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

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doi:10.3324/haematol.2020.246918

Supplementary Information

Materials and Methods

Fish husbandry

Zebrafish (*Danio rerio*) husbandry was performed as described (1), in zebrafish facilities at the Boston University (BU) Medical Campus following protocols approved by the Institutional Animal Care and Use Committee at BU.

Subcloning

The human cDNA of wild-type *CK2α* (*CK2αwt*) and mutant *CK2α* (*CK2αK68M*) was amplified by PCR from pZW6 and pGV15 vector, respectively (2). Mutant *CK2αK68M* was previously generated by mutating the lysine (K) codon at amino acid position 68 to methionine (M), resulting in the loss of kinase activity (3). PCR products were subsequently cloned into the *I-SceI-lck/pKS* or *I-SceI-rag2/pKS* vector, containing a zebrafish *lck* or *rag2* promoter sequence and flanked by the I-SceI endonuclease recognition sites. The forward primer contained an Age-I enzyme site and *CK2α* translation start sequence. The reverse primer contained the *CK2α* translation termination codon and a ClaI enzyme site. PCR-purified fragments were digested with AgeI-HF and ClaI restriction enzymes and then cloned into the respective *I-SceI/pKS* vector through AgeI-HF and ClaI sites. Clones containing inserts were fully sequenced. PCR primer pairs used for amplification of *CK2α* cDNA are shown in Supplementary Table 1.

Generation of stable *CK2αwt* and *CK2αK68M* transgenic fish

The *I-SceI-lck:CK2αwt-I-SceI*, *I-SceI-rag2:CK2αwt-I-SceI*, and *I-SceI-lck:CK2αK68M-I-SceI* constructs were microinjected with meganuclease (New England Biolabs, Ipswich, MA, USA)

into *nacre* (*mitfa*$^{-/-}$) zebrafish embryos at the one-cell stage of development, thereby generating mosaic F0 founder fish. The *I-SceI-lck:CK2αwt-I-SceI* construct was co-injected with the *I-SceI-rag2:mCherry-I-SceI* construct as previously described (4). The founder fish were outcrossed and their progeny were screened for the presence of human *CK2α* gene by gene-specific PCR.

4-hydroxytamoxifen treatment and ALL monitoring

The conditional zebrafish ALL model, *Tg(rag2:MYC-ER)*, was previously generated (5). In this transgenic fish line, the zebrafish *rag2* promoter drives the expression of human *MYC* fused to a modified estrogen receptor that binds to 4-hydroxytamoxifen (4HT, Sigma-Aldrich, St. Louis, MO, USA) but not endogenous estrogens (6). For all experiments described in this study, treatment with 4HT began at 5 dpf, and each clutch of larvae was placed in 1 L of water containing 50 μg/L 4HT with a weekly water change. Zebrafish raised in 4HT were imaged weekly by fluorescent microscopy beginning at 5 weeks of age to monitor the onset of ALL as previously described (5). Fish were imaged by brightfield and GFP/mCherry fluorescent microscopy using an Olympus fluorescent dissecting microscope (MVX10; Olympus, Center Valley, PA, USA). Fluorescent and brightfield images were merged using Adobe Photoshop (version 7.0). The onset of ALL was defined as the development of a fluorescent mass that was more than twice the size of a normal thymus or kidney together with fluorescent-labeled cell infiltration into local tissues. All fish were genotyped for *MYC-ER*, *CK2αwt,* or *CK2αK68M* transgenes using genomic DNA extracted from the individual fish fin upon the completion of tumor surveillance. Genotyping primers are listed in Supplementary Table 2.

Tumor regression was monitored after the removal of 4HT from the fish water. Tumor fish were placed in individual tanks and imaged weekly for a total of 8 weeks after the removal of 4HT.

Based on the diameter of the largest contiguous tumor mass at the end of the 8-week monitoring period, tumor phenotypes were classified into 4 groups as described in the results section. Fish that became moribund with leukemia <8 weeks after 4HT removal were placed in the progression category. Genotyping for fish was performed as described above.

Protein extraction and Western blotting

Whole-cell lysates were prepared from tumors in RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, and 1 mM EDTA) supplemented with 1x or 2x Halt proteinase and phosphatase inhibitor cocktail (Thermo Scientific, Cambridge, MA, USA). To visualize the levels of phosphorylated MYC, a phosphate-binding molecule (Phos-Tag, AAL-107; FUJIFILM Wako Pure Chemical Co., Richmond, VA, USA) was used. Specifically, the 8% resolving gel for the SDS-PAGE was freshly prepared according to the manufacture's instruction with 0.04 mM $MnCl_2$ (Sigma, St. Louis, MO, USA) and 0.02 mM Phos-tag (TM) Acrylamide AAL-107 (FUJIFILM Wako Pure Chemical Co., Richmond, VA, USA). Primary antibodies included anti-CK2 (SC-9030, Santa Cruz Biotechnology, Dallas, TX, USA), anti-MYC (5605S, Cell Signaling, Danvers, MA, USA), and anti-ACTIN (SC-47778, Santa Cruz Biotechnology, Dallas, TX, USA). Secondary antibodies included horseradish-peroxidase-conjugated anti-mouse (31430, Thermo Scientific, Waltham, MA, USA) or anti-rabbit (65-6120, Thermo Scientific, Waltham, MA, USA) antibody. Autoradiographs were obtained with a G:BOX Chemi XT4 (Syngene, Frederick, MD, USA) and a CCD camera, and quantification analysis were performed using Syngene GeneTools software (Syngene, Frederick, MD, USA).

Pulse-chase analysis

Premalignant thymi were isolated from *MYC-ER* and *MYC-ER;CK2αwt* zebrafish. Specifically, adult zebrafish were euthanized in ice-cold fish water for approximately 10 minutes. The thymus of each fish was immediately dissected with sterilized forceps and kept in ice-cold 1x RPMI-1640 medium (Corning, NY, USA) supplemented with 10% FBS (Life Technology, Carlsbad, CA, USA). Cell suspensions were made by pipetting several times in medium, then aliquoted into 12-well plates (Thermo Scientific, Waltham, MA, USA), treated with 50 μg/mL cycloheximide (Sigma, St. Louis, MO, USA), and collected at 0, 15, 30, 60, and 180 minutes post-treatment. Protein was then extracted for Western blotting analysis to detect MYC and Actin levels.

RT-PCR

The tumor was dissected from each fish under a fluorescent microscope and subjected to RNA extraction using Trizol reagent (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instruction. cDNA was synthesized with QuantiTect Reverse Transcription Kit (QIAGEN, Germantown, MA, USA). 100 ng of cDNA was used for semi-quantitative RT-PCR with gene-specific RT primers as previously described (7). Alternatively, 200 ng of cDNA was applied for qRT-PCR for anti-apoptotic genes with the SYBR green PCR master mix (Genecopoeia, Rockville, MD, USA) and a Step-One PCR instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. The primer sequences for qRT-PCR are included in Supplementary Table 2.

Statistical analyses

GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used to calculate *p*-values using a log-rank test for Kaplan-Meier curves and two-tailed t-test for tumor burden and

anti-apoptotic gene expression. A two-way ANOVA test was used to calculate significance for tumor changes among fish groups after 4HT removal. *P*-values less than or equal to 0.05 were considered statistically significant.

Supplementary Figure Legends

Supplementary Figure 1. Overexpression of *CK2α* promotes the survival of lymphocytes but does not induce leukemia in zebrafish. (A) CK2α protein expression in lymphocytes of wild-type (wt) and *Tg(lck:CK2αwt;rag2:mCherry)* zebrafish. (B-C) GFP and/or mCherry fluorescence in the transgenic *Tg(lck:EGFP)* (left panels), *Tg(rag2:mCherry)* (middle panels), and *Tg(lck:CK2αwt;rag2:mCherry)* (right panels) fish at 6 weeks (B) or 8 months (C) old. Scale bars in (B) = 1 mm and in (C) = 2 mm.

Supplementary Figure 2. Overexpression of human *CK2αwt* promotes leukemia aggressiveness in zebrafish. (A) Representative images of *MYC-ER* (left, overlay of brightfield and GFP channel) and *MYC-ER;CK2αwt* (middle and right panel, overlay of brightfield and RFP or GFP channel) fish at 8-month old. (B) Fluorescent intensity quantification of GFP channel of tumor from *MYC-ER* and *MYC-ER;CK2αwt* fish (*P=0.02*; n=6 and 8, respectively). Scale bars = 2 mm.

Supplementary Figure 3. Similar to *MYC-ER* fish, *MYC-ER;CK2αwt* fish develop both T and B-ALL. Semi-quantitative RT-PCR analysis show that tumors from both *MYC-ER* and *MYC-ER;CK2αwt* fish express T and B cell markers.

Supplementary Figure 4. CK2 phosphorylates and stabilizes MYC in leukemic cells from *MYC-ER;CK2αwt* zebrafish. (A) Western blotting analysis of CK2 and MYC (phosphorylated [upper bands] and unphosphorylated [the lowest band]) in tumors dissected from individual *MYC-ER* and *MYC-ER;CK2αwt* fish. The number above the upper bands of MYC are relative values of

the phosphorylated MYC versus the total MYC. (B) Cycloheximide (CHX) chase analysis of MYC stability in the premalignant thymus of *MYC-ER* and *MYC-ER;CK2αwt* fish. Levels of Actin were analyzed as a loading control. MYC band intensities were normalized to the corresponding Actin levels, and the value for cells isolated immediately before CHX treatment was set to 100 for ease of comparison.

Supplementary Figure 5. *CK2* overexpression does not increase the expression of anti-apoptotic genes. qRT-PCR analysis of the transcript levels of zebrafish anti-apoptotic genes in leukemic cells from *MYC-ER* or *MYC-ER;CK2αwt* fish (n=15 per group). Statistical analysis was performed using the two-tailed t-test.

Supplementary Tables

Supplementary Table 1. **PCR primer pairs used for amplification of *CK2α* cDNA for subcloning**

	Primer sequences (5′-3′)
Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCACCGGTATGTCGGGAC CCGTGCCAAGCAG
Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTATCGATTTACTGCTGAGCGCC AGCGGCAG

Supplementary Table 2. **Genotyping or qRT-PCR primers**

Target gene	Primer sequences (5'-3')
MYC-ER	Forward: AGTCCTGAGACAGATCAGCA
	Reverse: TCATCATGCGGAACCGACTT
CK2αwt	Forward: ATGTCGGGACCCGTGCCAAGCAG
	Reverse: TTACTGGCTTGAGAATTTTAAC
CK2αK68M	Forward: ATGTCGGGACCCGTGCCAAGCAG
	Reverse: TTACTGGCTTGAGAATCATAAC
bcl2a	Forward: AGATGGCGTCCCAGGTAGAT
	Reverse: GAAGGCATCCCAACCTCCAT
bcl2l1	Forward: AGGGCTTGTTTGCTTGGTTG
	Reverse: GCAATGGCTCATACCCATAACAC
mcl1a	Forward: GTCACTAACTGGGGCCGAAT
	Reverse: AACCCATGCCAGCTCTTGTT

Supplementary References

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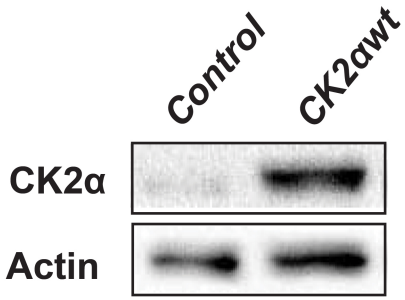
A



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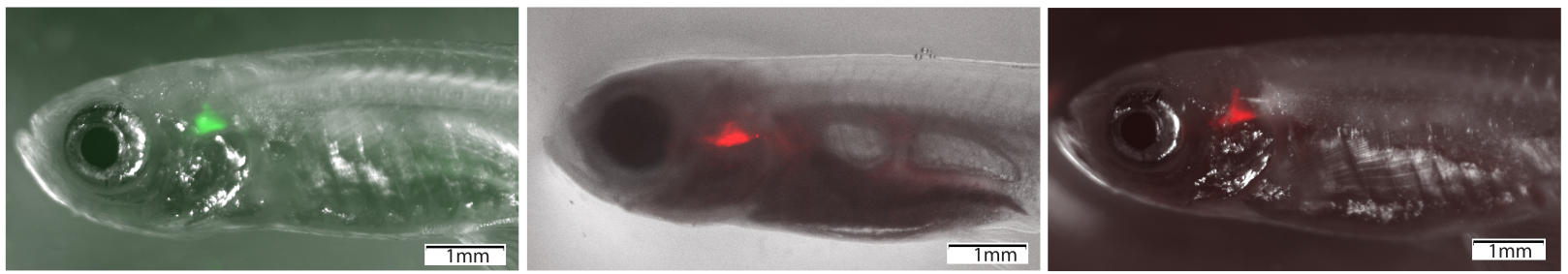

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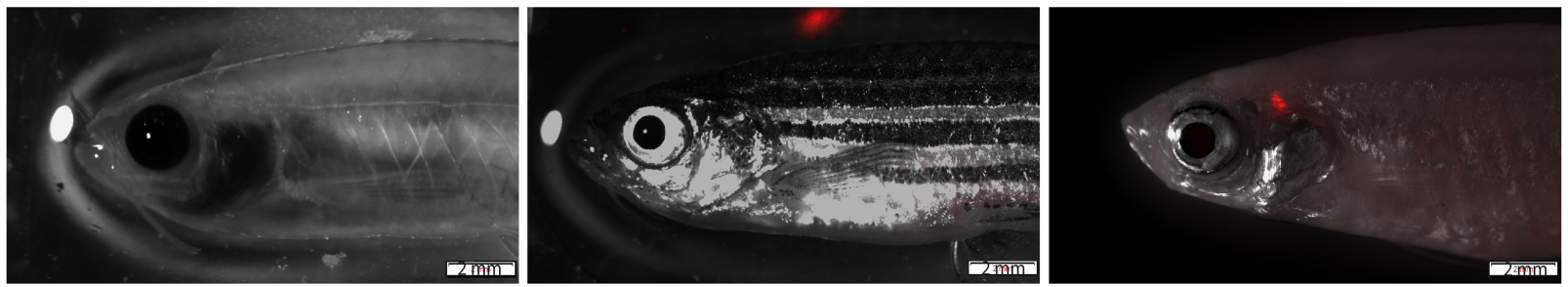
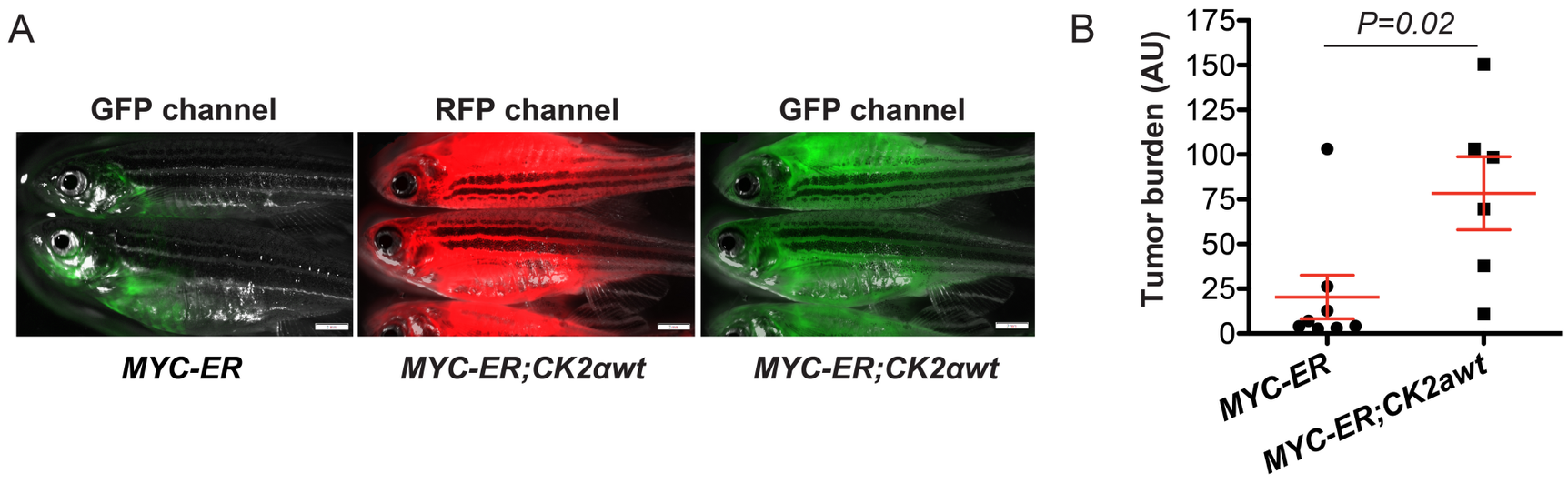


Supplementary Figure 1



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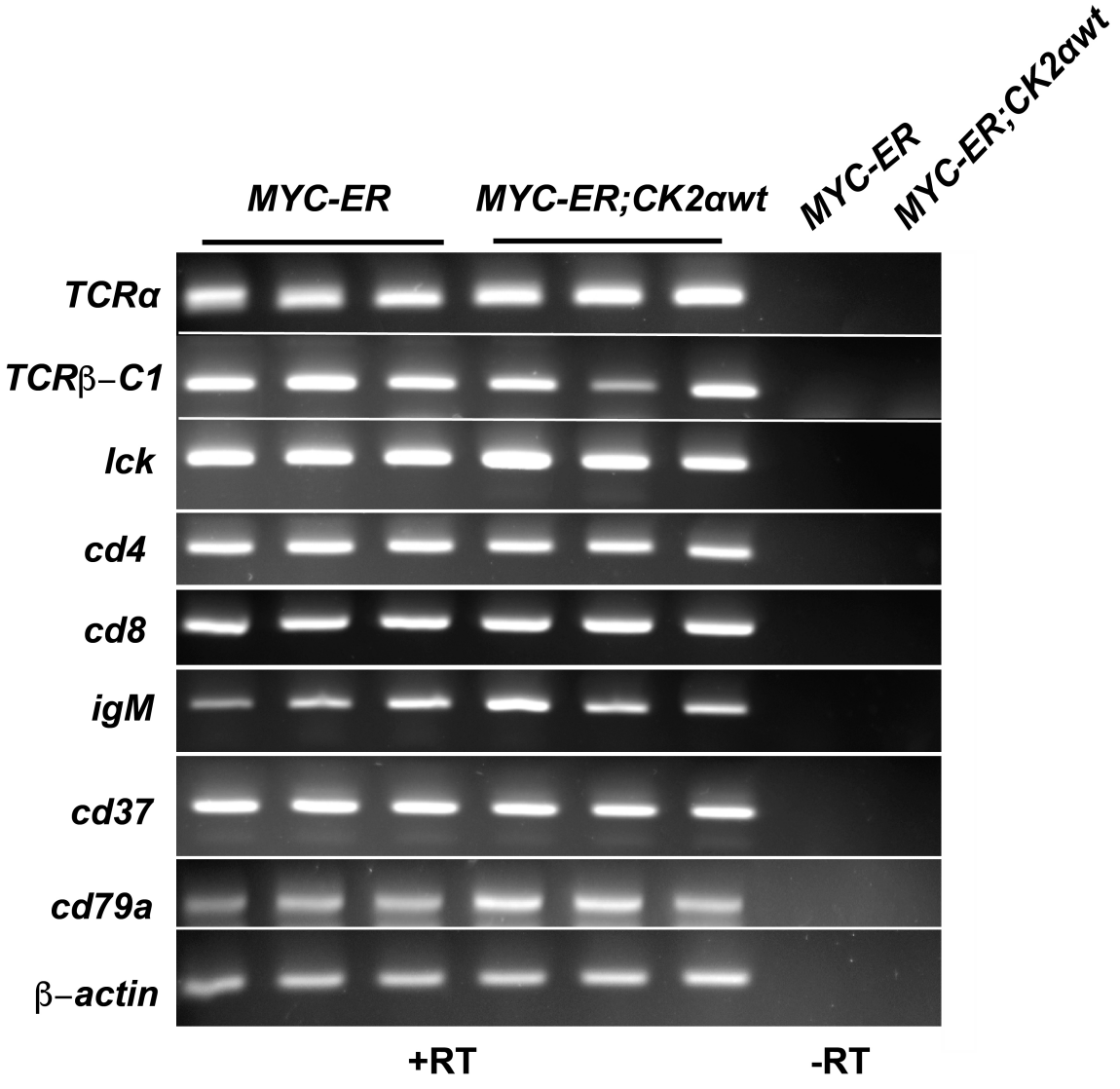
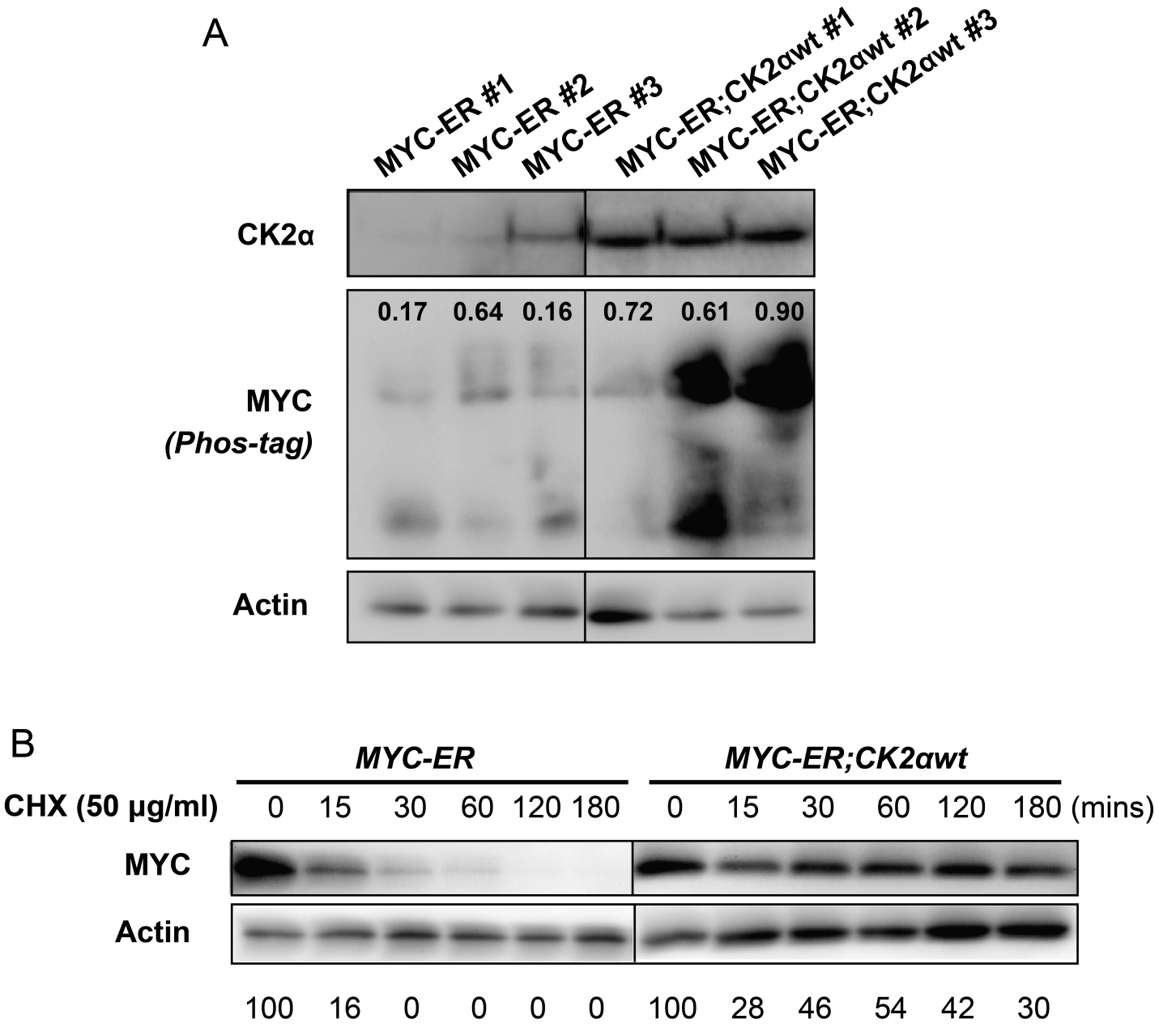


Supplementary Figure 3



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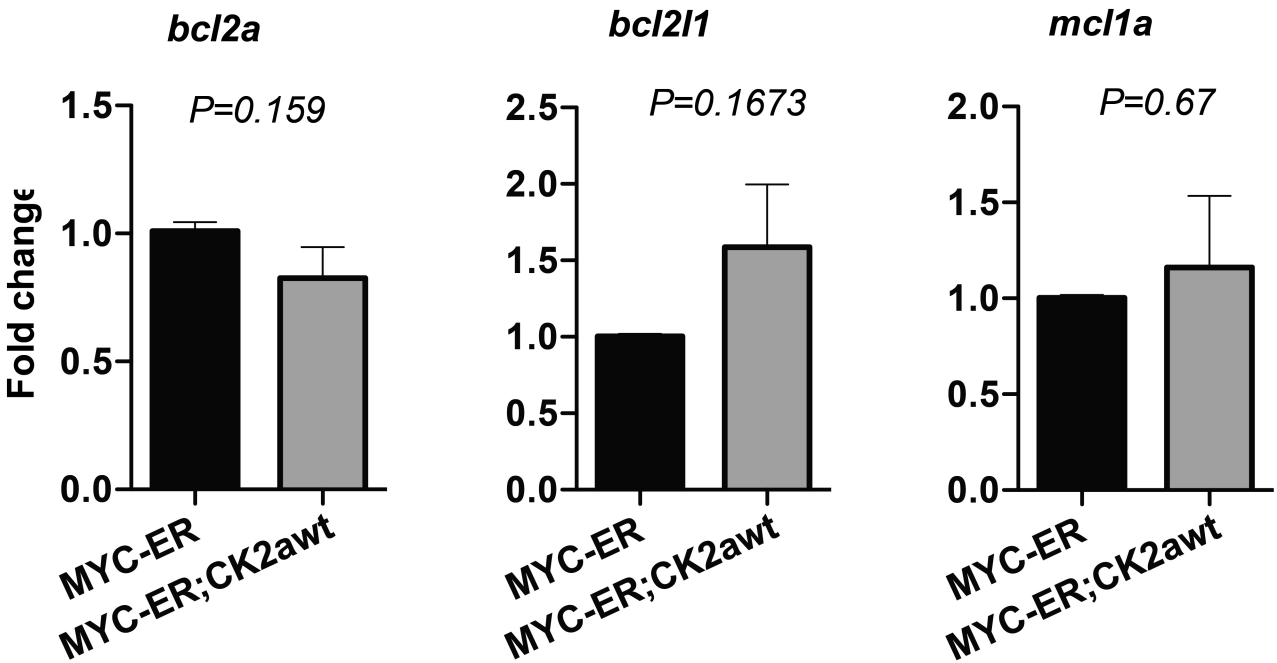

Supplementary Figure 5