Clinical Cancer Research

Therapeutic Implications for the Induced Levels of Chk1 in Myc-Expressing Cancer Cells

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Abstract

Purpose: The transcription factor c-Myc (or "Myc") is a master regulator of pathways driving cell growth and proliferation. *MYC* is deregulated in many human cancers, making its downstream target genes attractive candidates for drug development. We report the unexpected finding that B-cell lymphomas from mice and patients exhibit a striking correlation between high levels of Myc and checkpoint kinase 1 (Chk1).

Experimental Design: By *in vitro* cell biology studies as well as preclinical studies using a genetically engineered mouse model, we evaluated the role of Chk1 in Myc-overexpressing cells.

Results: We show that Myc indirectly induces *Chek1* transcript and protein expression, independently of DNA damage response proteins such as ATM and p53. Importantly, we show that inhibition of Chk1, by either RNA interference or a novel highly selective small molecule inhibitor, results in caspase-dependent apoptosis that affects Myc-overexpressing cells in both *in vitro* and *in vivo* mouse models of B-cell lymphoma.

Conclusion: Our data suggest that Chk1 inhibitors should be further evaluated as potential drugs against Myc-driven malignancies such as certain B-cell lymphoma/leukemia, neuroblastoma, and some breast and lung cancers. *Clin Cancer Res; 17(22); 7067–79. ©2011 AACR.*

Introduction

The *MYC* family of proto-oncogenes encodes 3 different transcription factors, c-Myc (hereafter Myc), N-Myc, and L-Myc, which are functionally similar. They regulate a vast number of genes involved in most aspects of cell growth and proliferation (1), explaining the selection for mutations that result in overexpression of Myc proteins in the majority of human cancers. However, Myc overexpression can be sensed as oncogenic stress in cells, which manifest as apoptosis or senescence (2, 3). This cellular defense against Myc-induced transformation involves apoptotic mediators, cell-cycle proteins, and tumor suppressors such as Arf, ataxia telangiectasia mutated (ATM), and p53 (4). As a consequence, these categories of genes are often deregulated during tumorigenesis, unleashing the oncogenic functions of Myc.

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Myc binds proteins in the prereplication complex and independent of its role as a transcription factor causes replication stress and DNA damage by excessive stimulation of replication fork firing (5, 6). This can trigger a DNA damage response (DDR), a process that depending on insult engages one or more of the proximal phosphoinositide-3-kinase–like proteins ATM, its related kinase ataxia telangiectasia and Rad3-related protein (ATR), and DNA-dependent protein kinase (DNA-PK). These kinases then initiate a signal transduction pathway feeding down to p53 that regulates the cell cycle or apoptosis depending on cell context (7).

Activation of p53 by the DDR causes a prolonged cell-cycle arrest in either the G_1 or the G_2 phase of the cell cycle. Checkpoint kinase 1 (Chk1) kinase, which is also activated via phosphorylation by ATM/ATR kinases (8), mediates a faster response and is known to play an important role in DNA damage checkpoint control, embryonic development, and tumor suppression (9). Activation of Chk1 occurs in response to improper DNA replication and various forms of genotoxic stress (8, 10). Chk1 then inactivates members of the Cdc25 family, which control cell-cycle transitions by dephosphorylating cyclin-dependent kinases (Cdk; ref. 11). In addition, Chk1 also phosphorylates proteins involved in stabilization of replication forks (12) and spindle checkpoint function (13).

Checkpoints in G_2 and M phase provide protection against a premature and lethal mitotic entry in case of damaged DNA. Tumors often exhibit genomic instability, making them very sensitive to disturbances in

Translational Relevance

The idea of using checkpoint kinase 1 (Chk1) inhibitors to treat cancer came from the observations that tumor cells get rid of DNA damage checkpoints during tumorigenesis or therapy, making them highly sensitive to additional genomic instability. By combining chemotherapy or radiotherapy with Chk1 inhibitors, tumors would be more sensitive than the normal surrounding cells. Here, we identify a possible means to stratify patients into groups that may benefit particularly well from the inclusion of Chk1 inhibitors in their treatment regimens. We also show efficacy of a novel Chk1 inhibitor, which could serve as the lead for the development of such treatments.

checkpoints, despite the fact that they might themselves have arisen because of checkpoint overrides. It thus seems that a low level of checkpoint override (e.g., *Chek1* haploinsuffiency) may be tumor promoting (9), whereas a high level of genomic instability (e.g., Chk1 inhibition) can be therapeutically beneficiary (14). Along the same line, we and others have recently shown that Myc overexpression selectively sensitizes cells to checkpoint override following inhibition of its transcriptional targets and mitotic regulators Cdk1 and Aurora B kinases (15, 16). Here, we report a novel and unanticipated connection between Myc and Chk1. Our findings suggest that Chk1 is essential for Myc to provoke illegitimate cell-cycle progression and suggest a novel therapeutic avenue against Myc-overexpressing tumors such as lymphomas, breast and lung cancers, and neuroblastoma.

Materials and Methods

Materials

Primary antibodies were obtained from Santa Cruz Biotechnology (Chk1, ER, Myc, and p19Ink4d), Sigma (β-actin and tubulin), Upstate (γH2AX), and Cell signalling (caspase-9). Horseradish peroxidase–conjugated antibodies against mouse and rabbit antibodies were from GE Healthcare Life Sciences.

Decitabine (5-aza-2′-deoxycytidine or Dacogen) was generously provided by MGI Pharma. The pan-caspase inhibitor Q-VD-OPH was obtained from BioVision.

Synthesis and evaluation of a selective Chk1 inhibitor

The Chk1 inhibitor "Chekin" was made in a structure–activity relationship series of position 8 of compound 2 described in a previous publication (17). Detailed description of the synthesis and the structure–activity relationship is presented elsewhere (18). Kinase assays in Table 1 were conducted as previously described (19). The selectivity of Chekin was further determined by a time-resolved fluorescence resonance energy transfer (TR-FRET) assay on the basis of the LanthaScreen Method (Invitrogen). Individual kinases (2–10 nmol/L) or probes (2× K_d of probe to the particular kinase; range of 6.25–200 nmol/L) were either manufactured in-house (Abbott Laboratories) or purchased from Invitrogen (kinases/probes) or CarnaBio (kinases). The inhibitor was tested across the range of 0.0001 to 10 μmol/L in a 10-fold dilution dose response. The control inhibitor staurosporine was used to assess the accuracy and precision of the system.

Cell culture

293T human kidney cells and NIH 3T3 fibroblasts were from American Type Culture Collection and cultured in

Table 1. Selectivity profile of Chekin

Kinase assay		TR-FRET kinome											
	IC_{50}, μmol/L		IC_{50}, μmol/L		IC_{50}, μmol/L		IC_{50}, μmol/L		IC_{50}, μmol/L		IC_{50}, μmol/L		IC_{50}, μmol/L
Chk1	0.0203	ACVR1	>10	CDK8/cyclin C	>10	Fyn	>10	Kdr	>10	PDGFRB	>10	TBK1	>10
AMPK	>10	ALK	>10	CDK9/cyclin K	>10	GRK5	>10	LTK	>10	PKA	>10	TNK2	>10
Aurora 2	>10	AMPK	>10	CLK2	>10	Gsk3a	>10	Lck	>10	PKCθ	>10	TTK	>10
CHK2	>10	Abl	>10	CLK4	>10	Gsk3b	>10	MEK1	>10	Pim1	>10	TYRO3	>10
CTAK-1	>10	Aurora1	>10	CSF1R	>10	IGF1R	>10	MEK2	>10	Prkcn	>10	TrkA	>10
EMK	>10	Aurora2	>10	Ck1α1	>10	IKKE	>10	MST1	>10	RET	>10	TrkB	>10
Gsk3b	>10	BTK	>10	DYRK1B	>10	InsR	>10	Map4k2	>10	Rock1	>10	TrkC	>10
IGF1R-Marme	>10	CAMK1D	>10	Dyrk1A	>10	JAK2	>10	Map4k4	>10	Rock2	>10	Wee1	>10
Kdr 789-1354	>10	CAMK2A	>10	FAK	>10	JAK3	>10	Nek2	>10	Rsk2	>10	ZipK	>10
MAPK-APK2	>10	CDK5/p25	>10	FGFR1	>10	JNK1	>10	PAK4KD	>10	Syk CatDom	>10	cMET	>10
Rsk2	>10	CDK7/cyclin H/Mat1	>10	Flt1	>10	JNK2	>10	PDGFRA V561D	>10	TAOK2	>10	p38 α	>10

Dulbecco's Modified Eagle's Medium with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and antibiotics. Human lymphoma cell lines P493-6, Akata, BJAB, DG75, KemI, and Raji were laboratory stocks and were cultured at a density between 10^5 and 10^6 cells per milliliter in RPMI-1640 medium with 10% FCS, 2 mmol/L L-glutamine, 50 μmol/L β-mercaptoethanol, 0.1875% sodium bicarbonate, and antibiotics. Mouse lymphoma cell lines established from tumors arising in either the λ-*Myc* or *Eμ-Myc* transgenic mice (15, 20) were cultured at a density between 10^5 and 10^6 cells per milliliter in RPMI-1640 medium with 5% FCS, 2 mmol/L L-glutamine, 50 μmol/L β-mercaptoethanol, 0.1875% sodium bicarbonate, and antibiotics. Mouse embryo fibroblasts (MEF) were generated from E13.5-E15 embryos from timed matings between *p53* heterozygous mice according to established methodology. All experiments were repeated with multiple MEF isolates.

Viral infections

Retroviruses were made by calcium phosphate–mediated cotransfection of 293T cells with pBABE-Hras^{G12V}-puro, MSCV-IRES-puro, or MSCV-IRES-GFP (with or without c-Myc or MycER) together with pCL-Eco (all from Addgene). Twenty-four hours posttransfection, media was changed and supernatants were harvested 4 times during 36 hours, filtered and used to infect *p53*$^{-/-}$ MEFs and bone marrow–derived B cells, or NIH 3T3 cells in the presence of 4 or 8 μg/mL polybrene, respectively. Cells infected with MSCV-IRES-puro–based retroviruses were selected in the presence of 4 to 6 μg puromycin.

Lentiviral infections were made by calcium phosphate–mediated cotransfection of 293T cells with packaging plasmids pCMV-dR8.2dvpr and pHCMV-Eco (Addgene) using 5 different MISSION short hairpin RNA (shRNA) constructs (Sigma) per gene directed against the *Chek1*, *ATM*, *ATR*, and *Prkdc* (encoding DNA-PK) mouse mRNA. Twenty-four hours posttransfection, media was changed and supernatants were harvested 4 times during 36 hours and used to infect target cells. Mouse lymphoma cells were infected by 3 rounds of spinoculation (20 minutes at 50 × *g*) during 24 hours in the presence of 2 μg/mL polybrene. MEFs or NIH 3T3 cells were infected by culturing the cells in the presence of viral particles and 4 to 8 μg/mL of polybrene. The cells were selected by culturing in the presence of 2 to 6 μg/mL puromycin.

Cell-cycle and apoptosis analyses

For cellular staining with propidium iodide (PI), mouse and human B cells were collected by centrifugation, and adherent cell cultures were gently detached from the dish with trypsin-EDTA and then collected by centrifugation together with its original culture supernatant. The cells were resuspended and incubated in Vindelov's reagent (10 mmol/L Tris, 10 mmol/L NaCl, 75 μmol/L PI, 0.1% Igepal, and 700 U/L RNase adjusted to pH 8.0) with a cell concentration not exceeding 10^6 cells per mL. The PI-stained cells were then analyzed with a FACSCalibur flow cytometer (BD Biosciences) using the FL3 channel in a linear scale for cell-cycle distribution. Apoptosis was determined on the basis of the number of cells that carried less than diploid DNA content (sub-G_1) in a logarithmic FL2 channel.

Western blot analysis

Cell pellets or tumors crushed in liquid nitrogen were lysed, and the debris was removed by centrifugation. A total of 30 to 50 μg protein per lane was separated on SDS-PAGE gels and subsequently transferred to nitrocellulose membranes (Schleicher-Schuell). Membranes were stained with Ponceau S red dye to verify equal loading. All subsequent steps were carried out in TBS-Tween (10 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, and 0.05% Tween-20) either containing 5% milk (blocking and antibody incubations) or 2% bovine serum albumin (phospho-specific antibody incubations). Antibody binding was visualized by enhanced chemiluminescence with the SuperSignal West Dura or Pico reagents from Pierce and either an X-ray film or a LAS4000 imaging system (Fujifilm Life Science). In the Figures, images with a dark tone are generated by autoradiography and those with a light tone are generated with the LAS4000 system.

RNA preparation and analysis

RNA was isolated with TRIzol reagent (Invitrogen) or NucleoSpin RNA II (Macherey-Nagel). cDNA synthesis was carried out on 1-μg RNA by iScript first strand synthesis kit (Bio-Rad). Quantitative PCR using gene-specific primers was carried out by the KAPA SYBR FAST qPCR Kit (Kapa Biosystems) on an iCycler iQ5 real-time PCR machine (Bio-Rad). Relative mRNA levels were calculated with the $\Delta\Delta C_t$ method.

Histology and immunohistochemistry

With Institutional Review Board approval, and following informed consent, paraffin blocks of tumors from Burkitt lymphoma, mantle cell lymphoma, and follicular lymphoma patients were banked. For immunohistochemistry, 2-μm sections were deparaffinized. Antigen retrival was carried out by pressure cooking in citrate buffer (pH 6) for 7 minutes. Binding of the Chk1 antibody was detected after an overnight incubation at 4°C by the DAKO REAL Detection Kit (DAKO) as per the manufacturer's protocol. Two independent operators analyzed $n = 3$ high-power fields (HPF; 40×) in $n = 5$ samples of Burkitt lymphoma or mantle cell lymphoma.

Mouse experiments

All animal experiments were carried out in accordance with the Regional Animal Ethic Committee Approval #A6-08 or #A18-08. The *p53*-knockout mice (21) and *Apc*^{Min} mice, both on a C57BL/6 background, were obtained from the Jackson laboratory. The λ-*Myc* mouse model (22) was a kind gift from Dr. Georg Bornkamm (GSF, Munich, Germany). All mice were observed daily for signs of disease. All moribund mice were immediately sacrificed.

When tumor-bearing mice were sacrificed, tumors and lymphoid organs were collected for analyses or tissue banking. Tumors were either snap frozen and/or dispersed into single-cell suspensions by scalpels and cell strainers.

To develop a *p53*-deficient Myc-driven *in vivo* model, we magnetically sorted bone marrow–derived B cells by labeling them with an anti-B220-R-PE antibody, followed by labelling with an anti-PE magnetic microbead and loading on a MACS column (Miltenyi). The purified B cells were cultured overnight in RPMI-1640 medium with 10% FCS, 2 mmol/L L-glutamine, 50 μmol/L β-mercaptoethanol, 0.1875% sodium bicarbonate, and antibiotics in the presence of MSCV-Myc-IRES-GFP retrovirus produced as described earlier and 4 μg/mL polybrene. Infected cells were injected into C57BL/6 mice, and tumor development was monitored. Tumors that developed were dispersed into single cells and were immunophenotyped to confirm B-cell origin and frozen down in medium containing 10% dimethyl sulfoxide (DMSO) for banking. For drug experiments, cells were thawed, and 150,000 cells were intravenously injected per mouse. After 1 week, Chekin (17 mg/kg; $n = 7$) or vehicle (PEG400:chremophore/70% EtOH:saline: cyclodextrin, 10:50:30:10; $n = 8$) was injected twice daily for 4 days after which tumor development was observed. For acute experiments in C57BL/6 transplanted with lymphoma cells or in healthy precancerous λ-*Myc* mice, 4 and 3 injections every 12 hours was carried out, respectively, after which tumors or splenic B cells, respectively, were harvested and analyzed by Western blot analysis.

Statistical analysis

Statistical analyses were conducted using the *t* test functions of Excel or GraphPad Prism (GraphPad Software). *, $P < 0.05$ and **, $P < 0.01$ were considered statistically significant. The bars shown represent the mean of triplicates ± SD.

Results

Myc regulates Chk1 *in vitro*

We have previously shown that Myc overexpression sensitizes *p53*-deficient cells to DNA damage (17, 23). To study the role of Myc in checkpoint override in more detail, we generated *p53*-null MEFs from E13.5 embryos and transduced them with a retrovirus engineered to express a fusion protein between c-Myc and the ligand-binding domain of the estrogen receptor (ER), the MycER protein. Addition of the estrogen analogue 4-hydroxytamoxifen (4-HT) to growth medium releases the MycER protein from binding to Hsp in the cytoplasm and exposes the nuclear localization signal of Myc to induce nuclear translocation. Activation of Myc resulted in override of the cell-cycle arrest exerted by γ-irradiation or decitabine, a DNA methyltransferase inhibitor that also induces DNA damage, resulting in cell death (data not shown; refs. 17, 23). Detailed analysis of *p53*-deficient cells at different time points following Myc activation and DNA damage surprisingly revealed that Chk1 is induced by Myc activation, even without prior DNA damage (Fig. 1A). To investigate at which level Chk1 was induced by Myc activation, we carried out quantitative reverse transcriptase PCR (qRT-PCR) on mRNA from cells that had been subjected to Myc activation. Interestingly, Chk1 upregulation upon 4-HT treatment required *de novo* protein synthesis, as the translation inhibitor cycloheximide blocked induction, in contrast to the direct Myc target *Odc* (Fig. 1B). This suggests that Myc upregulates Chk1 indirectly. In line with this, the regulatory sequences of the *Chek1* gene was devoid of conserved Myc-binding E-boxes (data not shown) and had not previously been discovered in screens for Myc-bound gene regulatory sequences (www.myccancergene.org). However, the Myc-dependent upregulation of Chk1 was evolutionarily conserved as activation of Myc in human P493-6 B cells, which carry a tetracycline-regulated *MYC* construct permitting conditional expression of Myc (24), also resulted in elevated levels of Chk1 protein (Fig. 1C). In essence, Chk1 expression correlated with the activation/reactivation of *MYC* and the associated increase in Myc protein.

Given that Chk1 has a dual role in promoting and inhibiting tumorigenesis, we were interested in studying this kinase in the context of Myc overexpression. Because supraphysiologic levels of Myc are known to both promote DNA damage, apoptosis, and tumorigenesis (25), we therefore assessed whether the induction of Chk1 by Myc was mediated by the DDR. To test this hypothesis, we carried out shRNA-mediated knockdown of *Atm*, *Atr*, and *Prkdc* (DNA-PK) in NIH 3T3 cells infected with MycER. qRT-PCR confirmed that potent knockdown was achieved for Atm and DNA-PK but to a lesser extent for ATR (Supplementary Fig. S1A). Induction of Chk1 upon Myc activation occurred normally in cells with decreased levels of these proximal kinases and their activity [measured by detection of the phosphorylated form of H2AX (γH2AX); ref. 26], suggesting either redundancy or independency of DDR (Fig. 1D and Supplementary Fig. S1B). To verify that Myc can induce Chk1 independently of downstream mediators of oncogenic stress, we also carried out these experiments in early-passage *p53*-deficient, *Arf*-deficient, or *Arf*/*Atm* double-knockout MEFs. Again, Myc activation caused an upregulation of total Chk1 protein levels, independent of *p53*, *Arf*, or *Atm* status (Supplementary Fig. S1C). Importantly, checkpoint activation occurred in these cells, as shown by the inactivating Tyr 15 phosphorylation of Cdc2 (Supplementary Fig. S1C).

Myc upregulates Chk1 *in vivo*

Next, we assessed whether Chk1 is a Myc-regulated gene also *in vivo*. To this end, we first analyzed λ-*Myc* transgenic mice which express *MYC* under the control of the immunoglobulin λ light chain enhancer and develop mature B-cell lymphomas (22). Splenic B cells from either precancerous λ-*Myc* mice or wild-type littermates were magnetically sorted using anti-IgM antibodies. These cells and palpable lymphomas harvested from sick λ-*Myc* animals were analyzed for Chk1 transcript and protein levels. All of the precancerous cells except one and all lymphomas exhibited high levels of Chk1 than wild-type

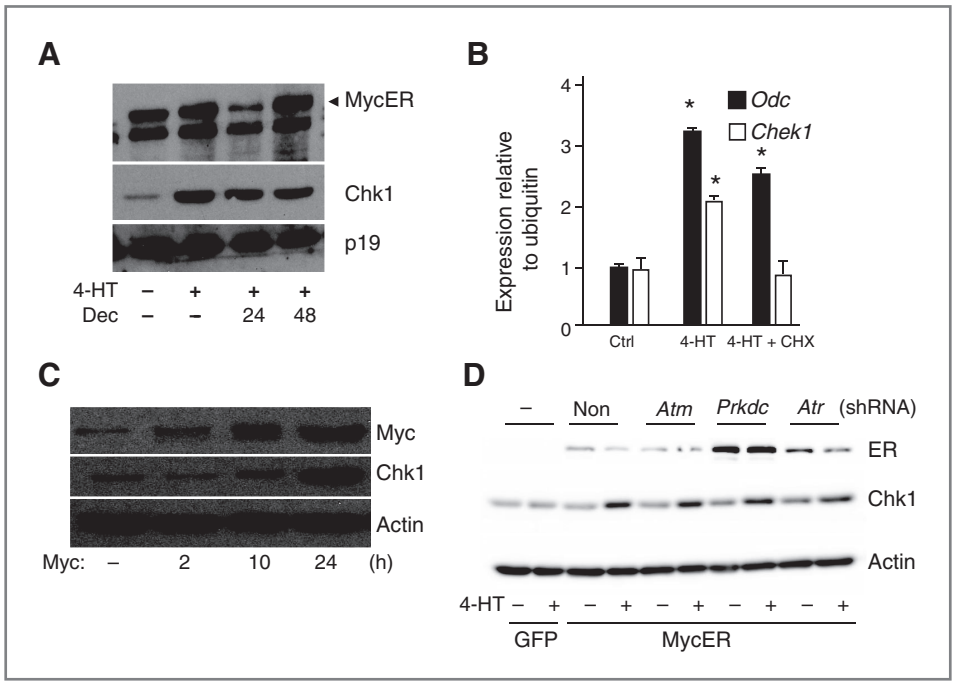

Figure 1. Myc upregulates Chk1 mRNA and protein. A, Western blot analysis of p53-knockout MEFs infected with an MSCV-MycER-IRES-puro retrovirus. The nuclear translocation of MycER was induced by 4-HT treatment for 24 hours, and then the cells were treated with decitabine for an additional 24 hours. Whole-cell lysates were harvested and analyzed using antibodies directed against the indicated proteins. The constitutively expressed cell-cycle protein $p19^{Ink4d}$ was used as a loading control (ctrl) as it is not regulated by Myc (51). B, qRT-PCR analysis of *Chek1* and *Odc* transcript levels in *p53*-knockout MEFs infected with MSCV-MycER-IRES-GFP retrovirus. 4-HT was added to the cells, and transcript expression was measured 24 hours later, with or without the presence of 1 μg/mL cycloheximide (CHX) in the growth media. *, $P < 0.05$; expression in control cells versus 4-HT or 4-HT and CHX. C, Western blot analysis of P493-6 cells after tetracycline removal from the growth media, leading to Myc induction. D, Western blot analysis of NIH 3T3 fibroblasts–expressing inducible MycER that were infected with *shRNA* against *Atm*, *Atr*, and *Prkdc*. Myc was activated, and 24 hours later whole-cell lysates were analyzed by Western blot analysis using antibodies directed against the indicated proteins. Dec, decitabine.

control cells (Fig. 2A and B), confirming that Myc induces Chk1 also *in vivo*.

Myc is deregulated in the majority of human cancers, frequently as a downstream event of an activated oncogenic pathway. There are, however, cancers like Burkitt lymphoma or neuroblastoma, and less frequently breast and lung cancer, where a member of the *MYC* gene family is directly activated either by translocation or gene amplification (1). Burkitt lymphoma is characterized by exhibiting 1 of 3 different translocations, which bring *MYC* on chromosome 8 under the control of enhancers of *IgK*, *IgH*, or *IgL* on chromosomes 2, 14, or 22 (27). This causes a massive transcriptional induction of *MYC* in B lymphocytes, resulting in apoptosis, hyperproliferation, or transformation. As shown earlier, Chk1 transcript and protein was induced in λ-*Myc* transgenic mice, an *in vivo* model of Burkitt lymphoma. We therefore next assessed *CHEK1*/Chk1 expression in human Burkitt lymphoma samples. By searching the web-based public database Oncomine (www.oncomine.org), we found expression profiling studies showing a highly significant correlation between *MYC* mRNA expression in Burkitt lymphoma with that of *CHEK1* (Supplementary Fig. S2A and S2B). To establish this correlation at the protein level, various lymphoma subtypes including Burkitt lymphoma were analyzed for Chk1 expression by immunohistochemistry. Again, samples from patients with Burkitt lymphoma exhibited a significantly higher level of Chk1-positive cells than other B-cell lymphoma subtypes (Fig. 2C). Thus, Myc activation is associated with high Chk1 positivity *in vivo*.

In most colon cancers, mutation of the adenomatous polyposis coli (*APC*) gene results in loss of function of the protein complex that controls degradation of β-catenin. The outcome of accumulation of nuclear β-catenin is activation of genes promoting proliferation, including c-*MYC* (28). To analyze whether cells with elevated Myc also would induce Chk1 in this context, we studied tumorigenesis of heterozygous Apc^{Min} mice. These mice lose the remaining wild-type *Apc* allele and develop spontaneous adenomas in the colon and small intestine macroscopically visible at around 120 days of age (29). By comparing expression of Chk1 in normal gut epithelium with that in adenomas by Western blot analysis, we observed elevated levels of Chk1 in 4 of 6 tumors (Supplementary Fig. S3A). Moreover, qRT-PCR analysis verified a striking correlation between *Myc* expression and *Chek1* (Supplementary Fig. S3B).

Despite the strong correlation between *Chek1* mRNA/protein expression and a deregulated Myc oncogene, we could not gain evidence that Myc directly induces *Chek1*.

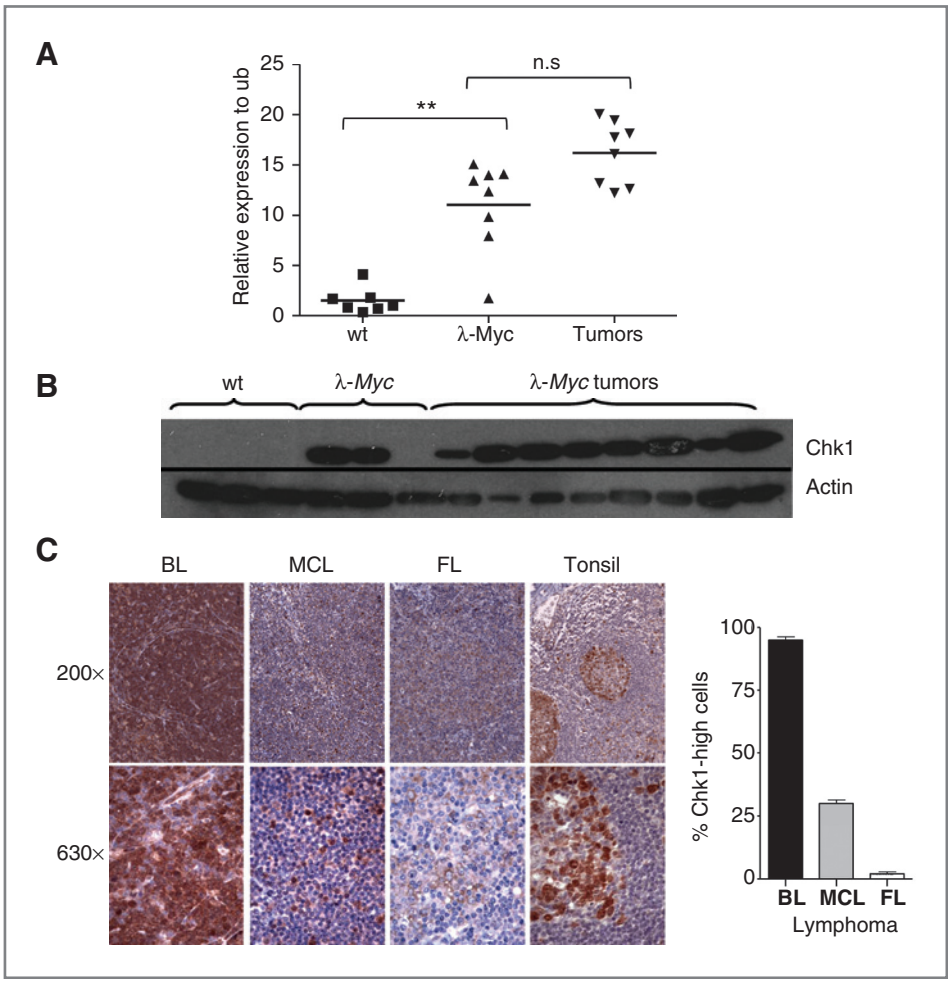

Figure 2. *Chek1* transcript and Chk1 protein is elevated in lymphomas from λ-Myc transgenic mice and in human Burkitt lymphoma (BL). A, qRT-PCR analysis of *Chek1* transcript levels in B cells from wild-type and λ-*Myc* mice as well as tumors developed in the λ-*Myc* transgenic animals. B, Western blot analysis of Chk1 protein levels in B cells from 4- to 6-week old wild-type (wt) and precancerous λ-*Myc* mice compared with palpable lymphomas harvested from sick animals. C, immunohistochemical analysis of Chk1 expression in Burkitt lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), and control tissue (tonsils). Left, representative samples at low power (200×) and high power (630×) views. Right, the percentage of positive staining in lymphomas for Chk1. Here, a grid ocular objective was used to count 400 cells over 3 HPFs (40×). The bars show the mean percentage of positive cells from 5 samples ± SD. ub, ubiquitin; n.s., not significant.

To test whether Myc regulated another transcription factor that could in turn induce Chek1, we screened the Basso lymphoma gene arrays (Supplementary Fig. S2) for genes encoding transcription factors involved in cell-cycle progression. Interestingly, expression of genes encoding transcription factors known to regulate S-phase progression such as *MYBL2*, *MYC*, and *FOXM1* was very high in Burkitt lymphoma. These had correlation *R* values to *CHEK1* of around 0.5, whereas expression of 2 other prominent S-phase regulators, *E2F1* and *MYBL1*, correlated slightly less to *CHEK1* expression (Supplementary Fig. S2B). This analysis suggests that Myc may regulate several transcription factors that could induce *CHEK1*.

Chk1 is crucial for the survival of Myc-overexpressing cells *in vitro*

Having established that Chk1 is regulated by Myc, we wanted to investigate whether Chk1 is a potential therapeutic target, or whether it rather acts as a tumor suppressor to a stress response provoked by Myc overexpression. Chk1 inhibitors are currently evaluated in clinical studies, testing the hypothesis that Chk1 constraint fortifies the activity of DNA-damaging agents (30). Indeed, several studies have shown that Chk1 inhibition, in combination with drug-induced replication stress or DNA damage, severely compromises tumor cell survival. To investigate the consequences of Chk1 inhibition in the context of Myc overexpression, we genetically removed *Chek1* mRNA using shRNA-expressing lentiviruses in NIH 3T3 fibroblasts and low passage *p53*-deficient MEFs that had been transduced with MSCV-Myc-IRES-GFP retroviruses (Fig. 3A). Interestingly, clonogenic survival assays over 10 days showed that knockdown of Chk1 severely compromised colony formation of Myc-overexpressing cells, with basically no cells surviving over this period of time (Fig. 3B). In contrast, cells infected with a lentivirus carrying a nontargeting shRNA did not die, but instead showed signs of overgrowth, suggesting that Myc triggers hyperproliferation which requires Chk1.

To analyze the consequence of Chk1 loss for Myc-induced tumorigenesis or tumor maintenance, we analyzed transformation efficiency of fibroblasts and growth of Myc-induced lymphomas *in vitro* and *in vivo*. Although Myc-overexpressing NIH 3T3 fibroblasts expressing the nontarget shRNA potently formed colonies in soft agar, a hallmark

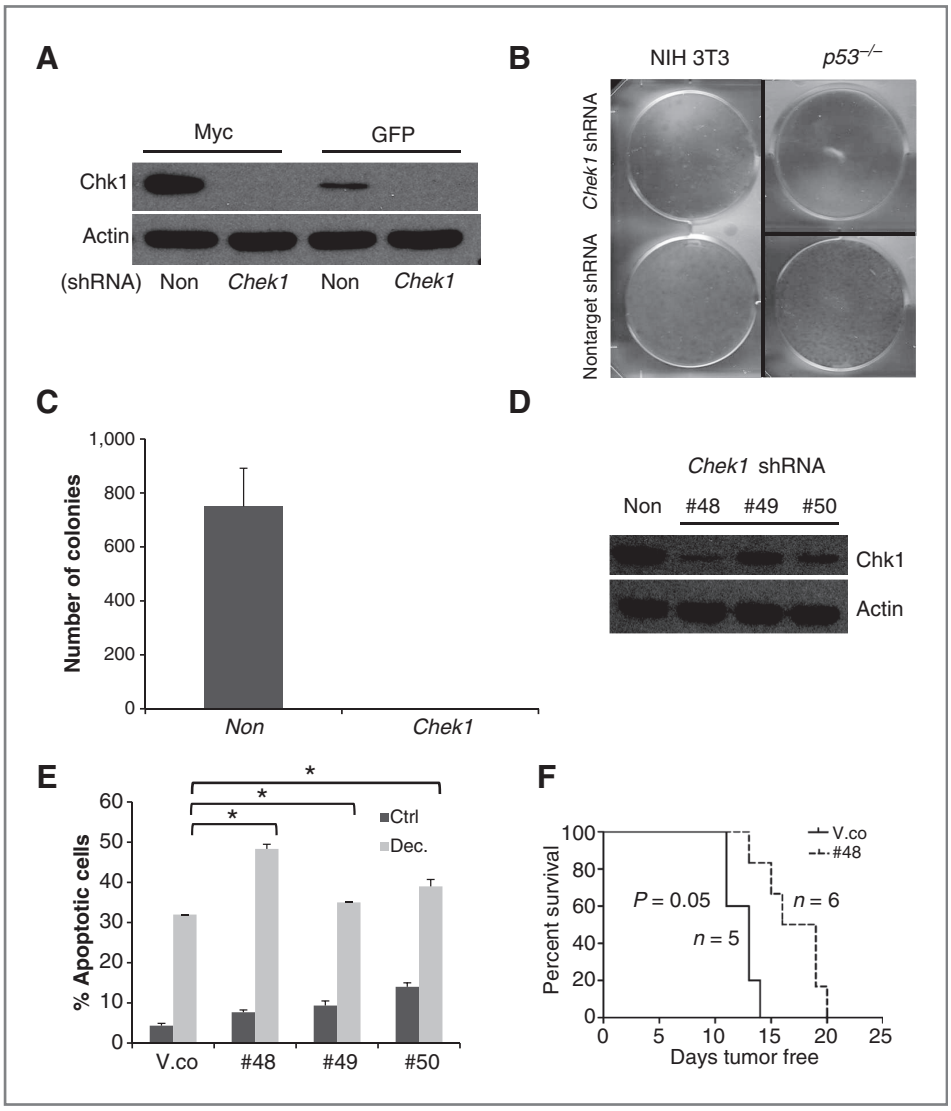

Figure 3. Myc-overexpressing cells are dependent on Chk1 for survival and transformation capacity. A, Western blot analysis of Chk1 protein levels in NIH 3T3 fibroblasts infected with MSCV-IRES-GFP or MSCV-Myc-IRES-GFP retroviruses and lentiviruses expressing *Chek1* or a nontarget shRNA. B, clonogenic survival assay of 10^4 NIH 3T3 fibroblasts (*p53* wild-type) or $p53^{-/-}$ MEFs expressing MSCV-Myc-IRES-GFP with or without expression of shRNA against *Chek1*. The cells were grown to confluence during the course of 10 days. Shown here is the result of 1 of 2 experiments yielding the same results. C, soft agar assay of NIH 3T3 fibroblasts expressing MSCV-Myc-IRES-GFP with or without *shRNA* directed against *Chek1*. D, a λ-*Myc* (*p53* mutant) mouse lymphoma cell line was infected with different shRNA hairpins against *Chek1*. These hairpins show a varying degree of *Chek1* knockdown as assessed by Western blot analysis. E, the cells in D were treated with 4 μmol/L of decitabine during 48 hours, stained with PI, and analyzed in a flow cytometer for apoptotic cells. F, cells in (D) expressing the nontargeting shRNA or the #48 *Chek1* shRNA against were transplanted via tail vein injection into recipient mice that were followed for signs of disease. When sick, the mice were euthanized, and the tumors were harvested for analysis by Western blot (Supplementary Fig. S4). V.co, vector control.

of malignant transformation, no colonies were observed in the cells expressing the *Chek1* shRNA (Fig. 3C). Introducing the same highly efficient shRNA into a *p53* mutant mouse lymphoma cell line generated from a sick λ-*Myc* mouse (20) selected against viable cells (data not shown). By infecting these lymphoma cells with *Chek1* shRNA virus that exhibited intermediate levels of *Chek1* knockdown (Fig. 3D), we were able to generate viable cells. A more detailed analysis of these Chk1-low cells revealed that they were clearly more sensitive to DNA damage induced by decitabine treatment (Fig. 3E), and they developed tumors slower upon transplantation into syngenic C57BL/6 recipients (Fig. 3F). Moreover, several of the tumors that did develop had regained high level expression of Chk1 (Supplementary Fig. S4), suggesting that they arose from puromycin-resistant Chk1-normal cells present in a diluted form in the Chk1-low culture. Taken together, these data strengthen the hypothesis that Myc requires appropriate levels of Chk1 for transformation and tumor cell survival. Chk1 therefore emerges as a potential pharmaceutical target rather than a tumor suppressor of Myc-induced tumorigenesis.

Small-molecule Chk1 inhibitors induce Chk1 degradation and cell death of Myc-transformed cells

To validate Chk1 as a therapeutic target, we tested the best compound of a new generation of Chk1 inhibitors developed by one of us (L.A. Hasvold; Abbott Laboratories). This compound, named Chekin (Fig. 4A inset), exhibits inhibition of Chk1 at low nanomolar concentrations and has shown Chk1 selectivity over 76 kinases tested in *in vitro* enzyme assays (Table 1). In one aspect Chekin is unique as other known selective Chk1 inhibitors also inhibit the related kinase Chk2 or one of the Cdks. Treating a panel of human B-cell lymphoma lines for 96 hours with Chekin induced an apoptotic phenotype in Akata and BJAB, whereas DG75 did not respond at all (Fig. 4A top, Supplementary Figs. S5 and S6). Raji primarily responded to Chekin by a slower growth (Supplementary Fig. S5A) and accumulation of cells in late S

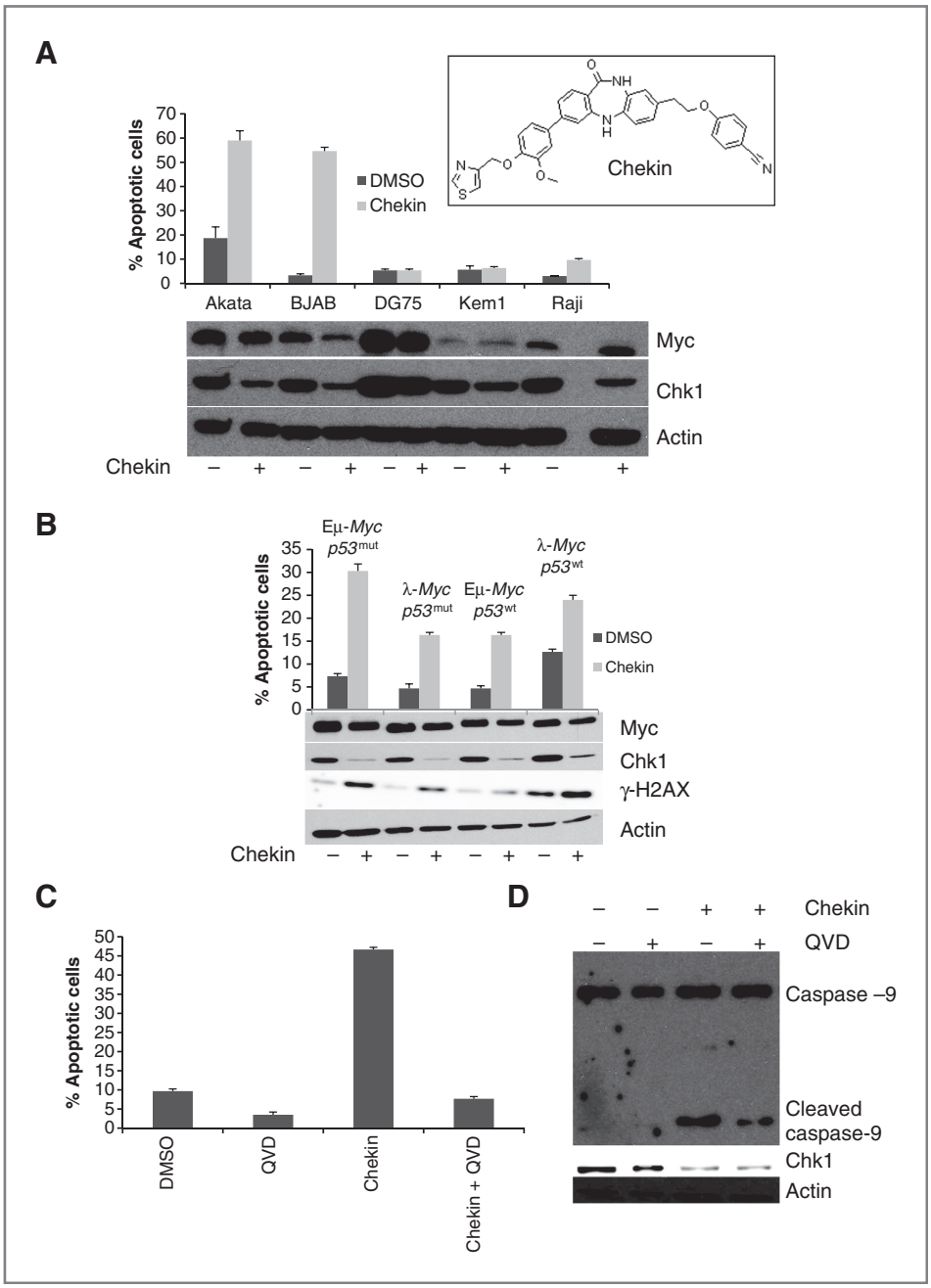

Figure 4. Chk1 inhibition induces cell death in human and mouse lymphoma cell lines. A, flow cytometry and Western blot analysis of human B-cell lymphoma cells. The Burkitt lymphoma cell lines Akata, DG75, Kem1, and Raji, and the non–Burkitt lymphoma cell line BJAB, were treated for 72 hours with 1 μmol/L of Chekin (see inset for structure and Table 1 for selectivity over other kinases). Apoptosis was scored by measurement of the sub-G_1 population after PI staining, and Western blot analysis was conducted with antibodies against Chk1 and Myc. B, flow cytometric and Western blot analysis of mouse lymphoma cell lines derived from Eμ-*Myc* or λ-*Myc* mice. The cells were treated with 1 μmol/L of Chekin during the course of 48 hours and scored for apoptosis by flow cytometry as described earlier. Western blot analysis was carried out with antibodies against Myc, Chk1, and γ-H2AX. C, flow cytometric analysis of a *p53* mutant λ-*Myc* mouse lymphoma cell line. Apoptosis was scored by analysis of the sub-G_1 population of PI-stained cells by flow cytometry. The cells were treated with 1 μmol/L of Chekin in combination with 10 μmol/L of the pan-caspase inhibitor QVD-OPH. D, Western blot analysis of samples described in (C) using antibodies against Chk1 and caspase-9.

and G_2 phase (Supplementary Fig. S6). KemI was only weakly affected in growth rate and cell-cycle distribution, which was further substantiated by a dose–response curve (Fig. 4A, Supplementary Figs. S5A and S5B and S6). All lines that were sensitive to Chekin either exhibited endogenous activation of DDR, as assessed by Western blot analysis of γH2AX (26), or had strong induction upon Chekin treatment (Supplementary Fig. S5C). Interestingly, total Chk1 protein levels following treatment were decreased in correlation to sensitivity to Chekin (Fig. 4A bottom, Supplementary Fig. S5C), suggesting mechanism-based degradation exerted by Chekin as reported in other studies using Chk1 inhibitors in combination with DNA-damaging agents (31, 32). This decrease is believed to occur via proteasome-mediated degradation and could indicate that Myc overexpression can replace DNA damage in the triggering of degradation upon Chk1 inhibition. Unexpectedly, however, the protein level of Myc was also decreased in the BJAB line, a non–Burkitt lymphoma line with a *MYC* amplification and which is known to lack stabilizing mutations of *MYC* (33).

The B-cell lymphoma lines tested are genetically diverse as they come from tumors of different patients and have been in culture for decades, resulting in secondary

mutations enabling adaptation to cell culture conditions. For instance, DG75, which did not respond to Chekin by either slower growth or apoptosis, is known to carry a biallelic frameshift mutation resulting in absence of proapoptotic Bax protein expression (34). To analyze the consequence of Chk1 inhibition on growth of cultured Myc-induced lymphomas arising in syngenic λ-*Myc* and Eμ-*Myc* transgenic animals, we made use of lines recently developed (15, 20). Lymphoma lines with either wild-type or mutant *p53* status were treated with Chekin for 48 hours. All of these cells showed sensitivity to Chk1 inhibition by inducing apoptosis (Fig. 4B top). This apoptosis was independent of their respective *p53* status but associated with induction of DNA damage as assessed by the phosphorylation of Histone H2AX (26). Again, we could observe degradation of Chk1 and of Myc in Chekin-treated cells (Fig. 4B bottom). However, as Myc-expressing cells are sensitive to aggresome formation in response to inhibition of the proteasome (ref. 35; and data not shown), we were precluded from treating the cells with proteasome inhibitors during the full course of Chekin treatment. Nevertheless, treatment with proteasome inhibitor for the last 3 hours of a 12-hour Chekin treatment period rescued the Chk1 degradation to a modest extent (Supplementary Fig. S7A).

Having established that both human and mouse cells transformed by Myc exhibit sensitivity to Chk1 inhibition, we wanted to elucidate whether the apoptotic response was mediated via mitochondria-mediated caspase cleavage. Pretreatment of a mouse lymphoma cell line with the pancaspase inhibitor QVD-OPH completely blocked Chekin-mediated apoptosis (Fig. 4C). Western blot analysis revealed that the inhibition of apoptosis was accompanied by a reduction in caspase-9 cleavage (Fig. 4D). Therefore, the activation of executioner caspase-3 (Supplementary Fig. S7B and S7C) is likely to be mediated by a pathway that involves mitochondria-mediated apoptosis. However, Chekin also impacted on cell-cycle progression as the cells grew slower (data not shown). Moreover, the cells had higher amount of cells in G_1/early S and G_2/M phase of the cell cycle when treated with Chekin while blocking apoptosis with QVD-OPH, suggesting that the cells died from these phases (Supplementary Fig. S7D).

Myc sensitizes cells to Chk1 inhibition *in vitro* and *in vivo*

On the basis of the expression patterns described earlier (Fig. 1 and 2 and Supplementary Figs. S2 and S3), there is a strong correlation between *MYC* and *CHEK1* expression. This implies that Myc proteins, more so than other oncogenes, would sensitize cells to Chk1 inhibition. To test this, we analyzed sensitivity to Chekin in low passage *p53*-deficient MEFs infected with retrovirus constructs engineered to express either Myc or oncogenic Ras. Both Myc and Ras-expressing cells induced apoptosis upon treatment with Chekin, but the Myc-expressing cells showed a higher degree of sensitivity (Fig. 5A). The level of Chk1 was highest in Myc-expressing cells, but Ras-transformed cells also exhibited a higher Chk1 levels than vector control cells (Fig. 5B). Analyzing Chk1 protein levels following inhibition revealed that the protein was degraded in a similar manner as in the Myc-induced lymphomas.

To test whether Myc would sensitize tumor cells to Chk1 inhibition, we transduced the switchable MycER protein into Ras-induced sarcoma cells that we had previously developed in tissue culture after they had grown subcutaneously in mice. Because MycER expression is leaky (36), resulting in some MycER translocation even without addition of 4-HT, we were able to test sensitivity to Chekin of cells expressing medium (Ras), high (MycER) and very high Myc (MycER plus 4-HT) activity. As seen in Fig. 5C, MycER sensitized the sarcoma cells to Chekin and addition of 4-HT, to induce MycER translocation, further stimulated death. Assessing previously characterized mediators of DNA damage-induced death revealed that Myc activation caused γH2AX phosphorylation and reduced levels of the antiapoptotic Bcl-2 family member Bcl-X_L (Fig. 5D). Interestingly, Bcl-X_L was even further downregulated by Chekin in Myc-expressing cells but not in the Ras sarcoma transduced with a control retrovirus. In Myc-overexpressing cells, it is thus likely that the mitochondrial death revealed by caspase induction (see Fig. 4 and Supplementary Fig. S7) occurs because of Bcl-X_L downregulation.

The ultimate goal with the generation of Chekin was to develop a potent anticancer drug that could work as a cancer therapeutic. To evaluate whether Chekin would function *in vivo*, we first treated 4-week-old wild-type or λ-*Myc* mice with Chekin and assessed the effect on the biomarker, Chk1 itself. As expected from Fig. 2A, sorted splenic B cells from precancerous λ-*Myc* mice exhibited higher levels of Chk1 protein than did B cells from wild-type mice. Reassuringly, B cells from mice treated with 17 mg/kg 3 times every 12 hours exhibited lower Chk1 levels 36 hours after the first injection (Fig. 6A). Confident that we were hitting the target, we developed a mouse lymphoma model for the assessment of the anticancer effect of Chekin. By infecting bone marrow–derived B cells from *p53*-knockout mice with an MSCV-Myc-IRES-GFP virus and then transplanting these into recipient syngenic C57BL/6 mice, we obtained mice that developed B-cell lymphomas (as assessed by fluorescence-activated cell sorting using antibodies directed against the B-cell marker B220, Supplementary Fig. S8). These lymphomas were transplanted into new animals that were divided into 2 groups receiving bidaily injections for 5 days of either vehicle ($n = 6$) or 17 mg/kg Chekin ($n = 7$), which was the highest dose possible to administer without compound precipitation. After these injections the mice were followed for signs of disease after which they were sacrificed or treated again (see later). Interestingly, Chekin was able to induce a significantly slower disease progress in this very aggressive lymphoma model (Fig. 6B). Moreover the therapeutic effect was associated with induction of Chk1 degradation and induction of DNA damage as assessed by γH2AX (Fig. 6C). Taken together these data suggest that Myc-overexpressing cells are sensitive to the new Chk1 inhibitor Chekin which therefore could serve as a lead for the development of new anticancer therapies.

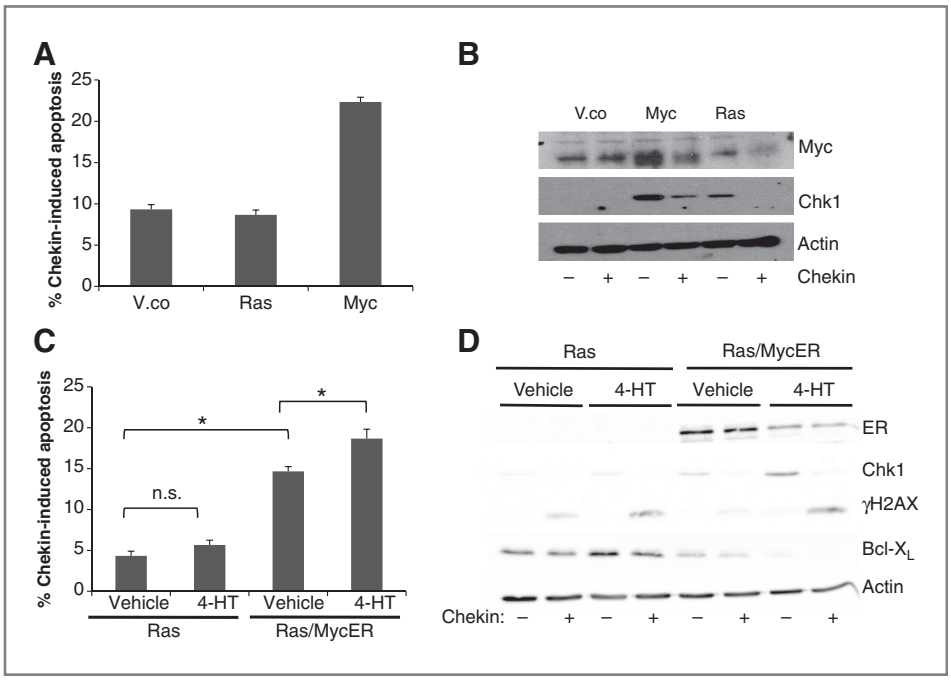

Figure 5. Myc sensitizes cells towards Chk1 inhibition. A, p53-deficient MEFs infected with MSCV-Myc-GFP, pBABE-H-RasG12V-puro, or control virus. The cells were treated with 1 μmol/L Chekin for 72 hours, and apoptosis was then scored by measuring the sub-G_1 population of PI-stained cells by flow cytometry. Results are shown as the mean difference in percentage of apoptosis between Chekin-treated cells and vehicle-treated cells (DMSO) in triplicates. B, Western blot analysis of samples described in (A) with antibodies against Chk1 and Myc. C, an H-RasG12V retrovirus–induced fibrosarcoma developed subcutaneously in C57BL/6 mice was established in culture. After transduction with a second retrovirus, either derived from MSCV-IRES-puro or MSCV-MycER-IRES-puro, cells were selected with puromycin. Cells were then treated with 4-HT or vehicle (EtOH) and 1 μmol/L Chekin or vehicle (DMSO) for 48 hours and assayed for apoptosis by measuring the percentage of PI-stained cells with less than diploid DNA content. Results are shown as the mean difference in percentage of apoptosis between Chekin-treated cells and vehicle-treated cells (DMSO and/or EtOH) in triplicates. D, the same types of cells as in (C) were also analyzed by Western blot for the expression of the indicated proteins following 48 hours of treatment. V.co, vector control.

To summarize, we show that Myc upregulates the expression of Chk1, and that Chk1 inhibition selectively contributes to apoptosis of Myc-transformed cells. This synthetic lethal interaction is p53 independent and therefore represents an attractive therapeutic approach in malignancies having Myc involvement.

Discussion

Targeting *MYC* for therapeutic reasons is conceivable based on its prevailing activation in cancer and the fact that Myc inhibition results in tumor regression of oncogene-addicted tumors, although sparing normal cells (37, 38). Myc proteins are not enzymes though, making the development of drugs targeting this class of proteins a challenge that has been undertaken by others but has not yet proven successful. An alternative approach is therefore to identify pathways and genes downstream or parallel to Myc that are druggable. To this end, we and others have determined the importance of Myc-regulated genes for Myc-induced lymphomagenesis. Although not all Myc-regulated genes are created equal, products of some genes encoding polyamine biosynthetic enzymes (39, 40), ribosomal proteins (41), and mitotic regulators seem to play important roles in either tumor development or tumor maintenance.

One would assume that the most important genes of Myc would be the direct transcriptional targets. However, we recently identified Aurora kinase B and Cks1 as essential products of Myc-regulated genes (15, 42). Herein, we identify Chk1, which is also regulated by Myc at the transcriptional level, as an additional essential kinase whose inhibition is synthetic lethal with Myc overexpression. These studies suggest that the Myc transcriptome may contain more indirectly regulated genes that remain to be found and that may encode possible therapeutic targets against Myc-driven cancer. At the moment, we do not know whether these genes are regulated by a common transcription factor downstream of Myc or whether other modes of regulation are operating. In the case of Chk1, very little is known about its transcriptional regulation but reports imply S-phase regulators such as E2F and FOXM1 transcription factors, as the promoter region of *CHEK1* harbors functional binding sites (43–45). Given that Myc regulates these transcription factors (www.myc-cancergene.org), it is possible that the observed *Chek1* mRNA induction by Myc occurs via these or other factors such as the *Myb* family of transcription factors. As shown

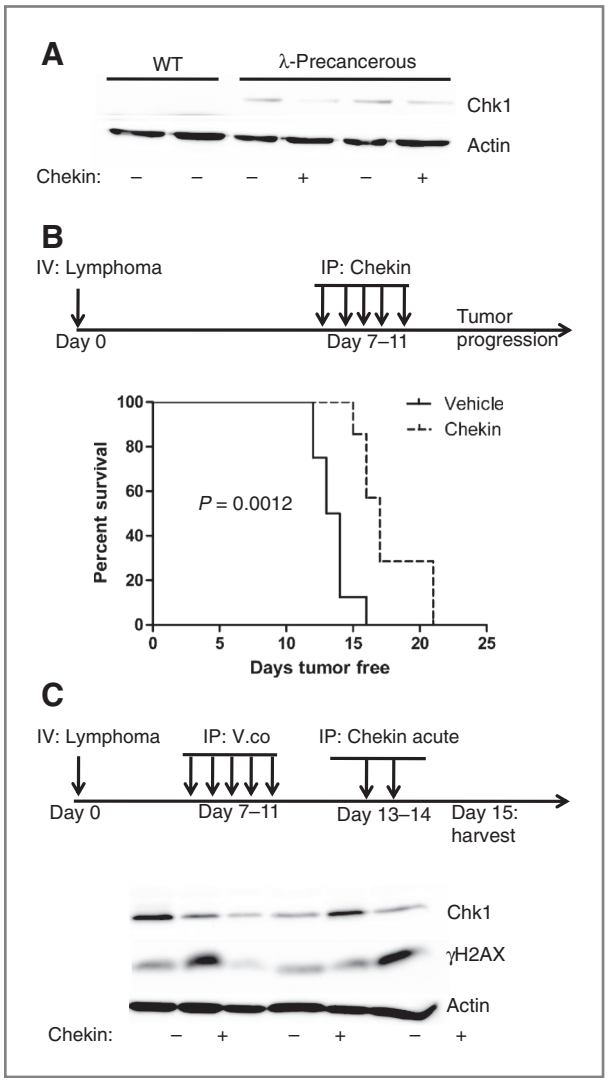

Figure 6. Chekin induces Chk1 degradation and reduces tumor growth *in vivo*. A, six precancerous λ-*Myc* mice were treated with either 3 doses of 17 mg/kg Chekin or 3 doses of vehicle control every 12 hours by intraperitoneal injections (IP). Thirty-six hours after the first injection, mice were sacrificed, and B cells were harvested from the mice for use in Western blot analysis of Chk1 expression. B, retroviral Myc-induced B-cell lymphomas were transplanted into recipient C57BL/6 mice by tail vein injection (IV). One week after transplantation, 7 mice (3 + 4 of 2 different original tumors) were treated bidaily for 5 days with 17 mg/kg Chekin, and 8 mice (4 + 4 of 2 different original tumors) were treated with vehicle control. Mice were monitored daily for signs of disease and were scored as sick when palpable lymphomas were apparent. C, three vehicle-treated mice were treated with Chekin when palpable tumors had developed. Tumors were harvested after 2 days of bidaily injections and analyzed by Western blot using the antibodies indicated. V.co, vehicle control.

here, there is a high degree of correlation between *MYC*, *CHEK1*, *MYBL2*, and *FOXM1* and to a lesser degree *E2F1*. It is thus plausible that several factors contribute to *CHEK1* expression in a redundant manner, which requires further studies to dissect.

Chk1 suppresses 2 main apoptotic pathways involving caspase-2 and -3. Following replication stress, Chk1 is crucial in blocking caspase-3–mediated apoptosis, independent of *p53* (46). Furthermore, Chk1 also inhibits caspase-2–mediated apoptosis following DNA damage in p53-deficient zebrafish, a pathway that is both mitochondria and death receptor independent (47). Although we observed caspase-3 activation in Chk1-inhibited cells, this does not answer which of these pathways is operational, perhaps both are. It is interesting to note, however, that the cells which survive Chk1 inhibition by Chekin continue to proliferate at a slower rate, suggesting that apoptosis inhibition by Chk1 may be uncoupled from cell-cycle functions of Chk1. Our study here provides a good model system to study this mechanism, which is important as it may predict pathways that may be deregulated in a relapse/disease progression situation following therapeutic Chk1 inhibition in the clinic. One potential relapse mechanism could be via the reactivation of antiapoptotic Bcl-2 family members, which are suppressed by Myc rendering cells sensitive to Chk1 inhibition and DNA damage (data herein and in work published previously by 2 of us; ref. 23).

Recently, inducible MycER mouse models showed that a deregulated level of Myc, corresponding only to that of mitogen stimulation, was sufficient to trigger ectopic proliferation and tumorigenesis whereas higher levels instead trigger activation of the tumor suppressors Arf and p53 and subsequently apoptosis (25, 48). The data shown herein suggest that Myc levels also dictate the level of induction of Chk1 and sensitivity to Chk1 inhibitors. It is thus tempting to speculate that tumors that develop in response to *MYC* translocation or amplification become "Chk1 addicts," as their expression level of Myc is so high. By forcing them to inactivate p53 to avoid apoptosis (4), the cells become dependent on Chk1 for genomic integrity. We therefore propose that *MYC* family members are biomarkers for Chk1 inhibitor–sensitive tumors, which would include large amount of breast cancers, lung cancers, neuroblastoma, and lymphoma. For other tumors, Chk1 inhibition is already considered for clinical trials as sensitizers to chemotherapy, but so far the results of trials are not reported.

Chk1 is a crucial gene *in vivo*, proven by the embryonic lethality of the Chk1-knockout animals (9, 49), and our data also show that cells lacking oncogene activation are hampered in cell division by removing Chk1. Extrapolating on these data to a potential clinical application of Chk1 inhibitors may foretell side effects that could counteract the benefits. Here, mouse models can help to guide the strategies for using Chk1 inhibitors. For instance, conditional deletion of *Chek1* from somatic epithelial cells in the small intestine was recently shown to induce DNA damage and genomic instability followed by apoptosis and crypt death (50). However, *Chek1*-proficient cells were able to repopulate suggesting that Chk1 targeted therapy shows non–tumor-specific side effect in the short term, but that a drug regime using windows of rest for the patients should get around this problem. Our newly discovered Myc–Chk1

connection offers an additional perception to the problem by offering patient selection possibilities.

Disclosure of Potential Conflicts of Interest

L.A. Hasvold and P. Merta are Abbott employees, and J.A. Nilsson has obtained a speaker fee from Abbott ($1,000). No potential conflicts of interest were disclosed by the other authors.

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