Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Real-time *in vivo* monitoring of circadian E-box enhancer activity: A robust and sensitive zebrafish reporter line for developmental, chemical and neural biology of the circadian clock

Meltem Weger [a], Benjamin D. Weger [a], Nicolas Diotel [a], Sepand Rastegar [a], Tsuyoshi Hirota [b], Steve A. Kay [b,c], Uwe Strähle [a], Thomas Dickmeis [a,*]

[a] Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Hermann-von-Helmholtz-Platz 1, D-76344 Eggenstein-Leopoldshafen, Germany
[b] Division of Biological Sciences, University of California San Diego, La Jolla, CA, USA
[c] Dana and David Dornsife College of Letters, Arts and Sciences, University of Southern California, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 15 November 2012
Received in revised form
11 April 2013
Accepted 30 April 2013

Keywords:
Circadian clock
Zebrafish
E-box
Luciferase reporter
Adult brain
Neurogenesis

ABSTRACT

The circadian clock co-ordinates physiology and behavior with the day/night cycle. It consists of a transcriptional–translational feedback loop that generates self-sustained oscillations in transcriptional activity with a roughly 24 h period via E-box enhancer elements. Numerous *in vivo* aspects of core clock feedback loop function are still incompletely understood, including its maturation during development, tissue-specific activity and perturbation in disease states. Zebrafish are promising models for biomedical research due to their high regenerative capacity and suitability for *in vivo* drug screens, and transgenic zebrafish lines are valuable tools to study transcriptional activity *in vivo* during development.

To monitor the activity of the core clock feedback loop *in vivo*, we created a transgenic zebrafish line expressing a luciferase reporter gene under the regulation of a minimal promoter and four E-boxes. This *Tg*(*4xE-box:Luc*) line shows robust oscillating reporter gene expression both under light-dark cycles and upon release into constant darkness. Luciferase activity starts to oscillate during the first days of development, indicating that the core clock loop is already functional at an early stage. To test whether the *Tg*(*4xE-box:Luc*) line could be used in drug screens aimed at identifying compounds that target the circadian clock *in vivo*, we examined drug effects on circadian period. We were readily able to detect period changes as low as 0.7 h upon treatment with the period-lengthening drugs lithium chloride and longdaysin in an assay set-up suitable for large-scale screens. Reporter gene mRNA expression is also detected in the adult brain and reveals differential clock activity across the brain, overlapping with endogenous clock gene expression. Notably, core clock activity is strongly correlated with brain regions where neurogenesis takes place and can be detected in several types of neural progenitors.

Our results demonstrate that the *Tg*(*4xE-box:Luc*) line is an excellent tool for studying the regulation of the circadian clock and its maturation *in vivo* and in real-time. Furthermore, it is highly suitable for *in vivo* screens targeting the core clock mechanism that take into account the complexity of an intact organism. Finally, it allows mapping of clock activity in the brain of a vertebrate model organism with prominent adult neurogenesis and high regeneration capacity.

Background

The circadian clock

The rotation of the earth around its axis results in a day/night or light/dark (LD) cycle of 24 h. Organisms can anticipate these cycles and the corresponding changes in environmental conditions by means of a biological timer, the so-called circadian clock (Dunlap et al., 2004). This molecular oscillator enables them to prepare appropriate behavioral and physiological changes across the day, such as the sleep/wake cycle or daily rhythms of hormone secretion, metabolism and cell division (Bass and Takahashi, 2010; Feng and Lazar, 2012; Prasai et al., 2011; Sehgal and Mignot, 2011; Takahashi et al., 2008). The oscillator mechanism is self-sustained, as it continues to function also when no time information can be obtained from the environment, such as under constant darkness (DD). The period length of the clock under these "free-running" conditions is close to, but not exactly 24 h, which is why it is called "circadian" (from Latin "*circa*"="around" and "dies"="day"). The clock uses regular environmental time cues ("Zeitgebers"), mainly

* Corresponding author.
E-mail address: thomas.dickmeis@kit.edu (T. Dickmeis).

0012-1606/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ydbio.2013.04.035

light or temperature changes, to be reset ("entrained") to the ambient 24 h LD cycle.

The molecular clockwork

The molecular mechanism of the vertebrate circadian clock is based on transcriptional–translational feedback loops (Feng and Lazar, 2012; Zhang and Kay, 2010). In the so-called "core loop" of the clock mechanism (Fig. 1A), a heterodimer of Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle Arnt-like protein (BMAL1) binds to E-box (CACGTG) regulatory elements of target genes and activates their transcription. Among the CLOCK–BMAL1 targets are also the *Cryptochrome* (*Cry*) and *Period* (*Per*) genes. PER and CRY proteins undergo posttranslational modifications and accumulate in the cytosol, then translocate into the nucleus and inhibit transcriptional activation by the CLOCK–BMAL1 complex. As a consequence, *Per* and *Cry* transcript and subsequently protein levels fall. Together with the degradation of PER and CRY by the 26S proteasomal pathway, this releases the inhibition and starts a new cycle of transcription. The posttranslational modifications of the feedback loop components have been recognized in recent years as crucial players in the clock mechanism (Brown et al., 2012; Mehra et al., 2009). Phosphorylation by various kinases has been shown to influence period length of the clock mechanism by regulating stability or nuclear translocation of feedback loop components (Reischl and Kramer, 2011).

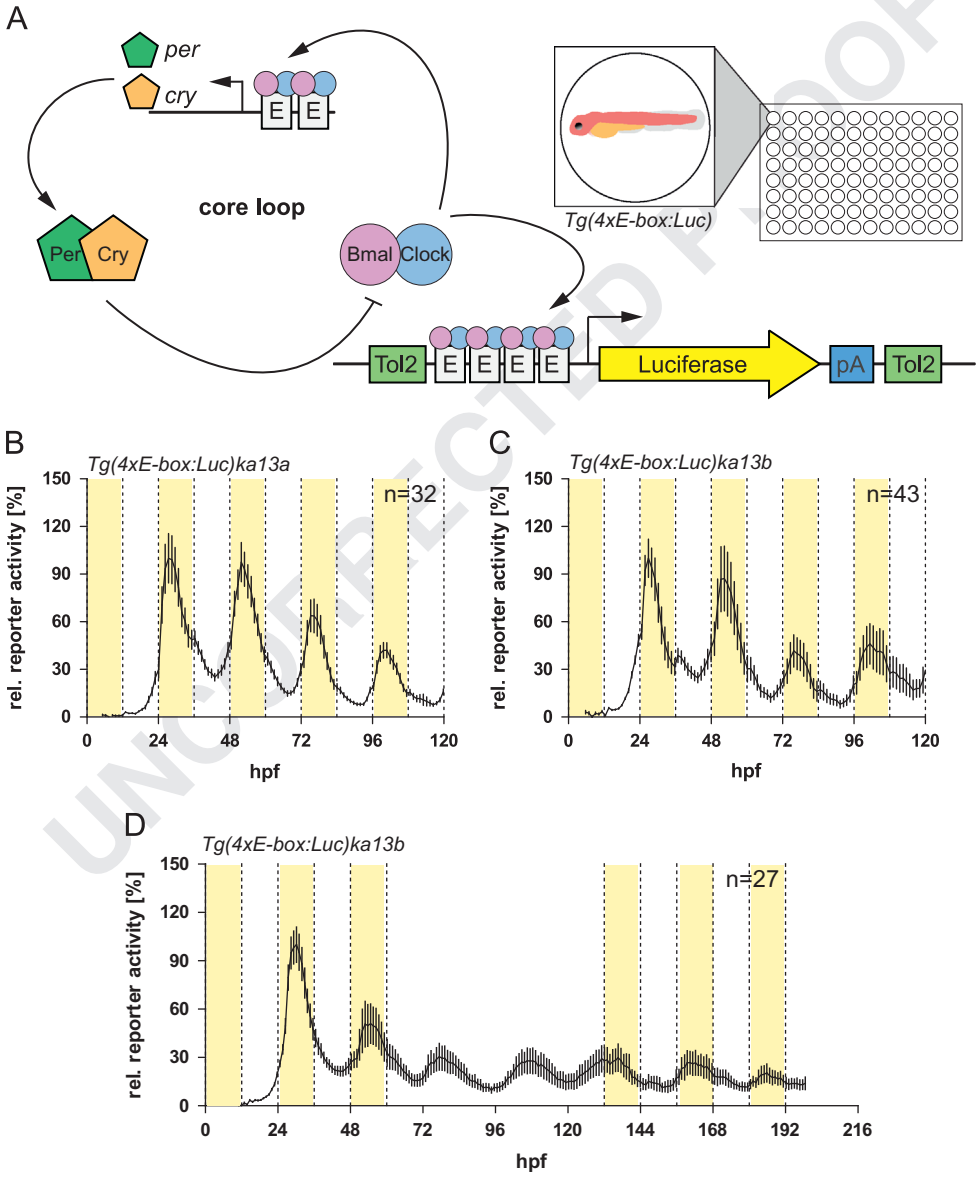

Fig. 1. The *Tg*(*4xE-box:Luc*) line monitors core clock activity. (A) Scheme of the functional principle of the 4xE-box:Luc assay. Clock (blue circle) and Bmal (purple circle) drive gene expression by binding to E-box (grey squares, E) enhancer elements. In the core feedback loop they bind to their own repressors *period* (*per*, green hexagon) and *cryptochrome* (*cry*, orange hexagon) that inhibit Clock–Bmal function later during the day. To monitor core clock activity *in vivo* and in real-time, four copies of E-box enhancer elements were cloned in front of a minimal promoter (TATA) of a luciferase reporter gene cassette (yellow), and injected into zebrafish to obtain a transgenic line via Tol2 mediated transgenesis (pA, polyA tag (blue); Tol2, Tol2 recognition site (green)). To measure reporter activity of the *Tg*(*4xE-box:Luc*) line, embryos/larvae were transferred into 96-well plates and bioluminescence was monitored for several days with a bioluminescence plate reader. ((B) and (C)) Two of a total of four *Tg*(*4xE-box:Luc*) lines, *ka*13*a* (B) and *ka*13*b* (C), showed robust rhythmic reporter activity upon light (L; yellow) dark (D; white) cycle exposure. Traces indicate the mean of relative reporter activity of n larvae as indicated in the scheme. Error bars represent mean values ± SEM. (D) Relative reporter activity of *Tg*(*4xE-box:Luc*)*ka*13*b* as detected over several days with different illumination conditions. Rhythmic reporter activity begins at 1 dpf and continues under constant darkness (white), demonstrating clock driven expression of the reporter. When illumination conditions are switched from LD to DL at 132 hpf, bioluminescence rhythms adapt to the new cycle, illustrating entrainment of the clock reporter.

Please cite this article as: Weger, M., et al., Real-time *in vivo* monitoring of circadian E-box enhancer activity: A robust and sensitive zebrafish reporter line for developmental, chemical and neural biology of the circadian clock. Dev. Biol. (2013), http://dx.doi.org/10.1016/j.ydbio.2013.04.035