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Role of Glycogen Synthase Kinase (GSK) in temperature compensation of the *Neurospora* circadian clock

presented by

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Rolle der Glykogen Synthase Kinase (GSK) in der Temperaturkompensation der circadianen Uhr von Neurospora crassa

vorgelegt von

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SUMMARY

Circadian clocks are biological oscillators that allow organisms to accurately predict and adjust to the rhythmic changes in the environment which increases their fitness. These oscillators are found in every cell and have three fundamental properties: they are endogenous, entrainable and temperature compensated. The former two properties of the clock are well studied. However, it is currently unknown how clocks accurately keep the time independent of the ambient temperature, a phenomenon known as “temperature compensation”. This is particularly important for poikilothermic organisms that cannot control their body temperature and yet still have accurate circadian clocks.

We used *Neurospora crassa* as a eukaryotic circadian clock model organism and showed that Glycogen synthase kinase (GSK) binds and specifically phosphorylates White Collar 1 (WC-1), which is the critical and rate-limiting positive element of the Neurospora clock. We found that these phosphorylations decrease the WC-1 stability in a temperature dependent manner. Our data completes the picture in our current understanding of temperature compensation of circadian clocks and shows that temperature compensation in *Neurospora crassa* is achieved by opposing functions of two kinases (GSK and CK2) on the positive (WCC) and negative (FRQ) elements of the clock, respectively. Since both kinases are well conserved among eukaryotes, it is also possible that this mechanism of temperature compensation is conserved among other eukaryotic circadian clocks.

SUMMARY

Circadiane Uhren sind biologische Oszillatoren, die es Organismen ermöglichen, rhythmische Änderungen in der Umwelt vorherzusagen und sich auf diese einzustellen. Diese Oszillatoren haben drei fundamentale Eigenschaften: sie sind endogen, trainierbar und temperaturkompensiert. Die ersten beiden Eigenschaften der Uhr wurden bereits eingehend studiert. Bis heute ist jedoch nicht bekannt, wie die zellulären Uhren unabhängig von der Umgebungstemperatur akkurat Zeit messen können, ein Phänomen, das als Temperaturkompensation bezeichnet wird. Vor allem für poikilotherme Lebewesen, die ihre Körpertemperatur nicht selbst regulieren können, ist diese Eigenschaft sehr wichtig.

Im eukaryontischen Modellorganismus, *Neurospora crassa*, haben wir gezeigt, dass Glykogen Synthase Kinase (GSK) den Transkriptionsfaktor White Collar 1 (WC-1) bindet, spezifisch phosphoryliert und damit temperaturabhängig dessen Stabilität reguliert. WC-1 ist das limitierende, positive Element in der *Neurospora* Uhr und bildet mit WC-2 den White Collar Complex (WCC). Bei erhöhten Temperaturen wird WC-1 durch GSK-vermittelte Phosphorylierung destabilisiert. Die vorliegenden Daten vervollständigen das Bild dessen, wie wir uns gegenwärtig das Prinzip der Temperaturkompensation circadianer Uhren vorstellen. Sie zeigen, dass Temperaturkompensation bei *Neurospora crassa* von zwei entgegengesetzt wirkenden Kinasen (GSK und CK2) bewerkstelligt wird, welche auf die jeweils positiven (GSK auf WCC) und negativen (CK2 auf FRQ) Elemente der Uhr einwirken. Da beide Kinasen in Eukaryonten gut konserviert sind, ist es sehr gut möglich, dass dieser Temperaturkompensationsmechanismus bei eukaryontischen circadianen Uhren ebenfalls konserviert und weit verbreitet ist.

1.1 Circadian clocks

1.1.1 Clocks in nature

Circadian rhythms are biological phenomena that occur with a period length of about 24 hours. These rhythms are driven by biochemical oscillators which are called circadian clocks. These clocks are found in most organisms ranging from cyanobacteria to mammals where it enables the organism to accurately predict rhythmic events in the environment and thereby increase its fitness. Accurate anticipation of dawn and dusk, for example, helps a nocturnal animal to avoid its diurnal predators. It also provides a safe window of opportunity for activities such as foraging, hunting or breeding. In plants, the circadian system provides cues that synchronize the opening and closing of leaves for maximum use of energy provided by sunlight. In single celled organisms such as algae, it gates growth and metabolic functions.

Circadian clocks not only drive a daily rhythm, but also aid in other aspects of life, sometimes in quite unexpected ways. For example, circadian clocks are essential for sun-compass navigation in insects, birds and mammals. It also provides time-of-day information to the brain which integrates this cue with visual input on the location of sun in the sky and calculates the correct direction for the organism. Without the circadian clock these organisms would not be able to navigate towards the correct destination, because the location of the sun in the sky changes throughout the day.

In addition to regulating daily rhythms, clocks also regulate annual rhythms such as breeding in animals or shedding of leaves in plants. They provide the organism with timing information on the avaliablity of resources or prey throughout the year.

Some sharks in the pacific, for example, travel thousands of miles and find a single island, the size of a football field, where their prey, birds, hatch only during a two week window in the year. In fact, they coordinate their feeding to the rhythmic availability of preys and hop from one island to another with precise timing during the year. Without the aid of circadian clock in navigation and timing, they would certainly not be able to utilize these resources.

Although the day-night cycle is the dominant environmental synchronizer for most organisms, many other aspects of the environment such as temperature, humidity and nutrition are also rhythmic. Circadian clocks enable the organism to anticipate changes in these variables also. It is therefore of great interest and importance to understand the mechanisms underlying such a common and important aspect of biology.

1.1.2 Organization of circadian hierarchy

Circadian clock in in multicellular organisms receives rhythmic environmental input and relays it to the rest of the organism in order to keep the individual components in harmony (Figure 1). In rodents, for example, the clock resides in brain region called the suprachiasmatic nuclei (SCN) located dorsally to the optic chiasm. SCN regulate rhythms of locomotor behavior, body temperature and many other physiological functions. The input to this clock, mainly light, is received by specialized photoreceptors in the retina and then conveyed to the SCN via neuronal fibers of the retino-hypothalamic tract (RHT). Other kinds of environmental stimuli such as social cues are also relayed to the SCN via various neuronal input pathways. These inputs are integrated in the SCN into timing information and then relayed to the rest of the organism via humoral or neuronal cues. Peripheral tissues also contain endogenous clocks. However, these clocks go out of synchrony without the input from SCN. Therefore, SCN is considered to be major clock and the orchestrator of peripheral clocks (Davidson, Yamazaki et al. 2003).

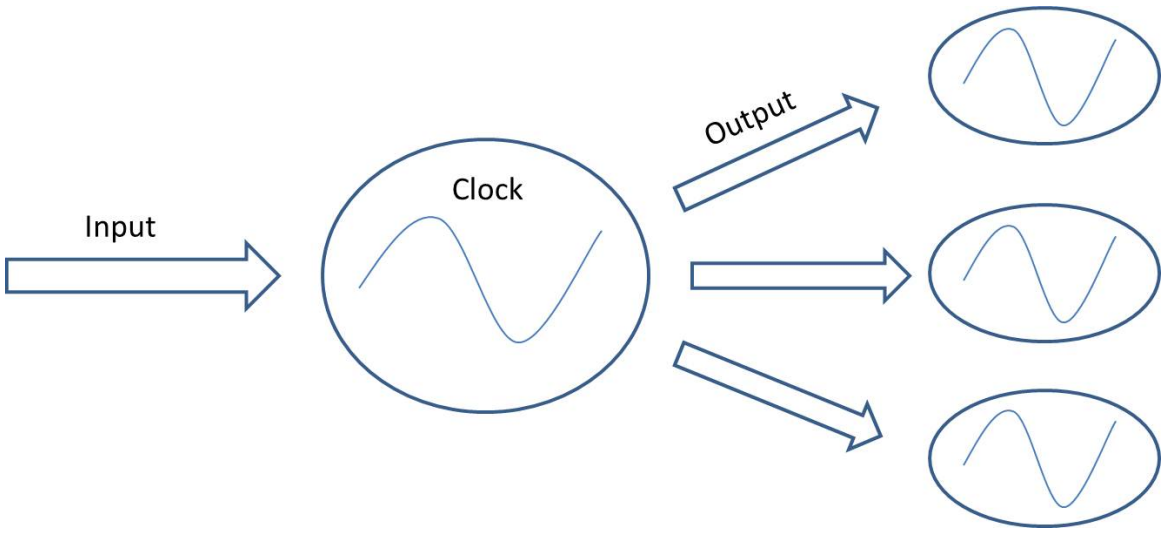

Figure 1
The circadian hierarchy. Clock receives environmental input such as light and temperature and relays to peripheral oscillators in multicellular organisms. In single cells the core clock mechanism drives many downstream genes.

1.2 Molecular mechanism of circadian clocks

1.2.1 The transcription-translation feedback loop (TTFL)

Circadian clocks exist not only in multicellular, but also in single celled organisms. In fact, the individual SCN neurons mentioned above are each considered a circadian clock cell. The rhythm in these cells is generated by a molecular transcription-translation feedback loop (TTFL). Although the individual components vary from one organism to another, the basic principle of how this mechanism functions is similar throughout taxa.

TTFL consists of positive and negative elements which feedback onto each other to generate the rhythm. The positive elements are transcription factors consisting of one or more subunits. The activity and sub-cellular localization of this transcription factor complex is highly regulated via post-translational mechanisms such as phosphorylation. In their active form, they translocate into nucleus where

they drive expression of many clock-controlled genes (ccgs). Products of one or more of these ccgs form a regulator complex, which are the negative elements of TTFL. This negative regulator complex associates with kinases or other proteins in the cell and regulate the activity, sub-cellular localization and degradation of the positive elements. One of their major roles is to keep the positive elements in inactive form in the cytosol. However, this negative regulator complex is also regulated via phosphorylation events and is gradually degraded throughout the day. Upon its depletion below threshold levels, the respression on the transcription factors is released and expression of ccgs resume, starting a new cycle. The temporal control of this cycle is achived by various mechanisms that introduce delays to the cycle such as phosphorylation dependent sub-cellular localization or degradation. This results in lengthening of the period of this cycle to roughly 24 hours, hence forms the circadian oscillator.

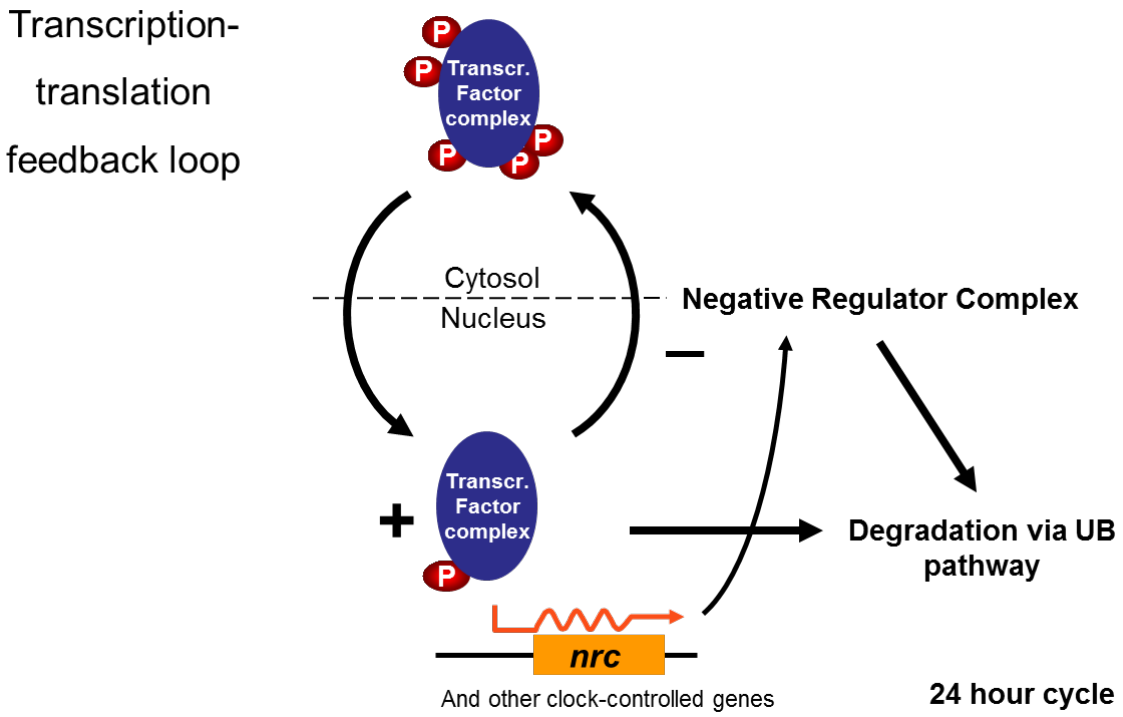

Figure 2
The molecular feedback loop. At the core of each clock cell is a molecular timer that consists of positive and negative elements that feedback to each other at the course of a day. Positive elements, namely transcription factors, drive clock controlled genes (ccgs). Some of these ccgs accumulate to high enough levels

and form the negative regulator complex, which eventually represses the transcription factors that drive them, thereby inhibiting their own synthesis. Throughout the day, the negative regulator complex is gradually degraded and the repression on the transcription factors is released and the cycle starts again. Although the components of these molecular clocks are different, the principle of feedback loops is conserved among eukaryotes.

1.2.2 Mammalian TTFL

In mammals, the positive elements of the TTFL are CLOCK and BMAL1 (Figure 3). These proteins form a heterodimer in the cytoplasm which translocate into the nucleus where they drive rhythmic expression of ccgs through binding to E-box elements on the DNA. Some of these ccgs are PERIOD (PER) and CRYPTOCHROME (CRY) proteins. PER has three isoforms (PER1, PER2 and PER3) and CRY has two (CRY1 and CRY2). These proteins form heterodimeric complexes in the cytosol with combinations of PER and CRY. Upon accumulating to threshold levels, these complexes translocate into the nucleus where they repress the activity of the CLOCK/BMAL1 transcription factor complex (Figure 3). Therefore, PER/CRY heterodimer is the negative regulator complex of the mammalian TTFL (Gallego and Virshup 2007). The stability and the activity of the PER/CRY heterodimer is regulated by phosphorylation through effects of kinases such as CASEIN KINASE1 epsilon (CK-1e) and GLYCOGEN SYNTHASE KINASE 3-beta (GSK-3b). Phosphorylation by these kinases throughout the day results in the degradation of the PER/CRY complex and releases the repression, starting a new cycle of CLOCK/BMAL1 expression. This molecular feedback loop is considered as the “main loop” of the mammalian TTFL. Another feedback mechanism called the stabilizing loop involves REVERB- alpha and ROR-alpha which are expressed by CLOCK/BMAL1. These proteins regulate the expression of BMAL1 which enhances the amplitude of the circadian oscillations (Lowrey and Takahashi 2004).

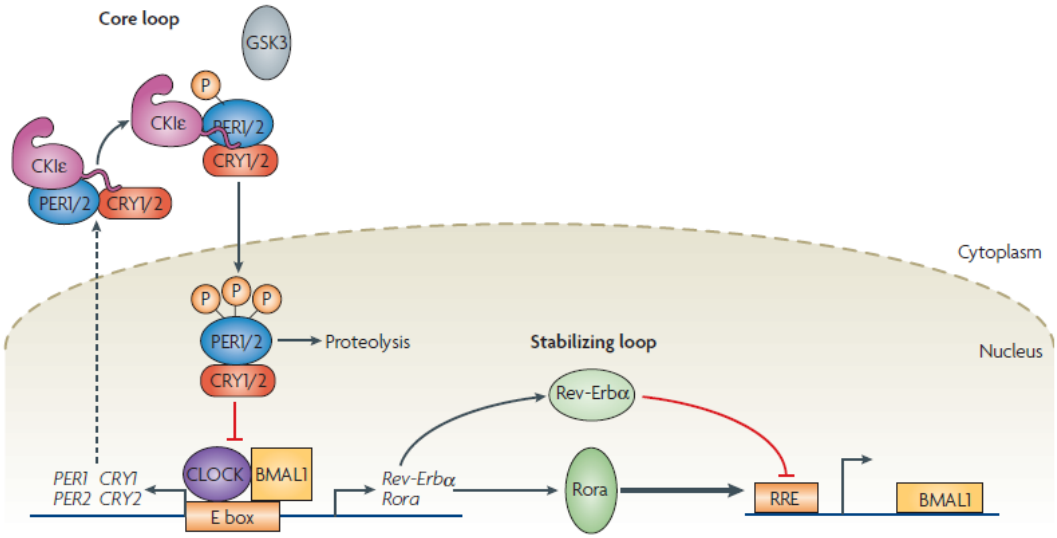

Figure 3
The mammalian molecular circadian oscillator. Positive elements CLOCK and BMAL1 drive expression of several ccgs including PERs and CRYs which in turn form heterodimeric complexes in the cytoplasm, translocate into the nucleus and inhibit the CLOCK/BMAL1 transctiptional activity. Several other ccgs form additional feedback loops that stabilize the clock by means of regulating its robustness through affecting the total levels of positive or negative elements. Figure from (Gallego and Virshup 2007).

1.2.3 Drosophila TTFL

The fly circadian clock is similar to mammals and also utilizes a TTFL to generate rhythms in individual cells (Figure 4). Although many components of the *Drosophila* TTFL are homologs of their mammalian counterparts, some are different. For example, instead of a BMAL1 there is CYCLE (CYC) in *Drosophila*. CYCLE interacts with CLOCK to form the positive transcription factor complex which drives ccgs such as PER and TIMELESS (TIM). These proteins are similar to PERs and CRYs in mammals and also form heterodimeric complexes which negatively regulate the activity of the positive element CLOCK/CYCLE heterodimer. Throughout the day, TIM is phosphorylated by SHAGGY (SGG), a homolog of the GSK in mammals, which results in its degradation. The stability of PER is regulated by kinases DOUBLETIME (DBT), a homolog of CK-1e, and

CASEIN KINASE 2 (CK2) which phosphorylate PER and target it for degradation. PER and TIM are become stable when they can form a heterodimer. This heterodimer translocate into the nucleus where it represses CLOCK/CYCLE activity, thereby inhibiting its own synthesis. PER/TIM complex is progressively phosphorylated and degraded throughout the day, similar to PER/CRY in mammals. When the levels of PER/TIM drop, the repression is released and a new cycle starts. The stabilizing loop in *Drosophila* consists of ccgs VRILLE (VRI) and PAR-domain protein 1 (PDP1) which regulate expression of CLOCK, similar to the actions of REVERB-alpha and ROR-alpha on BMAL1 in mammals.

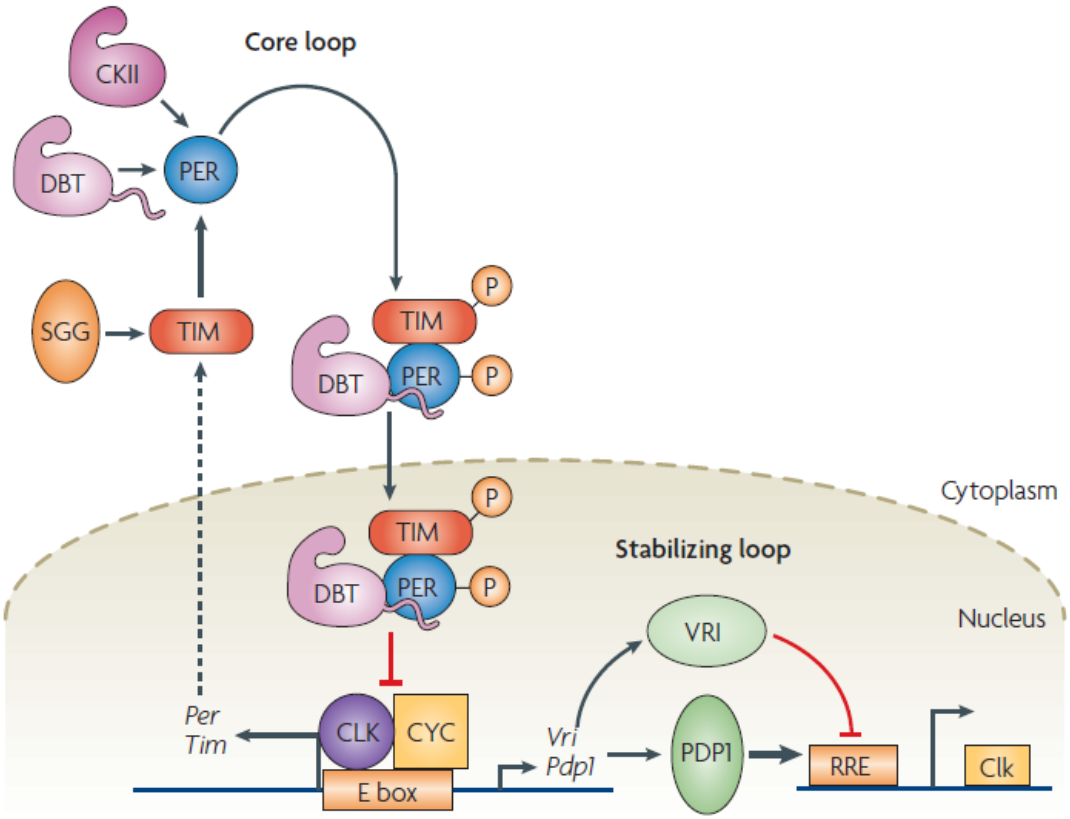

Figure 4
The Drosophila molecular circadian oscillator. CLOCK/CYCLE heterodimeric transcription factor drives expression of several genes in including *per* and *tim.* PER and TIM form heterodimers which translocate into nucleus where they inhibit the transcriptional activity of CLK/CYC, inhibiting their own synthesis. As the PER/TIM degrades during the day, this repression is released and the cycle starts again. Figure from (Gallego and Virshup 2007).

1.2.4 A model organism for over half a century: Neurospora crassa

During the history of the circadian field, *Neurospora Crassa,* a filamentous fungi, played a major role in our understanding of the circadian biology. *Neurospora* is a eukaryote and also harbors a molecular feedback oscillator which, in essence, is very similar to mammalian and fly clocks. It has been used as a model organism to study eukaryotic circadian clocks thanks to its ease of handling and powerful biochemistry and a circadian output measuring method called a “race-tube” assay (Figure 5). This assay is based on the inherent rhythmicity in the asexual production of spores which is directly driven by the circadian clock in *Neurospora*. It is easily visualized in a glass tube filled with solid supporting medium where the organism grows from one end to the other of the glass tube. As it grows, the rhythmic conidiation is observed as “bands”. This assay is sensitive to many inputs such as light and temperature and represents the functioning of the underlying circadian clock. Analysis of the period length, amplitude or phase of this behavior allowed circadian biologists to unravel many biochemical aspects of circadian clocks which were later on applied to mammalian and fly clocks. Although, the individual components are not homologous to their mammalian or fly counterparts, the molecular oscillator in Neurospora is very similar in its workings to these clocks. It also employs a TTFL where positive elements drive expression of ccgs which feed-back to repress their own transcription.

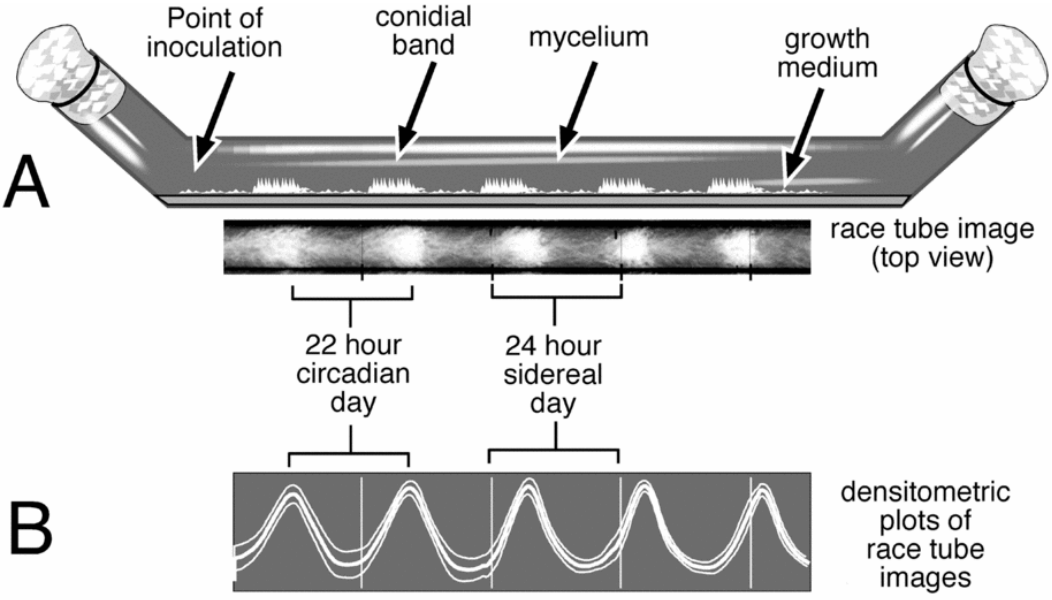

Figure 5
Rhythmic conidiation in *Neurospora crassa.* The output of the circadian clock in Neurospora is easily observed by analyzing the rhythmic banding on race-tubes. As the organism grows from one end to the other, it produces rhythmic conidiation which presents itself as “bands“ that can be analyzed using densitometry.

1.2.5 Neurospora TTFL

The positive element in the *Neurospora* TTFL is the White Collar Complex (WCC, Figure 6). It consists of two subunits, WHITE COLLAR 1 and 2 (WC-1 and WC-2) which are ZINC-finger transcription factors. Similar to BMAL1/CLOCK and CLOCK/CYCLE in mammals and flies, WC-1 and WC-2 also form heterodimers through their PAS domains. In addition, WC-1 also contains a light-oxygen-voltage (LOV) domain, which allows the WCC to act also as a light receptor. Therefore, the light input to the circadian clock is at the very core level in the TTFL of *Neurospora*.

Similar to regulation of CLOCK/BMAL1 and CLOCK/CYCLE, the activity and sub-cellular distribution of WCC is also regulated by its phosphorylation status. Active WCC is hypo-phosphorylated and resides in the nucleus. In this form, it is highly

unstable and DNA binding leads to its rapid degradation (Schafmeier, Haase et al. 2005; Schafmeier, Diernfellner et al. 2008).

One of the ccgs that WCC expresses is FREQUENCY (FRQ), which is the negative element in the *Neurospora* TTFL. The promoter of *frq* harbors one light-response element (LRE) and a clock-box. WCC recognizes these motifs and bind to them to drive expression of *frq*. LRE is involved in the light-dependent transcription of *frq* while the clock-box is required for rhythmicity in constant dark. Upon light exposure, WCC is activated through its LOV domain and binds to the LRE of *frq*. However, light-induced expression of *frq* gradually diminishes as the light induction continues. This is called light adaptation of the circadian clock and is necessary for proper entrainment to light/dark cycles in the nature. Recently, the mechanism of how this is achieved has been shown (Malzahn, Ciprianidis et al. 2010). The light-induced active WCC is counteracted by another light sensitive LOV-domain containing protein called VIVID (VVD). VVD is a small light-inducible protein, which binds to active WCC and inhibits its DNA-binding. Therefore, initially both *vvd* and *frq* are produced, but then VVD protein inhibits WCC to reduce expression of both proteins, achieving light-adaptation.

Hypo-phosphorylated FRQ forms complexes with several kinases such as Casein Kinase 1a (CK1a), casein kinase 2 (CK2) and protein kinase A (PKA) as well as FRQ-interacting helicase (FRH). This multimeric complex inhibits and stabilizes the WCC by promoting its inactivation and phosphorylation, thereby preventing further expression of *frq* RNA (Liu and Bell-Pedersen 2006; Pregueiro, Liu et al. 2006; Schafmeier, Kaldi et al. 2006). Similar to PERs in mammals and flies, FRQ is also degraded as the circadian cycle progresses. Over the course of the circadian cycle, it is progressively phosphorylated by CASEIN KINASE 1a and 2 (CK-1a and CK2). This leads to its accumulation in the cytosol and degradation via the proteasome by FWD-1 (F box and WD40 repeat-containing protein-1) (He, Cha et al. 2006; Diernfellner, Querfurth et al. 2009).
Phosphorylation of WCC by FRQ-dependent processes is in an equilibrium with the actions of protein phosphatases PROTEIN PHOSPHATASE 1 and 2A (PP1 and PP2A) on WCC. Therefore, as the levels of FRQ decrease during the day,

PP1 and PP2A-mediated dephosphorylation dominates which results in activation and translocation of WCC into nucleus, thereby initiating a new cycle (Brunner and Schafmeier 2006; Liu and Bell-Pedersen 2006; Schafmeier, Kaldi et al. 2006; Dunlap, Loros et al. 2007; Querfurth, Diernfellner et al. 2007; Schafmeier, Diernfellner et al. 2008).

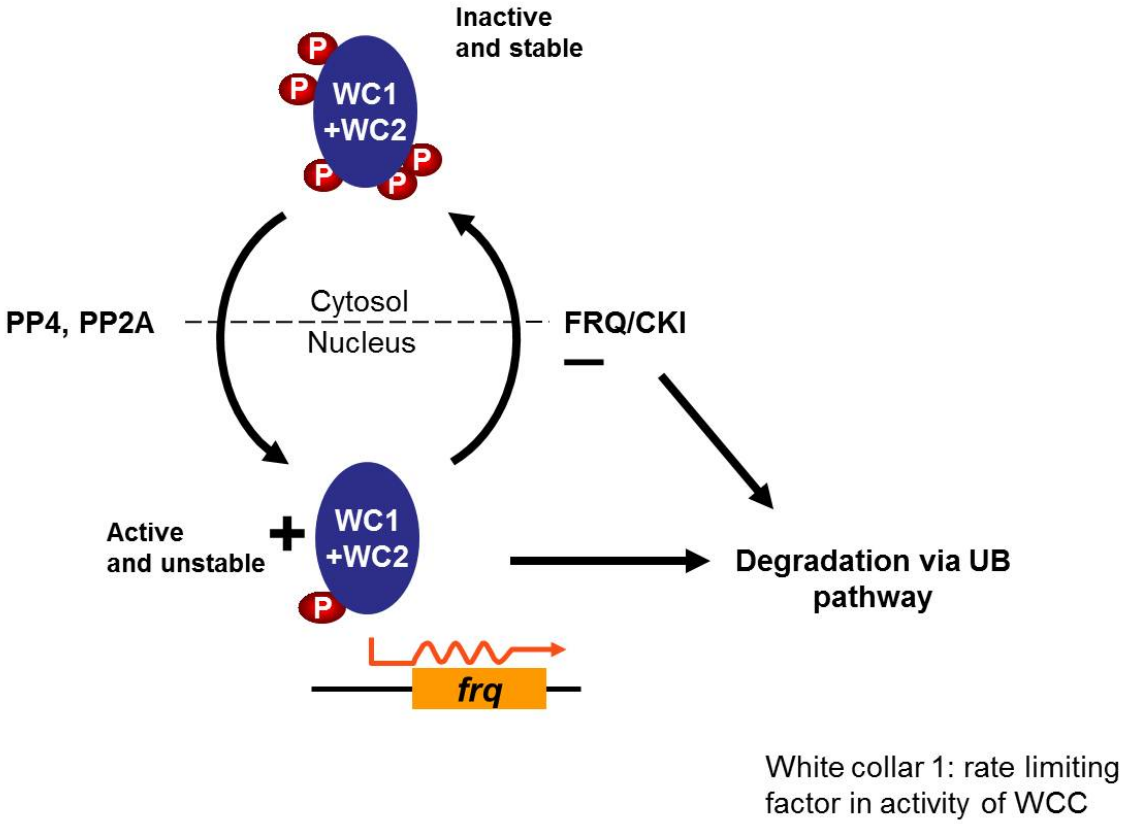

Figure 6
Neurospora TTFL. Positive elements WC-1 and WC2 form the heterodimeric transcription factor White Collar Complex (WCC) which drives many ccgs, including its own repressor FRQ. As FRQ accumulates, it forms complexes with many kinases, such as Casein Kinase 1a, and other proteins (eg. FRH) and form the negative regulator complex. This complex inhibits and stabilizes WCC via phosphorylation. As the levels of FRQ decrease during the day due to degradation, background activity of phosphatases PP4 and PP2A recruit WCC which translocates into the nucleus and starts a new cycle.

1.3 Temperature compensation of circadian clocks

1.3.1 Fundamental, but poorly understood

All circadian clocks share three fundamental properties: they are endogenous to the organism, can be influenced (entrained) by environmental stimuli (e.g. light) and are temperature compensated. The former two properties have been well studied. However, the mechanism underlying temperature compensation is poorly understood, albeit it's early history.

Temperature compensation of circadian rhythms was reported as early as the 1950s (Pittendrigh 1954; Bruce and Pittendrigh 1956). It is defined by the relative resistance of the speed of circadian clocks to changes in ambient temperature. This is actually an intriguing property for a biochemical system. The rates of most biochemical reactions increase when the ambient temperature rises. However, the output of the circadian clocks are largely unaffected by these changes, even though the molecular mechanisms underlying circadian oscillations are biochemical interactions themselves (Figure 7). Therefore, a poikilothermic organism with a period length of about 22 hours (such as *Neurospora*) will still have the same period length at 20 and 30 ^{o}C.

Interestingly, temperature compensation is observed both in poikilothermic and homoeothermic organisms (Enright 1967; Zimmerman, Pittendrigh et al. 1968; Jacklet 1980; Gardner and Feldman 1981; Anderson, Laval-Martin et al. 1985; Barrett and Takahashi 1995; Tosini and Menaker 1998; Izumo, Johnson et al. 2003; Tsuchiya, Akashi et al. 2003). Although the benefits of temperature compensation for a poikilothermic organism are obvious, it is not clear why this mechanism is still present in homotherms. One recent study suggested that in mammals, while the master clock, SCN, is temperature compensated, the peripheral organs are not. The authors suggested that fluctuations in the core body temperature which is driven by SCN may the cue for the entrainment of peripheral tissues to the master pacemaker (Buhr, Yoo et al. 2010).

Considering the natural fluctuations throughout the day, proper compensation of the circadian rhythm to changes in the ambient temperature is vital, especially for organisms that cannot regulate their own body temperature.

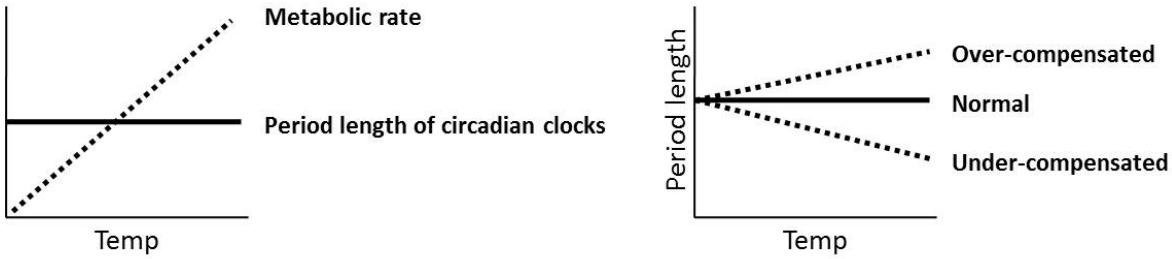

Figure 7
Temperature compensation of circadian clocks. Although the speed of most biochemical reactions increase with temperature, the period length (i.e. speed) of the circadian clock stays relatively constant. There have been many reports on mutant strains with defective compensation phenotypes such as over- or under-compensation where the clock ticks slower or faster (respectively) as the temperature increases. However, the molecular mechanism of temperature compensation is still unknown.

1.3.2 Mechanism of temperature compensation: Post-translational?

There have been several mechanisms suggested to explain the molecular mechanisms underlying temperature compensation based on the altered circadian proteins that give rise to temperature compensation phenotypes (Gardner and Feldman 1981; Mattern, Forman et al. 1982; Loros and Feldman 1986; Castiglione-Morelli, Guantieri et al. 1995; Huang, Curtin et al. 1995; Hong and Tyson 1997; Price 1997). For example, various temperature dependent responses of the Neurospora clock have been described to date, all affecting the core oscillator FRQ. By a posttranscriptional mechanism, FRQ steady-state levels rise

in response to temperature increases, which is critical for temperature dependent resetting of the clock and for maintaining robust rhythmicity over a wide physiological temperature range (Garceau, Liu et al. 1997; Liu, Garceau et al. 1997; Diernfellner, Schafmeier et al. 2005). In addition, two isoforms of FRQ mRNA are expressed via temperature sensitive alternative splicing (Colot, Loros et al. 2005). Temperature dependent changes in the ratio of these isoforms affect period length and tune the clock in response to ambient temperature (Diernfellner, Colot et al. 2007; Dunlap, Loros et al. 2007). Temperature dependent alternative splicing has also been described to occur in other clocks (Majercak, Sidote et al. 1999; Collins, Rosato et al. 2004). However, none of these mechanisms has been shown to have an effect on temperature compensation. In the cyanobacteria circadian clock, it has been shown that temperature compensation is achieved by similar kinetics of a kinase and phosphatase that affect the same core clock protein at various temperatures. However, this clock does not share the TTFL of eukaryotic clocks and can actually continue to run even in a test tube and is not considered to reflect the circadian clocks in eukaryotes (Nakajima, Imai et al. 2005; Nagai, Terada et al. 2010).

In summary, although many mechanisms have been proposed, it has not been possible to generalize these, because either the proteins whose mutations result in defective compensation are at the core of the circadian clock or the various suggested mechanisms are not shared among organisms while temperature compensation is.

Recently, the role CASEIN KINASE 2 (CK2) in the temperature compensation of the Neurospora Crassa circadian clock has been shown (Mehra, Shi et al. 2009). In their report, the authors showed that mutations in the two previously known strains with over-compensated phenotypes both map to subunits of CK2. They also showed that down-regulation of CK2 leads to over-compensation. When they analyzed the targets of CK2 phosphorylation, they found that it phosphorylates FRQ. Mutations of CK2 targets on FRQ also resulted in defective temperature compensation. The authors suggested that CK2-dependent phosphorylations regulate the stability of FRQ in a temperature dependent manner where the FRQ

protein is degraded faster upon phosphorylation by CK2 at high temperatures. This finding suggested that the mechanism of temperature compensation may be post-translational.

It is tempting to think that such a post-translational control of temperature compensation mechanism may be conserved among organisms. After all, post-translational modifications of clock proteins by kinases and phosphatases is a common feature of circadian clocks in all organisms (Naef 2005; Dunlap, Loros et al. 2007; Fang, Sathyanarayanan et al. 2007; Spengler, Kuropatwinski et al. 2009; Qin, Byrne et al. 2010). However, in the study of Mehra et al., the authors did not find another kinase or a phosphatase with a temperature compensation phenotype. Furthermore, the role of casein kinase 2 in the Neurospora circadian clock explained only the over-compensation phenotypes, where the period length of the clock increases as the temperature rises. This suggests that another kinase or a phosphatase must be involved that is not yet discovered. Therefore, elucidating the mechanism underlying under-compensation may reveal the molecular mechanism of temperature compensation.

1.3.3 Glycogen synthase kinase (GSK) and temperature compensation

A hint towards completing a picture of the post-translational mechanism of temperature compensation is provided in a study by Jolma *et al.* where the authors showed that addition of lithium results in loss of temperature compensation in *Neurospora Crassa* (Jolma, Falkeid et al. 2006). In this study, lithium lengthened the period at normal temperatures. However, when the temperature was raised, the period length became significantly shorter (Figure 8). This showed that lithium resulted in an under-compensated compensated phenotype in *Neurospora*.

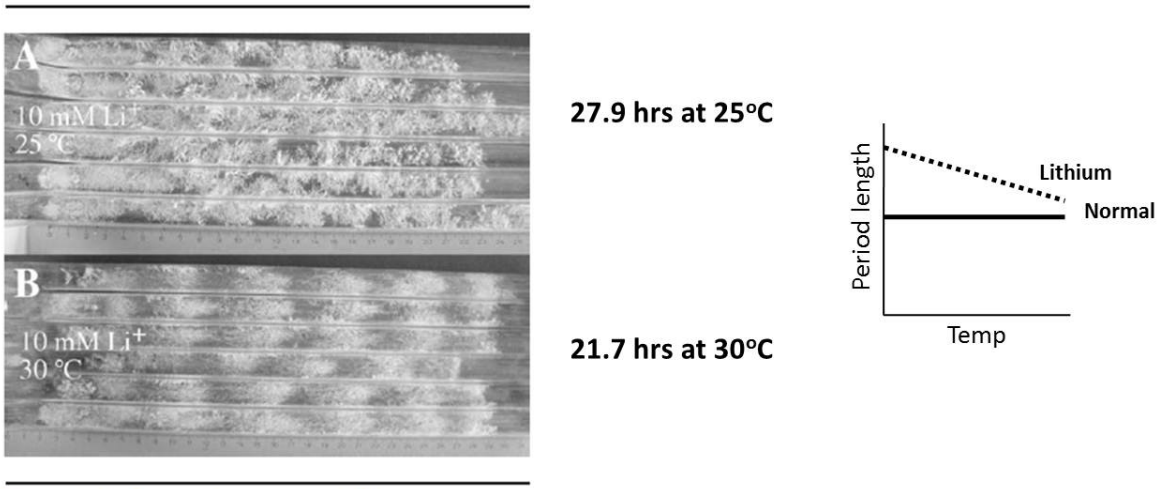

Figure 2. Influence of temperature. Higher temperatures diminish/reverse the effect of Li^+ ions on *Neurospora's* circadian rhythm by increasing growth rate and decreasing the period length. (A) 10 mM Li^+, 25 °C, growth rate 2.6 ± 0.3 cm/24 h, period 27.9 ± 4.0 h (n = 6). (B) 10 mM Li^+, 30.0 °C, growth rate 4.81 ± 0.85 cm/24 h, period 21.7 ± 0.5 h (n = 6). See also Figure 6B.

Jolma *et al. Journal of Biological Rhythms, October, 2006*

Figure 8

Addition of lithium results in loss of temperature compensation in *Neurospora crassa.* Lithium lengthens the period at normal temperatures which becomes shorter as the temperature increases, exibiting an under-compensated phenotype.

Lithium is a well-known inhibitor of GLYCOGEN SYNTHASE KINASE (GSK) (Stambolic, Ruel et al. 1996; Iwahana, Akiyama et al. 2004; Meijer, Flajolet et al. 2004; Padiath, Paranjpe et al. 2004; Gould and Manji 2005). It has also been shown to lengthen fungal, insect and mammalian circadian clocks (Engelmann, Bollig et al. 1976; Hofmann, Gunderoth-Palmowski et al. 1978; Subbaraj 1981; Hafen and Wollnik 1994; Jolma, Falkeid et al. 2006; Hirota, Lewis et al. 2008; Mohawk, Miranda-Anaya et al. 2009; O'Brien and Klein 2009).

GSK is the final enzyme in the biosynthesis of glycogen, where it was first discovered. It is an Mg^{2+} dependent kinase that phosphorylates its targets at consensus sequence S/T-xxx-S/T, where x is any amino acid. Presence of proline residues in the vicinity of this motif have been shown to promote GSK-dependent phosphorylations by increasing its catalytic efficiency. The activity of the kinase is

regulated by N-terminal phosphorylation of its pseudo-substrate domain which can be triggered by many pathways.

GSK is mostly known for its role in the wingless (WNT) signaling pathway which regulates segment polarity in flies. In this pathway, GSK is normally bound to beta-catenin where it phosphorylates and targets beta-catenin for ubiquitin-dependent degradation. It has also been shown to be involved in many other cellular processes such as insulin signaling, growth factors and TOR signaling pathways (Cook, Fry et al. 1996; Sakanaka, Sun et al. 2000; Cohen and Frame 2001; Frame and Cohen 2001; Papadopoulou, Bianchi et al. 2004). It has also been shown to be involved in inactivation and degradation of several transcription factors in response to environmental stimuli (Zhou, Deng et al. 2004; Zhou and Hung 2005; Punga, Bengoechea-Alonso et al. 2006; Bengoechea-Alonso and Ericsson 2009).

GSK is also a known component of the insect and mammalian circadian clocks (Figures 3 and 4) and it is highly conserved (Figure 9) and it's selective inhibitors alter the period length in these organisms (Martinek, Inonog et al. 2001; Harms, Young et al. 2003; Iitaka, Miyazaki et al. 2005; Kurabayashi, Hirota et al. 2006; Yin, Wang et al. 2006; Hirota, Lewis et al. 2008; Vougogiannopoulou, Ferandin et al. 2008; Mohawk, Miranda-Anaya et al. 2009; Kurabayashi, Hirota et al. 2010). In *Drosophila,* the GSK homolog SHAGGY (SGG) phosphorylates TIM and its overexpression promotes nuclear translocation of the PER/TIM heterodimer. SGG also phosphorylates PER *in vitro*, but its function is not known (Martinek, Inonog et al. 2001; Stanewsky 2002). In mammals, GSK interacts with PER2 and phosphorylates it *in vitro* and *in vivo* (Iitaka, Miyazaki et al. 2005). Furthermore, it affects the nuclear localization of PER2 and phosphorylates and stabilizes REVERB-alpha (Yin, Wang et al. 2006). Finally, in a recent study, it has been found that GSK phosphorylates BMAL1 and targets it for ubiquitin-dependent degradation, similar to its role in other transcription factors (Sahar, Zocchi et al. 2010).

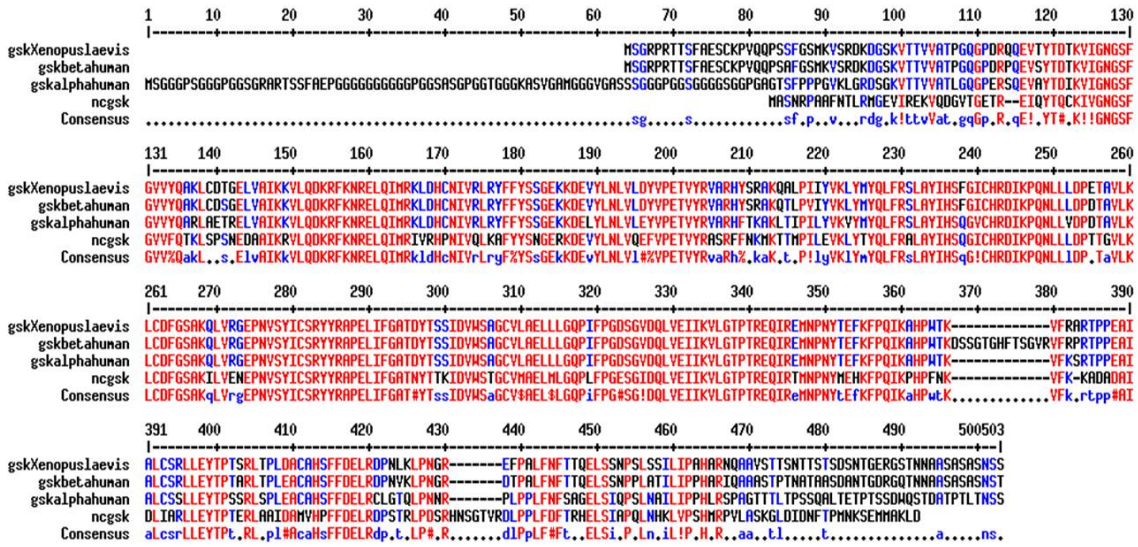

Figure 9
Alignment of GSK protein sequences from *Neurospora, Xenopus* and human showing that GSK is a highly conserved protein.

GSK is also involved in many signal transduction pathways where the inputs from multiple pathways converge on GSK to regulate its activity. Therefore, it is an ideal candidate to serve as an integrator of environmental stimuli that can affect many pathways, such as temperature (Figure 10). However, the role of GSK in the temperature compensation of circadian clocks have never been studied and it is one of the few kinases that was not tested in the (Mehra, Shi et al. 2009) study. Therefore, the role of GSK in the Neurospora clock or temperature compensation of circadian clocks is currently unknown. Considering that both GSK and CK2 are highly conserved and are components of the circadian clocks also in Drosophila and Mammals, it is possible that mechanism of temperature compensation is also conserved among clocks in eukaryotes and might employ GSK.

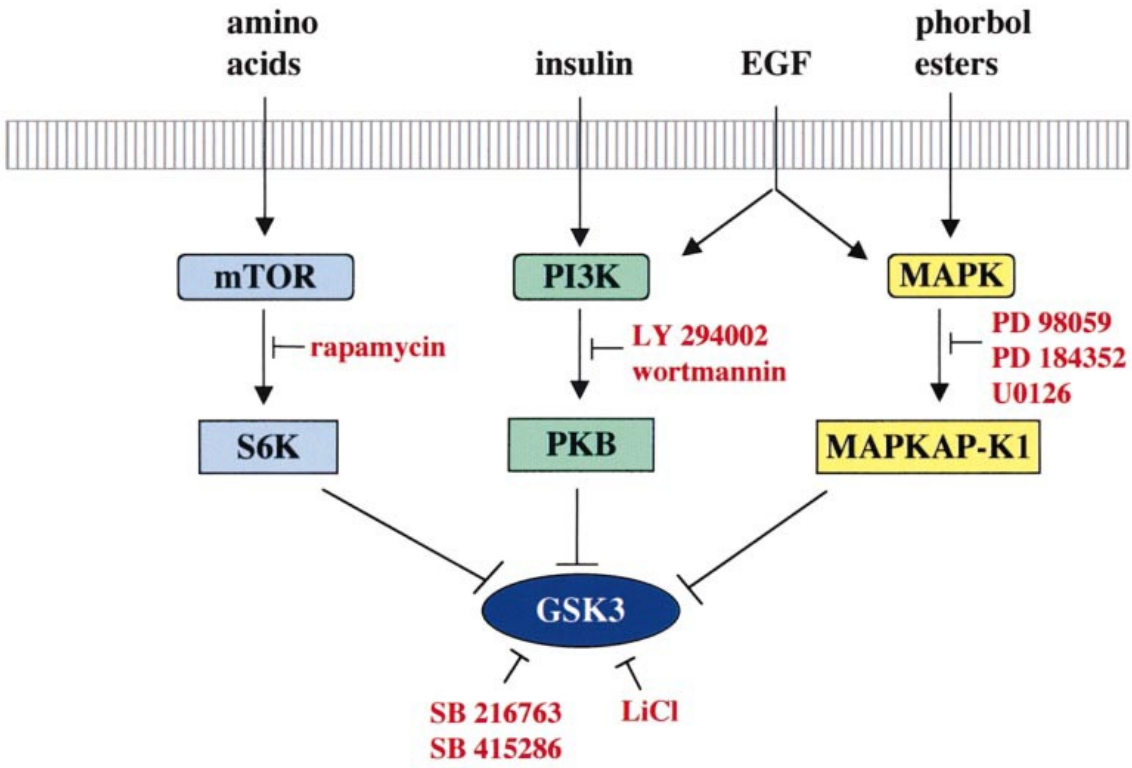

Figure 10
Activity of GSK is regulated its N-terminus phopshorylation which is triggered by various signals such as insulin, growth factors or nutrition. Therefore, GSK is in a good position to receive many environmental stimuli and integrate them into the activity of a single kinase and relay this information to the circadian clock.

I investigated the role of GSK in the circadian clock of Neurospora and in particular, its involvement in temperature compensation. The results showed that GSK binds and specifically phosphorylates the rate limiting element of the clock, the WHITE COLLAR COMPLEX (WCC) and regulate its stability in a temperature dependent manner. Either down-regulation of GSK or mutations of its phosphorylation sites on WCC both resulted in elevated levels of WC-1 and therefore higher WCC activity and under-compensated phenotypes. These data show that the mechanism of temperature compensation in Neurospora Crassa is post-translational where the effects of temperature are counter-acted by opposing functions of two kinases in the positive (GSK on WCC) and negative (CK2 on FRQ) elements of the circadian transcription-translation feedback loop.

2.1 Neurospora strains

Neurospora glycogen synthase kinase (GSK, NCU04185.2) heterokaryon knockout strain *Gsk het* and Abc transporter homokaryon knockout strain *Abc homo* (NCU?????) both contain hygromycin B resistance cassette "hph" in the corresponding gene loci and were obtained from the Fungal Genetics Stock Center along with their respective background control strains wt9718 and wt74. The strains *wt74 ras-1*^{bd}, Δ*wc-1,* Δ*wc-1/wc-1* Δ*C* (which lacks c-terminal domain of WC-1), *frq10/qa-frq, frq10* and Δ*vivid* all contain the ras-1^{bd} mutation (Belden et al. 2007). WC1 mutant strains *pWC1-wt, pWC1-8A* and *pWC1-8D* were created in this study. For a summary of strains used in this study see Figure 11.

2.2 Neurospora growth conditions

Conidial suspensions in 1M Sorbitol were prepared from samples grown on standard solid growth medium (2.2% Agar, 0.3% D+ Glucose monohydrate, 0.17% L-arginine, 1x Vogel's medium and 0.1% biotin). 200 µg/ml Hygromycin B (Applichem A5347) was added to the solid growth medium for *Gsk het* strain to enrich for knock-out conidia. Race tube medium were identical to solid growth medium with addition of 10 mM H_2O_2 or 80µg/ml Hygromycin where indicated. Liquid culture medium contained 2% Glucose, 2% Arginine and 1x Vogel's medium. Cyclohexiemide (CHX) was added at a concentration of 10 µg/ml where indicated.

2.3 Preparation of total cell lysates from Neurospora

Extraction of Neurospora protein, subcellular fractionation and extraction of nuclear proteins were performed as recently described (Schafmeier et al. 2005). Briefly, frozen mycelia were grinded using a mortar and pestle in presence of liquid nitrogen. Ground mycelia were incubated with bPEX buffer (25ml 1M Hepes

pH 7.4, 13.7ml 5M NaCl, 5ml 0.5M EDTA, pH8.0, 58.1ml 86%Glycerin in 500ml with water) in presence of protease inhibitors leupeptin (5µg/ml), pepstatin A (5µg/ml), PMSF (1mM), and PhosStop phosphatase inhibitor cocktail (Roche) for 30 minutes followed by centrifugation at 14000rpm. Supernatant was used as total cell lysates.

Strain	Phenotype	Source
wt9718	wild-type non-bd	Fungal genetics stock center
Gsk het	GSK down-regulation non-bd	Fungal genetics stock center
Abc homo	ABC knockout	Fungal genetics stock center
wt74 ras-1bd wt	wild-type bd	Fungal genetics stock center
wt74 ras-1bd Δwc-1	WC-1 knockout	Lee et al., 2003
wt74 ras-1bd Δwc-2	WC-2 knockout	Collett et al., 2001
wt74 ras-1bd Δvvd	VIVID knockout	Heintzen et al., 2001
wt74 ras-1bd frq10	FRQ knockout	Aronson et al., 1994)
wt74 ras-1bd Δwc-1, pWC1-wt	WC-1 wt expression from endog.promoter	this work
wt74 ras-1bd Δwc-1, pWC1-8A	WC-1 alanine mutant 8A	this work
wt74 ras-1bd Δwc-1, pWC1-8D	WC-1 aspartate mutant 8D	this work
wt74 ras-1bd Δwc-1, pWC1-ΔN-185	N-terminally truncated WC-1 up to a.a 185	K. Kaldi, Semmelweis University, Budapest
wt74 ras-1bd Δwc-1, pWC1-ΔN-287	N-terminally truncated WC-1 up to a.a 287	K. Kaldi, Semmelweis University, Budapest
wt74 ras-1bd Δwc-1, pWC1-ΔC-915	C-terminally truncated WC-1 up from a.a 915	K. Kaldi, Semmelweis University, Budapest

Figure 11
List of Neurospora strains used in this study

2.4 Sub-cellular fractionation of frozen mycelia

For sub-cellular fractionations, frozen mycelia was incubated with solution A (1 M sorbitol, 7% [w/v] Ficoll 400, 20% [v/v] glycerin, 5 mM, MgOAc2, 5 mM EDTA, 3 mM CaCl2, 3 mM DTT, 50 mM Tris/HCl pH 7.5) for 15 minutes. The sample was filtered using a cheese cloth and solution B (10% [v/v] glycerin, 5 mM MgOAc2, 5 mM EDTA, 25 mM Tris/HCl pH 7.5, protease inhibitors) was added in twice the volume. The mixture was gently poured over a solution containing solution A and B at a ratio of 1:1.7 and centrifuged at 1400rpm for 7 minutes in an Avanti J-26 XP (BeckmanCoulter Krefeld, Germany) rotor. An aliquot of the supernatant was taken as the TOTAL (T) sample. Rest of the sample was gently poured onto a solution containing 1 M saccharose, 10% [v/v] glycerin, 5 mM MgOAc2, 1 mM DTT, 25 mM Tris/HCl pH 7.5 and protease inhibitors and centrifuged for 20 minutes at 7000 rpm. Supernatant was collected as cytosol and the pellet (nuclear fraction) was dissolved in bPEX buffer and sonified.

2.5 Protein determination and analysis

Protein concentrations were determined by measuring absorption at 280 nm of an undiluted sample (NanoDrop®, PeqLab) and 200µg protein was loaded unless stated otherwise. Western blotting was performed as described (Gorl et al. 2001; Schafmeier et al. 2005). Enhanced chemiluminescence signals were detected with X-ray films. Series of exposures in the range of 10 s to 10 min were generated. Quantification of Western blots was performed using ImageJ software where indicated (Rasband, W.S., ImageJ, U. S. National Institutes of Health).

2.6 Generation of HIS-tagged CKI and GSK plasmids

Full length GSK and CKIa were amplified from a total cDNA preparation from *Neurospora* using primers with restriction sites SphI and PstI and inserted into the PQE30 plasmid (Qiagen) and transformed into M(15) Prep4 strain (Qiagen) of

E.Coli cells using heat shock along with an empty PQE30 plasmid (mock control). See figures 12 and 13 for plasmid maps.

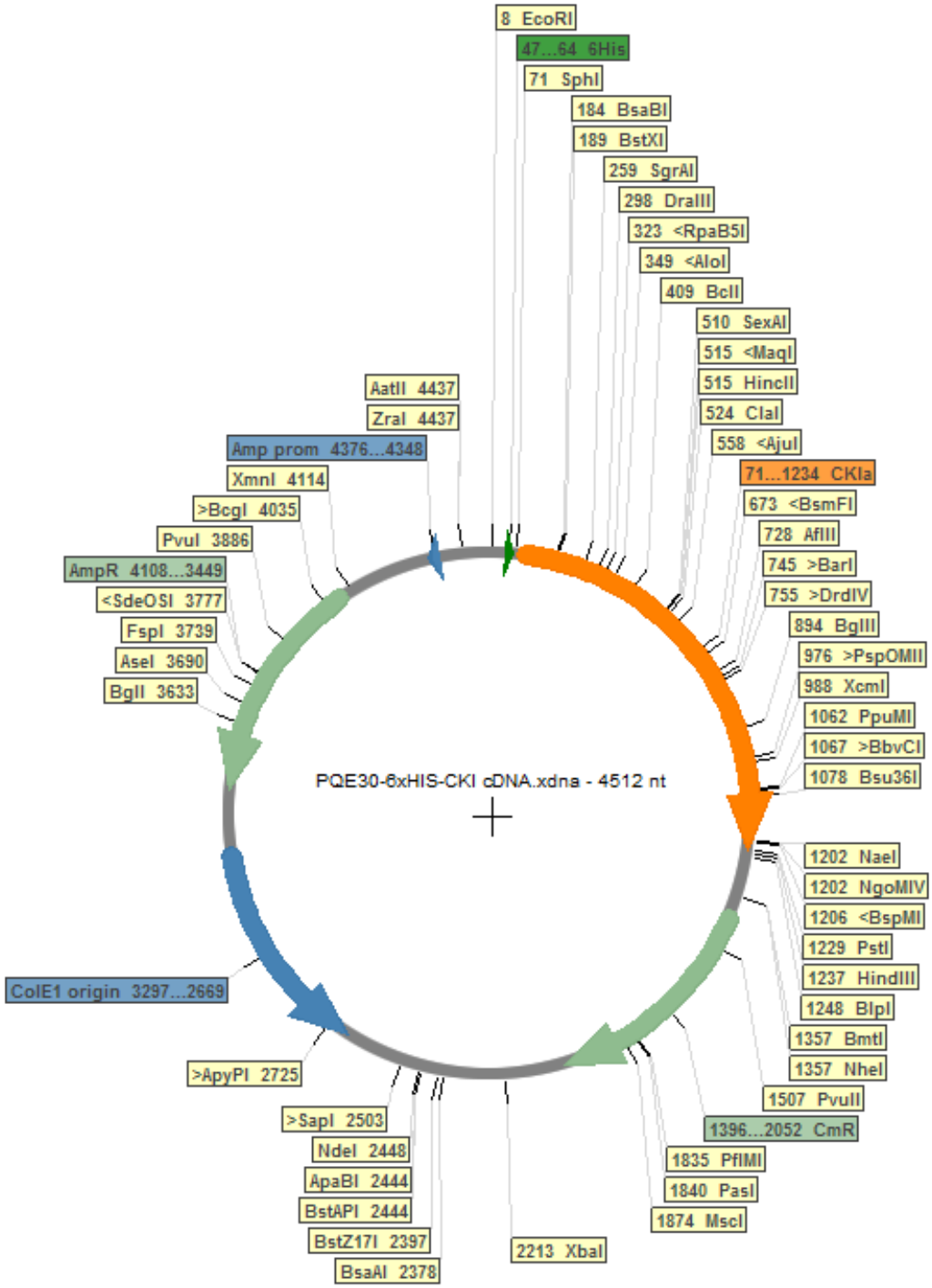

Figure 12

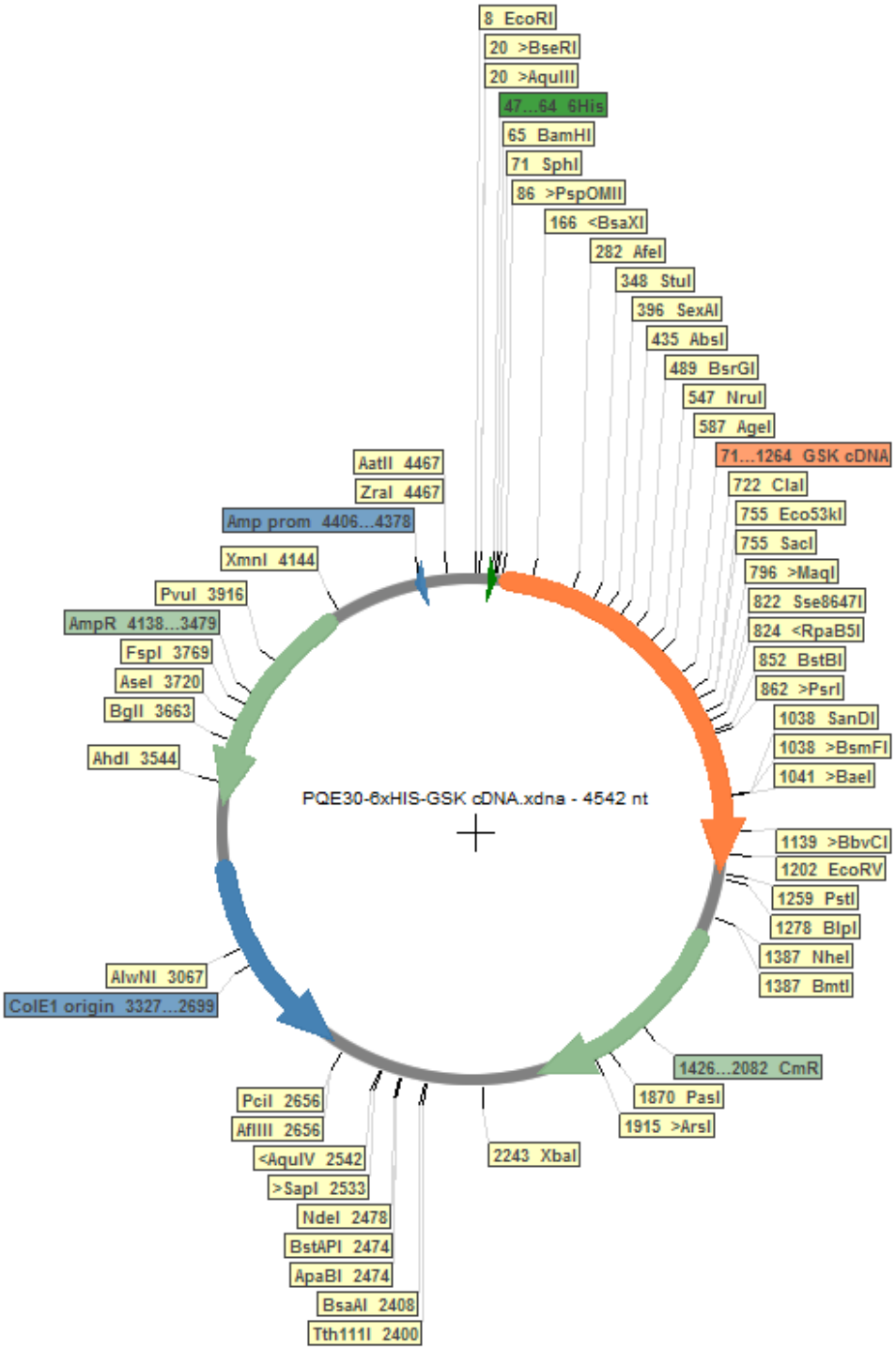
8 EcoRI
20 >BseRI
20 >AquIII
47...64 6His
65 BamHI
71 SphI
86 >PspOMII
166 <BsaXI
282 AfeI
348 StuI
396 SexAI
435 AbsI
489 BsrGI
547 NruI
587 AgeI
71...1264 GSK cDNA
722 ClaI
755 Eco53kI
755 SacI
796 >MaqI
822 Sse8647I
824 <RpaB5I
852 BstBI
862 >PsrI
1038 SanDI
1038 >BsmFI
1041 >BaeI
1139 >BbvCI
1202 EcoRV
1259 PstI
1278 BlpI
1387 NheI
1387 BmtI
1426...2082 CmR
1870 PasI
1915 >ArsI
2243 XbaI
PciI 2656
AflIII 2656
<AquIV 2542
>SapI 2533
NdeI 2478
BstAPI 2474
ApaBI 2474
BsaAI 2408
Tth111I 2400
AlwNI 3067
ColE1 origin 3327...2699
AhdI 3544
BglI 3663
AseI 3720
FspI 3769
AmpR 4138...3479
PvuI 3916
XmnI 4144
Amp prom 4406...4378
ZraI 4467
AatII 4467
PQE30-6xHIS-GSK cDNA.xdna - 4542 nt

Figure 13

2.7 Expression and purification of active kinases from *E.coli*

A preculture was inoculated into LB medium containing ampicillin (100ng/µl final) and grown at 37 degrees overnight (o/n, 180rpm shaking). Next day, a 2 liter (or more depending on the amount of protein needed) culture with 1:100 from the pre-culture was inoculated and grown at 37 degrees until OD600 is around 0.3 and then the temperature was lowered to 20 degrees. The culture was induced with 0.1mM IPTG overnight and harvested at 4000rpm for 20min.

Special care was given to perform all the remaining steps at 4 degrees celcius. HIS-tagged kinases were isolated using standard Qiagen protocol for PQE30 vectors (QIA expressionist) with the following changes. The lysate or any the solutions at any step never frozen to avoid precipitation of the proteins. The cell pellet was lysed in presence of protease inhibitors (EDTA-free since EDTA will interact with the NiNTA-column). Inhibitors were added right before lysing the cells. Special care was taken to keep the lysate cold and avoid heating up during lysing using a micro-fluidizer.

The lysed cells were centrifuged at 10 000 rpm for 20min at 4 degrees. During the centrifugation, a small column with 1ml NiNTA beads (bead volume) was prepared and washed twice with 15ml lysis buffer to equilibrate. The isolation of the HIS-tagged kinase was done in a cold room using gravity or a pump with max 0.5ml/min flow rate. Input fraction before running through the column and the flow-through (FT) after running the column was collected. The column was washed twice with 15ml wash buffer and fractions containing wash1 and wash 2 fractions (W1, W2) were collected. Elution was done in three steps with 1ml elution buffer with 1 min incubation between each step.

Three fractions of eluate were sampled for later use in SDS-PAGE. Since the final buffer contains a lot of Imizadole which may result precipitation, the sample was immediately re-buffered into the bPEX (without glycerol) buffer using PD-10 columns (GE Healthcare). Glycerol was added after re-buffering since it would interfere with the PD-10 column.

Aliquots from each fraction (Input,Ft, W1, W2, E1, E2 and E3) were tested with SDS-PAGE followed by coomassie staining and the purification was confirmed with the presence of a thick band near 40 KDa for the purified proteins. Protein concentration was measured and small aliquots for your future applications were frozen in liquid nitrogen and stored at -80 degrees. Repeated freeze and thaw resulted in reduction of activity, especially for GSK and therefore avoided.

2.8 In vitro phosphorylation in *Neurospora* total cell extracts

The amount of enzyme required for saturating phosphorylation was determined by a series of kinase dilutions and finally 18.24 µg CKIa, 38.88 µg GSK and 24 µg mock control proteins were added to 200 µg of total *Neurospora* cell lysate in a final reaction volume of 30 µl and incubated for 1 hour at 25 ^{o}C. Final reaction mixture contained 50mM HEPES/KOH pH 7.4, 150mM NaCl, 10mM MgCl2, 10mM ATP, leupeptin (5µg/ml), pepstatin A (5µg/ml), PMSF (1mM), and 1X PhosStop phosphatase inhibitor cocktail (Roche). Samples were analyzed with SDS-PAGE followed by western blotting with respective antibodies.

2.9 Co-immunoprecipitation in *Neurospora* total cell lysates

Antibody against Neurospora GSK was raised in rabbit using the C-terminal peptide sequence NH2-CDNFTPMNKSEMMAKLD-COOH (Pineda, Germany). 100 µl Protein A beads were washed 3X with 1ml TBS by inverting followed by centrifugation at 2000 rpm for 1 minute. 200µl GSK antibody or 40µl WC2 antibody (where indicated) was added and the mixture was completed to 1ml using TBS. The beads were incubated with the antibody at room temperature for 2 hours and then washed twice with 1ml bPEX and supernatant discarded. 5% Milk solution (buffered in TBS, pH 7.4) was added to beads for blocking and incubated at room temperature using a rotary shaker. During blocking, a total of 10mg protein from *Neurospora* cell extract was prepared in 2ml bPEX with fresh

protease inhibitors. 1ml of this solution was taken and stored as TOTAL (T). After blocking is complete, beads were washed 3X with 1ml bPEX. The remaining 1ml of the protein solution (contains 5mg protein) was added to the beads. Incubation was performed in a cold room overnight. Following day, the beads were centrifuged at 2000 rpm for 1 min and the supernatant was taken as UNBOUND (U). After 2 steps of washing with 1ml bPEX buffer, the BOUND (B) fraction was collected by cooking the beads with 250µl 1X Laemmli buffer at 95 degrees. The total and unbound fractions were also cooked at this time. The samples were loaded on SDS-PAGE and analyzed with western blotting. Successful immunoprecipitations were confirmed by depletion of GSK or WC2, depending on the antibody used.

2.10 Quantitative real-time PCR

Total mRNA from ground frozen mycelia was prepared using peqGOLD TriFAST™ (peqLab, Germany) and reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Germany) following the manufacturer's instructions. Transcript levels were analyzed by quantitative real time PCR as described previously (LIT). Primer and probes used in this study are as follows:

actin:	F	gat gac aca gat cgt ttt cga gac t
	R	cgg agg cgt aga gag aaa gga
	Probe	6-FAM- ccg cct tct acg tct cca tcc a - TAMRA
wc-1:	F	acc tcg ctg tcc tcg att tg
	R	tgc tgg gcc tct ttc aac tc
	Probe	6-FAM - ccg tcc gac atc gtg ccg g - TAMRA
frq:	F	ttg taa tga aag gtg tcc gaa ggt
	R	gga gga aga agc gga aaa cg
	Probe	6-FAM - acc tcc caa tct ccg aac tcg cct g - TAMRA
vvd :	F	acg tca tgc gct ctg att ctg
	R	aaa agc ttc cga ggc gta ca
	Probe	6-FAM - cga cct gaa gca aaa aga cac gcc a - TAMRA
wc-2 :	F	agt ttg cac cca atc cac aga

	R	agg gtc gag cca tca tga ac
	Probe	6-FAM – agt cgc ctt tct gcc agg ccg – TAMRA
gsk :	F	ccc gac tcg agg cac aac t
	R	tgg ata gct cat gac ggg taa agt
	Probe	6-FAM – acc gtt agg gat ctg cca ccg ctc tt– TAMRA

2.11 Generation of pWC1-WC1 plasmids and strains

A vector containing a DNA piece of the WC1 ORF from restriction sites XmaI to EagI with mutated serine and threonine residues were synthesized by Genscript (USA) in a PUC57 vector. A fragment of WC1 ORF from restriction sites PmlI to SexAI was cloned into an empty Pgem4 vector (promega). The source of this WC1 ORF fragment was a vector from K.Kaldi (Semmelweis University, Budapest). The fragments from the Genscript vector and the Pgem4 vector exchanged, creating a new Pgem4 vector with all eight amino acids exchanged into alanines or aspartates. Another vector containing the genomic locus (from -2280bp to +4980 bp from the ATG) of WC1 was cloned into pBM60 vector using restriction sites SpeI and NotI. This was is named pWC1-wt and contains both the 5‘ and the 3‘ UTRs of WC1. The mutant DNA piece from the created pGem4 vector was exchanged into this vector using restriction sites PmlI and SexAI, thus creating the plasmids labeled pWC1-8A and pWC1-8D.

See the charts below for the flowchart and plasmids of these clonings. All forward primers used are marked with red and reverse primers are marked with yellow and unique restriction sites indicated. ***All plasmid maps are also provided in digital format with more detail.***

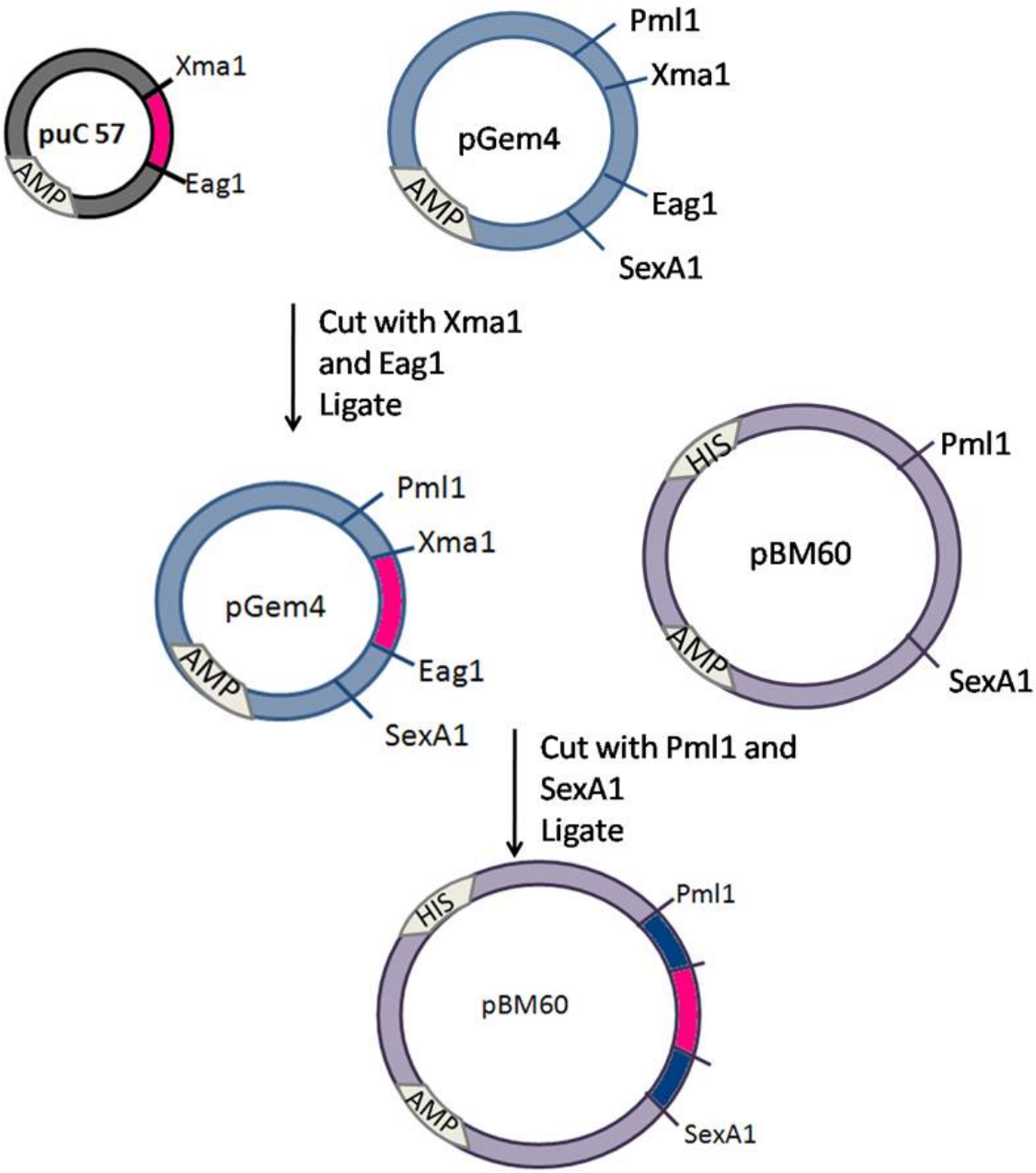
puC 57
Xma1
Eag1
AMP
pGem4
Pml1
Xma1
Eag1
SexA1
AMP
Cut with Xma1
and Eag1
Ligate
pGem4
Pml1
Xma1
Eag1
SexA1
AMP
pBM60
HIS
Pml1
SexA1
AMP
Cut with Pml1 and
SexA1
Ligate
pBM60
HIS
Pml1
SexA1
AMP

Figure 14

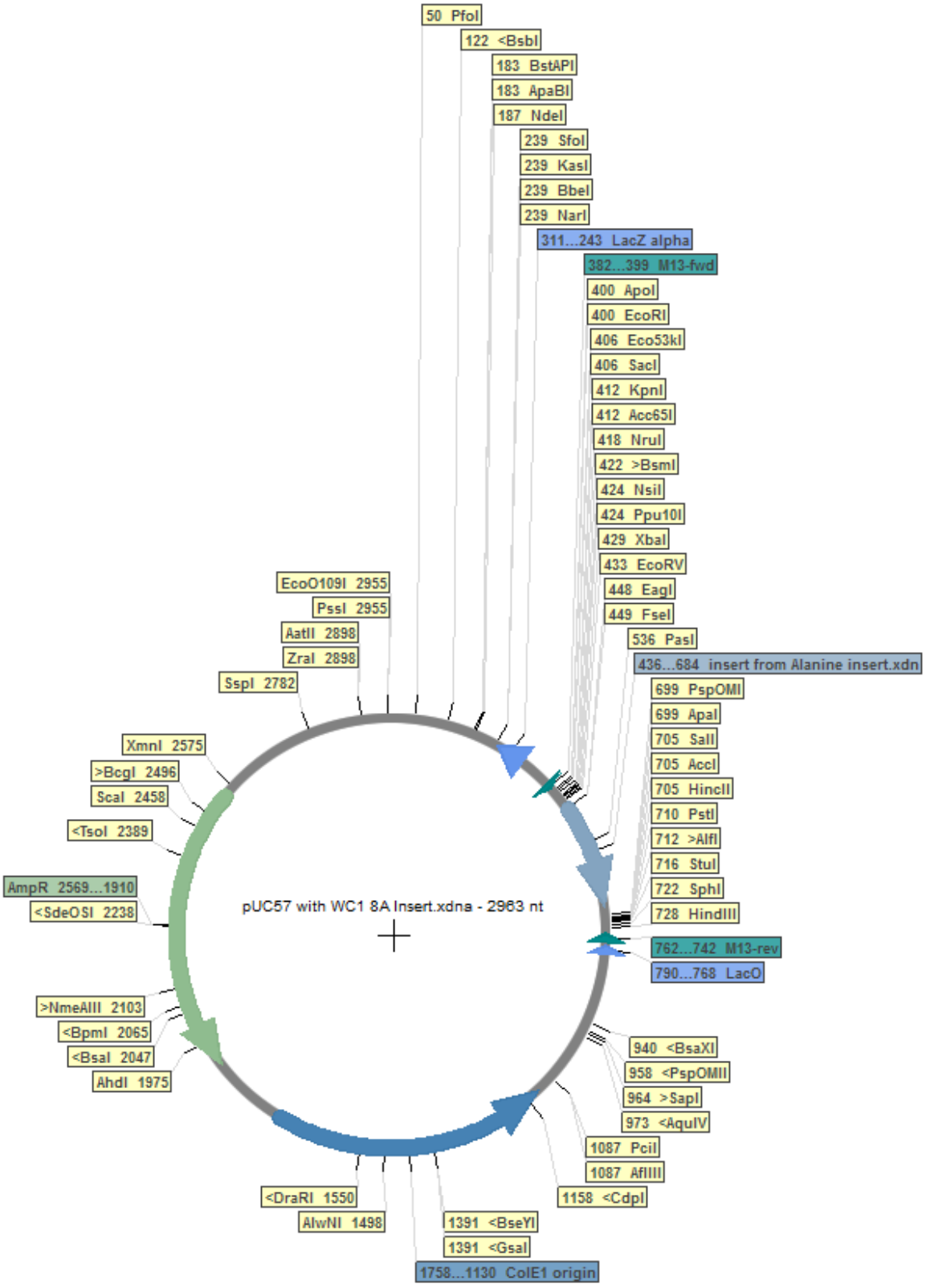
50 PfoI
122 <BsbI
183 BstAPI
183 ApaBI
187 NdeI
239 SfoI
239 KasI
239 BbeI
239 NarI
311...243 LacZ alpha
382...399 M13-fwd
400 ApoI
400 EcoRI
406 Eco53kI
406 SacI
412 KpnI
412 Acc65I
418 NruI
422 >BsmI
424 NsiI
424 Ppu10I
429 XbaI
433 EcoRV
448 EagI
449 FseI
536 PasI
436...684 insert from Alanine insert.xdn
699 PspOMI
699 ApaI
705 SalI
705 AccI
705 HincII
710 PstI
712 >AlfI
716 StuI
722 SphI
728 HindIII
762...742 M13-rev
790...768 LacO
940 <BsaXI
958 <PspOMII
964 >SapI
973 <AquIV
1087 PciI
1087 AflIII
1158 <CdpI
1391 <BseYI
1391 <GsaI
1758...1130 ColE1 origin
<DraRI 1550
AlwNI 1498
>NmeAIII 2103
<BpmI 2065
<BsaI 2047
AhdI 1975
AmpR 2569...1910
<SdeOSI 2238
<TsoI 2389
ScaI 2458
>BcgI 2496
XmnI 2575
SspI 2782
AatII 2898
ZraI 2898
PssI 2955
EcoO109I 2955
pUC57 with WC1 8A Insert.xdna - 2963 nt

Figure 15

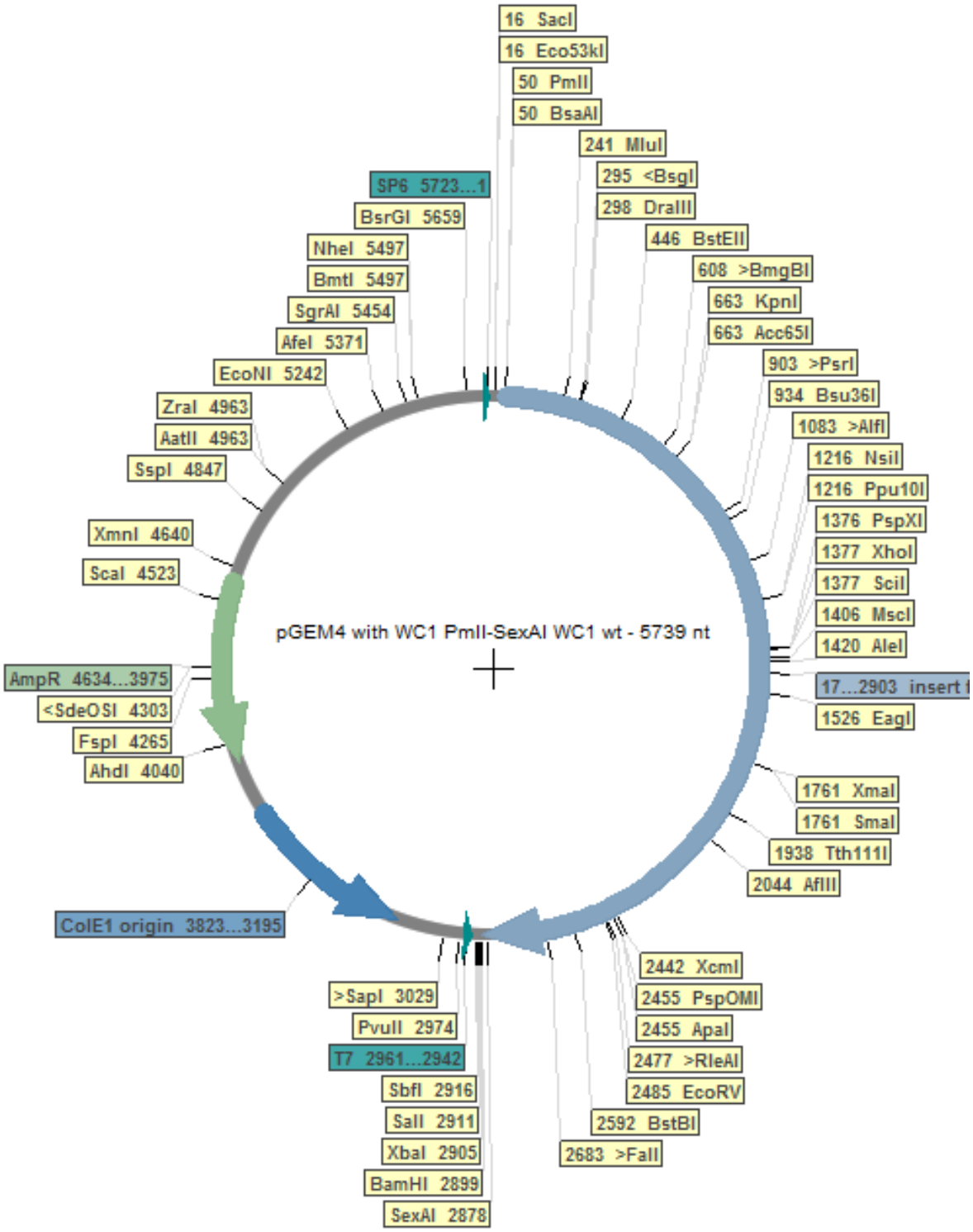
pGEM4 with WC1 PmlI-SexAI WC1 wt - 5739 nt
16 SacI
16 Eco53kI
50 PmlI
50 BsaAI
241 MluI
295 <BsgI
298 DraIII
446 BstEII
608 >BmgBI
663 KpnI
663 Acc65I
903 >PsrI
934 Bsu36I
1083 >AlfI
1216 NsiI
1216 Ppu10I
1376 PspXI
1377 XhoI
1377 SciI
1406 MscI
1420 AleI
17...2903 insert
1526 EagI
1761 XmaI
1761 SmaI
1938 Tth111I
2044 AflII
2442 XcmI
2455 PspOMI
2455 ApaI
2477 >RleAI
2485 EcoRV
2592 BstBI
2683 >FalI
SexAI 2878
BamHI 2899
XbaI 2905
SalI 2911
SbfI 2916
T7 2961...2942
PvuII 2974
>SapI 3029
ColE1 origin 3823...3195
AhdI 4040
FspI 4265
<SdeOSI 4303
AmpR 4634...3975
ScaI 4523
XmnI 4640
SspI 4847
AatII 4963
ZraI 4963
EcoNI 5242
AfeI 5371
SgrAI 5454
BmtI 5497
NheI 5497
BsrGI 5659
SP6 5723...1

Figure 16

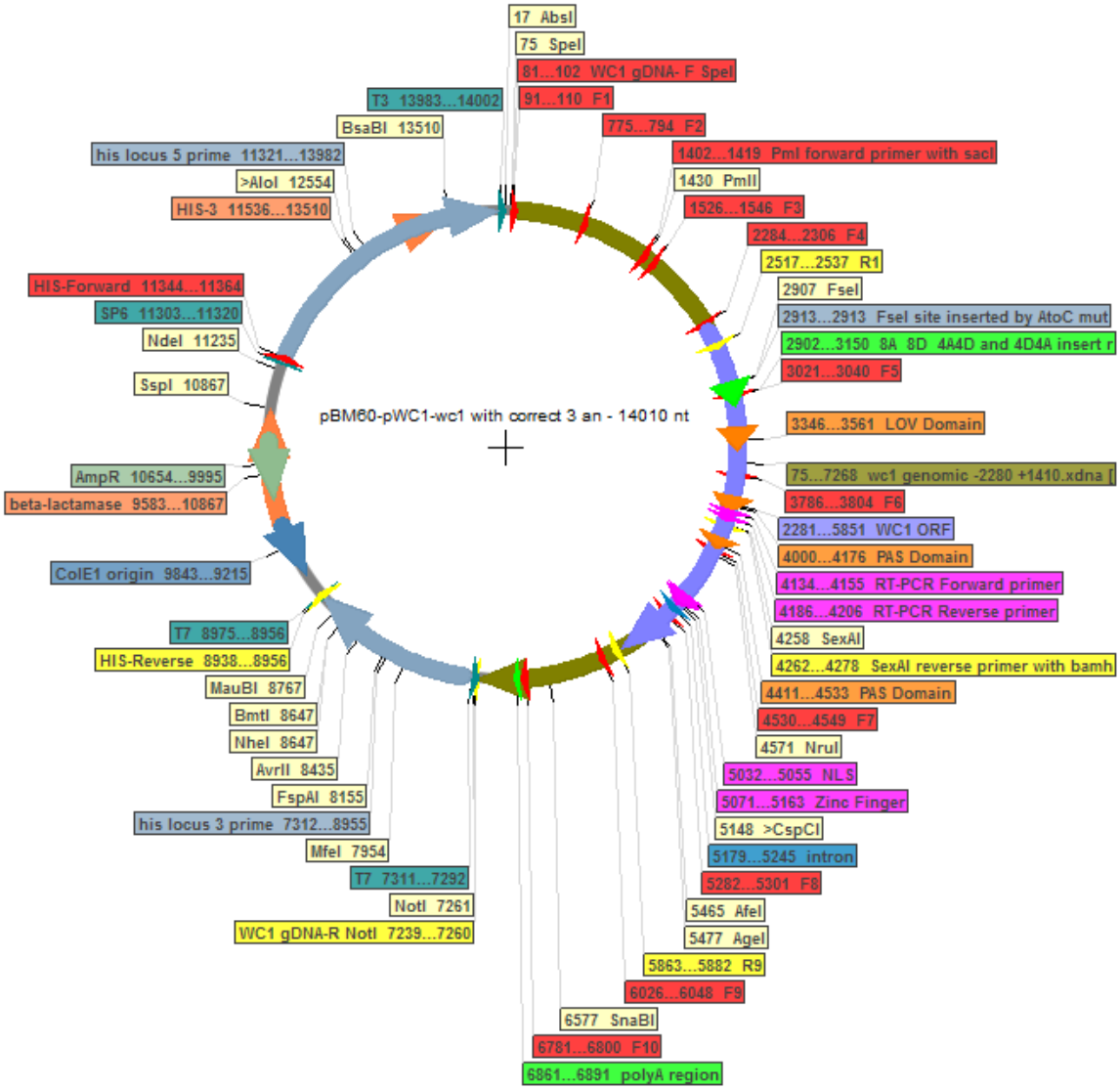
pBM60-pWC1-wc1 with correct 3 an - 14010 nt
17 AbsI
75 SpeI
81...102 WC1 gDNA- F SpeI
91...110 F1
775...794 F2
1402...1419 PmI forward primer with sacI
1430 PmlI
1526...1546 F3
2284...2306 F4
2517...2537 R1
2907 FseI
2913...2913 FseI site inserted by AtoC mut
2902...3150 8A 8D 4A4D and 4D4A insert r
3021...3040 F5
3346...3561 LOV Domain
75...7268 wc1 genomic -2280 +1410.xdna
3786...3804 F6
2281...5851 WC1 ORF
4000...4176 PAS Domain
4134...4155 RT-PCR Forward primer
4186...4206 RT-PCR Reverse primer
4258 SexAI
4262...4278 SexAI reverse primer with bamh
4411...4533 PAS Domain
4530...4549 F7
4571 NruI
5032...5055 NLS
5071...5163 Zinc Finger
5148 >CspCI
5179...5245 intron
5282...5301 F8
5465 AfeI
5477 AgeI
5863...5882 R9
6026...6048 F9
6577 SnaBI
6781...6800 F10
6861...6891 polyA region
T3 13983...14002
BsaBI 13510
his locus 5 prime 11321...13982
>AlwI 12554
HIS-3 11536...13510
HIS-Forward 11344...11364
SP6 11303...11320
NdeI 11235
SspI 10867
AmpR 10654...9995
beta-lactamase 9583...10867
ColE1 origin 9843...9215
T7 8975...8956
HIS-Reverse 8938...8956
MauBI 8767
BmtI 8647
NheI 8647
AvrII 8435
FspAI 8155
his locus 3 prime 7312...8955
MfeI 7954
T7 7311...7292
NotI 7261
WC1 gDNA-R NotI 7239...7260

Figure 17

3.1 Down-regulation of GSK results in loss of temperature compensation

GSK is an essential protein and complete knock-outs are not viable. Therefore, we obtained a GSK down-regulation strain (*Gsk*^{het}) to study the effects of altered GSK function on the circadian clock of *Neurospora*, in particular its role in temperature compensation. In this strain, g*sk* gene has been replaced by a Hygromycin B (Hyg) resistance cassette which allows dose dependent reduction of the *gsk* mRNA by positively selecting for knockout nuclei with increasing doses of Hyg. Under steady- state conditions (constant light, LL), the *Gsk*^{het} strain has reduced levels of GSK protein and mRNA compared to its background control strain *wt9718* and the levels of GSK can be further reduced by addition of Hygromycin B (Figure 18). Our preliminary experiments showed that addition of 80μM Hyg provides a balanced compromise between defective growth and reduction in GSK levels. Application of higher doses of Hyg simply resulted in defective growth and did not actually reduce the GSK levels further.

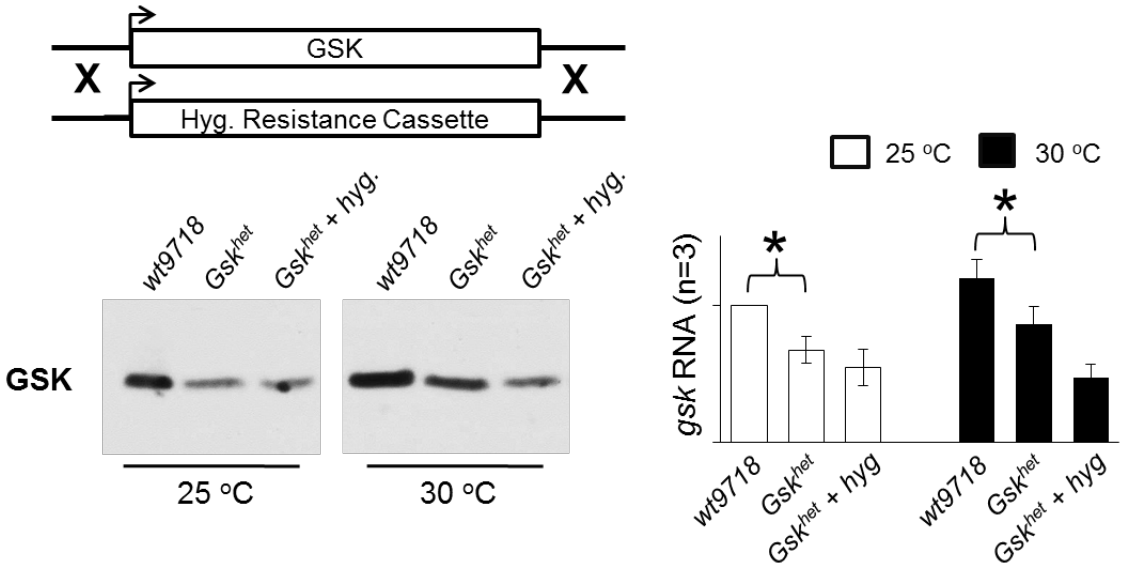

Figure 18
Total levels of GSK protein and mRNA in the Gskhet strain are lower compared to its background control strain *wt9718* at steady state conditions (constant light (LL), 25 and 30 ^{o}C). Note that addition of 80μM Hygromycin B results in further decrease in the *gsk* mRNA and protein.

Race-tube assays are a common and well established technique for measuring the period length of the circadian clock in *Neurospora*. The assay relies on a phenomenon called “banding” which is, in essence, rhythmic conidiation in a long glass tube. The tube is filled with a solid support medium and the strain of interest is inoculated from one end. As the *Neurospora* grows towards the other end, it exhibits rhythmic production of aerial hyphae which is seen as bands when the tube is scanned and analyzed (see introduction, figure 5). This phenomenon, albeit its common use, actually only occurs in a specific mutant strain of *Neurospora crassa*. Recently, this mutation has been identified and its role in the amplification of circadian rhythms has been shown to map to a hyper-active form of the RAS-1 protein (Belden, Larrondo et al. 2007).

Due to lethality of the complete loss of GSK, Gsk^{het} strain cannot be back-crossed to a this mutant ras^{bd} strain to allow for easy investigation of the circadian rhythm on race tubes. However, in the same study, it was shown that banding can also be induced in wild-type strains by applying oxidative stress using Menadione which also results in activation of the RAS pathway (Belden, Larrondo et al. 2007). After a series of trials where we also induced oxidative stress using various doses of Menadione or H_2O_2, we found that addition of 10 mM H_2O_2 to the race-tube medium resulted in a clear banding pattern in the Gsk^{het} strain as well as the *wt9718* strain (Figure 19). This method enabled us to investigate the period length of these strains at different temperatures in a reliable way.

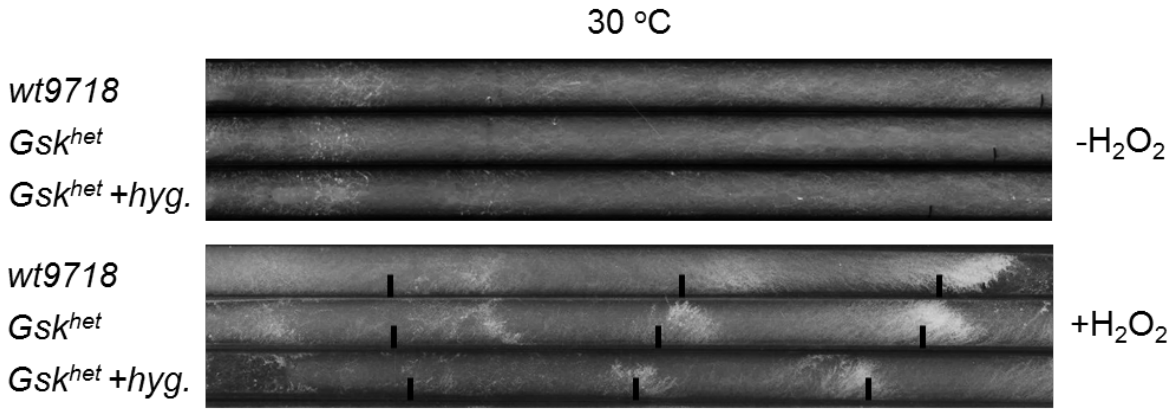

Figure 19
Representative banding patterns of the circadian conidiation rhythm in strains that are wild-type for *ras-1* with or without 10mM H_2O_2.

Using 10 mM H_2O_2, we performed various race tube experiments where we tested the effect of down-regulation of GSK on period length at different temperatures. Evaluation of these race tubes showed that there was no significant difference between the period lengths of the circadian clock of the *wt9718* and the *Gsk*^{het} strains at 25 °C (Figure 20). Furthermore, addition of 80µM Hyg also did not change the period length at this temperature.

When the temperature was raised to 30 °C, period length of the *wt9718* strain did not change significantly, showing that the strain is properly temperature compensated. However, period length of the *Gsk*^{het} strain was significantly shorter at 30 °C and this shortening was more pronounced when Hyg was added to the race-tube. This data showed that reduced levels of GSK result in loss of temperature compensation in the *Neurospora crassa* circadian clock.

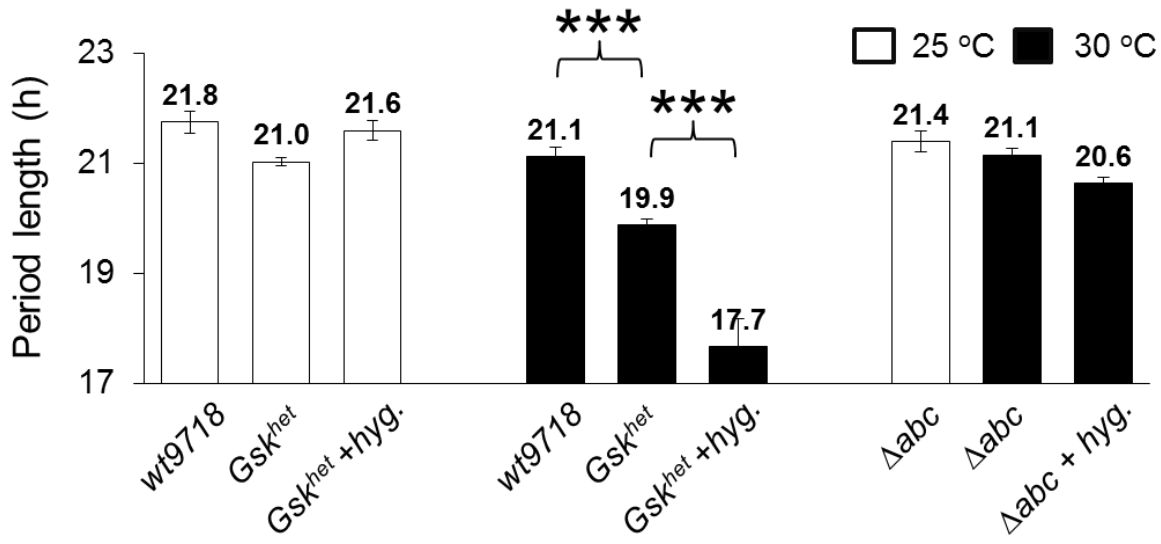

Figure 20
Summary of the average period lengths at 25 or 30 °C. Difference between groups were analyzed using two-tailed Student's t-Test and p-values ≤0.0005 are indicated (***). Hygromycin B was added to *Gsk*^{het} and *abc homo* strains at a dose of 50 µg/ml where indicated. Error bars show ±standard error of the mean (±SEM) from at least three independent experiments. Summary of period lengths of all strains used in this study are also summarized in figure 41 with ±SEM.

Application of both H_2O_2 and Hyg in race-tube assays was never done before. Therefore, we tested whether presence of both H_2O_2 and Hyg has any unspecific effect on the circadian clock. We used another Hyg resistant, non-banding strain called *abc homo*, because it also harbors a Hyg resistance cassette and our unpublished previous work showed that this gene has no effect on the circadian clock. The *abc* strain showed wild-type behavior at both temperatures showing that H_2O_2 alone or in combination with Hyg has no effect on period length or the temperature compensation of the circadian clock under these conditions (Figure 20). These findings show that reduction of GSK levels specifically result in loss of temperature compensation.

3.2 Down-regulation of GSK increases WC-1 levels

The results of the race tube assays showed that the clock is running faster in the *Gsk*^{het} strain at elevated temperatures, hence the shorter period length. This can be achieved by either increasing the activity of the positive element (WCC) or decreasing the effect of the negative element (FRQ) of the clock. Therefore, we investigated whether the levels of clock proteins change in the *Gsk*^{het} strain in a temperature dependent manner.

We did not observe a significant effect on the level of FRQ or WC-2 protein in the *Gsk*^{het} strain at either 25 or 30 degrees. However, the level of WC-1 protein was ca. 1.8 fold higher in the *Gsk*^{het} strain compared to its wild-type control at 30 degrees (Figure 21 and quantification in figure 22, left column).

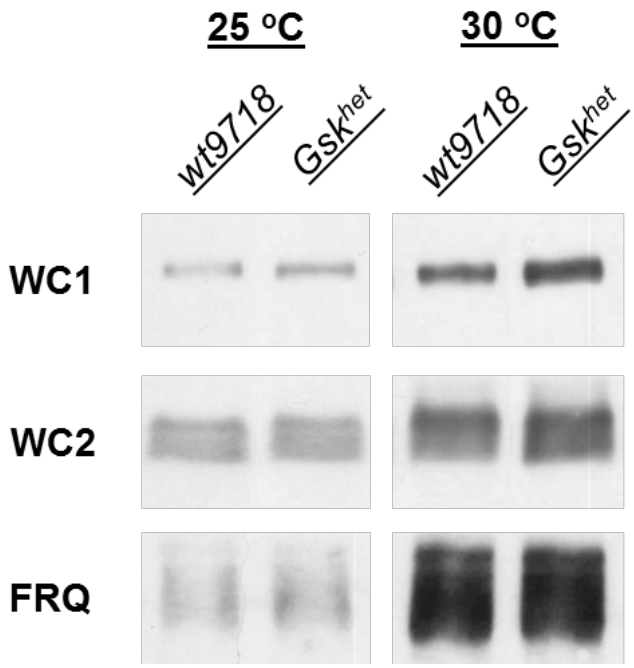

Figure 21
Representative western blots showing levels of clock proteins in the *Gsk*^{het} strain compared to its background control strain *wt9718* at steady state conditions (LL, 25 and 30 °C).

We asked whether this increase is due to elevated levels of *wc-1* mRNA. Our analysis revealed that the mRNA level of *wc2, frq* and *wc1* does not significantly increase when the temperature is elevated to 30°C. Furthermore, they were not altered by GSK down-regulation (Figure 22, right column).

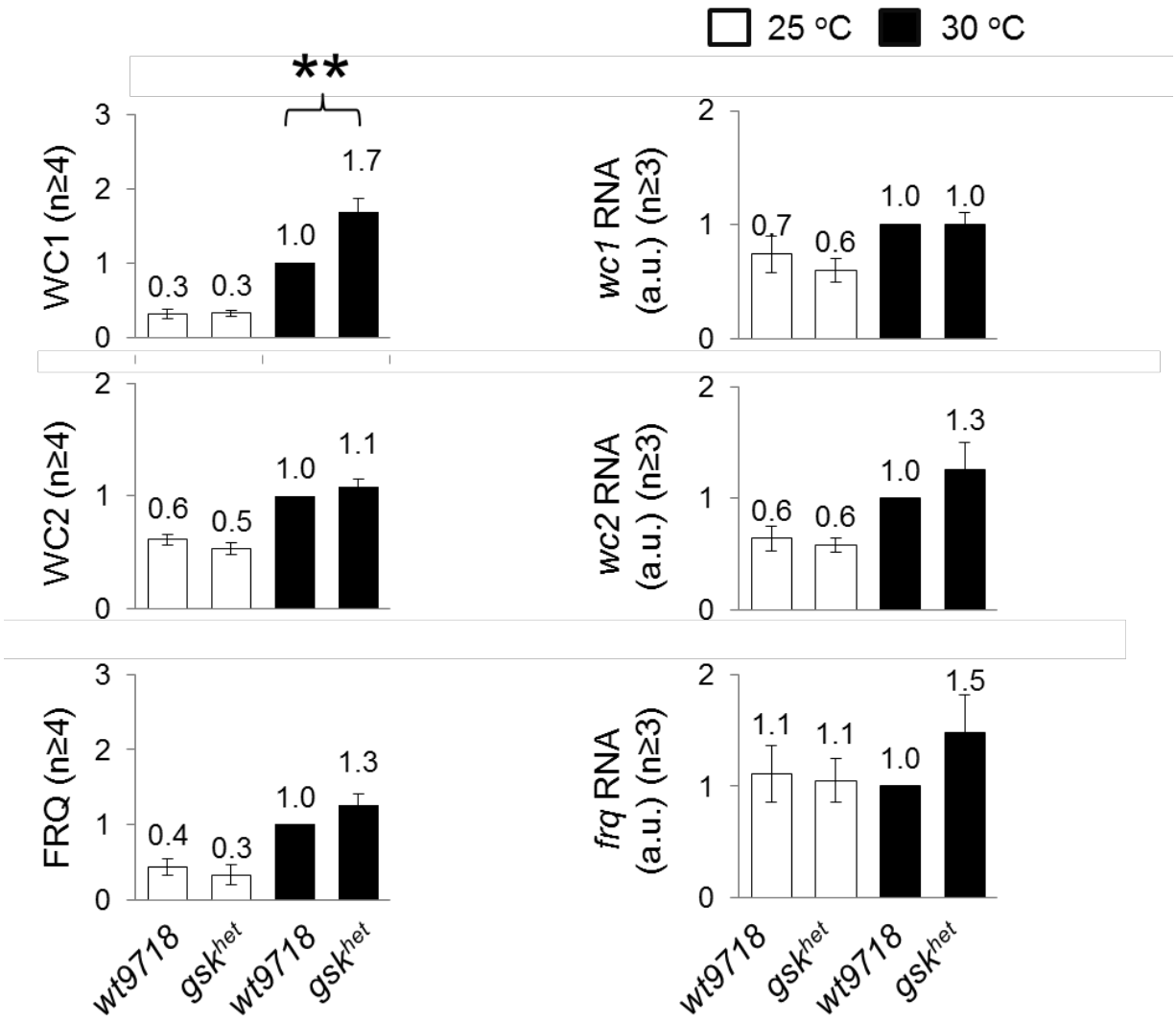

Figure 22
Quantification of steady-state levels of WC-1, WC-2 and FRQ proteins and their mRNA are shown with ±SEM. All quantifications were normalized to respective *wt9718* levels at 30 degrees. Difference between *wt9718* and *Gsk*^{het} within each temperature was analyzed using two-tailed Student's t-Test and p-values ≤0.0005 are indicated with ***.

The lack of a difference in the mRNA levels indicated that effects of GSK on WC-1 levels are post-transcriptional and that WC-1 protein may be more stable when GSK levels are lower. Therefore, we performed stability assays using Cyclohexiemide (CHX) under constant conditions. We did not see a significant difference between *wt9718* and *Gsk*^{het} strains at either temperature (Figure 23).

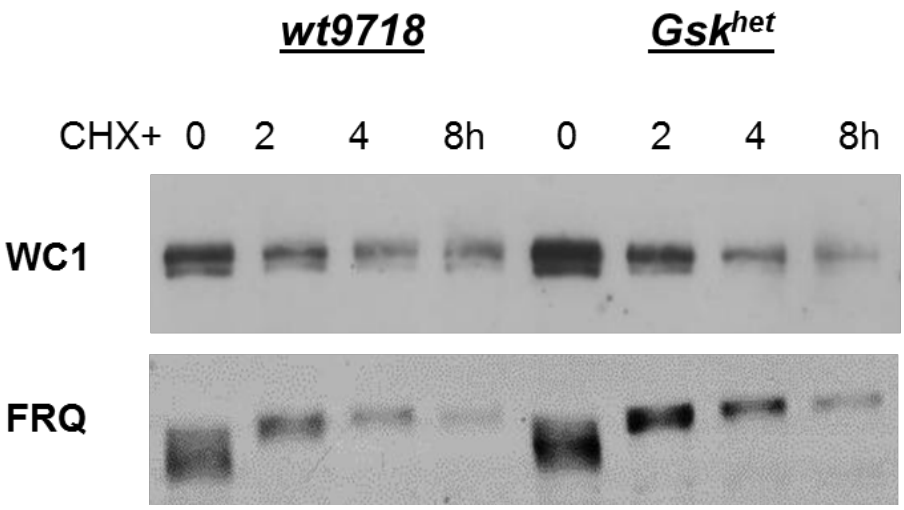

Figure 23
Representative western blots from samples grown at 30 degrees showing that the stability of WC-1 and FRQ are not affected in the GSK down-regulation strain under constant conditions (LL) after addition of CHX up to 8 hours.

GSK has been previously shown to have an effect on the subcellular distribution of clock proteins in Drosophila (Ko, Kim et al. 2010). Therefore, we tested whether such an effect is also present in *Neurospora crassa.* However, we did not see any significant effect of GSK down-regulation on the distribution of clock proteins WC-1, WC-2 or FRQ at normal or elevated temperatures in our sub-cellular fractionation assays (Figure 24).

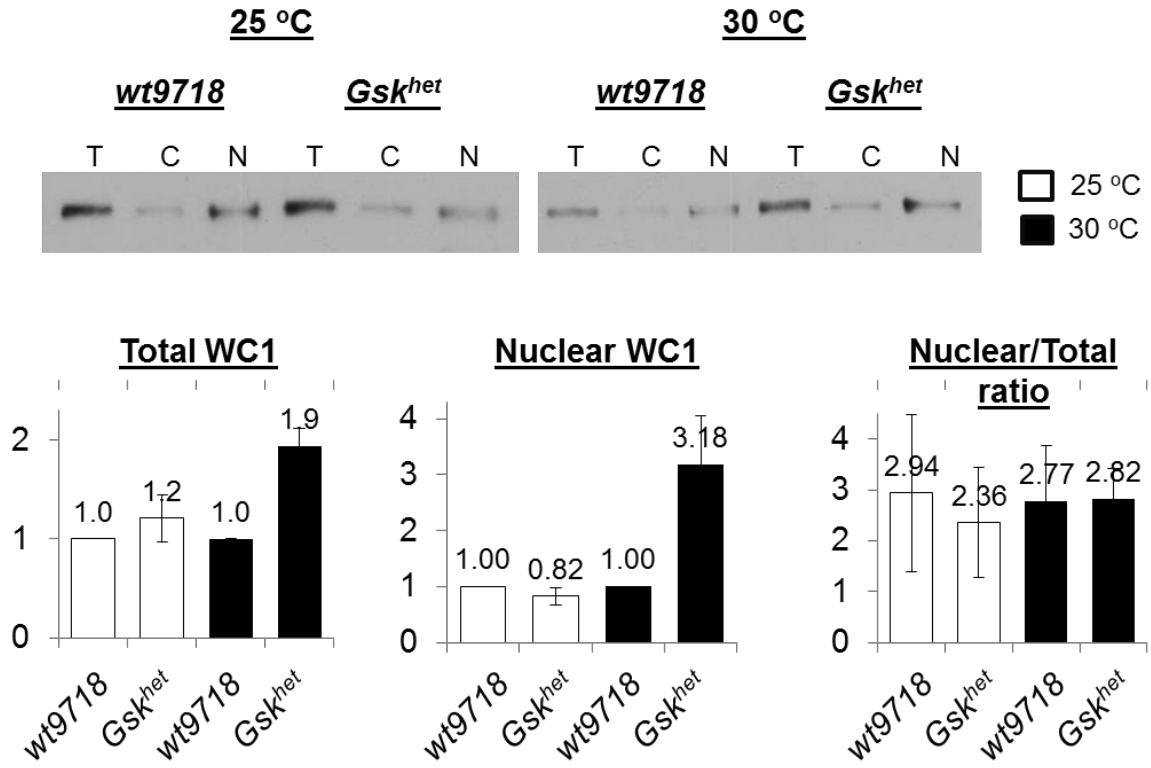

Figure 24
Sub-cellular fractionation of *Neurospora* cell extracts at 25 vs. 30 degrees from *wt9718* and *Gsk*^{het} strains. Protein levels in each fraction were normalized to its 25 degrees wild-type control and the averages of at least six independent experiments are shown with ±SEM. Note that although the total WC-1 levels increase in the Total and Nuclear fractions, their ratio does not differ between the *wt9718* and *Gsk*^{het} strains showing that GSK down-regulation does not affect the sub-cellular localization of WC1.

3.3 GSK binds to WCC *in vivo*

Since we observed that GSK down-regulation results in increased levels of WC-1, and therefore increased WCC activity, we tested whether GSK physically interacts with WCC *in vivo*. Our preliminary tests with commercially available antibodies from other organisms that may recognize *Neurospora* GSK failed (not shown). Therefore, we raised an antibody in rabbit against *Neurospora* GSK which

recognizes the C-terminal end of the *Neurospora* GSK protein. This antibody proved to be very efficient and clean and was used throughout this project.

We performed co-immunoprecipitation assays from whole cell extracts of *Neurospora* grown at 25°C or 30°C and found that both WC-1 and WC-2 co-immunoprecipitate with GSK (Figure 25). Interestingly, although the interaction of GSK with the WCC was stable (since the precipitation was done overnight and the WCC-GSK complex survived the process), the fraction we could precipitate was approximately only 10% of total WC-1. This shows that GSK is bound to only a small fraction of the total WCC at any given time in the cell.

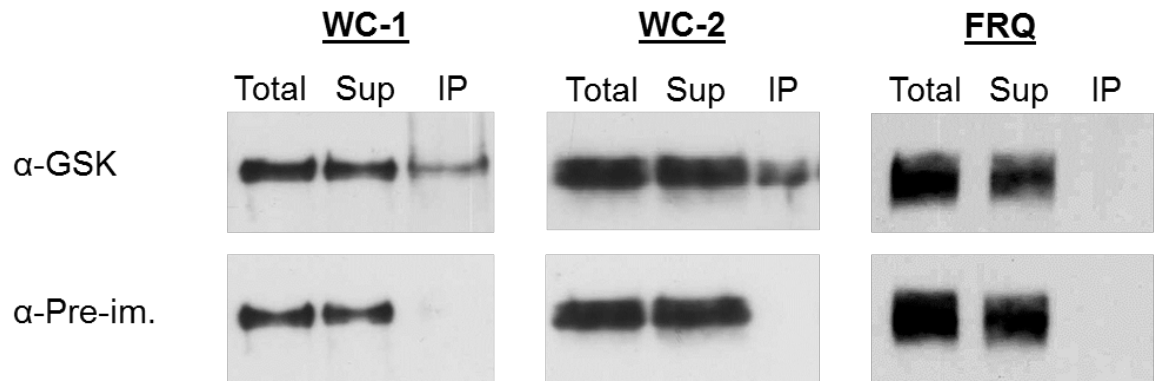

Figure 25
A representative co-**i**mmunoprecipitation assay using GSK antibody and pre-immune serum control in total cell extracts from *wt9718*. An equivalent of 200µg Total, 200µg supernatant and 800µg IP protein were loaded. Antibodies used for each blot are indicated on the top of each column and the antibodies used for the IP are shown on the left of each row.

Since WCC is comprised of WC-1 and WC-2, we tested whether GSK can bind directly to either WC-1 or WC-2 alone. We used a *ΔWC-1* strain where WC-1 gene is knocked out and found that GSK can bind to WC-2 in the absence of WC-1 (Figure 26). Unfortunately, WC-1 levels are undetectable in a ∆wc-2

background. Furthermore, in the only strain where WC-1 is stable by itself (because it lacks the ZINC finger motif); the WC-1 antibody produced excessive background with GSK pull-downs in spite of our numerous efforts. Therefore we were not able to measure whether GSK can bind WC-1 alone.

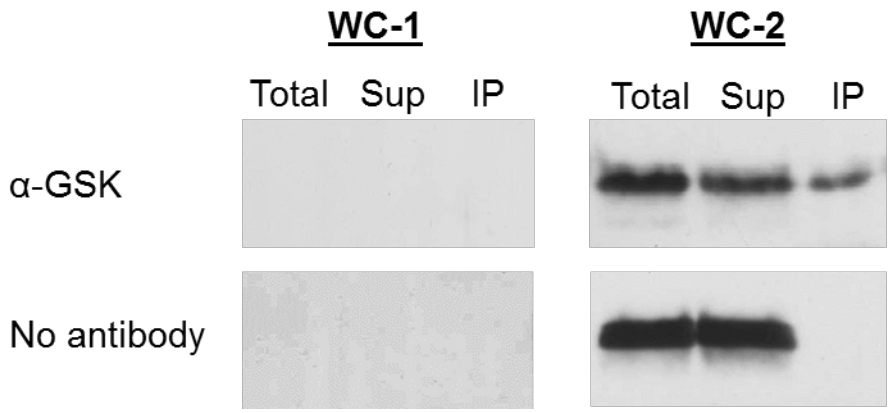

Figure 26
Co-**i**mmunoprecipitation assay using GSK antibody and no antibody control in total cell extracts from Δ*wc-1* strain. An equivalent of 200µg Total, 200µg supernatant and 800µg IP protein were loaded. Antibodies used for each blot are indicated on the top of each column and the antibodies used for the IP are shown on the left of each row.

FRQ was not detected in the bound fractions in any of the experiments (see Figure 25). Furthermore, both WC-1 and WC-2 proteins co-immunoprecipitated with GSK in the *frq*^{10} strain which lacks FRQ (not shown). This suggests that binding of GSK to the white collar complex (WCC) does not depend on FRQ.
We also did not see a significant effect of binding efficiency between cell lysates prepared from samples that were grown at either 25 or 30 ^{o}C. These data show that WCC is a direct target of GSK *in vivo* and that GSK is in a stable complex with a small population of WCC.

The majority of GSK is located in the cytosol, while WCC is predominantly nuclear (Figure 27 and 24). Therefore, we tested whether binding of GSK to WCC is compartment-specific. Co-immunoprecipitation assays using GSK antibody from cytosolic and nuclear fractions showed that WCC is associated with GSK in both compartments (Figure 27). Interestingly, the small fraction of GSK present in the nucleus was able to pull down more WCC than in the cytosol, suggesting that the abundance of GSK is not the rate limiting factor in the formation of the GSK/WC-1/ WC-2 complex.

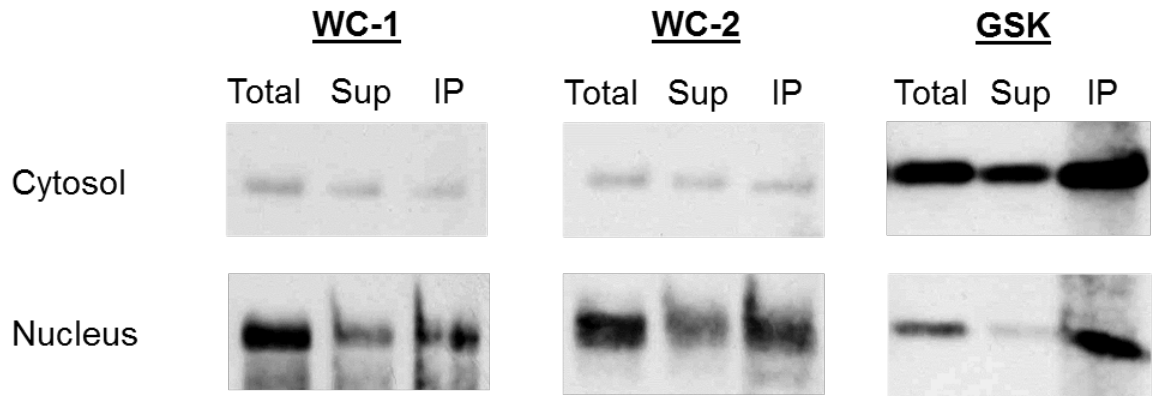

Figure 27
Co-immunoprecipitation assays using GSK antibody from Total, Nuclear and Cytosolic fractions of *wt9718* cell extract (LL) are shown. 200µg Total and an equivalent of 200µg supernatant and 1600µg IP protein were loaded. 1600µg IP protein was loaded to compensate for lower efficiency due to buffer conditions. Antibodies used for each blot are indicated on the top of each column.

3.4 GSK phosphorylates WCC, but not FRQ

We observed that levels of WC-1 protein are elevated in *Gsk*^{het} strain and GSK forms a stable complex with WCC and reasoned that this transcription factor complex might be a substrate for GSK phosphorylation. Therefore, we purified recombinant *Neurospora* GSK in its active form along with *Neurospora* Casein Kinase 1a (CK1a) and a mock control using HIS-tagged expression vectors in *E.Coli* and tested whether GSK is capable of phosphorylating WC-1 or other clock proteins *in vitro*. The mock control was simply an empty vector expressed and purified along with the kinases to account for the unspecific kinases or proteins that may come during the purification process and used as negative control. We used CK1a as a positive control for saturating phosphorylation states because it is essential for circadian clock function and known to phosphorylate clock proteins in *Neurospora* (Querfurth, Diernfellner et al. 2007).

In our preliminary experiments, we added the recombinant kinases to total cell lysates from *Neurospora* using various doses of ATP and buffer conditions and established the *in vitro* phosphorylation assay in such a complex mixture. Addition of ATP without the kinases under these conditions did not result in unspecific phosphorylation of clock protein. Furthermore, mock control did not result in significant phosphorylation of any of the proteins tested. To our knowledge, this is assay is the first of its kind in this organism.

The results of these i*n vitro* phosphorylation assays showed that CK1a can fully phosphorylate WC-1 as expected. Interestingly, GSK was also able to phosphorylate WC-1 to a similar extent, suggesting that WC-1 is a substrate of this kinase (Figure 28. GSK was also able phosphorylate WC-2, but the efficiency of WC-2 phosphorylations was lower compared to WC-1. This suggests that WC-1 is a better substrate for GSK.

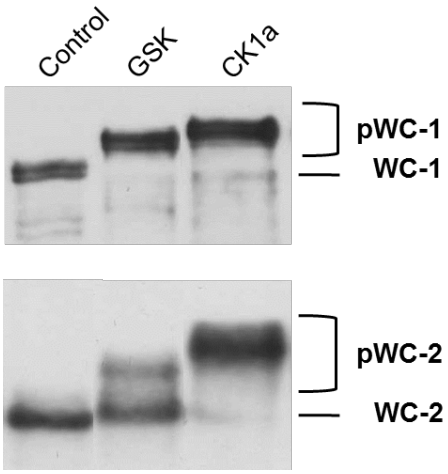

Figure 28
Representative western blots of WC-1 and WC-2 proteins from *in vitro* phosphorylation of LL grown whole cell extracts from *wt9718* strain. Saturating levels of recombinant *Neurospora* GSK, CK1a and control (mock purification) proteins were added and incubated for 1 hour in presence of 10 mM ATP at 25 degrees. Hyper-phosphorylated species are indicated as pWC-1, pWC-2.

Under steady-state conditions, FRQ is present in many phosphorylation states in *Neurospora* cell extracts. Therefore, in order to test whether GSK can phosphorylate FRQ, we induced FRQ from a Quinic acid (QA) inducible promoter and obtained a relatively hypo-phosphorylated pool of FRQ. Although FRQ harbors many putative GSK phosphorylation sites, to our surprise, it was not phosphorylated by GSK in any of our assays (Figure 29). In contrast, our positive control, CKI treatment resulted in hyper-phosphorylation of FRQ as well as WC-1 and WC-2.

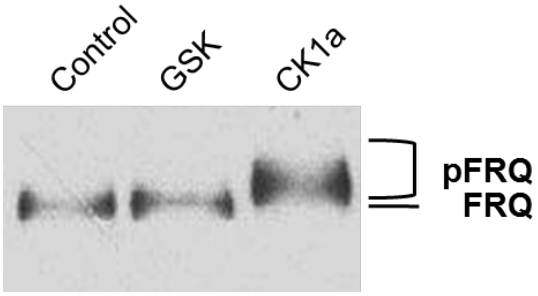

Figure 29

A relatively less phosphorylated population of FRQ was obtained by inducing FRQ from a quainic acid inducible promoter in the *frq*^{10} background (*frq*^{10}*/qa-frq*) for 4 hours. Saturating levels of recombinant *Neurospora* GSK, CK1a and control (mock purification) proteins were added and incubated for 1 hour in presence of 10 mM ATP at 25 degrees. Hyper-phosphorylated FRQ species are indicated as pFRQ.

Addition of purified kinases in such excess can lead to unspecific phosphorylation of proteins. Therefore, we analyzed a typical *in vitro* phosphorylation assay gel using coomassie staining. However, we did not observe any unspecific phosphorylation of proteins (Figure 30).

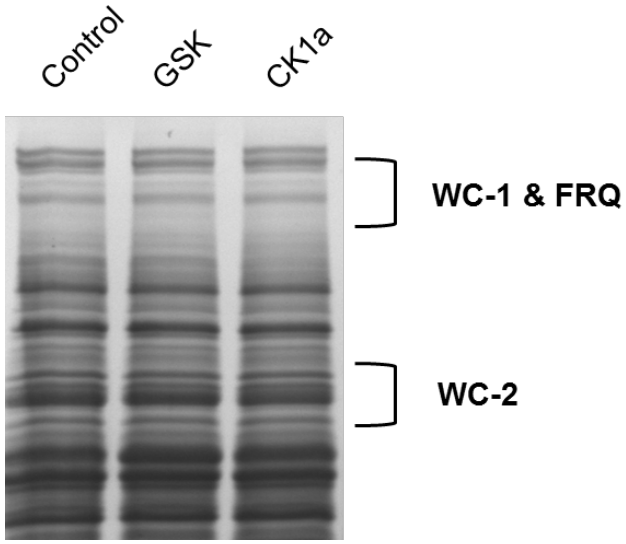

Figure 30

Representative Coomassie staining of an *in vitro* phosphorylation assay gel. Note that there is no overall phosphorylation of proteins in this assay. Approximate locations of WC-1, WC-2 and FRQ proteins are indicated.

To further test whether phosphorylation of WC-1 and WC-2 by GSK are specific, we used increasing amounts of GSK or specific GSK inhibitors to our *in vitro* assays. We observed that WC-1 and WC-2 phosphorylations by GSK were

dependent on the amount of recombinant GSK applied and could be inhibited by GSK inhibitors lithium, Kenpaullone (not shown) and Indirubin (Figure 31). These data suggest that the phosphorylations are specific to GSK and that WCC is a substrate of GSK while FRQ does not seem to be a target.

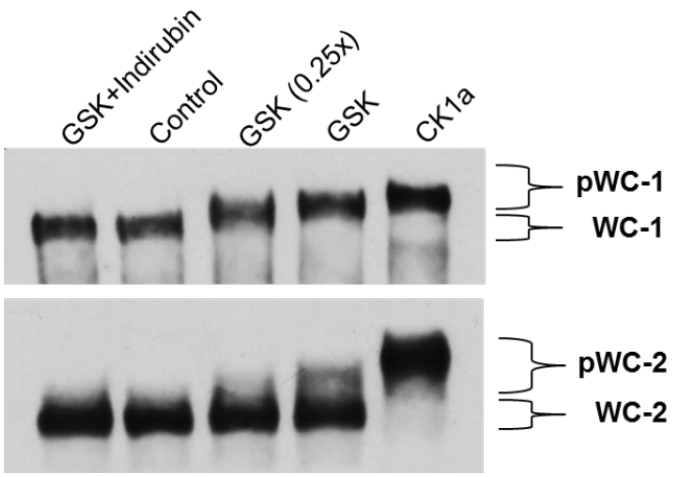

Figure 31
Phosphorylation of WCC by GSK is dependent on the amount of GSK and can be selectively inhibited by GSK inhibitor Indirubin.

3.5 GSK phosphorylates a specific region on WC-1

WC-1 is the rate limiting factor in the WCC activity and its levels are increased when GSK levels are lowered. Furthermore, GSK binds to WCC *in vivo* and WC-1 is a good substrate for GSK phosphorylation *in vitro*. Therefore, we explored whether GSK phosphorylates WC-1 at a specific region or if the phosphorylations are scattered along the WC-1 protein. WC-1 contains ~85 predicted GSK phosphorylation sites (S/T-X-X-X-S/T). Therefore, to test whether the phosphorylations can be narrowed down to specific region, we used various WC-1 truncation strains that were already available to us in our *in vitro* phosphorylation assays (Figure 32).

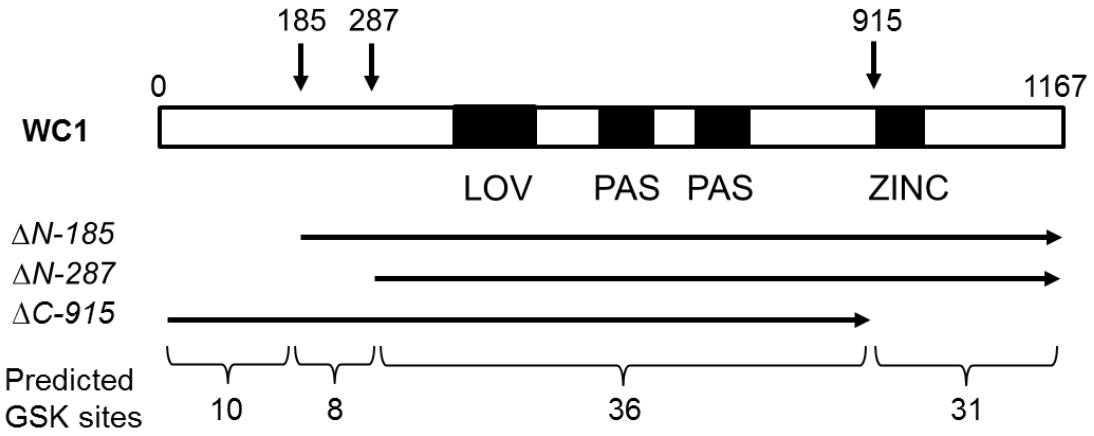

Figure 32
Full length and various WC1 truncation strains used in this study are depicted with respect to the domain structure of WC1. Number of predicted GSK phosphorylation sites (S/T-X-X-X-S/T) is also shown (Group-based prediction system, GPS software).

In our assays using truncation mutants, we observed that GSK was able to fully phosphorylate the C-terminal truncation mutant WC-1, the ΔC-915, even though the deleted region contains 31 predicted GSK phospho-sites. In addition, GSK was also able phosphorylate the N-terminal truncation mutant ΔN-185 which lacks 10 sites. However, the phosphorylations were significantly diminished in the N-terminal truncation strain Δ287 (Figure 33).

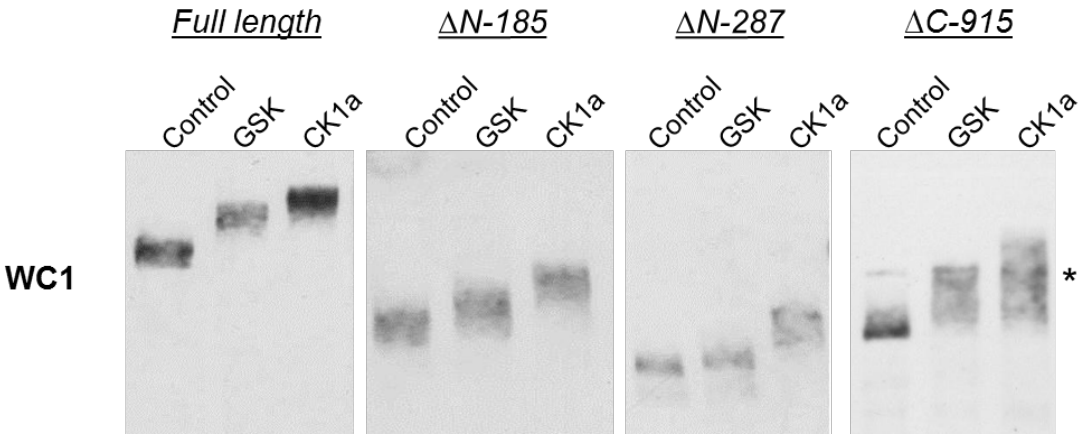

Figure 33
Western blots from representative *in vitro* phosphorylations of the WC1 truncation mutants. Note that the antibody used for ΔC-915 western blot is different from the others and shows and unspecific band (indicated with *).

Interestingly, the difference between the ΔN-185 and ΔN-287 strains is only 8 phospho-sites. This suggested that either these are the major GSK phospho-sites or that the GSK binding site lies between amino acid residues 185 and 287. Therefore, we tested whether GSK can still bind to the ΔN-287 WC-1.

Co-immunoprecipitation of WC-1 with GSK showed that GSK is able to bind to this truncated form of WC-1 equally well as it can to full-length WC-1 (Figure 34). This suggests that the region between amino acids 185 and 287 harbors the major GSK phosphorylation sites on the WC-1 protein. However, we cannot exclude other sites that are also predicted, since we observe diminished, but not lack of phosphorylation in this truncated form of WC-1

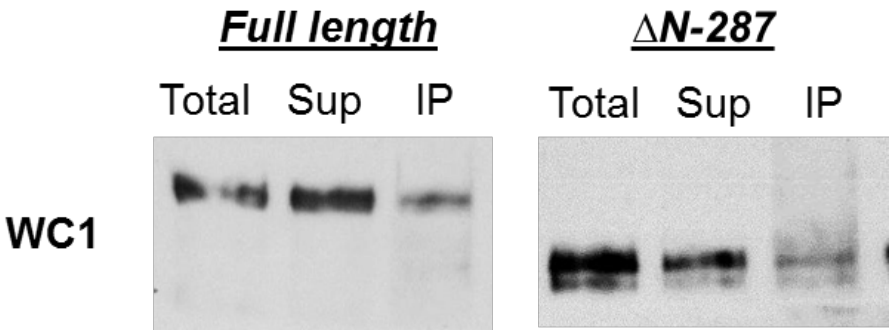

Figure 34
Co-immunoprecipitation of the full length WC1 and its truncation mutant Δ*N-287* with GSK antibody. Note that GSK can bind to this truncated form equally well.

3.6 Mutations of WC1 result in loss of temperature compensation

The finding that the deletion of only eight GSK phospho-sites from WC-1 resulted in diminished phosphorylation *in vitro* and that this was not due to lack of GSK binding to WC-1 suggested that this region is a major target for GSK. Therefore, we mutated these serine or threonine residues to alanines or aspartates to mimic unphosphorylated or phosphorylated states, respectively (Figure 35). We expressed mutant forms of WC-1 from the endogenous WC-1 promoter and created three strains that are wild-type, alanine or aspartate for these eight residues (*pWC1-wt, pWC1-8A* and *pWC1-8D*, respectively).

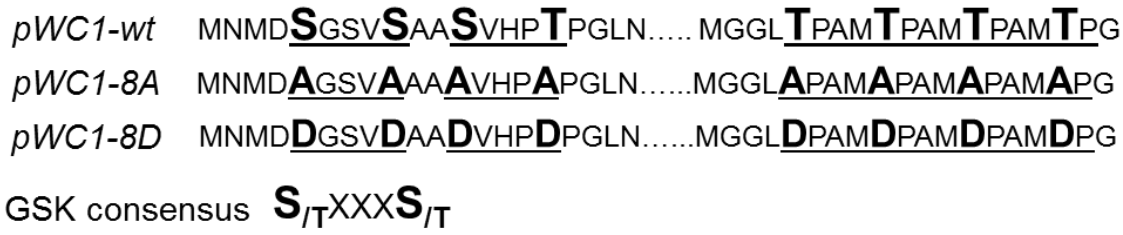

Figure 35
Predicted GSK phosphorylation sites between amino acids 223 and 289 of wild-type WC1 strain *pWC1-wt* and *pWC1-8A* and *pWC1-8D* mutant strains are shown with respect to GSK consensus phosphorylation motif.

We further tested whether these eight residues are indeed major targets of GSK by using *in vitro* phosphorylation assays. We predicted that if these sites are phosphorylated by GSK, the alanine mutant should mimic the phosphorylation defect we observed in the ΔN-287 WC-1. As expected, the alanine mutant showed diminished phosphorylation by GSK *in vitro*, while CK1a phosphorylated this protein fully (Figure 36). This showed that these sites are indeed major targets of GSK.

Aspartate mutant showed some phosphorylation by GSK; however, the extent of the phosphorylation is hard to measure since the protein is already shifted up on SDS-PAGE due to the nature of the mutations. We did not perform any co-immunoprecipitation assays since we observed that the strains are rhythmic on race-tubes, which requires WC-1 and WC-2 interaction (Figure 36).

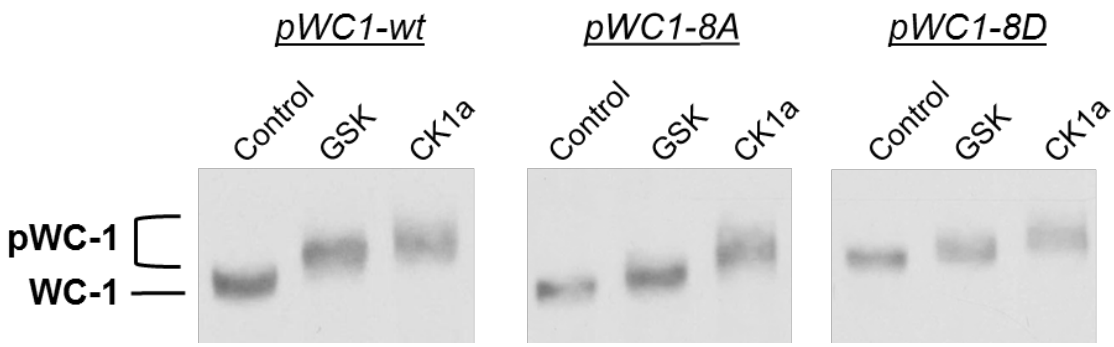

Figure 36
A representative western blot of *in vitro* phosphorylation of WC-1 in total cell lysates from *pWC1-wt, pWC1-8A* and *pWC1-8D* strains are shown. Hyper-phosphorylated WC-1 species is indicated as pWC-1

We hypothesized that since these sites are indeed the major GSK phosphorylation sites on WC-1, mutations of them to alanine should also result in loss of temperature compensation of the circadian clock and phenocopy the Gsk^{het} strain. Indeed, our race-tube assays showed that *pWC1-8A* have significantly lost temperature compensation with respect to the wild-type WC-1 expression strain pWC1-*wt* and had longer and shorter period lengths at 22 and 30 degrees, respectively (Figure 37). The aspartate mutant showed a slight effect on temperature compensation at 30 degrees, which was intermediate between the alanine and the wild-type.

The wild-type expression strain *pWC1-wt* is not actually as compensated as a *wt9718* non-banding strain. This is due to the background of these strains (*ras-1^{bd}*) which is known to shorten by up to 2 hours in its period length from 25 to 30 degrees. Unfortunately, a *ras-1^{wt}* strain where endogenous WC-1 is knocked-out does not exist which limited expression our WC-1 mutants into only the *ras-1^{bd}* strain. However, the defect in the temperature compensation in the alanine mutant was evident even on such a background (Figure 37).

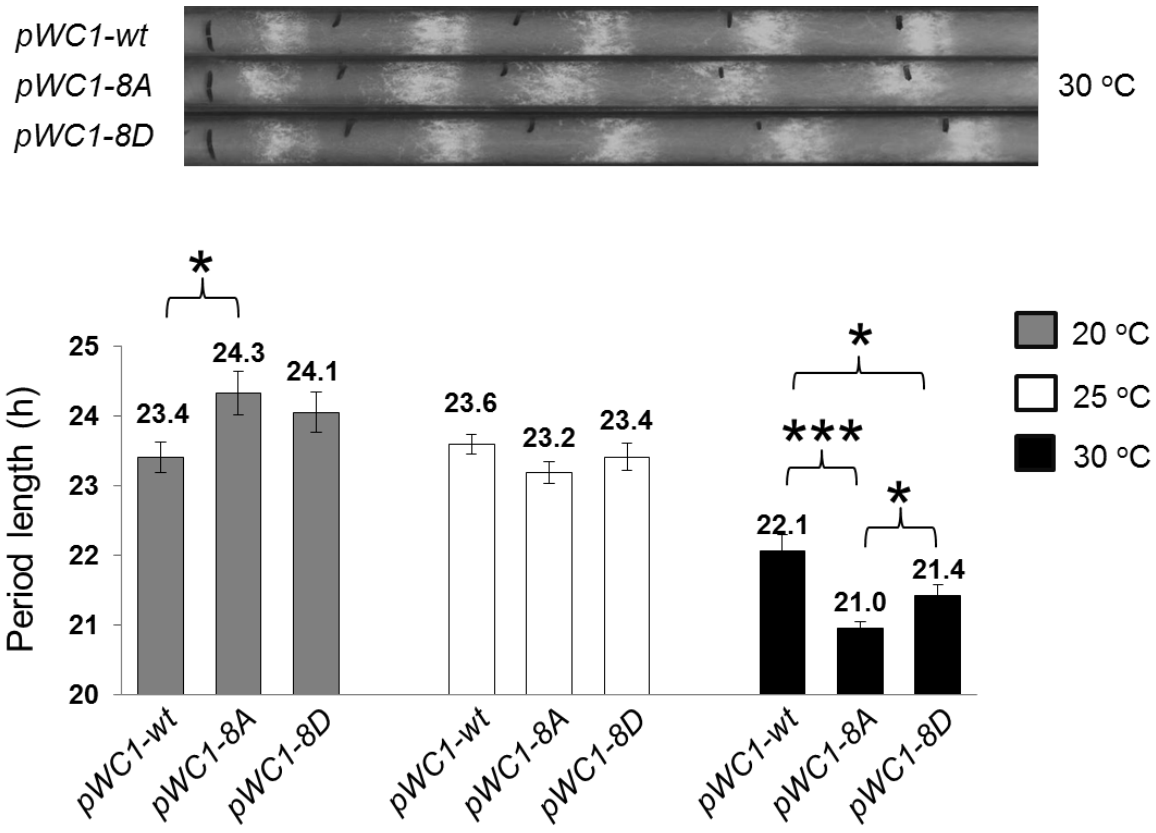

Figure 37
Representative race-tubes from the WC-1 mutant strains along with quantification of the average period lengths at 22, 25 and 30 °C are shown. Difference between groups were analyzed using two-tailed Student's t-Test and p-values less than 0.5 and 0.005 are indicated with * and ***, respectively. Error bars show ±standard error of the mean (±SEM) from at least three independent experiments (n≥18 for each group). See also Figure 41.

We expressed WC-1 from its endogenous promoter rather than an inducible promoter as it was shown in literature before due to its limitations on long term experiments which require repetitive addition of Quinic acid. This resulted in slightly lower levels of WC-1, hence a longer period length compared to other *ras-1^{bd}* strains. However, the banding pattern in these mutants was robust and the wild-type expression strain was temperature compensated just like a typical *ras-1^{bd}* strain (Figure 37).

3.7 Mutations of WC1 lead to higher levels of WC-1

Since we observed that alanine mutants of WC-1 also lack proper temperature compensation and phenocopy the GSK down-regulation strain, we tested whether the WC-1 protein levels were also elevated in this mutant similar to the *Gsk$^{het.}$*. Our results showed that levels of WC-1 and FRQ clock proteins were altered in the alanine and aspartate mutants of WC-1, but WC-2 was not affected (Figure 37)..

Alanine mutant showed elevated levels of WC-1 and FRQ at 30 degrees (but not 25 degrees) with respect to the wild-type control (Figure 37). The effect on WC-1 was much more pronounced than the effect on FRQ. This supports the race-tube data where we observed a shorter period length only at 30 degrees in the alanine mutant. In contrast, aspartate mutant showed slightly lower levels of WC-1 at 25 and 30 degrees. There was no effect of the aspartate mutant on FRQ levels. Furthermore, we did not observe any significant effect on the levels of WC-2 in either mutant. We did not test the aspartate mutant any further in this study since it wasn't significantly different from the wild-type.

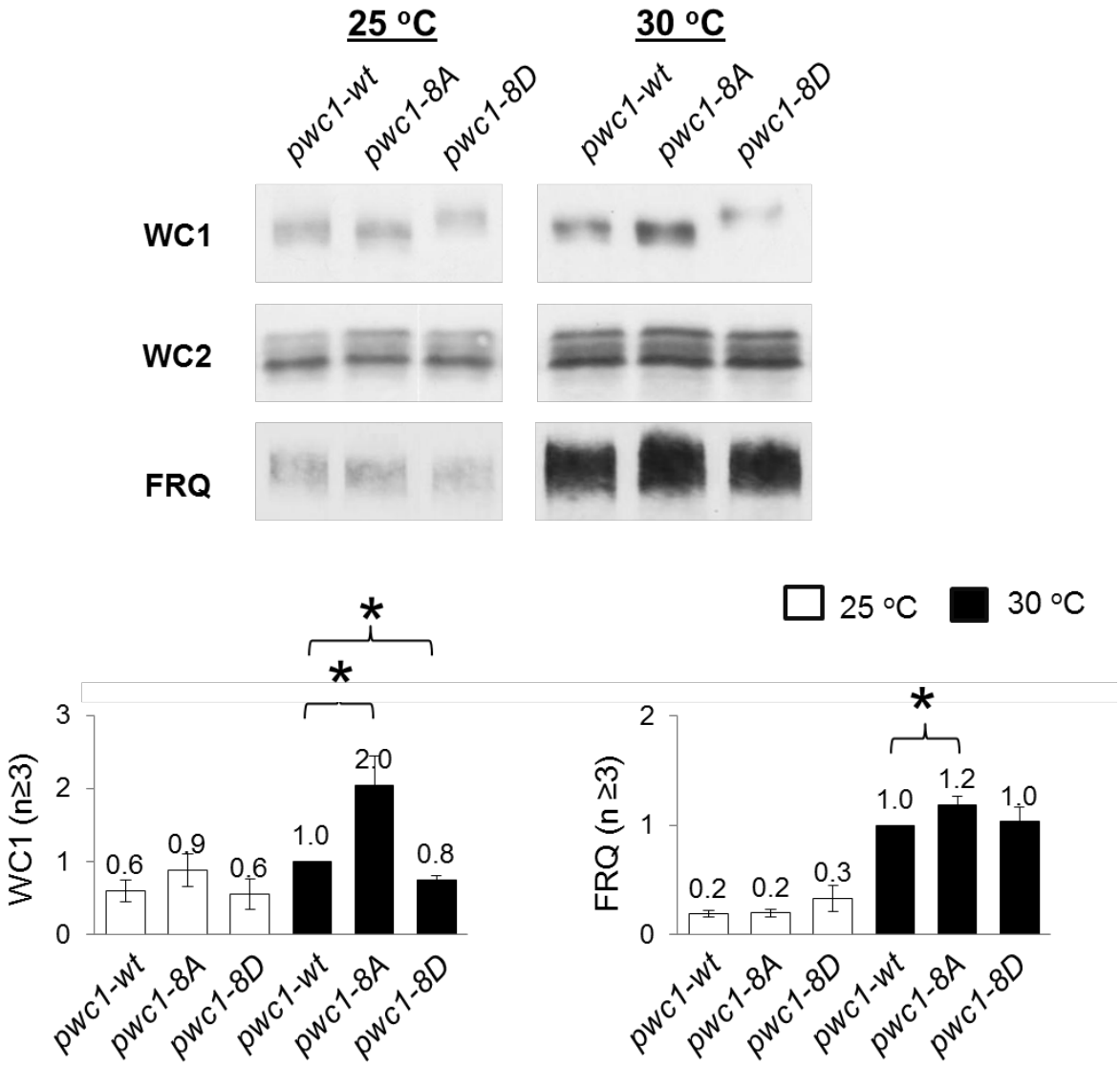

Figure 38

Representative western blots showing levels of clock proteins in the *pWC1-8A* and *pWC1-8D* mutant strains compared to their control strain *pWC1-wt* at steady state conditions (LL, 25 and 30 ^{o}C). Quantification of levels of WC-1 and FRQ proteins from at least three independent experiments are also shown with ±SEM. All values for quantifications were normalized to respective *pWC1-wt* level at 30 degrees. Difference between groups within each temperature was analyzed using two-tailed Student's t-Test and p-values less than 0.05 are indicated with *.

We further tested the effect of the alanine mutation on clock proteins and mRNA with temperature-shift experiments. We found that when the temperature is elevated from 25 to 30 degrees for 24 hours, the levels of WC-1 and FRQ

increase dramatically (Figure 39). The increase in FRQ was expected since it is known that FRQ levels increase at high temperatures due to alternative splicing (Diernfellner, Schafmeier et al. 2005; Diernfellner, Colot et al. 2007). However, we observed an additional increase on top of the increase in the wild-type in the alanine mutant.

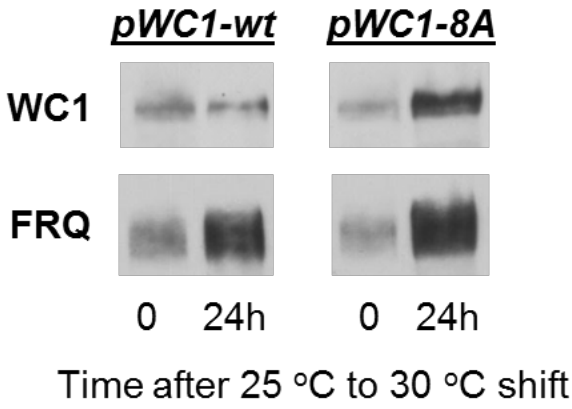

Figure 39
Representative western blot of a temperature shift experiment. Samples were grown in LL at 25 degrees and then temperature was raised to 30 for 24 hours. Note that WC-1 and FRQ levels increase to higher levels in the alanine mutant.

The levels of *wc-1* mRNA were not different between the wild-type and the alanine mutant (similar to the case in $Gsk^{het.}$). However, *vvd* mRNA was significantly elevated to ca. 3 fold at 30 degrees in the alanine mutant (Figure 40). The *frq* RNA was also elevated slightly by 1.4 fold. VVD and FRQ are well-known direct targets of active WCC and their mRNA levels are a good measure of the overall activity of WCC in the cell. Therefore, an increase in the *vvd* and *frq* mRNA is consistent with the elevated levels of WC-1 in the alanine mutant. This also supports the race-tube results where we observed a shorter period length in the alanine mutant, possibly due to higher levels of WC-1; hence higher WCC activity and shorter period.

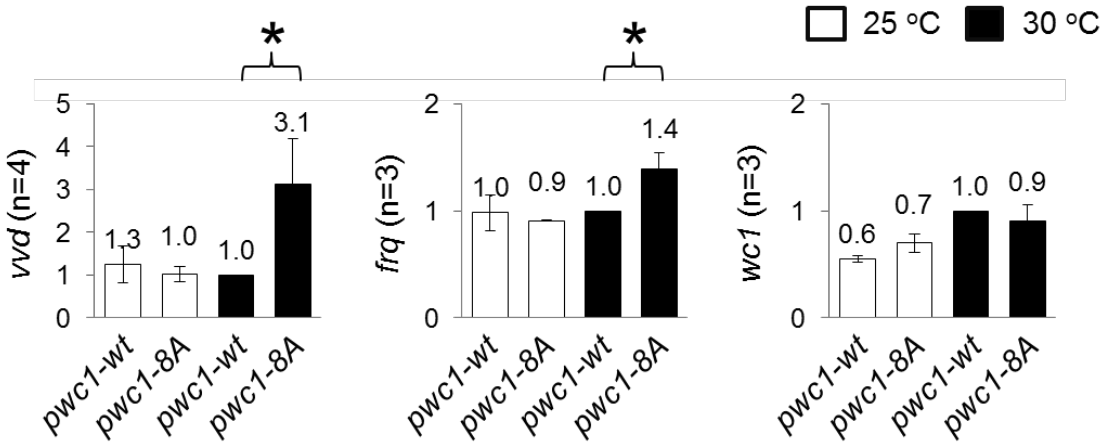

Figure 40
Levels of *vvd, frq and wc-1* mRNA after a shift from 25 to 30 degrees for 24 hours. *vvd* and *frq* mRNAs are markers of WCC activity and both are significantly elevated in the alanine mutant at high temperatures. Note that the increase in WC-1 protein is not due to an increase in the *wc-1* mRNA.

Strain	Hygromycin	Temperature (°C)	Period length (hours)	±SEM	n
wt9718	-	25	21.75	0.20	13
	-	30	21.11	0.17	16
Gsk het	-	25	21.02	0.08	28
	+	25	21.59	0.18	10
	-	30	19.87	0.11	26
	+	30	17.66	0.52	11
Abc homo	-	25	21.39	0.19	6
	-	30	21.15	0.12	9
	+	30	20.64	0.09	10
wt74	-	30	20.91	0.11	12
pwc1-wt	-	20	23.41	0.21	5
	-	25	23.60	0.14	24
	-	30	22.07	0.23	26
pwc1-8A	-	20	24.33	0.31	6
	-	25	23.19	0.15	27
	-	30	20.96	0.09	27
pwc1-8D	-	20	24.06	0.29	3
	-	25	23.42	0.19	18
	-	30	21.43	0.14	14

Figure 41

Summary of the average period lengths from at least three independent race-tube experiments are shown with ±SEM. n indicates total number of race tubes used. 50 µg/ml hygromycin B was added where indicated (+).

I investigated the role of Glycogen Synthase Kinase (GSK) in the *Neurospora crassa* circadian clock. In particular, it's role in the temperature compensation mechanism. We hypothesized that inhibition of GSK activity results in loss of temperature compensation based on a study by Jolma *et al.* (Jolma, Falkeid et al. 2006) where the authors applied a well-known GSK inhibitor, lithium, to race-tube assays and observed that the circadian clock runs faster at higher temperatures. To elucidate whether this effect is specific to GSK, we obtained a GSK down-regulation strain, the *Gsk*^{het}. However, this strain did not produce a banding pattern on race-tubes and due to the essential nature of GSK for the cell; it could not be back-crossed to a banding background (*ras-1*^{bd}). Therefore, we established a race-tube assay using H_2O_2 based on the findings of another recent paper (Belden, Larrondo et al. 2007). In this study, authors induced banding in wild-type (non-banding) strains of *Neurospora* using drugs such as Menadione that induce oxidative stress. However, we found that the use of H_2O_2 rather than Menadione was more reliable, especially at higher temperatures. Furthermore, Menadione is highly toxic and expensive, while H_2O_2 is not. We believe that this method will prove very useful in the study of heterokaryon strains of essential genes where the strain cannot be backcrossed to a banding background. The results of the race-tube assays clearly showed that down-regulation of GSK results in a loss of temperature compensation where the clock runs faster at higher temperatures. This fits well with the effects of lithium and prompted us to further investigate the role of GSK on the circadian clock in *Neurospora*.

4.1 GSK affects temperature compensation through stabilizing the WCC

Neurospora clock consists of two rate-limiting factors: the White Collar Complex (WCC) and FREQUENCY (FRQ). WCC is a transcription factor and drives

expression of *frq* mRNA as well as many other clock controlled genes (ccgs). FRQ protein, in turn, inhibits its own transcription by promoting the phosphorylation and inhibition of the WCC activity. Therefore, WCC and FRQ serve as positive and negative elements of the circadian clock, respectively. Our race-tube analysis showed that the clock runs faster when GSK is down-regulated. This could be achieved by either increasing the activity of WCC (positive element) or decreasing the activity of the FRQ (negative element). Analysis of the *Gsk*^{het} strain showed that the protein levels of White Collar 1, the critical and rate-limiting component in WCC, were increased by ca. 2 fold at higher temperatures while there were no effects on other clock proteins. This suggests that GSK affects the positive elements of the circadian clock rather than the negative.

This is in contrast to the effects of lithium. It was previously shown that application of lithium, a known inhibitor of GSK, increases the stability of FRQ and results in an under-compensated phenotype in *Neurospora* (Jolma, Falkeid et al. 2006). Although we also observed under-compensation in our experiments with the *Gsk*^{het} strain, we did not observe any significant effects on FRQ. Therefore, the effects of lithium on FRQ stability cannot be explained by GSK action. In fact, it was recently shown that inhibition of GSK activity by more specific GSK inhibitors or siRNA mediated knock-down results in shorter period lengths in the mammalian circadian clock in contrast to well-known period lengthening effect of lithium (Hirota, Lewis et al. 2008). Furthermore, lithium is known to have effects on other cellular compounds such as inositol monophosphatase and β-arrestin-2 (reviewed in (O'Brien and Klein 2009). This suggests that the effects of lithium on FRQ may be due to its effects on such pathways rather than GSK.

Elevated levels of WC-1 were previously shown to result in shorter period lengths of the *Neurospora* circadian clock (Cheng, Yang et al. 2001). When WC-1 is driven from an inducible and dose-dependent promoter, the period length of the clock gradually becomes shorter as the amount of WC-1 protein increases (Figure 42).

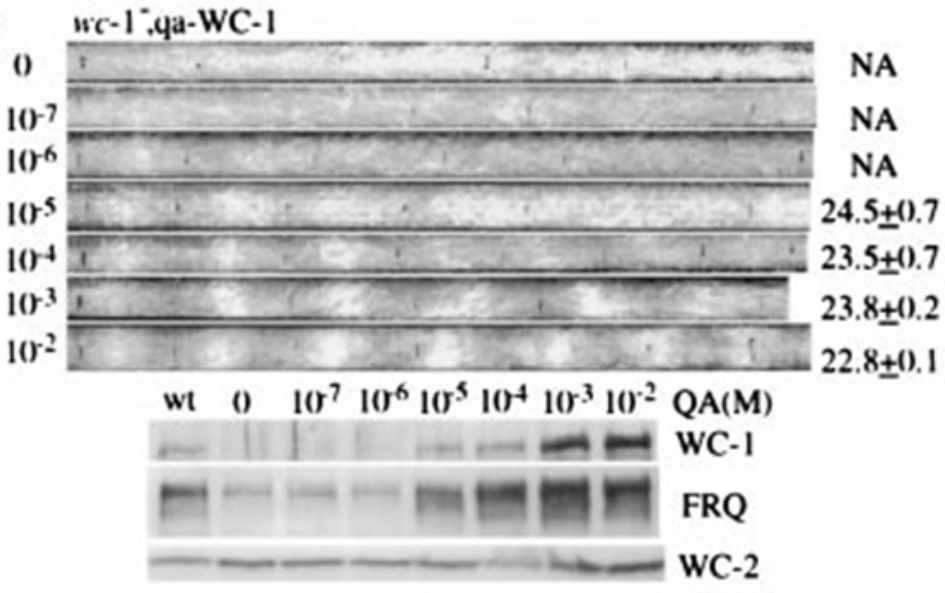

Figure 42
Effect of increasing amounts of WC-1 on the period length of the circadian clock. Amount of Quainic acid used to induce WC-1 is shown on the left and corresponding period lengths on the right. A representative western blot showing the increase in WC-1 and FRQ (due to WC-1) are also shown. Figure 3b from (Cheng, Yang et al. 2001)

In light of this study and our western blots, it is evident that the shorter period length that we observed at higher temperatures in the *Gsk*^{het} strain are due to elevated levels of WC-1 in this strain, but not due to reduced levels of FRQ. The effect of GSK on the abundance of WC-1 is particularly interesting because a role of GSK in the *Neurospora* circadian clock has never been shown. GSK is a house-keeping kinase and has many roles in the cell. Therefore, down-regulation of GSK may result in pleotropic effects that may interfere with WC-1 stability or the amount of the *wc-1* mRNA. Real-time PCR analysis showed that GSK does not have any effects on the *wc-1* mRNA. This suggests that GSK affects the stability of the WC-1 protein. To test this, we analyzed the half-life of WC-1 using Cyclohexiemide (CHX), but we did not see a significant effect.

At first, this seemed contradictory. However, the only published WC-1 variant whose stability can actually be resolved in a CHX assayis a C-terminally truncated

and transcriptionally inactive form, the ΔC-915 WC-1, which is stable for over 12 hours. Other mutants of WC-1 that effect the abundance of the protein were previously shown, but their stability kinetics were not resolved using CHX. For example, a similar limitation of CHX in resolving WC-1 stability has been observed before (He, Shu et al. 2005). In this study, the authors observed a 3-fold reduction in the abundance of WC-1 protein while *wc-1* RNA was not affected (Figure 43).

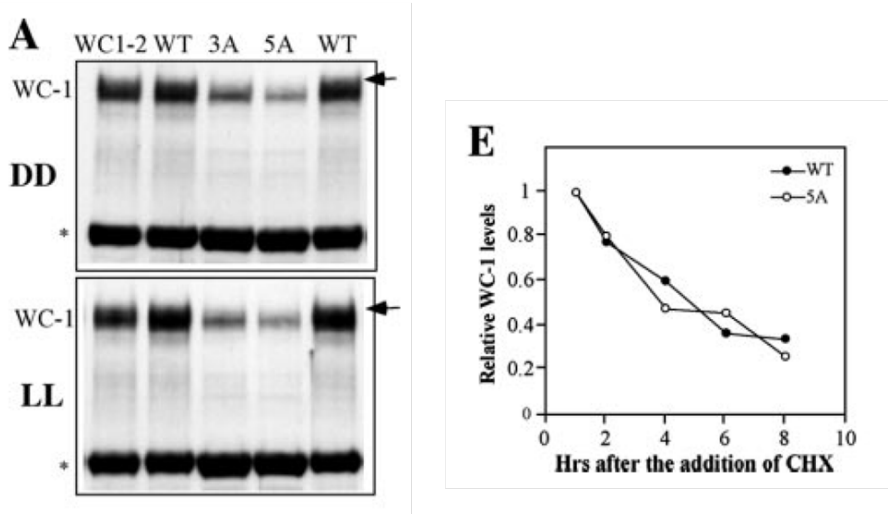

Figure 43
Cycloheximide assays are not a good tool to study the effect of WC-1 stability. Note that although there is ca. 3 fold difference in the abudance of WC-1 protein in constant dark (DD) or light (LL) this effect cannot be resolved using CHX. Total *wc-1* mRNA was not affected by the 3A and 5A mutants. Figure 2 from (He, Shu et al. 2005).

This showed that WC-1 protein was more stable but CHX assays did not resolve this difference. It is believed that the WC-1 species that is degraded is the active form which is a minor population (Schafmeier, Diernfellner et al. 2008). Therefore, application of a drug like CHX which affects many cellular processes may not be able to resolve the differences in this rapidly degraded minor population of WC-1. Our results are consistent with these data where we also don't see an effect using CHX, but the protein levels are higher and the mRNA is not affected. Therefore, we believe that GSK down-regulation increases the WC-1 stability.

GSK has a role in determining the sub-cellular localization of PER/TIM heterodimer in Drosophila (Ko, Kim et al. 2010). Phosphorylation of PER at specific residues by SGG, a GSK homolog in flies, promotes its nuclear entry. Therefore, we also investigated whether GSK down-regulation affects sub-cellular localization of clock proteins in *Neurospora*, in particular the WC-1. However, we did not find any evidence of an effect of GSK on nuclear translocation. This may be due to several reasons. First, such an effect of GSK on WCC or FRQ may not exist in the *Neurospora* circadian clock. Second, this could be due to differences in the kinetics or regulation of sub-cellular shuttling of circadian proteins where the rate is extremely fast in *Neurospora* (reviewed in(Tataroglu and Schafmeier 2010). Therefore, if GSK has any role in determining the rate of this fast shuttling of WCC, it would not be detected in our sub-cellular fractionation assays.

4.2 Recruitment of GSK to WCC modulates a phospho-degron on WC-1

We further tested the function of GSK with co-immunoprecipitation assays. Our results showed that GSK is bound to the WCC, but not to FRQ *in vivo*. We also observed that GSK is bound to WCC in the absence of FRQ. Although, the GSK-WCC interaction was stable, the amount of GSK-bound WCC was only a small fraction of total WCC (ca. 10%). This suggests that GSK is in a stable complex with a minor population of WCC. We further tested GSK binding to WCC using a WC-1 knockout strain and found that GSK can binds to WC-2 in the absence of WC-1. Unfortunately, we were not able to measure whether GSK can bind to WC-1 alone due to technical limitations. However, this suggests that WC-2 may be enough to recruit GSK to WCC. We also tested whether this binding is compartment specific and found that amount of GSK is not rate limiting in the formation of the GSK/WC-1/WC-2 complex. In summary, our results show that GSK forms a stable complex with a small population of the WCC *in vivo,* in both cytosol and nucleus.

Being a kinase, we hypothesized that if GSK can bind to WCC and affect its stability, it should also phosphorylate it. Indeed, our results from *in vitro* phosphorylation assays showed that GSK specifically phosphorylates WCC, but not FRQ *in vitro*. Efficiency of phosphorylations was quite high for WC-1, but slightly lower for WC-2. Furthermore, these phosphorylations were be prevented by application of several different GSK inhibitors. We did not observe any dependence of these phosphorylations on FRQ. This suggests that WCC is a direct and specific target of GSK in which WC-1 is the major substrate. This finding is particularly interesting, especially in light of our result that WC-1 levels are elevated post-transcriptionally in the Gsk^{het} strain, because this may be due to reduced phosphorylation of WC-1 when GSK is down-regulated.

We further analyzed WC-1 phosphorylation by GSK *in vitro* using various WC-1 truncation strains in an attempt to find the GSK phosphorylations sites on WC-1. The results of these assays showed that major phosphorylations of WC-1 by GSK occur at the N-terminal, in particular in a region between amino acids 185 and 287. We observed significant diminishing in the phosphorylations when this region was deleted. In addition, GSK was able to bind to this N-terminally truncated WC-1 form which suggests that diminished phosphorylation was not due to altered GSK binding. It should, however, be noted that we performed co-immoprecipitation assays in the presence of WC-2 and that may be the reason why we were able to pull-down WC-1 with GSK. This suggests that WC-2 may serve to recruit GSK to WCC where the kinase phosphorylates the WC-1 at this region and modulate its stability.

The region between amino acids 185 and 287 harbors eight consensus GSK phospho-sites which fit the GSK recognition motif S/T-X-X-X-S/T. In fact, these sites are oriented in such a way that allows for sequential phosphorylation by GSK which is known to happen in several known targets of GSK. One of these targets is Glycogen Synthase (Figure 44).

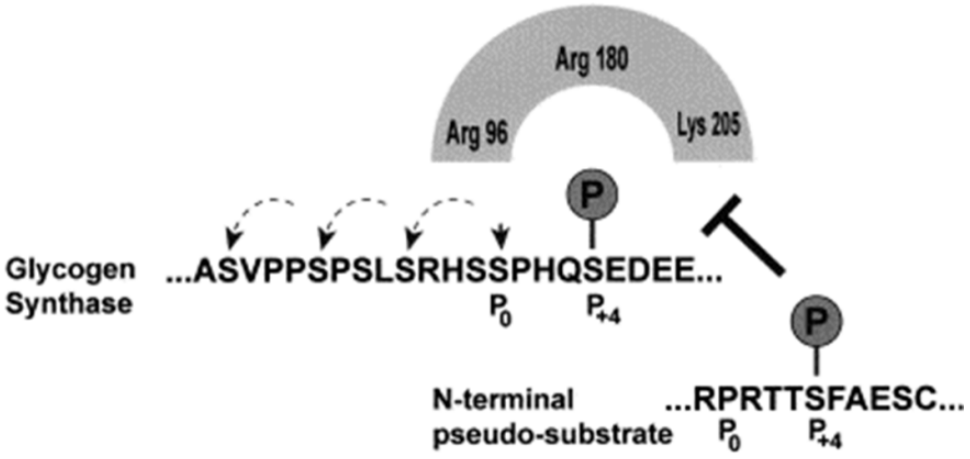

Figure 44
Sequential phosphorylation of a GSK target, the glycogen synthase. Note that the initial phosphorylation serves as the priming site for the remaining sites which leads to phosphorylation of all residues by GSK alone.

The substrate binding domain of GSK recognizes a phosphorylated serine or a threonine and phosphorylates the serine preceding that site by three amino acids. The initial phosphorylation can be primed by many kinases (Ferrarese, Marin et al. 2007). However, priming only increases the efficiency of phosphorylation and GSK can also phosphorylate without a priming kinase. Upon phosphorylation, the new phospho-site can now serve as the new priming site for efficient sequential phosphorylation of the remaining residues which eventually results in a hyper-phosphorylated region. Such sequential phosphorylation by GSK was shown to result in the degradation of proteins including several ZINC-finger transcription factors (Winston, Strack et al. 1999; Zhou, Deng et al. 2004; Jia, Zhang et al. 2005; Punga, Bengoechea-Alonso et al. 2006; Bengoechea-Alonso and Ericsson 2009; Sahar, Zocchi et al. 2010; Vinas-Castells, Beltran et al. 2010). The phosphorylated residues form a recognition motif called a phosphodegron to which other factors bind and target the protein for degradation. One such example is SNAIL. It is a highly unstable ZINC-finger transcription factor in mammals that regulates E-cadherin expression. Sequential phosphorylation of this transcription

factor results in its ß-TRCP mediated degradation ((Zhou, Deng et al. 2004) Figure 45).

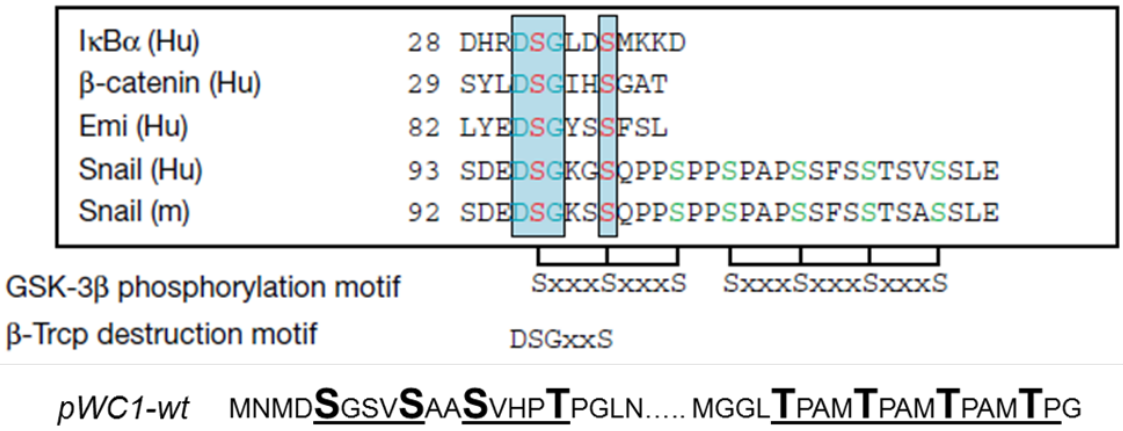

Figure 45
Sequential phosphorylation by GSK creates a phosphodegron on the transcription factor SNAIL. Alignment of several SNAILs and the WC-1 region between amino acids 223 and 289 are shown for reference. Note the similarity between the phosphodegron on SNAIL and the first two serines on WC-1 (DSGSVS).

4.3 Mutation of the phosphodegron shortens the period by stabilizing WC-1

To test whether the eight candidate GSK phosphosites on WC-1 are the major phosphorylation targets o GSK and whether if their phosphorylation status affects the stability of WC-1 protein, we created mutant forms of WC-1 where we replaced all eight candidate serine or threonine residues to aspartate or alanine to mimic phosphorylated and unphosphorylated states, respectively. We chose to drive WC-1 from its endogenous promoter to allow for *in vivo* regulation of its promoter and to avoid using repeated addition of Quinic acid in long term experiments which is used for inducible promoters in *Neurospora*. Although this resulted in slightly lower levels of WC-1 protein expression and longer period

length than wild-type, the strains were reliably rhythmic on race-tubes. Our analysis at 22, 25 and 30 degrees showed that the alanine mutant have lost temperature compensation, similar to the GSK down-regulation strain *Gsk*^{het}. Period length of this strain became gradually shorter as the temperature increased, showing an under-compensated phenotype. In contrast, the aspartate mutant showed an intermediate phenotype which suggests that these eight residues are regulated in a complex manner and that phosphates might be necessary.

Consistent with our observations in the race-tube assays, the levels of WC-1 protein were also higher in the alanine mutant. We also observed a slight effect on FRQ protein levels which we believe is due to elevated levels and activity of WC-1. The difference between the wild-type control strain and the alanine mutant was even more evident when we performed temperature-shift assays where we grew the cultures at 25 degrees and then increased the temperature to 30 degrees. We observed that the levels of WC-1 protein rose to much higher levels than the wild-type in the alanine mutant within 24 hours. This effect was post-translational since there was no effect on the *wc-1* mRNA. Furthermore, we also observed an increase in expression of two of the main targets of WCC, the *vivid* and *frq* mRNAs, consistent with elevated levels of WC-1. Both *vvd* and *frq* mRNA is widely used markers for WCC activity and their expression increases with increasing activity of WCC.

This data suggests that the mutated residues affect the stability of WC-1 at high temperatures. When we analyzed the disorder tendency of the WC-1 protein using prediction software, we found that the region between 185 and 287 is highly disordered, at least in prediction (Figure 46). This suggests that phosphorylation of these residues may occur more efficiently at higher temperatures since the region is flexible and unstructured. Alternatively, the effect of these eight residues can be observed only at a higher temperature. In fact, the regulation of this region seems complex, because our aspartate mutant did not simply result in an unstable

WC-1. This suggests that further work is needed to identify the roles of individual serines or threonines in this region on WC-1 stability and temperature compensation.

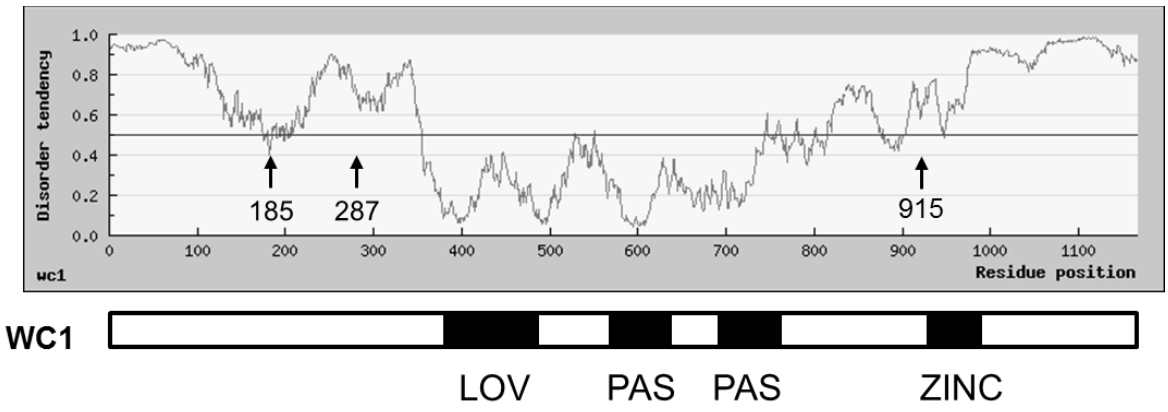

Figure 46
The region between amino acids 185 and 287 of WC-1 is highly flexible and disordered. Prediction was done by IUPred software. 0.5 was selected as the threshold for disorder. WC-1 protein domains are indicated at the bottom.

To test whether the phosphorylation by GSK can disrupt the WCC in total cell lysates, we performed *in vitro* phosphorylation by GSK followed by co-immunoprecipitation with WC-2 antibody. Under these conditions, the phosphorylated WC-1 forms were stably bound to WC-2 (Figure 47). However, it should be noted that the mechanisms required for dissociation of WC-1 from WC-2 may not be functional/present *in vitro* and may require the intact cell. This is supported by the fact that the hyper-phosphorylated forms of WC-1 which we see in the *in vitro* assays are not detectable under normal conditions. This suggests that these forms may be rapidly degraded in the intact cell. The fact that GSK is bound to only a minor fraction of WCC also supports this idea since it is believed that the active form of WCC constitutes only a small fraction of the total WC-1 and it is highly unstable.

In all assays, the alanine mutant WC-1 strain *pWC1-8A* successfully phenocopied the GSK down-regulation strain *Gsk*^{het}. In both strains, the stability of WC-1 protein was higher which resulted in shorter period lengths at higher temperatures. Therefore, our data clearly shows that GSK is essential for proper temperature compensation of the *Neurospora crassa* circadian clock.

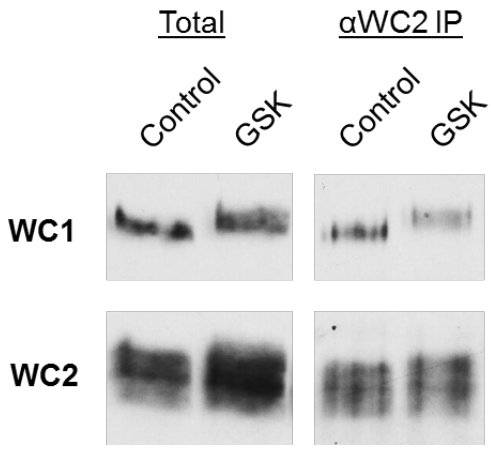

Figure 47
Phosphorylation by GSK *in vitro* does not disrupt the WCC. Total cell lysates from *Neurospora* were subjected to *in vitro* phosphorylation by GSK followed by co-immunoprecipitation with WC-2 antibody. Note that the hyper-phosphorylated WC-1 species co-immunoprecipitates with WC-2.

4.4 Opposing functions of GSK and CK2 regulate temperature compensation in *Neurospora crassa*

In a recently published paper from the laboratory of Jay Dunlap (Mehra, Shi et al. 2009), the authors investigated two previously known temperature compensation mutants, *chrono* and *period-3*. These mutants are known to have an over-compensated phenotype; they show longer period lengths as the temperature rises (opposite phenotype of the *Gsk*^{het} strain, Figure 48). They found that both of these strains contain mutations that map to two different subunits of Casein kinase 2 (CK2) which results in lower CK2 activity. Upon further study using radiolabelling, they also showed that CK2 phosphorylates FRQ *in vitro* and that

these phosphorylations result in destabilization of FRQ. When they mimicked these phosphorylation defects using alanine mutants of FRQ, they observed a more stable FRQ protein at higher temperatures. Interestingly, when they looked at other kinases that are known to be involved in phosphorylation/ dephosphorylation of FRQ, such as Protein kinase A (PKA), CK1a or protein phosphatase 1 (PPH-1), they did not find a temperature compensation phenotype.

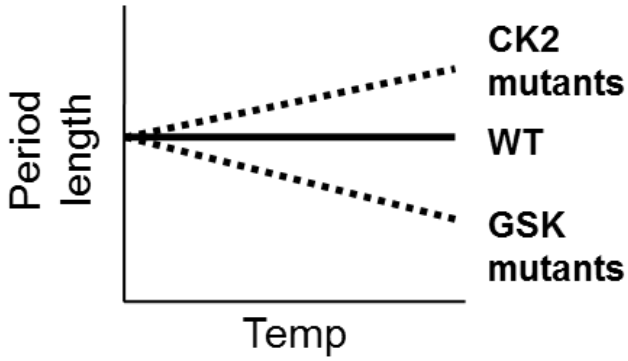

Figure 48
Reduction in the activity of two kinases GSK and Casein kinase 2 (CK2) results in opposite temperature compensation phenotypes. CK2 regulates FRQ stability and reduction of its activity leads to a more stable FRQ protein and slows down the clock at higher temperatures which is manifested as a longer period length.

This data fits very nicely with our results on the effects of GSK on WCC stability and its role in temperature compensation. At high temperatures, the levels of both FRQ and WCC increase. This increase is counteracted by the combined effects of two kinases, CK2 and GSK. CK2 phosphorylates FRQ and GSK phosphorylates WCC which increases the degradation of these negative and positive elements of the clock, respectively. Therefore, opposing functions of these two kinases regulate the temperature compensation mechanism in *Neurospora crassa* circadian clock. Such a mechanism also fits with the idea that circadian clocks evolved into existing cellular pathways where house-keeping kinases such as CK2 and GSK have many roles in the cell. Therefore, the clock achieved temperature

compensation by simply making its rate-limiting elements susceptible to degradation to the background activity of kinases already present in the cell. It is possible that this mechanism of temperature compensation is conserved in the higher eukaryotes since both GSK and CK2 are highly conserved kinases and have roles in other circadian clocks.

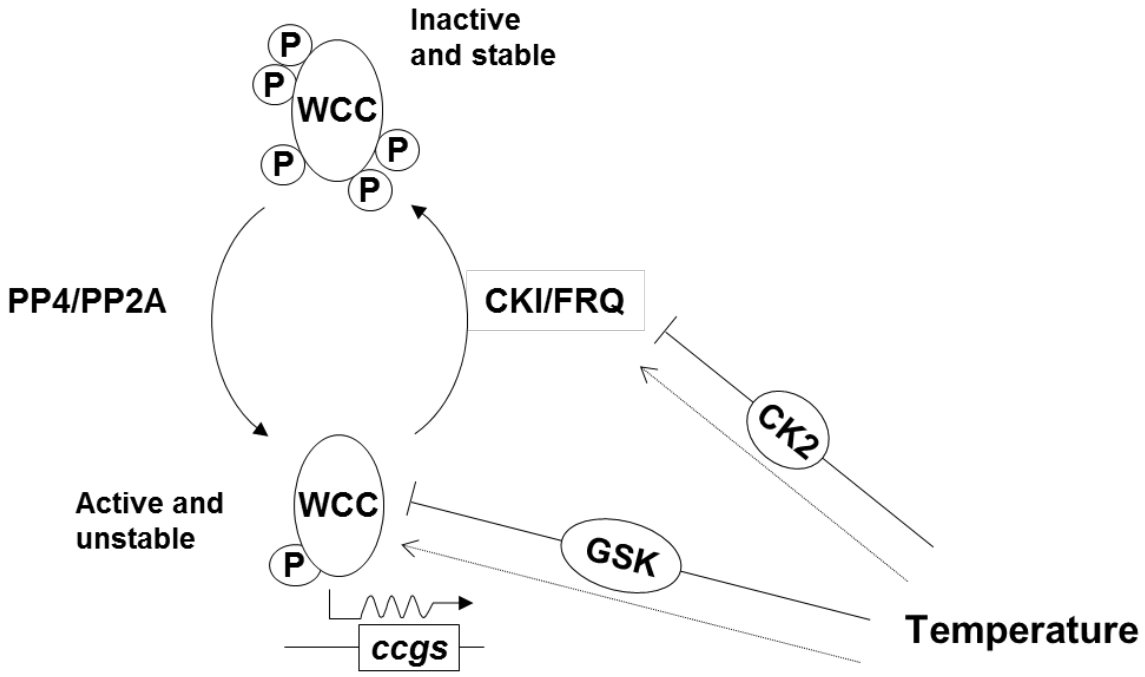

Figure 49
At elevated temperatures, the levels of both FRQ and WCC increase. This increase is counteracted by the combined effects of two kinases, CK2 and GSK. CK2 phosphorylates FRQ and GSK phosphorylates WCC which leads to increased degradation of the negative and positive elements of the clock, thereby achieving proper temperature compensation in a system where the individual components are temperature sensitive.

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