption of ticrr *impairs S-phase progression and causes premature chromatin condensation*

DNA damage checkpoint genes are often required for normal cell cycle control. For example, TopBP1 is required for both checkpoint activation in response to DNA damage and for the initiation of DNA replication in undamaged cells (Garcia et al. 2005). Consistent with this precedent, our analysis of the hi1573 and hi3202A mutant zebrafish revealed that *ticrr* is required for cell cycle control in the absence of DNA damage. FACS analysis of DNA content revealed that 40-hpf *ticrr* mutant embryos had a higher percentage of cells with between 2N and 4N DNA content than the wild-type control, suggesting an accumulation of cells in S phase (Fig. 3A). To further explore this possibility, we compared the ability of wild-type and *ticrr* mutant 40-hpf embryos to incorporate the nucleotide analog bromodeoxyuridine (BrdU) (Fig. 3B). The wild-type embryos had a high level of cells that had between 2N and 4N DNA content and were BrdU-positive. This is indicative of the high rate of cell proliferation at this early stage of zebrafish development. In contrast, the 40-hpf *ticrr* mutant embryos incorporated very little BrdU, even though a significant fraction of the cells had between 2N and 4N DNA content. This shows that the *ticrr*-deficient cells are impaired for DNA replication.

In addition to this replication defect, the 40-hpf *ticrr* mutants displayed defects in mitotic progression. First, quantification of the percentage of cells in each phase of mitosis by visual inspection of chromatin morphology of pH3-positive cells showed that the distribution of these populations differed between wild-type and *ticrr* mutant zebrafish (Fig. 3C). Specifically, the *ticrr* mutants had a much higher percentage of cells that appeared to be in

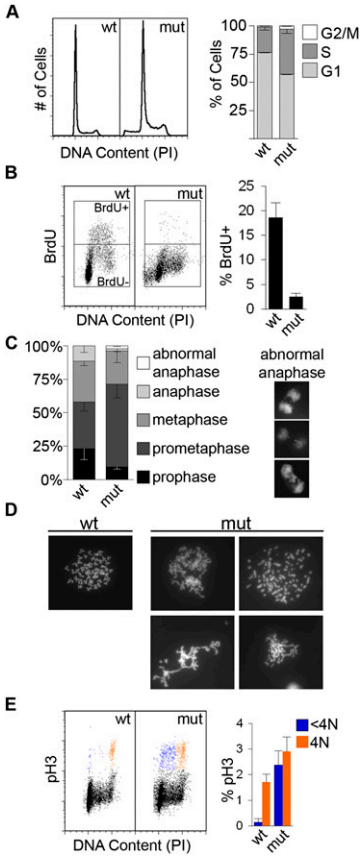

Figure 3. Ticrr is required for normal DNA replication and S/M checkpoint function. (*A*) Cell cycle profile from a representative pool of 40-hpf hi1573 wild-type (+/+ and +/−) and mutant (−/−) zebrafish embryos and quantification of cells with G1, S, or G2/M DNA content (mean ± SD, $n = 3$ biological replicates) showed an increase in S-phase cells in the *ticrr* mutants. (*B*) BrdU and propidium iodide (PI) FACS analysis of cells from pools of BrdU pulse-labeled wild-type and *ticrr* mutant embryos. Quantification of the $BrdU^+$ population (mean ± SD, $n = 3$ biological replicates) showed a dramatic reduction in replicating DNA in *ticrr* mutants. (*C*) Quantification of the proportion of pH3-positive cells in various mitotic phases (mean ± SD; $n = 100$ cells counted in each of four wild-type and mutant embryos) established a defect in mitotic progression and the presence of abnormal anaphase cells. Representative cells with anaphase bridges are shown. (*D*) Metaphase spreads of cells from colchicine-treated embryos showed mitotic cells with fragmented chromosomes. A representative wild-type metaphase spread and examples of abnormal metaphase spreads from mutants are shown. (*E*) FACS measurement of anti-pH3 and PI staining of cells from wild-type and mutant embryos showed a population of mutant pH3-positive cells with less than 4N DNA content (blue) in *ticrr* mutants, establishing entry into mitosis before completing DNA replication.

prometaphase, as judged by the presence of condensed chromatin that is not aligned on the metaphase plate. Moreover, they had a reduced percentage of anaphase cells and, within this population, anaphase bridges were prevalent (Fig. 3C). To explore this defect further, we analyzed chromosome spreads from colchicine-arrested cells (Fig. 3D). This revealed a low incidence of cells with highly fragmented, condensed chromosomes in the *ticrr* mutant, but never wild-type, zebrafish (Fig. 3D). This level of chromosomal fragmentation is consistent with cells that have undergone premature chromatin condensation. Given their DNA replication problems, we hypothesized that the *ticrr* mutants have an impaired ability to activate the S/M checkpoint and thus enter mitosis with partially replicated DNA. To address this possibility, we used FACS to determine the DNA content of pH3-positive cells (Fig. 3E). It is well established that histone H3 phosphorylation begins in late G2, peaks during metaphase, and then declines through anaphase (Hendzel et al. 1997). Accordingly, in the wild-type embryos, histone H3 phosphorylation is restricted to the cells with 4N DNA content (Fig. 3E). In contrast, we find that a high percentage of the pH3-positive cells in the *ticrr* mutants have less than 4N DNA content. Taken together, the cell cycle defects observed in 40-hpf *ticrr* mutants show that *ticrr* is required in the absence of exogenous DNA damage for S-phase progression and also for activation of the S/M checkpoint. We propose that the failure of these two processes causes cells to enter mitosis with partially replicated genomes, resulting in the chromosomal abnormalities and loss of anaphase cells observed in the *ticrr* mutants.

If *ticrr* mutants are defective in DNA replication and the S/M checkpoint, the DNA replication defect should be the primary defect and the mitotic abnormalities a secondary consequence. To test this, we turned to an earlier developmental time point, when the *ticrr* defects were just beginning to arise. Since we first detect apoptosis in the hi1573 mutants at 26 hpf, we used 24-hpf embryos to screen for cell cycle defects. At this time point, the *ticrr* mutant embryos are morphologically indistinguishable from the wild types. Thus, *ticrr* mutants were identified by PCR-genotyping a small fraction of cells dissociated from individual embryos. The remaining cells from 20 embryos of each genotype were then pooled for cell cycle analysis. FACS showed that there is already an increase in the percentage of cells with between 2N and 4N DNA content in the 24-hpf *ticrr* mutants compared with the wild-type controls, suggesting that cells are proceeding slowly through S phase (Fig. 4A). Moreover, the *ticrr* mutants had a lower average level of BrdU incorporation in these S-phase cells than the wild types (Fig. 4B). Thus, the defect in DNA replication clearly precedes the initiation of apoptosis. Notably, analysis of the DNA content of pH3-positive cells showed that, unlike the 40-hpf embryos, pH3-positive cells with sub-4N DNA content are not observed in the 24-hpf *ticrr* mutants, indicating that there is no premature mitotic entry at this time point (Fig. 4C). These data show that the DNA replication defect is the earliest detectable phenotype, and thus is not a consequence of the premature mitotic entry.

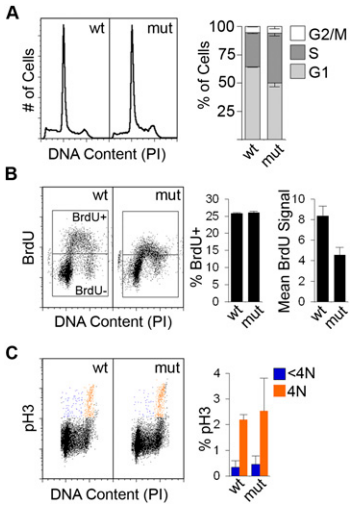

Figure 4. A DNA replication defect but not premature mitotic entry occurs in 24-hpf *ticrr* mutants. (*A*) Cell cycle profiles from 24-hpf hi1573 wild-type (+/+) and mutant (−/−) zebrafish embryos and quantitation of cells with G1, S, or G2/M DNA content (mean ± SD, $n = 3$ biological replicates), showed an increase in S-phase cells in mutants. (*B*) BrdU and PI FACS analysis of cells from pools of BrdU pulse-labeled, 24-hpf wild-type and *ticrr* mutant embryos. Quantification of the $BrdU^+$ population showed no change in the percentage of cells replicating DNA (mean ± SD, n = 20,000 cells in each of three biological replicates), but the level of BrdU incorporation per cell is significantly decreased in the mutants (mean BrdU signal = Mean[$BrdU^+$ signal]/Mean[BrdU− signal]; Student's t-test $P < 0.05$). (*C*) FACS measurement of anti-pH3 and PI staining of cells from 24-hpf wild-type and mutant embryos showed no evidence of an S/M defect in the *ticrr* mutants at this time point, as judged by the absence of pH3-positive cells with less than 4N DNA content.

TICRR binds TopBP1

The phenotypes of the *ticrr* mutant embryos are highly reminiscent of those resulting from depletion of TopBP1/Dbp11/Cut5/Mus101. Specifically, these deficiencies have been shown to disrupt DNA replication, the S/M checkpoint, and DNA damage checkpoints (Garcia et al. 2005). This raised the possibility that Ticrr acts in the TopBP1 pathway. In particular, we wondered whether Ticrr might be the vertebrate ortholog of the budding yeast protein Sld3, which interacts with Dpb11 and is required for DNA replication initiation (Kamimura et al. 2001; Tanaka et al. 2007; Zegerman and Diffley 2007). To test this hypothesis, we examined whether the Ticrr protein shows any sequence homology with Sld3 or any other protein that might yield insight into its biochemical function. The zebrafish *ticrr* gene is predicted to encode a 1824-residue protein (NM_001003887.1) with an anticipated molecular mass of 202 kDa. Genes encoding proteins with significant homology exist in other vertebrates, including humans, mice, and *Xenopus* (Table 1; Supplemental Fig. 1). We also found putative Ticrr

Table 1. *Conservation between* Danio rerio *Ticrr and orthologs identified by psiBLAST*

Species	Accession number	Length (amino acids)	psiBLAST results				ClustalW alignment
			HSP length (amino acids)	Percentage identity	Percentage positives	Percentage gaps	Percentage identity
D. rerio	NP_001003887.1[a]	1824					
Homo sapiens	NP_689472.3	1910	1126	35	52	8	30
Mus musculus	NP_084111.1	1889	1114	35	52	7	28
Gallus gallus	XP_413862.2	1679 (1937[b])	1113	34	50	13	29
X. laevis	AAH73061.1	1394	1098	36	54	6	30
B. floridae	XP_002215127.1	1911	1147	22	38	12	16
C. intestinalis	XP_002120860.1	1441	1172	19	34	16	14
S. purpuratus	XP_792617.1	2305 (1768[b])	654	22	37	16	11
I. scapularis	XP_002416131.1	1495	1111	20	35	19	16
C. elegans	NP_491752.1	919	472	21	37	15	11
N. vectensis	XP_001628252.1	1845	1125	22	37	14	14
T. adhaerens	XP_002111061.1	1069	515	18	37	17	14

(HSP) High-scoring segment pairs.
[a]Sequence used in Psi-BLAST was truncated at amino acid 1181 before a long stretch of low complexity proline-rich sequence.
[b]Length of predicted Xl and Sp proteins from manually refined gene models.

orthologs in other metazoans, including the primitive chordates *Branchiostoma floridae* and *Ciona intestinalis*, the echinoderm *Strongylocentrotus purpuratus*, the arthropod *Ixodes scapularis*, the nematode *Caenorhabditis elegans*, the cnidarian *Nematostella vectensis*, and the placozoan *Trichoplax adhaerens*. The conservation across these proteins maps to specific stretches, yielding high confidence in its significance, but the overall conservation is poor (Table 1; Supplemental Fig. 1). We were unable to detect putative Ticrr orthologs in *Drosophila*, and there was no significant homology with any yeast protein, including Sld3. However, given how rapidly the conservation falls off among the metazoan Ticrr proteins, and also the poor sequence conservation of Sld3 across the fungi kingdom, it seemed plausible that Ticrr could be a functional analog of Sld3, and yet bear no significant sequence similarity. Notably, despite their large size, the Ticrr proteins have no known functional motifs that could help to infer their biochemical activity.

In the absence of any functional insight from the protein sequence, we investigated the biochemical properties of the Ticrr protein. For these studies, we switched to human cells because of the availability of reagents for known replication and checkpoint regulators. Human *TICRR* is an uncharacterized gene on chromosome 15 (C15orf42). The NCBI Reference Sequence database contained a predicted full-length *TICRR* mRNA sequence (NM_152259.3), inferred from partial cDNA and genomic sequences, and the encoded human TICRR protein is predicted to be 211 kDa. We generated a full-length 5753-bp *TICRR* ORF by amplifying two overlapping cDNA fragments from HeLa cell mRNA. We also used a 30-kDa, C-terminal fragment of the TICRR protein to raise multiple polyclonal antisera in mice and rabbits. These successfully recognized overexpressed TICRR and also an endogenous protein of ~250 kDa by both Western blotting and immunoprecipitation (Fig. 5; data not shown). This band was verified to be TICRR by partial knockdown using *TICRR* shRNAs (data not shown). Unfortunately, we were unable to achieve full knockdown of the endogenous TICRR to allow assessment of its cell cycle role in human cells.

Having established that we can detect the endogenous TICRR protein, we assessed its subcellular localization through biochemical fractionation of human cells. TICRR was entirely recovered from nuclear extracts of asynchronously growing HeLa-S3 cells and was not present in cytosolic extracts. Moreover, TICRR fractionated with the insoluble nuclear material and was resistant to extraction from nuclei by low salt (150 mM NaCl) and nonionic detergent (1% NP-40) but could be extracted with high salt (0.3 M NaCl) or ionic detergents (0.1% SDS or 0.5% deoxycholate) (data not shown). These results strongly suggested that TICRR associates with chromatin. Dpb11^{TopBP1} and Sld3 are both chromatin-associated; thus, we further tested whether TICRR was bound to chromatin by treating the insoluble nuclear material with nuclease. For this analysis, we used a component of the ORC, ORC2, as a positive control. We found that treatment of the chromatin/nuclear matrix material with micrococcal nuclease caused partial release of TICRR, and this mirrored the level of release of ORC2 (Fig. 5A). These results demonstrate that a large fraction of TICRR is associated with chromatin, as has been described previously for TopBP1 (Garcia et al. 2005).

We next asked whether the endogenous TICRR and TopBP1 proteins interacted. For this analysis, proteins were extracted from nuclei using ionic detergent (0.1% sodium dodecyl sulfate) and then immunoprecipitated with either preimmune or anti-TICRR polyclonal rabbit antisera. Subsequent Western blotting established that TopBP1 was present in the anti-TICRR, but not in preimmune, immunoprecipitates (Fig. 5B). Moreover, we were able to conduct a reciprocal immunoprecipitation/immunoblotting experiment to show that TICRR coimmunoprecipitated with TopBP1 (Fig. 5C). Since both

TICRR and TopBP1 are chromatin-associated proteins, it seemed possible that DNA bridged the interaction between these two proteins. However, we were able to show that these two proteins continued to coimmunoprecipitate even if the lysates were pretreated with ethidium bromide or DNase I (Fig. 5B). Thus, we conclude that human TICRR and TopBP1 are associated proteins, and this interaction can occur in the absence of DNA.

Given the relatively poor sequence homology of TICRR across species, it was important to determine whether the TopBP1-binding ability of human TICRR was conserved in the zebrafish Ticrr protein. Since antibodies were not available for zebrafish Ticrr, we cloned the full-length zebrafish Ticrr coding sequence and overexpressed GFP-tagged versions of either zebrafish Ticrr (GFP-zTicrr) or human TICRR (GFP-hTICRR) in human cells along with human TopBP1. Notably, human TopBP1 was recovered with similar efficiency in GFP-zTicrr and GFP-hTICRR immunoprecipitates, even though the GFP-zTicrr protein was poorly expressed (Fig. 5D). This cross-species binding shows unequivocally that the Ticrr–TopBP1 interaction is conserved between humans and zebrafish.

The human TopBP1 is a large multifunctional protein whose predominant feature is the presence of eight BRCT domains. The yeast homologs of TopBP1, Dpb11, and Cut5 have only four BRCT domains that are highly conserved with BRCTs I, II, IV, and V of the vertebrate TopBP1 proteins (Hashimoto et al. 2006). Prior studies have shown that individual or pairs of BRCT domains mediate interactions with specific proteins, and various known TopBP1 functions have been mapped to specific BRCT domains. The N-terminal half of *Xenopus laevis* TopBP1, which includes the four highly conserved BRCTs, is both necessary and sufficient for the initiation of DNA replication (Hashimoto et al. 2006). Moreover, BRCT motifs I and II appear to be particularly important in both replication and checkpoint functions: These are required for Dpb11^{TopBP1} to bind to Sld3 (Tanaka et al. 2007; Zegerman and Diffley 2007) and for interaction with the 9–1–1 checkpoint complex (Furuya et al. 2004; Delacroix et al. 2007; Lee et al. 2007). If the interaction between TopBP1 and TICRR is relevant to their DNA replication functions, then TICRR would be predicted to interact with the N-terminal BRCTs of TopBP1. To address this, we created a panel of human TopBP1 mutants in which specific BRCT domains were deleted either singly or pairwise (ΔI + II, ΔIII, ΔIV + V, ΔVI, and ΔVII + VIII), and tested their ability to bind to TICRR in cotransfection assays (Fig. 5E). All five of the TopBP1

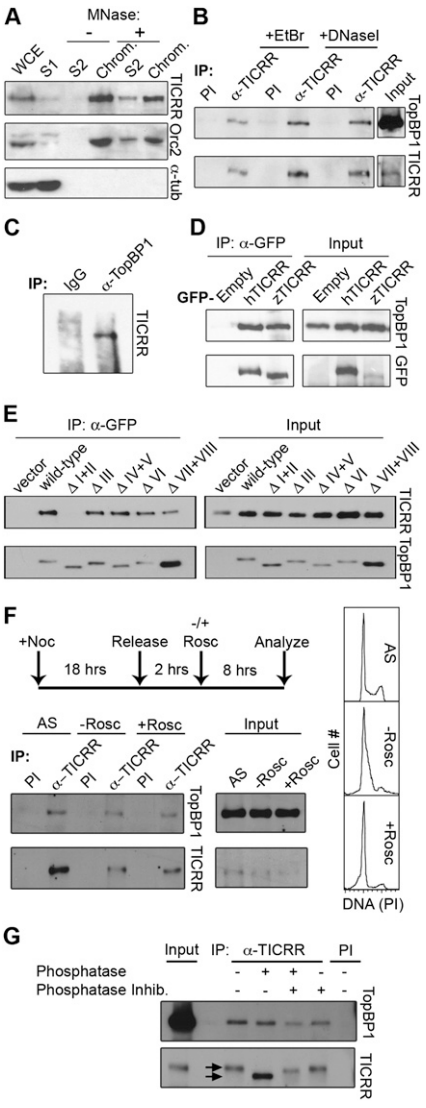

Figure 5. Human TICRR is a chromatin-associated protein that interacts with TopBP1. (*A*) Western blotting of biochemical fractions from HeLa cells showed that the majority of TICRR is present in the chromatin-enriched fraction (chrom) and can be partially released by micrococcal nuclease (Mnase) treatment. Orc2 and α-tubulin are chromatin and cytoplasmic markers. (WCE) Whole-cell extract; (S1) soluble cytoplasmic fraction; (S2) soluble nuclear fraction. (*B*) Immunoblotting showed TopBP1 coimmunoprecipitates with TICRR but not preimmune (PI) antibodies. The association was not affected by treatment with ethidium bromide (EtBr) or DNase I, indicating its independence from chromatin binding. (*C*) Reciprocal immunoprecipitation-Western blotting confirmed that TICRR is present in TopBP1, but not IgG, immunoprecipitates. (*D*) Immunoprecipitation-Western blotting showed that GFP-tagged zebrafish Ticrr associated with human TopBP1 when coexpressed in human cells. (*E*) Anti-GFP immunoprecipitates and whole-cell lysates (input) from cells transfected with GFP-tagged TopBP1 deletion mutants were screened for TICRR and TopBP1 proteins by immunoblotting. (*F*) HeLa cells were synchronized in mitosis and then allowed to re-enter the cell cycle in the absence (−Rosc) or presence (+Rosc) of roscovitine as depicted. FACS analysis showed that the −Rosc cells were entering S phase, while the +Rosc cells remained in G1. Immunoprecipitation-Western analysis shows that the TICRR–TopBP1 complex was recovered at similar levels in the −Rosc and +Rosc samples. (AS) Asynchronous. (*G*) Asynchronous HeLaS3 cell extracts were immunoprecipitated with anti-TICRR antibodies and then incubated with either λ phosphatase, phosphatase inhibitors, or both. The mobility of the TICRR protein was significantly increased in the phosphatase-treated sample versus the controls, but showed no difference in the levels of associated TopBP1.

mutants were expressed at similar levels to the wild-type TopBP1. TICRR coimmunoprecipitated with four of these mutants (ΔIII, ΔIV + V, ΔVI, and ΔVII + VIII) and with wild-type TopBP1. In contrast, no TICRR was recovered in the immunoprecipitate of the ΔI + II deletion mutant, even though TICRR was present at high levels in these cells. Taken together, our data show that TICRR associates with TopBP1 in vivo, and this interaction requires the two N-terminal BRCT domains that have been associated with TopBP1's role in both checkpoint signaling and DNA replication.

We were intrigued to find that TICRR association maps to the region of TopBP1 that is required for Sld3 binding. In yeast, formation of the Sld3–Dpb11 complex is known to require CDK phosphorylation of Sld3, and this, together with the phosphorylation of Sld2, accounts for the CDK dependence of DNA replication in this organism (Tanaka et al. 2007; Zegerman and Diffley 2007). CDK is also essential for replication in human cells, but there is some doubt as to whether pre-IC components will be the relevant target (DeGregori et al. 1995). Given these questions, we used two complementary approaches to examine the role of CDK phosphorylation in the TICRR–TopBP1 interaction. First, we generated a population of HeLa cells that were synchronously re-entering G1 from mitosis, and then we cultured them in the absence or presence of the pan-CDK inhibitor roscovitine for 8 h (Fig. 5F). Consistent with the known CDK dependence of S-phase entry, FACS analysis showed that the untreated cells (−ROSC) were beginning to enter S phase, while the roscovitine-treated cells (+ROSC) remained blocked in G1 (Fig. 5F). Notably, TopBP1 was recovered at comparable levels in the TICRR immunoprecipitates of both the untreated and treated cells (Fig. 5F), suggesting that binding occurs in the presence of CDK inhibition. In the second approach, we incubated TICRR–TopBP1 immunoprecipitates from asynchronous cell extracts with or without λ phosphatase, and then assayed the association of TICRR and TopBP1 by Western blotting. Notably, phosphatase treatment significantly increased the mobility of the TICRR protein (Fig. 5G), indicating that TICRR is phosphorylated in vivo and that we successfully removed this modification. Despite this change, there was no detectable difference in the levels of the TICRR–TopBP1 complex in phosphatase-treated versus untreated cells (Fig. 5G). Importantly, this result was not altered by DNase treatment of the extracts prior to immunoprecipitation (data not shown), indicating that this complex formation occurs in the absence of chromatin binding. Taken together, these synchronization and phosphatase experiments strongly suggest that the interaction between TICRR and TopBP1 can occur in a CDK-independent manner.

Ticrr deficiency inhibits pre-IC formation

The TopBP1 orthologs in *X. laevis* and budding and fission yeast are known to be essential for the transition of the pre-RC into the pre-IC, an intermediate in the initiation of DNA replication (Garcia et al. 2005). Having established that *ticrr* is essential for normal DNA replication in zebrafish, and that TICRR and TopBP1 associate in the absence of DNA, we asked whether *ticrr* is similarly required to form the pre-IC. To address this question, we employed the wild-type and *ticrr* mutant zebrafish. For this analysis, we used embryos at 40 hpf, the developmental time point at which the *ticrr*-deficient cells have a profound replication defect, as judged by S-phase accumulation and strongly reduced BrdU incorporation. Pools of wild-type and mutant embryos were dissociated and used to generate extracts from whole-cell or chromatin-enriched fractions. These cells were then assayed by Western blotting for chromatin association of the Mcm2–7 complex (using a pan-MCM monoclonal antibody) and the GINS complex (using an antibody against Psf1), which are core components of the pre-RC and pre-IC, respectively (Fig. 6). There was no difference in the chromatin association of the Mcm2–7 complex in wild-type versus *ticrr* mutant embryos. In contrast, Psf1 showed a significant level of chromatin association in the wild-type embryos, but was nearly absent (although still detectable on long exposure) from the chromatin-enriched fraction in the *ticrr* mutant, even though the Psf1 protein was present at normal levels in the whole-cell extracts. Taken together, our data show that, in concert with TopBP1, Ticrr is required for the transition from pre-RC to pre-IC, explaining its essential role during DNA replication.

Discussion

Using a screen for zebrafish mutants that fail to arrest mitotic entry after exposure to IR, we identified two mutant lines, each having a different mutation in the *ticrr* gene. In addition to the G2/M checkpoint defect, these lines have a profound apoptotic phenotype in the absence of exogenous damage that revealed a more general role in cell survival during development. Consistent with this observation, we found that *ticrr* mutants at 40 hpf failed to incorporate BrdU, demonstrating that loss of *ticrr* also causes a defect in DNA replication. Instead of arresting in S phase, many cells in the *ticrr* mutants proceed into mitosis and display an array of chromosomal

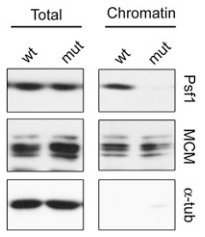

Figure 6. Ticrr is essential for the chromatin association of the pre-IC component Psf1. Total cell lysates and chromatin-enriched fractions were prepared from 40-hpf wild-type (+/+ and +/−) and *ticrr* mutant (−/−) zebrafish embryos. Immunoblotting for core pre-RC and pre-IC components, the MCMs and Psf1, respectively, showed that these are expressed at normal levels, but only the MCMs are chromatin-loaded, in the *ticrr* mutants.

abnormalities—including fragmented chromosomes and anaphase bridges—that likely account for the loss of anaphase cells through mitotic catastrophe. Thus, *ticrr* is required to prevent mitotic entry both following exogenous DNA damage and also when DNA replication is impaired. These phenotypes are highly reminiscent of those arising when TopBP1 and its orthologs are inactivated. Consistent with this observation, we found that the human and zebrafish TICRR proteins both interact with TopBP1 in human cells, and *ticrr* is required for transformation of the pre-RC to the pre-IC in zebrafish. Taken together, these data suggest that TICRR functions with TopBP1 in DNA replication and both the S/M and G2/M checkpoints.

TICRR in DNA replication

The replication machinery and its mechanisms of regulation have been studied in numerous organisms. These processes are generally conserved among eukaryotes, but a unifying model remains elusive because the order of assembly of pre-IC proteins seems to differ between *X. laevis* and yeast, and known key components such as Sld3 have not been identified in higher eukaryotes. The initiation of DNA replication is best understood in *S. cerevisiae*. Here, $Dpb11^{TopBP1}$, Sld2, Sld3, GINS, and Cdc45 are all required to transform the pre-RC into the pre-IC. Sld3 and $Dpb11^{TopBP1}$ form a complex that is induced by the CDK phosphorylation of Sld3 and requires the two N-terminal BRCT domains of Dpb11 (Tanaka et al. 2007; Zegerman and Diffley 2007). Although the formation of the Dpb11–Sld3 complex is critical for replication initiation in *S. cerevisiae*, an analogous event has not been defined in higher eukaryotes.

Much of our understanding of vertebrate DNA replication comes from studies in *X. laevis* (Bell and Dutta 2002; Sclafani and Holzen 2007). In this organism, $Cut5^{TopBP1}$ is also required for the recruitment of DNA polymerases onto chromatin (Hashimoto and Takisawa 2003; Kubota et al. 2003). Thus, the general function of Dpb11 in pre-IC formation seems to be conserved in vertebrate TopBP1. Our data show that TICRR displays some of the core properties of the Sld3 protein. First, Ticrr is essential for DNA replication in unperturbed cells, and it is specifically required for formation of pre-ICs but not pre-RCs. Second, TICRR associates with TopBP1 through the N-terminal BRCT motifs I and II that are conserved with the Sld3-interacting BRCTs of Dpb11. However, our data do not address whether the interaction between TICRR and TopBP1 is direct, and other observations are less consistent with the idea that TICRR is a true ortholog of Sld3. First, we note that there is no detectable sequence similarity between these proteins. This is not a particularly telling finding, since the Sld3 and TICRR proteins are both poorly conserved even within their own kingdoms, but it does raise questions about both the relationship and mechanism(s) of action of these proteins. The second, and more striking, finding is the apparent discrepancy in the role of CDK phosphorylation in Sld3 versus TICRR regulation. Specifically, CDK phosphorylation of Sld3 is required for it to bind to Dpb11, and this, together with the phosphorylation of Sld2, accounts for the CDK dependence of DNA replication in yeast (Tanaka et al. 2007; Zegerman and Diffley 2007). In contrast, we find that the TICRR–TopBP1 interaction is completely unaffected by culturing in the presence of the pan-CDK inhibitor roscovitine or treatment with phosphatase. We note that there are potential limitations to these approaches: The roscovitine-induced block to S-phase entry (Fig. 5F) could reflect a reduction, but not full loss, of CDK activity, and/or the phosphatase may be unable to access the phosphorylated residue(s) mediating the TICRR–TopBP1 interaction, even though it effectively targets other phosphorylation sites (Fig. 5G). Despite these caveats, our data are most consistent with the notion that TICRR associates with TopBP1 in a CDK-independent manner. On first consideration, this finding seems to imply that TICRR is not human Sld3. However, there is evidence to suggest that the underlying basis for the CDK dependence of DNA replication differs in human versus yeast cells. First, DeGregori et al. (1995) have shown that E2F1 expression completely bypasses the CDK dependence of S phase in human cells. This argues that there is no absolute requirement for CDK phosphorylation of either pre-RC or pre-IC components in this organism. Consistent with this conclusion, CDK phosphorylation is required for formation of the yeast Sld2–Dpb11 complex but not the vertebrate counterpart RECQL4–TopBP1 (Matsuno et al. 2006; Tanaka et al. 2007; Zegerman and Diffley 2007). Clearly, additional studies will be required to explore how similar or different Sld3 and TICRR are to one another in the context of both DNA replication and, as described below, checkpoint response.

TICRR in the S/M and G2/M checkpoints

The S/M checkpoint plays a vital role in ensuring that the genome is fully replicated prior to mitotic entry. Our data clearly show that Ticrr is essential for the integrity of this checkpoint in vivo. Thus, Ticrr joins a short list of proteins that play a dual role in both replication regulation and S/M checkpoint response. Notably, the existing dual replication/checkpoint proteins can be divided into two different subclasses, based on their role in the S/M checkpoint. The Mcm2–7 complex, Cdt1, and Cdc45 are representative members of the first subclass. The analysis of yeast conditional mutants shows that the loss of MCMs, Cdt1, or Cdc45 prior to the initiation of replication allows cells with unreplicated DNA to enter mitosis, but the loss of these proteins in replicating cells does not impair the S/M checkpoint (Tercero et al. 2000; Labib et al. 2001). These findings suggest that these pre-RC and pre-IC components are not involved directly in the S/M checkpoint, but they are required to create the replication structures that signal that S phase is ongoing and not yet complete. Studies using *X. laevis* extracts confirm that replication structures are a prerequisite for S-phase checkpoint signaling in vertebrates, and further suggest that the necessary feature is the RNA primer generated by DNA polymerase α (Michael et al. 2000).

TopBP1 is an example of the second class of dual replication/checkpoint protein. Studies with conditional and deletion mutants show that Dpb11/TopBP1's checkpoint function can be separated from its role in pre-IC formation (McFarlane et al. 1997; Saka et al. 1997; Hashimoto et al. 2006; Yan et al. 2006). Biochemical data show that TopBP1 is recruited to the site of replication stress/DNA damage by the 9–1–1 complex, dependent on the same two BRCT motifs that are required for TopBP1's replication function (Furuya et al. 2004; Delacroix et al. 2007; Lee et al. 2007). Once recruited, TopBP1 promotes ATR activation (Kumagai et al. 2006). Thus, TopBP1 is a bona fide checkpoint protein.

Given the precedent of the existing dual replication/checkpoint proteins, it remains an open question whether Ticrr is directly involved in the S/M checkpoint or whether this function simply reflects its role as an essential replication regulator. We favor the former hypothesis, based on the following observations. First, our data show that TICRR interacts with TopBP1, raising the possibility that it cooperates in TopBP1-dependent processes beyond DNA replication, such as checkpoint signaling. Second, our FACS data show that *ticrr*-deficient cells enter mitosis with partially replicated DNA. This clearly differs from the yeast MCM, Cdt1, and Cdc45 mutants, which do not prematurely enter mitosis once S phase has begun (Tercero et al. 2000; Labib et al. 2001). Third, we originally identified *ticrr* through a screen for G2/M checkpoint regulators. This showed that *ticrr*-deficient cells fail to arrest in mitosis in response to treatment with IR. Importantly, our FACS analysis shows that these mitotic cells have 4N DNA content. Thus, we believe that this MAI phenotype reflects a bona fide defect in the G2/M checkpoint and is not simply an indirect consequence of the replication and/or S/M checkpoint defects. Consistent with this view, there is no evidence in the literature that the G2/M checkpoint is dependent on appropriate replication initiation. Moreover, included in our zebrafish screen were a large number of known replication gene mutants that did not display the MAI phenotype. Finally, we again note that TopBP1 has a well-documented role in the radiation-induced G2/M checkpoint (Garcia et al. 2005). Given all of these observations, we speculate that TICRR is a previously unknown partner for TopBP1 in its myriad roles as a core regulator of the DNA replication, S/M checkpoint, and the G2/M checkpoint machinery.

Materials and methods

Zebrafish maintenance, collection, genotyping, and expression analysis

Zebrafish were maintained as described previously (Amsterdam et al. 2004). For the screen, heterozygous insertion carriers were intercrossed and, at 24 hpf, the embryos were dechorionated and 1-phenyl-2-thiourea (PTU, 0.003%) was added to suppress pigmentation. At 32 hpf, 60 embryos from each clutch were subjected to G2/M checkpoint analysis. Primers used for genotyping and mRNA level analysis are described in the Supplemental Material.

G2/M checkpoint assays

Embryos were exposed to 15 Gy IR from a ^{60}Co source and analyzed for pH3 1 h later as described (Sansam et al. 2006). For the nocodazole trapping experiment, embryos were exposed to IR, immediately placed in nocodazole (150 ng/mL + 1% DMSO), and incubated for 2 h at 28.5°C before analysis of pH3/DNA content by FACS.

In vitro kinase assay

Fifty embryos for each data point were treated with 15 Gy IR and incubated for 30 min at 28.5°C. Embryos were then dechorionated, deyolked, and homogenized in 400 μL of KLB (50 mM Tris at pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 4 mM EDTA, 0.1% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 100 μM leupeptin, 5 μg/mL aprotonin, 1 mM PMSF). The homogenate was adjusted to 1 mg/mL prior to precipitation with anti-Cyclin B1. The beads were washed twice with KLB and KAB (50 mM Tris at pH 8.0, 10 mM $MgCl_2$, 1 mM EGTA) and incubated for 15 min at 30°C in 20 μL of kinase assay mix (KAB with 40 μM cold ATP, 100 μM peptide [HATPPKKKRK], 1 mM DTT, 0.5μCi/μl γ-^{32}PATP [6000Ci/mmol]). Substrate phosphorylation was quantified by filter binding and scintillation counting.

FACS analysis

For BrdU labeling in zebrafish, dechorionated embryos were incubated with 10 mM BrdU/15% DMSO for 15 min on ice, washed, and incubated for 15 min at 28.5°C. Mutant embryos were identified by either developmental phenotype or PCR-genotyping of a fraction of individual, fixed embryos. Cells were disaggregated by triturating embryos in 0.25% Trypsin/1 mM EDTA using a p200, and fixed in 70% ethanol overnight at −20°C. Suspensions of cells from 20 wild-type or mutant embryos were pooled and prepared for pH3/propidium iodide (PI) or BrdU/PI FACS analysis as described (Pozarowski and Darzynkiewicz 2004). FACS analysis was conducted by FACScan (Becton-Dickinson). DNA content was quantified by ModFit LT (Verity Software), and pH3 and BrdU was quantified by FlowJo (Tree Star, Inc.).

Plasmid construction

The construction of expression vectors for human TICRR, zebrafish Ticrr, and human TopBP1 is described in the Supplemental Material. The TopBP1 BRCT domain deletion mutants carry the following in-frame deletions within the 1522-amino-acid ORF: Δ98–306 (BRCTs I + II), Δ354–452 (BRCT III), Δ547–760 (BRCTs IV + V), Δ922–1011 (BRCT VI), Δ1267–1489 (BRCTs VII–VIII). Except for Δ922–1011, the deleted amino acids were replaced by two glycines.

Human cell culture and chromatin fractionation

HeLa, HeLa-S3, and 293FS cells were grown in DMEM with 10% FBS. For the synchronization experiments, HeLa cells were cultured in the presence of 100 ng/mL nocodazole (Calbiochem) for 18 h. Synchronized mitotic cells were recovered by shake-off, replated in DMEM containing 10% FBS for 2 h, and then incubated for a further 8 h in the presence or absence of 20 μM roscovitine (Calbiochem). The isolation of soluble fraction (S1), soluble nuclear fraction (S2), and chromatin-enriched fractions were conducted as described (Mendez and Stillman 2000). To solubilize the chromatin-bound proteins, nuclei were treated with 50 U of micrococcal nuclease (Worthington) for 2 min at 37°C. When indicated, lysates were treated with 2000 U/mL

DNase I (30 min at 25°C; Roche) or 20 μg/mL ethidium bromide (30 min on ice) prior to TICRR immunoprecipitation. For phosphatase treatment, precipitates were resuspended in λ phosphatase buffer (NEB) with or without 400 U of λ phosphatase (NEB) and phosphatase inhibitors (50 mM NaF, 10 mM Na_3VO_4), and incubated for 30 min at 30°C. The immunoprecipitates were washed four times with RIPA and were resuspended in 2× Laemmli Buffer.

Zebrafish chromatin preparation

Deyolked zebrafish embryos were triturated in 0.25% Trypsin/1 mM EDTA using a p200. Disaggregated cells were filtered through a 35-μm nylon mesh and washed once with PBS. The chromatin-enriched fraction was prepared essentially as described (Aparicio et al. 2009). Briefly, cells were lysed in 10 mM HEPES (pH 7.9), 0.2 M KOAc, 0.1% Triton X-100, 0.34 M sucrose, 10% glycerol, 1 mM 1,4-DTT, protease, and phosphatase inhibitors. The chromatin-associated fraction was recovered by spinning at 18,000*g* for 10 min and was washed twice in lysis buffer before suspension in Laemmli Buffer.

Antibodies

A 6-His-tagged N-terminal fragment of human TICRR (NP_689472.3 amino acids 1094–1348) was expressed in bacteria, purified over Ni^{2+} NTA-agarose resin (Qiagen), and used to immunize BALB/c mice or New Zealand White rabbits (Pocono Rabbit Farm). Other antibodies were phosho-H3 (sc-8656-R, Santa Cruz Biotechnologies), mouse anti-goldfish Cyclin-B1 (B112) (Katsu et al. 1993), PSTAIRE-Cdc2 (sc-53, Santa Cruz Biotechnologies), tubulin (T9026, Sigma), ORC2 (sc-13238, Santa Cruz Biotechnologies), LaminA/C (2032, Cell Signaling), TopBP1 (NB100-217, Novus Biologicals; and sc-32923, Santa Cruz Biotechnologies), rabbit polyclonal anti-human Psf1 (Aparicio et al. 2009), mouse monoclonal anti-human Pan-MCM (Austin et al. 1999), normal mouse IgG (sc-2025, Santa Cruz Biotechnologies), and GFP (11814460001, Roche).

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