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PAPER

Genes for iron metabolism influence circadian rhythms in *Drosophila melanogaster*†

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Haem has been previously implicated in the function of the circadian clock, but whether iron homeostasis is integrated with circadian rhythms is unknown. Here we describe an RNA interference (RNAi) screen using clock neurons of *Drosophila melanogaster*. RNAi is targeted to iron metabolism genes, including those involved in haem biosynthesis and degradation. The results indicate that *Ferritin 2 Light Chain Homologue* (*Fer2LCH*) is required for the circadian activity of flies kept in constant darkness. Oscillations of the core components in the molecular clock, PER and TIM, were also disrupted following *Fer2LCH* silencing. Other genes with a putative function in circadian biology include *Transferrin-3*, *CG1358* (which has homology to the FLVCR haem export protein) and five genes implicated in iron–sulfur cluster biosynthesis: the *Drosophila* homologues of IscS (*CG12264*), IscU (*CG9836*), IscA1 (*CG8198*), Iba57 (*CG8043*) and Nubp2 (*CG4858*). Therefore, *Drosophila* genes involved in iron metabolism are required for a functional biological clock.

Introduction

The surface of Earth is exposed periodically to fluctuations in solar radiation, the predominant source of energy for most living ecosystems. Circadian and metabolic rhythms characteristic of individual species can be viewed as adaptations to predictable environmental alterations of conditions such as temperature or light. The molecular connections between time-keeping mechanisms and key metabolic pathways ensure the succession and regulation of physiological processes during the day–night cycle.[1–4] Proteins requiring iron-containing cofactors are involved in such metabolic pathways, including intermediary metabolism and aerobic respiration.[5] Haem is the cofactor to which iron is coordinated in protoporphyrin IX and has been implicated in the molecular clock mechanism.[6] Injection of haem into mice was shown to alter the rhythmic expression of the core molecular clock transcription factors PER1 and PER2.[7] Haem is a prosthetic group for clock transcription factors NPAS2/BMAL1,[6] REV-ERBα and REV-ERBβ[8,9] and PER2,[10–12] though whether the binding of haem to PER2 is physiological has been questioned.[13] NPAS2/BMAL1 heterodimers regulate the expression of Alas1, the rate-limiting enzyme of the haem biosynthetic pathway, thus providing an example of clock regulation of a metabolic pathway that itself participates in clock molecular feedback loops.[7] In addition, REV-ERB acts as a transcriptional repressor, inhibiting transcription of *Bmal1* in a cell-autonomous feedback loop.[14–16] REV-ERB activity may be regulated *in vivo* by gas molecules, such as CO or NO, which can bind directly to the haem moiety.[17] The single *Drosophila* homologue of REV-ERB, known as Nuclear Receptor E75, also known as Ecdysone-induced protein 75B (Eip75B) contains haem and is also gas-responsive.[18] Despite multiple putative haem binding sites present in clock transcription factors, a role for haem in the circadian clock of *Drosophila* has not been demonstrated to date.

Drosophila melanogaster is an established model organism for studies of circadian behaviour.[19] The rhythmic behaviour of *Drosophila* is mediated by a group of about 150 circadian neurons in the central brain: ventral Lateral Neurons (LNvs; further subdivided into 5 small and 5 large neurons), dorsal Lateral Neurons (6 LNds; further subdivided by expression of neuropeptide F or Mai^{179}), Lateral Posterior neurons (LPNs) and Dorsal Neurons (DNs; present in three clusters).[20–23] Within these neurons the principal molecular feedback loop that times neural activity and behaviour is composed of the heterodimeric transcription factor CLOCK/CYCLE (CLK/CYC), which activates PERIOD (PER) and TIMELESS (TIM), which in turn feedback to repress CLK/CYC activity.[24] One common way of probing the cellular architecture underlying clock function involves elimination of neurons *via* apoptosis or *via* synaptic inhibition of a subset of clock neurons. For example, elimination of *s*-LNvs results in severely reduced amplitude of per mRNA oscillations, impaired synchronization of PER cycling within different groups of circadian neurons and compromised

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self-sustained rhythms.[25,26] Using similar experimental approaches, the morning peak of activity was shown to depend on the small (s-) LNvs, while the evening peak is thought to be governed by a group of neurons that include the LNds and a subset of the DN1s.[21,27,28] To date, the one group best understood encompasses the *s*-LNvs, five cells, four of which express the neuropeptide pigment dispersing factor (PDF). *s*-LNvs are believed to maintain circadian rhythms in constant darkness (DD) and to synchronize other groups of circadian neurons through rhythmic secretion of PDF.[29,30] In contrast, l-LNvs receive light inputs from the Hofbauer–Buchner eyelet and may be less involved in time-keeping in DD.[23]

We have previously shown that transgene-derived overexpression of the iron storage protein ferritin in glial cells of *Drosophila melanogaster* results in a late-onset loss of circadian rhythms in constant darkness, but there are no mechanistic insights on why this is the case.[31] Here we report on the use of targeted RNA interference (RNAi)[32] in order to address systematically the contribution of iron metabolism to the regulation of circadian rhythms. We took advantage of the detailed knowledge of the circadian clock's molecular and cellular circuitry in this model. Individual *Drosophila* iron metabolism genes were silenced specifically in pacemaker cells. Effects on circadian rhythmicity of individual flies in DD were then assessed.

Materials and methods

Drosophila stocks

All flies were reared as previously described at 25 °C under 12 : 12 h light–dark conditions (LD).[33] The source of RNAi lines is indicated in Table 1 and their construction is detailed in the Vienna *Drosophila* RNAi Centre website. The *UAS-Dicer2* transgene[32] was crossed into selected *Gal4* lines and into the *UAS-Fer1HCH-RNAi* and *UAS-Fer2LCH-RNAi* lines to enhance the potency of RNAi. *UAS-CD8GFP* was recombined with *UAS-Fer2LCH-RNAi* to evaluate the morphology of clock neurons following RNAi. Expression patterns for the drivers we have used have been described previously for *tim^{27}-Gal4; tim^{62}-Gal4; tim^{82}-Gal4; tim^{86}-Gal4; cry^{39}-Gal4; cry^{19}-Gal4; Mai^{179}-Gal4; $clk^{4.1M}$-Gal4; $clk^{4.5F}$-Gal4; pdf-Gal4; tim-Gal4, cry-Gal80; tim-Gal4, pdf-Gal80* (references in the text). Lines available from the Bloomington Stock center include: *Elav-Gal4* (pan-neuronal; #458); *GMR-Gal4* (photoreceptors; #9146); *RG-Gal4* (ring gland; #6986); *Actin-Gal4* (ubiquitous; #4414). *Midgut-Gal4* (intestine; NP3084) was a gift by Christoph Metzendorf (Uppsala University); *TH-Gal4* (dopaminergic neurons) was a gift by Amanda Freeman (Emory University); *Nrv2-Gal4* (adult glia only) was a gift by Makis Skoulakis (Alexander Fleming Institute); *cry^{17b}-Gal4* was a gift by François Rouyer (Institut de Neurobiologie Alfred Fessard) and is characterized for the first time here by crossing to *UAS-GFP* (Fig. 2B).

Locomotor activity measurements

The locomotor activities of individual one-week-old, age matched male flies were measured using *Drosophila* activity monitors (TriKinetics, U.S.A). Monitoring conditions included LD cycles for 2 days, followed by DD cycles for another 7 days unless otherwise indicated. Data were analyzed using autocorrelation (Rhythmic Strength was assessed by the autocorrelation 'rhythmicity index'[34] multiplied by 10) and Matlab software as described previously.[31] Flies with period values in the circadian range and with a rhythmic strength value greater than 1.5 were considered rhythmic.

Immunofluorescent staining with *anti*-TIM

Adult flies were decapitated and their heads fixed for 4 hours in phosphate-buffered saline (PBS) containing 4% formaldehyde, before brain dissections were performed in PBS. Brains were permeabilized with PBS containing 0.5% Triton X-100 (PBT), blocked in PBT containing 2% bovine serum albumin and incubated for 48-hours with primary antibodies against TIM at a 1 : 10 000 dilution in blocking solution at 4 °C. After 3 washes, brains were incubated with goat-anti-rat antibody conjugated with fluorophore AlexaFluor 488 nm (Molecular Probes) diluted 1 : 500 in PBT for 24 hours, washed extensively and mounted on slides using Vectashield (Vector Laboratories, UK) in preparation for microscopy. Eight brains were assessed for controls and twelve brains for the experimental flies at each time point. Each image shown is a combination of 3 consecutive confocal sections generated on a Leica microscope.

Western blotting

For each indicated time point, protein extracts from 20 fly heads were prepared in 115 µl of extraction buffer HEPES 10 mM (pH 7.5), KCl 50 mM, 5% Glycerol, EDTA 10 mM, 0.1% Triton X-100. Homogenates were centrifuged at 12 000 × *g* for 15 minutes at 4 °C and supernatants collected. Protein samples were denatured by addition of 1 mM DTT and heating at 60 °C for 10 min and loaded into 6% SDS–polyacrylamide gels (29.6 : 0.4, acrylamide : bis-acrylamide ratio). Following electrophoresis, gels were electroblotted onto nitrocellulose for 1 hour at 0.25 A using a semidry blotting apparatus (Hoefer, USA). PER protein abundance was analyzed by immunoblot using rabbit *anti*-PER[35] (1 : 10 000). The antibody against Fer2LCH has been previously described.[36] To ensure that equivalent amounts of protein were loaded in each lane, we used equal numbers of heads per protein sample and confirmed by Ponceau S staining. Three independent biological replicates were run and quantified using NIH imageJ software.

RNA extraction, reverse transcription and quantitative PCR

Total RNA was isolated from frozen whole heads using a TRI reagent (0.5 ml; Invitrogen, UK), and purified with a LiCl solution according to the manufacturer's instructions (Ambion, UK). cDNA synthesis was performed with a reverse transcription reagents kit (Applied Biosystems, UK) in 10 µl reactions according to the manufacturer's instructions. Quantitative-PCR reactions were prepared as follows: 7.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems, UK), 4.0 µl of cDNA, each primer at 0.5 µM final concentration and H_2O to a 15 µl final volume reaction. Reactions were performed in a Taqman 7900HT platform (Applied Biosystems, UK) under the following temperature conditions: hot start at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds; 60 °C for 30 seconds; and 10 seconds at a reading temperature. The relative quantification was determined using the comparative 2-ΔΔCT method[37,38] using *Rp49* as control for each gene. Data were collected and analyzed using SDS

Table 1 Circadian behaviour of transgenic flies kept in DD. Iron metabolism gene expression was suppressed by clock-cell-specific RNAi

Gene			*UAS-Dicer2; tim^{27}-Gal4*				*UAS-Dicer2; cry^{17b}-Gal4*				
Name/function	Flybase (CG)	VDRC ID	N	% Rhythmic	Period (h)	R.S.	N	% Rhythmic	Period (h)	R.S.	Ref.
Iron trafficking											
Tsf1/Transferrin	6186	14 666	8	**100**	25.1 ± 0.2	5.2 ± 0.3	10	**90**	24.2 ± 0.2	4.6 ± 0.8	40
Tsf2/Melanotransferrin	10 620	5236	7	**100**	24.6 ± 0.6	4.0 ± 0.6	12	**100**	23.6 ± 0.3	3.9 ± 0.3	41
***Tsf3*/Transferrin**	3666	108 470	15	**46**	**26.1 ± 1.0**	**2.5 ± 0.4**	28	**86**	**26.5 ± 0.3**	2.7 ± 0.6	40
Mvl/Divalent metal transporter	3671	44 000	12	**91**	24.2 ± 0.7	3.2 ± 0.9	12	**100**	24.1 ± 0.7	4.5 ± 0.4	42
Nemy/DcytB-like	8776	40 803	23	**91**	24.7 ± 0.6	3.5 ± 1.2	12	**83**	23.8 ± 0.2	3.9 ± 0.4	43
CG1275/DcytB-like	1275	105 418	16	**88**	24.3 ± 0.7	3.5 ± 0.8	10	**100**	23.8 ± 0.5	3.7 ± 0.4	43
MCO3/Multicopper oxidase	5959	43 288	6	**100**	23.4 ± 0.3	3.5 ± 0.6	8	**100**	24.1 ± 0.2	3.9 ± 0.3	42
Zip3/Zinc-iron transporter	6898	37 358	10	**100**	25.5 ± 0.2	4.5 ± 0.8	8	**88**	24.1 ± 0.3	3.7 ± 0.5	
CG10505/ABC transporter	10 505	107 842	12	**100**	23.7 ± 0.4	3.9 ± 1.3	8	**100**	23.8 ± 0.6	3.1 ± 0.2	44
Mfrn/Mitoferrin	4963	12 342	10	**100**	24.7 ± 0.2	4.7 ± 0.7	12	**100**	23.4 ± 0.3	4.9 ± 0.8	45
Fer1HCH/Ferritin H	2216	12 925	**L**	—	—	—	12	**100**	23.3 ± 0.5	3.8 ± 0.6	46
		102 406	**L**	—	—	—	26	**76**	23.8 ± 0.6	4.1 ± 1.4	
***Fer2LCH*/Ferritin L**	1469	14 991	40	**40**	**26.3 ± 06**	**1.9 ± 0.3**	12	**83**	23.8 ± 0.5	4.6 ± 0.9	46
		106 960	54	**33**	24.3 ± 1.7	**2.0 ± 0.4**	29	**37**	24.4 ± 0.8	**2.1 ± 0.5**	
Fer3HCH/mitoch. ferritin	4349	40 505	28	**85**	24.5 ± 0.5	3.9 ± 0.7	20	**90**	23.7 ± 0.3	3.9 ± 0.3	36
mub/poly (rC) binding protein	7437	105 495	16	**81**	23.7 ± 0.3	**2.5 ± 0.3**	23	**87**	24.3 ± 0.5	3.4 ± 0.4	47
CG32702/TMPRSS6 iron sensing	32 702	14 623	13	**100**	24.2 ± 0.3	3.0 ± 0.3	13	**84**	23.6 ± 0.5	3.1 ± 0.3	48
CG9003/Fbxl5 iron sensing	9003	23 482	11	**100**	24.3 ± 0.3	3.8 ± 0.5	12	**100**	24.1 ± 0.3	4.2 ± 0.4	49
Haem biosynthesis and catabolism											
Alas/5′-Aminolevulinate synthase	3017	48 774	23	**96**	24.9 ± 0.6	3.9 ± 1.1-	16	**93**	23.9 ± 0.5	2.8 ± 0.8	50
		105 958	**L**	—	—	—	**L**	—	—	—	
Pbgs/Porphobilinogen synthase	10 335	40 612	**L**	—	—	—	16	**100**	23.9 ± 0.4	4.6 ± 1.0	51
Coprox/Corpoporphyrinogen III oxidase	3433	105 125	**L**	—	—	—	20	**80**	24.9 ± 0.5	**2.2 ± 0.4**	
Ppox/Protoporphyrinogen oxidase	5796	40 607	L	—	—	—	10	**100**	24.1 ± 0.2	4.3 ± 1.3	
Ferrochelatase/Ferrochelatase	2098	101 496	22	**90**	**27.3 ± 1.0**	3.3 ± 1.0	16	**93**	24.1 ± 0.2	3.5 ± 1.4	52
		20 804	12	**83**	24.5 ± 0.6	3.4 ± 0.9	6	**100**	23.3 ± 0.5	3.6 ± 0.3	
Ho/Haem oxygenase	14 716	R1 III[a]	38	**89**	24.0 ± 0.8	3.4 ± 0.7	16	**75**	24.6 ± 0.4	2.7 ± 0.5	53
		R3 II[a]	6	**83**	24.7 ± 0.6	3.9 ± 1.1	8	**100**	23.8 ± 0.5	3.9 ± 0.6	
CG9471/Biliverdin reductase	9471	24 042	10	**100**	24.1 ± 0.3	4.9 ± 0.9	16	**93**	23.7 ± 0.2	3.6 ± 1.1	
Cchl/Cytochrome *c* haem lyase	6022	45 020	12	**100**	23.7 ± 0.6	3.5 ± 0.6	12	**100**	24.0 ± 0.5	3.3 ± 0.6	54
***CG1358*/FLVCR haem transporter**	1358	101 453	6	**67**	23.9 ± 0.4	**2.4 ± 0.4**	23	**60**	23.8 ± 0.7	2.7 ± 0.7	55
Cat/Catalase	6871	6283	10	**100**	**27.0 ± 0.7**	**2.0 ± 1.0**	12	**100**	23.7 ± 0.3	3.3 ± 0.2	56
Eip75B/Rev-Erb	8127	44 851	**L**	—	-	-	**L**	—	—	—	18
Iron–sulfur cluster biosynthesis											
fh/frataxin homologue	8971	From ref.	22	**90**	**27.3 ± 1.0**	3.4 ± 1.0	10	**90**	23.6 ± 0.4	3.3 ± 0.3	57
			9	**88**	**27.6 ± 0.3**	3.3 ± 0.9	9	**88**	23.5 ± 0.2	3.5 ± 0.4	58
***CG12264*/IscS homologue**	12 264	105 106	20	**80**	**26.9 ± 0.8**	**2.5 ± 1.1**	61	**28**	25.0 ± 1.3	**2.1 ± 0.5**	59
***CG9836*/IscU homologue**	9836	29 295	**L**	—	-	-	59	**33**	24.9 ± 1.4	**2.3 ± 0.7**	60
***CG8198*/IscA1 homologue**	8198	104 791	26	**65**	23.2 ± 0.8	2.9 ± 0.9	10	**100**	23.5 ± 0.8	3.9 ± 0.4	61
CG13623/IscA2 homologue	13 623	110 643	11	**91**	23.5 ± 0.3	4.1 ± 0.5	11	**100**	23.6 ± 0.5	3.1 ± 0.4	62
CG6523/Grx3, Grx4	6523	101 433	20	**75**	24.0 ± 0.3	2.9 ± 0.4	12	**100**	23.8 ± 0.3	3.9 ± 0.3	63
CG14407/Grx5	14 407	43 020	7	**87**	24.3 ± 0.3	5.2 ± 0.5	10	**90**	24.1 ± 0.8	4.7 ± 0.3	64
CG7995/ABCB7	7955	40 838	10	**100**	24.7 ± 0.2	5.1 ± 0.7	12	**100**	23.3 ± 0.4	3.7 ± 0.3	45
CG7349/Ferredoxin	7349	51 482	10	**100**	24.6 ± 0.2	3.9 ± 1.0	10	**100**	23.9 ± 0.5	3.5 ± 0.6	
CG17904/Nubp1	17 904	103 384	16	**88**	23.9 ± 0.4	2.9 ± 0.5	9	**88**	23.5 ± 0.3	3.3 ± 0.6	65
***CG4858*/Nubp2**	4858	106 917	28	**78**	23.9 ± 0.9	**2.2 ± 0.7**	13	**46**	23.4 ± 0.5	**2.1 ± 0.4**	66
CG1783/NAR1p	17 683	110 003	**L**	—	-	-	12	**100**	24.0 ± 0.4	3.8 ± 0.5	67
CG30152/FAM96a	30 152	105 959	**L**	—	-	-	12	**100**	23.0 ± 0.4	3.2 ± 0.5	
CG12797/CiaO1	12 797	105 939	17	**77**	24.3 ± 0.7	3.5 ± 0.8	24	**75**	23.3 ± 0.8	2.9 ± 0.7	68
***CG8043*/Iba57**	8043	104 355	31	**38**	**22.8 ± 0.6**	**1.9 ± 0.7**	22	**63**	24.6 ± 0.3	2.9 ± 0.7	69
CG17843/Erv1	17 843	102 838	8	**75**	23.9 ± 0.4	3.0 ± 0.5	14	**100**	23.9 ± 0.3	4.4 ± 0.8	70
CG1458/MitoNEET	1458	104 501	6	**100**	24.1 ± 0.2	2.9 ± 0.4	12	**100**	23.4 ± 0.5	3.9 ± 0.4	71
Irp-1B/cytosolic aconitase	6342	30 153	8	**100**	24.6 ± 0.9	4.1 ± 1.4	10	**100**	23.4 ± 0.6	3.5 ± 0.5	72
		110 637	21	**76**	24.0 ± 0.6	3.3 ± 0.6	12	**100**	23.6 ± 0.7	3.9 ± 0.4	
Acon/mitochondrial aconitase	9244	103 809	10	**100**	24.5 ± 0.4	3.9 ± 1.3	12	**92**	23.8 ± 0.6	3.3 ± 0.4	73
Sod1/Cu–Zn superoxide dismutase	11 793	From ref.	9	**100**	24.1 ± 0.2	4.4 ± 0.5	16	**100**	23.4 ± 0.8	4.1 ± 0.7	74
			10	**80**	25.3 ± 0.3	4.0 ± 0.7	16	**100**	23.3 ± 1.0	4.0 ± 0.9	
Sod2/Mn superoxide dismutase	8905	From ref.	11	**100**	24.0 ± 0.5	3.9 ± 1.2	10	**100**	23.9 ± 0.9	4.4 ± 0.9	75
			10	**100**	23.7 ± 0.3	3.5 ± 1.3	16	**93**	23.8 ± 0.6	3.1 ± 1.4	

[a] Obtained from Kyoto Stock Centre.

software version 2.2.1. Primer sequences used were as follows (5′ → 3′ orientation):

Rp49 sense CGATATGCTAAGCTGTCGCACA,
Rp49 antisense CGCTTGTTCGATCCGTAACC,
per sense CAACAAGTCGGTGTACACGAC,
per antisense GTCTTGACGGATGCGCTCTG,
tim sense AATGCAATCATCGCACAG,
tim antisense GCCAAATCCCTCATCGTC,

Fer2LCH sense GAACACTGTAATCACCGC,
Fer2LCH antisense CAGATACTCGTCGAACAG.

Results

A targeted genetic screen reveals candidate iron metabolism genes with roles in the circadian behaviour of *Drosophila*

We asked whether iron metabolism genes are required in the clock pacemaker neuronal circuitry for the regulation of rhythmic activity of flies kept in constant darkness. Our approach used the Gal4/UAS-RNAi transgenic system.[32,39] Forty-eight genes functioning in iron trafficking, haem and iron–sulfur (Fe–S) cluster biosynthesis and catabolism were selected either because of their prior implication in iron homeostasis of *Drosophila* or because they are clear homologues of genes implicated in mammalian iron metabolism (Table 1).[18,36,40–75] The *tim*^{27}*-Gal4* driver[76] was used to down-regulate the expression of each selected gene in all clock cells. Flies from the F1 progeny of all crosses that carried the *tim*^{27}*-Gal4*, the *UAS-RNAi* and the *UAS-Dicer2* transgenes, the latter of which was included in an attempt to enhance the potency of gene silencing,[32,77] were tested for circadian rhythmicity in locomotor activity assays performed under DD. We repeated the screen with *cry*^{17b}*-Gal4*, which was provided by François Rouyer (Institut de Neurobiologie Alfred Fessard, France) and expressed in a narrow clock-specific pattern.

Table 1 summarizes the behavioural attributes of viable flies carrying Gal4 and RNAi transgenes. For each fly tested the rhythmic strength (R.S.) value was determined, providing an objective statistical measure of rhythmicity.[34] Flies were first scored as rhythmic if R.S. $>$ 1.5 or arrhythmic if R.S. $<$ 1.5 and the percentage of rhythmic flies is tabled. The mean percentage value of rhythmic flies in this screen was 88 $\pm$ 18%; hence we have noted any genetic combination that resulted in fewer than 70% demonstrable rhythmic activity. The average R.S. value for the flies classified as rhythmic in this screen was 3.5 $\pm$ 0.9 for the RNAi strains crossed to the *tim*^{27}*-Gal4* driver line and 3.6 $\pm$ 0.7 when crossed to *cry*^{17b}*-Gal4*. We have therefore considered low rhythmic strength values when R.S. $\leq$ 2.5 (Table 1). Finally, the average period of the rhythmic pattern of flies carrying the RNAi transgene in trans with *tim*^{27}*-Gal4* was 24.6 $\pm$ 1.1 h and 23.6 $\pm$ 0.6 h for flies carrying the RNAi transgene in trans with *cry*^{17b}*-Gal4*. The latter value is consistent with that expected from wild type flies. We also note that the slightly higher value obtained with *tim*^{27}*-Gal4* may be attributable to the effect of RNAi on a small number of genes leading to a significantly increased period ($>$26 h).

Our specific findings were as follows: RNAi against thirty-eight genes resulted in rhythmic flies; silencing of nine genes with *tim*^{27}*-Gal4* resulted in lethality; two genes – *Aminolevulinate synthase* (*Alas*) and the *Drosophila* REV-ERB homologue *Eip75B* – were also lethal when silenced with *cry*^{17b}*-Gal4*; whereas RNAi of *Transferrin 3* (*Tsf3*), *Ferritin 2 Light Chain Homologue* (*Fer2LCH*), *CG1358* (which has homology to the FLVCR haem export protein) and of five genes implicated in iron–sulfur cluster biosynthesis, *IscS*/*CG12264, IscU*/*CG9836, IscA1*/*CG8198, Iba57*/*CG8043* and *Nubp2*/*CG4858*, resulted in a robust reduction of the number of flies showing rhythmic behaviour in constant darkness and a concomitant reduction in the R.S. value of flies classified rhythmic with both drivers (Table 1). *tim*^{27}*-Gal4* driven RNAi against *Tsf3*, *Fer2LCH* and *IscS* showed a prolonged period whereas RNAi of *Iba57* showed a reduced period. Further genotypes that showed a prolonged period included *tim*^{27}*-Gal4*-RNAi against *Ferrochelatase*, *Catalase* and *frataxin* (Table 1). We are mindful that RNAi may reduce expression differentially across the genes, nevertheless our results suggest that genes involved in the biosynthesis of iron-containing protein cofactors are involved in the endogenous time-keeping process in the absence of an external *zeitgeber*.

Fer2LCH silencing leads to disrupted oscillations of the core molecular clock components PER and TIM in DD

To validate the findings of our screen, we performed a detailed analysis of the phenotypes present in *UAS-Dicer2; tim*^{27}*-Gal4*/*UAS-Fer2LCH-RNAi* flies. We chose to analyse RNAi against *Fer2LCH* because it was the only gene already implicated in the circadian clock, albeit in an overexpression system,[31] it gave the strongest phenotype with both Gal4 driver lines and specific reagents were immediately available to us for further experimentation.

Under 12 : 12 hour alternating light and dark cycles (LD), *y, w* control flies and *UAS-Dicer2; tim*^{27}*-Gal4*/*UAS-Fer2LCH-RNAi* flies show no apparent differences (Fig. 1A, B). In contrast, the rhythmic pattern observed with control *y, w* flies under DD conditions (Fig. 1A) completely breaks down in *UAS-Dicer2; tim*^{27}*-Gal4*/*UAS-Fer2LCH-RNAi* (Fig. 1B). The inability of these flies to sustain rhythmic circadian activity in DD suggested that the molecular oscillations of clock transcription factors may have been disrupted. Upon re-establishment of LD cycles *UAS-Dicer2; tim*^{27}*-Gal4*/*UAS-Fer2LCH-RNAi* flies readily entrain to light cues and rhythmic behaviour is restored (Fig. 1B) further suggesting a specific disruption in the endogenous clock machinery under DD for the given genotype.

To establish that RNAi against *Fer2LCH* did not kill the clock neurons, we marked the cells where RNAi was induced with a membrane-bound green fluorescent protein (GFP). All clock neurons could be identified in *UAS-Dicer2; tim*^{27}*-Gal4*/*UAS-Fer2LCH-RNAi, UAS-CD8GFP* flies (Fig. 1C). These flies were also tested in our behaviour assays and were as expected arrhythmic in DD. Next, we monitored *per, tim* and *Fer2LCH* mRNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR; Fig. 1D,E), PER and Fer2LCH protein by Western blot analysis (Fig. 1F,G) and TIM protein by immuno-fluorescent staining of whole mount preparations of adult *Drosophila* brains (Fig. 1H,I). In these assays, we compared the control genotype *y, w* to *UAS-Dicer2; tim*^{27}*-Gal4*/*UAS-Fer2LCH-RNAi*. Both groups of flies were entrained under 12 : 12 LD conditions for 3 days and transferred into DD conditions for another day before flies were killed at indicated circadian time (CT) points and their heads used to isolate total mRNA or protein. As expected, qRT-PCR measurements of *per* and *tim* mRNA in control *y, w* flies showed low expression at CT2 (perceived morning) and high expression at CT18 (perceived night), consistent with the notion that cyclic expression of the *per* and *tim* genes underlies

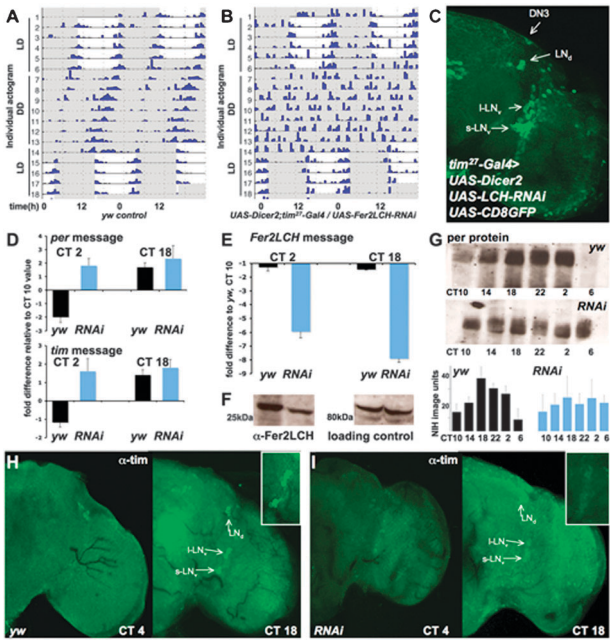

Fig. 1 Silencing of Fer2LCH in clock neurons leads to the loss of circadian rhythms in DD and deregulation of core clock transcription factors. (A) *y, w* and (B) *UAS-Dicer2;tim*^{27}*-Gal4/UAS-Fer2LCH-RNAi* genotypes shown in double-plotted locomotor actograms showing representative individual male flies spanning 5 days in LD, 7 days in DD and 4 further days in LD. (C) Visualization of clock neurons in dissected brains from *UAS-Dicer2;tim*^{27}*-Gal4,UAS-CD8-GFP/ UAS-Fer2LCH-RNAi* flies. These flies were behaviourally arrhythmic, but showed no apparent signs of neuronal degeneration. (D) qRT-PCR experiments showing relative expression levels for the *per* and *tim* genes at two different CTs normalized against CT10, from control *y, w* (black bars) and *UAS-Dicer2;tim*^{27}*-Gal4/UAS-Fer2LCH-RNAi* (blue bars) heads. (E) qRT-PCR for the *Fer2LCH* gene in heads. (F) Western blot using α-Fer2LCH and samples from whole flies. (G) Western blots using α-PER. Note PER accumulation during the subjective night timepoints in *y, w* head samples (upper blot), but constitutive PER accumulation in *Fer2LCH* RNAi samples (lower blot). Plots depict the quantification of three independent experimental replicates. (H) Immunofluorescent detection of the TIM protein during subjective morning (CT4) and night (CT18). Only during the latter timepoint is accumulation of TIM detected in the nuclei of clock neurons of control flies (inset shows *s*-LNvs and l-LNvs). (I) The staining is largely decreased and localization appears in the cytoplasm in brains from *Fer2LCH* RNAi flies. Neuronal clusters are indicated with arrows.

the rhythmic behaviour of these flies (Fig. 1D, black bars). In contrast, *per* and *tim* gene expression was not significantly different between CT2 and CT18 in samples from *UAS-Dicer2; tim*^{27}*-Gal4/UAS-Fer2LCH-RNAi* heads (Fig. 1D, blue bars). Our results indicated that RNAi flies had inadvertently higher levels of *per* and *tim* mRNA at CT2 relative to *y, w* controls, suggesting impaired regulation of gene expression.

Fer2LCH RNAi was effective in reducing *Fer2LCH* mRNA levels in fly heads (Fig. 1E) and total body Fer2LCH protein accumulation is also reduced in *UAS-Dicer2; tim*^{27}*-Gal4/UAS-Fer2LCH-RNAi* flies compared to *y, w* controls (Fig. 1F). We also confirmed that in lysates prepared from heads of *y, w* flies kept under DD, PER protein accumulates during the subjective night with a few hours of delay relative to mRNA expression peaks (Fig. 1G, upper blot and black bars in quantification of Western repeats). In contrast, PER protein did not show consistent differences in amounts during the different time points in samples from *UAS-Dicer2; tim*^{27}*-Gal4/UAS-Fer2LCH-RNAi* heads (Fig. 1G, lower blot and blue bars in quantifications), further corroborating the evidence that the molecular clock is disrupted in these flies.

To directly assess the accumulation of TIM protein in the different groups of pacemaker cells, we dissected brains from flies kept for 3 days under DD conditions and compared TIM-dependent immuno-fluorescent signals between the control and the arrhythmic flies at two key CTs. In control *y, w* flies, a weak signal for TIM was detected in the cytoplasm of *s*-LNvs and l-LNvs at CT4 and a strong nuclear signal for TIM at CT18 (Fig. 1H). In contrast a weak TIM signal in the cytoplasm was observed in the *UAS-Dicer2; tim*^{27}*-Gal4/UAS-Fer2LCH-RNAi* flies at both time points (Fig. 1I), suggesting that the lack of circadian activity patterns in these flies results from defects in the key transcription factor oscillations normally present in core central pacemaker neurons. Considering all the findings presented in Fig. 1, we conclude that RNAi of *Fer2LCH* in all clock cells results in a robust disruption of molecular rhythms known to sustain rhythmic activity in DD. These results provide a validation for the targeted genetic screen presented above.

RNAi of *Fer2LCH* in seven neurons on each brain hemisphere disrupts maintenance of a functional circadian clock

There are about 150 clock neurons in the adult brain of *Drosophila*[19]. The *tim*^{27}*-Gal4* transgene has been shown to drive gene expression in all six groups of them (*s*-LNvs, l-LNvs, LNds, and DNs1-3). To identify which neurons required *Fer2LCH* expression for maintenance of the circadian clock, we drove RNAi with a variety of spatially restricted clock Gal4 lines (Table 2). The most relevant expression patterns and the corresponding behavioural phenotypes following RNAi are shown in Fig. 2.

As with *tim*^{27}*-Gal4*, the *cry*^{17b}*-Gal4* drives expression in a broad subset of clock neurons and when used to silence *Fer2LCH* the resulting flies exhibit an arrhythmic phenotype (Fig. 2A, B). Consistently, *cry-Gal80* (Stoleru *et al.* 2004) suppression of *tim*^{27}*-Gal4*-induced *Fer2LCH* RNAi rescued the circadian rhythms (Fig. 2C), further supporting the notion that *cry*+ neurons are involved in the observed phenotype. In contrast, neither *Fer2LCH* RNAi driven by *Pdf-Gal4*, which drives expression in the majority of *s*-LNvs and l-LNvs (Fig. 2D), nor *Fer2LCH* RNAi driven by *cry*^{19}-Gal4, which drives expression in one of the three LNds and the majority of l-LNvs, but not in *s*-LNvs (Fig. 2E) impaired circadian behaviour. Collectively, these data suggest that RNAi of *Fer2LCH* would disrupt circadian rhythms when driven simultaneously in *cry* +-LNds and sLNvs, but not when silencing occurs separately in these clusters of neurons. To test this suggestion, *Fer2LCH* RNAi was driven with *Mai*^{179}*-Gal4*, which drives expression only in three *cry*+-LNds and four *s*-LNvs (Fig. 2F). This genotype resulted in the majority of the flies lacking any detectable circadian rhythms (for quantification see Table 2) providing experimental support to the idea that *Fer2LCH* has

Table 2 Analysis of circadian behaviour in DD for *Fer1HCH* and *Fer2LCH* RNAi driven by a variety of *Gal4* drivers

	UAS-Dicer2; UAS-Fer1HCH-RNAi				*UAS-Dicer2; UAS-Fer2LCH-RNAi*			
Gal4 drivers	N	% Rhythmic	Period (h)	R.S.	N	% Rhythmic	Period (h)	R.S.
Clock-cell specific								
***tim*^{62}**	Lethal	—	—	—	15	**13**	23.4 ± 2.6	**1.6 ± 0.1**
***tim*^{82}**	Lethal	—	—	—	16	**12**	24.3 ± 1.7	**1.7 ± 0.3**
***tim*^{86}**	Lethal	—	—	—	14	**21**	**22.3 ± 2.4**	**1.8 ± 0.2**
***cry*^{17b}**	26	**76**	23.8 ± 0.6	4.1 ± 1.4	46	**22**	23.8 ± 1.3	**1.8 ± 0.9**
***cry*^{39}**	32	**90**	24.1 ± 0.6	3.6 ± 1.0	58	**41**	24.1 ± 1.1	**2.1 ± 0.8**
cry^{19}	32	**100**	23.9 ± 0.4	3.2 ± 0.7	26	**100**	24.5 ± 0.2	3.6 ± 1.5
***Mai*^{179}**	40	**75**	23.2 ± 4.1	3.0 ± 1.1	52	**40**	23.5 ± 2.4	**2.1 ± 0.9**
clk$^{4.1M}$	32	**75**	24.1 ± 1.7	2.9 ± 1.2	32	**78**	24.1 ± 0.6	2.7 ± 0.7
clk$^{4.5F}$	40	**85**	24.4 ± 1.2	3.4 ± 1.3	14	**85**	23.8 ± 0.6	2.9 ± 0.7
pdf	28	**96**	23.9 ± 0.9	3.6 ± 1.2	22	**100**	23.9 ± 0.7	3.3 ± 0.8
tim, cry-Gal80	44	**90**	24.4 ± 0.5	3.6 ± 1.0	68	**72**	**26.0 ± 1.8**	**2.3 ± 0.6**
tim, pdf-Gal80	40	**82**	24.1 ± 1.0	2.6 ± 0.7	71	**51**	24.2 ± 2.5	**2.3 ± 0.8**
Other								
Elav, UAS-Dicer2[a]	9	**55**	23.4 ± 0.3	**2.5 ± 0.6**	15	**40**	23.3 ± 0.3	**2.4 ± 0.5**
Tyrosine Hydroxylase	12	**100**	24.0 ± 0.6	2.9 ± 0.6	16	**88**	23.4 ± 0.6	2.9 ± 0.8
GMR	32	**100**	23.2 ± 0.3	4.2 ± 1.1	20	**100**	23.2 ± 0.3	4.1 ± 1.1
Repo	Lethal	—	—	—	Lethal	—	—	—
Nrv2	16	**88**	23.9 ± 0.2	3.5 ± 0.3	16	**94**	23.6 ± 0.5	3.7 ± 0.4
Actin5C	Lethal	—	—	—	Lethal	—	—	—
Midgut	10	**80**	23.4 ± 0.4	2.6 ± 0.3	16	**93**	23.6 ± 0.3	2.9 ± 0.9
Ring Gland	30	**87**	23.4 ± 0.3	3.7 ± 1.0	36	**86**	23.8 ± 0.5	2.7 ± 0.8

[a] Because this *Gal4* insertion is on the X-chromosome, we generated a recombinant chromosome and used females from the driver for the cross.

a hitherto unknown function in a small subset of clock neurons supporting circadian activity of flies in DD.

Three additional insertions of *tim-Gal4* lines and *cry*^{39}*-Gal4* all confirmed the arrhythmic phenotype when crossed to *UAS-Fer2LCH-RNAi* (Table 2). Conversely, *Fer2LCH* RNAi with drivers specific to the posterior DN1s, *clk*$^{4.5F}$*-Gal4* and *clk*$^{4.1M}$*-Gal4* resulted in rhythmic flies (Table 2). This result is consistent with the alternative function of synchronization of rhythms attributed to a subset of DNs.[78] Pan-neuronal RNAi of *Fer2LCH* with *Elav-Gal4* also resulted in arrhythmic flies as expected. Given a prior implication of glia in circadian rhythms, we tested two glial drivers, *Repo-Gal4* and *Nrv2-Gal4*, but whereas *Fer2LCH* RNAi driven by *Repo-Gal4* resulted in lethality, likely due to strong Gal4 expression during development, *Nrv2-Gal4*-driven RNAi resulted in rhythmic flies (Table 2).

To rule out the involvement of peripheral tissues, we used drivers that are highly specific to the ring gland and to the intestine (tissues where *tim*^{27}*-Gal4* and *cry*^{17b}*-Gal4* drive expression but *Mai*^{179}*-Gal4* does not). *Fer2LCH* RNAi with these drivers resulted in rhythmic flies. We also tested circadian behaviour following *Fer2LCH* RNAi in photoreceptors (with *GMR-Gal4*) and in dopaminergic neurons (with *TH-Gal4*) and confirmed that circadian behaviour was normal (Table 2).

RNAi against *Fer1HCH* in the same clock neurons does not disrupt circadian rhythms

One of the nine genes for which *tim*^{27}*-Gal4* driven RNAi resulted in lethality in our initial screen was *Fer1HCH*, a gene encoding for the second chain required for the formation of the functional iron-loaded ferritin heteropolymer.[46] The lethality of this genotype was confirmed with three independent *tim-Gal4* drivers (Table 2). However, *Fer1HCH* RNAi driven with all other clock-specific drivers resulted in viable flies, which were able to maintain circadian rhythms (Table 2). These included *cry*^{17b}*-Gal4, cry*^{39}*-Gal4* and *Mai*^{179}*-Gal4*, all of which resulted in rhythmic flies when crossed to *Fer1HCH* RNAi. In addition, both *pdf-Gal80* and *cry-Gal80* rescued the lethality induced by *tim*^{27}*-Gal4*, but resulted in individual flies able to retain circadian rhythms in DD (Table 2). Although this result would at face value indicate that *Fer1HCH* is required in pdf+ neurons, we also noted that peripheral expression seen with *tim*^{27}*-Gal4* in the intestine and fat bodies was clear in *tim-Gal4, pdf-Gal80* flies, suggesting that the lethality effect seen in the *tim*^{27}*-Gal4, UAS-Fer1HCH-RNAi* genotype might be due to a *Fer1HCH* function in the intestine. The only indication that *Fer1HCH* may be required for the maintenance of circadian rhythm was seen when the strong pan-neural driver *Elav-Gal4* was used (Table 2).

Discussion

A novel function for *Drosophila Fer2LCH* in the circadian clock

One key finding of this study is best summarized in Fig. 2F, which shows that RNAi of *Fer2LCH* in 14 neurons (7 in each brain hemisphere) results in flies unable to maintain circadian activity in the absence of external cues. These *cry*+ neurons (LNds and *s*-LNvs) have been previously implicated as the central pacemaker neurons under DD conditions.[28,79] As ferritin expression elsewhere in the brain and in the body is unaffected in this strain, the profound behavioural consequences that follow interference with *Fer2LCH* in a small subset of neurons can be attributed to a dysfunction of previously described oscillations in neuronal activity of the central pacemaker circuitry that governs circadian behaviour.[19] Accumulation of the key transcription factors PER and TIM, whose cyclic accumulation and degradation normally defines the major molecular rhythms in these neurons, was no longer regulated in a circadian manner

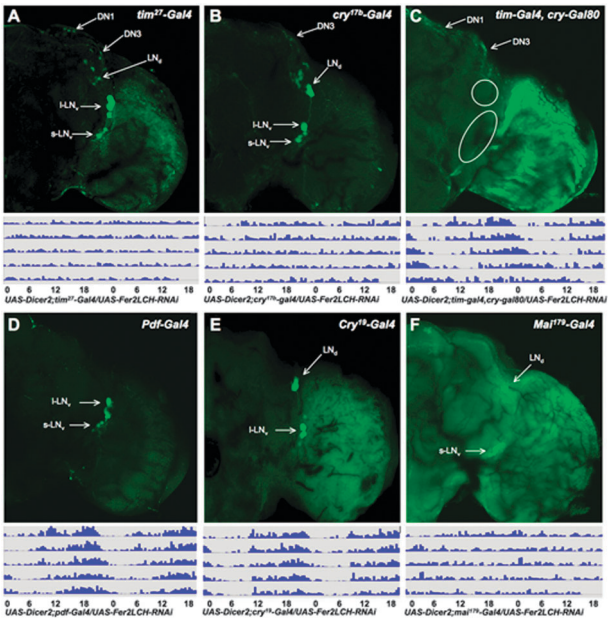

Fig. 2 Restricted RNAi of Fer2LCH reveals the subset of clock neurons where Fer2LCH is required for circadian timing. (A–F) Upper panels show the *Gal4* driver line crossed to *UAS-GFP* to reveal the expression pattern. Neuronal types are indicated with arrows. Lower panels show double-plotted locomotor actograms showing average activity from five representative individual male flies spanning 5 days in DD, which were previously entrained for 3 days in LD. (A) *UAS-Dicer2; tim*^{27}*-Gal4|UAS-Fer2LCH-RNAi* are arrhythmic and target *Fer2LCH* for RNAi in all clock cells. (B) *UAS-Dicer2;cry*^{17b}*-Gal4|UAS-Fer2LCH-RNAi* silence *Fer2LCH* in many clock cells and also result in arrhythmic flies (C) *cry-Gal80* suppresses *tim-Gal4* in LNvs and LNds (circles), rescuing circadian rhythmicity. (D) *pdf-Gal4* silencing of *Fer2LCH* in *s*-LNvs and l-LNvs resulted in flies that maintained circadian rhythms under DD conditions. (E) *cry*^{19}*-Gal4* expresses in l-LNvs and LNds, but not in *s*-LNvs and when crossed to *UAS-Fer2LCH-RNAi* did not result in arrhythmia. (F) In contrast, *Mai*^{179}*-Gal4* expresses exclusively in 3 LNds and 4 *s*-LNvs and when crossed to *UAS-Fer2LCH-RNAi* resulted in arrhythmic flies.

following *Fer2LCH* RNAi, implicating the ferritin subunit in the time-keeping process.

Is the function of Fer2LCH in iron storage relevant for its function in the circadian clock?

The major iron storage protein complex in *Drosophila melanogaster* is ferritin, which as in many other insects,[80] is predominantly found in the hemolymph or within the secretory pathway of cells.[46] For a ferritin molecule to form and store iron, 12 Fer1HCH and 12 Fer2LCH subunits are joined together by molecular interactions that include disulfide bonds.[81] Formation of the ferritin heteropolymer is induced by iron in a cell-type specific manner,[82] however several aspects of ferritin gene and protein regulation remain unclear to date.[46,83–85]

The demonstration that overexpression of both ferritin subunits in glia cells of *Drosophila* resulted in iron-loaded ferritin accumulation and in a late-onset loss of circadian activity[31] led us to think that iron storage influenced the molecular time-keeping machinery. However, the observation that RNAi against *Fer1HCH* resulted in rhythmic flies (Table 2) casts some doubt on this speculation. Indeed, if functional ferritin heteropolymers, and hence iron storage, were to be implicated in the phenotype one needs to explain why *Fer1HCH* RNAi in the clock neurons did not result in arrhythmia. One possibility could be that Fer1HCH subunits were being produced and trafficked from different cells, most likely other neurons given that *Elav-Gal4, UAS-Dicer2; UAS-Fer1HCH-RNAi|+* flies showed compromised circadian activity.

Although ferritins are best known for their role in iron storage, during their long evolutionary history it is not too surprising that they have been adapted to serve other functions as well.[86] Some of the previously proposed functions attributed to ferritin are notably subunit-specific; for example nuclear ferritin is thought to be H chain specific[87] and failure of nuclear translocation was recently proposed to contribute to triple A syndrome—a rare and poorly understood neurological disorder.[88] Other H chain specific functions have been proposed in the regulation of folate metabolism[89] and of the CXC chemokine receptor 4.[90] Interestingly, L chain has been previously implicated in the maturation of tyrosinase, a copper dependent enzyme required for melanin production.[91] A recent report has implicated iron homeostasis in a fly model of the circadian-related Retstless Legs syndrome.[92] Our study suggests a novel requirement of Ferritin L chain in circadian rhythms in *Drosophila melanogaster*.

Drosophila transferrins

The only transferrin that has been analysed functionally from *Drosophila melanogaster* is the melanotransferrin homologue *Tsf2* (also known as *MTf*). Tsf2 is a component of the septate junction and indeed septate junction assembly during epithelial maturation was shown to rely on endocytosis and apicolateral recycling of iron-bound Tsf2.[41] Consistently, *Tsf2* is highly expressed during embryogenesis and mutants are embryonic lethal. This is in contrast to *Tsf1* expression, which is upregulated from entry into the larval stages and induced upon an immune challenge.[40,93] Tsf1 is the main form of transferrin found in hemolymph. However, only RNAi of *Tsf3*, the third of *Drosophila* transferrin homologues,[94] resulted in disrupted circadian rhythms, especially when driven with *tim*^{27}*-Gal4* (Table 1). To our knowledge, *Tsf3* has not been studied experimentally to date.

Is haem required for a functional circadian clock in *Drosophila*?

Based on the demonstration that the single *Drosophila* homologue of REV-ERB, Eip75B (also known as Nuclear Receptor E75) contains haem[18] and the implication of haem in the circadian clock of mammals,[6,7] we hypothesized that haem biosynthetic enzymes might be involved in generating endogenous circadian rhythms. We were encouraged by observations from others of cyclic activity in gene expression of *Alas* and *Haem Oxygenase,*[95] findings that we independently reproduced with qRT-PCR experiments using samples from fly heads. In view of the above, it was intriguing that four genes for which *tim*^{27}*-Gal4* driven RNAi resulted in lethality were directly implicated in haem biosynthesis (*Alas, Porphobilinogen synthase, Corpoporphyrinogen III oxidase, Protoporphyrinogen oxidase*) and a fifth was *Eip75B* (Table 1). However, whenever we could generate viable adults following RNAi with more restricted Gal4 drivers we found no

evidence of disrupted circadian activity with any of the genes related to the haem biosynthetic pathway. Nevertheless, it would be premature to conclude based on these limited experiments that there is no cell-autonomous requirement within the clock neurons of the haem biosynthetic or degradation pathways for the maintenance of circadian rhythm. Indeed, RNAi against the only gene identified as a putative haem transporter *FLVCR* (*CG1358*) suggested some impairment in circadian behaviour, which should be further investigated (Table 1).

Are iron–sulfur clusters required for a functional circadian clock in *Drosophila*?

The cysteine desulfurase IscS operates in complex with IscU in the iron–sulfur cluster assembly. IscS provides the sulfur from cysteine and IscU is a scaffold protein for the build up of the cluster.[59,60] Subsequent steps in this pathway are currently under intense scrutiny in many laboratories. IscA1 may be functioning in complex with Iba57 in the maturation of the clusters.[96] Intriguingly, RNAi against *Drosophila IscS, IscU, Iba57* and to a smaller extent of *IscA1* resulted in visible disruptions of circadian behaviour in DD (Table 1). These results strongly implicate the iron–sulfur cluster biosynthetic pathway in the function of the circadian pacemaker. Whether an iron–sulfur cluster protein mediates this effect remains at present unclear. One obvious canditate would be the cytosolic iron–sulfur cluster protein IRP1-A,[72] which we could not test at this time due to the unavailability of the corresponding transgenic RNAi line. The disruption of rhythmic activity following RNAi of *Nupb2*, whose gene product has been proposed to work in complex with Nupb1 in the maturation of cytosolic iron–sulfur clusters,[66] would be consistent with this idea.

Conclusions

RNAi of *Fer2LCH* in a subset of *Drosophila melanogaster* clock neurons leads to disrupted circadian oscillations of PER and TIM and, as a consequence, to the disruption of circadian activity in the absence of external cues. Our targeted genetic screen has uncovered a number of other iron metabolism genes implicated in circadian biology, notably genes involved in iron–sulfur cluster biosynthesis and haem transport.

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