MECHANISMS OF DEVELOPMENT 126 (2009) 503–516

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journal homepage: www.elsevier.com/locate/modo

MECHANISMS OF DEVELOPMENT

Bruno negatively regulates *germ cell-less* expression in a BRE-independent manner

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ARTICLE INFO

Article history:
Received 5 December 2008
Received in revised form
8 April 2009
Accepted 10 April 2009
Available online 22 April 2009

Keywords:
Drosophila
Germ cell-less
Bruno, Oogenesis
Germline
Regulation
Translation
RNA-binding

ABSTRACT

Mechanisms of post-transcriptional control are essential during *Drosophila* oogenesis and embryogenesis to sequester gene products in discrete regions and ultimately achieve embryonic asymmetry. Maternal *germ cell-less* (*gcl*) mRNA accumulates in the pole plasm of the embryo before Gcl protein is detectable. *gcl* mRNA, but not Gcl protein, can also be detected in somatic regions of the embryo, suggesting that *gcl* RNA is subject to translational control. We find that Gcl is expressed during oogenesis, and that it is regulated by the translational repressor Bruno (Bru). Increased levels of Gcl are observed in the oocyte when Bru level is reduced, and overexpression of Bru reduces Gcl expression. Consistently, reduction of the maternal dosage of Bruno leads to ectopic Gcl expression in the embryo, which, in turn, represses anterior *hückebein* (*hkb*) expression. Bru binds directly to the *gcl* 3'UTR in vitro, but, surprisingly, this binding is independent of a BRE (Bruno response element)-like motif. This motif is also not required for in vivo repression of Gcl expression during oogenesis or early embryogenesis. Bru binds the *gcl* 3'UTR via its C-terminal domain, which includes RNA recognition motif 3 (RRM3), with little or no contribution from the remainder of the protein. We conclude that repression by Bruno during oogenesis is required to restrict Gcl expression in the early embryo and that Bru represses *gcl* expression in a manner that involves RRM3 and a sequence unrelated to the BRE.

1. Introduction

The polarization of body axes in the *Drosophila* embryo and the segregation of the germline both require that the expression of specific maternal transcripts is restricted to limited temporal and spatial patterns. Multiple levels of post-transcriptional regulation of localized transcripts underlie this process, as has been characterized for *oskar* (*osk*) and *nanos* (*nos*), two mRNAs required for posterior patterning and germ cell development (Vardy and Orr-Weaver, 2007). These mRNAs become enriched in the pole plasm during oogenesis by processes that include biased localization along the microtubule network and localized trapping by actin microfilaments (Forrest and Gavis, 2003; Zimyanin et al., 2008). Restriction of Osk and Nos proteins to the posterior pole is accomplished primarily by complex translational regulation, whereby the mRNA component present in the pole plasm is translationally active, while the rest is translationally repressed (Wilhelm and Smibert, 2005; Vardy and Orr-Weaver, 2007).

Bruno (Bru) is an RNA-binding protein with three RNA recognition motifs (RRMs) that regulates the translation of several germline mRNAs, including *osk*. Bru binds directly to discrete sequence elements in the *osk* 3'UTR known as Bruno response elements (BREs), using both the N-terminal and C-terminal RRMs (Kim-Ha et al., 1995; Webster et al., 1997; Snee et al., 2008). Bru negatively regulates *osk* translation through two distinct mechanisms: first by blocking recognition of the 5' cap structure by recruiting Cup, a eukaryotic

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0925-4773/$ - see front matter
doi:10.1016/j.mod.2009.04.002

initiation factor 4E (eIF4E) binding protein that competitively inhibits recruitment of eIF4G (Nakamura et al. 2004), and second, in a cap-independent manner by packaging *osk* RNA into heavy particles that render *osk* inaccessible to the translational machinery (Nakamura et al., 2004; Chekulaeva et al., 2006). Three more targets of Bruno-mediated translational repression are also known: *gurken* (*grk*), *Sex lethal* (*Sxl*) and *Cyclin A* (*CycA*). Bruno binds directly to the *grk* 3'UTR, and Bru overexpression leads to defects in dorsal-ventral polarity due to a reduction of Gurken in the oocyte (Filardo and Ephrussi, 2003). Regulation of *Sxl* translation by Bru is required in the germarium for cystoblast differentiation, and repression of *CycA* RNA is essential to maintain mitotic quiescence during meiosis and to prevent overproliferation of germ cells (Parisi et al., 2001; Sugimura and Lilly, 2006; Wang and Lin, 2007).

Maternal *germ cell-less* (*gcl*) activity is required for germ cell specification, although, unlike *osk* and *nos*, *gcl* is not involved in posterior somatic patterning. Females lacking *gcl* function produce viable embryos with few or no pole cells, but with no detectable somatic defects (Robertson et al., 1999). Gcl accumulates at the interior face of the nuclear membrane of pole cell nuclei, where it is required to establish transcriptional silencing in the germline (Robertson et al., 1999; Leatherman et al., 2002). Ectopic expression of Gcl in the anterior of the embryo, achieved by fusing the *gcl* coding region to the *bicoid* (*bcd*) 3'UTR, results in reduced transcription of anterior somatic genes, *sisterlessA*, *tailless* and *hückebein* (*hkb*), suggesting a shift towards germline fate (Leatherman et al., 2002).

gcl RNA is first detected in the nurse cells at stage 5 of oogenesis and is abundant in the oocyte at stage 10 (Jongens et al., 1992; Nakamura et al., 2001). *gcl* RNA is also present throughout the early embryo and it accumulates in the pole plasm (Jongens et al., 1992). Gcl protein is not present outside of the pole plasm, and is first detected in the pole plasm after *gcl* RNA has accumulated there. Gcl is readily detectable at the time of pole bud and pole cell formation, when it is associated with the nuclear envelopes of nuclei that contact the pole plasm (Jongens et al., 1992). The distributions of *gcl* RNA and protein support the hypothesis that *gcl* mRNA is translationally repressed in the embryonic soma, and that this repression is relieved by pole plasm components. This hypothesis has, however, not heretofore been directly tested.

Here, we implicate Bruno in the translational regulation of *gcl*. We determined that Gcl protein is expressed during oogenesis, where it localizes to the nuclear envelope in both nurse cells and the oocyte, and that Gcl expression in the oocyte cytoplasm is regulated by Bru. This regulation is required to restrict Gcl expression to the germline in the early embryo and Gcl protein is detectable outside of the germ plasm in embryos from females heterozygous for *arrest* (*aret*, which encodes Bru). These embryos also show reduced zygotic *hkb* expression. We identified a sequence in the *gcl* 3'UTR that matches the BRE consensus sequence and is conserved among several *Drosophila* species. Recombinant Bru binds to the *gcl* 3'UTR in vitro, but surprisingly, this binding is independent of the *gcl* BRE and dependent upon a different region of the 3'UTR. The *gcl* BRE is also not required to repress Gcl expression during oogenesis or embryogenesis. In contrast to Bru binding to *osk*, *gcl* is not bound by an N-terminal Bru construct that includes RRM1 and 2. However, a C-terminal Bru construct binds the *gcl* 3'UTR efficiently. We conclude that Bruno represses *gcl* translation during oogenesis in order to refine Gcl expression in early embryos and that this regulation occurs through a novel interaction involving RRM3 and operating independently of the BRE.

2. Results

2.1. *Germ cell-less protein is expressed during oogenesis*

Four independent polyclonal antibodies were raised against a GST-Gcl fusion protein and used to characterize Gcl expression during oogenesis. Pre-adsorbed αGcl antisera identified a single band on immunoblots at the predicted molecular weight of 65 kDa in protein extracts made from wildtype ovaries, but not in protein extracts made from $gcl^{\Delta 1}$ ovaries (Fig. 1A). Similar results were obtained with embryonic extracts (data not shown). Using these antisera, we detected Gcl at the nuclear membrane of germline cells in the germarium as early as stage 2b (staging according to King, 1970). Gcl accumulates in the oocyte cytoplasm (Fig. 1D and D') and colocalizes with nuclear pore complex proteins in the nurse cells (Fig. 1B). In contrast, Gcl does not colocalize with Vasa (Vas), a component of the perinuclear nuage (Lasko and Ashburner, 1990), but is detected immediately adjacent to it toward the interior of the nuclei (Fig. 1C). This indicates that, as in cells in the early embryo, Gcl localizes predominantly at the interior of the nuclear envelope in nurse cells. Starting at stage 8, Gcl becomes slightly enriched at the oocyte cortex, particularly at the anterior and posterior poles, which persists at the posterior at least until the end of stage 10 (Fig. 1D and D'). We also detected Gcl expression in embryogenesis as previously described (Jongens et al., 1992). All four independent antisera we produced gave essentially identical results for Gcl expression in ovaries, as did a previously published antiserum (Jongens et al., 1992).

2.2. *Gcl expression in the oocyte depends on Bruno*

We assessed Gcl distribution in ovaries from females mutant for *osk* ($osk^{54}/Df(3R)p\text{-}XT103$), *vas* ($vas^{1}$) and *tud* ($tud^{tux}$), respectively (Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986, 1991; Thomson and Lasko, 2004). *osk* and *tud* mutations had no effect on Gcl distribution during oogenesis ($osk^{54}/Df(3R)p\text{-}XT103$, Fig. 1G and G' and data not shown) indicating that Gcl accumulation is independent of *osk* and *tud*. However, in vas^{1} oocytes Gcl frequently accumulates in an irregular pattern at the anterior and lateral cortex and cortical Gcl appears more prominent (Fig. 1H–J). As *gcl* RNA localization is not significantly altered in egg chambers from pole plasm mutants (Fig. 1K–M and data not shown), this suggests that posterior enrichment of Gcl protein in stage 8–10 oocytes is independent of the canonical pole plasm assembly pathway, but may depend specifically on *vas* for maintenance at the lateral and anterior cortex.

We next investigated whether known regulators of translation affect Gcl expression during oogenesis. *gcl* RNA colocalizes with Me31B (Nakamura et al., 2001), but we observed no change in Gcl expression in $me31B^{\Delta 1}$ germline clones (data