CHAPTER ONE

Studying Circadian Rhythm and Sleep Using Genetic Screens in *Drosophila*

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Contents

Abstract

The power of *Drosophila melanogaster* as a model organism lies in its ability to be used for large-scale genetic screens with the capacity to uncover the genetic basis of biological processes. In particular, genetic screens for circadian behavior, which have been performed since 1971, allowed researchers to make groundbreaking discoveries on multiple levels: they discovered that there is a genetic basis for circadian behavior, they identified the so-called core clock genes that govern this process, and they started to paint a detailed picture of the molecular functions of these clock genes and their encoded proteins. Since the discovery that fruit flies sleep in 2000, researchers have successfully been using genetic screening to elucidate the many questions surrounding this basic animal behavior. In this chapter, we briefly recall the history of circadian rhythm and sleep screens and then move on to describe techniques currently employed for mutagenesis and genetic screening in the field. The emphasis lies on comparing the newer approaches of transgenic RNA interference (RNAi) to classical forms of mutagenesis, in particular in their application to circadian behavior and sleep.

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We discuss the different screening approaches in light of the literature and published and unpublished sleep and rhythm screens utilizing ethyl methanesulfonate mutagenesis and transgenic RNAi from our lab.

1. INTRODUCTION: STUDYING CIRCADIAN BEHAVIOR IN THE FRUIT FLY, *DROSOPHILA MELANOGASTER*

Drosophila exhibits a multitude of innate and adaptive behaviors that allow researchers to study complex behaviors in a genetically tractable organism. Fruit flies, like all animals, need to correctly interpret and respond to their environment.

All life on earth is subject to the changes in light and temperature due to the earth's rotation. Many animals and plants exhibit diurnal or nocturnal behavior depending on their habitat and lifestyle. French scientist Jean-Jaques d'Ortous de Mairan discovered in 1729 that the daily opening and closing of plant leaves persisted in a dark room, indicating that this circadian behavior was not merely a reaction to light, but was effected by internal processes (de Mairan, 1729). It was not until over 200 years later that Konopka and Benzer analyzed the role of endogenous forces—genes—on the daily eclosion rhythm of the fruit fly *Drosophila melanogaster* (Konopka & Benzer, 1971). Since then, studies in *Drosophila* have played a prominent role in elucidating the genes and molecular mechanisms driving circadian behavior (Blau et al., 2007; Stanewsky, 2003). Analogous studies in mammals have revealed that these genes and mechanisms are largely conserved through evolution, indicating that these mechanisms are fundamental and underlie the conservation of animal behavior across evolution (Wager-Smith & Kay, 2000). Insights from *Drosophila* continue to have a broad impact on our understanding of circadian biology in vertebrates, including mechanisms of human circadian dysfunction that alter core clock components homologous to those characterized in *Drosophila* (Toh, Jones, He, Eide, & Hinz, 2001; Xu, Padiath, Shapiro, Jones, & Wu, 2005).

More recently, *Drosophila* has been used to study sleep, a behavior that is functionally linked to the circadian clock. Like other invertebrates that have been carefully examined (Campbell & Tobler, 1984), *Drosophila* displays the key behavioral attributes of sleep (Hendricks, Finn, Panckeri, & Chavkin, 2000; Shaw, Cirelli, Greenspan, & Tononi, 2000). These attributes include postural changes specific to sleep, immobility correlated with an increased arousal threshold, a homeostatic rebound in sleep duration and intensity after

sleep deprivation, changes in brain electrical activity during sleep (Nitz, van Swinderen, Tononi, & Greenspan, 2002), and alterations in sleep by stimulants and hypnotics that parallel their effects in mammals (Hendricks et al., 2000; Shaw et al., 2000). Recently, it has been suggested that sleep in fruit flies, like that of humans, has different stages of depth during the sleep cycle (van Alphen, Yap, Kirszenblat, Kottler, & van Swinderen, 2013).

Although the adoption of *Drosophila* as a model organism to study sleep is relatively recent, considerable enthusiasm exists for its potential impact on our understanding of the molecular underpinnings of sleep regulation and function. Despite intensive studies over the past several decades, many aspects of sleep have remained elusive.

How sleep is regulated by circadian inputs and in a homeostatic manner (Borbély, 1982) is one focus of investigation. A second focus concerns the essential functions of sleep, as well as how sleep or lack thereof affects other physiological and behavioral processes. Theories for the functions of sleep invoke memory consolidation, synaptic downscaling, cell repair, metabolic and immune augmentation, and removal of toxins from the brain (Crocker & Sehgal, 2010; Xie et al., 2013). How sleep might function within the brain and somatic tissues to achieve these functions is still unclear, particularly at a molecular and cellular level, and these questions are the subject of several studies in *Drosophila*.

The impact of *Drosophila* in studies of circadian rhythms and sleep, as in other areas of biology, stems from the ability to perform large-scale and unbiased forward genetic screens and from powerful genetic tools that enable the fruits of these screens to be exploited (St Johnston, 2002). This chapter reviews recent genetic screens to gain further insight into the molecular basis circadian rhythm and sleep. We touch briefly on prior screens for rhythm and sleep mutants and proceed to the genetic screens for circadian rhythm and sleep that have been performed in recent years with an emphasis on transgenic mutagenesis in comparison with classical methods of genomic mutagenesis.

2. SCREENING FOR CIRCADIAN RHYTHM AND SLEEP MUTANTS

2.1. History of circadian rhythm screens

In their landmark 1971 study, Konopka and Benzer isolated the first mutants altering the rhythmicity of *Drosophila* circadian behavior (Konopka & Benzer, 1971). They conducted a screen with the goal of identifying genes

for so-called free-running behavior in constant darkness (dark:dark, DD) and described mutants of a locus they named *period* (*per*), which shortened, lengthened, or abolished the rhythmicity of eclosion and locomotor activity in constant darkness. The cloning of the *per* gene in 1984 (Bargiello, Jackson, & Young, 1984; Zehring et al., 1984) marked the onset of a "clockwork explosion" in genetic screens identifying the genetic basis and molecular characteristics of the circadian clock. It has been over 15 years since most of these screens were completed and uncovered the majority of the circadian components. Extensive review of these earlier screens is not the subject of this review and can be found elsewhere (Blau et al., 2007, Price, 2005, Stanewsky, 2003).

While the first rhythm screens utilized measurement of eclosion behavior to identify mutants, later higher throughput screens monitored the rhythmicity of locomotor behavior in individual animals and its persistence in free-running conditions (Stanewsky, 2003). *Drosophila* means "dew-loving," and when put in a 12 h light–12 h dark cycle (12:12 LD), flies are indeed most active during dawn and dusk, and sleep most of the day and night (Fig. 1). In free-running conditions without any light or temperature cues, flies continue to wake at the beginning of the subjective day and sleep during the subjective night. Mutants deficient in clock components cannot maintain wild-type (~24 h) rhythmicity in DD and, depending on the type of mutation, display shortened or lengthened rhythms, or become completely arrhythmic.

A variety of mutagenesis methods have been used to identify clock mutants in *Drosophila*, reviewed in Stanewsky (2003). Chemical mutagenesis by feeding ethyl methanesulfonate (EMS) has been widely used to induce circadian mutants (Stanewsky, 2003). Other screens utilized gamma irradiation as a mutagen to screen for genes affecting the clock (Newby et al., 1991; van Swinderen & Hall, 1995). The advent of P-element screens, in which the integration of a transposable element disrupts a gene's expression, saw their adoption to identify circadian mutants (Kloss et al., 1998; Newby & Jackson, 1993; Price et al., 1998; Sehgal, Price, Man, & Young, 1994). Subsequent gain-of-function screens employed modified P-elements containing *UAS* elements (Rørth, 1996), enabling adjacent genes to be transcribed and overexpressed in the presence of the *Gal4* transcriptional activator (Brand & Perrimon, 1993). A neuroanatomically restricted screen in cells expressing the core clock gene *timeless* (*tim*) was performed using a *tim-Gal4* promoter (Martinek, Inonog, Manoukian, & Young, 2001), identifying a circadian role for the *glycogen synthase*

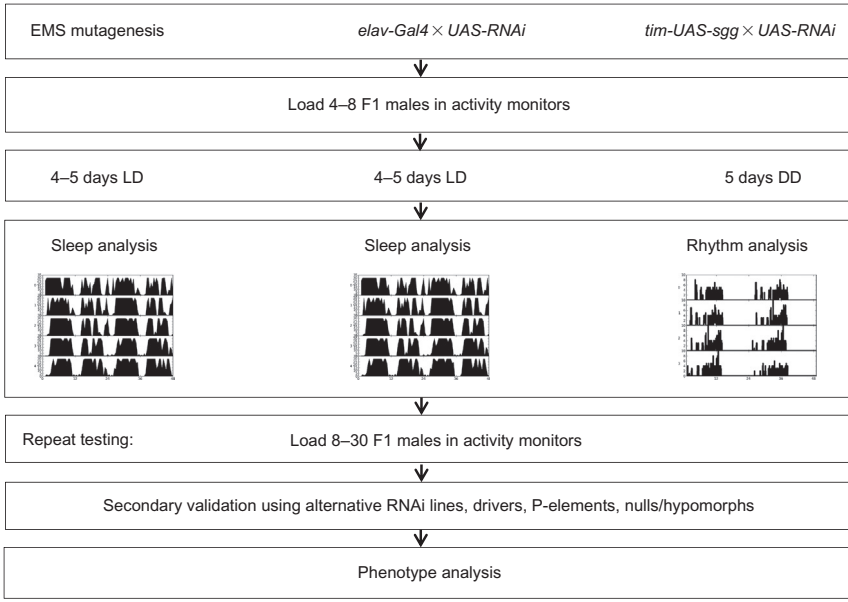

Figure 1 Workflow of three recent screens for circadian behavior. The workflow depicts differences and similarities in the screening process. We employed different strategies to obtain genetic nulls and hypomorphs, either in the whole fly or in specific cell types. Depending on the goal of the screen, behavioral assays were focused either on recording sleep, which is carried out in LD, or rhythmic behavior, which is conducted in DD. While circadian rhythms can be detected with data collections every 5 min or even every 30 min, measuring sleep requires a higher data resolution of at least 1 min bins. In all three screens, data were acquired in 1 min bins. Candidate genes were subjected to rounds of rescreening and secondary validation including available genetic tools. Phenotypic analysis of confirmed candidates includes identifying a gene's expression pattern, cells in which its function is required, effects on other behaviors, and molecular analyses of the protein function.

kinase-3 (*gsk-3*)*/shaggy* (*sgg*). As discussed below, recent screens for circadian rhythms and sleep continue to exploit the *Gal4/UAS* system in conjunction with current genetic tools for the manipulation of gene function.

2.2. History of sleep screens

Many years of research in mammals have begun to elucidate different aspects of sleep. These include the discovery of different sleep stages using electroencephalography (EEG), the role of various neurotransmitters and genetic pathways in promoting or inhibiting sleep, and the identification of brain loci important for sleep regulation (Crocker & Sehgal, 2010). The discovery that flies display key behavioral criteria for sleep has been followed by both

reverse and forward genetic strategies to address the mechanisms underlying sleep regulation and function. Studies employing reverse genetics have focused on candidate genes and pathways, many of which are known to impact sleep in mammals (Bushey & Cirelli, 2011; Crocker & Sehgal, 2010). Genetic manipulation of neurotransmitter systems and neuropeptide signaling in *Drosophila* has shown that neurochemical modulation of sleep is similar in *Drosophila* and vertebrates. Neurotransmitters including dopamine (Kume, 2005), gamma-aminobutyric acid (GABA, Agosto et al., 2008), octopamine/norepinephrine (Crocker & Sehgal, 2008), serotonin (Yuan, Joiner, & Sehgal, 2006), histamine (Oh, Jang, Sonn, & Choe, 2013; Yi et al., 2013), and acetylcholine (Yi et al., 2013) impact sleep in *Drosophila*. Neuropeptide Y/F (He, Yang, Zhang, Price, & Zhao, 2013; Shang et al., 2013) and various signaling pathways including the CREB (Hendricks et al., 2001), extracellular signal-regulated kinase (ERK, Foltenyi, Greenspan, & Newport, 2007), and protein kinase A (Hendricks et al., 2001; Joiner, Crocker, White, & Sehgal, 2006) pathways impact sleep in *Drosophila* as in mammals. Alongside these candidate gene approaches, expression profiling (Cirelli, LaVaute, & Tononi, 2005; Williams, Sathyanarayanan, Hendricks, & Sehgal, 2007) and selective breeding (Seugnet, Suzuki, & Thimgan, 2009) have been used to identify genes that impact sleep, while neuroanatomically driven strategies, utilizing electrical and genetic manipulations, have implicated various neuronal populations in the regulation of sleep (Cavanaugh et al., 2014; Joiner et al., 2006; Pitman, McGill, Keegan, & Allada, 2006).

Several large-scale screens for sleep mutants have been reported (Cirelli, Bushey, et al., 2005; Koh et al., 2008; Liu et al., 2014; Pfeiffenberger & Allada, 2012; Rogulja & Young, 2012; Shi, Yue, Kuryatov, Lindstrom, & Sehgal, 2014; Stavropoulos & Young, 2011). These screens have largely focused on sleep duration and have led to the isolation of mutants that strongly reduce the length and consolidation of sleep. Two screens have yielded mutations in genes that regulate neuronal excitability, including the *Shaker* potassium channel (Cirelli, Bushey, et al., 2005) and *quiver/sleepless* (Koh et al., 2008), an extracellular membrane-linked peptide that alters *Shaker* expression and trafficking (Wu, Robinson, & Joiner, 2014). Mutations in *Hyperkinetic*, the beta-subunit of the *Shaker* channel, reduce sleep duration similarly (Bushey, Huber, Tononi, & Cirelli, 2007). The isolation of a short sleep mutant in the *redeye* allele of the α4 subunit of the nicotinic acetylcholine receptor (Shi et al., 2014) provides additional evidence for the modulation of arousal and sleep/wake states by neuronal excitability.

Other mutants implicate novel mechanisms by which sleep may be regulated. Cloning and characterization of *insomniac* have raised the possibility that neuronal protein degradation pathways may contribute to the control of sleep duration (Stavropoulos & Young, 2011), and the isolation of *cyclin A* (Rogulja & Young, 2012) has indicated a neuronal function of a broadly essential gene in regulating sleep.

Sleep timing is considered to be regulated by two inputs: the circadian clock determining a sensible daily sleep time and a yet elusive homeostat, which measures sleep pressure and could override circadian sleep timing (Borbély, 1982). This idea is supported by the observation that animals exhibit rebound sleep after sleep deprivation, which occurs independently of circadian timing and is accompanied with EEG changes in humans (Borberly & Achermann, 1999). Along with total daily sleep, researchers now can measure various sleep parameters including (a) number of sleep bouts and their length, which indicate sleep fragmentation; (b) sleep latency, pointing to difficulty to initiate sleep or circadian components; and (c) sleep homeostasis, which is measured as the amount of rebound sleep following sleep deprivation.

The first generation of screens identified mutants based on a reduction of total daily sleep, and recovered a handful of mutants that exhibit a reduction of daily sleep greater than 50% (Cirelli, Bushey, et al., 2005; Koh et al., 2008; Liu et al., 2014; Pfeiffenberger & Allada, 2012; Rogulja & Young, 2012; Shi et al., 2014; Stavropoulos & Young, 2011). The observed sleep reduction could be the result of either circadian or homeostatic disturbance. Recent studies are focusing on specific sleep parameters including sleep latency (Liu et al., 2014) and sleep homeostasis (Bushey & Cirelli, 2011) to tease apart each pathway's contribution to sleep. While the sleep patterns of published short-sleeping mutants appear fragmented compared to wild-type flies, they still show a normal circadian rhythm in constant darkness, suggesting that the circadian input into the timing of sleep is not affected (Cirelli, Bushey, et al., 2005; Koh et al., 2008; Liu et al., 2014; Rogulja & Young, 2012; Shi et al., 2014; Stavropoulos & Young, 2011). However, a detailed analysis of the short-sleeping mutant *wide awake* revealed an increased sleep latency (Liu et al., 2014). *wide awake* is strongly expressed in clock cells and its effect on latency is dependent on the core circadian clock gene *Clock*, despite exhibiting rhythmicity in constant darkness, pointing toward separate pathways for circadian activity and sleep mediated by *Clock*. Despite large-scale screening specifically looking for sleep homeostasis, so far no mutant has been found to specifically affect

rebound sleep (Bushey & Cirelli, 2011). Of the published short-sleeping mutants, *sleepless* as well as *cyclinA* show reduced rebound sleep after sleep deprivation, suggesting a function in sleep homeostasis (Koh et al., 2008; Rogulja & Young, 2012).

For the most part, the precise mechanisms of the sleep genes' function remain unknown, although some mutants have been linked to mechanisms regulating synaptic transmission (Wu et al., 2014), to the GABA pathway (Chen et al., 2014), and to the circadian clock (Liu et al., 2014).

3. SCREENING TECHNIQUES

Various techniques have been used to induce genetic lesions in *Drosophila*. We are briefly reviewing EMS and transposon mutagenesis in the context of rhythm and sleep screens and then focus on transgenic techniques of gene inactivation. To illustrate some of the mutagenesis techniques, we discuss one EMS and two RNAi screens, one of which is unpublished, in more detail (Fig. 1).

3.1. EMS mutagenesis

EMS mutagenesis has been used for over 45 years to analyze gene function (Lewis & Bacher, 1968). Its power lies in the simplicity of administration, by feeding, and its ability to induce high mutation rates (Greenspan, 2004). Although there is increased lesion frequency in "hotspots" (Bentley, MacLennan, Calvo, & Dearolf, 2000), its ability to create various types of genomic lesions in an unbiased manner is paramount (Bökel, 2008). To illustrate the approach and workflow of EMS mutagenesis, we discuss a recent EMS screen for sleep mutants in the following section.

3.1.1 X-linked EMS screen for sleep mutants

We conducted a chemical mutagenesis screen to find novel genes affecting the flies' sleep. We selected a recently isogenized Canton S (CS) strain displaying well-consolidated nighttime sleep and screened the X-chromosome using a mating scheme in which we screened four F2 males from each mutagenized line (Fig. 2). Screening four animals for each mutant line permits increased screening throughput at the cost of additional rescreening. We screened more than 3500 lines and rescreened 471 lines (13.5%) assessed as exhibiting potentially different sleep patterns than wild-type animals. In most cases, a single round of rescreening is sufficient to discard lines. After multiple rounds of rescreening, approximately 50 stocks were assessed to

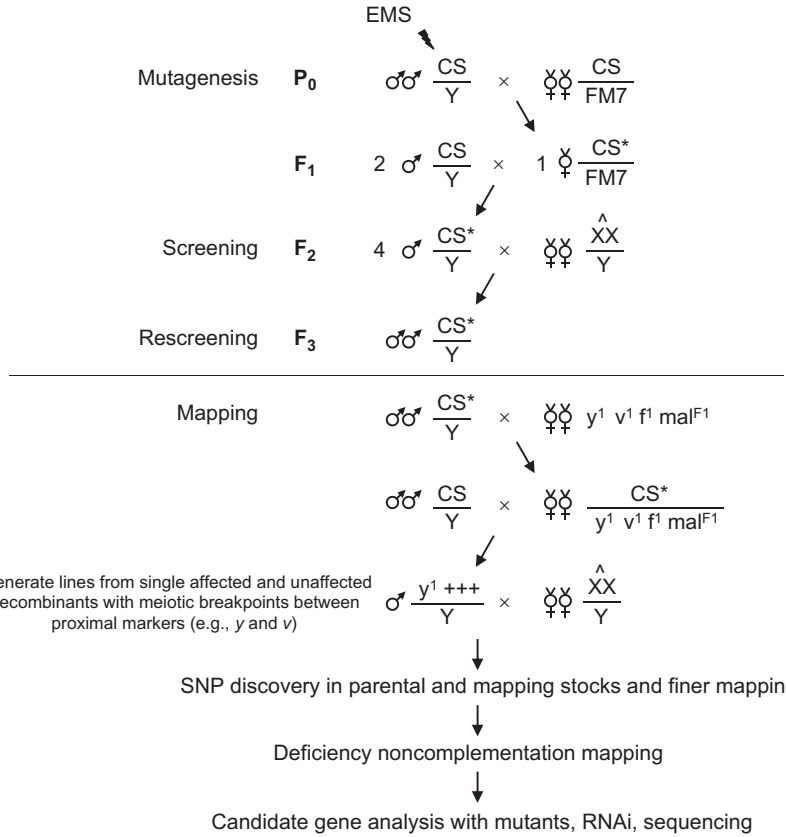

Figure 2 Crossing and mapping scheme employed in the EMS mutagenesis screen for sleep mutants. Wild-type Canton S (CS) males were mutagenized with 25–40 m*M* EMS and crossed en masse to FM7/CS virgins. F1 virgins were backcrossed to wild-type CS males. Four F2 males were assayed for sleep behavior and potential hits were backcrossed to isogenized attached-X virgins for rescreening. For genomic mapping of the *inc* mutation, balanced males were crossed to virgins from a mapping stock. Recombination analysis revealed that *inc* lies proximal to *y*. Further polymorphism mapping narrowed down the cytological location to a 250-kb to 1-mb window. Finally, deficiency noncomplementation analysis of the genomic region identified a 190-kb stretch that failed to complement the *inc* mutation and was used for further sequencing analysis. Asterisk (*) indicated mutated chromosome; iso, isogenized; *y*, *yellow*; *v*, *vermillion*; *f*, *forked*; *mal*, *maroon-like*.

potentially bear X-linked phenotypes of interest (1.4%). Sixteen candidates have extremely short sleep with less than 508 min of daily sleep, which is more than three standard deviations from the mean of 901 min/day, and five flies had extremely long sleep (>3 standard deviations above the mean) with

more than 1295 min of daily sleep. Three mutant lines with a severe reduction of sleep were selected for further analysis, two of which are alleles of the already described *Shaker* mutation (Cirelli, Bushey, et al., 2005) as assessed by noncomplementation and shaking under ether anesthesia (data not shown). The third mutant was further characterized and mapped to an intergenic region between CG14795 and CG32810 (Fig. 2). Using a line carrying a transposable element disrupting CG32810 and replicating the phenotype, it was shown that this is the gene responsible for the sleep phenotype. We called the gene *insomniac* (*inc*) and published a detailed description of this novel gene, its phenotype, and cellular and molecular characteristics (Stavropoulos & Young, 2011).

3.2. Transposon mutagenesis

Mutagenesis via P-element transposition appeared in the 1990s and had the advantage that P-elements carry an identifiable sequence allowing for direct gene cloning. Such screens yielded the two clock mutants *tim0* (which, however, turned out to be unrelated to the P-element insertion, Sehgal et al., 1994) and *lark* (Newby & Jackson, 1993). These screens mobilized existing P-elements on the X, second, and third chromosomes by crossing P-element containing flies to flies harboring a transposase transgene (for explicit methodology and mating scheme, see Price, 2005). Since 1991, the *Drosophila* gene disruption project has been aiming to generate transposon insertions in all *Drosophila* genes and is now covering at least two-thirds of the *Drosophila* genome (Bellen et al., 2011). Ordering flies from this (http://flystocks.bio.indiana.edu/Browse/in/GDPtop.htm) and other (Kim et al., 2010; Ryder, 2004; Thibault et al., 2004) libraries has been allowing researchers to directly screen flies containing different P-element insertions for phenotypes without having to map or clone the mutation because P-elements' positions are annotated with single-nucleotide resolution (http://flybase.org/). Such screens identified one of the alleles of the clock gene *double-time* (Price et al., 1998) as well as the sleep genes *sleepless* and *wide awake* (Koh et al., 2008; Liu et al., 2014).

Some of the P-elements used to create libraries carry *UAS* gene activation sequences, which can be used to express neighboring DNA sequences if a *Gal4*-driver is also present. Martinek et al. used such a library of 2300 so-called EP lines (Rørth, 1996) to screen for circadian genes (Martinek et al., 2001). Crossing the EP lines to a *Gal4* driver line containing a *tim* promoter fused to *Gal4* (*tim*(*UAS*)-*Gal4*) led to F1 progeny where the DNA

adjacent to the EP transgene was only expressed in *tim*-expressing clock neurons. This screen discovered a circadian function for the kinase *glycogen synthase kinase-3/shaggy* (*gsk-3/sgg*).

In addition to genomic mutants as produced by chemical and insertional mutagenesis, certain scientific questions benefit from a more targeted approach in which a gene of interest is removed from specific cells or at a specific time. To this end, different techniques have been developed. While EMS and P-element mutagenesis have been used for decades and extensively reviewed, we focus our discussion of the transgenic techniques of gene manipulation and inactivation.

3.3. Tools for conditional transgene expression

Many *Gal4* drivers have been created to drive transgene expression only in specific cells and tissues. For circadian research, a number of drivers exist that drive transgene expression in different subsets of neurons, including clock gene-expressing neurons (Yoshii, Rieger, & Helfrich-Frster, 2012). Moreover, researchers now have access to vast driver libraries containing drivers for randomly selected genes (8000 lines, Kvon et al., 2014) and many neuronal subgroups (7000 lines, Jenett et al., 2012). By combining *Gal4* expression with neuronal inactivation, researchers can screen for neuronal subpopulations required for a specific function (Cavanaugh et al., 2014; Joiner et al., 2006; Pitman et al., 2006). By using *Gal80ts*, a temperature-sensitive inhibitor of the gene expression driver *Gal4*, *Gal4* expression can be temporally and spatially limited (McGuire, 2003). Another method for conditional gene expression is the modified *Gal4* driver GeneSwitch, which is only activated after binding of the activator RU486, which is fed to flies (Osterwalder, Yoon, White, & Keshishian, 2001). In addition to *Gal4*, other systems exist for transgene expression, and all systems can be used interjectionally (Venken, Simpson, & Bellen, 2011) to express transgenes only in specific cells and/or at a specific time.

For genetic screens in specific tissues, transgenic RNA interference (RNAi) is widely used to study clock and sleep function on a genome-wide scale, both in *Drosophila* and other organisms (Chung, Kilman, Keath, Pitman, & Allada, 2009; Itoh & Matsumoto, 2012; Mandilaras & Missirlis, 2012; Rogulja & Young, 2012; Zhang et al., 2009).

It is believed that RNAi evolved to fight transposons and RNA viruses and comprises an intracellular machinery for the targeted destruction of specific mRNAs (Shabalina & Koonin, 2008). The discovery that this

machinery can be exploited by researchers to specifically remove mRNAs of specific genes opened a new posttranscriptional method to analyze gene function without having to modify the genome itself (Dykxhoorn & Lieberman, 2005).

RNAi is based on the complementarity of a double-stranded RNA (dsRNA) molecule to an endogenous mRNA, which leads to destruction of complementary mRNA molecules. While in mammalian systems short interfering RNAs (siRNAs) have been used for RNAi, in *Drosophila* mostly long dsRNAs (300–600 bp long) have proven efficient. In addition to cell-based RNAi, researchers also have been making use of transgenic RNAi *in vivo* by fusing the complementary sequence to a *UAS* element thereby allowing knockdown of a given gene using tissue-specific *Gal4* drivers (Perrimon, Ni, & Perkins, 2010).

3.4. *Drosophila* RNAi libraries and screens

Now we have vast libraries with commercially available RNAi lines containing transgenic *UAS-RNAi* constructs, which are used for genome-wide RNAi screens. We want to provide an overview of the resources available for such screens as well as juxtapose RNAi with other types of mutagenesis.

To date, there are three main sources for RNAi lines: VDRC Vienna, Nig-Fly Japan, and TriP Harvard in Boston (Table 1). The VDRC stock center in Vienna consists of two libraries: the GD and the newer KK library together targeting ca. 12,000 genes or ca. 90% of the *Drosophila* protein-coding genome. The GD library was generated using random P-element-mediated transformation, regularly yielding multiple lines per construct, and covers 84% of the genome. The second-generation KK library covers 67% of genes and was generated using targeted insertion with the PhiC31 integrase into a defined attP landing site on the second chromosome to minimize position effects. These lines are considered in general more efficient than the GD lines (S. Axelrod, data not shown). The Japanese library was generated using random P-element-mediated transformation, with possible insertion sites on all three chromosomes. For many genes, multiple lines exist with variable RNAi efficiency depending on the genomic environment of the insertion site creating position effects suppressing or enhancing transgene expression. To test RNAi efficiency, all available RNAi lines have been crossed to an *actin-Gal4* driver thereby leading to ubiquitous RNAi expression. By assessing the survival of the next generation, NIG-Fly assessed the efficiency of a given RNAi line, lethality indicating strong expression of RNAi transgenes. *actin-Gal4*-induced lethality provides

Table 1 Resources for *in vivo* RNAi

Library	Number of lines	Type of insertion	Reference	Comments
Nig-Fly Japan	11,000	Random	http://www.shigen.nig.ac.jp/fly/nigfly	Multiple lines per gene, all lines crossed to *actin-Gal4* for lethality assessment
VDRC Vienna	25,259		http://stockcenter.vdrc.at, Dietzl et al. (2007)	
GD library	16,442	Random		Multiple lines per gene
KK library	9817	Site directed		One line per gene
TRiP Harvard	9128	Site directed	http://flyrnai.org/TRiP-HOME.html, Ni et al. (2009, 2011)	
TRiPSoma	2486			No germline expression
TRiPGermline	6638			1605 Expressed only in germline, 5033 in both

valuable information about the efficiency of a given RNAi line. The Harvard TriP library was generated using targeted insertion with the PhiC31 integrase in two characterized landing sites on the second and third chromosome. The landing sites had been chosen both for minimizing leaky expression of the *UAS-RNAi* transgenes in absence of *Gal4* and for maximizing RNAi expression in the presence of *Gal4*. There are two collections of lines, TriPSoma and TriPGermline, utilizing different vectors optimized for either somatic (TriPSoma) or germline/germline and somatic expression (TriPGermline) of RNAi (Table 1). While the TriPSoma lines utilize the regular long dsRNA method to induce RNAi, the second-generation TriPGermline lines utilize microRNA-mediated knockdown.

To illustrate the methodology and approach of RNAi, in the next section we outline two recent RNAi screens, one for rhythm and one for sleep mutants.

3.4.1 RNAi screen for suppressors and enhancers of shaggy

Many genes have been identified to be required for clock function, and progress has been made in delineating the molecular functions of the proteins

involved. However, it is still largely unclear how different clock proteins interact with each other to achieve their effect. One of the main mysteries of the clock is how the exact timing of 24 h is established and how delays are built into the clock to prevent clock proteins from advancing the period. Posttranslational modifications such as phosphorylation and glycosylation are generally good candidates for such regulation of protein function (Petsko & Ringe, 2004). Indeed, three kinases have been identified to be required for correct clock timing: Double-time (Price et al., 1998), Casein kinase II (CKII, Akten et al., 2003), and Glycogen synthase kinase-3, Shaggy (GSK-3, SGG, Martinek et al., 2001). In addition, it has been shown that the core clock genes PER and Clock are glycosylated, which has been proposed as a mechanism for fine-tuning the clock (Kaasik et al., 2013; Kim et al., 2012; Li et al., 2013).

sgg was found in a screen that employed a library of transgenic lines called EP lines carrying P-element insertions (Martinek et al., 2001). DNA adjacent to the P-element insertion site was expressed in clock neurons using *tim*(*UAS*)-*Gal4*. One of the EP lines, when expressed in clock neurons, shortened the period by roughly 3–20.3 h. Inverse PCR analysis mapped the insertion to the *sgg* gene. *sgg* is an essential gene and null flies die during development, restricting analyses of *sgg*'s function in the circadian rhythm to conditional misexpression experiments. While overexpression of *sgg* shortens the period length, reducing *sgg* function lengthens the rhythms to 26 h. In addition, *sgg* overexpression advances nuclear entry and leads to increased phosphorylation of TIM, indicating that SGG advances the circadian clock through acting on TIM. However, in a 2007 paper, Stoleru et al. (2007) showed that when the circadian photoreceptor, *cryptochrome* (*cry*), is mutated, *sgg* overexpression only shortens the period by 1 h, instead of 3 h. How *sgg* function is affected by the presence of the light receptor *cry* is unclear. In 2010, Edery's group showed that SGG specifically phosphorylates a serine in PER, and that abolishing that site leads to longer behavioral rhythms (Ko et al., 2010).

The kinase encoded by *sgg* is intriguing because it is involved in several cellular and developmental processes including metabolism (Garofalo, 2002), growth (Woodgett, Plyte, Pulverer, Mitchell, & Hughes, 1993), cell fate determination (Siegfried, Chou, & Perrimon, 1992), and as the main target of lithium, a pharmaceutical drug used to treat bipolar disorder, potentially linking this mental illness to circadian dysfunction.

We are currently conducting a suppressor/enhancer screen to find genetic *sgg* interactors (Fig. 3). Such a modifier screen has been successfully

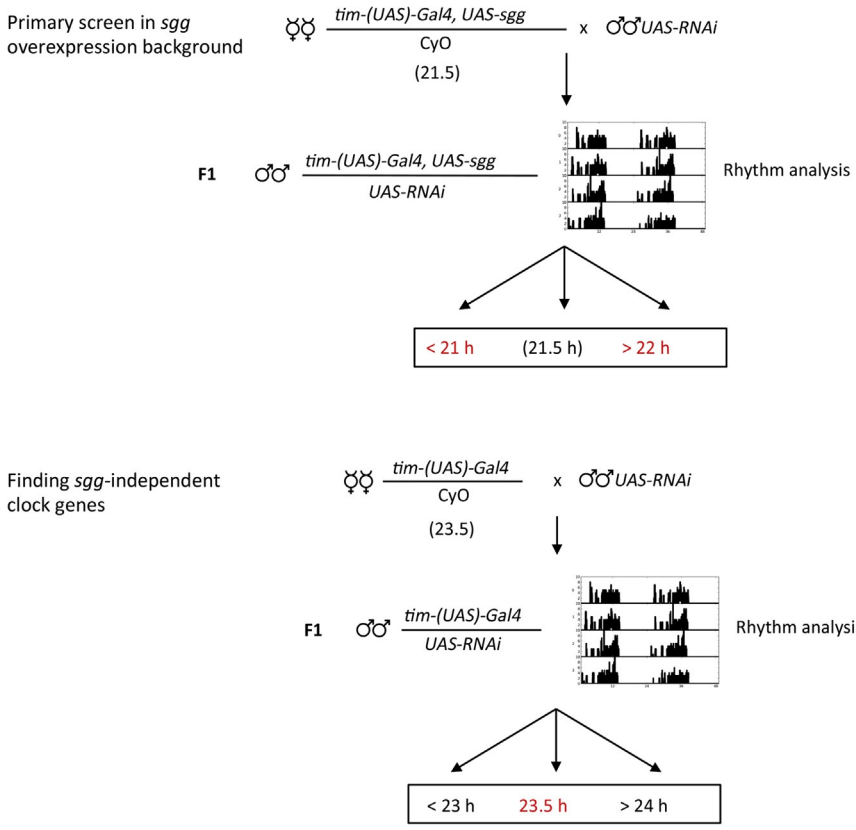

Figure 3 Screen for interactors of *sgg*. In a first step, random genes are knocked down in *tim*-expressing flies in a *sgg*-overexpression background. RNAi lines producing lengthening or shortening of this period are in a second stop recrossed to the *tim-Gal4* line alone to identify sgg-independent clock genes.

used in the past to find specific interactors of short and long period producing alleles of *per* (Rutila et al., 1996). We are using a *tim*(*UAS*)-*Gal4*, *UAS*-*sgg* line to overexpress *sgg* in *tim*-expressing neurons, which produces a period of 21.5 h. We then use this line to drive expression of RNAi in the same cells and monitor free-running activity rhythms in constant darkness. Gene knockdowns that change the period length in this assay (below 21 h or above 22 h) are candidates for *sgg*-specific suppressor or enhancers. To identify whether these candidates genes are general clock mutants, RNAi lines are retested without *sgg* overexpression. In some cases, the phenotype persists without *sgg* overexpression indicating potential direct involvement of such genes in the clock. To this day, we screened ca.

3000 lines representing ca. 2500 genes. In around 110 lines, we observed a change in rhythm (ca. 3% of tested lines). Notably, we recovered 10 known clock genes from the screen so far, which serves as proof of principle: *circadian trip*, *cycle*, *PAR-domain protein 1*, *per*, *ckIIα*, *ckIIβ*, *Clock*, *resistant to dieldrin*, *ras homolog enriched in brain ortholog*, and *cullin3*, a gene described to be involved in both sleep and circadian rhythms (Grima, Dognon, Lamouroux, Chélot, & Rouyer, 2012; Stavropoulos & Young, 2011). Among the other lines with changed rhythm are genes affecting several cellular processes including transcription, chromatin remodeling, ribosomal function, odorant binding, cytoskeleton, phosphorylation and dephosphorylation, Zn binding, splicing, and many genes whose function is unknown. This screen is proving to be a useful source to find new clock genes, as well as identify specific *sgg* interactors. To validate the results, we are using alternative RNAi lines both from Japan and VDRC. To narrow down the candidate genes' requirement in specific cells, we use different overlapping and non-overlapping *Gal4* drivers.

3.4.2 Neuronal RNAi screen for sleep mutants

While EMS mutagenesis provides an unbiased approach to finding any genes potentially involved in the process of sleep, we also wanted to take a more targeted approach. Intuitively, the brain seems to be the most likely source of potential sleep signals, and therefore, we tried to find genes required for sleep in neurons. To this end, virgins from the strain *elav-Gal4;UAS-dicer2* (Lin & Goodman, 1994) were crossed to males from randomly selected RNAi lines (NIG-Fly, Japan).

If there were multiple RNAi lines available, we chose lines producing lethality when crossed to *actin-Gal4* because that indicates stronger RNAi efficiency than lines not producing lethal phenotypes. Approximately, 4000 lines covering ca. 3500 genes were tested. Males were collected after eclosion and aged for 1–5 days, and four males of each genotype were loaded into *Drosophila* Activity Monitoring Systems (Trikinetics). The screen was performed at a slightly elevated temperature of 26 °C/27 °C to reduce background levels of activity, and flies were assayed for at least 4 days in 12 h light, 12 h dark cycles (LD). To validate screen results, we retested initial hits three times with 8–16 flies. For interesting candidates, alternative RNAi lines were tested, which produced varying results. If available, null mutants were tested. Around 10% of screened neuronal genes produced lethality when knocked out in the nervous system. Of the nonlethal hits that survived the multiple rounds of retesting, 20 genes remain that show robust and

reproducible phenotypes. Genes from this screen fall into various categories including membrane proteins, transcription factors, and RNA-binding proteins. Of the 20 genes, 14 show a reduction of sleep, 4 show an increase, and 2 show other activity changes, e.g., predominantly nocturnal activity. One of the genes whose knockdown in neurons severely reduced sleep is the *regulator of cyclin A1* as well as its target, *cyclin A*. We published an in-depth analysis of these phenotypes in 2012 (Rogulja & Young, 2012).

This screen demonstrates the usefulness of transgenic RNAi for *in vivo* studies of behavior and sleep in a spatially defined manner: while the genes discovered in this screen might have other functions or even be developmentally required, only removing their expression in specific neurons enables us to address their function in this specific context.

3.5. Advantages and drawbacks of screening with RNAi in comparison to chemical and transposon mutagenesis

The creation of *UAS-RNAi* libraries provides a quick and simple means to reduce gene function in conjunction with *Gal4* drivers. Large-scale RNAi screens offer several advantages over classical chemical and transposon mutagenesis screens. First, RNAi screens can be performed with a single cross of a *Gal4* driver of interest to *UAS-RNAi* lines, enabling F1 progeny to be screened. In contrast, chemical mutagenesis (Cirelli, Bushey, et al., 2005; Shi et al., 2014; Stavropoulos & Young, 2011) and transposon screens (Koh et al., 2008; Liu et al., 2014) require additional generations and longer breeding schemes to obtain progeny of interest. For "shelf" screens using existing transposon insertions, the sensitivity of sleep to genetic background and the possible accumulation of suppressor mutations require additional generations of backcrossing prior to homozygosis (Koh et al., 2008). A second, and important, advantage of RNAi screens is that they provide the immediate identity of targeted genes. Chemical mutagenesis, in contrast, generates unmarked lesions whose mapping and positional cloning is laborious and time consuming (Stavropoulos & Young, 2011). Mapping and positional cloning EMS-induced sleep mutants in the absence of other phenotypic hints (e.g., Cirelli, Bushey, et al., 2005) remain a challenge, even with the availability of polymorphism libraries (Berger et al., 2001) and whole-genome sequencing (Shi et al., 2014). A third advantage of RNAi screens is that they can be directed in an anatomically and temporally restricted manner as dictated by *Gal4* drivers. In particular, anatomically restricted RNAi screens can identify tissue-specific contributions of essential genes, as the lethality of null mutations in these genes would preclude their

isolation from chemical mutagenesis screens (Rogulja & Young, 2012). A substantial fraction (25–30%) of all genes in *Drosophila* are essential (Miklos & Rubin, 1996).

Alongside these advantages, RNAi screens have several potential drawbacks that should be considered.

1. *False positives*. The long dsRNA is transcribed from the RNAi construct and processed by the cellular RNAi machinery to create the specific siRNAs targeting the desired mRNA. siRNAs can have off-target effects by binding not (only) to the desired sequence of the target gene but also to other homologous sequences in other genes. The observed phenotypic effect could then be the result of creating unwanted knockdowns in other genes and not of the gene in question (Kulkarni et al., 2006). A recent report from Green, Fedele, Giorgini, and Kyriacou (2014) shows that the host strain for the KK library from VDRC contains not one but two landing sites and that multiple stocks contain two transgenes creating nonspecific phenotypes.

   To mitigate against the possibility of false positives, different RNAi lines that target nonoverlapping portions of a transcript of interest should be used wherever possible (Yamamoto-Hino & Goto, 2013). Also, validation of RNAi at the level of reduced protein or mRNA levels as well as other means of secondary validation should be employed to rule out false positives (Echeverri et al., 2006; Perrimon et al., 2010). In particular, to ascertain that a phenotype stems from knockdown of a particular gene, RNAi can be combined with a recessive genomic loss-of-function allele of the same gene, with enhancement of the phenotype of RNAi pointing to further transcript loss of the same gene (Rogulja & Young, 2012). Also knockdown of genes in the same pathway yielding a similar phenotype is indicative of an on-target effect (Rogulja & Young, 2012). Another validation approach is rescuing the RNAi phenotype by expressing an RNAi-resistant version of the target gene (Yamamoto-Hino & Goto, 2013).
2. *False negatives*. The efficiency of RNAi is variable and may not reduce protein abundance sufficiently to induce phenotypes, even when genes have a role in the process under study.

   Only 60% of lines from the first GD library from VDRC reportedly produced a knockdown. Overexpression of the enzyme *dicer2*, which produces the siRNAs targeting specific mRNAs for degradation (Lee et al., 2004), enhances RNAi efficiency by ca. 50% and is routinely used by researchers to increase knockdown effects (e.g., Neely et al., 2010;

Neumüller et al., 2011; Rogulja & Young, 2012). Although newer KK and TRiP lines incorporate design elements for enhanced expression and RNAi transgenes are inserted in optimized genomic landing sites (Ni et al., 2009; Yamamoto-Hino & Goto, 2013), RNAi efficiency remains variable as it depends on many factors, only one of which is the actual siRNA production and their target affinity (Booker et al., 2011). Knockdown efficiency also depends on the transcript levels of a given gene, as well as the reduction of protein necessary to achieve a phenotype (Mohr & Perrimon, 2011). Protein turnover varies for different types of proteins, potentially hampering knockdown of very stable proteins (Scott et al., 2013). Using RNAi lines differing in construct sequence and/or insertion site (Yamamoto-Hino & Goto, 2013), enhancement of RNAi efficiency by adding *UAS-dicer2* (Dietzl et al., 2007) or using multiple copies of *Gal4* and/or *UAS* (S. Axelrod, data not shown) can be used to increase RNAi efficiency. To study highly redundant processes or paradigms where partial loss of function is unlikely to yield phenotypes, performing screens in a sensitized background could be useful. To validate candidate genes, comparing the phenotype to that of animals carrying null mutations or disruptive P-element insertions can be used to identify false-negative results.

3. *Target limitations*. All present RNAi libraries target protein-coding genes. For analysis of noncoding DNA regions, including regulatory regions and the various species of noncoding RNA, this approach cannot be employed and in this field the EMS and transposon mutagenesis methods are more useful (Sarin et al., 2010).

By combining unbiased and targeted approaches, investigators in the circadian rhythm and sleep fields are currently trying to expand our knowledge of behavior in different and complementary ways. Sleep screens help us to shed light on the molecular mechanisms required for proper sleep. Modifier screens like the *sgg* screen go back to the old question of how the clock works, and in particular how the 24 h rhythm is so precisely established. This systematic approach—utilizing the genetic power of the fruit fly to uncover the molecular and cellular basis of circadian rhythm and sleep—gives researchers the opportunity to gain mechanistic insight into two behaviors that are fundamental to all living organisms.

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REFERENCES

Agosto, J., Choi, J. C., Parisky, K. M., Stilwell, G., Rosbash, M., & Griffith, L. C. (2008). Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. *Nature Neuroscience*, *11*(3), 354–359.

Akten, B., Jauch, E., Genova, G. K., Kim, E. Y., Edery, I., Raabe, T., et al. (2003). A role for CK2 in the Drosophila circadian oscillator. *Nature Neuroscience*, *6*(3), 251–257.

Bargiello, T. A., Jackson, F. R., & Young, M. W. (1984). Restoration of circadian behavioural rhythms by gene transfer in Drosophila. *Nature*, *312*(5996), 752–754.

Bellen, H. J., Levis, R. W., He, Y., Carlson, J. W., Evans-Holm, M., Bae, E., et al. (2011). The Drosophila gene disruption project: Progress using transposons with distinctive site specificities. *Genetics*, *188*(3), 731–743.

Bentley, A., MacLennan, B., Calvo, J., & Dearolf, C. R. (2000). Targeted recovery of mutations in Drosophila. *Genetics*, *156*(3), 1169–1173.

Berger, J., Suzuki, T., Senti, K.-A., Stubbs, J., Schaffner, G., & Dickson, B. J. (2001). Genetic mapping with SNP markers in Drosophila. *Nature Genetics*, *29*(4), 475–481.

Blau, J., Blanchard, F., Collins, B., Dahdal, D., Knowles, A., Mizrak, D., et al. (2007). What is there left to learn about the Drosophila clock? *Cold Spring Harbor Symposia on Quantitative Biology*, *72*(1), 243–250.

Bökel, C. (2008). EMS screens: From mutagenesis to screening and mapping. *Methods in Molecular Biology (Clifton, N.J.)*, *420*, 119–138.

Booker, M., Samsonova, A. A., Kwon, Y., Flockhart, I., Mohr, S. E., & Perrimon, N. (2011). False negative rates in Drosophila cell-based RNAi screens: A case study. *BMC Genomics*, *12*(1), 50.

Borbély, A. A. (1982). A two process model of sleep regulation. *Human Neurobiology*, *1*(3), 195–204.

Borberly, A. A., & Achermann, P. (1999). Sleep homeostasis and models of sleep regulation. *Journal of Biological Rhythms*, *14*(6), 559–570.

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development (Cambridge, England)*, *118*(2), 401–415.

Bushey, D., & Cirelli, C. (2011). From genetics to structure to function: Exploring sleep in Drosophila. *International Review of Neurobiology*, *99*, 213–244.

Bushey, D., Huber, R., Tononi, G., & Cirelli, C. (2007). Drosophila hyperkinetic mutants have reduced sleep and impaired memory. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *27*(20), 5384–5393.

Campbell, S. S., & Tobler, I. (1984). Animal sleep: A review of sleep duration across phylogeny. *Neuroscience and Biobehavioral Reviews*, *8*(3), 269–300.

Cavanaugh, D. J., Geratowski, J. D., Wooltorton, J. R. A., Spaethling, J. M., Hector, C. E., Zheng, X., et al. (2014). Identification of a circadian output circuit for rest: Activity rhythmsin Drosophila. *Cell*, *157*(3), 689–701.

Chen, W.-F., Maguire, S., Sowcik, M., Luo, W., Koh, K., & Sehgal, A. (2014). Glia interaction involving GABA transaminase contributes to sleep loss in sleepless mutants. *Molecular Psychiatry*, advance online publication, 18 March 2014, 1–12.

Chung, B. Y., Kilman, V. L., Keath, J. R., Pitman, J. L., & Allada, R. (2009). The GABAA receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. *Current Biology*, *19*(5), 386–390.

Cirelli, C., Bushey, D., Hill, S., Huber, R., Kreber, R., Ganetzky, B., et al. (2005). Reduced sleep in Drosophila Shaker mutants. *Nature*, *434*(7037), 1087–1092.

Cirelli, C., LaVaute, T. M., & Tononi, G. (2005). Sleep and wakefulness modulate gene expression in Drosophila. *Journal of Neurochemistry*, *94*(5), 1411–1419.

Crocker, A., & Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *28*(38), 9377–9385.

Crocker, A., & Sehgal, A. (2010). Genetic analysis of sleep. *Genes & Development*, *24*(12), 1220–1235.

de Mairan, J. J. D. (1729). Observation botanique. *Histoire de Academie Royale Sciences*, pp. 35–36.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*, *448*(7150), 151–156.

Dykxhoorn, D. M., & Lieberman, J. (2005). The silent revolution: RNA interference as basic biology, research tool, and therapeutic. *Annual Review of Medicine*, *56*(1), 401–423.

Echeverri, C. J., Beachy, P. A., Baum, B., Boutros, M., Buchholz, F., Chanda, S. K., et al. (2006). Minimizing the risk of reporting false positives in large-scale RNAi screens. *Nature Methods*, *3*(10), 777–779.

Foltenyi, K., Greenspan, R. J., & Newport, J. W. (2007). Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in Drosophila. *Nature Neuroscience*, *10*(9), 1160–1167.

Garofalo, R. S. (2002). Genetic analysis of insulin signaling in Drosophila. *Trends in Endocrinology and Metabolism*, *13*(4), 156–162.

Green, E. W., Fedele, G., Giorgini, F., & Kyriacou, C. P. (2014). A Drosophila RNAi collection is subject to dominant phenotypic effects. *Nature Methods*, *11*(3), 222–223.

Greenspan, R. J. (2004). *Fly pushing: The theory and practice of Drosophila genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Grima, B., Dognon, A., Lamouroux, A., Chélot, E., & Rouyer, F. (2012). CULLIN-3 controls TIMELESS oscillations in the Drosophila circadian clock. *PLoS Biology*, *10*(8), e1001367.

He, C., Yang, Y., Zhang, M., Price, J. L., & Zhao, Z. (2013). Regulation of sleep by neuropeptide Y-like system in *Drosophila melanogaster*. *PLoS One*, *8*(9), e74237.

Hendricks, J. C., Finn, S. M., Panckeri, K. A., & Chavkin, J. (2000). Rest in Drosophila is a sleep-like state. *Neuron*, *25*(1), 129–138.

Hendricks, J. C., Williams, J. A., Panckeri, K., Kirk, D., Tello, M., Yin, J. C., et al. (2001). A non-circadian role for cAMP signaling and CREB activity in Drosophila rest homeostasis. *Nature Neuroscience*, *4*(11), 1108–1115.

Itoh, T. Q., & Matsumoto, A. (2012). Genome-wide RNA interference screening for the clock-related gene of ATP-binding cassette transporters in *Drosophila melanogaster* (diptera: Drosophilidae). *Applied Entomology and Zoology*, *47*(2), 79–86.

Jenett, A., Rubin, G. M., Ngo, T.-T. B., Shepherd, D., Murphy, C., Dionne, H., et al. (2012). A GAL4-driver line resource for Drosophila neurobiology. *Cell Reports*, *2*(4), 991–1001.

Joiner, W. J., Crocker, A., White, B. H., & Sehgal, A. (2006). Sleep in Drosophila is regulated by adult mushroom bodies. *Nature*, *441*(7094), 757–760.

Kaasik, K., Kivimäe, S., Allen, J. J., Chalkley, R. J., Huang, Y., Baer, K., et al. (2013). Glucose sensor O-GlcNAcylation coordinates with phosphorylation to regulate circadian clock. *Cell Metabolism*, *17*(2), 291–302.

Kim, E. Y., Jeong, E. H., Park, S., Jeong, H. J., Edery, I., & Cho, J. W. (2012). A role for O-GlcNAcylation in setting circadian clock speed. *Genes & Development*, *26*(5), 490–502.

Kim, Y.-I., Ryu, T., Lee, J., Heo, Y.-S., Ahnn, J., Lee, S.-J., et al. (2010). A genetic screen for modifiers of Drosophila caspase Dcp-1 reveals caspase involvement in autophagy and novel caspase-related genes. *BMC Cell Biology*, *11*(1), 9.

Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S., et al. (1998). The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iε. *Cell*, *94*(1), 97–107.

Ko, H. W., Kim, E. Y., Chiu, J., Vanselow, J. T., Kramer, A., & Edery, I. (2010). A hierarchical phosphorylation cascade that regulates the timing of PERIOD nuclear entry reveals novel roles for proline-directed kinases and GSK-3/SGG in circadian clocks. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *30*(38), 12664–12675.

Koh, K., Joiner, W. J., Wu, M. N., Yue, Z., Smith, C. J., & Sehgal, A. (2008). Identification of SLEEPLESS, a sleep-promoting factor. *Science (New York, N.Y.)*, *321*(5887), 372–376.

Konopka, R. J., & Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, *68*(9), 2112–2116.

Kulkarni, M. M., Booker, M., Silver, S. J., Friedman, A., Hong, P., Perrimon, N., et al. (2006). Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. *Nature Methods*, *33*(1010), 833–838.

Kume, K. (2005). Dopamine is a regulator of arousal in the fruit fly. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *25*(32), 7377–7384.

Kvon, E. Z., Kazmar, T., Stampfel, G., Yáñez-Cuna, J. O., Pagani, M., Schernhuber, K., et al. (2014). Genome-scale functional characterization of Drosophila developmental enhancers in vivo. *Nature*, *512*(7512), 91–95.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., et al. (2004). Distinct roles for Drosophila dicer-1 and dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, *117*(1), 69–81.

Lewis, E. B., & Bacher, F. (1968). Method of feeding ethyl methane sulfonate (EMS) to Drosophila males. *Drosophila Information Service*, *43*, 193.

Li, M.-D., Ruan, H.-B., Hughes, M. E., Lee, J.-S., Singh, J. P., Jones, S. P., et al. (2013). GlcNAc signaling entrains the circadian clock by inhibiting BMAL1/CLOCK ubiquitination. *Cell Metabolism*, *17*(2), 303–310.

Lin, D. M., & Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron*, *13*(3), 507–523.

Liu, S., Lamaze, A., Liu, Q., Tabuchi, M., Yang, Y., Fowler, M., et al. (2014). WIDE AWAKE mediates the circadian timing of sleep onset. *Neuron*, *82*(1), 151–166.

Mandilaras, K., & Missirlis, F. (2012). Genes for iron metabolism influence circadian rhythms in *Drosophila melanogaster*. *Metallomics*, *4*(9), 928.

Martinek, S., Inonog, S., Manoukian, A. S., & Young, M. W. (2001). A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. *Cell*, *105*(6), 769–779.

McGuire, S. E. (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. *Science (New York, N.Y.)*, *302*(5651), 1765–1768. http://dx.doi.org/10.1126/science.1089035.

Miklos, G., & Rubin, G. M. (1996). The role of the genome project in determining gene function: Insights from model organisms. *Cell*, *86*(4), 521–529.

Mohr, S. E., & Perrimon, N. (2011). RNAi screening: New approaches, understandings, and organisms. *Wiley Interdisciplinary Reviews. RNA*, *3*(2), 145–158.

Neely, G. G., Hess, A., Costigan, M., Keene, A. C., Goulas, S., Langeslag, M., et al. (2010). A genome-wide Drosophila screen for heat nociception identifies a2d3as an evolutionarily conserved pain gene. *Cell*, *143*(4), 628–638.

Neumüller, R. A., Richter, C., Fischer, A., Novatchkova, M., Neumüller, K. G., & Knoblich, J. A. (2011). Genome-wide analysis of self-renewalin Drosophila neural stem cells by transgenic RNAi. *Cell Stem Cell*, *8*(5), 580–593.

Newby, L. M., & Jackson, F. R. (1993). A new biological rhythm mutant of *Drosophila melanogaster* that identifies a gene with an essential embryonic function. *Genetics*, *135*(4), 1077–1090.

Newby, L. M., White, L., DiBartolomeis, S. M., Walker, B. J., Dowse, H. B., Ringo, J. M., et al. (1991). Mutational analysis of the Drosophila miniature-dusky (m-dy) locus: Effects on cell size and circadian rhythms. *Genetics*, *128*(3), 571–582.

Ni, J. Q., Liu, L. P., Binari, R., Hardy, R., Shim, H. S., Cavallaro, A., et al. (2009). A Drosophila resource of transgenic RNAi lines for neurogenetics. *Genetics*, *182*(4), 1089–1100.

Ni, J.-Q., Zhou, R., Czech, B., Liu, L.-P., Holderbaum, L., Yang-Zhou, D., et al. (2011). A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nature Methods*, *8*(5), 405–407.

Nitz, D. A., van Swinderen, B., Tononi, G., & Greenspan, R. J. (2002). Electrophysiological correlates of rest and activity in *Drosophila melanogaster*. *Current Biology*, *12*(22), 1934–1940.

Oh, Y., Jang, D., Sonn, J. Y., & Choe, J. (2013). Histamine-HisCl1 receptor axis regulates wake-promoting signals in *Drosophila melanogaster*. *PLoS One*, *8*(7), e68269.

Osterwalder, T., Yoon, K. S., White, B. H., & Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(22), 12596–12601.

Perrimon, N., Ni, J.-Q., & Perkins, L. (2010). In vivo RNAi: Today and tomorrow. *Cold Spring Harbor Perspectives in Biology*, *2*(8), a003640.

Petsko, G. A., & Ringe, D. (2004). *Protein structure and function*. London: New Science Press.

Pfeiffenberger, C., & Allada, R. (2012). Cul3 and the BTB adaptor insomniac are key regulators of sleep homeostasis and a dopamine arousal pathway in Drosophila. *PLoS Genetics*, *8*(10), e1003003.

Pitman, J. L., McGill, J. J., Keegan, K. P., & Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in Drosophila. *Nature*, *441*(7094), 753–756.

Price, J. L. (2005). Genetic screens for clock mutants in Drosophila. *Methods in Enzymology*, *393*, 35–60.

Price, J. L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., & Young, M. W. (1998). double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. *Cell*, *94*(1), 83–95.

Rogulja, D., & Young, M. W. (2012). Control of sleep by cyclin a and its regulator. *Science (New York, N.Y.)*, *335*(6076), 1617–1621.

Rørth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(22), 12418–12422.

Rutila, J. E., Zeng, H., Le, M., Curtin, K. D., Hall, J. C., & Rosbash, M. (1996). The timSL mutant of the Drosophila rhythm gene timeless manifests allele-specific interactions with period gene mutants. *Neuron*, *17*(5), 921–929.

Ryder, E. (2004). The DrosDel collection: A set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster*. *Genetics*, *167*(2), 797–813.

Sarin, S., Bertrand, V., Bigelow, H., Boyanov, A., Doitsidou, M., Poole, R. J., et al. (2010). Analysis of multiple ethyl methanesulfonate-mutagenized caenorhabditis elegans strains by whole-genome sequencing. *Genetics*, *185*(2), 417–430.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., et al. (2013). Towards the elements of successful insect RNAi. *Journal of Insect Physiology*, *59*(12), 1212–1221.

Sehgal, A., Price, J. L., Man, B., & Young, M. W. (1994). Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless. *Science*, *263*(5153), 1603–1606.

Seugnet, L., Suzuki, Y., & Thimgan, M. (2009). Identifying sleep regulatory genes using a Drosophila model of insomnia. *The Journal of Neuroscience*, *29*(22), 7148–7157.

Shabalina, S., & Koonin, E. (2008). Origins and evolution of eukaryotic RNA interference. *Trends in Ecology & Evolution*, *23*(10), 578–587. http://dx.doi.org/10.1016/j.tree.2008.06.005.

Shang, Y., Donelson, N. C., Vecsey, C. G., Guo, F., Rosbash, M., & Griffith, L. C. (2013). Short neuropeptide F is a sleep-promoting inhibitory modulator. *Neuron*, *80*(1), 171–183.

Shaw, P. J., Cirelli, C., Greenspan, R. J., & Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. *Science*, *287*(5459), 1834–1837.

Shi, M., Yue, Z., Kuryatov, A., Lindstrom, J. M., & Sehgal, A. (2014). Identification of redeye, a new sleep-regulating protein whose expression is modulated by sleep amount. *Elife*, *3*, e01473.

Siegfried, E., Chou, T. B., & Perrimon, N. (1992). Wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. *Cell*, *71*(7), 1167–1179.

Stanewsky, R. (2003). Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *Journal of Neurobiology*, *54*(1), 111–147.

Stavropoulos, N., & Young, M. W. (2011). Insomniac and cullin-3 regulate sleep and wakefulness in Drosophila. *Neuron*, *72*(6), 964–976.

St Johnston, D. (2002). The art and design of genetic screens: *Drosophila melanogaster*. *Nature Reviews. Genetics*, *3*(3), 176–188.

Stoleru, D., Nawathean, P., Fernández, M. P., Menet, J. S., Ceriani, M. F., & Rosbash, M. (2007). The Drosophila circadian network is a seasonal timer. *Cell*, *129*(1), 207–219.

Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., et al. (2004). A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nature Genetics*, *36*(3), 283–287.

Toh, K. L., Jones, C. R., He, Y., Eide, E. J., & Hinz, W. A. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science*, *291*(5506), 1040–1043.

van Alphen, B., Yap, M. H. W., Kirszenblat, L., Kottler, B., & van Swinderen, B. (2013). A dynamic deep sleep stage in Drosophila. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *33*(16), 6917–6927.

van Swinderen, B., & Hall, J. C. (1995). Analysis of conditioned courtship in dusky-andante rhythm mutants of Drosophila. *Learning & Memory*, *2*(2), 49–61.

Venken, K. J. T., Simpson, J. H., & Bellen, H. J. (2011). Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron*, *72*(2), 202–230.

Wager-Smith, K., & Kay, S. A. (2000). Circadian rhythm genetics: From flies to mice to humans. *Nature Genetics*, *26*(1), 23–27.

Williams, J. A., Sathyanarayanan, S., Hendricks, J. C., & Sehgal, A. (2007). Interaction between sleep and the immune response in Drosophila: A role for the NFkappaB relish. *Sleep*, *30*(4), 389–400.

Woodgett, J. R., Plyte, S. E., Pulverer, B. J., Mitchell, J. A., & Hughes, K. (1993). Roles of glycogen synthase kinase-3 in signal transduction. *Biochemical Society Transactions*, *21*(4), 905–907.

Wu, M., Robinson, J. E., & Joiner, W. J. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. *Current Biology*, *24*(6), 621–629.

Xie, L., Kang, H., Xu, Q., Chen, M. J., Liao, Y., Thiyagarajan, M., et al. (2013). Sleep drives metabolite clearance from the adult brain. *Science (New York, N.Y.)*, *342*(6156), 373–377.

Xu, Y., Padiath, Q. S., Shapiro, R. E., Jones, C. R., & Wu, S. C. (2005). Functional consequences of a CKIδ mutation causing familial advanced sleep phase syndrome. *Nature*, *434*(7033), 640–644.

Yamamoto-Hino, M., & Goto, S. (2013). In vivo RNAi-based screens: Studies in model organisms. *Genes*, *4*(4), 646–665.

Yi, W., Zhang, Y., Tian, Y., Guo, J., Li, Y., & Guo, A. (2013). A subset of cholinergic mushroom body neurons requires go signaling to regulate sleep in Drosophila. *Sleep*, *36*(12), 1809–1821.

Yoshii, T., Rieger, D., & Helfrich-Frster, C. (2012). *Two clocks in the brain: An update of the morning and evening oscillator model in Drosophila The neurobiology of circadian timing*. (1st ed., Vol. 199, pp. 59–82).

Yuan, Q., Joiner, W. J., & Sehgal, A. (2006). A sleep-promoting role for the Drosophila serotonin receptor 1A. *Current Biology*, *16*(11), 1051–1062.

Zehring, W. A., Wheeler, D. A., Reddy, P., Konopka, R. J., Kyriacou, C. P., Rosbash, M., et al. (1984). P-element transformation with period locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. *Cell*, *39*(2 Pt. 1), 369–376.

Zhang, E. E., Liu, A. C., Hirota, T., Miraglia, L. J., Welch, G., Pongsawakul, P. Y., et al. (2009). A genome-wide RNAi screen for modifiers of the circadian clock in human cells. *Cell*, *139*(1), 199–210.