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Small molecule inhibitors and a kinase-dead expressing mouse model demonstrate that the kinase activity of Chk1 is essential for mouse embryos and cancer cells

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Running title: Chk1 kinase activity is essential for embryogenesis, cell survival and tumorigenesis

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## Abstract

Chk1 kinase is downstream of the ATR kinase in the sensing of improper replication. Previous cell culture studies have demonstrated that Chk1 is essential for replication and Chk1 inhibitors are efficacious against tumors with high-level replication stress such as Myc-induced lymphoma cells. Treatment with Chk1 inhibitors also combines well with certain chemotherapeutic drugs and effects associates with induction of DNA damage and reduction of Chk1 protein levels. Most studies of Chk1 function has relied on the use of inhibitors. Whether or not a mouse or cancer cells could survive if a kinase-dead form of Chk1 is expressed has not been investigated before. Here we generate a mouse model that expresses a kinase-dead (D130A) allele in the mouse germline. We find that this mouse is overtly normal and does not have problems with erythropoiesis with ageing as previously been shown for a mouse expressing one null allele. However, similar to a null allele, homozygous kinase-dead mice cannot be generated and timed pregnancies of heterozygous mice suggest lethality of homozygous blastocysts at around the time of implantation. By breeding the kinase-dead Chk1 mouse with a conditional allele we are able to demonstrate that expression of only one kinase-dead allele, but no wildtype allele, of *Chk1* is lethal for Myc-induced cancer cells. Finally, treatment of melanoma cells with tumor-infiltrating T cells or CAR-T cells is effective even if Chk1 is inhibited, suggesting that Chk1 inhibitors can be safely administered in patients where immunotherapy is an essential component of the arsenal against cancer.

## Introduction

Replication is tightly controlled to ensure accurate copying of the large DNA molecule (1). Not only the initiation of replication but also the progression of the replication fork is regulated by kinases (2). If the cell experiences e.g. replication stress, UV induced DNA damage and enzymatic and helicase remodeling following DNA inter-strand crosslinking chemotherapy or environmental agents, the replication fork is stalled (3). This causes single-strand DNA (ssDNA) that induces accumulation of replication protein A which recruits the ATR kinase (4-6). ATR phosphorylates Chk1 kinase and other proteins to ensure replication is being blocked as to avoid replication fork collapse and DNA damage (7-9). Both ATR and Chk1 are regarded essential kinases (10-12), albeit Chk1 deficient fission yeast and chicken lymphoma cells can be generated (13, 14). These display defects in DNA damage response and mitosis, since Chk1 also have a role there (15).

Oncogenes such as Myc and Ras can cause replication stress (16), which makes cancer cells sensitive to ATR or Chk1 inhibition (17-22). Insertion of an extra copy of the *Chk1* gene in mice protects cells from replication stress, prolongs survival in *Atr* hypomorphic (Seckel) mice and facilitate transformation (23). Furthermore, we previously showed that Chk1 levels are elevated in Myc-induced cancer cells and both

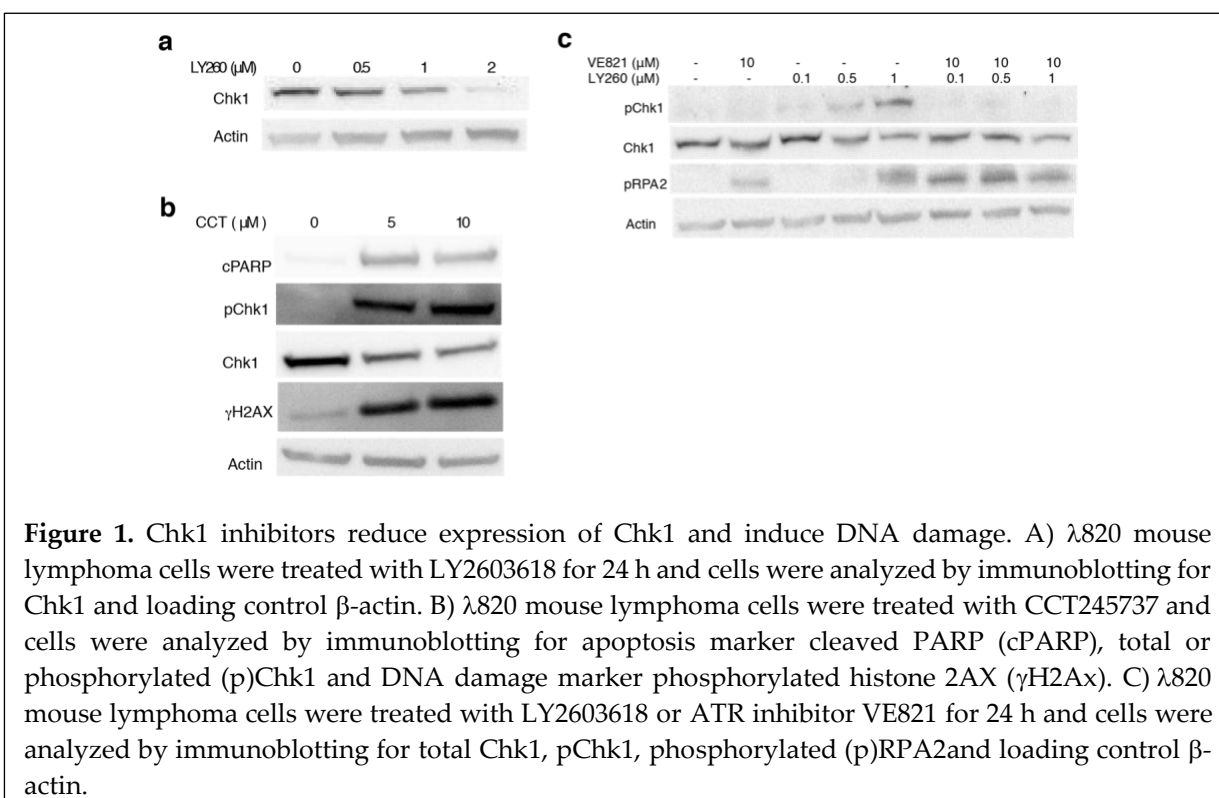
genetic and pharmacological inhibition of Chk1 resulted in cell death of cancer cells in vivo with no evident toxicity in mice (18). These data points to that there may be a therapeutic window targeting Atr and Chk1, despite they are essential.

A surprising effect of Chk1 inhibition in Myc-induced lymphoma cells was also the fact that the protein levels of Chk1 were reduced in Chk1 inhibitor treated cancer cells (18). Because of this, an outstanding question remained whether or not the effect of Chk1 inhibitors was only due to inhibition of Chk1 kinase activity or a lack of the Chk1 protein, or both. To model this, and the importance of Chk1 activity in embryogenesis and tumorigenesis, we here describe the phenotypes of Chk1 kinase-dead mice. We also investigate the role of Chk1 in lymphoma, in erythropoiesis and in T-cell mediated tumor cell killing, using inhibitors and/or mice expressing a kinase-dead *Chk1* allele.

## Results

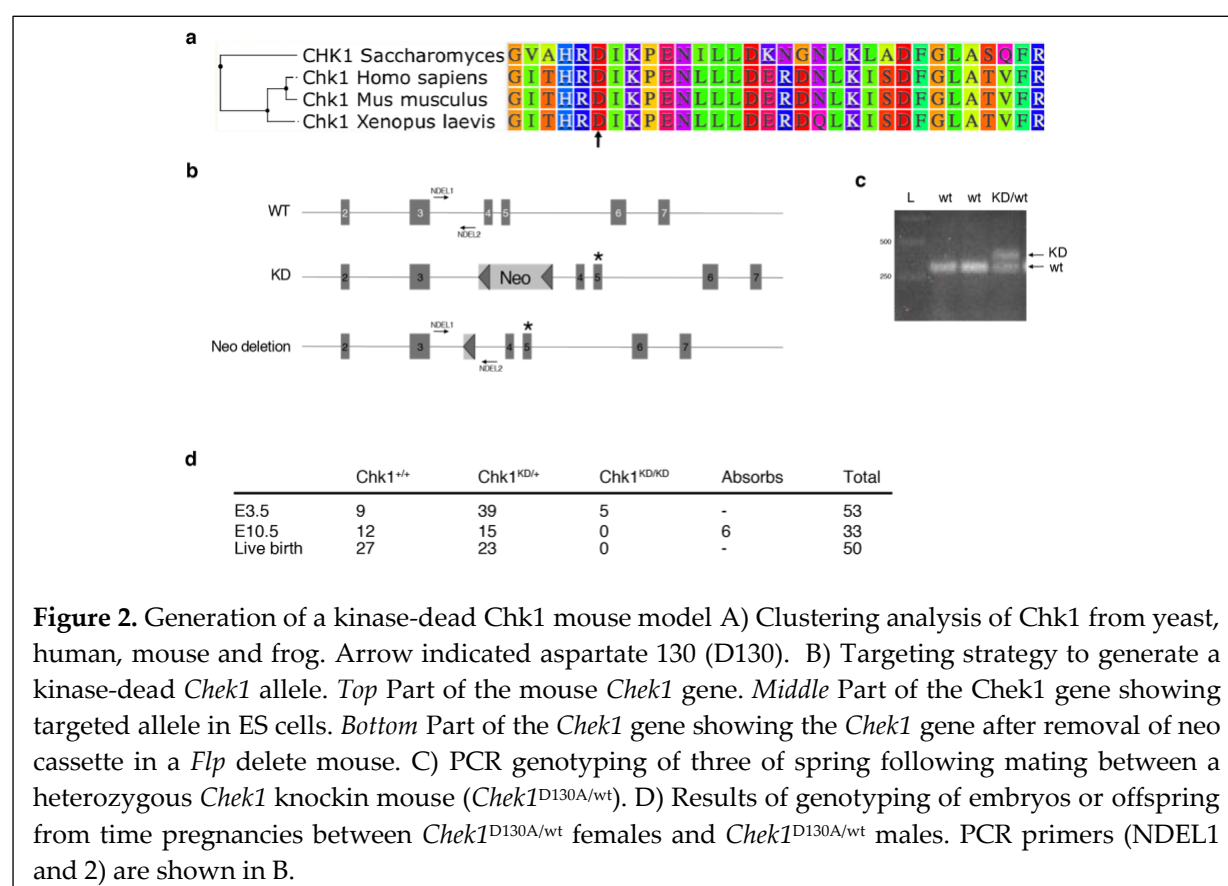
### *Chk1 kinase activity is essential for embryogenesis*

We have previously shown that the highly selective Chk1 inhibitor Chekin can kill Myc-induced lymphoma cells (18). This coincides with an induction of DNA damage and a reduction in Chk1 levels as assessed by immunoblotting. To investigate if these effects can be reproduced using Chk1 inhibitors under clinical development we repeated these experiments using LY2603618 and CCT245737. Both compounds induced DNA damage and cell death, which coincided with a reduction in Chk1 levels (Figure 1A-B). We also observed that levels of phosphorylated Chk1 increased at serine



345, and this phosphorylation appears to be mediated by ATR since the ATR inhibitor VE821 ameliorated the S345 phosphorylation induced by Chk1 inhibitor (Figure 1C).

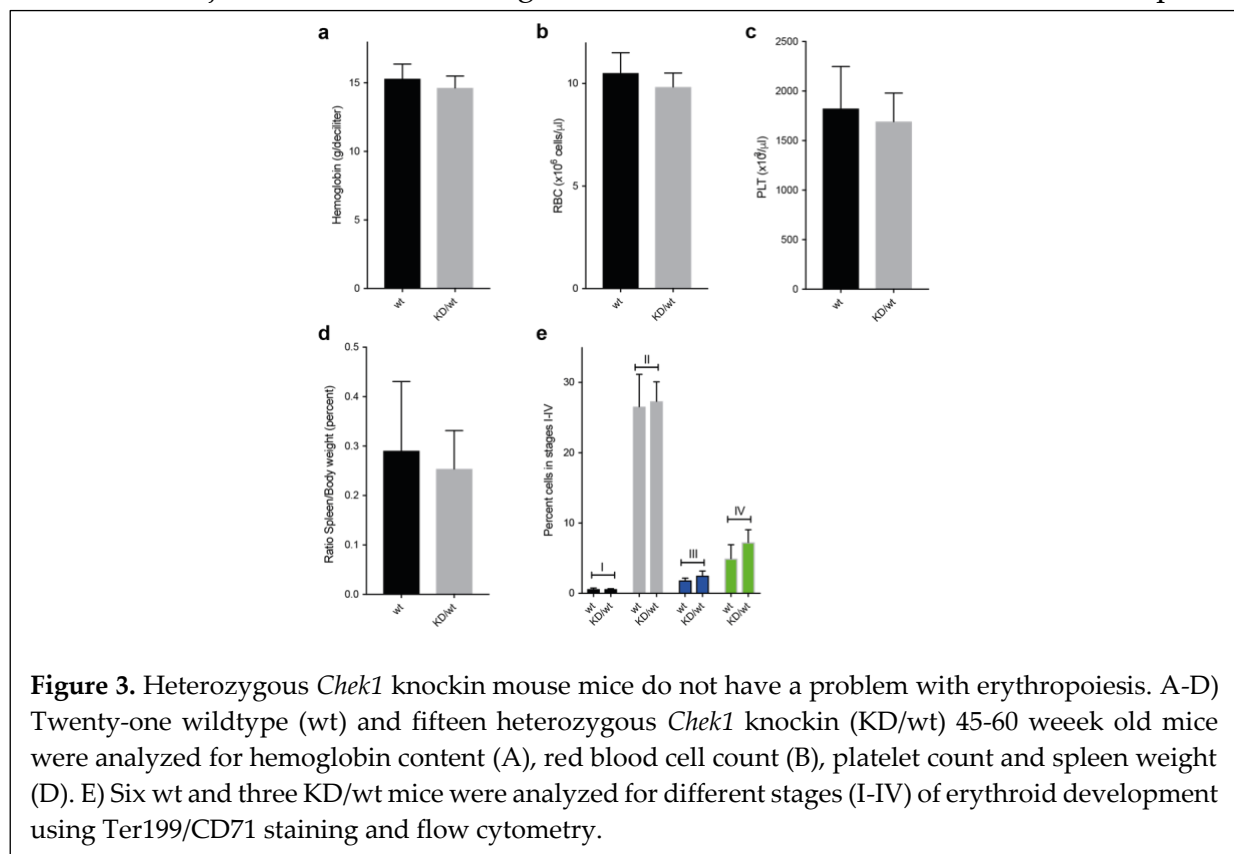
Small molecule inhibitors can potentially have off-target effects since their small size results in that they fit many pockets of proteins (24). They can also be pumped out of cells or remain inaccessible for tissues and developing embryos (25). To model Chk1 inhibition *in vivo* we generated a mouse carrying a point mutation in the *Chk1* gene which is calculated to result in a substitution of aspartate 130 into an alanine in the mouse Chk1. This aspartate is essential for the kinase activity of Chk1, resides in a highly homologous region of the protein (Figure 2A) and a substitution to an alanine creates a kinase-dead form of Chk1 (26). The targeting vector (Figure 2B) used was transfected into mouse C57BL/6- ES cells and correct targeting was confirmed by PCR and Southern blot. Two correctly targeted ES cell clone were injected into blastocysts and several high chimera founders were born. These appeared normal and breeding to C57BL/6 mice generated offspring that were heterozygous or wildtype for the *Chk1*<sup>D130A</sup> allele (Figure 2C) and that were also normal. Generation of mouse embryo fibroblasts (MEFs) from wildtype and heterozygous *Chk1*<sup>D130A/wt</sup> embryos resulted in growth patterns that were similar irrespective of genotype (Figure S1A-B). Interbreeding between heterozygous *Chk1*<sup>D130A</sup> mice generated a non-Mendelian ratio of offspring. No mice were born that were homozygous for the *Chk1*<sup>D130A</sup> allele (Figure 2D), suggesting that the kinase activity of Chk1 is essential for embryogenesis. To verify that the PCR strategy was correct we performed timed pregnancies between



heterozygous *Chk1*<sup>D130A</sup> mice and genotyped embryos at different times post-coitus. The only stage where we were able to detect homozygous *Chk1*<sup>D130A</sup> embryos were at embryonic day 3.5 (E3.5; Figure 2D). The amount of heterozygous *Chk1*<sup>D130A</sup> embryos at E10.5 and at birth was not at a Mendelian ratio (Figure 2D), suggesting that heterozygous oocytes or sperms have adverse phenotypes that are not fully penetrant, or that there are placental defects associated with the heterozygous *Chk1*<sup>D130A</sup> females or the embryos.

### *Half the level of Chk1 kinase activity does not induce anemia in aged mice*

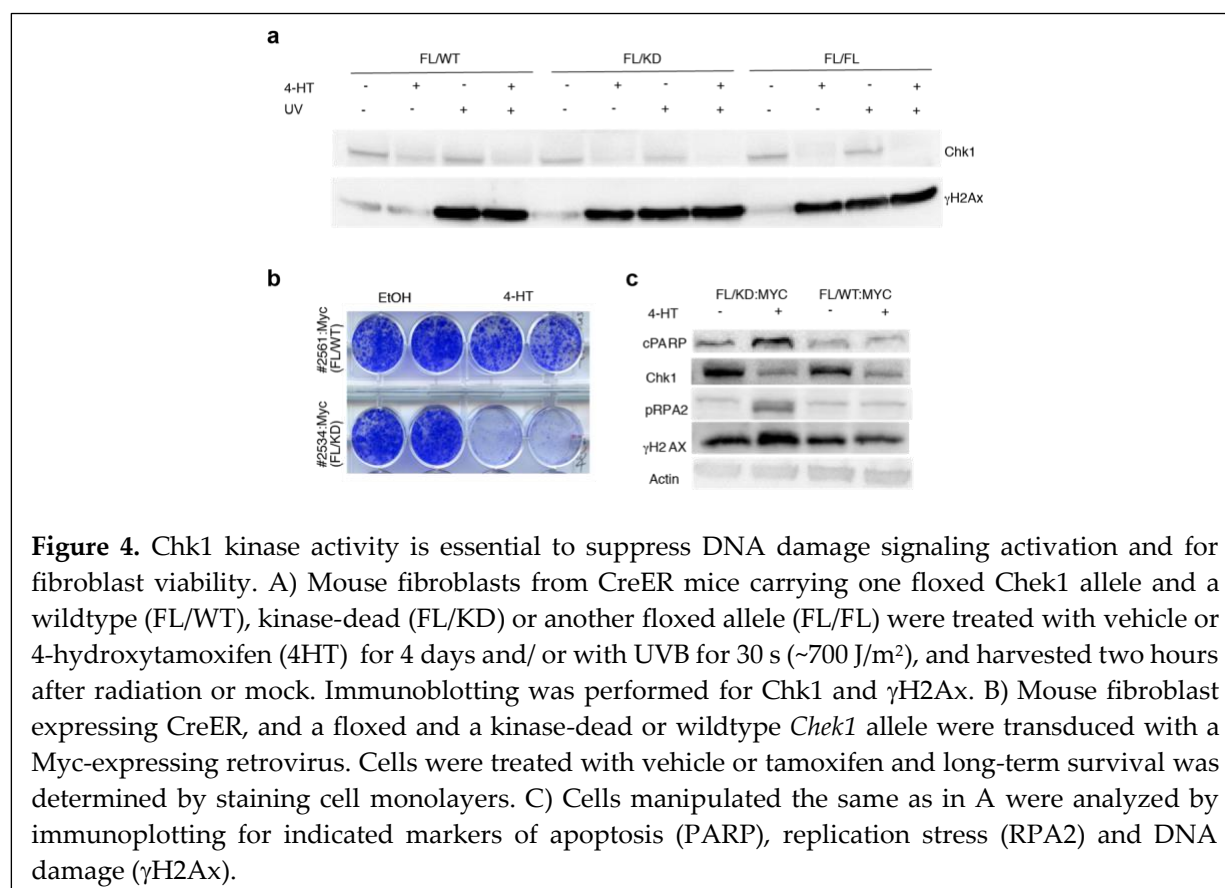
A previous study using heterozygous *Chk1* knockout mice demonstrated that *Chk1* appeared to be 'haploinsufficient' for red blood cell production (27). To investigate if it was the kinase activity, or the Chk1 protein, that constituted this phenotype we aged *Chk1*<sup>D130A/wt</sup> mice for one year and monitored their blood status. Surprisingly, aged mice from both lines of mice carrying one kinase-dead copy of Chk1 displayed normal values on all parameters analyzed. We assessed hemoglobin (Hb, Figure 3A), red blood cells (RBC, Figure 3B) as well as platelets (PLT, Figure 3C) on mice 45-60 weeks of age, both females and males. Additionally, we compared spleen weights of the same cohorts by ages 50-65 weeks, and also here the groups are indistinguishable from each other (Figure 3D). Boles et al describe specific defects during erythropoiesis in Chk1 heterozygote mice. To gain more insight into erythropoiesis in our mice carrying one copy of kinase-dead Chk1, we stained cells isolated from bone marrow of our aged mice and subjected these to staining with Ter119-APC and CD71-PE and subsequent



flow cytometry analysis. Also in this assay, we fail to detect any differences between the two groups (Figure 3E).

### *Chk1 kinase activity is essential for cell survival*

Since kinase-dead Chk1 mice cannot be generated by interbreeding we obtained *Chek1* conditional knockout mice (*Chek1*<sup>Flox/Flox</sup>) and mice expressing a fusion between the Cre recombinase and the ligand binding domain of the estrogen receptor (*CreER* mice). By breeding these mice to heterozygous kinase-dead Chk1 mice we would be able to delete Chk1 by Cre activation with the estrogen analog Tamoxifen after the mice were born, thereby leaving only the kinase-dead allele to be expressed. Administration of tamoxifen to *CreER;Chek1*<sup>Flox/D130A</sup> mice, but not to *CreER;Chek1*<sup>Flox/wt</sup> mice resulted in toxicity (data not shown) and the ethical limitations prohibited us from further experiments using this system. We therefore interbred mice carrying *CreER* and floxed or kinase-dead *Chek1* alleles with *Cdkn2a*<sup>-/-</sup> mice and generated mouse fibroblasts from interbreedings. We could then investigate the effect of deletion of the wildtype allele of cultured cells since *Cdkn2a*<sup>-/-</sup> mouse embryo fibroblasts do not undergo culture-induced senescence. Addition of the estrogen analog 4-hydroxytamoxifen (4-HT) to MEFs of different genotypes demonstrated that MEFs relying on just a kinase-dead form of Chk1 triggers DNA damage as revealed by phosphorylation of H2AX ( $\gamma$ H2AX, Figure 4A). The induction of  $\gamma$ H2AX upon loss of active Chk1 was equal to that of UV irradiation. To investigate if cells expressing oncogenes such as Myc would be sensitive to a kinase-dead Chk1 allele we transduced *CreER;Chek1*<sup>Flox/D130A</sup>; *Cdkn2a*<sup>-/-</sup> or

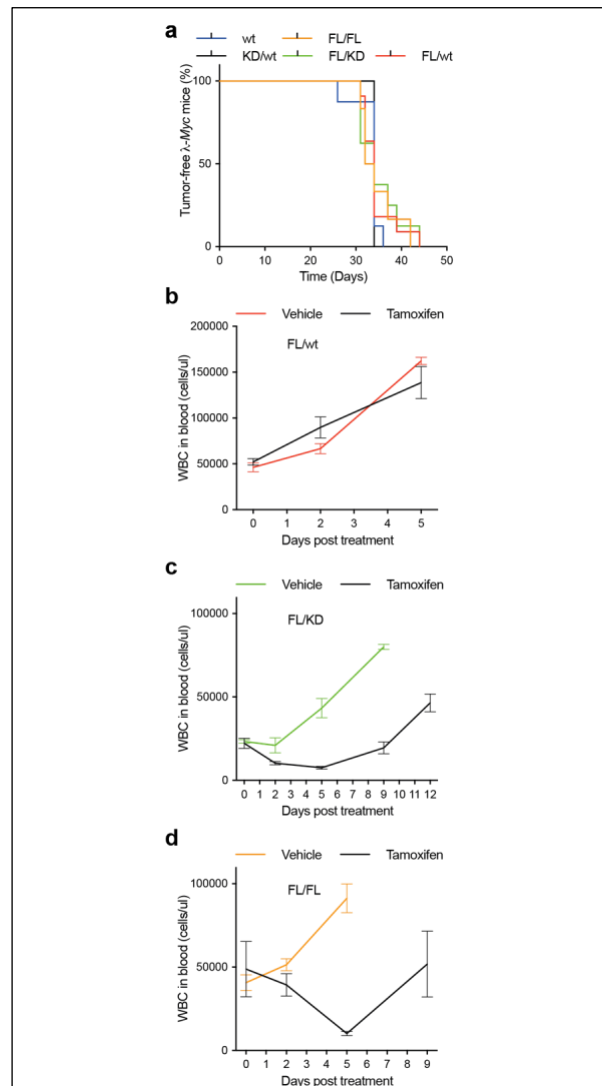




*CreER;Chk1<sup>Flox/wt</sup>;Cdkn2a<sup>-/-</sup>* cells with a retrovirus expressing c-Myc. Cre activation with 4-HT resulted in a loss of viability of Myc-expressing fibroblasts (Figure 4B). This correlated with an induction of replication stress, ie phosphorylation of replication protein A (p-RPA), induction of DNA damage ( $\gamma$ H2AX) and cleavage of PARP, a marker of apoptosis (Figure 4C).

### *The Chk1 kinase activity is essential for Myc-induced lymphoma survival*

We and others have previously shown that Chk1 inhibitors are very efficacious in killing Myc-induced lymphoma cells. To assess if kinase inhibitor effects can be modeled genetically we interbred the *CreER;Chk1<sup>Flox/D130A</sup>* or *CreER;Chk1<sup>Flox/wt</sup>* with the  $\lambda$ -Myc mouse, a transgenic mouse model where c-Myc is under the control of the immunoglobulin  $\lambda$  light chain promoter (28).  $\lambda$ -Myc mice develop B-cell lymphoma by 100 days of age. To accelerate the process of lymphomagenesis we interbred to *Cdkn2a<sup>-/-</sup>* mice. Heterozygous  $\lambda$ -Myc; *Cdkn2a<sup>+/-</sup>* mice develop tumors 30-50 days of age and the tumors lose the remaining wildtype *Cdkn2a* allele (29). One kinase-dead allele did not delay this acceleration (Figure 5A). When tumors developed in the offspring, we harvested lymphoma from  $\lambda$ -Myc; *CreER;Chk1<sup>Flox/D130A</sup>;Cdkn2a<sup>+/-</sup>*,  $\lambda$ -Myc; *CreER;Chk1<sup>Flox/Flox</sup>;Cdkn2a<sup>+/-</sup>* or  $\lambda$ -Myc; *CreER;Chk1<sup>Flox/wt</sup>;Cdkn2a<sup>+/-</sup>* mice. These tumors could be transplanted into recipient C57BL/6 mice. These mice were monitored for lymphoma manifestation by measuring white blood cells in the blood. When mice had higher than normal blood counts (>12 white cells per nl), each cohort was divided in two and these groups received vehicle or tamoxifen. Mice transplanted with  $\lambda$ -Myc; *CreER;Chk1<sup>Flox/wt</sup>;Cdkn2a<sup>+/-</sup>* lymphoma cells did not respond at all to tamoxifen (Figure 5B), again



**Figure 5.** Expression of only a kinase-dead allele of *Chk1* suppresses lymphoma growth. A) Kaplan-Meier survival analysis of  $\lambda$ -Myc; *Cdkn2a<sup>+/-</sup>* mice carrying the indicated *Chk1* genotypes. B-D) Lymphomas from mice generated from mice in A were transplanted into eight C57BL/6 mice per indicated genotype. When white blood counts (WBC) reached between 20-50x10<sup>3</sup> cells/ $\mu$ l four mice received vehicle or tamoxifen by ip injection. WBC was followed until relapse or when palpable tumors required sacrifice of the animals.

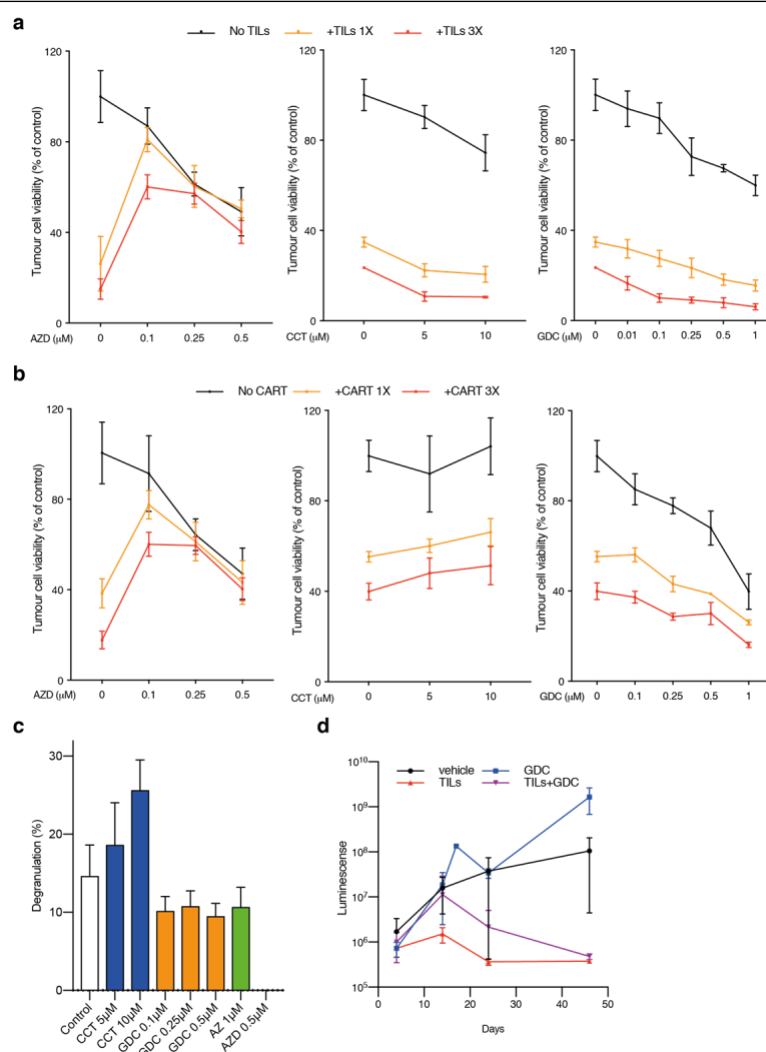
demonstrating that one allele of *Chek1* is sufficient for lymphoma cells to survive. On the other hand, mice transplanted with lymphoma cells from  $\lambda$ -Myc;CreER;*Chek1*<sup>Flox/D130A</sup>;*Cdkn2a*<sup>+/-</sup> mice (Figure 5C), and to a lesser extent lymphoma cells from  $\lambda$ -Myc;CreER;*Chek1*<sup>Flox/Flox</sup>;*Cdkn2a*<sup>+/-</sup> mice (Figure 5D), exhibited a rapid decrease in white blood cell count upon tamoxifen treatment. Despite the acute therapeutic effects in lymphoma by deleting the wildtype alleles of *Chek1*, lymphomas eventually relapsed in the mice.

#### *Anti-tumoral immunity mediated by T cells is not impaired by Chk1 inhibition*

The fact that Myc-induced B-lymphoma cells are sensitive to Chk1 inhibition is promising for lymphoma treatment but recent data suggest that the sensitivity reflects a B-cell lineage sensitivity (30). Importantly though, other lymphocytes, such as T cells, are essential for anti-tumor immunity of solid tumors. Therefore, we investigated the impact of Chk1 inhibition on T cell activity. We investigated the anti-tumoral function of human T cells during Chk1 inhibition. We cultured melanoma cells labelled with luciferase in the absence or presence of melanoma tumor-infiltrating cells (TILs) and increasing concentrations of the Chk1 inhibitors CCT245737, AZD7762 and GDC-0575. AZD7762 blocked the ability of the TILs to kill the melanoma cells, whereas CCT245737 and GDC-0575 did not inhibit but enhanced the killing of melanoma cells by TILs (Figure 6A). We previously showed that melanoma cells are sensitive to HER2 CAR-T cells (31). When we treated melanoma cells with HER2 CAR-T cells, AZD7762 inhibited the CAR-T cells from killing melanoma cells, whereas CCT245737 and GDC-0575 enhanced the CAR-T-mediated killing of melanoma cells (Figure 6B). The inability of T cells to kill melanoma cells in the presence of AZD7762 correlated with an impaired ability to degranulate (Figure 6C). On the other hand, neither CCT245737, GDC-0575 nor the ATR inhibitor AZ20 negatively affected degranulation. We therefore hypothesized that inhibition of both Chk1 and Chk2 by AZD7762 is less optimal for T-cell combination therapies than inhibiting the ATR/Chk1 axis.

GDC-0575 is an orally available Chk1 inhibitor under clinical development. To test if the inhibitor is compatible with immunotherapy we used a recently developed model, the PDXv2 model (32), where human melanoma is grown in an immunocompromised mouse transgenic for human IL-2 (hIL2-NOG mice). Infusion of autologous T cells into this mouse model can be used to assess quality of the patient's TILs and responses in these mice mimic the responses to adoptive T cell transfer in the same patients from which the PDXv2 model is developed. We transplanted the same luciferase labelled melanoma cells used in experiments depicted in Figure 6A-C into hIL2-NOG mice. When tumor growth was confirmed by bioluminescence imaging we transplanted the tumor-bearing mice with vehicle or TILs. The mice were thereafter treated with vehicle or GDC-0575. As seen in Figure 6D, all mice receiving TILs had a regression of the tumor, irrespective of GDC-0575 co-treatment, whereas GDC-0575 monotherapy was ineffective. We therefore conclude that Chk1 inhibitors could be used in cancer treatment by not negatively impact tumor immunity.





**Figure 6.** Chk1 inhibition does not interfere with T-cell mediated killing of melanoma cells. A) 33F8-luciferase expressing melanoma cells were treated with indicated Chk1 inhibitors in the absence or presence of autologous TILs (1:1 or 3:1 TILs vs melanoma cells). Luciferin was added and viability was measured in a 96-well plate luminometer. B) The same experimental setup as in A but using HER2-directed CAR-T cells instead of TILs. C) 33F8 cells were mixed with autologous TILs in the presence of indicated inhibitors. Surface expression of CD107a as a marker of T cell degranulation was determined by flow cytometry. D) NOD/SCID/IL2 receptor gamma knockout mice transgenic for human interleukin-2 (hIL2-NOG mice) were transplanted with 33F8-luciferase melanoma cells. When tumors were detectable by IVIS imaging, TILs were injected and mice were dosed in indicated groups of animals (n=4). Mice were followed with IVIS imaging until tumor size reached the ethics limit (10 mm on the shortest side). Shown are median IVIS signal and standard error of the mean.

## Discussion

Here we present the phenotypes of a mouse where one of the alleles have been mutated to express a kinase-dead variant of Chk1. We developed this model to complement our studies of Chk1 kinase inhibition using drugs. The main novel conclusions and implications are as follows: First, heterozygosity of the D130A allele is compatible with viability, tumorigenesis and even erythropoiesis. This suggests that

a kinase-dead form of Chk1 is a non-functional protein which does not endow the protein with additional problems. This is in contrast to the kinase-dead forms of both ATM and ATR, where the kinase-dead expressing mouse models behave differently than the knockout and the heterozygous mice respectively (33, 34). In these models the dynamics of the kinase-dead proteins is hindered by their inability to phosphorylate other proteins. We find here that a kinase-dead form of Chk1 does not appear to operate as a dominant negative form. We do however find that there is a difference between the kinase-dead allele and a null allele with respect to erythropoiesis in the heterozygous state. We reason that this is either due to that Chk1 has a kinase activity independent function in reticulocytes, or that the targeting constructs differ significantly. In our mouse model, a single amino acid change has been introduced, and the selection cassette used during ES targeting has been removed. In Boles et al, where it is claimed that Chk1 is 'haploinsufficient' for erythropoiesis, the original *Chek1* knockout mouse was used (27). In this mouse, exons 2-5 of *Chek1* is replaced by a neomycin cassette (neo), which would pose a risk that other genes are affected by the presence of an ectopic promoter driving neomycin. It is of interest to note that the erythropoietin receptor gene *Epor* resides in the vicinity of *Chek1* in the mouse genome. It is thus tempting to speculate that the neo cassette in *Chek1* heterozygous mice could result in a hypomorphic allele of *Epor* resulting in a defect in erythropoiesis in this animal model. Unfortunately, we were unable to confirm this since *Chek1* heterozygous mice was not available at the time when we found that a heterozygous kinase-dead expressing Chk1 mouse did not exhibit any ageing-related defects in erythropoiesis.

The second important conclusion we make is that a homozygous *Chek1*<sup>D130A/D130A</sup> mouse embryo does not survive after implantation, suggesting that the kinase activity of Chk1 is essential for early embryogenesis. This is in accordance with data using the *Chek1* knockout mouse. Our acute deletion using tamoxifen of the wildtype allele while leaving one kinase-dead allele of *Chek1* intact suggested that also some adult cells required Chk1. Indeed, the clinical trials done so far have indicated dose-limiting side-effects using Chk1 inhibitors but given that many drugs have side-effects it has been impossible to tie these to Chk1 inhibition. Our new animal model offers a unique possibility to study both therapeutic effects and side-effects of Chk1 inhibitors.

The third set of conclusions of this study is that we can confirm that Myc overexpressing cells are sensitive to Chk1 inhibition and that this sensitivity is associated with kinase inhibition and not just a loss of protein. We show that cancer cells are sensitive to Chk1 inhibition but the activity of tumor-killing T cells are not inhibited by Chk1 inhibition. This is an important finding since cancer immunotherapies have become mainstay in many diagnoses including melanoma, lung cancer and renal cell carcinoma. Since T cells in our animal model are proliferating because of constitutive IL2 signaling our data suggest that Chk1 is not as essential for this lineage as the B cell lineage (30). This is in line with recent studies using inhibitors of the upstream kinase ATR, the kinase that activates Chk1. ATR

inhibitors synergize with radiotherapy in mouse models by suppressing PD-L1 expression and reducing amounts of T regulatory cells promoting anti-tumor immunity (2). Trials combining ATR inhibitors and PD-1 inhibitors are on the way (e.g. NCT04095273). Our new data and previous studies showing that ATR and Chk1 inhibitors synergize [(35) and own unpublished data] support that Chk1 inhibitors could be combined with immunotherapy and/or ATR inhibitors in the clinical management of solid tumors such as melanoma.

## Methods

### *Animal ethics*

All animal experiments were conducted in accordance with Directive 2010/63/EU and was approved by the regional animal ethics committee of Gothenburg. Approval numbers were #98-2015, #36-2014 and #1183-2018.

### *Generation of a mouse expressing a kinase-dead form of Chk1*

The *Chk1*<sup>D130A/+</sup> mouse model was made at inGenious Targeting Laboratory (Ronkonkoma, NY, USA). Briefly, the targeting construct to generate the genomic D130A substitution was generated by cloning a 2.45 kb 5' homology arm and a 5.8 kb 3' homology arm of Chk1 from a BAC clone upstream and downstream of a FRT-NEO-FRT cassette, respectively. The 3' homology arm contained an A->C substitution compared to the wildtype sequence. The targeting construct was electroporated into C57BL/6 ES cells and 300 clones were picked after selection. Five of these were selected after PCR confirmation for verification by sequencing of the mutation, Southern blot and karyotyping. All passed with the correct mutation, only one integration and ≥ 89 % euploidy. Two of the five clones were used for blastocyst injection into Balb/c blastocysts, called line 1 and line 2. Line 1 generated three high and two medium percentage chimeras. Lines 2 generated two high and one medium percentage chimeras. The chimeric mice were bred to B6 FLP mice, to remove the neo cassette. Offspring were genotyped to confirm loss of neo cassette and a region containing the D130A mutation was amplified by PCR and sequenced. These were sent to University of Gothenburg and were backcrossed to C57BL/6 once and genotyped to confirm neo deletion, before interbreeding heterozygous mice. Neither heterozygous mice derived blastocyst injection ES cell line 1 nor from line 2 blastocyst injections were able to generate viable homozygous offspring (Figure 2 and data not shown). For the rest of the experiments we used only mice and cells derived from line 1.

To be able to assess the effects of expression of a kinase-dead form of Chk1, we interbred the *Chk1*<sup>D130A/+</sup> mouse to a conditional floxed *Chk1* mouse (10) and to a transgenic mouse expressing CreER both from the Jackson Laboratories. The latter mouse expresses a Cre recombinase fused to the ligand binding domain of the estrogen receptor. Treatment of mice or cells expressing CreER results in an ability of Cre to translocate to the nucleus and to recombine DNA elements flanked by lox P sites. The

compound mice were also bred to a  $\lambda$ -Myc transgenic mouse (The European Mouse Mutant Archive repository) and to *Cdkn2a* knockout mouse (Jackson Laboratories).

### *Cell Culture*

Mouse embryonic fibroblasts (MEFs) were cultured in DMEM with stable glutamate (Gibco) supplemented with 10 % heat-inactivated FBS, Gentamycin, sodium pyruvate and  $\beta$ -Mercapto-ethanol. TILs were cultured in RPMI with stable glutamate, supplemented with 10 % human serum, gentamycin and 6000 units of human IL-2 (Pepro Tech, USA). 33F8-Luc cells were cultured in RPMI supplemented with 10% heat inactivated FBS and Gentamycin. All cells were tested for mycoplasma by PCR and were cultured at 37°C and 5% CO<sub>2</sub>.

### *Inhibitors*

ATR inhibitor AZ20 was purchased from MedChem express, while Chk1 inhibitors, CCT245737 and AZD7762, GDC-0575 were purchased from Selleckchem. Inhibitors were dissolved in DMSO at 10mM and stored at -20 °C or -80°C.

### *Cell viability*

For cell viability measurements, cells were cultured in 96 well plates and metabolic activity was measured using Cell-Titer-Glo assay or CellTiter-Fluor™ Cell Viability Assay (Promega, USA), as per manufacturer's protocol, in GloMax plate reader. Killing assay was performed in 96 well plates. For killing assay, 33F8 cells expressing Luciferase were seeded in 96 well plates and 33F8 TILs were added to them at varying ratios, in the presence or absence of various Chk1 inhibitors. Luciferase assay was carried out 24 and 48 hours post treatment to assess viability of the tumor cells.

### *Immuno-blotting*

Cell pellets were lysed in Arf lysis buffer as described earlier (36). In all, 50  $\mu$ g of proteins were resolved on 4-20% Biorad Mini-PROTEAN TGX gels and then transferred onto nitrocellulose membrane, following which, the membrane was blocked in 5% BSA or milk. Blocked membranes were then blotted with antibodies directed against the following proteins: p-Chk1, cleaved PARP (Cell Signaling Technology, Danvers, MA, USA), Chk1 (Santa Cruz Biotechnology, Dallas, TX, USA), Actin (Sigma-Aldrich) or phosphorylated-histone-H2AX (S139,  $\gamma$ H2AX; Merck Millipore).

### *Cell cycle analysis*

Around one million cells per ml were lysed in Vindelov's solution ((20 mmol/L Tris, 100 mmol/L NaCl, 1  $\mu$ g/mL 7-AAD, 20  $\mu$ g/mL RNase, and 0.1 % NP40) for 30 minutes at 37 degrees. Stained nuclei were analyzed using BD accuri C6. Apoptosis was measured on logarithmic scale on FL3 channel, whereas cell cycle of viable cells was analyzed on linear scale.

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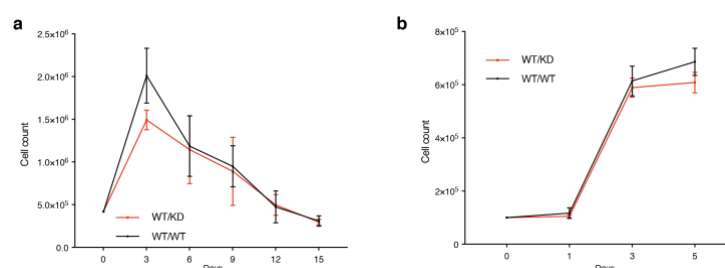
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## Supplemental data



**Supplemental Figure 1.** Heterozygosity of a kinase-dead Chk1 expressing allele does impact growth of mouse embryo fibroblasts (MEFs). A) Timed pregnancies between *Chek1*<sup>D130A/+</sup> mice and C57BL/6 mice were used to make MEFs. Passage 3 MEFs were seeded at 0.5 million cells in a 5 cm plates and cultured for three days. Cells were counted and 0.5 million cells were plated and cultured for three days again. The procedure was continued until the cells no longer were able to proliferate in three days. B) Growth curves of MEFs grown for five days.