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Evolution of Developmental Control Mechanisms

The Gcm/Glide molecular and cellular pathway: New actors and new lineages

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ABSTRACT

In *Drosophila*, the transcription factor Gcm/Glide plays a key role in cell fate determination and cellular differentiation. In light of its crucial biological impact, major efforts have been put for analyzing its properties as master regulator, from both structural and functional points of view. However, the lack of efficient antibodies specific to the Gcm/Glide protein precluded thorough analyses of its regulation and activity *in vivo*.

In order to relieve such restraints, we designed an epitope-tagging approach to "FLAG"-recognize and analyze the functional protein both *in vitro* (exogenous Gcm/Glide) and *in vivo* (endogenous protein).

We here (i) reveal a tight interconnection between the small RNA and the Gcm/Glide pathways. *AGO*1 and *miR*-1 are Gcm/Glide targets whereas *miR-279* negatively controls Gcm/Glide expression (ii) identify a novel cell population, peritracheal cells, expressing and requiring Gcm/Glide. Peritracheal cells are non-neuronal neurosecretory cells that are essential in ecdysis.

In addition to emphasizing the importance of following the distribution and the activity of endogenous proteins *in vivo*, this study provides new insights and a novel frame to understand the Gcm/Glide biology.

Introduction

The differentiation of the Nervous System (NS) relies on the coordinated expression of cell-specific transcription factors that allocate distinct fates during development. In *Drosophila*, the Gcm/Glide gene (*gcm* in the following text) codes for a potent transcription factor of the Zinc finger family that is expressed in the glial lineages (reviewed in Trebuchet and Giangrande, 2012). Its role is to dictate the choice between neurons and glia in multipotent neural precursors, as most glia are converted into neurons in animals lacking the *gcm* gene (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Miller et al., 1998; Flici et al., 2011). Moreover, its threshold levels are sufficient to promote gliogenesis when ectopically expressed within and outside the neurogenic region. Finally, *gcm* is required in additional cell types, including postembryonic neuronal subpopulations (Yoshida et al., 2005; Chotard et al., 2005; Colonques et al., 2007; Soustelle and Giangrande, 2007a; Soustelle et al., 2007), tendons (Soustelle et al., 2004) and blood cells (Bernardoni et al., 1997; Alfonso and Jones, 2002; Bataillé et al., 2005; Jacques et al., 2009).

Gcm requires a tight, multi-layered modulation of its activity, exerted through (i) a fine transcriptional control which benefits from a modular organization of the *gcm* promoter, where distinct factors act in a spatio-temporal specific fashion (Jones et al., 2004; Ragone et al., 2003), (ii) an asymmetric segregation of *gcm* RNA between neural precursor daughter cells, thus committed to be neuron or glia, (Akiyama-Oda et al., 1999; Bernardoni et al., 1999; Ragone et al., 2001), (iii) a short-lasting activity. This last feature relies on the intrinsic instability of both *gcm* RNA (Soustelle et al., 2008) and protein (Akiyama et al., 1996; Miller et al., 1998; Yang et al., 2005). In particular, Gcm protein degradation has been recently pointed out as a crucial post-translational process: mutations in the proteasome affect Gcm kinetics and, as a consequence, glial proliferation (Ho et al., 2009). Moreover, different cofactors modulate Gcm activity (Jacques et al., 2009; De Iaco et al., 2006; Popkova et al., in press). Altogether, these observations stress the importance of following Gcm expression at the protein level.

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These reasons, together with the accumulating evidence that post-translational regulation plays an essential role in signaling cascades, made it ineluctable to set up approaches for detecting Gcm. The high inefficiency of the α-Gcm antibodies, prompted us to design an alternative, epitope-tagging based strategy to visualize the Gcm protein *in vivo* and *in vitro*. We also develop a flagged BAC transgenic stock (Venken et al., 2006; Bischof et al., 2007) fully rescuing the *gcm* mutation and allowing us to characterize unexplored aspects of the Gcm pathway.

We first validate that the FLAG-Gcm fusion is active. We provide the embryonic developmental profile of the transiently expressed Gcm protein and we identify peritracheal cells, a component of the neurosecretory system involved in ecdysis, as a novel cell type expressing and requiring Gcm. Finally, we reveal *gcm*/Gcm post-transcriptional regulation via the microRNA *miR*-279 and show that the Gcm protein affects the miRNA pathway, controlling the expression of *AGO*1 and *miR*-1.

In sum, the present work explores new aspects of a pathway that is essential in several developmental processes and opens novel perspectives in the field.

Material and methods

Flies and Genotypes

Flies were kept at 25 °C, w^{1118} was used as a wild-type (*WT*) strain. The gcm^{26}/*CyO, twist-lacZ* allele, the gcm^{rA87} enhancer trap line (carrying a *lacZ* insertion into the *gcm* promoter) and the point mutant gcm^{N7-4}/*CyO, twist-lacZ* are described in (Vincent et al., 1996) gcm^{34}/*CyO, twist-lacZ* was used as a hypomorphic allele (Bernardoni et al., 1997). The *Df*(2*L*)132/*CyO, twist-lacZ* strain (carrying a large deletion encompassing the *gcm* and *gcm*2 loci (Lane and Kalderon, 1993)) is described in Kammerer and Giangrande (2001).

The *UAS–gcm* (*M24A*) line was used for ectopic expression of two doses of *gcm* (Bernardoni et al., 1998) and the *UAS–miR*-279 line (gift of E. Lai, (Cayirlioglu et al., 2008)) for overexpression *sca-Gal*4 was used as a driver in the neurogenic territories, *gcm–Gal*4 as a driver in all *gcm*-expressing lineages, *hs-Gal*4 as a ubiquitous, inducible driver (heat shock experiments were performed like in Flici et al. 2011). All the drivers were from the Bloomington *Drosophila* Stock Center. *UAS–mCD8GFP* (targeting GFP to the membrane), UAS-*luc*ORF$^{WT luc3'UTR}$ and UAS-*luc*ORF$^{WT gcm3'UTR}$ were utilized as reporters. Luc-based flies are described in (Soustelle et al., 2008). The rescued stocks are: gcm^{26}/gcm^{26}; gcm^{BAC}/gcm^{BAC} and gcm^{26}/gcm^{26}; *gcm–FLAG*^{BAC}/*TM6, Tb*. Furthermore a double homozygous strain was established: *Df*(2*L*)132/*Df*(2*L*)132; gcm^{BAC}/gcm^{BAC}.

The following genotypes were used in the rescue tests: gcm^{N7-4}/*CyO, twi-lacZ*; $gcm^{BAC}/+$ was crossed with *Df(2L)132/CyO, twist-lacZ*; $gcm^{BAC}/+$ and gcm^{N7-4}/*CyO, twist-lacZ*; *gcm–FLAG*$^{BAC}/+$ was crossed with *Df(2L)132/CyO, twist-lacZ*; *gcm–FLAG*$^{BAC}/+$. gcm^{26}/*CyO, twist-lacZ*; $gcm^{BAC}/+$ was crossed with *Df(2L)132/CyO, twist-lacZ*; $gcm^{BAC}/+$ and gcm^{26}/gcm^{26}; *gcm–FLAG*^{BAC}/*TM6, Tb was* crossed with *Df(2L)132/CyO, twist-lacZ*; *gcm–FLAG*^{BAC}/*gcm–FLAG*^{BAC}.

UAS–gcm–FLAG flies were generated by classic germline transformation, gcm^{BAC} and *gcm–FLAG*^{BAC} flies by targeted transgenesis into the ZH-attP-68E genomic site (III chromosome), according to Bischof et al. (2007).

Oligonucleotides

All the oligonucleotides employed are listed in Supplementary Table 1.

Plasmids

The *pUAST–gcm–FLAG* construct is described in (Jacques et al., 2009).

pPac5c–miR-279. A region encoding for *miR*-279 was amplified through oligonucleotides (oligos) *miR*-279 *clon* 100 *FW* and *miR*-279 *clon* 100 *RV* from a genomic DNA template and cloned into the *pPac5c* expression vector. The construct was then modified removing the *Actin* termination signal from the backbone of *pPac5c* by restriction enzyme digestion. A similar procedure was used for cloning *miR*-286, using the oligos *miR*-286 *clon* 100 *FW* and *miR*-286 *clon* 100 *RV*.

pPac5c–gcm–FLAG/3′UTR. The 3′UTR of *gcm* was amplified from genomic DNA with the oligos *gcm* 3′*UTR Kpn FW* and *gcm* 3′*UTR Not RV*, and cloned downstream to the FLAG cassette of the *pPac5c–gcm–FLAG* vector (Ho et al., 2009). A mutant version of *pPac5c–gcm–FLAG/3′UTR* construct (*pPac5c–gcm–FLAG/3′UTR mut*) was derived by inverse PCR with oligos *gcm* 3′*UTR mut FW* and *gcm* 3′*UTR mut RV*.

4.3 kb *repo–RFP*. A fragment of 4.3 kb of the *repo* promoter (Jones et al., 2004) was digested from the *pCasper-hs-lacZ* plasmid (gift of B.W. Jones) and subcloned into the *pRed*and *pGreen-Hpelican* vector (KpnI-NotI restriction). A GFP reporter was derived similarly (4.3 kb *repo–RFP*).

6 kb *gcm–Gal*4.6 kb of the *gcm* upstream region were isolated from a recombinant phage (Sorrentino and Giangrande, unpublished data) and cloned in the *pCasper-Gal*4 vector (see Ragone et al., 2003).

Cell culture and transfections

Schneider's 2 (S2) cells were grown at a density comprised between 3 and $10*10^6$ cells/ml in Schneider's conditioned medium, complemented with 10% heat inactivated Fetal Calf Serum and 0.5% Penicillin/Streptomicine.

1–3 μg of total DNA mix was transfected using the Effectene Kit (Qiagen), according to manifacturer's instructions. 0.5–1 μg of *Actin-Gal*4 construct (*pBSK-Actin-Gal*4) was utilized as inducer of the UAS-mediated gene expression. *pUAST–gcm–FLAG* was used for Gcm–FLAG ectopic expression, *pUAST–gcm* (Bernardoni et al., 1997), for WT Gcm overexpression and *pUAST–eGFP* or *pPac*5*C-lacZ* as transfection controls. Cells were harvested at specific timepoints in Lysis Buffer for extract preparation (see below).

Western blot assay

Protein expression was detected from 10 to 20 μg of total protein lysate (extracted by freezing-thawing cell pellets in 400 mM KCl, 25 mM Tris HCl, pH 7.9, 10% glycerol) by using the following primary antibodies: mouse-α-FLAG (F3165, Sigma Aldrich, 1:5000 working dilution), rabbit-α–GFP (A-11122, Molecular Probes, 1:2000) rabbit-α-actin (A2066 Sigma Aldrich, 1:5000), mouse-α-β-Gal (G-4644 Sigma Aldrich, 1:1000), rabbit-α–AGO1 (ab5070, Abcam 1:2000). Donkey-α-mouse HRP and goat-α-rabbit HRP (Jackson ImmunoResearch) were used as secondary antibodies (1:10000). 200 μg of embryonic extracts (produced from the crosses *sca-Gal4/+;gcm–FLAG*^{BAC}/*UAS–mCD8GFP* X *UAS–miR*-279 and *sca-Gal4/+; gcm–FLAG*^{BAC}/*UAS–mCD8GFP* X w^{1118}, as a control) were fractionated on gradient acrylamide gels (Invitrogen) and Gcm–FLAG was detected by using 1:500 diluted F3165 antibody.

Immunocytochemistry (ICC), Immunohistochemistry (IHC) and in situ hybridization

S2 cells (seeded onto coverslips), dechorionated embryos or manually dissected tracheae from third instar larvae were fixed in