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DNA Replication Stalling Attenuates Tyrosine Kinase Signaling to Suppress S Phase Progression

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SUMMARY

Here we report that T cell protein tyrosine phosphatase (TCPTP)-dependent and -independent pathways attenuate the JAK and Src protein tyrosine kinases (PTKs) and STAT3 phosphorylation to suppress cyclin D1 expression and S phase progression in response to DNA replication stress. Cells that lack TCPTP fail to suppress JAK1, Src, and STAT3, allowing for sustained cyclin D1 levels and progression through S phase despite continued replication stress. Cells that bypass the checkpoint undergo aberrant mitoses with lagging chromosomes that stain for the DNA damage marker γH2AX. Therefore, inactivating JAK, Src, and STAT3 signaling pathways in response to DNA replication stress may be essential for the suppression of S phase progression and the maintenance of genomic stability.

INTRODUCTION

During cellular division, specific and intricate surveillance mechanisms, known as checkpoints, facilitate orderly cell-cycle progression and ensure the faithful transmission of replicated DNA to daughter cells. Failure of such checkpoints can lead to genomic instability that is associated with many human cancers. The mammalian phosphatidylinositol 3-kinase (PI3K)-related serine/threonine protein kinases ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia Rad3-related) are integral regulators of cell-cycle checkpoints. In S phase, they are activated in response to varied forms of cellular stress: ATM is activated primarily by DNA double-strand breaks (DSBs) caused by ionizing radiation or radiomimetic drugs, whereas ATR is activated by replication stalls as well as a broad range of other forms of DNA damage. ATM and ATR phosphorylate numerous substrates, including histone H2AX and the protein kinases Chk1 and 2, which in turn phosphorylate other proteins, including the transcription factor p53 and the Cdc25 phosphatases, to mediate checkpoint responses (Kastan and Bartek, 2004).

During S phase, the genome is under constant scrutiny to ensure that DNA is copied accurately and completely before cells enter mitosis. Both DNA damage and DNA replication checkpoint mechanisms serve to maintain the fidelity of DNA. The DNA damage checkpoints suppress S phase progression and coordinate the repair of DNA in response to genotoxic insults that cause damage away from active replicons. The DNA replication checkpoint is ATR dependent and functions specifically in response to stalls in DNA synthesis to inhibit origin firing, stabilize replication forks, and orchestrate DNA repair (Bartek et al., 2004; Osborn et al., 2002). The yeast ATM/ATR homologs Mec1 (*S. cerevisiae*) and Rad3 (*S. pombe*) are essential for S phase arrest in response to replication inhibitors; *Mec1* and *Rad3* mutant yeast exposed to replication inhibitors fail to arrest in S phase and proceed to partition unreplicated chromosomes (al-Khodairy and Carr, 1992; Enoch et al., 1992; Weinert et al., 1994). In contrast, mouse embryonic fibroblasts (MEFs) deficient in ATR, ATM and ATR, or ATR and p53 remain arrested in S phase after the inhibition of DNA synthesis with the polymerase α inhibitor aphidicolin (Brown and Baltimore, 2003). Thus,

SIGNIFICANCE

The JAK and Src protein tyrosine kinases (PTKs) and their substrates STAT3 and cyclin D1 are overexpressed and/or activated in a wide variety of human tumors contributing to the proliferative drive. Here we report that the inactivation of PTK pathways and the depletion of cyclin D1 can contribute to the delay in cell division when DNA replication is compromised. When PTK pathways are not turned off and cyclin D1 is sustained, unscheduled cell division can ensue wherein cells partition their DNA unevenly. Our results identify a mechanism by which oncogenic PTK signaling may bypass the replication checkpoint and contribute to genetic instability in cancer.

additional pathways contribute to the suppression of S phase progression when DNA replication is compromised in mammalian cells.

The T cell protein tyrosine phosphatase (TCPTP; *Ptpn2*) is an intracellular nontransmembrane tyrosine-specific phosphatase (Tiganis and Bennett, 2007). Although most abundant in the hematopoietic compartment, TCPTP is present in all tissues and at all stages of mammalian development. TCPTP can specifically regulate growth factor- and cytokine-induced protein tyrosine kinase (PTK)-mediated signaling pathways by dephosphorylating distinct substrates that include the Janus-activated PTKs (JAKs) 1 and 3 and the JAK substrates signal transducer and activator of transcription (STAT) 1, 3, and 6 (Lu et al., 2007; Tiganis and Bennett, 2007). More recently, our laboratory also identified Src-family PTKs (SFKs) as cellular substrates for TCPTP and reported increased SFK activation in TCPTP-deficient cells (van Vliet et al., 2005). The JAKs, SFKs, and their common substrate STAT3 are frequently hyperactivated in a wide variety of human malignancies promoting cell-cycle progression and proliferation (Bromberg et al., 1999; Valentino and Pierre, 2006; Yeatman, 2004). Here we report that the inactivation of the SFK and JAK PTKs and STAT3 by TCPTP, for the inhibition of cyclin D1 expression, contributes to the suppression of S phase progression in response to DNA replication stress. Furthermore, we identify a mechanism by which oncogenic PTK pathways may bypass the replication checkpoint and contribute to genetic instability and tumorigenesis.

RESULTS

TCPTP Attenuates PTK Signaling in Response to DNA Replication Stress

To determine TCPTP's role in PTK signaling in response to replication stress, we used spontaneously immortalized *Ptpn2*$^{-/-}$ MEFs versus those reconstituted stably with physiological amounts of TCPTP (Galic et al., 2003). Cells were synchronized in G0 by serum deprivation and released into the cell cycle by the readdition of serum. In late G1 (12 hr release), cells were treated with replication inhibitors for 6–36 hr (S phase entry normally occurred at 15–18 hr release), and PTK signaling was assessed by immunoblot analysis using antibodies against the phosphorylated and activated forms of c-Src (Tyr418), JAK1 (Tyr1022/Tyr1023), and STAT3 (Tyr705). Two replication inhibitors were used: aphidicolin, which inhibits polymerase α, and thymidine, which indirectly inhibits ribonucleotide reductase. Previous studies have established that thymidine-induced stalls can result in the generation of poorly defined replication intermediates but not DSBs (Liu and Lim, 2005; Lundin et al., 2002; Saleh-Gohari et al., 2005; Xie et al., 2004), whereas aphidicolin-induced stalls collapse to form DSBs (Brown and Baltimore, 2003; Rothkamm et al., 2003; Saintigny et al., 2001). Consistent with this, both agents activated the DNA replication checkpoint as assessed by the phosphorylation and retarded electrophoretic mobility of the ATR substrate Chk1, but only aphidicolin activated ATM (Ser1981 phosphorylation) (Figure 1A). Also, p53 activation (accumulation and Ser18 phosphorylation) and histone H2AX phosphorylation (Ser139; γH2AX) were more pronounced in the presence of aphidicolin (Figure 1A), in line with aphidicolin's propensity to induce DSBs.

In TCPTP-reconstituted MEFs, thymidine- or aphidicolin-instigated S phase arrest attenuated SFK, JAK1, and STAT3 phosphorylation (Figure 1B). This could not be ascribed to changes in PTK signaling during normal G1/S and G2/M progression, as SFK, JAK1, and STAT3 phosphorylation remained unaltered or increased modestly in cells otherwise proceeding through G1 into S and thereon into G2 (data not shown). The inactivation of PTK pathways in response to thymidine or aphidicolin occurred when ATR/Chk1 signaling had waned (after 12 hr) and ATM activation, p53 induction, and γH2AX were greatest (Figures 1A and 1B). PTK inactivation in response to DNA replication stress induced by thymidine was also noted in other cells including HeLa cells and normal human 7625 (Figure 1C) and MRC5 fibroblasts (data not shown). Since thymidine and aphidicolin act indirectly to slow S phase progression, we asked whether PTK pathway suppression also occurred in response to agents that modify DNA to directly impede the advancing fork. Methyl methanesulfonate (MMS) is a DNA-alkylating agent that has been used widely as a radiomimetic. However, recent evidence indicates that MMS does not induce DSBs (Lundin et al., 2005) but instead slows fork progression, increases stalling, and inhibits origin firing in yeast and mammals (Merrick et al., 2004; Tercero and Diffley, 2001). Accordingly, we examined the status of PTK signaling pathways in response to MMS. MEFs were treated with MMS either at 15 hr release, at the onset of S phase entry, or at 18 hr release, when the cells were in S phase. In both cases, S phase progression was inhibited (data not shown) and JAK1, STAT3, and to a lesser extent SFKs were inactivated, coinciding with the induction of Chk1 signaling (Figure 1D); MMS treatment also resulted in JAK1 and STAT3 inactivation in HeLa cells during S phase (data not shown). Addition of MMS to MEFs at earlier times, during G1, did not inactivate PTK pathways until the equivalent of 15 hr release, when the cells would otherwise be entering S phase (see Figure S1 available online). Thus, these results indicate that the inactivation of PTKs may be a specific and active response to DNA replication stress. Consistent with this interpretation, we found that exposing S phase cells (G1/S synchronized and released into S) to ultraviolet light (UV), which generates pyrimidine dimers that stall the advancing replication fork, also suppressed PTK and STAT3 signaling (Figure 1E). In contrast, we found that the topoisomerase II inhibitor etoposide, which is capable of promoting replicon-independent damage (Tanaka et al., 2007), accumulated cells in G1 and S but did not inhibit SFK or STAT3 signaling even after 36 hr of treatment (Figure 1F). PTK pathways also remained unaltered in response to other genotoxic agents, including adriamycin (data not shown), which generates DSBs in G1. Thus, the inactivation of PTK pathways may be a specific response to replicative stress.

In TCPTP-deficient MEFs, SFKs, JAK1, and STAT3 remained phosphorylated and activated even at 36 hr of thymidine treatment (Figure 1B). Indeed, rather than being inactivated, STAT3 phosphorylation increased over time to >4-fold when compared to untreated TCPTP-deficient cells proceeding through S phase (Figure 1G). Importantly, the increase in STAT3 phosphorylation was evident within hours of S phase onset (9 hr thymidine treatment, equivalent to 21 hr release) in a time frame similar to ATR/Chk1 signaling (Figure 1G). STAT3 phosphorylation did not otherwise increase in TCPTP-deficient cells proceeding through S

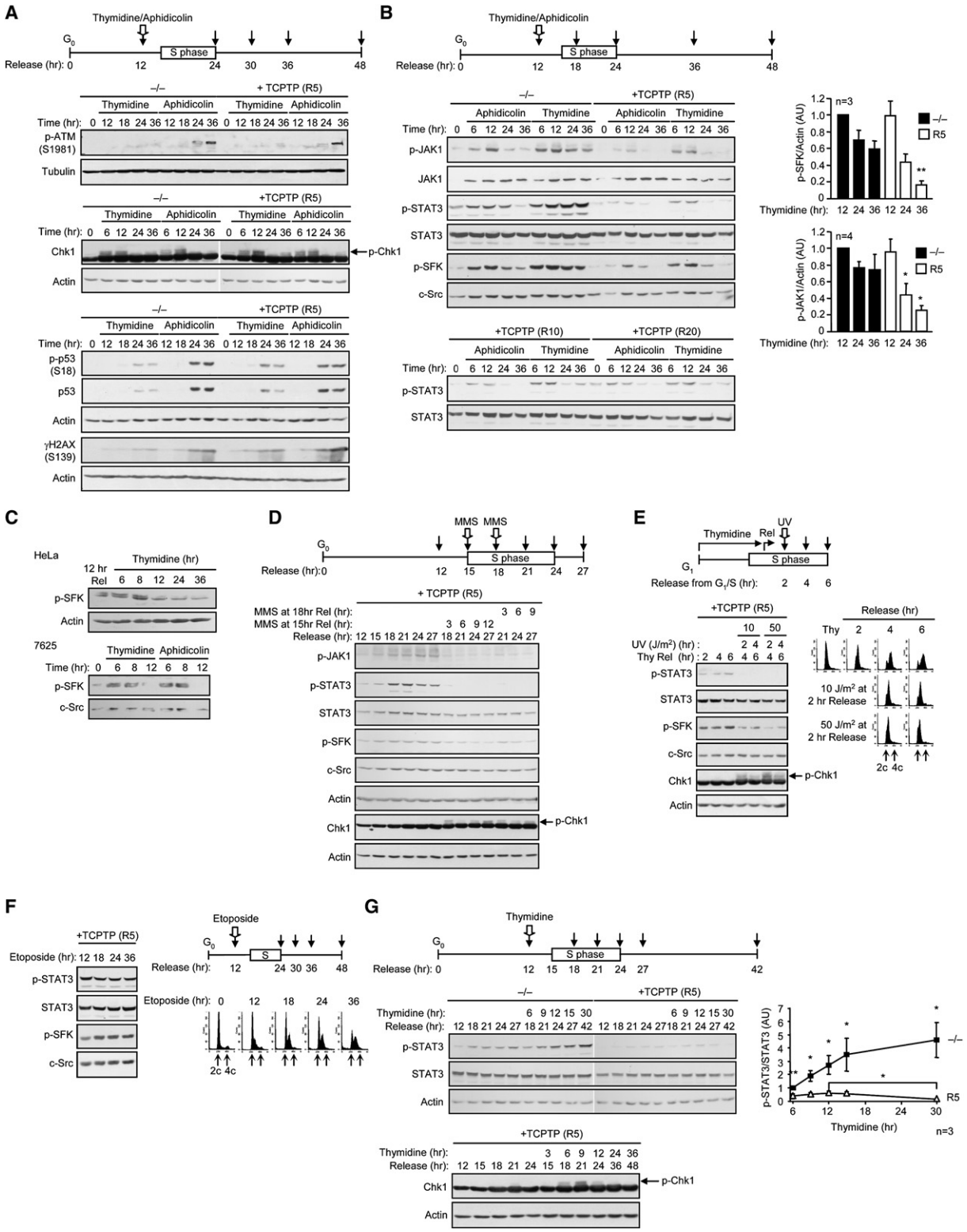

Figure 1. SFKs, JAK1, and STAT3 Are Attenuated in Response to DNA Replication Stress

(A and B) G0-synchronized and released *Ptpn2*$^{-/-}$ (–/–) and 45 kDa TCPTP-reconstituted MEFs (+TCPTP; clones R5, R10, and R20) were treated with thymidine (2 mM) or aphidicolin (2 μM), and lysates were processed for immunoblot analysis.

(C) HeLa and 7625 cells were synchronized by double thymidine block and released for 12 hr so that cells returned to G1 and then treated with thymidine (5 mM) or aphidicolin (2 μM) as indicated, and lysates were processed for immunoblot analysis.

(see 18–24 hr release in Figure 1G), indicating that the hyperactivation was a specific response to replication stress. Therefore, these results suggest that TCPTP may be required to prevent STAT3 hyperactivation when replication is stalled. In response to aphidicolin, the SFK, JAK1, and STAT3 pathways were also elevated when compared to TCPTP-reconstituted cells treated with aphidicolin (Figure 1B), and STAT3 activation increased over time until 24–36 hr of treatment (Figure S2), when ATM signaling ensued (Figure 1A). However, in response to MMS or UV, PTK pathway inactivation/suppression occurred irrespective of TCPTP status (Figure S3; data not shown). Therefore, both TCPTP-dependent and -independent mechanisms may exist for the attenuation of SFK, JAK1, and STAT3 signaling in response to DNA replication stress.

DNA Replication Checkpoint Bypass in TCPTP-Deficient Cells

Next we assessed the possible impact of TCPTP deficiency on the DNA replication checkpoint. We saw no difference in the activation of ATM/ATR checkpoint pathways as monitored by ATM Ser1981 phosphorylation, Chk1 phosphorylation, p53 induction, and γH2AX in TCPTP-deficient versus -reconstituted cells arrested in S phase with thymidine, aphidicolin (Figure 1A), UV, or MMS (data not shown). Consistent with this, TCPTP-deficient and -reconstituted cells responded similarly to genotoxic agents that instigated ATM/ATR-mediated checkpoint arrests in G1, S, or G2 (data not shown). Thus, ATM/ATR checkpoint pathways and responses are not generally altered in the absence of TCPTP. Despite this, we found that TCPTP-deficient cells (or vector control cells; see Figure S4) treated with thymidine bypassed the replication checkpoint, traversing S phase and progressing into G2 as determined by an increase in DNA content (Figures 2A and 2B) and an earlier peak in cyclin B1 expression (Figure 2C). In contrast, TCPTP-reconstituted cells remained arrested at G1/S in the presence of thymidine until approximately 36 hr treatment, when they began to progress through S phase (Figures 2A and 2B). Furthermore, the replication checkpoint bypass was not a mere consequence of enhanced G1 or S phase progression, since the G1 restriction point was not altered and cells synchronized in S phase by brief thymidine or aphidicolin treatment progressed similarly after release (data not shown).

The checkpoint bypass and progression of TCPTP null MEFs through S phase in the presence of thymidine was apparent within 12 hr of treatment, when the cells would otherwise be completing S phase (Figures 2A and 2B). By 20–24 hr of treatment, TCPTP-deficient cells that had bypassed the thymidine-induced arrest appeared to have completed mitosis and reentered G1 of the next cell cycle (Figure 2A). Consistent with this, we noted an increase in phosphorylated CDK1 (S/T)P epitopes (MPM-2), an increase in histone H3 phosphorylation, and the presence of mitotic figures (Figures 2D–2F). Roughly one-third of all mitoses occurring in the presence of thymidine were aberrant and characterized predominantly by lagging chromosomes (Figures 3A and 3B); mitotic aberrations were not evident in untreated TCPTP-deficient cells or in TCPTP-reconstituted cells treated with thymidine (data not shown). The incidence of lagging chromosomes in cells that had been arrested in S phase with thymidine for 24 hr and then allowed to proceed into mitosis was reduced by approximately 50% (data not shown), consistent with the aberrations being attributable to checkpoint bypass. Notably, in many cases, the unaligned and lagging chromosomes stained for γH2AX (Figure 3C), a marker for DSBs and other forms of DNA damage (Liu and Lim, 2005; Lundin et al., 2002; Saleh-Gohari et al., 2005; Ward and Chen, 2001; Xie et al., 2004). Thymidine-induced stalls are known to result in replication intermediates that involve γH2AX recruitment (Bolderson et al., 2004; Liu and Lim, 2005; Lundin et al., 2002; Saleh-Gohari et al., 2005; Xie et al., 2004), and γH2AX foci were readily discernable in thymidine-treated TCPTP-deficient and -reconstituted cells (data not shown). Therefore, one possibility is that the γH2AX-positive lagging chromosomes in TCPTP-deficient cells may have resulted from unresolved replication intermediates.

We also tested the response of TCPTP-deficient cells to aphidicolin and another widely used replication inhibitor, hydroxyurea. Although PTK pathways were elevated in TCPTP-deficient cells when compared to TCPTP-reconstituted cells treated with aphidicolin, TCPTP-deficient cells remained arrested in S phase and did not progress into G2 (data not shown). Similarly, we found that TCPTP-deficient cells did not bypass the checkpoint induced by hydroxyurea (data not shown), which inhibits ribonucleotide reductase to deplete all cellular deoxyribonucleotide triphosphates (thymidine only depletes deoxycytidine triphosphate). Previous studies have established that stalls induced by aphidicolin and hydroxyurea can collapse to form DSBs (Lundin et al., 2002; Rothkamm et al., 2003; Saintigny et al., 2001). In contrast, thymidine-induced stalls do not result in DSBs (Lundin et al., 2002). Given that DNA damage signaling pathways and responses may not be altered in the absence of TCPTP (Figure 1A; data not shown), we reasoned that the inability of TCPTP-deficient cells to bypass the aphidicolin-induced checkpoint might be due to the DSB-associated activation of ATM signaling pathways. To test this, we determined whether the inhibition of ATM signaling might permit aphidicolin-treated TCPTP-deficient cells to bypass S phase and progress into G2/M. To this end, we inhibited the ATM and ATR targets

(D) G0-synchronized and released R5 cells were treated with methyl methanesulfonate (MMS; 0.0075%) as indicated, and lysates were processed for immunoblot analysis.
(E) G0-synchronized R5 cells were released for 11 hr, synchronized at G1/S with thymidine (2 mM) for 9 hr, and then released again and either left untreated or exposed to ultraviolet light (UV) at 2 hr release. Cells were processed for flow cytometry to monitor DNA content (2c and 4c cells are indicated) and for immunoblot analysis.
(F) G0-synchronized and released R5 cells were treated with etoposide (25 μg/ml) as indicated and processed for flow cytometry and immunoblot analysis.
(G) G0-synchronized and released −/− and R5 cells were treated with thymidine (2 mM) at the indicated times and processed for immunoblot analysis.
Results are representative of three or more independent experiments in each case. The indicated timelines highlight the method of synchronization, the cell-cycle stage, treatments, and the collection time points for immunoblot analysis. In (A), (D), (E), and (G), the retarded electrophoretic mobility of Chk1 was used as an indicator of phosphorylation and activation (p-Chk1). p-JAK1 and p-SFK in (B) and p-STAT3 in (G) were quantified by densitometric analysis and normalized for actin or STAT3; values are given in arbitrary units (AU) as means ± SEM (significance values determined using Student's t test: *$p < 0.05$, **$p < 0.01$).

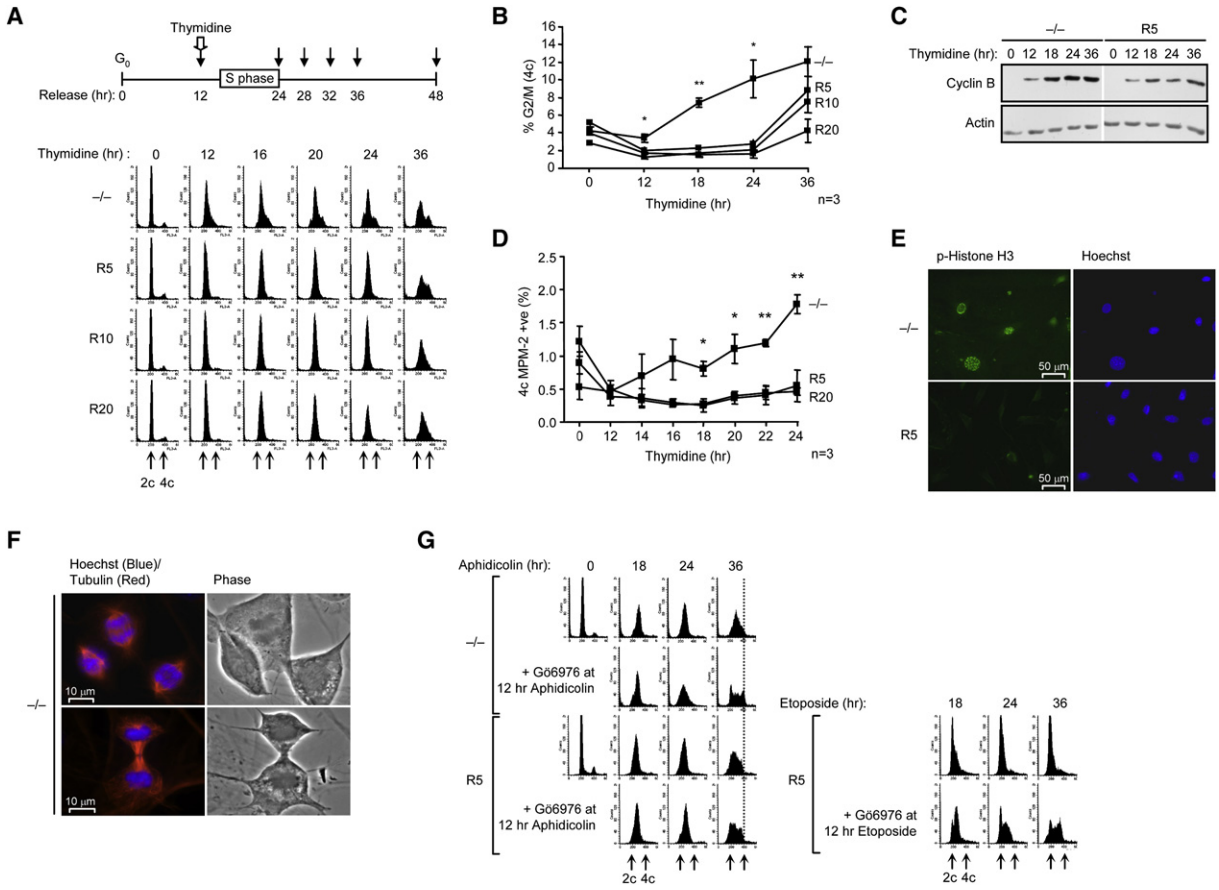

Figure 2. The DNA Replication Checkpoint Response Is Defective in TCPTP-Deficient Cells

(A and B) G0-synchronized and released −/− and TCPTP-reconstituted MEFs (clones R5, R10, and R20) were treated with thymidine (2 mM) for the indicated times and processed for flow cytometry (2c and 4c cells are indicated; 4c cells were quantified and means ± SEM of three independent experiments are shown; *$p < 0.05$, **$p < 0.01$).

(C) G0-synchronized and released −/− and R5 cells were treated with thymidine (2 mM) for the indicated times. Lysates were processed for immunoblotting with antibodies to cyclin B1 (expression highest in metaphase of mitosis) and actin.

(D) G0-synchronized and released −/− and TCPTP-reconstituted MEFs (clones R5 and R20) were treated with thymidine (2 mM) for the indicated times and processed for flow cytometry (4c MPM-2-positive cells were quantified and means ± SEM of three independent experiments are shown; *$p < 0.05$, **$p < 0.01$).

(E and F) G0-synchronized and released −/− and R5 cells were treated with thymidine (2 mM) for 24 hr and processed for immunofluorescence microscopy after staining for DNA (Hoechst) and either phosphorylated (Ser10) histone H3 (p-histone H3; occurs in prophase) or tubulin.

(G) G0-synchronized and released −/− and R5 cells were treated with aphidicolin (0.5 μM) or etoposide (25 μg/ml) for 12 hr, at which point vehicle control or Gö6976 (30 nM) was added and cells were processed for flow cytometry.

Results shown are representative of three independent experiments.

Chk1 and Chk2 with the pharmacological inhibitor Gö6976 (Kohn et al., 2003). Consistent with our supposition, we found that Gö6976-treated TCPTP-deficient MEFs, otherwise arrested in S phase by the replication inhibitor aphidicolin, progressed into G2/M and thereon into G1 of the next cell cycle (Figure 2G). In contrast, Gö6976 did not abrogate the checkpoint response and the suppression of S phase progression induced by aphidicolin in TCPTP-reconstituted cells but allowed TCPTP-reconstituted cells arrested in G1 and S with the DNA-damaging agent etoposide to progress into S and G2, respectively (Figure 2G). Thus, neither deficiencies in ATM/ATR signaling nor TCPTP are in themselves sufficient to abrogate the aphidicolin-induced arrest; instead, both ATM/ATR inactivation and sustained PTK signaling are necessary for checkpoint bypass. Accordingly, we propose that TCPTP/PTK pathways act in concert with ATM/ATR to coordinate the suppression of S phase progression in response to replication stress.

ATR Antagonizes TCPTP

Our results indicated that ATM/ATR pathways are not required for the TCPTP-mediated suppression of S phase progression: neither pharmacological inhibition of ATM/ATR (with caffeine) or Chk1 (with Gö6976) nor deficiencies in ATR (short hairpin RNA [shRNA]-mediated ATR knockdown) or ATM (ataxia-telangiectasia fibroblasts) allowed cells to bypass the replication checkpoint induced by thymidine or aphidicolin, but they readily abrogated the DNA damage checkpoint instigated by etoposide (data not shown). Consistent with this, we found that ATM/ATR inhibition with caffeine (Figure 4A) or Chk inhibition with Gö6976 (Figure 4B) did not prevent the suppression of PTKs

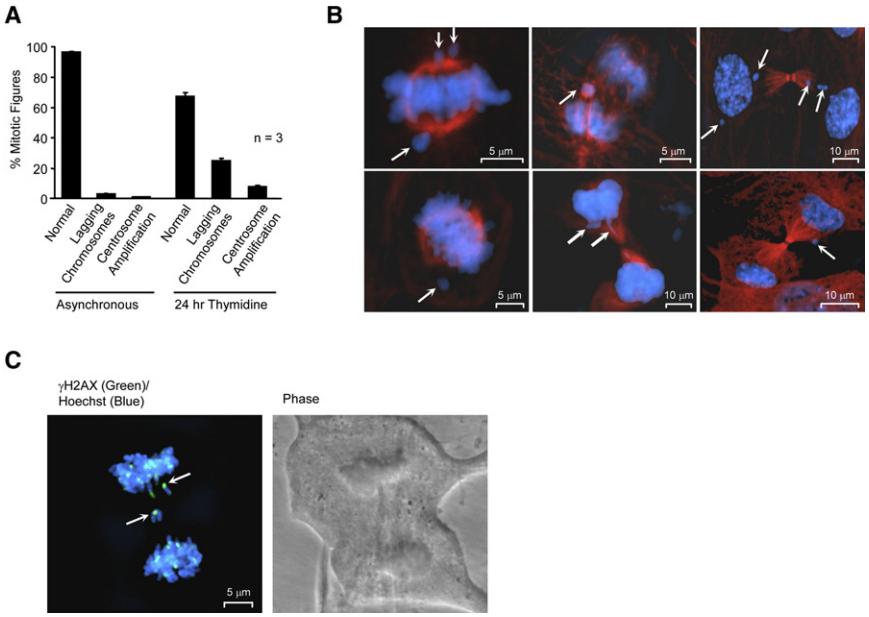

Figure 3. TCPTP-Deficient Cells that Have Bypassed the Replication Checkpoint Exhibit Lagging Chromosomes

Asynchronous −/− MEFs or G0-synchronized −/− MEFs released into the cell cycle for 12 hr and treated with thymidine (2 mM) for 24 hr were processed for immunofluorescence microscopy and stained for DNA (Hoechst) and either tubulin or γH2AX as indicated.

(A) Mitotic figures were scored for lagging chromosomes in metaphase, anaphase, and telophase and for amplified centrosomes as assessed by the presence of asymmetric mitotic spindles. Results are shown as means ± SEM of three independent experiments counting 300–500 cells in each case.

(B and C) Compressed deconvolved (inverse filter) Z stacks depicting cells with lagging chromosomes (white arrows).

and STAT3 in TCPTP-reconstituted cells in response to thymidine. On the contrary, we found that the inhibition of ATM/ATR signaling with caffeine or Gö6976 resulted in a more immediate and pronounced suppression of PTK signaling in TCPTP-reconstituted cells (Figures 4A and 4B), suggesting that ATM/ATR may antagonize TCPTP to prevent the complete inactivation of PTK signaling when the checkpoint is first instigated. Importantly, caffeine did not promote the suppression of STAT3 signaling in TCPTP-reconstituted cells treated with the DNA-damaging agent etoposide (data not shown). Thus, the effects of caffeine on PTK pathway suppression may be specific to the replication checkpoint. In addition, caffeine had no effect on STAT3 signaling in TCPTP-deficient cells treated with thymidine (Figure 4C), indicating that TCPTP is required for the suppression of PTK signaling after ATM/ATR inhibition. Finally, shRNA-mediated ATR knockdown in TCPTP-reconstituted cells enhanced the suppression of STAT3 signaling in response to thymidine (Figure 4D), providing a link between ATR and the status of PTK signaling during replication stress. Taken together, these results indicate that ATR specifically antagonizes TCPTP to coordinate the timing and degree of PTK pathway inactivation in response to replication stress.

PTK Signaling Is Required for DNA Replication Checkpoint Bypass

Next we determined whether the elevated PTK signaling in TCPTP-deficient cells was responsible for the replication checkpoint bypass and subsequent mitotic progression. We delineated the specific contributions of the JAK PTKs and STAT3 using RNA interference (RNAi) and asked whether the stable knockdown of JAK1, JAK2, or STAT3 with shRNAs could prevent S phase progression in the presence of thymidine. Suppression of JAK1 protein levels in TCPTP-deficient cells with two different JAK1-specific shRNAs suppressed STAT3 phosphorylation and largely prevented the replication checkpoint bypass (Figure 5A; data not shown). In contrast, the suppression of JAK2 had no significant effect on STAT3 phosphorylation or S phase progression (data not shown). Consistent with this, we found that the inhibition of

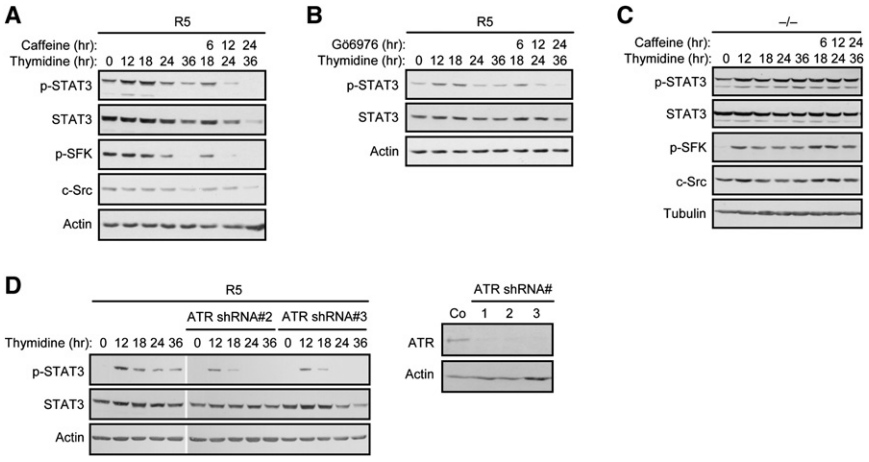

Figure 4. ATR Antagonizes the TCPTP-Mediated Suppression of PTK Signaling

(A–C) G0-synchronized and released TCPTP-reconstituted MEFs (R5) (A and B) or −/− MEFs (C) were treated with thymidine (2 mM) for 12 hr, caffeine (2 mM) or the Chk1 inhibitor Gö6976 (30 nM) was added, and the incubations were continued. Cells were collected at the indicated times and processed for immunoblot analysis.

(D) G0-synchronized TCPTP-reconstituted MEFs (R5) stably transduced with ATR shRNA lentiviral particles were released for 12 hr, treated with thymidine (2 mM), and processed for immunoblot analysis.

Results shown are representative of three independent experiments.

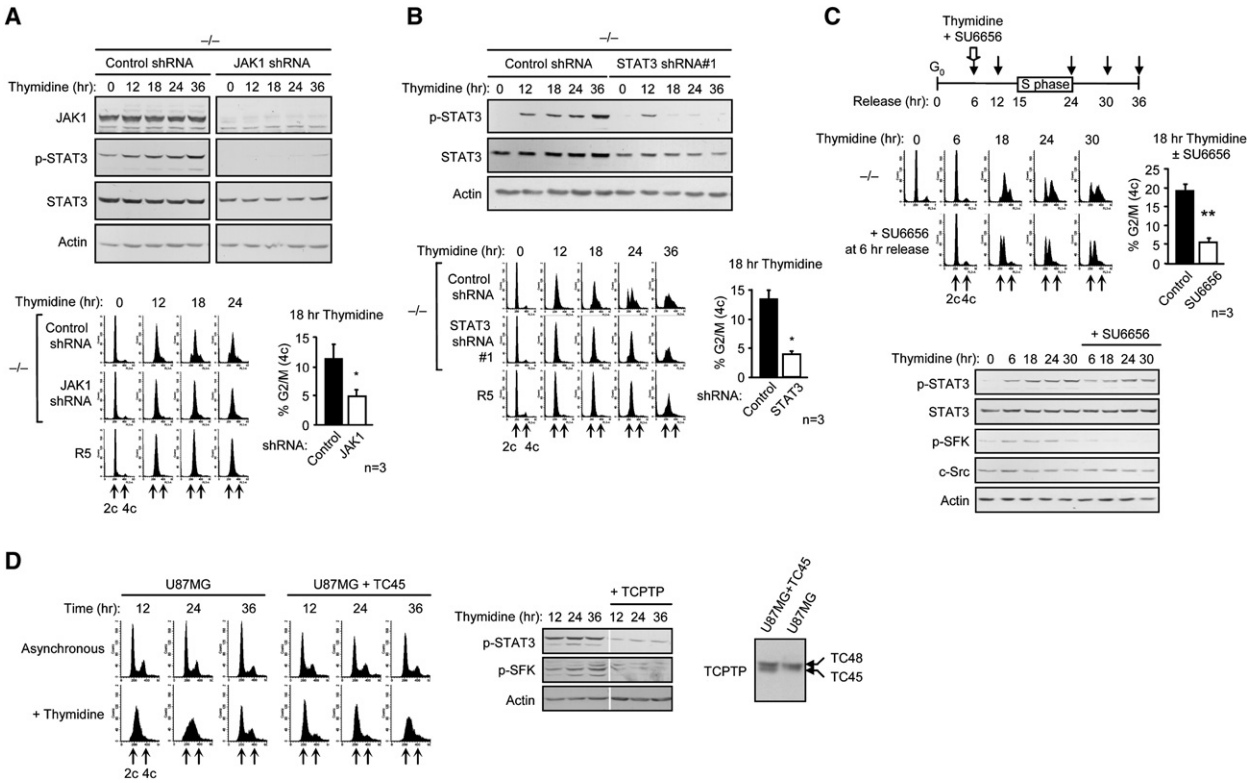

Figure 5. Elevated STAT3 Signaling Is Required for DNA Replication Checkpoint Bypass

(A and B) −/− MEFs were transduced with control and either JAK1 (A) or STAT3 (B) shRNA lentiviral particles. G0-synchronized cells were released for 10 hr, treated with thymidine (2 mM), and processed for immunoblot analysis or flow cytometry.

(C) −/− MEFs were released into the cell cycle for 6 hr, treated with thymidine and vehicle control or the SFK inhibitor SU6656 (10 μM), and processed for immunoblot analysis or flow cytometry (2c and 4c cells are indicated).

(D) Asynchronous U87MG cells or those expressing the 45 kDa TCPTP (TC45) were treated with thymidine and processed for immunoblot analysis or flow cytometry at the indicated times.

Results shown are representative of at least three independent experiments. In (A)–(C), cells with a 4c DNA content were quantified at the indicated times, and means ± SEM are shown (significance values determined using Student's t test: *$p < 0.05$, **$p < 0.001$).

JAK PTKs and downstream STAT3 signaling prior to or during S phase with the JAK-selective inhibitor CMP6 (Thompson et al., 2002) also suppressed replication checkpoint bypass (Figures S5A–S5C). Furthermore, we found that the knockdown of STAT3 with specific shRNAs prevented progression through S phase in the presence of thymidine (Figure 5B; Figure S5D). Neither JAK1 knockdown nor STAT3 knockdown had any significant effect on normal G1 or S phase progression in TCPTP-deficient cells (data not shown). Therefore, these results indicate that enhanced JAK1/STAT3 signaling may be essential for the DNA replication checkpoint bypass in TCPTP-deficient cells.

To assess whether sustained SFK signaling may also contribute to the abrogated checkpoint response, G0-synchronized TCPTP-deficient cells were released into G1 and treated with thymidine with or without the SFK inhibitor SU6656 (Blake et al., 2000), a 10 μM concentration of which was necessary for the inactivation of SFKs in TCPTP-deficient MEFs (data not shown). Addition of SU6656 at 6 hr release so that SFKs were inhibited by the time the cells reached S phase (15–18 hr release) delayed G1 progression as expected but also gradually accumulated cells in early S phase in the presence of thymidine (Figure 5C); this was accompanied by the suppression of STAT3 phosphorylation. Addition of SU6656 at 15 hr release so that SFKs were inhibited after the majority of TCPTP-deficient cells had already traversed S phase accumulated cells in G2 (4c DNA content) and prevented mitotic entry (Figure S6). Thus, these results are consistent with sustained SFK signaling contributing to the replication checkpoint bypass and the subsequent progression into mitosis.

Next, we sought to establish an independent model by which to assess the impact of tyrosine phosphorylation-dependent signaling on the DNA replication checkpoint. Human U87MG glioblastoma cells have inactivated *PTEN* and *INK4a/ARF* and also overexpress IL-6 to promote constitutive JAK/STAT3 signaling (Goswami et al., 1998; Lee et al., 2000; Li et al., 1997). In addition, U87MG cells express the endoplasmic reticulum-targeted 48 kDa TCPTP variant, but not the 45 kDa form (Figure 5D) that shuttles in and out of the nucleus to dephosphorylate both cytoplasmic and nuclear substrates such as STAT3 (Tiganis and Bennett, 2007). Therefore, one may expect that these cells should readily bypass the replication checkpoint. Consistent with this, we found that although the progression of asynchronous cells was somewhat delayed by thymidine, U87MG bypassed the thymidine-induced S phase arrest and proceeded into G2/M and subsequently G1 of the next cell cycle (Figure 5D). The replication checkpoint bypass

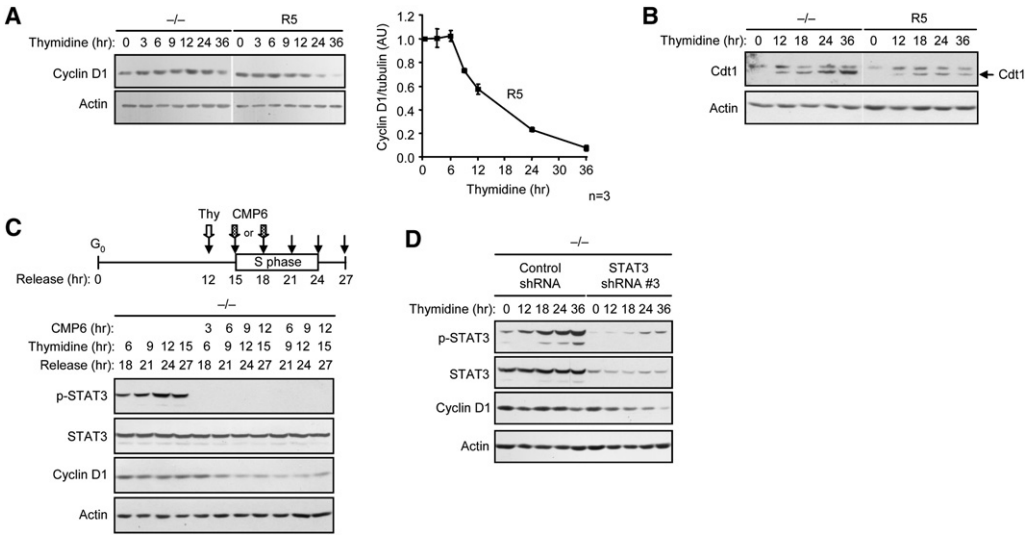

Figure 6. Sustained STAT3-Mediated Cyclin D1 Expression in TCPTP-Deficient Cells

(A and B) G0-synchronized −/− and TCPTP-reconstituted MEFs (R5) were released into the cell cycle for 12 hr, treated with thymidine (2 mM) or aphidicolin (2 μM), and processed for immunoblot analysis. In (A), cyclin D1 in R5 cells was quantified by densitometric analysis and normalized for tubulin; data are given in arbitrary units (AU) as means ± SEM of three independent experiments.

(C) G0-synchronized −/− MEFs were released into the cell cycle for 12 hr, treated with thymidine and then with either vehicle control or the JAK inhibitor CMP6 (5 μM) at the equivalent of 15 or 18 hr release, and processed for immunoblot analysis at the indicated times.

(D) G0-synchronized −/− MEFs transduced with either control or STAT3-specific shRNA lentiviral particles were released into the cell cycle for 12 hr, treated with thymidine (2 mM), and processed for immunoblot analysis.

Results shown are representative of at least three independent experiments.

was delayed by the pharmacological inhibition of the SFK or JAK PTKs and the consequent suppression of STAT3 (data not shown). Furthermore, stable expression of the 45 kDa TCPTP form resulted in the suppression of SFK and STAT3 signaling and prevented the replication checkpoint bypass, maintaining U87MG cells arrested in S phase (Figure 5D); TCPTP expression did not alter the cell-cycle progression (Figure 5D) or proliferation in untreated cells (Klingler-Hoffmann et al., 2001). These results are consistent with TCPTP-regulated PTK pathways being specifically linked to the DNA replication checkpoint.

STAT3-Induced Cyclin D1 Is Required for DNA Replication Checkpoint Bypass

Our studies indicate that the regulation of STAT3 is integral to the DNA replication checkpoint response. A key STAT3 transcriptional target is *CCND1*, which encodes cyclin D1 (Bromberg et al., 1999; Fukada et al., 1998). Cyclin D1 is an allosteric regulator of CDK4/6 and promotes G1/S progression through the CDK4/6-mediated phosphorylation and inactivation of the retinoblastoma protein (pRb) (Sherr, 1995). We found that cyclin D1 protein declined in TCPTP-reconstituted cells treated with either thymidine (Figure 6A) or aphidicolin (data not shown) to almost negligible levels by 24–36 hr of treatment. As expected, the decline of cyclin D1 in thymidine-treated cells was associated with a decrease in pRb phosphorylation on the CDK4 target site Ser807/Ser811 (Figure S7). In marked contrast, cyclin D1 expression (Figure 6A) and pRb phosphorylation (Figure S7) were sustained in TCPTP-deficient cells treated with thymidine. Recent studies have reported that the nuclear accumulation of a cancer-derived oncogenic cyclin D1 mutant during S phase can inhibit the proteolysis of the origin licensing factor Cdt1, resulting in rereplication (Aggarwal et al., 2007). In line with the maintenance of cyclin D1 and sustained pRb phosphorylation, we found that Cdt1 levels were also elevated in TCPTP-deficient cells (Figure 6B). Notably, we found that the maintenance of cyclin D1 protein in thymidine-treated TCPTP-deficient cells was attributable to the sustained/elevated PTK/STAT3 signaling, since pharmacological inhibition of the SFK (data not shown) or JAK PTKs during S phase (at the equivalent of 15 or 18 hr release) and the consequent suppression of STAT3 (Figure 6C; Figure S5C) or the knockdown of STAT3 by RNAi resulted in the replication checkpoint-induced depletion of cyclin D1 without altering cyclin D1 levels in untreated cells in G1 (0 hr thymidine, Figure 6D; data not shown). Therefore, these results suggest that sustained STAT3 signaling and checkpoint bypass might be associated with the maintenance of cyclin D1 levels in S phase.

Previous studies have reported that cyclin D1 accumulates in G1, plateaus in S, and then increases again in G2 (Baldin et al., 1993; Guo et al., 2005; Hitomi and Stacey, 1999; Pagano et al., 1994). Furthermore, cyclin D1 has been shown translocate from the nucleus to the cytoplasm during S phase so that it is sequestered away from nuclear substrates and is degraded (Baldin et al., 1993; Guo et al., 2005; Hitomi and Stacey, 1999; Pagano et al., 1994). This prompted us to examine the levels and subcellular localization of cyclin D1 in normal S phase and under conditions of replication stress in more detail. First, cyclin D1 levels were examined in cells proceeding through S phase. In G0-synchronized and released MEFs, cyclin D1 increased from 12 hr (G1) to 15 hr (G1/S) release and then remained constant

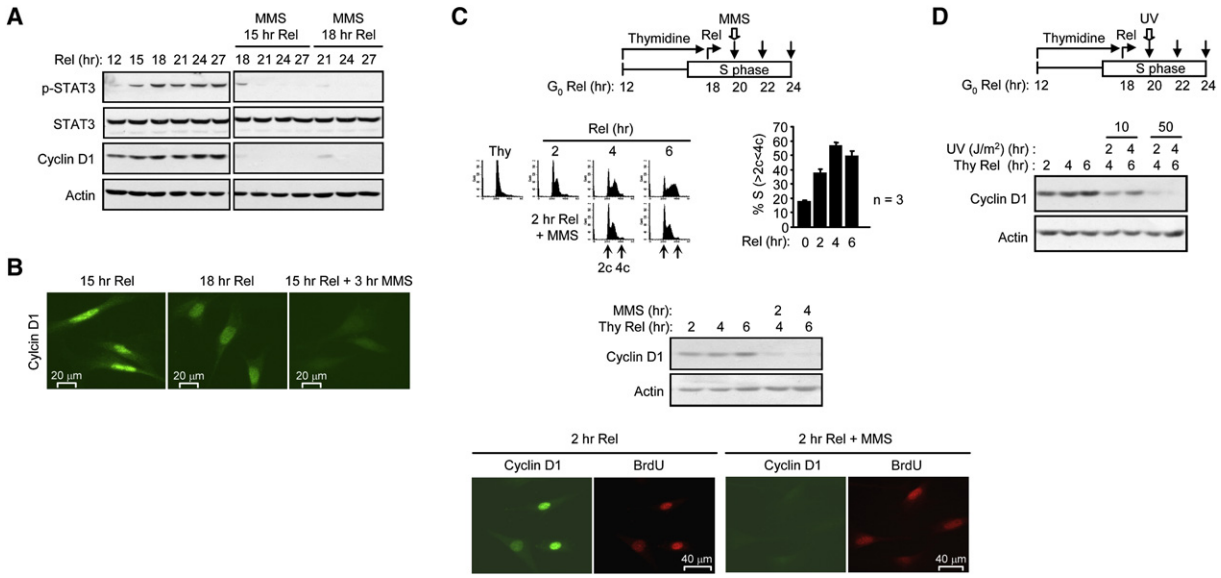

Figure 7. The Replication Checkpoint Attenuates Cyclin D1 Expression

(A and B) G0-synchronized TCPTP-reconstituted MEFs (R5) were released (Rel) into the cell cycle, either left untreated or treated with MMS (0.0075%) at 15 or 18 hr release, and processed for immunoblot analysis (A) or immunofluorescence microscopy with cyclin D1-specific antibodies (B) at the indicated times.

(C and D) G0-synchronized R5 cells were released for 12 hr, treated with thymidine (Thy; 2 mM) for 6 hr to synchronize cells at G1/S, and then released and left untreated or treated at 2 hr release with MMS (0.0075%) or UV for the indicated times. Cells were processed for immunoblot analysis, DNA content analysis by flow cytometry (2c and 4c cells indicated), or immunofluorescence microscopy (S phase cells identified by the incorporation of BrdU) as indicated.

Results shown are representative of three independent experiments in each case. In (C), S phase entry (>2c <4c) of untreated cells was quantified for three independent experiments, and means ± SEM are shown.

until cells entered G2 at 24–27 hr (Figure 7A). In MEFs released from G0, synchronized briefly at G1/S with thymidine, and then released back into S phase (so that >50% were in S phase by 4 hr release; Figure 7C), we noted an apparent decline in cyclin D1 protein after thymidine synchronization and an increase after release into S phase; the increase in cyclin D1 occurred prior to G2 onset (Figure 7C; Figure S8). By immunofluorescence microscopy, we noted that nuclear cyclin D1 was highest at the G1/S transition (15 hr release) and was reduced but nevertheless present in the nuclei of S phase cells (18 hr release) (Figure 7B) incorporating bromodeoxyuridine (BrdU) (data not shown). Similarly, nuclear cyclin D1 was evident in cells that were G1/S synchronized (treated at 12 hr release with thymidine for 6 hr) and released for 2–4 hr into S phase (prior to G2 onset; Figure 7C). Thus, these results indicate that cyclin D1 protein is present in S phase cells and that at least some of this protein persists in the nuclei of cells replicating DNA. Next, we determined the impact of replication stress on cyclin D1 levels and localization. G0-synchronized and released MEFs or cells synchronized at G1/S by brief thymidine treatment and released back into S phase were exposed to replication inhibitors. Treatment of cells at G1/S (15 hr release) or during S phase (18 hr release) (Figure 7A) or cells synchronized at G1/S (with brief thymidine treatment) and released back into S phase with MMS (Figure 7C; data not shown) or UV (Figure 1E; Figure 7D; Figure S3) resulted in the inactivation of STAT3 and concomitant depletion of cyclin D1. Importantly, treatment with MMS completely eliminated cyclin D1 from the nucleus in S phase cells (Figure 7C). Taken together, these results indicate that nuclear cyclin D1 is present during S phase, albeit at considerably lower levels than those found in late G1, and that replication stress results in its rapid elimination.

Having established that cyclin D1 is present in S phase nuclei and that it is eliminated by replication stress, we next determined whether sustained nuclear cyclin D1 may be responsible for the checkpoint bypass of TCPTP-deficient cells. The biochemical activity of cyclin D1 is mediated by its interaction with CDK4/6 in the nucleus (Sherr, 1995). Accordingly, we determined whether the CDK4-selective pharmacological inhibitor R00505124-000 (CDK4i) (Burgess et al., 2006) could prevent S phase progression in the presence of thymidine. CDK4i (1–2 μM) suppressed pRb phosphorylation on the CDK4 target site Ser807/Ser811 and inhibited the replication checkpoint bypass irrespective of whether CDK4i was added prior to (Figure 8A) or at the onset of S phase (Figure S9). We also asked whether promoting cyclin D1 degradation could prevent the replication checkpoint bypass of TCPTP-deficient cells. Inhibition of PI3K/Akt signaling targets cyclin D1 for proteasomal degradation (Diehl et al., 1998). We found that the PI3K inhibitor wortmannin reduced cyclin D1 protein levels and suppressed S phase progression in thymidine-treated TCPTP-deficient cells (Figure S10). Furthermore, the knockdown of cyclin D1 with two different cyclin D1-specific shRNAs prevented the replication checkpoint bypass of TCPTP-deficient cells (Figure 8B) without having any overt effect on normal G1/S progression (Figure S11). Finally, we asked whether preventing the decline in cyclin D1 in response to replication stress could promote replication checkpoint bypass in TCPTP-reconstituted cells. To this end, we asked whether

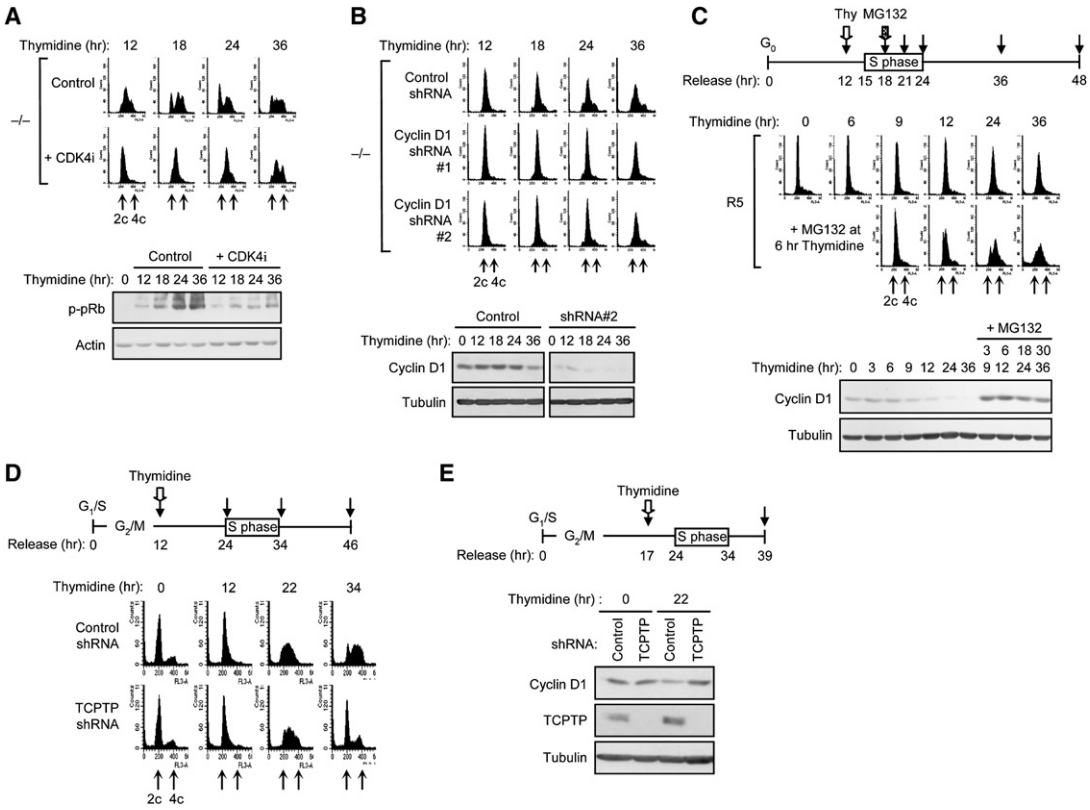

Figure 8. Sustained Cyclin D1 Expression Is Necessary for Replication Checkpoint Bypass

(A) G0-synchronized −/− MEFs were released into the cell cycle for 12 hr, treated with thymidine (2 mM) and either vehicle control (DMSO) or a CDK4 inhibitor (CDK4i; 2 μM), and processed for flow cytometry and immunoblot analysis.

(B) G0-synchronized −/− MEFs stably transduced with cyclin D1-specific shRNAs were released for 12 hr, treated with thymidine, and processed for flow cytometry and immunoblot analysis at the indicated times.

(C) R5 MEFs were released into the cell cycle, treated with thymidine (Thy) and then MG132 (5 μM) at the indicated times, and processed for flow cytometry and immunoblot analysis.

(D and E) HeLa cells transduced stably with control or TCPTP shRNAs were synchronized by double thymidine block, released and treated with thymidine (1 mM) as indicated, and collected for flow cytometry (D) and immunoblot (E) analysis.

Results shown are representative of three independent experiments in (A) and (C)–(E) and two independent experiments in (B).

blocking the degradation of cyclin D1 in TCPTP-reconstituted cells with the proteasome inhibitor MG132 could promote replication checkpoint bypass. Addition of MG132 at 6 hr thymidine treatment (equivalent to 18 hr release) increased the overall levels of cyclin D1 and promoted the progression of thymidine-treated TCPTP-reconstituted cells through S phase, with some cells progressing into G2/M (Figure 8C). Taken together, these results are consistent with the regulation of cyclin D1 expression and stability being integral to the DNA replication checkpoint response.

One possibility is that sustained cyclin D1-CDK4 activity and the subsequent sustained phosphorylation and inactivation of pRb in TCPTP-deficient cells may be responsible for the replication checkpoint bypass. To examine this directly, we asked whether the shRNA-mediated knockdown of pRb in TCPTP-reconstituted cells could compromise the integrity of the DNA replication checkpoint and allow TCPTP-reconstituted cells to progress into G2. Stable suppression of pRb protein levels by as much as 80% did not abrogate the checkpoint response (Figure S12). Consistent with this, we found that the replication checkpoint was largely intact in HeLa cells (Figure 8D; data not shown), in which pRb-family pocket proteins are inactivated by the papillomavirus E7 oncoprotein. As in fibroblasts, we found that cyclin D1 protein declined in thymidine-treated HeLa cells (Figure 8E). Taken together, these results indicate that the replication checkpoint may be regulated by cyclin D1-CDK4 independently of pRb. Consistent with this, we found that the shRNA-mediated suppression of TCPTP in HeLa cells resulted in elevated SFK and STAT3 signaling (Figure S13; data not shown), sustained cyclin D1 protein levels, and bypass of the thymidine-induced replication checkpoint (Figures 8D and 8E; data not shown). Importantly, S phase progression per se was not altered in HeLa cells after TCPTP knockdown (Figure S13), indicating that the bypass was not simply due to enhanced G1 or S progression. Thus, these results affirm TCPTP's role in the cell-cycle-dependent regulation of SFK and STAT3 signaling and in the specific regulation of the DNA replication checkpoint response by tyrosine phosphorylation and cyclin D1-dependent but pRb-independent pathways.

DISCUSSION

Given the complexity and scale of DNA replication, it is not surprising that complex S phase checkpoint mechanisms have evolved to ensure that this process occurs accurately and in a timely manner (Bartek et al., 2004). In this study, we have shown that the JAK and SFK PTKs and their target STAT3 are attenuated specifically in response to stalls in DNA replication to coordinately suppress S phase and mitotic progression. Specifically, our studies show that the attenuation of STAT3-mediated cyclin D1 transcription allows for proteasome-mediated depletion of cyclin D1 to facilitate the suppression of S phase progression. We found that TCPTP was essential for the suppression of the SFK and JAK PTKs and STAT3 in response to stalls caused by thymidine or aphidicolin. Importantly, in TCPTP-deficient MEFs, STAT3 phosphorylation increased significantly in response to thymidine or aphidicolin, with similar albeit slightly delayed kinetics to the induction of the ATR-instigated Chk1-mediated checkpoint. Therefore, we propose that TCPTP is required upon replication fork stalling to prevent STAT3 hyperactivation and the associated checkpoint bypass. Although TCPTP can suppress STAT3 signaling by inactivating the SFK and JAK PTKs, previous studies have reported that TCPTP can also act directly on STAT family members (Lu et al., 2007; Tiganis and Bennett, 2007), including STAT3 (Yamamoto et al., 2002). Therefore, it is possible that TCPTP might act both directly and indirectly to inactivate STAT3 upon replication stress.

The degree and timing of PTK pathway inactivation in TCPTP-expressing cells varied depending on the replication stress. The response to thymidine or aphidicolin was biphasic, with STAT3 signaling being at first attenuated and then inactivated. We found that the control of PTK signaling in response to thymidine or aphidicolin, which slow fork progression and may for example mimic the response to fragile sites or slow replication zones (Glover et al., 1984; Lemoine et al., 2005), was mediated by TCPTP with the biphasic nature being ascribed to the antagonistic regulation by ATR. We showed that TCPTP prevented STAT3 hyperactivation and the associated checkpoint bypass early in the response to replication stress but that ATR antagonized the complete inactivation of STAT3 by TCPTP. This raises the question as to why it may be necessary to prevent the complete inactivation of STAT3. Although further studies are needed to address this, we propose that the maintenance of STAT3 signaling may facilitate recovery after replication stress is alleviated, since S phase progression was compromised in TCPTP-reconstituted but not -deficient cells released from prolonged thymidine treatment (unpublished data). Our studies indicate that the trigger for the complete inactivation of PTK signaling by TCPTP is the waning of ATR/Chk1 signaling (occurring when the cells would otherwise exit S phase; see Figure 1G). In *Xenopus*, Chk1 inactivation resulting from the degradation of claspin may be associated with the adaptation of cells from the DNA replication checkpoint (Yoo et al., 2004), whereas in mammalian cells, claspin degradation may be required for recovery when replication/genotoxic stresses are removed (Mailand et al., 2006; Peschiaroli et al., 2006). In our experiments, we found that MEFs remained arrested in S phase long after the thymidine- or aphidicolin-induced activation of Chk1 subsided. Therefore, we propose that PTK pathway inactivation when ATR/Chk1 signaling subsides may be critical to the maintenance of S phase arrest under conditions of prolonged replicative stress that result in the accumulation of replication intermediates and damaged DNA.

MMS and UV, which modify DNA to directly stall replication forks, caused the immediate and complete inactivation of PTK signaling upon S phase entry, independent of TCPTP status, and this coincided with the induction of ATR/Chk1 checkpoint signaling. Thus, DNA damage specifically at replication forks may be the trigger for an overriding TCPTP-independent pathway for the suppression of PTK signaling. Consistent with this notion, we found that after prolonged treatment with aphidicolin (which collapses forks to generate DSBs), PTK signaling was also attenuated in TCPTP-deficient cells, albeit remaining elevated when compared to TCPTP-reconstituted cells at the same time points; the suppression of PTK signaling at these later time points coincided with the activation of ATM. Thus, we propose that the suppression of PTK signaling may work in concert with DNA damage response pathways activated at stalled or damaged replication forks to suppress S phase progression. In line with this, we found that inhibiting ATM/ATR signaling permitted aphidicolin-treated TCPTP-deficient cells, but not TCPTP-reconstituted cells, otherwise arrested in S phase, to bypass the checkpoint and progress into G2/M.

Our studies indicate that the coordination of cyclin D1 production and degradation may be integral to the DNA replication checkpoint response and that failure to eliminate cyclin D1 due to sustained STAT3-mediated transcription may allow for checkpoint bypass. Previous studies have shown that nuclear cyclin D1 is reduced in S phase, and this has been attributed to nuclear exit and degradation (Baldin et al., 1993; Guo et al., 2005; Hitomi and Stacey, 1999; Pagano et al., 1994). In our studies, we found that, although reduced, nuclear cyclin D1 continued to be present in S phase cells coinciding with incorporated BrdU; nuclear cyclin D1 was also noted in MRC5 and IMR90 fibroblasts during S phase (unpublished data). Thus, although high levels of nuclear cyclin D1 may be deleterious to DNA replication and repair (Pagano et al., 1994), we surmise that low levels of cyclin D1 might continue to be necessary in the nuclei of S phase cells. Previous studies have shown that cyclin D1 can be degraded in response to UV-, γ irradiation-, or cisplatin-mediated DNA damage in MEFs, human fibroblasts, and HeLa cells and that this may be essential for DNA repair (Agami and Bernards, 2000; Lan et al., 2002; Pagano et al., 1994). Notably, the γ irradiation-instigated reduction in cyclin D1 has been shown to precede the activation of p53 and to be essential for the induction of G1 arrest (Agami and Bernards, 2000). Our studies show that the depletion of cyclin D1 may also be integral to the DNA replication checkpoint and that this may be reliant not only on instigated degradation but also on the suppression of STAT3-mediated cyclin D1 expression.

Recent studies have shown that hyperplasia and premature origin firing associated with the expression of oncoproteins such as c-Mos, cyclin E, or activated STAT5 can result in replication-associated damage and the activation of DNA damage checkpoints in normal fibroblasts as well as U2OS osteosarcoma cells (Bartkova et al., 2005, 2006; Di Micco et al., 2006; Mallette et al., 2007). The activation of DNA damage checkpoint pathways in precancerous lesions consequently promotes

cellular senescence; when DNA damage checkpoints are compromised, genomic instability and senescence bypass ensue, resulting in tumorigenesis (Bartkova et al., 2005, 2006; Collado et al., 2005; Di Micco et al., 2006; Gorgoulis et al., 2005; Mallette et al., 2007). In our studies, we saw no evidence for the inappropriate activation of DNA damage checkpoint pathways. However, overall proliferation and S phase progression were not increased in TCPTP-deficient cells. Therefore, the activation of oncogenic PTKs per se may not be sufficient to instigate DNA damage checkpoints and associated senescence, at least not without first inducing hyperproliferation. Thus, our studies provide insight into an alternative means by which oncogenic PTK pathways may contribute to tumorigenesis.

The aberrant activation of PTK signaling in tumor cells confers selective growth advantages that include increased proliferation and survival. However, information as to the involvement of specific PTPs in tumorigenesis has only recently started to emerge (Bentires-Alj et al., 2004; Julien et al., 2007). In the case of TCPTP, although polymorphisms in *Ptpn2* have been linked with the development of several inflammatory disorders (WTCCC, 2007; Todd et al., 2007), similar polymorphisms or mutations have not been identified in human tumors. However, we have shown previously that the downregulation of TCPTP can contribute to the resistance of CML cells to the ABL inhibitor STI571 in vitro (Shimizu et al., 2004), whereas differences in TCPTP expression may contribute to the distinct biological characteristics of diffuse large B cell lymphoma subtypes (Lu et al., 2007). In addition, TCPTP is expressed to varying degrees in breast cancer cell lines (unpublished data). Therefore, it is possible that TCPTP deficiency may contribute to tumorigenicity by abrogating the replication checkpoint. Nevertheless, irrespective of TCPTP status, the results of this study highlight the potential for oncogenic PTK pathways to perturb the replication checkpoint response and contribute to genomic instability and tumor progression.

EXPERIMENTAL PROCEDURES

Reagents

CMP6 (2-*tert*-butyl-9-fluoro-3,6-dihydro-7*H*-benz[h]-imidaz[4,5-*f*]isoquinoline-7-one), aphidicolin, Gö6976, thymidine, and anti-v-Src were from Calbiochem; SU6656, etoposide, MMS, MG132, wortmannin, and caffeine were from Sigma-Aldrich; adriamycin was from ICN; p53, phospho (p)-p53 (Ser15), p-STAT3 (Tyr705), p-SFK (Tyr418), p-ATM (Ser1981), p-SFK (Tyr418), Chk1, and STAT3 antibodies were from Cell Signaling; JAK1, JAK2, Cdt1, Cyclin D1, and ATR antibodies were from Santa Cruz; p-SFK (Tyr418) and p-JAK1 (Tyr1022/Tyr1023) were from Biosource; cyclin B1 and D1 antibodies were from BD Biosciences; and p-histone H3 (Ser10) and MPM-2 antibodies were from Upstate. The CDK4 inhibitor RO0505124 was provided by Hoffmann-La Roche Inc., anti-TCPTP CF4 was provided by N.K. Tonks (Cold Spring Harbor Laboratory), anti-γH2AX was provided by J. Heierhorst (St. Vincent's Institute), and *Ptpn2*$^{-/-}$ cells were provided by M.L. Tremblay (McGill University).

Cell Culture

Generation and culture conditions of spontaneously immortalized *Ptpn2*$^{-/-}$ MEFs and those reconstituted with the 45 kDa form of TCPTP (clones R5, R10, and R20) (Galic et al., 2003; Ibarra-Sanchez et al., 2001), U87MG cells and those expressing TCPTP, and HeLa cells were as described previously (Klingler-Hoffmann et al., 2001). For G0 synchronization, MEFs were seeded at 0.5–1.0 × 10^6 cells per 10 cm dish and cultured for 48 hr, serum starved in medium containing 0.1% (v/v) FBS for 48 hr, and released into the cell cycle by readdition of FBS. For stable knockdown, control (MISSION pLKO.1-Puro control particles) and STAT3 (#1: ID71455; #2: ID71457; #3: ID71453), ATR (#1: ID23909; #2: ID23910; #3: ID23911; #4: ID23912; #5: ID23913), JAK1 (#1: ID23289; #2: ID23290), TCPTP (ID2783), or cyclin D1 (#1: ID55233; #2: ID55236) specific shRNA lentiviral particles (Sigma-Aldrich) were used to transduce *Ptpn2*$^{-/-}$ immortalized MEFs, TCPTP-reconstituted MEFs, or HeLa cells according to the manufacturer's instructions, and cells were selected in 2 μg/ml puromycin.

Microscopy and Flow Cytometry

Cells were fixed with 95% (v/v) ethanol, and fluorescence-activated cell sorting analysis was performed on propidium iodide-stained cells; where indicated, cells were also stained with MPM-2. Immunofluorescence microscopy was performed as described previously (Tiganis et al., 1998) using a Zeiss Axioskop 2 mot plus microscope and Zeiss AxioVision software. BrdU incorporation was assayed using a kit (Roche) according to the manufacturer's instructions.

Statistical Analysis

A one-tailed Student's t test was used to test for differences between genotypes or treatments.

SUPPLEMENTAL DATA

The Supplemental Data include thirteen figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/2/166/DC1/.

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