The multifaceted role of protein kinase CK2 in high-risk acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is an aggressive malignancy of developing lymphocytes. Despite outstanding overall cure rates, patients with the refractory or relapsed disease have a poor prognosis.[1] In order to improve treatments for these high-risk (HR)-ALL patients, it is critical to gain an in-depth understanding of the disease pathogenesis. The enhanced expression of the protein kinase *CK2* gene and proto-oncogene *MYC* are common in T cell ALL (T-ALL) and B cell ALL (B-ALL).[2-6] CK2 is a constitutively active serine/threonine kinase composed of two catalytic (α or α') and two regulatory (β) subunits that are overexpressed in a broad spectrum of human cancers.[7] Despite the demonstrated antileukemic efficacy of CK2 inhibitors,[8] how CK2 contributes to HR-ALL development remains incompletely understood. Here we utilized transgenic zebrafish models to elaborate the multifaceted role of CK2 in HR-ALL pathogenesis, providing therapeutic implications for this stubborn disease.

Overexpression of the *CK2α* subunit under the immunoglobulin gene promoter induces low penetrance of T-cell lymphomas in a murine model.[9] In order to further understand the oncogenic potential of CK2 in T and B lineages, we generated transgenic zebrafish that overexpress the wild-type or the kinase-dead version (*K68M*) of the human *CK2α* gene in T and B cells through the zebrafish tyrosine kinase gene (*lck*) promoter.[2,10] Western blotting analysis revealed elevated expression of CK2α in transgenic *CK2* fish, compared to age-matched wild-type (wt) fish (*Online Supplementary Figure S1A*). Despite relatively normal thymus development and no observable difference in fish survival (*Online Supplementary Figure S1B*), lymphocytes in *Tg(lck:CK2 wt;rag2:mCherry)* fish survived much longer than the control *Tg(lck:EGFP)* or *Tg(rag2:mCherry)* transgenic fish (*Online Supplementary Figure S1C*). By 8 months, *CK2* transgenic fish still had clearly defined red-fluorescent thymi, while control transgenic fish began to lose their thymic fluorescence as early as 5 months of age (*Online Supplementary Figure S1C* and *data not shown*). Strikingly, *CK2* transgenic fish can retain their thymic fluorescence till 18 months. In order to determine the effect of CK2α in inducing lymphoid malignancies in zebrafish, starting at 21 days post-fertilization (dpf), we monitored both wt and mutant *CK2* transgenic fish at least once a month until 2 years of age and found no tumor development in these fish (*Online Supplementary Figure S1C* and *data not shown*). Additionally, we also overexpressed *CK2α* under the zebrafish *rag2* promoter and also failed to observe tumor development in this fish line. These results indicate that *CK2* overexpression alone has very limited oncogenic potentials.

Despite the early knowledge that CK2 accelerates MYC-induced T-ALL,[9] several questions remain: i) can CK2 and MYC synergize to promote B-ALL?, ii) does CK2's tumor-promoting effect solely depend on its enzymatic activity?, and iii) how does CK2 contribute to different stages of ALL development? To this end, we bred our *lck*-promoter-driven wt or kinase-dead *CK2* transgenic fish to conditional *Tg(rag2:MYC-ER;lck:EGFP)* fish, in which aberrant MYC activity is regulated by tamoxifen and induces leukemia in both T and B lineages.[2,11] We raised their offspring in fish water containing 4-hydroxytamoxifen (4HT) beginning at 5 dpf when thymic fluorescence was first visible (Figure 1A), and monitored the fish for tumor onset using previously defined criteria.[12,13] At 4 weeks of age, all groups showed normal-sized thymi. However, by 6 weeks, all three fish lines exhibited evidence of tumor initiation compared to *Tg(lck:EGFP)* and *Tg(rag2:mCherry)* controls (Figure 1C; *Online Supplementary Figure S1B*). By 12 weeks of life, tumors developed in more than 90% of *Tg(rag2:MYC-ER;lck:EGFP;lck:CK2αwt;rag2:mCherry*) fish, referred to as *MYC-ER;CK2αwt* (Figure 1B and C). However, tumors developed in less than 60% of *Tg(rag2:MYC-ER;lck:EGFP*) fish, referred to as MYC-ER (Figure 1B and C).

Interestingly, overexpressing the enzyme-dead version of *CK2α* in *Tg(rag2:MYC-ER;lck:EGFP;lck:CK2αK68M;rag2:mCherry*) fish, referred to as *MYC-ER;CK2αK68M*, failed to accelerate the disease, with approximately 50% of fish developing tumors at 12 weeks of life (Figure 1B and C). These results demonstrate that the HR-ALL development depends on the enzymatic activity of CK2 since wt, but not kinase-dead *CK2α*, significantly accelerated the onset of MYC-induced ALL.

Next, we questioned whether *CK2α* could hasten the progression of MYC-induced ALL by quantifying the tumor burden in the above three groups of fish. We found that ALL developed in *MYC-ER;CK2αwt* fish much more aggressively, as demonstrated by a significantly heavier tumor burden in these fish compared to those in *MYC-ER* sibling fish (*Online Supplementary Figure S2A* and *B*). However, overexpression of *CK2αK68M* failed to enhance disease aggression as the tumor burden in *MYC-ER;CK2αK68M* fish was similar to those in *MYC-ER* fish (*data not shown*). Since MYC-ER fish develop both T- and B-ALL,[2,11] we then asked which types of leukemia *MYC-ER;CK2αwt* fish developed by performing semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using zebrafish T- and B-cell specific primers.[14] Our results show that *MYC-ER;CK2αwt* fish also developed ALL of T and B lineages (*Online Supplementary Figure S3*).

In order to determine whether MYC-induced transformation is restricted to the particular stages of lymphocyte development, we treated *MYC-ER* fish with 4HT at 30 dpf instead of 5 dpf, and monitored fish for tumor development with weekly fluorescent imaging (Figure 2A). Surprisingly, none of these *MYC-ER* fish developed tumors after 8 weeks of 4HT treatment (Figure 2B and C). However, if these fish were treated with 4HT at 5 dpf, more than 30% of *MYC-ER* fish had already developed tumors at this time (Figure 1C). Next, we determined if the enhanced *CK2α* expression could overcome this temporal restriction of lymphocyte transformation. In order to do so, we bred *CK2α* transgenic fish to *MYC-ER* fish and treated the fish with 4HT at 30 dpf. Strikingly, tumors started to arise in the *MYC-ER;CK2αwt* fish within 1 week of 4HT treatment (Figure 2C). Within less than 2 weeks of 4HT treatment, approximately 80% of *MYC-ER;CK2αwt* fish developed aggressive ALL (Figure 2B and C). These results demonstrate that CK2 can overcome the temporal restriction of *MYC*-mediated lymphocyte transformation and induce ALL at a later developmental stage.

Since the aggressive nature of leukemia in *MYC-ER;CK2αwt* fish depends on the kinase activity of CK2 (Figure 1), we next performed phos-tag western blotting to determine whether enforced CK2 expression increases MYC phosphorylation *in vivo*. Compared to tumors in *MYC-ER* fish, we detected increased CK2α and relatively more phosphorylated MYC (upper bands) protein levels

in tumors from *MYC-ER;CK2αwt* fish (*Online Supplementary Figure S4A*). In order to determine whether increased phosphorylation of MYC led to the stabilization of MYC protein *in vivo*, we analyzed the half-life of MYC-ER protein in the presence or absence of *CK2α* overexpression in zebrafish developing lymphocytes. We isolated premalignant thymocytes from 5-week-old MYC-ER and *MYC-ER;CK2αwt* fish, dissociated the thymocytes, and treated them with cycloheximide (CHX) to inhibit protein synthesis. Western blotting analysis was then performed to measure MYC-ER protein levels at different time points. We found that MYC-ER was stabilized in lymphocytes with *CK2α* overexpression, compared to those without *CK2α* overexpression (*Online Supplementary Figure S4B*). In order to understand whether CK2 can promote MYC-mediated leukemogenesis through other mechanisms, we performed quantitative RT-PCR analysis of zebrafish homologs of human anti-apoptotic genes, *BCL2, BCL-XL*, and *MCL1*. No significant difference was found in leukemic cells from MYC-ER *versus MYC-ER;CK2αwt* fish (*Online Supplementary Figure S5*). Together, these data indicate that CK2's ability in phosphorylating and stabilizing MYC *in vivo* serves as one mechanism to promote leukemia initiation and aggressiveness.

In order to determine whether overexpression of *CK2α* alleviates the necessity of MYC in established tumors, we treated fish with 4HT starting at 5 dpf for 11 weeks to induce tumor development. We then removed 4HT from MYC-ER and *MYC-ER;CK2αwt* tumor fish to inactivate MYC and monitored disease regression for 8 weeks by fluorescent imaging (Figure 3A). We categorized tumor phenotypes based on the extent of change in tumor size as previously described: complete regression, partial

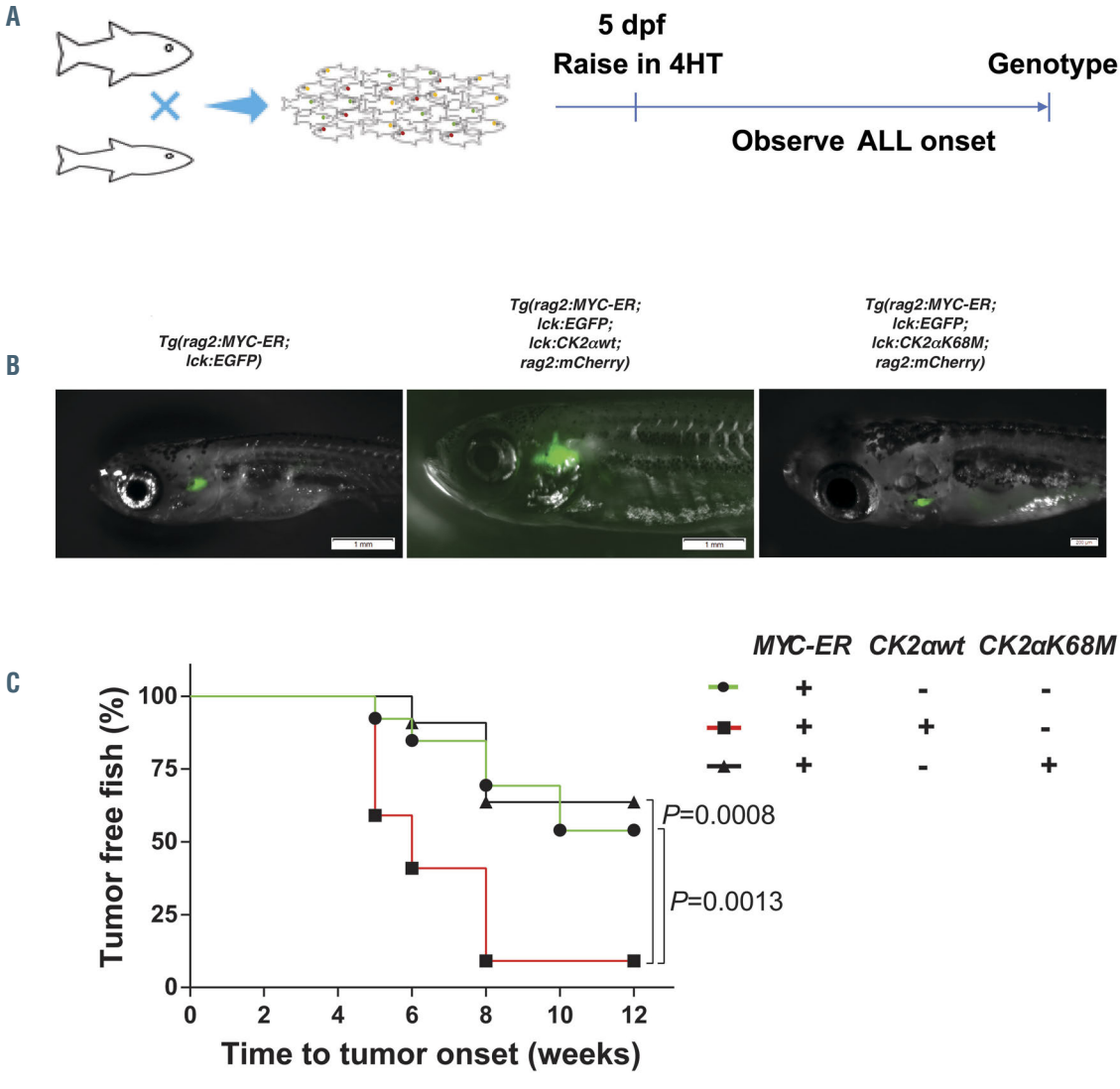

Figure 1. Overexpression of wild-type but not enzyme dead *CK2α* promotes the onset of MYC-induced acute lymphoblastic leukemia in zebrafish. (A) Diagram of the experimental design. (B) Thymic fluorescence in the *Tg(rag2:MYC-ER;lck:EGFP)* (left), *Tg(rag2:MYC-ER;lck:EGFP;lck:CK2αwt; rag2:mCherry)* (middle), and *Tg(rag2:MYC-ER;lck:EGFP;lck:CK2αK68M;rag2:mCherry)* (right) fish raised in 129 nM 4-hydroxytamoxifen (4HT) at 12 weeks of life. One representative fish is shown for each group. (C) Kaplan-Meier analysis of tumor-free fish revealed that overexpression of *CK2αwt* but not *CK2αK68M* significantly accelerated the onset of MYC-induced acute lymphoblastic leukemia (ALL) (*P*=0.0013 for *Tg(rag2:MYC-ER;lck:EGFP)* [*MYC-ER*; green line] *vs. Tg(rag2:MYC-ER;lck:EGFP;lck:CK2αwt; rag2:mCherry);* [*MYC-ER;CK2αwt;* red line] n=19 and n=22, respectively; and *P*=0.0008 for *MYC-ER;CK2αwt* [red line] *vs.Tg(rag2:MYC-ER;lck:EGFP;lck:CK2αK68M;rag2:mCherry)* [*MYC-ER;CK2αK68M*; black line], n=22 and n=13, respectively). There was no statistical significance between *MYC-ER* and *MYC-ER;CK2αK68M* fish. Statistical analysis was performed using the log-rank test. The scale bar in the left and middle panel of Figure 1B =1 mm and in the right panel =200 μm.

regression, stable disease, and progression.[11] By 4 weeks post-withdrawal of 4HT, approximately 35% of MYC-ER fish and approximately 50% of *MYC-ER;CK2αwt* fish had already exhibited complete tumor regression (Figure 3B). We found that there were no statistically significant differences between MYC-ER *versus MYC-ER;CK2αwt* fish for the changes of tumor status at both 4 and 8 weeks post 4HT removal (Figure 3C and *data not shown*). These results demonstrate that *CK2* overexpression alone cannot substitute for aberrant MYC activity in maintaining the established disease.

In this study, we elaborated on the contribution of CK2 to different stages of HR-ALL development using the tamoxifen-regulated zebrafish model of MYC-induced ALL. Our data show that the kinase activity of CK2 promotes both the onset and progression of T- and B-ALL in the presence of aberrant MYC activation, but cannot maintain the disease upon MYC inactivation through the removal of 4HT. When *MYC-ER* fish are treated with 4HT to activate MYC at a later stage of development, these fish can no longer develop leukemia, indicating a temporal restriction of MYC-induced lymphocyte trans-

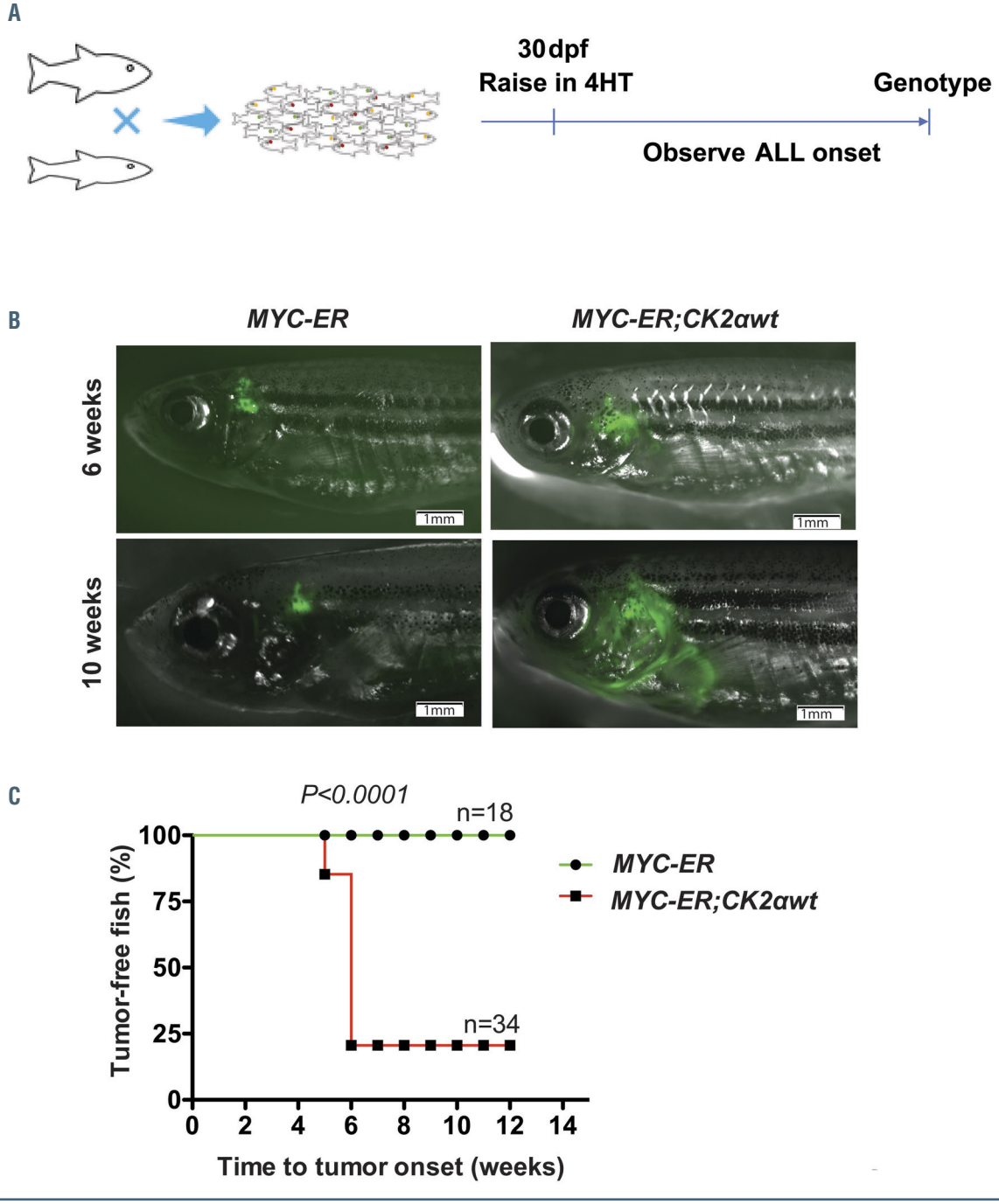

Figure 2. *CK2α* overexpression overcomes temporal restriction of MYC-induced lymphocyte transformation and induces leukemia at the later stage of development. (A) Diagram of the experimental design. (B) Thymic fluorescence in *MYC-ER* and *MYC-ER;CK2αwt* zebrafish that were raised in 129 nM 4-hydroxytamoxifen (4HT) beginning at 30 dpf. (C) Kaplan-Meier analysis of tumor-free fish based on genotype ($P<0.0001$; n=18 for *MYC-ER* and n=34 for *MYC-ER;CK2αwt* fish). Statistical analysis was performed using the log-rank test and scale bars =1 mm.

formation. Strikingly, however, this temporal restriction can be overcome by enforced *CK2* expression, leading to high penetrance of leukemia development. Although *CK2α* overexpression alone cannot induce leukemia, it promotes the survival of lymphocytes. Hence, it is likely that MYC activation at a later stage of development induces apoptosis in lymphocytes that is overcome by *CK2* overexpression, enabling the rapid induction of leukemia in these fish.

As CK2 inhibition with the selective and potent inhibitor, CX-4945, exhibits anti-tumor activities,[7] CX-4945 has been included in clinical testing to treat hematological malignancies (clinicaltrials.gov. Identifier: NCT01199718) and solid cancers (clinicaltrials.gov

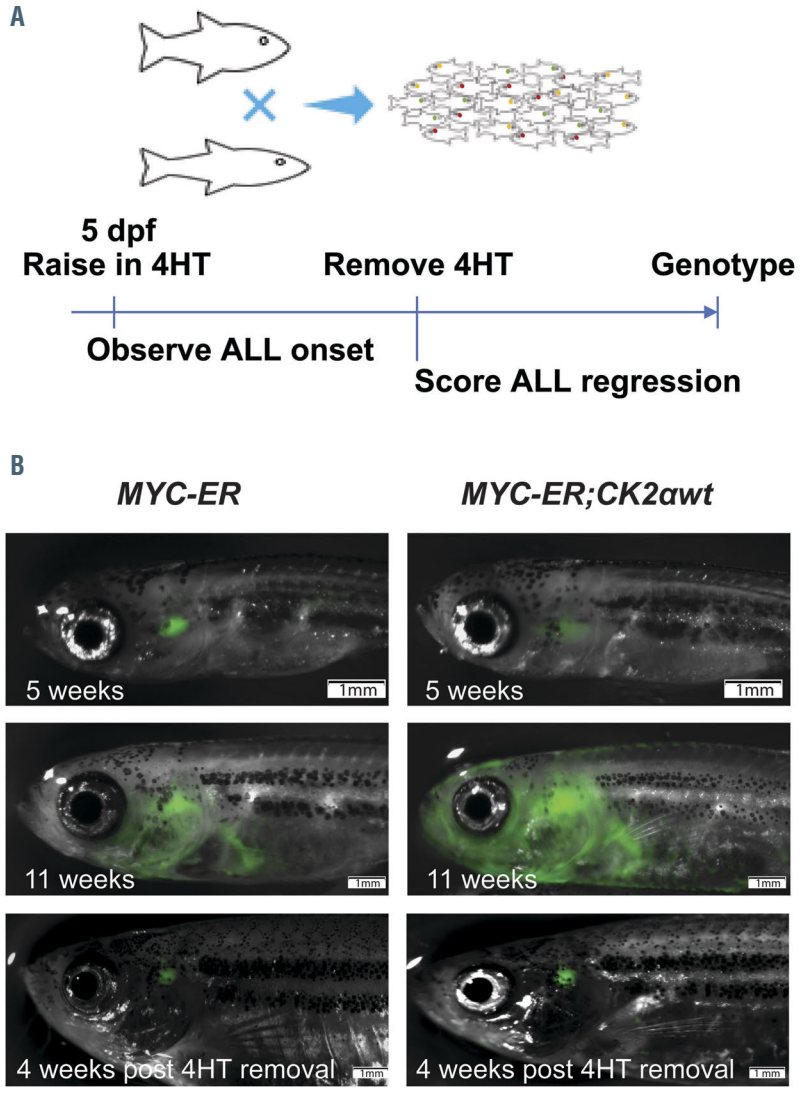

Figure 3. *CK2α* overexpression alone cannot maintain acute lymphoblastic leukemia in the absence of aberrant MYC activation. (A) Diagram of the experimental design. (B, top panel) Thymic fluorescence in *MYC-ER* (left) and *MYC-ER;CK2αwt* (right) zebrafish raised in 129 nM 4-hydroxytamoxifen (4HT) for 5 weeks showing tumor initiation in *MYC-ER;CK2αwt* fish. (B, middle panel) shows both *MYC-ER* (left) and *MYC-ER;CK2αwt* (right) with aggressive disease at 11 weeks although *MYC-ER;CK2αwt* fish developed more aggressive ALL than *MYC-ER* fish. (B, bottom panel) shows thymic fluorescence 4 weeks after 4HT withdrawal. One representative fish is shown for each group. (C) Zebrafish were classified by the indicated tumor phenotype at 8 weeks post 4HT removal (P=0.13; *MYC-ER vs. MYC-ER;CK2αwt*; n=11 per group). The difference in observed tumor phenotypes between each group as a whole was statistically insignificant, as calculated by a two-way ANOVA test. Scale bars =1 mm. ALL: acute lymphoblastic leukemia.

Identifier: NCT03897036, NCT03904862, NCT00891280, and NCT0357143). Based on our findings that MYC, but not CK2, is the key factor for HR-ALL maintenance, it is important to simultaneously target MYC and CK2. Although directly targeting MYC remains challenging, combination treatment of CX4945 with inhibitors targeting MYC-regulated oncogenic pathways, such as metabolism and stress response pathways, may be highly effective and beneficial to patients with HR-ALL, and possibly other cancers with high expression of MYC and CK2.

Yun Zhou,[1,2] Haiwei Lian,[2,3*] Ning Shen,[2,4] Sovannarith Korm,[2] Andrew Kwok Ping Lam,[2] Olivia Layton,[2] Leah N. Huiting,[2] Dun Li,[2] Kelly Miao,[2] Aozhuo Zeng,[2] Esther Landesman-Bollag,[4] David C. Seldin,[4] Hui Fu,[3] Li Hong[1] and Hui Feng[2,4]*

[1]*Department of Gynecology, Wuhan University Renmin Hospital, Wuhan, Hubei, P. R. China;* [2]*Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA, USA;* [3]*Department of Anatomy and Embryology, Wuhan University School of Basic Medical Sciences, Wuhan, Hubei, P. R. China and* [4]*Department of Medicine, Section of Hematology and Medical Oncology, Boston University School of Medicine, Boston, MA, USA*

**YZ and HL contributed equally as co-first authors*

Correspondence: HUI FENG - huifeng@bu.edu

HONG LI - drhongli1011@yeah.net

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References

1. Ko RH, Ji L, Barnette P, et al. Outcome of patients treated for relapsed or refractory acute lymphoblastic leukemia: a Therapeutic Advances in Childhood Leukemia Consortium study. J Clin Oncol. 2010;28(4):648-654.
2. Borga C, Foster CA, Iyer S, et al. Molecularly distinct models of zebrafish Myc-induced B cell leukemia. Leukemia. 2019;33(2):559-562.
3. Bonaccorso P, La Rosa M, Andriano N, et al. Clinical significance of Ck2 (CSNK2) and C-Myc expression in childhood acute lymphoblastic leukemia. Blood. 2016;128(22):5269.
4. Borgo C, Ruzzene M. Role of protein kinase CK2 in antitumor drug resistance. J Exp Clin Cancer Res. 2019;38(1):287.
5. Piazza F, Manni S, Ruzzene M, et al. Protein kinase CK2 in hematologic malignancies: reliance on a pivotal cell survival regulator by oncogenic signaling pathways. Leukemia. 2012;26(6):1174-1179.
6. Piazza F. Protein kinase CK2 in normal and malignant hematopoiesis. In: Pinna LA, editor. Protein Kinase CK2. 2013. Chapter 13:344-362.
7. Chua MM, Ortega CE, Sheikh A, et al. CK2 in cancer: cellular and biochemical mechanisms and potential therapeutic target. Pharmaceuticals (Basel). 2017;10(1):18.
8. Gowda C, Sachdev M, Muthusami S, et al. Casein kinase II (CK2) as a therapeutic target for hematological malignancies. Curr Pharm Des. 2017;23(1):95-107.
9. Seldin DC, Leder P. Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle. Science. 1995;267(5199):894-897.
10. Penner CG, Wang Z, Litchfield DW. Expression and localization of epitope-tagged protein kinase CK2. J Cell Biochem. 1997;64(4):525-537.
11. Gutierrez A, Grebliunaite R, Feng H, et al. Pten mediates Myc oncogene dependence in a conditional zebrafish model of T cell acute lymphoblastic leukemia. J Exp Med. 2011;208(8):1595-1603.
12. Feng H, Langenau DM, Madge JA, et al. Heat-shock induction of T-cell lymphoma/leukaemia in conditional Cre/lox-regulated transgenic zebrafish. Br J Haematol. 2007;138(2):169-175.
13. Borga C, Park G, Foster C, et al. Simultaneous B and T cell acute lymphoblastic leukemias in zebrafish driven by transgenic MYC: implications for oncogenesis and lymphopoiesis. Leukemia. 2019;33(2):333-347.
14. Garcia EG, Iyer S, Garcia SP, et al. Cell of origin dictates aggression and stem cell number in acute lymphoblastic leukemia. Leukemia. 2018;32(8):1860-1865.