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Transformation/Transcription Domain-Associated Protein (TRRAP)-Mediated Regulation of Wee1

Teresa M. Calonge,* Majid Eshaghi,† Jianhua Liu,† Ze'ev Ronai‡ and Matthew J. O'Connell*,[1]

**Department of Oncological Sciences, Mount Sinai School of Medicine, New York, New York 10029,* †*Genome Institute of Singapore, Singapore 138672, Singapore, and* ‡*Burnham Institute for Medical Research, La Jolla, California 92037*

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ABSTRACT

The G2 DNA damage checkpoint inhibits Cdc2 and mitotic entry through the dual regulation of Wee1 and Cdc25 by the Chk1 effector kinase. Upregulation of Chk1 by mutation or overexpression bypasses the requirement for upstream regulators or DNA damage to promote a G2 cell cycle arrest. We screened in fission yeast for mutations that rendered cells resistant to overexpressed *chk1*$^{+}$. We identified a mutation in *tra1*, which encodes one of two homologs of transformation/transcription domain-associated protein (TRRAP), an ATM/R-related pseudokinase that scaffolds several histone acetyltransferase (HAT) complexes. Inhibition of histone deacetylases reverts the resistance to overexpressed *chk1*$^{+}$, suggesting this phenotype is due to a HAT activity, although expression of checkpoint and cell cycle genes is not greatly affected. Cells with mutant or deleted *tra1* activate Chk1 normally and are checkpoint proficient. However, these cells are semi-wee even when overexpressing *chk1*$^{+}$ and accumulate inactive Wee1 protein. The changed division response (Cdr) kinases Cdr1 and Cdr2 are negative regulators of Wee1, and we show that they are required for the Tra1-dependent alterations to Wee1 function. This identifies Tra1 as another component controlling the timing of entry into mitosis via Cdc2 activation.

THE control of the transition from G2 into mitosis is highly conserved and ancient in origin, being effectively an universal process in all eukaryotic cells (Nurse 1990). The key mitotic inducer is the mitotic cyclin-dependent kinase Cdc2, whose activity is controlled not only by binding to its cyclin partners, but also by a finely tuned and reversible inhibitory phosphorylation on tyrosine 15 (Y15) (Dunphy 1994). This phosphorylation is catalyzed by the Wee1 family of kinases, which maintains Cdc2 in its inactive state throughout interphase. For mitotic entry to occur, the Cdc25 family of phosphatases dephosphorylates Y15, rapidly activating Cdc2 to enable phosphorylation of proteins that promote mitosis.

The timing of Cdc2 activation is influenced by multiple checkpoint pathways that monitor the order and fidelity of cell cycle events, thus ensuring the readiness for chromosome segregation to proceed. Upon detection of DNA damage, the G2 DNA damage checkpoint delays entry into mitosis, enabling time for DNA repair prior to chromosome segregation (O'Connell *et al.* 2000; O'Connell and Cimprich 2005). Failure to establish this checkpoint results in catastrophic mitoses, where acentric chromosome fragments are lost and incompletely repaired chromosomes fail to segregate. This results in gross chromosomal rearrangements that can lead to cell death or, when less severe, tumorigenesis via activation of oncogenes and loss of tumor suppressors.

The effector kinase of the G2 DNA damage checkpoint, Chk1, elicits this delay through dual regulation of the Cdc25 phosphatases and Wee1 kinases that modulate Cdc2 activation (Raleigh and O'Connell 2000; O'Connell and Cimprich 2005). As with the core cell cycle machine, this checkpoint is also conserved from the fission yeast *Schizosaccharomyces pombe* to humans, and a detailed description of the molecular events leading to Chk1 activation has emerged from studies in multiple experimental systems (Kuntz and O'Connell 2009). The PI3-K-related ATM and ATR (ATM/R) protein kinases are targeted to sites of DNA damage that are processed into replication-protein-A–coated single-stranded DNA by binding their partners, the Mre11-Rad50-Nbs1 (MRN) complex and ATR-interacting protein (Rad26 in *S. pombe*), respectively (Falck *et al.* 2005). Independently, PCNA-related 9-1-1 complexes, composed of Rad9, Rad1, and Hus1, are loaded to sites of DNA damage by a replication factor C (RFC)-related complex where Rad17 replaces the large RFC1 component (Bermudez *et al.* 2003; Parrilla-Castellar *et al.* 2004). The assembly of these complexes and several ATM/R-catalyzed phosphorylation events recruits BRCT-domain mediator proteins (Canman 2003), which in turn recruit Chk1 to enable activating phosphorylation on residues in the C-terminal

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[1]*Corresponding author:* Department of Oncological Sciences, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029. E-mail: matthew.oconnell@mssm.edu

regulatory domain (Liu *et al.* 2000; Lopez-Girona *et al.* 2001b; Capasso *et al.* 2002; Gatei *et al.* 2003). The duration, rather than magnitude, of Chk1 activation is dependent on the extent of DNA damage (Latif *et al.* 2004), and the inactivation of Chk1 by dephosphorylation is necessary and sufficient for relief of the checkpoint-mediated arrest to allow mitotic entry (den Elzen *et al.* 2004; den Elzen and O'Connell 2004).

Precisely how phosphorylation activates Chk1 is not yet clear, although it may relieve *in cis* auto-inhibition of the N-terminal kinase domain by the C-terminal regulatory domain (Katsuragi and Sagata 2004). However, while deletion of the regulatory domain increases Chk1 activity *in vitro* (Chen *et al.* 2000), it is essential for Chk1 function *in vivo* (Kosoy and O'Connell 2008). Further, mutations in the C-terminal domain can either inactivate or super-activate Chk1 function *in vivo* (Wang and Dunphy 2000; Kosoy and O'Connell 2008; Palermo *et al.* 2008; Pereira *et al.* 2009), suggesting that it contributes more than an inhibitory function to the catalytic domain (Tapia-Alveal *et al.* 2009).

In *S. pombe*, Chk1 activated in G2 and then inactivated for mitotic entry is reactivated in the following cell cycle without apparent DNA damage and does not delay the progression of that cell cycle (den Elzen and O'Connell 2004; Harvey *et al.* 2004). Further, several DNA repair mutants cycle normally with active Chk1 (our unpublished observations), suggesting that additional signaling may be regulated by DNA damage that impacts on cell cycle progression. Indeed, we have shown that the changed division response (Cdr) kinases, Cdr1 and Cdr2, act as Chk1 antagonists through their negative regulation of Wee1 (Calonge and O'Connell 2006), and thus it is possible that other pathways controlling cell cycle progression await identification. Cdr1, and possibly Cdr2, directly inhibit Wee1 by phosphorylation (Coleman *et al.* 1993; Kanoh and Russell 1998). Recent observations implicate these kinases in the coordination of cell growth with division, where they are regulated within a spatial gradient controlled by another kinase, Pom1 (Martin and Berthelot-Grosjean 2009; Moseley *et al.* 2009). Notably, limited nutrition reduces the size at division, and Cdr1 and Cdr2 are particularly important in advancing cell cycle progression under these conditions. However, *cdr1*Δ and *cdr2*Δ cells are delayed in progression through G2 under normal exponential growth conditions (Feilotter *et al.* 1991; Breeding *et al.* 1998; Kanoh and Russell 1998), suggesting that the regulation of Wee1 by Cdr1 and Cdr2 may extend to other conditions and/or other stresses when there is no limitation to nutrition.

The overexpression of *chk1⁺* is sufficient to cause a G2 cell cycle arrest without Chk1 C-terminal phosphorylation, DNA damage, or the upstream checkpoint components (Walworth *et al.* 1993; O'Connell *et al.* 1997; Lopez-Girona *et al.* 2001b). Here, we have taken a novel screening approach to search for genes that render cells resistant to overexpressed *chk1⁺*. The screen identified one of two *S. pombe* transformation/transcription domain-associated protein (TRRAP) homologs, Tra1, which is required for cell cycle arrest mediated by *chk1⁺* overexpression, but is not required for Chk1 activation or G2 arrest following DNA damage. TRRAP proteins scaffold several histone acetyltransferase (HAT) complexes (Grant *et al.* 1998; Allard *et al.* 1999; Cai *et al.* 2003). They are proteins closely related to the ATM/R kinases, but lack critical residues required for ATP binding. TRRAP proteins therefore lack kinase activity and are thus referred to as pseudokinases (Boudeau *et al.* 2006). We show here that Tra1 is required for the positive regulation of Wee1 and appears to work in opposition to the Cdr1 and Cdr2 kinases. These data define another mode of Wee1 regulation important for integrating signals controlling the G2/M transition.

MATERIALS AND METHODS

General *S. pombe* methods: All strains are derivatives of *972h⁻* and *975h⁺*. Standard media and methods were employed for strain construction, the propagation of cultures, the introduction of plasmids by transformation, and FACS analysis of DNA content (Moreno *et al.* 1991; Outwin *et al.* 2009). Survival assays on plates containing methyl methanesulfonate (MMS) used 10-fold serial dilutions of cultures starting at 1×10^6 cells/ml, and 5 μl of each dilution plated for 4 days at 30°. Microscopy was performed on a Nikon E800 microscope and images were captured on a Spot XE camera. Cell-length measurements were made using an eye-piece micrometer, using septated cells or, if cell cycle arrested, no length was recorded and these cultures were listed as "arrested." For derepression of the *nmt1* promoter (Basi *et al.* 1993; Maundrell 1993), exponential cultures growing in minimal media supplemented with 10 μg/ml thiamine were washed three times in thiamine-free medium and then grown for the indicated times. For nitrogen starvation assays, exponential cultures growing in supplemented EMM2 medium were extensively washed in nitrogen-free medium and reinoculated into EMM2 containing 100%, 10%, or 0% nitrogen and cultured for a further 16 hr before fixing in 70% ethanol and being processed for FACS analysis of DNA content.

Western blotting: Whole-cell extracts for Western blotting were prepared in 8 M urea, 50 mM $NaPO_4$, 10 mM Tris, pH 8.0, separated by SDS–PAGE and transferred to nitrocellulose in 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid, pH 11, 10% methanol. Overproduced Chk1 was detected with rabbit anti-Chk1 polyclonal antibodies (O'Connell *et al.* 1997). Endogenous expression of Chk1 (HA_3), Wee1 (HA_3), Cdc25 (Myc_{13}), Cdr1 ($Flag_3$), and Cdr2 (HA_3) were detected using 12CA5 (HA, Roche), 9E10 (Myc, Santa Cruz Biotechnology), and M2 (Flag, Sigma) monoclonal antibodies. All epitope-tagged alleles were confirmed to retain wild-type function. Total Cdc2 was detected with anti-PSTAIRE antibodies (Santa Cruz Biotechnology), and Y15 phosphorylated Cdc2 was detected with phospho-specific antibodies (Cell Signaling Technologies). Actin was detected with HRP-coupled anti-actin antibodies (Santa Cruz Biotechnology). Band intensities were quantified by densitometry (BioRad GS-800 with Quantity One software).

Screen for Chk1-resistant mutants: A 1.8-kb *Hin*dIII fragment containing the *ura4⁺* gene was isolated and used to

transform wild-type *S. pombe* to generate a library of stable and random Ura+ integrants via three successive rounds of selection for Ura+ and selection relief by growth in yeast extract plus supplements. The library was transformed with pREP41-*chk1-E472D* (Kosoy and O'Connell 2008), and Chk1-resistant colonies were selected on media lacking thiamine. The pREP41-*chk1-E472D* was then lost from 500 independent colonies by selection relief, and the plasmid was reintroduced by mating from a wild-type carrier strain. From this, 2 of the 500 colonies were shown to harbor chromosomal mutations leading to Chk1 resistance, with the remainder being due to plasmid-borne mutations. Of the two, one colony showed Chk1 resistance linked to the *ura4*$^+$ marker. The resistance was also observed for pREP1-Chk1, which was used in subsequent assays.

Cloning of Tra1: Using the Chk1-resistant strain from the screen above, Southern blot analysis with a Ura4 probe was performed with a panel of restriction enzymes, and the smallest fragment containing *ura4*$^+$ was generated by digestion with *Msp*I (2.4 kb). *Msp*I-digested genomic DNA was then religated into circular molecules, and the *ura4*$^+$ fragment was recovered by inverse PCR using the following primers within the *ura4*$^+$ sequence: forward—GCGTTTTATGTCAGAAGGC; reverse—GAGGTTCTTGGTAGGACA. The PCR fragment was purified and the site of insertion determined by DNA sequencing, which was within chromosome 2 at the 3′ end of the *tra1*$^+$ gene, truncating Tra1 at residue 3559 of 3699. This allele was denoted *tra1-1*. The entire *tra1* ORF was also deleted and replaced with *ura4*$^+$ by homologous recombination, creating *tra1*Δ.

Analysis of gene expression profiles: cDNA was prepared from RNA acid extracted from exponentially growing wild-type and *tra1-1* cells and labeled with Cy3 or Cy5 as previously described (Bimbo *et al.* 2005). This was hybridized onto oligonucleotide-based microarray slides covering all predicted genes, and signals were quantified as described (Bimbo *et al.* 2005). A full data set of relative expression ratios, derived from three independent experiments, is presented in Table S1.

Protein kinase assays: Extracts were made in Chk1 IP buffer (Capasso *et al.* 2002), and Chk1 was immunoprecipitated with polyclonal anti-Chk1 antibodies (O'Connell *et al.* 1997). Following extensive washing in Chk1 IP buffer, the beads were washed into Chk1 assay buffer, and activity was determined on a peptide substrate as described (Harvey *et al.* 2004; Latif *et al.* 2004) and quantified by scintillation counting. For Cdc2 assays, extracts were made in histone-kinase buffer, and Cdc2 was captured by immunoprecipitation of the B-type cyclin Cdc13 using anti-Cdc13 monoclonal antibody (O'Connell *et al.* 1997). Cdc2 activity was determined using histone H1 as a substrate and quantified by Phosphorimager analysis of dried gels. In both cases, assays were performed in triplicate.

RESULTS

Tra1 is required for G2 arrest mediated by Chk1 overexpression: Activation of Chk1 is essential for checkpoint arrest following DNA damage. *chk1*$^+$ overexpression causes a G2 cell cycle arrest without the requirement for DNA damage or activating phosphorylation, enabling Chk1-mediated control of cell cycle progression to become uncoupled from other DNA-damage–regulated events. We hypothesized that other signaling pathways that control cell cycle progression may be regulated in concert with Chk1 in response to DNA damage. Previously, we had obtained evidence that this was the case for the negative regulators of Wee1,

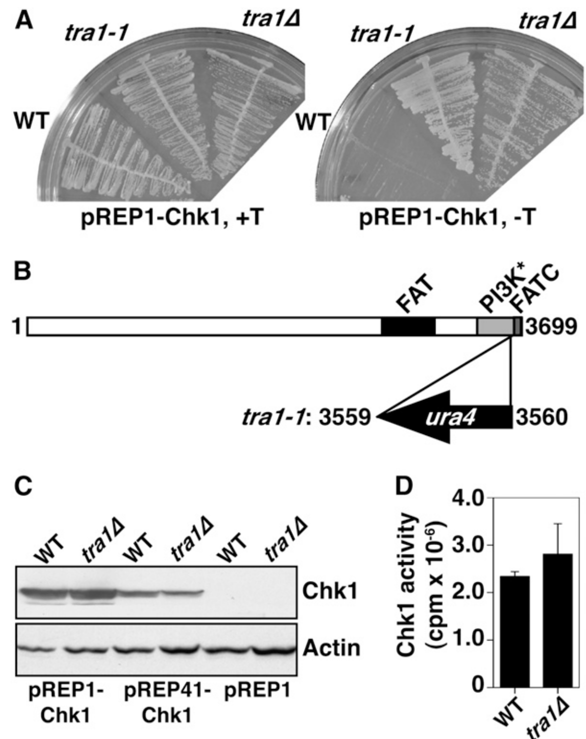

Figure 1.—Tra1 is required for cell cycle arrest by Chk1 overexpression. (A) The indicated strains containing pREP1-Chk1 were grown on media in the presence (promoter repressed) or absence (promoter derepressed) of thiamine for 4 days at 30°. (B) Tra1 is a 3699-amino-acid protein containing the FAT and FATC domains characteristic of the ATM and ATR kinases, but lacks ATP coordinating residues in the PI3K domain (labeled PI3K*) required for kinase activity. *ura4* truncates Tra1 at residue 3559 and is expressed in the opposite direction. (C) Western blot analysis shows that expression levels of Chk1 (rabbit anti-Chk1) are unaffected in *tra1*Δ cells. Actin was used as a loading control. (D) Chk1 activity is also unaffected in *tra1*Δ cells. Data are mean ± SE; $n = 3$.

the Cdr1 and Cdr2 kinases (Calonge and O'Connell 2006). To search for additional components that modulate the G2/M transition relevant to Chk1 signaling, we undertook an insertional mutagenesis screen, using the *ura4*$^+$ gene as the insertional mutagen, to identify genes required for cell cycle arrest caused by *chk1*$^+$ overexpression (see materials and methods).

We identified one strain that was totally nonresponsive to *chk1*$^+$ overexpression and detected the point of insertion by inverse PCR. This strain contained the *ura4*$^+$ gene inserted near the 3′ end of a gene encoding one of two *S. pombe* TRRAP homologs, *tra1* (Kanoh and Yanagida 2007), and this insertion allele was denoted *tra1-1* (Figure 1, A and B). TRRAP proteins are large pseudokinases highly related to ATM, ATR, and DNA-PK$_{cs}$ (Boudeau *et al.* 2006). The insertion in *tra1-1* truncates Tra1 at residue 3559 of 3699, deleting the C-terminal FATC domain, known to be critical in the function of the active kinases (Jiang *et al.* 2006). We deleted the entire open reading frame of *tra1*, replacing it with *ura4*$^+$, but in all assays this allele (*tra1*Δ) phenocop-

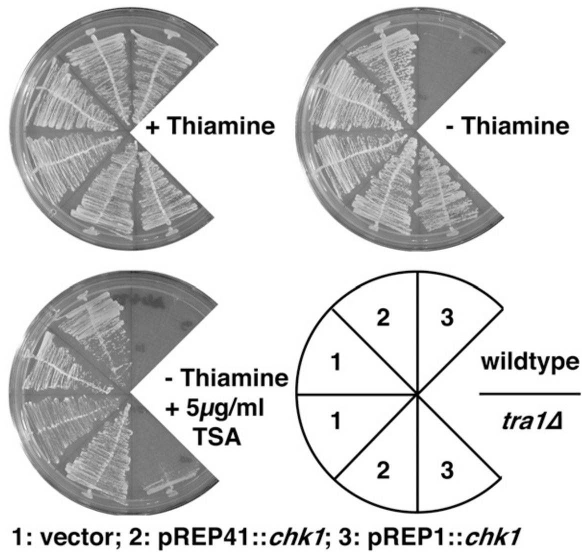

FIGURE 2.—The histone deacetylase inhibitor trichostatin A suppresses the Chk1 resistance of *tra1*Δ. Plates were incubated for 4 days at 30°.

TABLE 1

Suppression of resistance to Chk1 overexpression of *tra1*Δ by HDAC mutants and TSA

Genotype	Vector	*nmt1::chk1*
Wild type	13.8 ± 1.0	Arrested
Wild type + 5 μg/ml TSA	14.7 ± 1.1	Arrested
*tra1*Δ	10.3 ± 1.1	12.4 ± 1.3
*tra1*Δ + 5 μg/ml TSA	13.5 ± 0.8	Arrested
*sir2*Δ	14.4 ± 1.3	Arrested
*sir2*Δ *tra1*Δ	10.7 ± 0.9	12.1 ± 2.0
*hos2*Δ	13.2 ± 1.2	Arrested
*hos2*Δ *tra1*Δ	13.6 ± 2.9	23.4 ± 4.4
*hst2*Δ	14.0 ± 1.2	Arrested
*hst2*Δ *tra1*Δ	12.6 ± 1.6	12.4 ± 2.2
*hst4*Δ	14.4 ± 1.0	Arrested
*hst4*Δ *tra1*Δ	16.6 ± 2.4	23.9 ± 6.0
*clr3*Δ	13.4 ± 0.8	Arrested
*clr3*Δ *tra1*Δ	12.1 ± 1.2	Arrested
clr6-1	16.2 ± 1.5	Arrested
*clr6-1tra1*Δ	16.1 ± 2.7	Arrested

Numbers are cell length at division following 20 hr growth in the absence of thiamine at 30°, except for *clr6-1*, which is 30 hr at 25°. Cell cycle arrest was determined by nondividing highly elongated cells. Data are mean ± SD; $n = 50$.

ied *tra1-1*, highlighting the importance of the FATC domain in Tra1. We confirmed that the resistance to *chk1*⁺ overexpression was not an artifact of loss of expression (Figure 1C) nor a lack of kinase activity (Figure 1D), and thus we concluded Tra1 is required for overexpressed *chk1*⁺ to elicit a cell cycle arrest.

Inhibiting histone deacetylases blocks tra1Δ resistance to Chk1 overexpression: TRRAP proteins scaffold several HAT complexes. We therefore tested whether reducing histone deacetylase (HDAC) activity would reverse the resistance of *tra1*Δ cells to Chk1 overexpression. *S. pombe* cells have six HDACs representing the three classes of these enzymes encoded by *hos2*, *clr6* (class I), *clr3* (class II), and *sir2*, *hst2*, and *hst4* (class III) (EKWALL 2005). Of these, only *clr6* is essential, but this number of genes and redundancy of function makes genetic downregulation of total HDAC activities problematic. Therefore, to this end, we used the HDAC inhibitor trichostatin A (TSA) to dampen total HDAC activity. We measured growth with *chk1*⁺ expressed from the wild-type *nmt1* promoter (pREP1) or from the attenuated *nmt1* promoter (pREP41), which directs expression to ~60-fold lower levels than wild type (FORSBURG 1993). Expression of *chk1*⁺ from pREP41 causes a modest G2 cell delay (CALONGE and O'CONNELL 2006) and was tolerated by both wild-type and *tra1*Δ cells. Colony formation in both strains under these conditions was not affected by addition of TSA (Figure 2). Expression of *chk1*⁺ from pREP1 is lethal to wild type but does not affect cell cycle progression in *tra1*Δ. However, addition of TSA to the medium restored pREP1::*chk1*-mediated cell cycle arrest to *tra1*Δ cells (Figure 2 and Table 1). This is consistent with the resistance to *chk1*⁺ overexpression being due to a reduction in Tra1-dependent HAT activity, which is balanced by TSA-mediated HDAC inhibition.

We then asked whether inactivation of any of the individual HDAC genes could similarly suppress the resistance to *chk1*⁺ overexpression in *tra1*Δ cells (Table 1; supporting information, Figure S1). We employed null alleles of each gene except the essential *clr6*, for which we used *clr6-1*, a temperature-sensitive lethal mutation that is significantly compromised at 25° (GREWAL *et al.* 1998). Deletion of *sir2* or *hst2*, which encode members of the class III NAD-dependent family of HDACs, had no effect on the Chk1 resistance of *tra1*Δ cells. Deletion of the other class III HDAC, *hst4*, partially suppressed the Chk1 resistance of *tra1*Δ cells; colonies still formed, but the cells were delayed in cell cycle progression and thus are significantly elongated. Deletion of the class I HDAC gene *hos2* conferred a similar partial suppression, whereas mutation of the other class I gene, *clr6*, or deletion of the class II HDAC gene *clr3*, completely suppressed the Chk1 resistance of *tra1*Δ cells. As these different HDACs have specificity for different genomic regions, and for different lysines on histones and nonhistone proteins (EKWALL 2005), these data suggest that a HAT deficiency in *tra1*Δ cells may reduce the acetylation at multiple residues and loci, and thus it is possible that phenotypes of *tra1*Δ cells are pleiotrophic in origin.

We also tested whether mutation in other nonessential components of HAT complexes conferred resistance to *chk1*⁺ overexpression. For this we utilized cells deleted for *gcn5*⁺ (YAMADA *et al.* 2004) and *mst2*⁺ (GOMEZ *et al.* 2005), which encode components of the SAGA and NuA3 HATs,

TABLE 2

Genes with ≥2-fold reduction in expression in *tra1-1*

Gene	Protein	Fold change
SPBPB10D8.01	Cysteine transporter (predicted)	−5.20
obr1	Ubiquitinated histone-like protein	−4.04
gst2	Glutathione *S*-transferase	−3.48
abp2	ARS binding protein	−3.07
SPBPB10D8.04c	Membrane transporter (predicted)	−2.94
SPAC869.05c	Sulfate transporter (predicted)	−2.77
urg2	Uracil phosphoribosyltransferase (predicted)	−2.74
hsp16	Heat-shock protein	−2.74
SPBC428.11	Homocysteine synthase	−2.72
tra1	TRAPP homolog	−2.70
SPAC1039.02	Phosphoprotein phosphatase (predicted)	−2.60
SPAC869.02c	Nitric oxide dioxygenase (predicted)	−2.60
SPCC70.08c	Methyltransferase (predicted)	−2.56
cnp3	CENP-C	−2.55
SPBC1271.07c	*N*-acetyltransferase (predicted)	−2.49
SPAC5H10.10	NADPH dehydrogenase (predicted)	−2.46
adg1	Dequence orphan	−2.42
vht1	Vitamin H transporter	−2.41
SPAC869.10c	Proline-specific permease (predicted)	−2.40
plr1	Pyridoxal reductase	−2.35
SPBPB7E8.01	Sequence orphan	−2.35
SPCC569.05c	Spermidine family transporter (predicted)	−2.31
mik1	Mitotic inhibitor kinase	−2.24
SPAC11D3.13	ThiJ domain protein	−2.16
SPAC11D3.01c	Conserved fungal protein	−2.15
SPAC5H10.03	Phosphoglycerate mutase family	−2.15
SPBC359.03c	amino acid permease	−2.13
urg1	GTP cyclohydrolase II (predicted)	−2.12
SPAC977.14c	Aldo/keto reductase	−2.09
arg4	Carbamoyl-phosphate synthase	−2.03
SPCC569.07	Aromatic aminotransferase (predicted)	−2.01

Fold change is calculated compared to wild-type cells. Table S1 includes the full data set. Note that only *mik1* (down 2.24-fold) has been implicated in Cdc2 regulation. See also Figure 3. Predicted protein functions are based on homology and assigned by GeneDB.

respectively. In both cases, these null mutants were sensitive to *chk1*$^+$ overexpression (Figure S2), suggesting that *tra1*Δ affects either both classes of HAT complexes or the function on other subunits encoded by essential genes.

Finally, Tra1 has also been implicated as a member of the ASTRA complex (Shevchenko *et al.* 2008), which is essential for telomere maintenance. The deletion of each of the genes for the other members of this complex is lethal, presumably due to telomere erosion, and thus it is not possible to test if these genes are required for resistance to *chk1*$^+$ overexpression. Nevertheless, we tested *tel2*Δ and *tti1*Δ heterozygous diploid strains for sensitivity to *chk1*$^+$ overexpression, and both strains were wild type for this phenotype (Figure S3). Combined with the nonessential nature of *tra1*, the data suggest that the effects of *tra1*Δ are more likely via HAT function rather than telomere maintenance.

Gene expression profiles controlled by Tra1: As the resistance to *chk1*$^+$ overexpression appeared to be dependent on HAT activity, we presumed that this was due to altered gene expression affecting the response to Chk1. Global expression profiles were determined by microarray analysis, comparing wild type to *tra1-1* cells, and a complete data set is presented in Table S1. Only 57 genes showed a ≥2-fold change in gene expression; 31 genes were downregulated (Table 2) and an additional 26 genes upregulated (Table 3). Interestingly, expression of *tra1* itself was reduced 2.7-fold in *tra1-1* cells ($P = 0.005$), suggesting that Tra1 controls its own expression.

Inspection of the known or homology-based predicted functions for these genes identified only one gene that is implicated in Chk1 signaling, encoding the Wee1 kinase family member, Mik1. *mik1*$^+$ expression was 2.24-fold lower in *tra1-1* ($P = 0.002$), and although *mik1*$^+$ expression is extremely low in cycling cells, it is upregulated during S-phase (Christensen *et al.* 2000), and this is essential for cell viability in the absence of Wee1 (Lundgren *et al.* 1991). Further, the deletion of *mik1* has been reported to render cells resistant to Chk1 overexpression (Baber-

TABLE 3

Genes with ≥2-fold increase in expression in *tra1-1*

Gene	Protein	Fold change
isp6	Vacuolar serine protease	+2.01
SPAC5H10.04	NADPH dehydrogenase (predicted)	+2.06
ste4	Adaptor protein	+2.07
SPBC409.08	Spermine family transporter (predicted)	+2.09
SPBC1773.12	Transcription factor (predicted)	+2.10
SPBPB2B2.12c	UDP-glucose 4-epimerase	+2.10
gas2	1,3-β-glucanosyltransferase Gas2 (predicted)	+2.13
gpx1	Glutathione peroxidase	+2.14
itr2	Myo-inositol transporter	+2.18
SPBC8E4.01c	Inorganic phosphate transporter (predicted)	+2.26
SPCC70.03c	Proline dehydrogenase (predicted)	+2.29
mei2	RNA-binding protein	+2.29
SPAC23D3.12	Inorganic phosphate transporter (predicted)	+2.47
zym1	Metallothionein	+2.59
SPAC15E1.02c	DUF1761 family protein	+2.79
SPAC186.07c	Hydroxyacid dehydrogenase (predicted)	+2.88
mfm2	M-factor precursor	+2.90
ste11	Transcription factor	+2.93
SPBPB2B2.10c	Galactose-1-phosphate uridylyltransferase (predicted)	+3.48
pho1	Acid phosphatase	+4.26
SPBC725.10	tspO homolog	+4.79
ght5	Hexose transporter	+5.51
hsp9	Heat-shock protein	+6.00
SPAC27D7.10c	But2 family protein	+7.10
gpd3	Glyceraldehyde 3-phosphate dehydrogenase	+8.45
SPAC27D7.11c	But2 family protein	+8.73

Fold change is calculated compared to wild-type cells. Table S1 includes the full data set. Predicted protein functions are based on homology and assigned by GeneDB. None of these genes have been implicated in the progression of the mitotic cell cycle.

Furnari *et al.* 2000; Rhind and Russell 2001), and thus we thought this a good candidate to explain the resistance of *tra1* mutants to Chk1.

We therefore measured Mik1 protein levels in wild-type and *tra1-1* cells, including conditions of replication arrest with the ribonucleotide reductase inhibitor hydroxyurea (HU) (Figure 3A). Mik1 levels were very difficult to detect in cycling cells, but were dramatically increased in HU-treated cells (Figure 3A). However, no significant difference was observed between wild type and *tra1-1*, and thus the observed reduction in *mik1* mRNA levels in *tra1-1* may represent subtle differences in S-phase progression (although no evidence for this is seen by FACS analysis) or may be an effect of the extremely low expression levels in cycling cells.

Next we tested the sensitivity of *mik1*Δ cells to *chk1*$^+$ overexpression (Figure 3B). Growth of cells expressing *chk1*$^+$ from pREP1 was severely impaired in *mik1*Δ cells, although it was slightly better than in wild-type cells. However, if cells were further sensitized to *chk1*$^+$ overexpression through Chk1 activation by sublethal DNA damage (0.005% MMS), *mik1*Δ cells were completely growth inhibited, whereas the growth of *tra1-1* and *tra1*Δ cells was unaffected. Thus, we cannot explain the complete resistance of *tra1* mutant cells to *chk1*$^+$ overexpression due to perturbations to Mik1.

Tra1 cells are defective in G2/M cell cycle control: There are two possible explanations for the resistance of *tra1-1* and *tra1*Δ cells to Chk1 overexpression. First, they could be specifically affected in some aspect of Chk1 function, although because expression and *in vitro* kinase activity are unaffected, this would be accounted for by an alternative explanation such as nuclear exclusion or the inability to interact with the substrate. Such a defect should cause a defect in the DNA damage checkpoint. Alternatively, *tra1* mutants may have a defect in the regulation of mitotic entry downstream of Chk1. In this case, evidence for perturbation to negative regulation of cell cycle progression should exist, which in *S. pombe* is evident as division at a reduced cell size, the "wee" phenotype.

To assess the first possibility, we asked if Tra1 was required for Chk1-dependent cell cycle delay in response to DNA damage. Neither *tra1-1* nor *tra1*Δ cells were sensitive to DNA damage or replication arrest, (Figure 4, A and C; Figure S4). *tra1*Δ and *tra1-1* cells delayed cell cycle progression with wild-type kinetics following DNA damage and did not alter the sensitivity

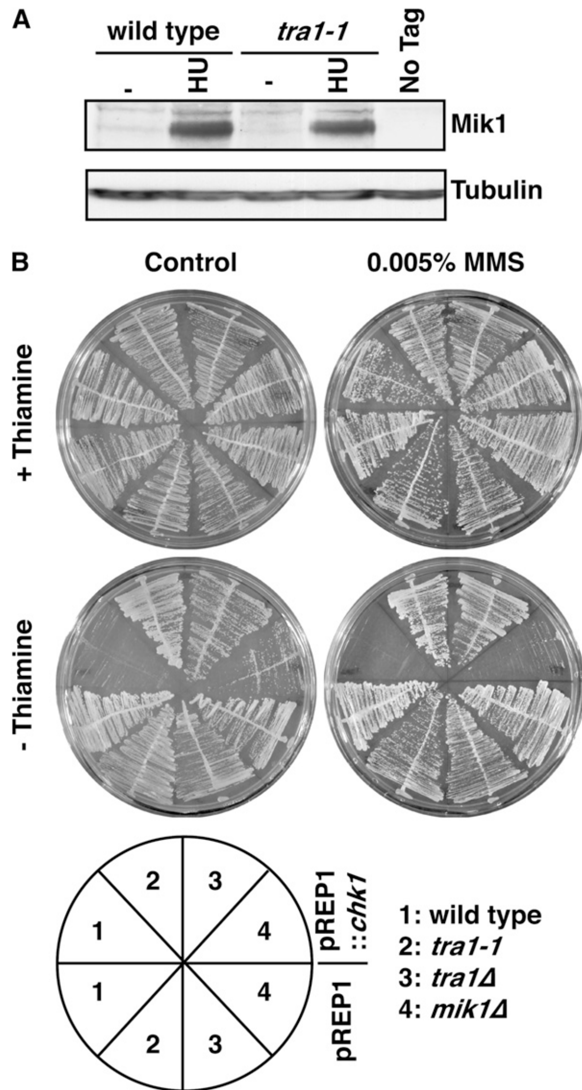

FIGURE 3.—Chk1 resistance in *tra1*Δ is independent of Mik1. (A) Western blot of extracts from cells expressing Myc-tagged Mik1 or an untagged control (no tag) from untreated cells (−) or cells treated with 11 mM HU for 4 hr at 30°. Antitubulin antibodies were used as a loading control. (B) Cells harboring vector (pREP1) or pREP1-Chk1 were grown in the presence or absence of thiamine/MMS for 4 days at 30°. Note that *mik1*Δ cells are severely growth inhibited by Chk1 overexpression, and this is further exacerbated by MMS.

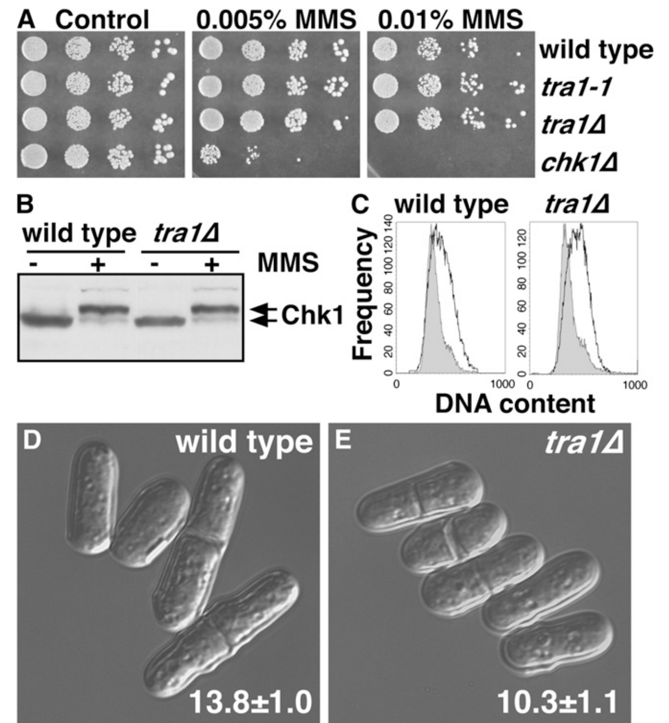

FIGURE 4.—Tra1 is not required for checkpoint arrest, but is required for regulation of the G2/M transition. (A) Tra1 is not required for resistance to MMS. YES plates containing the indicated concentrations of MMS or no drug (control) were inoculated with spots of 10-fold serial dilutions of the indicated strains and were grown at 30° for 4 days. (B) Tra1 is not required for activating phosphorylation on Chk1 in the presence of MMS, showing that signaling through endogenous Chk1 is intact in *tra1*Δ cells. (C) FACS profiles of DNA content in cycling cells (shaded) or in MMS-treated cells (open). The cell cycle delay (cell elongation) in MMS broadens the profiles of these samples. The lengths of 50 exponentially growing (D) wild-type and (E) *tra1*Δ cells were determined by microscopy, and representative images are shown. Data are mean ± SD.

of multiple DNA-damage-sensitive mutants including *chk1*Δ (data not shown). Moreover, Tra1 was not required for Chk1 activation by phosphorylation, assayed as a mobility shift on Western blots (Figure 4B). Further, *tra1*Δ did not render cells resistant to the lethal effects of overexpression of *mad2*$^+$, *cds1*$^+$, or *fin1*$^+$, which signal cell cycle delay/arrest by different mechanisms (data not shown). Therefore, as *tra1*Δ cells are responsive to Chk1 activated by DNA damage, the resistance to overexpressed *chk1*$^+$ in the absence of DNA damage might be due to a defect in signaling downstream of Chk1 that enables progression into mitosis when *chk1*$^+$ is overexpressed, but not when Chk1 is fully activated by the DNA damage checkpoint.

Microscopic observation of *tra1*Δ cells indicated that these cells have a semi-wee phenotype, dividing at only 10.3 μm, compared to the 13.8 μm of wild-type controls (Table 1 and Table 4; Figure 4, C and D). This semi-wee phenotype is less severe than a complete wee phenotype, where cells divide at ∼8 μm, but nevertheless is indicative of a shortened G2 period of the cell cycle in *tra1*Δ cells. Cells with wee or semi-wee phenotypes are checkpoint proficient but resistant to *chk1*$^+$ overexpression (WALWORTH *et al.* 1993; O'CONNELL *et al.* 1997; RALEIGH and O'CONNELL 2000; CALONGE and O'CONNELL 2006), and with the normal response to DNA damage, we propose this is the reason for Chk1 resistance in *tra1* mutants. Notably, both TSA and several of the HDAC mutants also suppressed the semi-wee phenotype of *tra1*Δ cells (Table 1).

Altered regulation of Wee1 in tra1Δ cells: Chk1 signals cell cycle arrest by enforcing the inhibitory Y15

TABLE 4

***cdr1* and *cdr2* are required for resistance to *chk1*$^+$ overexpression**

Genotype	Vector	*nmt1::chk1*
Wild type	13.8 ± 1.0	Arrested
tra1-1	11.1 ± 1.2	11.0 ± 1.0
tra1Δ	10.3 ± 1.1	12.4 ± 1.3
cdr1Δ	18.1 ± 0.9	Arrested
cdr2Δ	17.9 ± 1.1	Arrested
tra1-1 cdr1Δ	17.0 ± 1.4	Arrested
tra1Δ cdr1Δ	17.1 ± 1.4	Arrested
tra1-1 cdr2Δ	12.8 ± 1.2	17.2 ± 1.8
tra1Δ cdr2Δ	12.8 ± 1.1	18.3 ± 2.2

Numbers are cell length at division following 20 hr growth in the absence of thiamine. Cell cycle arrest was determined by nondividing highly elongated cells. Data are mean ± SD from three samples of 50 cells.

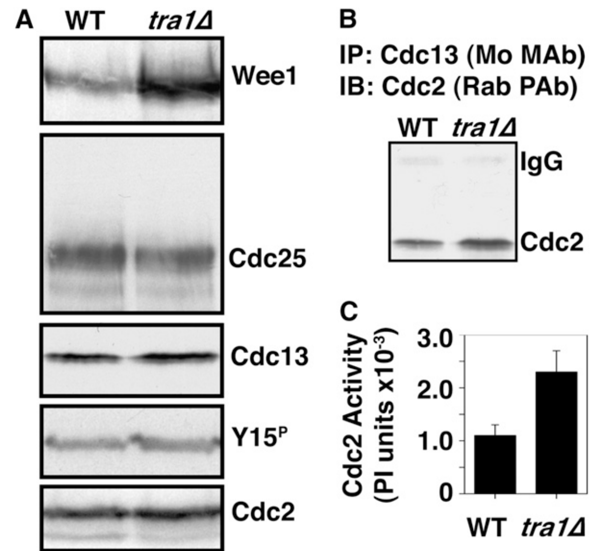

Figure 5.—Wee1 levels accumulate in *tra1Δ* cells. (A) Western blotting shows that Wee1 protein accumulates in *tra1Δ* cells, but the levels of Cdc25, Cdc13, tyrosine-15 phosphorylated (Y15^P), and total Cdc2 are not affected. Note that *tra1Δ* cells are semi-wee (Table 3) and that Y15^P does not accumulate, indicating that the excess Wee1 in *tra1Δ* cells is not fully active. (B) Cdc13 was immunoprecipitated with a mouse monoclonal anti-Cdc13 antibody and that coprecipitating Cdc2 was detected by Western blotting with a rabbit polyclonal anti-Cdc2 antibody. The anti-rabbit secondary antibody weakly cross-reacts with the mouse IgG heavy chain. Immunoprecipitated Cdc13 comigrates with the IgG heavy chain, which precludes its detection by IP or Western blots, both mouse antibodies. (C) Cdc2 kinase activity in Cdc13 IPs is increased by approximately twofold in *tra1Δ* cells. Data are mean ± SE; $n = 3$.

phosphorylation on Cdc2 (O'Connell *et al.* 1997; Rhind *et al.* 1997). This is achieved through the dual regulation of Wee1 and Cdc25 (Raleigh and O'Connell 2000). We therefore asked whether these proteins were altered in *tra1Δ* cells (Figure 5A). We observed that Cdc25 levels were unaffected in *tra1Δ* cells. Cdc25 activation is normally restricted to mitosis and is associated with a phosphorylation-dependent mobility shift (Moreno *et al.* 1990; Wolfe and Gould 2004), which we did not observe. Further, although *chk1*$^+$ overexpression results in a net nuclear exclusion of Cdc25 (Lopez-Girona *et al.* 1999), this is not required for Chk1-dependent cell cycle arrest (Lopez-Girona *et al.* 2001a) and thus cannot explain the resistance of *tra1Δ* cells to *chk1*$^+$ overexpression.

Conversely, Wee1 protein levels were significantly upregulated in *tra1Δ* cells (approximately fivefold by densitometry). This was a surprising result, as wild-type cells are extremely sensitive to increased levels of Wee1. An enforced approximately fivefold increase in expression levels leads to a doubling of cell cycle duration, with cells dividing at 28 μm, compared to 14 μm for wild-type cells (Russell and Nurse 1987). As *tra1Δ* cells are semi-wee, the accumulated Wee1 protein cannot be fully active, suggesting that Tra1 is required for full Wee1 activity. Further, as Wee1 mRNA levels are unaffected in *tra1-1* cells (Table S1), in this context Wee1 activity negatively correlates with Wee1 protein stability.

Consistent with semi-wee phenotype, exponentially growing *tra1Δ* cells had twofold higher Cdc2 kinase activity measured with IP of the major B-type cyclin, Cdc13 (Figure 5C). Although (inactive) Y15 phosphorylated Cdc2 levels were the same in wild-type and *tra1Δ* cells (Figure 5A), this inhibitory phosphorylation occurs only on Cdc2 molecules that are bound to a cyclin (Parker *et al.* 1991, 1992; Parker and Piwnica-Worms 1992), and consistently there was more Cdc2 in anti-Cdc13 IPs in *tra1Δ* than in wild-type cells (Figure 5B). Therefore, these complexes contain more dephosphorylated (active) Cdc2. The same phenomenon may account for unaltered Y15 phosphorylation levels in other "wee" mutants (Raleigh and O'Connell 2000). Cdc13 accumulates during G2 phase (Alfa *et al.* 1989), and despite the shortened G2 of the semi-wee *tra1Δ* cells, the steady-state levels of Cdc13 are unaffected (Figure 5A). Therefore, this increase in Cdc13–Cdc2 complexes could arise from more efficient complex formation in interphase, less efficient complex destruction in mitosis, or a combination of both these events.

Deletion of Cdr kinases suppresses cell cycle defects in *tra1* mutants: Cdr1 and Cdr2 are serine/threonine kinases that act as negative regulators of Wee1 (Coleman *et al.* 1993; Wu and Russell 1993; Breeding *et al.* 1998; Kanoh and Russell 1998). Consequently, *cdr1Δ* and *cdr2Δ* cells are delayed in cell cycle progression (Young and Fantes 1987). Dominant-negative alleles of *cdr1*, which interfere with both Cdr1 and Cdr2, render cells hyper-sensitive to *chk1*$^+$ overexpression, whereas *cdr1*$^+$ overexpression results in a wee phenotype and resistance to *chk1*$^+$ overexpression (Calonge and O'Connell 2006). Given this relationship to the phenotypes of *tra1Δ* cells, we assayed whether Wee1 accumulation in *tra1Δ* was dependent on Cdr1 and/or Cdr2. Western

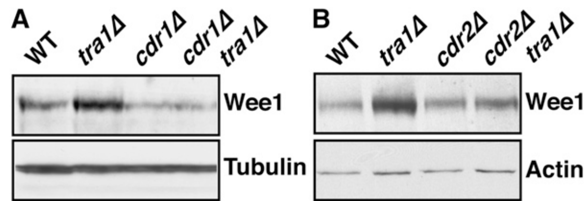

FIGURE 6.—Wee1 accumulation in *tra1*Δ cells is dependent on Cdr1 and Cdr2. (A and B) Western blot for HA-tagged Wee1 in the indicated strains grown to mid-logarithmic phase at 30°. Antitubulin and anti-actin were used as loading controls. Note that increased Wee1 levels are suppressed by *cdr1*Δ and *cdr2*Δ.

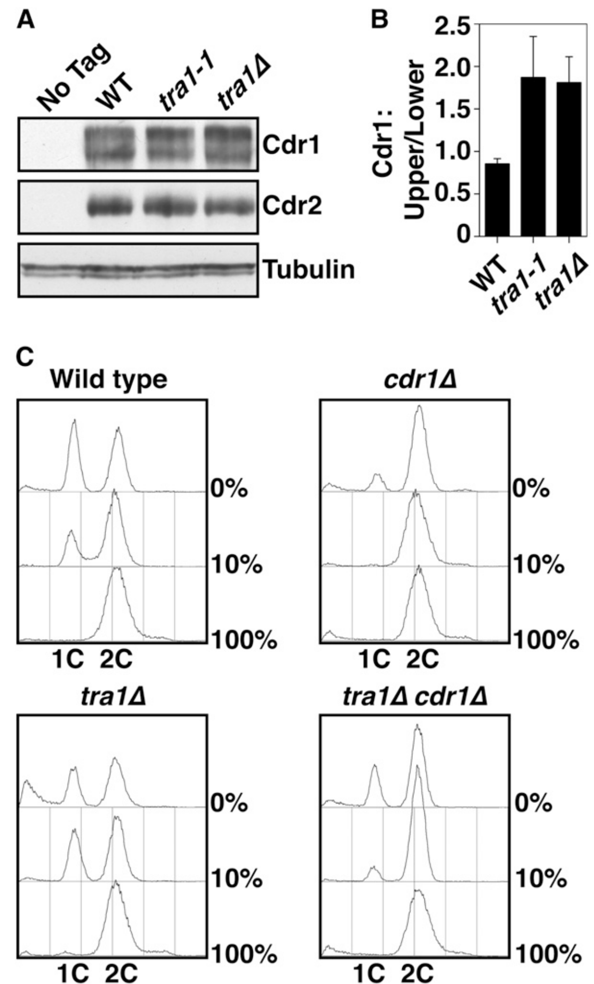

FIGURE 7.—Accumulation of active Cdr1 in *tra1* mutants. (A) Western blot analysis of Cdr1 (Flag-tagged) and Cdr2 (HA-tagged) levels. Tubulin and actin are used as loading controls. (B) The ratio of the upper (phosphorylated) to lower (unphosphorylated) Cdr1 was determined by densitometry. Data are mean ± SD; $n = 3$. (C) *tra1*Δ cells have an enhanced nitrogen starvation response. FACS profiles of cells grown in 100%, 10%, and 0% nitrogen for 16 hr at 30°.

blotting showed that this is indeed the case (Figure 6, A and B). In the case of *cdr1*Δ cells (wild type for *tra1*), Wee1 levels were also reduced compared to wild type, and yet *cdr1*Δ cells are delayed in cell cycle progression in a Wee1-dependent manner (Young and Fantes 1987). This is consistent with the negative correlation between Wee1 levels and activity and suggests that Cdr1 also regulates Wee1 levels in cycling cells.

We next asked whether Cdr1 or Cdr2 affected the semi-wee and *chk1*$^+$ overexpression resistance phenotypes of *tra1* mutants. Deletion of *cdr1* suppressed both phenotypes for *tra1*Δ and *tra1-1*, whereas deletion of *cdr2* partially suppressed these phenotypes (Table 4). Therefore, these Wee1 regulators either directly or indirectly influence *tra1* mutant phenotypes, which firmly establishes altered regulation of Wee1 as the root of these effects. Further, as upregulation of Cdc25 suppresses the cell cycle delay of *cdr* mutants (Kanoh and Russell 1998), it is unlikely that Cdc25 is altered in the *tra1* mutants. However, as *cdr2*Δ fully rescues the elevated Wee1 levels, but only partially rescues the cellular phenotypes, these must be affected by more than just the amount of Wee1 protein.

The phosphorylation of Wee1 by Chk1 also stabilizes Wee1 (Raleigh and O'Connell 2000), and although this increases the cellular pool of Wee1 by approximately twofold, it does not alter the specific activity of the enzyme (O'Connell *et al.* 1997). We tested the effects of *chk1*$^+$ overexpression in cells lacking Tra1 and/or Cdr1. As in wild-type cells, *chk1*$^+$ overexpression increased Wee1 levels in both *cdr1*Δ and *cdr1*Δ *tra1*Δ cells, which are responsive to Chk1. However, in *tra1*Δ cells, Wee1 levels are already higher than in wild-type cells overexpressing *chk1*$^+$ and did not significantly increase upon *chk1*$^+$ overexpression (Figure S5); presumably *chk1*$^+$ overexpression has no effect on Wee1 activity in *tra1*Δ cells, as they are nonresponsive to overexpressed *chk1*$^+$, and thus the effects of lacking Tra1 on Wee1 are epistatic to those derived by Chk1-catalyzed phosphorylation.

We next assayed if Cdr1 and/or Cdr2 were upregulated in *tra1* mutant cells. Western blotting showed that the overall levels of each protein was unaffected by *tra1-1* or *tra1*Δ (Figure 7, A and B). All published assays of Cdr1 and Cdr2 kinase activity in *S. pombe* have utilized recombinant (baculoviral) or overexpressed protein (Coleman *et al.* 1993; Wu and Russell 1993; Kanoh and Russell 1998). We attempted to assay the activities of endogenous (immunoprecipitated) Cdr1 and Cdr2 using Wee1 as a substrate and published conditions for the recombinant proteins, but did not find significant activity. We have extensively explored variations in assay conditions, including pH range, cation requirement, alternative substrates, and surfactants, and still are yet to find a robust *in vitro* activity. However, the autophosphorylation of Cdr1 is accompanied by a mobility shift visible on Western blots (Calonge and O'Connell 2006), and the upregulation of Cdr1 is itself sufficient to confer a wee phenotype (Calonge and O'Connell 2006). In both *tra1-1* and *tra1*Δ cells, there was an ~50–80% increase in phosphor-

ylated (active) Cdr1 (Figure 7, A and B), suggesting that Cdr1 is indeed upregulated in the absence of Tra1.

Under conditions of nitrogen starvation, wild-type *S. pombe* cells advance entry into mitosis at reduced cell size and arrest in the G1 phase of the cell cycle (Young and Fantes 1987). This is achieved, at least in part, by negative regulation of Wee1 by the Cdr kinases (Coleman *et al.* 1993; Parker *et al.* 1993; Wu and Russell 1993; Breeding *et al.* 1998; Kanoh and Russell 1998). Cells lacking Cdr1 are defective in this response and alternatively arrest in G2 phase. We assayed the response to nitrogen starvation in *tra1*Δ cells and observed an enhanced G1 arrest (1C DNA content) in 10% nitrogen and a combination of G1 arrest and dead cells (<1C DNA, although we do not suggest that this is apoptosis) in 0% nitrogen (Figure 7C). Both enhanced responses were largely suppressed by deletion of *cdr1*, although *tra1*Δ *cdr1*Δ cells had a residual starvation response not present in *cdr1*Δ.

Together, these observations suggest that Tra1 may, directly or indirectly, downregulate Cdr1 and, possibly, Cdr2 activity; thus, in the absence of Tra1, increased Cdr kinase activity results in a semi-wee phenotype, an enhanced nitrogen starvation response, and a resistance to *chk1*$^+$ overexpression.

DISCUSSION

Orderly progression through the cell cycle is essential to maintain ploidy and stability of the genome. For the transition from G2 into mitosis, upstream checkpoint proteins signal the timing of mitotic entry. Among these are checkpoints to detect completion of DNA replication, the absence of genomic lesions, the doubling of cell mass, and the synthesis of macromolecules. Ultimately, these signals up- or downregulate the inhibitory Y15 phosphorylation of Cdc2, the universal switch for the transition from G2 into mitosis. Through controlling the kinases and phosphatases that phosphorylate and dephosphorylate Y15, these checkpoint-signaling pathways work together to ensure that mitosis is initiated only when it will result in two viable and identical daughters. Although most checkpoints halt cell cycle progression in response to an insult, osmotic stress and limited nutrition actually advance mitotic entry in *S. pombe* (Young and Fantes 1987; Shiozaki and Russell 1995). It is therefore likely that there must be coregulation of checkpoints such that cell cycle delay can occur in the face of other signals promoting entry into mitosis.

The DNA damage checkpoint, via its effector kinase Chk1, inhibits mitotic entry through direct regulation of Cdc25 and Wee1. In the case of Wee1, phosphorylation by Chk1 stabilizes this otherwise labile protein, increasing the total Wee1 activity in the cell (Raleigh and O'Connell 2000), but does not alter the specific activity of Wee1 (O'Connell *et al.* 1997). Under conditions of limited nutrition, the Cdr kinases Cdr1 and Cdr2 advance mitotic entry by negatively regulating Wee1, and *in vitro* they inhibit recombinant Wee1 by phosphorylation (Coleman *et al.* 1993; Wu and Russell 1993; Breeding *et al.* 1998; Kanoh and Russell 1998). We have previously shown that these Cdr kinases act as Chk1 antagonists. Blocking Cdr kinase activity with dominant-negative *cdr1* alleles, which interfere with both Cdr1 and Cdr2 function, greatly sensitizes cellular sensitivity to overexpressed *chk1*$^+$. Presumably Cdr-mediated inhibitory phosphorylation negates Chk1-mediated Wee1 stability (Calonge and O'Connell 2006). Here, we have identified a new positive regulator of Wee1, the TRRAP homolog Tra1. Cells lacking Tra1-mediated Wee1 regulation have a shortened G2 period of the cell cycle, resulting in a semi-wee phenotype. This renders cells resistant to the overexpression of *chk1*$^+$.

*tra1*Δ cells accumulate Wee1 protein to levels approximately fivefold over wild-type cells without any change in *wee1* mRNA. Wee1 homologs are short-lived PEST sequence proteins, which are subjected to ubiquitin-dependent proteolysis (Michael and Newport 1998; Watanabe *et al.* 2004, 2005). We were not able to demonstrate an increase in Wee1 half-life in *tra1* mutants because a cycloheximide chase actually stabilizes Wee1 protein, which is thought to be a physiological response to protein synthesis rates (Suda *et al.* 2000), although it is not known whether Wee1 is active under these conditions. Further, the very low levels of Wee1 rendered ^{35}S-methionine chase experiments below the level at which we could detect expression. Nevertheless, it remains likely that the increased levels of Wee1 in *tra1*Δ cells is due to a block in ubiquitin-dependent proteolysis.

However, *tra1*Δ cells are actually semi-wee (10–11 μm at division) and have increased Cdc2 activity. Therefore, the Wee1 molecules in these cells must have only residual activity; complete lack of activity would result in a full wee phenotype (division at ~8 μm), while wild-type–specific activity for the increased levels of Wee1 would at least double the size at division (~28 μm), as wild-type cells are very sensitive to increased Wee1 expression (Russell and Nurse 1987). We have not directly measured endogenous Wee1 activity in *tra1*Δ cells because we (and others) have managed this only with recombinant protein (O'Connell *et al.* 1997), which is still a very challenging assay.

How, then, does Tra1 affect the regulation of Wee1? As a component of HAT complexes, Tra1 presumably has an indirect effect on the regulation of Wee1 through altered gene expression, and this is consistent with the TSA- and HDAC mutation-mediated suppression of the Chk1 resistance. However, it is not clear from our expression profiling which genes may be having a direct effect or, indeed, whether this may be a complex and pleiotrophic effect of small changes to the expression of many genes. It is notable that *S. pombe* contains a second TRRAP homolog, Tra2 (SPAC1F5.11c), which, like the single TRRAP gene in *Saccharomyces cerevisiae* (*TRA1*), is

essential for cell viability (Antony M. Carr, personal communication). Therefore, Tra2 may have a more profound effect on gene expression in *S. pombe*, and consistent with this, mass spectrometry analysis shows that Tra2 predominates in Tip60 HAT complexes in *S. pombe* (Shevchenko *et al.* 2008).

Most phenotypes of *tra1*Δ and *tra1-1* are completely suppressed by *cdr1*Δ and partially suppressed by *cdr2*Δ. Therefore, signals resulting in reduced Wee1 activity in the *tra1* mutants must involve increased signaling through the Cdr kinases. The accumulation of hyperphosphorylated Cdr1 and the enhanced nitrogen starvation response in the *tra1*Δ mutants is consistent with this. However, further biochemical characterization will require the development of sensitive and quantitative assays for these kinases, which has yet to be achieved with the endogenous proteins. Further, the precise signals that control Cdr kinase activity are not known, and advances in this regard will inform further analysis of the gene expression profiles controlled by Tra1. We note that Cdr kinases are critical for starvation responses and that many of the genes downregulated by ≥2-fold in *tra1-1* cells encode predicted transporter proteins and nutrient permeases. Therefore, the gene expression changes in *tra1*Δ may potentiate or mimic a starvation response that, via the Cdr kinases, regulates Wee1 and, hence, sensitivity to Chk1.

Importantly, we note that the mechanisms underlying Wee1 inhibition and Wee1 accumulation may not be the same. *cdr1*Δ only partially suppresses the enhanced starvation response of *tra1*Δ cells. Further, although *cdr1* overexpression inhibits Wee1, this does not lead to Wee1 accumulation (Calonge and O'Connell 2006). These observations are consistent with Wee1 inactivation and stabilization being separately regulated events.

TRRAP homologs have also been shown to interact with the MRN complex (Robert *et al.* 2006) and with Tel2, an essential protein that also interacts with ATM, ATR, and DNA-PK$_{cs}$ (Kanoh and Yanagida 2007; Takai *et al.* 2007; Anderson *et al.* 2008) and is part of the ASTRA complex involved in telomere maintenance (Shevchenko *et al.* 2008). It cannot be ruled out that these and other yet to be uncovered molecular interactions also impact on the regulation of Wee1. Furthermore, acetylation of histones and other nonhistone proteins can have effects other than changes in gene expression, for example, in the establishment of epigenetically controlled chromosome segregation (Dunleavy *et al.* 2005; Pidoux and Allshire 2005), and these events could also affect Wee1 activity via stress signaling. We have assayed for the acetylation of immunoprecipitated Chk1, Wee1, and Cdr1 with anti-acetyl lysine antibodies, which we did not observe (not shown). Furthermore, we cannot detect a physical interaction between Tra1 and these proteins, although we treat these data with caution because a C-terminal epitope tag completely inactivates Tra1 and an N-terminal tag partially inactivates the protein (as measured by cell size and response to *chk1*$^{+}$ overexpression). Therefore, additional biochemical tools need to be developed to pursue these studies.

Inhibitors of both Chk1 and HDACs have been developed and are in trial for use as anticancer agents. This work links these two biological processes and opens a window for investigation in areas where these types of therapeutics might be used together in targeted therapies. Like Chk1 and Wee1, TRRAP is highly conserved across species, and we anticipate that human TRRAP will impact on the Chk1-Wee1 pathway of Cdc2 regulation in humans that may prove to be useful in designing these therapeutic regimens.

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LITERATURE CITED

Alfa, C. E., R. Booher, D. Beach and J. S. Hyams, 1989 Fission yeast cyclin: subcellular localisation and cell cycle regulation. J Cell Sci Suppl **12:** 9–19.

Allard, S., R. T. Utley, J. Savard, A. Clarke, P. Grant *et al.*, 1999 NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p. EMBO J. **18:** 5108–5119.

Anderson, C. M., D. Korkin, D. L. Smith, S. Makovets, J. J. Seidel *et al.*, 2008 Tel2 mediates activation and localization of ATM/Tel1 kinase to a double-strand break. Genes Dev. **22:** 854–859.

Baber-Furnari, B. A., N. Rhind, M. N. Boddy, P. Shanahan, A. Lopez-Girona *et al.*, 2000 Regulation of mitotic inhibitor *mik1* helps to enforce the DNA damage checkpoint. Mol. Biol. Cell **11:** 1–11.

Basi, G., E. Schmid and K. Maundrell, 1993 TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene **123:** 131–136.

Bermudez, V. P., L. A. Lindsey-Boltz, A. J. Cesare, Y. Maniwa, J. D. Griffith *et al.*, 2003 Loading of the human 9–1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro. Proc. Natl. Acad. Sci. USA **100:** 1633–1638.

Bimbo, A., Y. Jia, S. L. Poh, R. K. Karuturi, N. den Elzen *et al.*, 2005 Systematic deletion analysis of fission yeast protein kinases. Eukaryot. Cell **4:** 799–813.

Boudeau, J., D. Miranda-Saavedra, G. J. Barton and D. R. Alessi, 2006 Emerging roles of pseudokinases. Trends Cell Biol. **16:** 443–452.

Breeding, C. S., J. Hudson, M. K. Balasubramanian, S. M. Hemmingsen, P. G. Young *et al.*, 1998 The cdr2(+) gene encodes a regulator of G2/M progression and cytokinesis in Schizosaccharomyces pombe. Mol. Biol. Cell **9:** 3399–3415.

Cai, Y., J. Jin, C. Tomomori-Sato, S. Sato, I. Sorokina *et al.*, 2003 Identification of new subunits of the multiprotein mammalian TRRAP/TIP60-containing histone acetyltransferase complex. J. Biol. Chem. **278:** 42733–42736.

Calonge, T. M., and M. J. O'Connell, 2006 Antagonism of Chk1 signaling in the G2 DNA damage checkpoint by dominant alleles of Cdr1. Genetics **174:** 113–123.

Canman, C. E., 2003 Checkpoint mediators: relaying signals from DNA strand breaks. Curr. Biol. **13:** R488–R490.

Capasso, H., C. Palermo, S. Wan, H. Rao, U. P. John *et al.*, 2002 Phosphorylation activates Chk1 and is required for checkpoint-mediated cell cycle arrest. J. Cell Sci. **115:** 4555–4564.

Chen, P., C. Luo, Y. Deng, K. Ryan, J. Register *et al.*, 2000 The 1.7 A crystal structure of human cell cycle checkpoint kinase Chk1: implications for Chk1 regulation. Cell **100:** 681–692.

Christensen, P. U., N. J. Bentley, R. G. Martinho, O. Nielsen and A. M. Carr, 2000 Mik1 levels accumulate in S phase and may mediate an intrinsic link between S phase and mitosis. Proc. Natl. Acad. Sci. USA **97:** 2579–2584.

Coleman, T. R., Z. Tang and W. G. Dunphy, 1993 Negative regulation of the wee1 protein kinase by direct action of the nim1/cdr1 mitotic inducer. Cell **72:** 919–929.

den Elzen, N. R., and M. J. O'Connell, 2004 Recovery from DNA damage checkpoint arrest by PP1-mediated inhibition of Chk1. EMBO J. **23:** 908–918.

den Elzen, N., A. Kosoy, H. Christopoulos and M. J. O'Connell, 2004 Resisting arrest: recovery from checkpoint arrest through dephosphorylation of Chk1 by PP1. Cell Cycle **3:** 529–533.

Dunleavy, E., A. Pidoux and R. Allshire, 2005 Centromeric chromatin makes its mark. Trends Biochem. Sci. **30:** 172–175.

Dunphy, W. G., 1994 The decision to enter mitosis. Trends Cell Biol. **4:** 202–207.

Ekwall, K., 2005 Genome-wide analysis of HDAC function. Trends Genet. **21:** 608–615.

Falck, J., J. Coates and S. P. Jackson, 2005 Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature **434:** 605–611.

Feilotter, H., P. Nurse and P. G. Young, 1991 Genetic and molecular analysis of *cdr1/nim1* in *Schizosaccharomyces pombe*. Genetics **127:** 309–318.

Forsburg, S. L., 1993 Comparison of *Schizosaccharomyces pombe* expression systems. Nucleic Acids Res. **21:** 2955–2956.

Gatei, M., K. Sloper, C. Sorensen, R. Syljuasen, J. Falck *et al.*, 2003 Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. J. Biol. Chem. **278:** 14806–14811.

Gomez, E. B., J. M. Espinosa and S. L. Forsburg, 2005 *Schizosaccharomyces pombe mst2+* encodes a MYST family histone acetyltransferase that negatively regulates telomere silencing. Mol. Cell. Biol. **25:** 8887–8903.

Grant, P. A., D. Schieltz, M. G. Pray-Grant, J. R. Yates, III and J. L. Workman, 1998 The ATM-related cofactor Tra1 is a component of the purified SAGA complex. Mol. Cell **2:** 863–867.

Grewal, S. I., M. J. Bonaduce and A. J. Klar, 1998 Histone deacetylase homologs regulate epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. Genetics **150:** 563–576.

Harvey, S. H., D. M. Sheedy, A. R. Cuddihy and M. J. O'Connell, 2004 Coordination of DNA damage responses via the Smc5/Smc6 complex. Mol. Cell. Biol. **24:** 662–674.

Jiang, X., Y. Sun, S. Chen, K. Roy and B. D. Price, 2006 The FATC domains of PIKK proteins are functionally equivalent and participate in the Tip60-dependent activation of DNA-PKcs and ATM. J. Biol. Chem. **281:** 15741–15746.

Kanoh, J., and P. Russell, 1998 The protein kinase Cdr2, related to Nim1/Cdr1 mitotic inducer, regulates the onset of mitosis in fission yeast. Mol. Biol. Cell **9:** 3321–3334.

Kanoh, J., and M. Yanagida, 2007 Tel2: A common partner of PIK-related kinases and a link between DNA checkpoint and nutritional response? Genes Cells **12:** 1301–1304.

Katsuragi, Y., and N. Sagata, 2004 Regulation of Chk1 kinase by autoinhibition and ATR-mediated phosphorylation. Mol. Biol. Cell **15:** 1680–1689.

Kosoy, A., and M. J. O'Connell, 2008 Regulation of Chk1 by its C-terminal domain. Mol. Biol. Cell **19:** 4546–4553.

Kuntz, K., and M. J. O'Connell, 2009 The G(2) DNA damage checkpoint: Could this ancient regulator be the achilles heel of cancer? Cancer Biol. Ther. **8:** 1433–1439.

Latif, C., N. R. Elzen and M. J. O'Connell, 2004 DNA damage checkpoint maintenance through sustained Chk1 activity. J. Cell Sci. **117:** 3489–3498.

Liu, Q., S. Guntuku, X. S. Cui, S. Matsuoka, D. Cortez *et al.*, 2000 Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. **14:** 1448–1459.

Lopez-Girona, A., B. Furnari, O. Mondesert and P. Russell, 1999 Nuclear localization of Cdc25 is regulated by DNA damage and a 14–3-3 protein. Nature **397:** 172–175.

Lopez-Girona, A., J. Kanoh and P. Russell, 2001a Nuclear exclusion of Cdc25 is not required for the DNA damage checkpoint in fission yeast. Curr. Biol. **11:** 50–54.

Lopez-Girona, A., K. Tanaka, X. B. Chen, B. A. Baber, C. H. McGowan *et al.*, 2001b Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast. Proc. Natl. Acad. Sci. USA **98:** 11289–11294.

Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner *et al.*, 1991 mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell **64:** 1111–1122.

Martin, S. G., and M. Berthelot-Grosjean, 2009 Polar gradients of the DYRK-family kinase Pom1 couple cell length with the cell cycle. Nature **459:** 852–856.

Maundrell, K., 1993 Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123:** 127–130.

Michael, W. M., and J. Newport, 1998 Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1. Science **282:** 1886–1889.

Moreno, S., P. Nurse and P. Russell, 1990 Regulation of mitosis by cyclic accumulation of p80cdc25 mitotic inducer in fission yeast. Nature **344:** 549–552.

Moreno, S., A. Klar and P. Nurse, 1991 Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194:** 795–823.

Moseley, J. B., A. Mayeux, A. Paoletti and P. Nurse, 2009 A spatial gradient coordinates cell size and mitotic entry in fission yeast. Nature **459:** 857–860.

Nurse, P., 1990 Universal control mechanism regulating onset of M-phase. Nature **344:** 503–508.

O'Connell, M. J., and K. A. Cimprich, 2005 G2 damage checkpoints: What is the turn-on? J. Cell Sci. **118:** 1–6.

O'Connell, M. J., J. M. Raleigh, H. M. Verkade and P. Nurse, 1997 Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. EMBO J. **16:** 545–554.

O'Connell, M. J., N. C. Walworth and A. M. Carr, 2000 The G2-phase DNA-damage checkpoint. Trends Cell Biol. **10:** 296–303.

Outwin, E. A., A. Irmisch, J. M. Murray and M. J. O'Connell, 2009 Smc5-Smc6-dependent removal of cohesin from mitotic chromosomes. Mol. Cell. Biol. **29:** 4363–4375.

Palermo, C., J. C. Hope, G. A. Freyer, H. Rao and N. C. Walworth, 2008 Importance of a C-terminal conserved region of chk1 for checkpoint function. PLoS ONE **3:** e1427.

Parker, L. L., and H. Piwnica-Worms, 1992 Inactivation of the $p34^{cdc2}$-cyclin B complex by the human WEE1 tyrosine kinase. Science **257:** 1955–1957.

Parker, L. L., S. Atherton-Fessler, M. S. Lee, S. Ogg, J. L. Falk *et al.*, 1991 Cyclin promotes the tyrosine phosphorylation of $p34^{cdc2}$ in a $wee1^{+}$ dependent manner. EMBO J. **10:** 1255–1263.

Parker, L. L., S. Atherton-Fessler and H. Piwnica-Worms, 1992 $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. Proc. Natl. Acad. Sci. USA **89:** 2917–2921.

Parker, L. L., S. A. Walter, P. G. Young and H. Piwnica-Worms, 1993 Phosphorylation and inactivation of the mitotic inhibitor wee1 by the *nim1/cdr1* kinase. Nature **363:** 736–738.

Parrilla-Castellar, E. R., S. J. Arlander and L. Karnitz, 2004 Dial 9–1-1 for DNA damage: the Rad9-Hus1-Rad1 (9–1-1) clamp complex. DNA Repair (Amst.) **3:** 1009–1014.

Pereira, E., Y. Chen and Y. Sanchez, 2009 Conserved ATRMec1 phosphorylation-independent activation of Chk1 by single amino acid substitution in the GD domain. Cell Cycle **8:** 1788–1793.

Pidoux, A. L., and R. C. Allshire, 2005 The role of heterochromatin in centromere function. Philos. Trans. R. Soc. Lond. B Biol. Sci. **360:** 569–579.

Raleigh, J. M., and M. J. O'Connell, 2000 The G(2) DNA damage checkpoint targets both Wee1 and Cdc25. J. Cell Sci. **113:** 1727–1736.

Rhind, N., and P. Russell, 2001 Roles of the mitotic inhibitors Wee1 and Mik1 in the G(2) DNA damage and replication checkpoints. Mol. Cell. Biol. **21:** 1499–1508.

Rhind, N., B. Furnari and P. Russell, 1997 Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. Genes Dev. **11:** 504–511.

Robert, F., S. Hardy, Z. Nagy, C. Baldeyron, R. Murr *et al.*, 2006 The transcriptional histone acetyltransferase cofactor TRRAP associates with the MRN repair complex and plays a role in DNA double-strand break repair. Mol. Cell. Biol. **26:** 402–412.

Russell, P., and P. Nurse, 1987 Negative regulation of mitosis by *wee1*+, a gene encoding a protein kinase homolog. Cell **49:** 559–567.

Shevchenko, A., A. Roguev, D. Schaft, L. Buchanan, B. Habermann *et al.*, 2008 Chromatin Central: towards the comparative proteome by accurate mapping of the yeast proteomic environment. Genome Biol **9:** R167.

Shiozaki, K., and P. Russell, 1995 Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature **378:** 739–743.

Suda, M., S. Yamada, T. Toda, T. Miyakawa and D. Hirata, 2000 Regulation of Wee1 kinase in response to protein synthesis inhibition. FEBS Lett. **486:** 305–309.

Takai, H., R. C. Wang, K. K. Takai, H. Yang and T. de Lange, 2007 Tel2 regulates the stability of PI3K-related protein kinases. Cell **131:** 1248–1259.

Tapia-Alveal, C., T. M. Calonge and M. J. O'Connell, 2009 Regulation of Chk1. Cell Div. **4:** 8.

Walworth, N., S. Davey and D. Beach, 1993 Fission yeast *chk1* protein kinase links the rad checkpoint pathway to *cdc2*. Nature **363:** 368–371.

Wang, S. X., and W. G. Dunphy, 2000 Activation of Xenopus Chk1 by mutagenesis of threonine-377. FEBS Lett. **487:** 277–281.

Watanabe, N., H. Arai, Y. Nishihara, M. Taniguchi, T. Hunter *et al.*, 2004 M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. Proc. Natl. Acad. Sci. USA **101:** 4419–4424.

Watanabe, N., H. Arai, J. Iwasaki, M. Shiina, K. Ogata *et al.*, 2005 Cyclin-dependent kinase (CDK) phosphorylation destabilizes somatic Wee1 via multiple pathways. Proc. Natl. Acad. Sci. USA **102:** 11663–11668.

Wolfe, B. A., and K. L. Gould, 2004 Fission yeast Clp1p phosphatase affects G2/M transition and mitotic exit through Cdc25p inactivation. EMBO J. **23:** 919–929.

Wu, L., and P. Russell, 1993 Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. Nature **363:** 738–741.

Yamada, T., K. Mizuno, K. Hirota, N. Kon, W. P. Wahls *et al.*, 2004 Roles of histone acetylation and chromatin remodeling factor in a meiotic recombination hotspot. EMBO J. **23:** 1792–1803.

Young, P., and P. Fantes, 1987 *Schizosaccharomyces pombe* mutants affected in their division response to starvation. J. Cell Sci. **88:** 295–304.

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GENETICS

Supporting Information
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Transformation/Transcription Domain-Associated Protein (TRRAP)-Mediated Regulation of Wee1

Teresa M. Calonge, Majid Eshaghi, Jianhua Liu, Ze'ev Ronai and Matthew J. O'Connell

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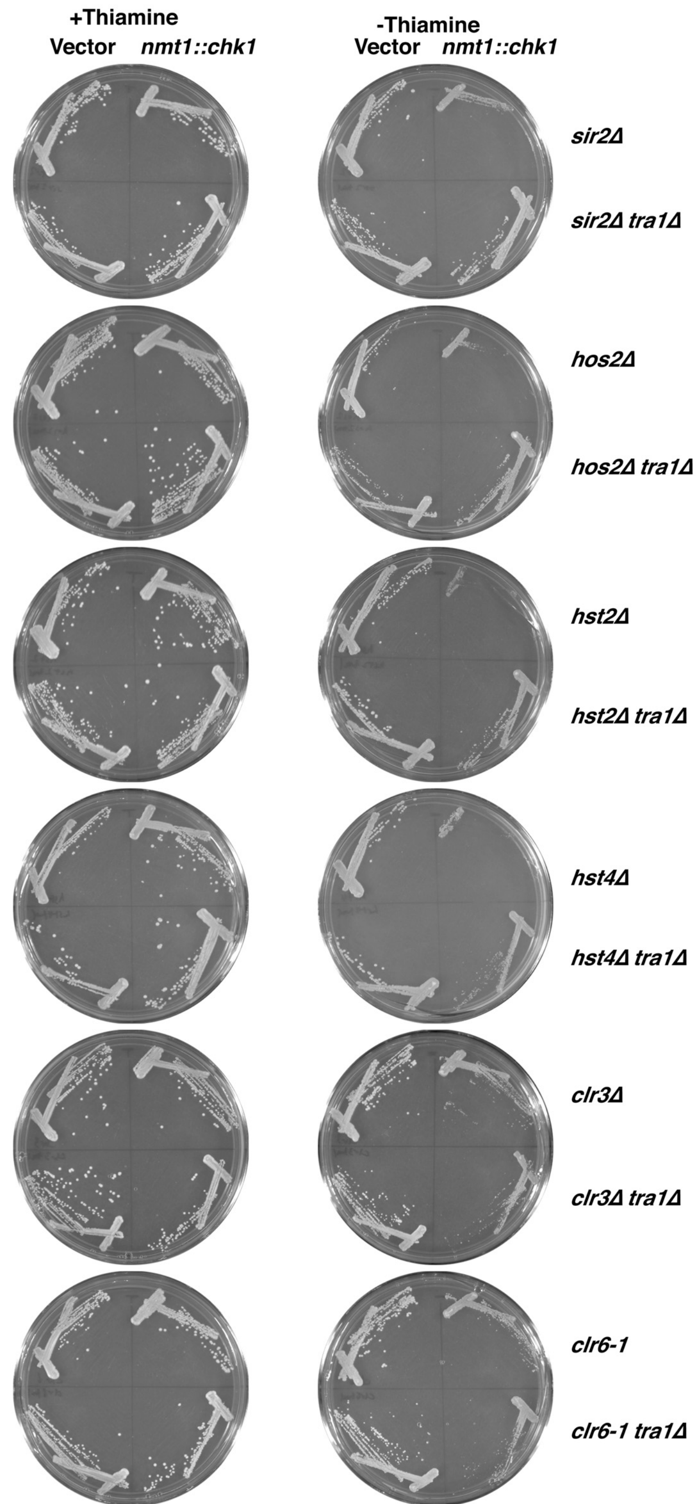

FIGURE S1.—Effect of HDAC mutations on sensitivity to *chk1*$^{+}$ overexpression. The indicated strains were transformed with pREP1 (vector) or pREP1-Chk1 (*nmt1::chk1*) and streaked on plates either with thiamine (promoter repressed), or without thiamine (promoter derepressed). Plates were incubated at 30°C for 4 days, except for *clr6-1* strains, which were incubated at 25°C for 6 days.

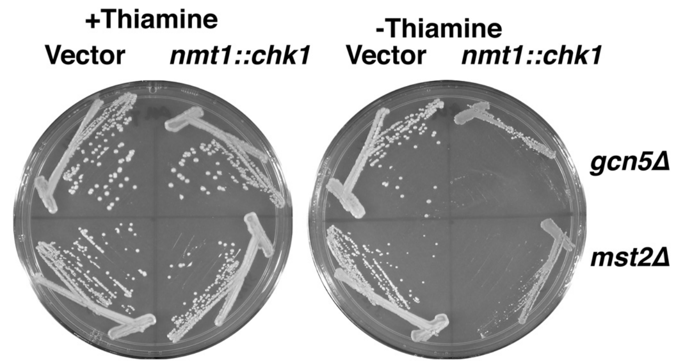

FIGURE S2.—Effect of HAT mutations on sensitivity to *chk1⁺* overexpression. The indicated strains were transformed with pREP1 (vector) or pREP1-Chk1 (*nmt1::chk1*) and streaked on plates either with thiamine (promoter repressed), or without thiamine (promoter derepressed). Plates were incubated at 30°C for 4 days.

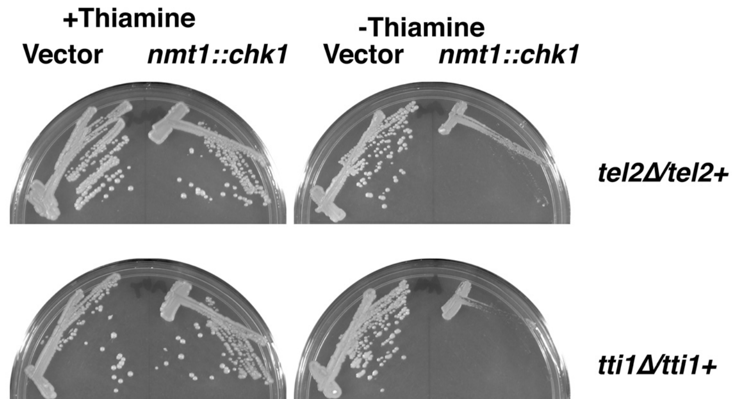

FIGURE S3.—Effect of ASTRA complex mutations on sensitivity to *chk1+* overexpression. The indicated diploid strains, heterozygous for null alleles of components of the ASTRA complex, were transformed with pREP1 (vector) or pREP1-Chk1 (*nmt1::chk1*) and streaked on plates either with thiamine (promoter repressed), or without thiamine (promoter derepressed). Plates were incubated at 30°C for 4 days.

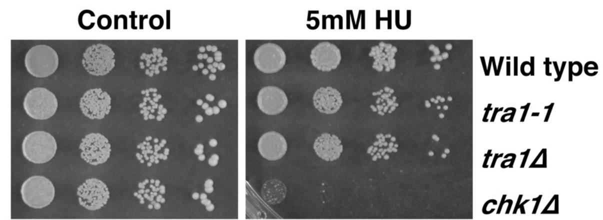

FIGURE S4.—*Tra1* mutants are not sensitive to the ribonucleotide reductase inhibitor hydroxyurea (HU). Ten-fold serial dilutions were spotted onto control plates, or plates containing 5mM HU, and incubated at 30°C for 4 days.

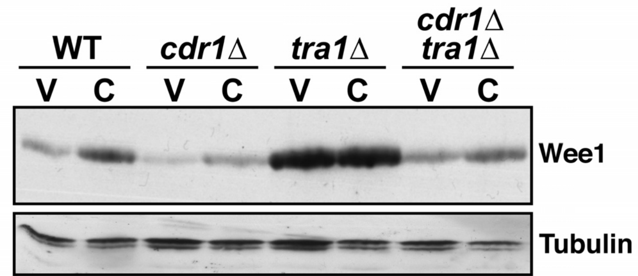

FIGURE S5.—Regulation of Wee1 levels. The indicated strains expressing an HA-tagged allele of Wee1 were transformed with pREP1 (V) or pREP1-Chk1 (C), and grown in media lacking thiamine for 20 hours at 30°C. Wee1 levels were detected by western blotting with an anti-HA antibody (12CA5), and tubulin was used as a loading control.

TABLE S1

Full data set for microarray analysis, wildtype vs *tra1-1*

Table S1 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.110.114769/DC1.

Data are mean (log2) fold change tra1-1 versus wildtype (n=3) compared to mean of wild type versus wildtype (n=2). P values were obtained by T-test.