The Light-Inducible Genes \textit{Per2}, \textit{Cry1a}, and \textit{Cry2a} Regulate Oxidative Status in Zebrafish

Yikelamu Alifu, Satoshi Kofuji, Sachi Sunaga, Mizuki Kusaba, Jun Hirayama, and Hiroshi Nishina

\footnote{These authors contributed equally to this work.}

\textsuperscript{a}Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University (TMDU); Tokyo 113–8510, Japan; and \textsuperscript{b}Department of Clinical Engineering, Faculty of Health Sciences, Komatsu University; Komatsu, Ishikawa 923–0961, Japan.

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The circadian clock is a highly conserved 24 h biological oscillation mechanism and is affected by environmental stimuli such as light, food and temperature. Disruption of the circadian clock results in disorders of diverse biological processes, including the sleep-wake cycle and metabolism. Although we previously identified several components of the circadian clock in zebrafish, our understanding of the relationship between light-inducible clock genes and metabolism remains incomplete. To investigate how light-inducible clock genes regulate metabolism, we performed transcriptomic and metabolomic analyses of the light-inducible clock genes \textit{zPer2}, \textit{zCry1a}, and \textit{zCry2a} in zebrafish. Transcriptomic analysis of \textit{zPer2} and \textit{zCry2a} in zebrafish showed that their gene expression profiles differed from that of wild type (WT) zebrafish. In particular, mRNA levels of \textit{zKeap1}, which encodes an oxidative stress sensor, were increased in DKO and TKO mutants. Metabolomic analysis showed genotype-dependent alteration of metabolomic profiles. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) showed the alteration of cysteine/methionine metabolism and glutathione metabolism. Specifically, cysteine and glutathione were decreased but methionine sulfoxide was increased in TKO zebrafish. These results indicate that the light-inducible genes \textit{zPer2}, \textit{zCry1a}, and \textit{zCry2a} are involved in regulating the oxidative status of zebrafish.

Key words \textit{Period2}; \textit{Cryptochromela}; \textit{Cryptochrome2a}; \textit{Keap1}; metabolism; oxidative status

INTRODUCTION

The circadian clock directs the homeostasis of metabolic and physiological functions and is composed of an endogenous time maintenance system called the cellular clock.\textsuperscript{1)} The cellular clock consists of clock genes encoding positive regulators (BMAL and CLOCK) and negative regulators (PER and CRY), which form a transcription-translation loop.

The zebrafish is a useful animal model for circadian clock research. Exposure to light synchronizes cellular clocks to form the rhythm of zebrafish behavior by inducing circadian clock genes such as \textit{zPer2}, \textit{zCry1a}, and \textit{zCry2a}.\textsuperscript{2)} Especially, \textit{zCry2a} is induced after 12 h light exposure and can restore normal circadian rhythms even in the absence of \textit{zPer2} and \textit{zCry1a}, indicating the coordinated regulation of behavioral rhythms by \textit{zPer2}, \textit{zCry1a}, and \textit{zCry2a}.\textsuperscript{2)} Importantly, both double knockout (DKO) and triple knockout (TKO) zebrafish showed decreased total locomotor activity and reduced ATP levels compared to wild type (WT), suggesting the disruption of cellular metabolism. We therefore undertook the present study to investigate differences in metabolism among WT, DKO, and TKO zebrafish. To this end, we first performed a transcriptomic analysis to identify genes whose mRNA levels were altered in DKO and/or TKO zebrafish compared to the WT. We then conducted a metabolomic analysis to pinpoint alterations to metabolites that were specific to the DKO and/or TKO mutants. Our results implicate \textit{zPer2}, \textit{zCry1a}, and \textit{zCry2a} in the light-induced regulation of the oxidative status in zebrafish.

MATERIALS AND METHODS

\textbf{Fish} Adult TL strain was used in this study. DKO and TKO zebrafish were generated previously.\textsuperscript{2)} All zebrafish were maintained as described previously.\textsuperscript{2)} For transcriptomic and metabolomic analyses, approx. 60 larvae/sample (about 20 mg of total weight) were raised under constant dark conditions after hatching and were exposed to light for 3 or 12 h at the beginning of 6 days post-fertilization (dpf). All experimental protocols were approved by the Animal Welfare Committee of Tokyo Medical and Dental University.

\textbf{RNA-Sequencing Analysis} Total RNA was extracted from zebrafish larvae as described previously.\textsuperscript{2)} Strand-specific RNA-sequencing analysis was performed at GENEWIZ, Inc. (Tokyo, Japan) following their standard protocol. RNA-sequencing was performed at the Illumina NovaSeq platform. Normalized estimations of gene expression were calculated as fragments per kilobase of exon model per million reads mapped (FPKM) and are available upon request.

\textbf{Metabolomic Data Acquisition and Statistical Analysis} Metabolomic analysis was performed by Human Metabolome Technologies (Tsuruoka, Yamagata, Japan). Metabolites were detected using a capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) system (Agilent Technologies, Santa Clara, CA, U.S.A.). Identified metabolites and their relative areas were provided in Supplementary Table S1.
MetaboAnalyst 5.0 was used for metabolite analyses including principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), enrichment analysis, and pathway analysis. The contributions of each metabolite in PCA and variable importance in projection (VIP) scores in PLS-DA were provided in Supplementary Tables S2 and S3, respectively.
RESULTS

Loss of zPer2, zCry1a, and/or zCry2a Induces the Expression of zKeap1a  To investigate gene expression differences among WT, DKO, and TKO zebrafish, we performed RNA-sequencing analysis of whole larval bodies collected after 12 h light exposure starting at 6 dpf. PCA with whole data sets and clustering analysis revealed a clear separation of DKO and TKO gene expression patterns from those of the WT (Figs. 1A, B), suggesting a definite influence of the deletion of zPer2 and zCry1a on gene expression.

To investigate differences between the DKO and TKO mutants in more detail, we analyzed our RNA-sequencing data with a fold-change (FC) >1.5 and q value <0.05. By comparing WT vs. DKO, and WT vs. TKO, we identified zKeap1a, but not zKeap1b, both of which encodes oxidative stress sensors, as being significantly upregulated in DKO and TKO zebrafish (Fig. 1C). Keap1 downmodulates protein levels of nuclear factor-E2-related factor 2 (Nrf2), which drives gene expression in response to oxidative stress. Of note, mRNA levels of zNrf2a and zNrf2b were not changed in our mutants.

Taken together, these data suggest that loss of zPer2, zCry1a,
Metabolomic Analysis Reveals Altered Cysteine and Glutathione Metabolism in the Absence of zPer2, zCry1a, and/or zCry2a

To examine metabolic differences in WT, DKO, and TKO zebrafish, we performed metabolomic analyses of larvae exposed to light (Supplementary Fig. 1A). For this purpose, extracts of larvae were subjected to CE-TOFMS to identify total metabolites (Supplementary Fig. 1B). The data obtained were analyzed by PCA, PLS-DA, enrichment analysis, and pathway analysis using MetaboAnalyst 5.0 (Supplementary Fig. 1C). We found that PCA highlighted genotype-specific changes to metabolism in our mutants (Fig. 2A). Enrichment analysis of the top 30 metabolites contributing to the PC1 axis revealed altered amino acid metabolism, particularly that of cysteine/methionine and glutathione (Fig. 2B). Glutathione is a redox-related metabolite synthesized from cysteine. These results were consistent with the result of Fig. 1C.

The PLS-DA method clarified the above differences among WT, DKO, and TKO zebrafish by showing that these three genotypes were clearly separated along the Component 1 axis (Fig. 3A). Among the top 20 VIP scores from our PLS-DA, we found that methionine sulfoxide, glutathione (GSH), and cysteine were among the most highly ranked (Fig. 3B). In both enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG)-based pathway analysis of the top 20 VIP score metabolites, those related to metabolism of cysteine/methionine and glutathione were prominent (Figs. 3C, D). These results indicate that a key feature affected by loss of zPer2,
Oxidative Status Is Increased in TKO Zebrafish

To further investigate effects of loss of zPer2, zCry1a, and/or zCry2a on oxidative status, we compared levels of cysteine, GSH, and methionine sulfoxide among WT, DKO, and TKO zebrafish using the metabolomic analysis. In DKO zebrafish, both cysteine and GSH levels fell below WT levels by 3 h light exposure, but fully recovered to normal levels by 12 h light exposure (Fig. 4A). In contrast, in TKO zebrafish, both cysteine and GSH levels were low at all time points (Fig. 4A). Conversely, levels of methionine sulfoxide, an oxidized form of methionine, were increased in TKO mutants independently of light exposure, suggesting that TKO zebrafish are generally stuck in a state of enhanced oxidation.

Taken together, our data suggest that the circadian clock components zPer2, zCry1a, and zCry2a cooperate to regulate the intracellular oxidative status via effects on the Keap1–Nrf2 pathway. A model illustrating this concept appears in Fig. 4B.
DISCUSSION

In this study, we performed transcriptomic analysis and metabolomic analysis to evaluate the consequences of cellular clock gene deletion in zebrafish. Transcriptomic analysis showed that *zKeap1a* was upregulated in both DKO and TKO zebrafish in a manner showing linearity with the number of clock genes deleted (Fig. 1C). In contrast, our metabolomic analysis indicated that methionine sulfoxide levels were increased only in TKO zebrafish (Fig. 4A). These two independent results suggest that, in the case of *zKeap1a* and its downstream oxidative metabolites, there seems to be a threshold of gene expression required to produce an effect on oxidative status. Thus, both transcriptomic and metabolomic analyses are useful and necessary to gain a true picture of the effects of genetic modifications.

Previous studies have suggested a regulation of NRF2 expression by positive regulators, BMAL1/CLOCK. However, the contribution of negative regulators, PER/CRY, to antioxidant activity was less understood. Our finding that light-inducible Per and Cry control Keap1 expression in zebrafish reveals a previously unknown mode of regulation of the Keap1–Nrf2 pathway.

The model in Fig. 4B brings together our prior and present results. We previously showed that light-induced hydrogen peroxide (H$_2$O$_2$) enhances the expression of *zPer2* and *zCryla* in zebrafish, establishing a relationship between circadian clock and oxidative stress. Our present results indicate that *zPer2*, *zCryla*, and *zCry2a*, which are upregulated by light-induced reactive oxygen species (ROS), increase antioxidant activity by downmodulating zKeap1 and thereby upregulating zNrf2. We therefore propose that the circadian clock is involved in negative feedback regulation of intracellular oxidative status. There is a difference in light-induced behavior rhythm between DKO and TKO zebrafish due to impaired cellular clock synchronization. Thus, oxidative status might be connected with behavioral rhythm. In conclusion, a finding may have profound implications for studies of circadian rhythm disorders in higher vertebrates such as humans.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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