The clock in the cell
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Introduction
Circadian clocks

Organisms on earth are influenced by the alternation of day and night caused by the rotation of the earth. Many – if not all - species have developed strategies to cope with daily changes in the environment, which are to a large extent systematic and predictable. One of the evolved coping strategies is an endogenous, temporal program that regulates daily rhythms of physiology and behavior. This program functions as a clock to control endogenous daily rhythms with high precision. The so-called circadian clock (from Latin “circa diem”, about a day) is endogenous, as was proven by experiments that released plants, animals, fungi and cyanobacteria into constant conditions yielding oscillations with an approximately 24 h period. These are called circadian rhythms.

The internal clock, which regulates aspects of molecular biology, physiology and social interactions across phyla, generates a temporal program that optimizes the sequence of daily events and prepares the organism for upcoming events. Locomotor activity (Pittendrigh & Daan, 1976a) and photoreception are regulated by the circadian system (Freedman et al, 1999) in animals. Leaf movement (Darwin, 1880), growth (McClung, 1992; McClung et al, 1992; Quail, 2002), opening of stomata, photosynthesis, cell metabolism (Lüttge, 2000) and gene regulation (Bognar et al, 1999) in plants or spore release in fungi (Merrow & Dunlap, 1994; Roenneberg & Merrow, 2001a) are examples of circadian ‘behaviour’ in sessile organisms. Even unicellular organisms, e.g. cyanobacteria (Kondo et al, 1994) or the alga Gonyaulax polyedra, show circadian rhythmicity (Roenneberg & Morse, 1993): in a daily repeating cycle Gonyaulax travels from the ocean's surface (during the day to gather photosynthetic energy) to great depths (during the night to harvest nutrients).

That the circadian clock also confers an adaptive advantage has been described experimentally (DeCoursey et al, 2000; Johnson & Golden, 1999; Yan et al, 1998). Therefore selective pressure might be the main driving force for the evolution of circadian rhythms.

One of the reasons that circadian biology is a relevant question is that it concerns our lives in immediately recognizable ways. There are hundreds of examples for how circadian rhythms control physiology (and hence potentially lead to pathology) in humans by influencing circadian variations in hormone levels, body temperature, mental and physical performance and pharmacokinetics (McFadden, 1988; Moore-Ede et al, 1982a; Moore-Ede et al, 1982b; Rocco et al, 1987).

The huge impact that circadian rhythmicity has on human biology and human society (Moore, 1997) can be seen in the example of shift work: about 20% of all employees in developed countries work in night shifts. The problem here arises
from the fact that during a night shift, the circadian clock-regulated physiology of the worker usually remains entrained to local time instead of adjusting to the night shift. So, while physiology and psychology are saying “sleep!”, shift workers are forced to be active and alert (Scott, 2000; Waterhouse et al, 1997).

Clock mechanisms

Given the pervasive effect of circadian biology on life on earth, there is much interest in understanding molecular mechanisms using genetic tools. Though the molecular components of the circadian clock are significantly different among animals, plants, fungi and cyanobacteria, important features are common across phyla: According to Eskin’s model – which very simply describes circadian systems for essentially all living things – circadian clocks consist of three major functional components: input pathways, a rhythm generator (central oscillator) and output pathways (Fig. 1.1) (Eskin, 1979). Zeitgeber (German for time-giver) signals (the most important of which are light or/and temperature) are transduced to the central oscillator via the input pathway.

One of the most important zeitgebers for the circadian system is light. While photoreception for vision requires high time- and high spatial-resolution, circadian photoreception must integrate the amount of light over the course of a day, comparable to a scintillation counter (Roenneberg & Foster, 1997; Roenneberg & Merrow, 2000). The use of additional light qualities to tell time-of-day (e.g., wavelength/color) has not yet been described, even though both blue and red light have been shown to feed into the circadian clock of plants as inputs.

**Figure 1.1** Cartoon of a basic circadian system, that can be described as input pathways that perceive and transduce external, entraining signals from a zeitgeber (e.g. light, temperature), and a rhythm generator or central oscillator that generates rhythms and regulates various output pathways creating overt rhythms. (re-drawn after Roenneberg and Merrow, 1998; Eskin, 1979).
Photoreceptors are the best characterized components of circadian input pathways. They will either deliver a signal directly (e.g., WC-1 in *Neurospora crassa*) or indirectly (e.g., melanopsin in mice) to the rhythm generator/central oscillator. Concerning cellular clocks (the former case), there will be diverse strategies to carry signals that hold exogenous time information to the endogenous circadian program. Plants and fungi and some examples of transparent animals (like *Drosophila*) can harvest light intracellularly, allowing for an efficient (i.e. comprising only few steps) transfer of information downstream. Concerning complex, hierarchical clocks (the latter example), animals receive light exclusively through the eyes, and must send signals via the retino-hypothalamic tract to the circadian pacemaker in the brain, called the suprachiasmatic nucleus (SCN) (Berson et al, 2002; Menaker, 2003). These examples show how broadly the Eskin model applies to circadian systems.

The SCN is made up of neurons that display a circadian rhythm in gene expression and neurophysiology, even when dissociated into single cells (Welsh et al, 1995). Thus, the complexity of the system is revealed: the SCN functions as one oscillator that orchestrates others such as liver and lung clocks, but it itself is made up of individual cells that show self-sustained free running and entrainable circadian rhythms. Hence, the cellular system (input-oscillator-output) parallels that of the organism.

Concerning the molecular oscillator mechanism, animals with altered circadian properties were generated in mutagenesis experiments, eventually leading to the discovery of clock genes. The first clock genes were discovered in *Drosophila* (Konopka & Benzer, 1971), with *Neurospora* following soon after (Feldman & Hoyle, 1973). Mammalian clock genes were finally revealed, too (King et al, 1997; Lowrey et al, 2000; Ralph & Menaker, 1988). The discovered components were modeled into a network based on genetic experiments. The cartoons drawn of circadian networks (Reppert & Weaver, 2002; Schwartz et al, 2001) reveal the complexity of circadian systems.

The central oscillator generates self-sustained rhythmicity (see below) of the clock, and then it is the job of output pathways to transduce this oscillatory signal downstream. One mechanism is via gene expression (transcriptional regulation). This was first shown for *Neurospora*, specifically with the discovery of clock controlled genes (ccg’s) (Loros et al, 1989). Microarray studies helped to identify 145 ccg’s, whose predicted or known functions in development, metabolism, cell signaling and stress responses suggest a contribution of the circadian clock in a wide range of cell processes (Correa et al, 2003). An example of an output pathway in mammals is the one that leads to induction of the *vip* (vasoactive intestinal peptide)-gene (Hurst et al, 2002; Silver et al, 1999). This gene together with the genes coding for other neuropeptides (vasopressin, cholecys-
tokinin and substance P) are used as molecular readouts for circadian rhythms and represent examples of cgg’s in mammals. Furthermore, more than a hundred genes have been shown to be under direct clock control (Oishi et al, 2003), and many more might be, as many microarray analyses in mammals (Delaunay & Laudet, 2002; Duffield, 2003), but also in Arabidopsis (Schaffer et al, 2001), and Drosophila (McDonald & Rosbash, 2001) suggest.

Clock properties

By analyzing the behavior of organisms, properties of their clocks can be deduced, shared features defined and characteristics of circadian clocks in general described (Gwinner, 1986; Pittendrigh, 1960; Roenneberg & Merrow, 1998). These include at least the following:

- **Rhythmicity.**
  There must be a quantifiable ‘up’ and ‘down’.

- **Circadian range.**
  The oscillation has a free-running period (FRP) in the circadian range in constant conditions, i.e. one full cycle takes approximately 24 hours

- **Robustness of the amplitude.**
  The amplitude of the oscillation has to be sufficiently robust to drive output rhythms

- **Self-sustainment.**
  In constant conditions (without zeitgebers) the rhythmicity continues unabated, and is therefore self-sustained and endogenous. In some organisms the endogenous circadian rhythmicity can continue over years. (Gwinner, 1986; Richter, 1978)

- **Entrainability.**
  Circadian systems must be synchronizable to zeitgeber cycles, a property called entrainment (Roenneberg et al, 2003). Hereby, the organism entrains with a specific relationship, the phase angle, to external cues (like natural light and temperature cycles, but also food or certain chemicals) keeping its physiological functions synchronized with the environment. Circadian systems are able to entrain to cycle lengths different from 24 hours, but only within a certain range. This property is called the range of entrainment, defined by the minimum and the maximum cycle length (called ‘T’) to which the system is still able to entrain. Being exposed to very short or long cycle lengths, an organism can show a frequency demultiplication (e.g., only one conidial band every two 12 h cycles in Neurospora (Merrow et al., 1999) or a frequency multiplication ((Pittendrigh & Daan, 1976b), e.g., two conidial
bands per cycle)). Entrainment differs from driven-ness (a reaction to a zeitgeber stimulus that is uniform in different zeitgeber conditions and does not necessarily require a circadian clock) in being an active process where the influence of timing information on the circadian clock depends on the state of the circadian clock at the time of exposure.

- **Temperature compensation.**
  Circadian rhythms are highly temperature compensated, i.e. the period is roughly unaltered even when the (constant) temperatures applied vary over a rather wide (10°C difference or more) range (Pittendrigh, 1954). This phenomenon extends to other parameters like pH inside a cell, nutrition and social interaction, as well, and could therefore be termed noise compensation.

### The TTO (Transcription-Translation-Oscillator) as a model to describe molecular clock mechanisms

As mentioned above, clock genes have been identified through mutant screens and they have been constructed in various configurations based largely on molecular genetic and genetic experiments. The predominant theory explaining the molecular mechanism of circadian rhythms is that of a Transcription-Translation-regulated-Oscillator (TTO). According to this theory some of the so-called “canonical clock genes” are rhythmically transcribed. Their protein products negatively feed back to regulate their own transcription (see Fig. 1.2). In Neurospora crassa, the negative element FREQUENCY (FRQ) feeds back via the WHITE-COLLAR-COMPLEX (WCC) to an element within the promoter region of the frq gene (Loros & Dunlap, 2001a). Via posttranslational protein modifications, additional interlocked loops and nuclear import the molecular feedback process is slowed down to occur once per circa 24h period (Lakin-Thomas, 2006b). Recent modeling efforts (Roenneberg & Merrow, 2002) show that a freerunning period (FRP) of around 24 hours can be achieved by forming a network of several interconnected short-period TTOs. This model furthermore mimics all of the circadian clock properties mentioned above, suggesting that this is one possibility for how molecular clocks are put together.

The TTO model, as it stands, still fails to explain much of the circadian mechanism. Anomalies have been accumulating over the last years, including the demonstration of rhythmicity in organisms with constant clock gene transcription, and rhythmicity in clock gene knock-out mutants (Bell-Pedersen et al, 2005; Loros & Feldman, 1986; Yang & Sehgal, 2001). It has been suggested that rhythmic transcription may have other functions in the circadian system (e.g. participating in input and output pathways and providing robustness to the oscil-
lations) and that circadian systems might use a non-circadian oscillator consisting of metabolic feedback loops, which acquires its circadian properties from additional regulatory molecules such as the products of canonical clock genes (Lakin-Thomas, 2006b). Rhythmic de-/phosphorylation of clock components, a hypothesized ‘phoscillator’, might be common to all circadian systems, as suggested by the pervasive and prominent role played by kinases and phosphatases in eukaryotic clocks (Merrow et al., 2006).

**Figure 1.2** A simplified circadian transcription/translation oscillator (TTO) model with 2 interlocked loops: Clock gene A is transcribed into RNA and translated into protein. Clock protein B positively regulates transcription of clock gene A. Clock protein A negatively regulates its own transcription by interfering with the positive effect of clock protein B. Clock protein A also positively regulates production of clock protein 2, via either transcription or translation. Biosynthetic pathways are shown as solid lines with arrowheads. Positive influence is shown as a circle with plus sign. Negative influence is shown as a dashed line together with a square filled with a minus sign. Nuclear/cytoplasmic compartmentation, phosphorylation, degradation pathways, environmental inputs, and outputs to clock-controlled genes and observed rhythms have been omitted. (re-drawn after Lakin-Thomas, 2006)
The biology of *Neurospora crassa*

This thesis employs the model genetic organism *Neurospora crassa* as an experimental tool. Here, I describe basic features of Neurospora ecology.

The filamentous fungus *N. crassa* belongs to the phylum Ascomycota or ‘sac fungi’, due to the sac-like ascospore containers that are built during sexual propagation. Depending on environmental conditions this fungus can propagate asexually or reproduce sexually. Most *Neurospora* species are haploid and spend most of their life cycle in this state, because the diploid nuclei formed during the sexual phase are only transient (information taken from http://www.fgsc.net/Neurospora/sectionB2.htm). In its asexual stage, *Neurospora* forms a mycelium, a network of tubular filaments with multiple haploid nuclei (syncytial hyphae), whereas macroconidia (hereafter called conidia) are formed from aerial hyphae. Conidia do not survive for a long time in nature, but allow for rapid spreading due to their huge number. Upon environmental signals (e.g., hydration) conidia germinate to form hyphae, which grow by tip extension and branch to form mycelia.

Historically, the genus *Neurospora* was thought to be predominant in moist, tropical and subtropical areas (information taken from http://www.fgsc.net/Neurospora/sectionB4.htm), but recent collection initiatives revealed that *Neurospora* even habituates many temperate zones as far North as Alaska (Jacobson et al, 2004). In nature, *Neurospora* is one of the first colonists in areas of burnt-over vegetation (Jacobson et al, 2006; Perkins & Turner, 1988), and has as such been described already in 1925 after the fire of Tokyo (Kitazima, 1925). It grows easily indoors on food or food waste, accounting for its commonly used name “red bread mold”.

Evolving in and adapting to an exposed natural habitat, *Neurospora* has developed a variety of light responses including mycelial carotenoid production (Harding & Turner, 1981), formation of sexual structures (perithecia), their phototropism (Degli-Innocenti et al, 1984; Harding & Melles, 1984), gene expression (Arpaia et al, 1993; Collett et al, 2002; Crosthwaite et al, 1995a; Li & Schmidhauser, 1995; Sommer et al, 1989) and entrainment of its circadian rhythm.

*Neurospora crassa* as a tool to study the circadian clock

*Neurospora*, which spawned the “One gene - One enzyme” hypothesis in the early 1940s, is an excellent research tool for several reasons:

- It exists predominantly in a haploid state, e.g. no backcross is needed to screen *Neurospora* progeny, which makes reverse and forward genetics easier.
• It has a fully sequenced genome (Galagan et al, 2003) making molecular research systematic.
• A wealth of genetic and biochemical tools are available from the decades of basic genetics work that it has been used for.
• Additionally, *Neurospora* has a short generation time of a few weeks, and potentially many progeny and much tissue can be grown in a few days.

However, the key for circadian research is the easily detectable circadian output behavior of *Neurospora*. The standard phenotypic assay to assess circadian rhythmicity in *Neurospora* is the ‘race tube assay’, where cultures are grown on solid agar media in glass tubes. Thus grown, *N. crassa* shows a free running circadian rhythm in conidia formation (banding) of about 22h in darkness. The bands are easily visualized on solid agar medium (Pittendrigh et al, 1959). Under entraining conditions (e.g., light or temperature cycles), the bands show a distinct phase relationship to external time (Chang & Nakashima, 1997; Lakin-Thomas & Brody, 2000; Merrow et al, 1999b; Roenneberg and Merrow, 2001). In constant light, discrete banding is mostly absent, with conidia being produced continuously (Roenneberg & Merrow, 2001; Pittendrigh et al, 1959).

Starting with mutant screening for strains with altered free running periods (FRP’s) in *Neurospora*, the first *Neurospora* clock gene, *frequency* (*frq*), was found in the early 1970s (Feldman & Hoyle, 1973). Genetic analyses resulted in the description of more than 30 mutant alleles influencing the clock (Feldman & Dunlap, 1983; Lakin-Thomas et al, 1990). Further screening showed that seven of these 30 mutants were alleles of the *frq* gene, conferring shorter (e.g. *frq*1, FRP=16.5 h) or longer period lengths (e.g. *frq*7, FRP=27 h) than the normal 22h. Also, arrhythmic strains, like *frq*9, carrying a recessive, loss-of-function mutation, were found (Loros & Feldman, 1986) or subsequently generated (e.g., *frq*10, where almost the whole open reading frame (ORF) of the *frq*-gene is removed). In the FRQ-deficient mutants light entrainment of the conidiation rhythm is impaired, indicating an additional role for the FRQ-protein in the light input system (Roenneberg & Merrow, 2001; Pittendrigh et al, 1959).

The expression of *frq* is regulated through transcriptional and posttranscriptional control mechanisms (Fig. 1.3). Briefly, the transcription of *frq* is positively regulated by the WHITE-COLLAR-1 (WC-1) and WHITE-COLLAR-2 (WC-2) proteins, and the FRQ protein feeds back negatively on its own transcription (Aronson et al, 1994; Crosthwaite et al, 1997a). Nuclear localization of FRQ is essential for rhythmicity. FRQ enters the nucleus as it is made and represses accumulation of *frq* mRNA (Luo et al, 1998). As mentioned, phosphorylation is a crucial player in the generation of circadian rhythms and FRQ is progressively phosphorylated throughout the day and controls the activity of WC-1 and WC-2.
by regulating their phosphorylation states (Schafmeier et al, 2005). If this phosphorylation is inhibited experimentally, then the rate of FRQ turnover decreases and period length increases (Liu et al, 2000). Several kinases, e.g CASEIN KINASES I and II or the calcium/calmodulin-dependent kinase (CAMK) as well as PROTEIN PHOSPHATASE 2A (PP2A) and PROTEIN PHOSPHATASE 1 (PP1), regulate the stability of the FRQ protein and the length of the free-running period (Görl et al, 2001; Liu et al, 2000; Yang et al, 2002).
Light reception in *Neurospora*

Several mutations have been reported to affect light responsiveness in *Neurospora crassa* (Linden et al., 1997). For example mutations in the *white collar-1* and *white collar-2* (*wc-1* and *wc-2*) genes have been shown to impair light-regulated carotenogenesis. Many other light responses are also abolished in these mutants (Ninnemann, 1991; Perkins et al., 1982; Russo, 1988), which made the WC-1 and WC-2-proteins possible candidates for photoreceptors (Harding & Shropshire, 1980). That *wc-1* - and *wc-2*-mutants are also clock mutants was shown later (Crosthwaite et al., 1997) and makes any light input pathway mutant potentially interesting to use for understanding the mechanisms of the circadian clock in *Neurospora*. An interesting case is the cytoplasmic blue light photoreceptor and flavoprotein VIVID (VVD) (Schwerdtfeger & Linden, 2003): although the mutant does not display a difference in free running period in several conditions (Shrode et al., 2001), it regulates entrainment (Elvin et al., 2005; Heintzen et al., 2001; Madeti, unpublished data), apparently through its impact on photoadaptation.

In addition to VVD, WC-1 and WC-2, the fully sequenced and annotated genome (Galagan et al., 2003) of *Neurospora crassa* (available at: http://www.broad.mit.edu/annotation/genome/neurospora/Home.html) provides additional photoreceptor candidates:

- A possible green light photoreceptor protein with high homology to bacteriorhodopsin, *novel opsin-1* (*nop-1*), was identified (Bieszke et al., 1999a; Bieszke et al., 1999b). NOP-1 binds retinal and forms a photochemically active pigment (Brown et al., 2001) but neither the physiological function, in general, nor the involvement of this fungal opsin in the circadian system is known.

- The same is true for the homolog to archaean rhodopsins, ORP-1 (OPSIN-RELATED-PROTEIN 1). Being regulated by heat-shock, it appears to be involved in responses to pH, organic solvents and stress (Nemcovic & Borkovich, 2003).

- The genome sequence also reveals a *cryptochrome* homologue (Daiyasu et al., 2004) and two homologues of bacterial *phytochromes* (Catlett et al., 2003), possible candidates for Red/Far Red photoreceptor genes. *phy1* mRNA levels have been described to be under clock control (Froehlich et al., 2005), whereas knockouts of *phy-1* and *phy-2* are described not to have an effect on any -so far- known photoresponses. In the same publication, the putative *Neurospora* blue light photoreceptor protein CRYPTOCHROME (nCRY) is said to be photo-regulated by the WC-Complex (Froehlich et al., 2005), but its function is not known yet.
• Also, a homolog of the Aspergillus nidulans gene velvet is present in Neurospora. In Aspergillus, this gene is involved in the signal transduction of both red and blue light (Yager et al, 1998). The presence of three genes (velvet, phy-1 and phy-2) possibly involved in red light photoreception is surprising given the fact that red light responses have not been described yet in Neurospora – it is thought to be blind for red light (e.g.(Dharmananda, 1980; Froehlich et al, 2005) - and suggests that the photobiology in Neurospora might be more complex than recognized, so far.

Action spectra show how much light of a given wavelength is required for synthesis of mycelial carotenoids (De Fabo et al., 1976), phase shifting of the conidiation rhythm (Dharmananda, 1980), photosuppression of self-sustained rhythmicity in conidial band formation (Sargent and Briggs, 1967) and the in vitro light induced binding of the WC-1 protein to the promotor of the frq gene (Froehlich et al., 2002). In Neurospora crassa, the aforementioned responses could not be stimulated by wavelengths longer than 520 nm and all of them show a maximal activity around 460 nm with sensitivity extending into the UVA region (De Fabo et al, 1976; Froehlich et al, 2002; Sargent & Briggs, 1967). All this indicates that flavins or carotenoids are involved as chromophores. However, since a triple albino mutant (al-1, al-2, al-3) containing less than 0.5% of wt carotenoids is still able to exhibit normal sensitivity for other light responses, photoreception in N. crassa is probably not based on carotenoids (Russo, 1988). Furthermore, mutants deficient in biosynthesis of riboflavin exhibit a decreased light sensitivity, which makes flavin species (as have been identified cofactors for WC-1 and VVD) the best candidates for Neurospora photoreceptor chromophores.

Prospect of this thesis

As a young student I was fascinated by fungi and was inspired by a lecture of Professor Agerer from the Botanical Institute of the University of Munich. He described the complex factors interacting to make mushrooms grow in the autumn. Still, I would have never imagined writing a thesis on fungi (especially the „red bread mold“ Neurospora crassa) and the complex factors that influence their daily timing system. Even more improbable – even though my family took me on mushroom collection trips as a child - was that one day I would be part of an international team searching for the first wild Neurospora isolates in temperate climates in Europe.

In my first months as a doctoral student, I had the rare opportunity to see the object of my further studies in nature after the huge and devastating fires in Europe in 2003. Neurospora crassa is a colonist found on burnt trees after forest
fires and was, until 2003, apart from reports from French bakeries, not described in temperate climates, but rather designated to be an inhabitant of the tropics and subtropics. Chapter 2 of this thesis describes the findings of the 2003 collection trip in Europe and compares the strain prevalence and growth patterns to those of the previously known strains.

Chapter 3 describes chronobiological experiments done with a collection of wild type Neurospora crassa strains from the whole world. These experiments include the newly collected strains from Europe together with the older ones from the rest of the world. Strains collected from different latitudes were used to assess the correlations of latitude-of-origin and phase of entrainment or free running period.

Chapter 4 also characterizes clock properties in wild type strains, but this time, with an eye to genetics. Two wild type strains were crossed and 200 to 500 progeny were selected for a quantitative trait analysis experiment. While our collaborators at U. C. Berkeley generated genetic markers for the strains, we worked on phenotyping them for the circadian clock. Earlier QTL-studies in mice and Arabidopsis indicated that more genes than expected were involved in the clock quantitative traits phase and free-running period. Given this and Neurospora crassa’s optimal prerequisites for a QTL-study, it is surprising that no earlier studies existed.

The following chapters set up much of the remainder of the thesis. Whereas the work with wild type strains, especially using QTL, is one approach to find novel clock genes, using entrainment for phenotyping a mutant is another way to reach the same end. This approach is ongoing in the lab. For my thesis, however, I took this a step further, combining a functional genetics approach with using entrainment to reveal new clock genes.

Chapter 5 describes assaying a cryptochrome mutant in Neurospora crassa. It displays the same free running period as the wild type/background strain bda, but it shows differences in the phase of entrainment in blue or white light cycles. What does this tell us about entrainment and the dogma of „longer period-later entrainment and shorter period –earlier entrainment“?

In Chapter 6 formal entrainment properties of (a lab strain of) Neurospora crassa are discussed.

In Chapter 7, I describe the entrainment of N. crassa in a ,circadian surface‘ using temperature as a zeitgeber.

Chapter 8 reviews a recent publication describing the cloning of the ,band‘ gene. Having been used as a basically universal standard and background strain for circadian experiments, the discovery of its identity as a ras-1-mutant raises many questions about its applicability. The most important –and still not solved-question is: is bd/ras-1 a clock mutant itself?
Chapter 9, a review on circadian clocks in human fibroblasts gives an insight into relatively new circadian research in human tissues. Do in vitro experiments using human fibroblasts together with questionnaires have the potential to make the challenging bunker or constant routine experiments unnecessary? Is Neurospora a parallel relative to human fibroblasts, with respect to circadian systems? Much of the work that was built up using Neurospora was done before tissue culture systems were developed for mammalian cells.

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