

## **Circadian gene *cry* controls glioblastoma tumorigenesis through modulation of *myc* expression.**

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### **Abstract**

Glioblastoma (GB) is the most frequent malignant brain tumor among adults and currently there is no effective treatment. It is a very aggressive tumor that grows fast and spreads through the brain causing the death of patients in 15 months. GB cells mutate frequently and generate a heterogeneous population of tumoral cells genetically distinct. Thus, the contribution of genes and signaling pathways relevant for GB progression is of great relevance. We use a *Drosophila* model of GB that reproduces the features of human GB, and describe the upregulation of the circadian gene *cry* in GB patients and in a *Drosophila* GB model. We study the contribution of *cry* to the expansion of GB cells, to the neurodegeneration caused by GB, and to premature death and determine that *cry* is required for GB progression. Moreover, we analyze the mechanisms that regulate *cry* expression by the PI3K pathway. Finally, we conclude that *cry* is necessary and sufficient to regulate *myc* expression in GB. These results contribute to the understanding of the signals that impulse GB malignancy and lethality and open novel opportunities for the treatment of GB patients.

**Keywords:** Cancer, neurodegeneration, glioma, *Drosophila*, disease model, PI3K, EGFR, genetics.

## Introduction

Glioblastoma (GB) is the most common and aggressive type of glioma of all brain tumors, accounting for 57.3% of all gliomas (Ostrom *et al.* 2020). It is classified as a WHO grade IV diffuse oligodendroglial and astrocytic brain tumor. It is more frequent in the adult population between 75-84 years of age. Treatment is based on radiotherapy accompanied by chemotherapy with temozolamide (TMZ) after surgical tumor resection. Despite current treatments, the mean survival of the patients is around 15 months (Wirsching and Weller 2016). And it is estimated that only 6.8% of patients survive five years after diagnosis (Ostrom *et al.* 2020). Understanding the genetic, molecular and cellular bases of gliomagenesis processes is fundamental for the development of effective therapies against these types of tumors. GB is a very heterogeneous type of tumor in terms of histopathology and genetic expression, even within the same tumor (Rich and Bigner 2004). However, there are common mutations in GB affecting different pathways that show mutual exclusivity: the p53 pathway, the Rb pathway, as well as components of the PI3K pathway (Brennan *et al.* 2013).

Previous studies from our lab used a GB model developed by Read and collaborators in 2009 in *Drosophila* that recapitulates key aspects of the disease (Read *et al.* 2009; Portela *et al.* 2019a; b, 2020; Portela and Casas-Tintó 2020; Formica *et al.* 2021; Jarabo *et al.* 2021; Vigneswaran *et al.* 2021) both genetically and phenotypically, such as the proliferation of glial cells, invasion, inappropriate differentiation and the interaction between genetic routes and the components of the signaling pathways. The model is based on the expression of the constitutively active forms of EGFR and dp110 (orthologues of EGFR and PI3K catalytic subunit in *Drosophila*, respectively) specifically in glial cells using the control of *repo* driver (Read *et al.* 2009). The co-activation of these signaling pathways in *Drosophila* glial cells increases Myc levels, essential for tumor transformation characterized by an increase in the number of glial cells, in the volume of the membrane, a reduction in the number of synapses and a reduction in the survival. Furthermore, this co-activation regulates processes such as progression and entry into the cell cycle and protein synthesis (Read *et al.* 2009; Portela *et al.* 2020).

*c-myc* is one of the oncogenes most amplified in human cancer, including GB. About 60-80% of human GB cases have elevated Myc levels (Annibali *et al.* 2014). Myc regulates cell proliferation, transcription, differentiation, apoptosis and cell migration and plays an essential role in the progression of GB as it is the point where EGFR and PI3K pathways converge, as well as being essential for tumor transformation (Read *et al.* 2009; Annibali *et al.* 2014). Furthermore, *in vitro* and *in vivo* studies have shown that Myc inhibition prevents glioma formation, inhibits cell proliferation and survival, and even induces disease regression (Annibali *et al.* 2014). These features are conserved in *Drosophila* (Read *et al.* 2009).

In recent years, the study of alterations in circadian rhythm genes has emerged in different types of cancer, including GB. Previous reports suggested that circadian rhythm genes have an important role in different aspects of tumor progression. The central clock organizes the oscillations and rhythmicity of the physiological processes by controlling the expression of a high number of ubiquitously expressed genes. Among them, there are genes related to cell proliferation or differentiation, such as cell cycle components (Lahti *et al.* 2012), proto-oncogenes and tumor suppressors (Kettner *et al.* 2014). Besides, CRY1 expression is androgen-responsive, CRY1 regulates DNA repair and the G2/M transition and it is associated with poor outcome in prostate cancer and colorectal cancer.

In *Drosophila*, the molecular mechanisms that govern circadian behaviors are based on transcriptional feedback loops evolutionarily conserved from insects to vertebrates. Simplified, *clock* (*clk*) and *cycle* (*cyc*) encode for proteins that form heterodimers and bind to the E-box sequences, which are found in promoter regions of circadian genes. Among many other effectors, Clk/Cyc induce the expression of their own repressors, *period* (*per*) and *timeless* (*tim*), which also dimerize in the cytoplasm. These proteins accumulate during night and interact with Clk/Cyc inhibiting their binding to DNA (Allada and Chung 2009; Peschel and Helfrich-Förster 2011), thus impeding their own expression. In turn, decreases Per/Tim levels and increases Clk/Cyc. In multicellular organisms, although all cells have their own circadian rhythm, there is a so-called "central clock", which is the structure responsible for coordinating circadian behavior

throughout the body. In mammals, it is the suprachiasmatic nucleus (SCN), located in the anterior region of the hypothalamus and made up of about 50,000 neurons in humans (Jarabo and Martin 2017). There are other tissues involved in the maintenance of circadian rhythms apart from the central clock, which are called “peripheral clocks”, which help to synchronize the central clock (Kettner *et al.* 2014). For example, there are studies in mammals that also give certain glial cells a fundamental role in maintaining circadian rhythms. These cells are called “glial clocks” and they in turn depend on the SCN for their resynchronization (Chi-Castañeda and Ortega 2016). All the neurons that compose the central clock express these genes to develop the oscillations that organize the cycles of the whole organism in absence of environmental cues. Furthermore, synchronization of the internal clock with light/dark cycles relies on cryptochrome protein (Cry), a blue light photopigment expressed in certain subsets of clock neurons. Cry binds Tim, triggering its degradation when activated by light. Cry is a receptor of near-UV/blue light and a regulator of gene expression that belongs to the group of DNA photolyases. It was suggested that the last universal common ancestor (LUCA) had one or several photolyases, supporting the evolutionary conservation of cryptochrome genes (Vechtomova *et al.*, 2020). In mammals, the molecular circadian clock is composed of Clk/Bmal1 (instead of Clk/Cyc) and Per (Per1, 2, 3)/Cry (Cry1, 2) (instead of Per/Tim) (Jarabo and Martin 2017). However, the mammalian gene that plays the role of *Drosophila* cry remains unknown. Interestingly, *Drosophila* Cry also acts as a transcriptional repressor and binds to Per when expressed in peripheral clocks (Collins *et al.* 2006).

Regarding GB, studies in patients with primary gliomas found an association between a specific *per1* variant with overall glioma risk. Several circadian genes including *cry1*, exhibited differential expression in GB samples compared to control brains as described in the literature (Madden *et al.* 2014; Wang *et al.* 2021) and human databases (<https://www.proteinatlas.org>; <https://cancer.sanger.ac.uk/>). Besides, *clk* expression was found significantly enhanced in high grade gliomas and correlated to tumor progression (Chen *et al.* 2013). High *per1* and *per2* expression increases the efficacy of radiotherapy also in GB cells (Zhanfeng *et al.* 2015).

Furthermore, high levels of *cry1* inversely correlate with median survival in GB patients, acting as signal of poor prognosis (<http://gepia.cancer-pku.cn/detail.php?gene=CRY1>) Still, a functional mechanism of Cry in cancer susceptibility and carcinogenesis remains unsolved.

Different studies show a relationship between Cry and Myc. C-Myc levels have been found to decrease in mice in *cry1/cry2* null mutants (Liu *et al.* 2020). Besides, Cry expression is induced by Myc in GB cells in culture (Altman *et al.* 2015).

Taking into account the deregulation in the expression of circadian rhythm genes in tumor tissues in GB, and the pre-established relationship between *cry* and *myc*, which is a key player in GB, here we validate the role of Cry in the tumorigenesis and progression of GB.

## Results

### Expression of *cry* in glioblastoma

To determine if *cry* expression was affected in glioma samples, we extracted RNA from heads of 7 days old adult control flies, expressing *LacZ* in glial cells (*repo-Gal4*), or *PI3K+EGFR* constitutively active forms to generate a glioma. Quantitative RT-PCR results indicate that *cry* mRNA level is 50 times higher in glioma samples as compared to controls (Figure 1A). However, to determine if *cry* upregulation occurs in glial cells we used a specific fluorescent *GFP-cry* reporter line that generates a GFP tagged form of Cry, and visualized larvae brains in confocal microscopy. The images show that GFP signal in glial cells is higher in glioma samples than in controls (Figure 1B, C), and this signal is restored to control levels upon *cry* RNAi in glial cells (Figure 1D). We quantified the GFP signal that overlaps with glial membrane (red, mRFP, red in Figure 1B', C', D' and E') and the quantifications indicate that *GFP-cry* signals is higher in glioma, and this increase is prevented upon *cry* knockdown in glioma cells (Figure 1F).

We analyzed human mRNA expression databases for Glioblastoma multiforme (<http://gliovis.bioinfo.cnio.es/>). The results indicate that *cry* in GB patients (CRY) is transcriptionally upregulated (Figure 1G). Besides, *CRY* upregulation correlates with worse prognosis (Figure 1H). All together, these results indicate

that *cry* is transcriptionally upregulated in GB cells in *Drosophila* and patients, and suggest a role in GB malignancy and aggressiveness.

### **Cry mediates GB progression and neurodegeneration**

To determine the contribution of *cry* to GB progression and the consequences, we used a previously validated protocol (Portela *et al.* 2019b, 2020; Jarabo *et al.* 2021) to quantify tumor growth and the associated neurodegeneration. We stained control adult brains, and compared with GB, GB and *cry RNAi*, and wt brains expressing *cry RNAi* in glial cells. We used a specific antibody against repo to visualize the nuclei of all glial cells, and quantified the fluorescent confocal images to count the number of glial cells (Figure 2A-D, E). The results indicate that GB samples have a significant increase in the number of glial cells compared to control samples, but this increase depends on *cry* expression (Figure 2A, B, C and E). Besides, knockdown of *cry* in normal glia does not alter the number of glial cells (Figure 2D and E). In addition, we quantified the volume that the glial membrane occupies in the brain. We measured with Imaris software the volume of red signal that corresponds to a myristoylated form of RFP (mRFP) expressed in glial cells. The quantification of the volume indicates that GB samples have a significant expansion of glial membrane compared to control samples, but this increase depends on *cry* expression. Again, knockdown of *cry* in normal glia does not alter the volume of glial membrane (Figure 2A'-D', F). These results suggest that *cry* expression is required for tumor progression, but not for normal glia development.

Next we studied the impact of GB progression and *cry* expression in neighboring neurons. We counted the number of synapses in motor neurons of adult neuromuscular junction (NMJ), a standardized tissue to study neurodegeneration (Portela *et al.* 2019b; Arnés *et al.* 2020; Jarabo *et al.* 2021). To visualize synapses, we used an anti-repo to detect active zones (presynaptic section), and counted the number of synapses in control samples, GB, GB+*cry* knockdown and normal glia + *cry* knockdown (Figure 2G-J). The quantification of synapse number (Figure 2K) shows that GB induction provokes a significant reduction in the number of synapses as compared to control samples, compatible with a neurodegenerative process. This effect was previously described (Portela *et al.* 2019b, 2020; Jarabo *et al.* 2021) as a consequence of

GB progression on neighboring neurons. Moreover, *cry* knockdown in GB prevents the reduction in the number of synapses, and *cry RNAi* in normal glial cells does not cause any detectable change in the number of synapses. Finally, we aimed to determine the systemic effect of *cry* silencing in GB or in normal glia, thus we analyzed the life span of adult flies. The results show that GB causes a significant reduction of life span and a premature death, this phenomenon is prevented if *cry RNAi* is expressed in GB cells and rescue lifespan to control values. Moreover, *cry RNAi* in normal glial cells does not reduce the lifespan but causes a significant increase in the average lifespan (Figure 2L).

### Signaling pathway to control *cry* upregulation

Glioblastoma condition triggers *cry* upregulation in glial cells but we aimed to decipher which signaling pathway is responsible for *cry* transcriptional activation. EGFR and PI3K are the two main pathways activated in this model of GB, and converge in *Myc* (Figure 3A). Thus, we analyzed the contribution of PI3K, EGFR and *Myc* to *cry* upregulation. We measured the fluorescent signal of *cry* transcriptional reporter in control adult brains (Figure 3B-B'), and compared with adult brains upon expression of the constitutively active forms of *PI3K* (Figure 3C-C') or *EGFR* (Figure 3D-D') in glial cells under the control of *repo-Gal4*. In addition we expressed *Myc* (Figure 3E-E') in glial cells as the convergence point of both pathways. We quantified the GFP signal of the *GFP-cry* reporter from confocal images (Figure 3F) and the results indicate that *PI3K* expression is sufficient to increase *GFP-cry* signal, but nor *EGFR* or *Myc* overexpression. These results suggest that PI3K controls *cry* transcription in a *Myc* independent signaling pathway, and EGFR does not participate in *cry* regulation in glial cells.

### Cry regulates *Myc* expression in glial cells

Next, to determine the epistatic relation between Cry and *Myc*, we analyzed *Myc* protein levels upon modulation of *cry* expression. First, to analyze if Cry is sufficient to drive *Myc* expression, we used a specific antibody against *Myc* and compared *Myc* protein levels in control brains, samples upon *cry* overexpression, *myc* overexpression or *cry* + *myc* overexpression in glial cells (Figure 4A-D'). The quantification of *Myc* signal surface that coincides with repo



signal (glial cells nuclei) showed that *cry* expression in glia is sufficient to trigger *myc* expression in glial cells, comparable to *myc* upregulation. In addition, *cry* + *myc* upregulation have a summation effect on *myc* upregulation (Figure 4E). To conclude if *cry* is required for *myc* expression in GB, we quantified glial Myc signal in control, *cry RNAi*, GB, GB+*cry RNAi* and *cry* upregulation (Figure 4F-J'). The quantifications indicate that *cry RNAi* in glial cells does not reduce the amount of Myc in glial cells (Figure 4K). In addition, GB condition triggers the number of Myc positive glial cells, as well as *cry* upregulation in glial cells (Figure 4K). Finally, *cry RNAi* in GB cells prevents the accumulation of Myc in GB cells. Taking all these results together, we conclude that *cry* is sufficient to trigger Myc accumulation in glial cells, and *cry* expression is necessary for Myc accumulation in GB condition.

### **Cry contribution to gliomagenesis**

To investigate the contribution of *cry* to gliomagenesis and glioma progression, we determined the number of glial cells and volume of glial membrane network in control adult brain, GB (*PI3K+EGFR*), *PI3K+cry*, *EGFP+cry* or *Myc+cry* expressed in glial cells (Figure 5A-E'). The quantification showed that all these genetic combinations cause an increase in the number of glial cells as compared to control brains (Figure 5F). However, only GB condition provoked an expansion of the glial membrane volume, and the combination of *PI3K+cry*, *EGFP+cry* or *Myc+cry* showed a volume of glial membrane comparable to control brains (Figure 5G). To further determine the contribution of *cry* to GB expansion, we analyzed the contribution of single gene upregulation in glial cells for *cry* or *Myc* and the combination of *cry+Myc* (Figure 5H-K'). The quantification of glial cell number showed that *cry* or *Myc* expression alone, or in combination, is sufficient to increase the number of glial cells with respect to control samples (Figure 5L). Nevertheless, none of these genetic modifications is sufficient to expand glial membrane volume (Figure 5M). These results suggest that *cry* or *Myc* is sufficient to trigger glial cell number increase in adult brains, but not to expand the volume of glial membrane network.

### **Cry upregulation in glial cells causes synapse loss and premature death**

It was previously described that GB progression induces synapse loss, an early symptom of neurodegeneration. To determine the contribution of *cry* to synapse



loss, we counted the number of active zones in motor neurons of adult neuromuscular junction in control, GB (*PI3K+EGFR*), *PI3K+cry*, *EGFP+cry* or *Myc+cry* samples (Figure 5N-R). The quantification of the number of active zones show that the expression in glial cells of GB (*PI3K+EGFR*), *PI3K+cry*, *EGFP+cry* or *Myc+cry* is sufficient to reduce the number of synapses in motoneurons (Figure 5S).

Finally, to evaluate the systemic effect of GB and glial expression of *PI3K+cry*, *EGFP+cry* or *Myc+cry*, we analyzed the lifespan of adult individuals. The results show that GB causes a premature death, as previously described (REFERENCE), glial upregulation of *EGFP+cry* or *Myc+cry* causes a significant reduction of lifespan but less aggressive than GB, and *PI3K+cry* upregulation in glial cells does not reduce lifespan (Figure 5T).

## Figure Legends

**Figure 1. Circadian gene *cry* is up-regulated in both human GB samples and GB *Drosophila* model.** A) RT-qPCR analysis of complete brains of 7 day old adult flies from *repo-Gal4>UAS-LacZ* (Control) and *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma) genotypes in LD conditions at ZT6 for the circadian gene *cry* (t-test). B-E) Confocal microscopy images of brains of 7 day old adult flies from B) *repo-Gal4>UAS-LacZ* (Control), C) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma), D) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>, UAS-cryRNAi* (Glioma CryRNAi) and E) *repo-Gal4>UAS-cryRNAi* (Glia CryRNAi) after using B'-E') the reporter GFP-Cry in green and B''-E'') the glial membrane is marked in red. F) Colocalization between GFP-Cry and the glial membrane and statistical analysis in at least N = 16 (ANOVA, post-hoc Bonferroni). G) Data on overexpression of *cry1* in human GB against normal tissue (T: tumor; N: normal). H) Graph showing a lower life expectancy in those patients with GB and *cry1* overexpressed compared to patients with GB with low expression of *cry1*. (Images obtained from gliovis.bioinfo.cnio.es) (scale bar, 30μm) (\**p*-value<0,05, \*\**p*-value<0,01, \*\*\**p*-value<0,001).

**Figure 2. Ectopic down-regulation of *cry* prevents GB tumorigenesis and effects.** A-D) Confocal microscopy images of brains of 7 day old adult flies from A) *repo-Gal4>UAS-LacZ* (Control), B) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma), C) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>, UAS-cryRNAi* (Glioma CryRNAi) and D) *repo-Gal4>UAS-cryRNAi* (Glia CryRNAi) with glial nuclei marked in green (scale bar, 100 μm). A'-D') Glial membrane is shown in red. E) Glial cells number and F) glial membrane volume quantification and statistical analysis for at least N = 11 per genotype (ANOVA, post-hoc Bonferroni). G-J) Confocal images of adult NMJ of 7 day old flies from G) *repo-Gal4>UAS-LacZ* (Control) H) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma), I) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>, UAS-cryRNAi* (Glioma CryRNAi) and J) *repo-Gal4> UAS-cryRNAi* (Glia CryRNAi) genotypes. Active zones are marked in green (scale bar, 25μm). K) Quantification and statistical analysis of active zones in at least N = 17 per genotype (ANOVA, post-hoc Bonferroni). L) Graph shows a survival assay of *repo-Gal4>UAS-LacZ* (Control, grey), *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma, black), *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-cryRNAi*, (Glioma CryRNAi, dark green) and *repo-Gal4>UAS-cryRNAi*, (GliaCryRNAi, light green) flies and statistical analysis in N = 90 (Mantel-Cox test) (\*\*p-value<0,001).

**Figure 3. PI3K up-regulates the levels of *cry*.** A) Scheme of EGFR (blue) and PI3K (yellow) signaling pathways involved in GB tumoral transformation with Myc as convergence player (green) (modified from (Read *et al.* 2009)). B-E) Confocal microscopy images of brains 7 day old adult flies from B) *repo-Gal4>UAS-LacZ* (Control), C) *repo-Gal4>UAS-dp110<sup>CAAX</sup>* (PI3K), C) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>* (EGFR), and D) *repo-Gal4>UAS-dmyc* (Myc) using the *GFP-cry* reporter in marked in green (scale bar, 100 μm) and B'-E') the glial membrane is marked in red. F) Colocalization between *GFP-cry* and the glial membrane and statistical analysis in at least N = 16 (ANOVA, post-hoc Bonferroni). (\*p-value<0,05, \*\*p-value<0,01, \*\*\*p-value<0,001).

**Figure 4. Cry increases glial myc levels in physiological and GB conditions.** A-D) Confocal microscopy images of brains 7 day old adult flies from A) *repo-Gal4>UAS-LacZ* (Control), B) *repo-Gal4>UAS-cry* (Cry), C) *repo-Gal4>UAS-dmyc* (Myc), D) *repo-Gal4>UAS-dmyc, UAS-cry* (MycCry) with Myc

marked in magenta A'-D') and glial nuclei marked in green (scale bar, 25  $\mu$ m). E) Glial Myc quantification and statistical analysis for at least N = 9 per genotype (ANOVA, post-hoc Bonferroni). F-J) Confocal microscopy images of brains 7 day old adult flies from F) *repo-Gal4>UAS-LacZ* (Control), G) *repo-Gal4>UAS-cryRNAi* (CryRNAi), H) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>, UAS-cryRNAi* (Glioma CryRNAi), I) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma) and J) *repo-Gal4>UAS-cry* (Cry), with Myc marked in magenta F'-J') and glial nuclei marked in green (scale bar, 25  $\mu$ m). K) Glial Myc quantification and statistical analysis for at least N = 12 per genotype (ANOVA, post-hoc Bonferroni). (\**p*-value<0,05, \*\**p*-value<0,01, \*\*\**p*-value<0,001).

**Figure 5. EGFR-cry coexpression induces glial proliferation, synapse number and survival reduction.** A-E) Confocal microscopy images of brains of 7 day old adult flies from A) *repo-Gal4>UAS-LacZ* (Control), B) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma), C) *repo-Gal4> UAS-dp110<sup>CAAX</sup>, UAS-cry* (PI3KCry) D) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-cry* (EGFRCry), and E) *repo-Gal4>UAS-dmyc, UAS-cry* (MycCry) with glial nuclei marked in green (scale bar, 100  $\mu$ m). A'-E') Glial membrane is shown in red. F) Glial cells number and G) glial membrane volume quantification and statistical analysis for at least N = 12 per genotype (ANOVA, post-hoc Bonferroni). H-K) Confocal images of adult brains of 7 day old flies from H) *repo-Gal4>UAS-LacZ* (Control) I) *repo-Gal4>UAS-cry* (Cry), J) *repo-Gal4>UAS-dmyc* (Myc) and K) *repo-Gal4> UAS-cry, UAS-dmyc* (MycCry) genotypes with glial nuclei marked in green (scale bar, 100  $\mu$ m) H'-K') and glial membrane shown in red. L) Glial cells number and M) glial membrane volume quantification and statistical analysis for at least N = 9 per genotype (ANOVA, post-hoc Bonferroni). N-R) Confocal microscopy images of NMJ of 7 day old adult flies from N) *repo-Gal4>UAS-LacZ* (Control), O) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma), P) *repo-Gal4>UAS-dp110<sup>CAAX</sup>, UAS-cry* (PI3KCry), Q) *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-cry* (EGFRCry), and R) *repo-Gal4>UAS-dmyc, UAS-cry* (MycCry). Active zones are marked in green (scale bar, 25  $\mu$ m). S) Quantification and statistical analysis of active zones in at least N = 13 per genotype (ANOVA, post-hoc Bonferroni). T) Graph shows a survival assay of *repo-Gal4>UAS-LacZ* (Control, grey), *repo-Gal4>UAS-dEGFR<sup>Δ</sup>, UAS-dp110<sup>CAAX</sup>* (Glioma, black), *repo-Gal4> UAS-*

*dp110<sup>CAAX</sup>*, *UAS-cry* (PI3KCry, blue), *repo-Gal4>UAS-dEGFR<sup>Δ</sup>*, *UAS-cry*, (EGFRCry, red) and *repo-Gal4>UAS-cry*, *UAS-dmyc* (MycCry, green) flies and statistical analysis in N = 90 (Mantel-Cox test)(\*\**p*-value<0,01\*\*\**p*-value<0,001).

## Discussion

Different studies have established a relation between alterations in circadian rhythm genes and cancer. Specifically, one of the genes associated with different types of cancer is *cry* (Shafi *et al.* 2021; Yang *et al.* 2021; Mampay *et al.* 2021). So this study aims to investigate the role of *cry* in a *Drosophila* GB model.

We have described the upregulation of *cry1* in human GB samples, and in a well studied *Drosophila* model of GB. This model is based on the two most frequent mutated pathways in GB, PI3K and EGFR, which converge in Myc as a convergence point. This pathway is of great relevance to promote GB cells expansion, GB progression and in consequence, the deterioration of neighboring neurons and a premature death.

The results indicate that *cry* upregulation in GB cells depends on PI3K expression, and is required for GB cells increase and synapse loss. Cry is sufficient to increase the number of glial cells. However, *cry* expression is expendable for normal wt glial growth during development, which makes it a potential target for GB treatment.

In addition, we show that Cry is necessary to induce *myc* expression in GB cells, and *cry* expression is sufficient to induce *myc* upregulation. This agrees with *in vitro* studies that revealed an increase in Myc levels as a result of *cry* upregulation (Altman *et al.* 2015). Therefore, we propose that *cry* is part of the PI3K-Myc signaling pathway in GB, where *cry* upregulation would be associated with glial proliferation. However, PI3K is a highly promiscuous enzyme that participates in numerous signaling pathways, and the results suggest that Cry contribution is restricted to the malignant features of GB dependent on *myc* such as GB cell number increase and neurodegeneration. However, *cry* expression is independent of expansion of glial membrane characteristic of GB progression.

In addition, *cry* expression in glial cells partially reduces life span, but is less aggressive than GB. This result suggests that Cry plays a central role in GB and it is required for GB formation, and *cry* mutations might be responsible for many features of GB. The human gene expression databases indicate that *cry1* expression levels correlate negatively with life span, and it is associated with a

poor prognosis. In consequence, these results suggest that further studies on the contribution of *Cry1* to human GB progression could lead to novel strategies to treat GB patients.

The studies of other groups describe the effect of haloperidol on *cry1* expression in GB cells. But these results obtained in cell culture suggest that the doses required to treat patients might be toxic, in consequence specific delivery strategies combined with haloperidol are worth of study. We have observed significant effects of *cry* knockdown in normal glial cells, in line with Bolukbaso and cols that recently described the extension of lifespan by *foxo* upregulation in glial cells (Bolukbasi *et al.* 2021). We have observed an effect of *cry* RNAi expression in the number of glial cells (Figure 5L). Given that *cry* and *foxo* respond to PI3K pathway, it is tempting to speculate that *cry* expression is relevant for life span extension by PI3K pathway, and associated behaviours such as diet restriction.

In consequence, the classical definition of *Cry* as a regulator of circadian rhythms can now be expanded to glial cells biology, GB progression and life span. This plethora of different phenotypes associated with one gene, is now a common feature previously described for TroponinI (Casas-Tintó *et al.* 2016a; b; Casas-Tintó and Ferrús 2019), Caspases (Baena-Lopez 2018; Baena-Lopez *et al.* 2018; Arthurton *et al.* 2020) or even other circadian genes as *per1* (Zhang *et al.* 2013; Kwapis *et al.* 2018) and contributes to the explanation of the multiple phenotypes observed in patients.

## Material and methods

### Fly stocks and genetics

All fly stocks were maintained at 25°C (unless otherwise specified) on a 12/12 h light/dark cycles at constant humidity in a standard medium. The stocks used from Bloomington Stock Center were *tub-Gal80<sup>ts</sup>* (BL-7019), *Repo-Gal4* (BL-7415), *UAS-LacZ* (BL-8529). Other fly stocks used were *UAS-dEGFR<sup>Δ</sup>*; *UAS-dp110<sup>CAAX</sup>* (gift from R Read), *UAS-cry* (gift from F.Royer), *GFP-cry* (gift from PE.Hardin), *UAS-PI3K* (gift from J.Botas), *UAS-cryRNAi* (gift from F.Royer), *UAS-dMyc* (gift from E.Moreno).

The glioma-inducing line contains the *UAS-dEGFR<sup>Δ</sup>*, *UAS-dp110<sup>CAAX</sup>* transgenes that encodes for the constitutively active forms of the human orthologs PI3K and EGFR, respectively (Read *et al.* 2009). *Repo-Gal4* line drives the *Gal4* expression to glial cells and precursors (Lee and Jones 2005; Casas-Tintó *et al.* 2017) combined with the *UAS-dEGFR<sup>Δ</sup>*, *UAS-dp110<sup>CAAX</sup>* line allow us to generate a glioma thanks to the Gal4 system (Brand and Perrimon 1993). *Elav-LexA* line drives the expression to neurons, allowing us to manipulate neurons in a glioma combining *LexA* and *Gal4* expression systems (Lai and Lee 2006).

Gal80<sup>TS</sup> is a repressor of the Gal4 activity at 18°C, although at 29°C is inactivated (McGuire *et al.* 2003). The *tub-Gal80<sup>ts</sup>* construct was used in all the crosses to avoid the lethality caused by the glioma development during the larval stage. The crosses were kept at 17°C until the adult flies emerged. To inactivate the Gal80<sup>ts</sup> protein and activate the Gal4/UAS system to allow the expression of our genes of interest, the adult flies were maintained at 29°C for 7 d except in the survival assay (flies were at 29°C until death).

### Immunostaining and image acquisition

Adult brains were dissected and fixed with 4% formaldehyde in phosphate-buffered saline for 20 min whereas adult NMJ were fixed 10 min; in both cases, samples were washed 3 × 15 min with PBS+0.4% triton, blocked for 1 h with PBS+0.4% triton+ BSA 5%, incubated overnight with primary antibodies, washed 3 × 15 min, incubated with secondary antibodies for 2 h, and mounted in Vectashield mounting medium, with DAPI in the case of the brains. The primary antibodies used were anti-repo mouse (1/200; DSHB) to recognize glial nuclei, anti-bruchpilot-NC82-mouse (1/50; DSHB) to recognize the presynaptic protein bruchpilot, anti-HRP rabbit (1/400; Cell Signaling) to recognize membranes, anti-GFP rabbit (1:500; DSHB), anti-Myc guinea pig (1/100; DSHB) to recognize the nuclear protein Myc,. The secondary antibodies used were Alexa 488 or 647 (1/500; Life Technologies). Images were taken by a Leica SP5 confocal microscopy.



## qRT-PCR

cDNA samples from 1:5 dilutions were used for real-time PCR reactions. Transcription levels were determined in a 14-ml volume in duplicate using SYBR Green (Applied Biosystem) and 7500 qPCR (Thermo Fisher Scientific). We analyzed transcription levels of *cry* using Rp49 as housekeeping gene reference.

Sequences of primers were: Rp49 F: GCATACAGGCCCAAGATCGT R: AACCGATGTTGGGCATCAGA; *cry* F: TTCTTCCCATCAAACTGG R: AAACGCATCCGATTGTAACC.

After completing each real-time PCR run, with cycling conditions of 95°C for 10 min, 40 cycles of 95°C for 15 seconds and 55°C for 1 min, outlier data were analyzed using 7500 software (Applied Biosystems). Ct values by triplicate of duplicates from 3 biological samples were analyzed calculating 2DDCt.

## Survival assays

Lifespan was determined under 12:12 h LD cycles at 29°C conditions. Three replicates of 30 1- to 4-d-old male adults were collected in vials containing standard *Drosophila* media and transferred every 2–3 d to fresh *Drosophila* media.

## Quantification

Fluorescent reporter-relative *cry* signals within brains were determined from images taken at the same confocal settings avoiding saturation. For the analysis of co-localization rates, “co-localization” tool from LAS AF Lite software (Leica) was used taking the co-localization rate data for the statistics analyzing the co-localization between green signal (both cases) and signal coming from glial tissue from three slices per brain in similar positions of the z axis.

Glial network was marked by a *UAS-myristoylated-RFP* reporter specifically expressed under the control of *repo-Gal4*. The total volume was quantified using Imaris surface tool (Imaris 6.3.1 software). Glial nuclei were marked by staining with the anti-Repo (DSHB). The number of Repo+ cells and number of

synapses (anti-nc82; DSHB) were quantified by using the spots tool in Imaris 6.3.1 software. We selected a minimum size and threshold for the spot in the control samples of each experiment: 0.5  $\mu\text{m}$  for active zones and 2  $\mu\text{m}$  for glial cell nuclei. Myc glial signal was quantified using Imaris surface tool (Imaris 6.3.1 software) creating a mask for the glial nuclei signal and selecting exclusively the myc signal corresponding to glial nuclei. Then we applied the same conditions to the analysis of the corresponding experimental sample.

## Statistics

The results were analyzed using the GraphPad Prism 5 software ([www.graphpad.com](http://www.graphpad.com)). Quantitative parameters were divided into parametric and nonparametric using the D'Agostino and Pearson omnibus normality test, and the variances were analyzed with F test. *t* test and ANOVA test with Bonferroni's post hoc were used in parametric parameters, using Welch's correction when necessary. The survival assays were analyzed with Mantel–Cox test. The *P* limit value for rejecting the null hypothesis and considering the differences between cases as statistically significant was  $P < 0.05$  (\*). Other *P*-values are indicated as \*\* when  $P < 0.01$  and \*\*\* when  $P < 0.001$ .

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