Developmental Biology 350 (2011) 169–182

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

JAK-STAT signalling is required throughout telotrophic oogenesis and short-germ embryogenesis of the beetle *Tribolium*

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ARTICLE INFO

Article history:
Received for publication 24 February 2010
Revised 4 October 2010
Accepted 15 October 2010
Available online 23 October 2010

Keywords:
Telotrophic oogenesis
Tribolium castaneum
Epithelial patterning
Stalk cells
JAK-STAT signalling
Domeless
SOCS
Segment polarity
Pair-rule genes
Gap genes
Short germ
Segmentation

ABSTRACT

In *Drosophila*, the JAK-STAT signalling pathway regulates a broad array of developmental functions including segmentation and oogenesis. Here we analysed the functions of *Tribolium* JAK-STAT signalling factors and of *Suppressor Of Cytokine Signalling* (*SOCS*) orthologues, which are known to function as negative regulators of JAK-STAT signalling, during telotrophic oogenesis and short-germ embryogenesis. The beetle *Tribolium* features telotrophic ovaries, which differ fundamentally from the polytrophic ovary of *Drosophila*. While we found the requirement for JAK-STAT signalling in specifying the interfollicular stalk to be principally conserved, we demonstrate that these genes also have early and presumably telotrophic specific functions. Moreover, we show that the *SOCS* genes crucially contribute to telotrophic *Tribolium* oogenesis, as their inactivation by RNAi results in compound follicles. During short-germ embryogenesis, JAK-STAT signalling is required in the maintenance of segment primordia, indicating that this signalling cascade acts in the framework of the segment-polarity network. In addition, we demonstrate that JAK-STAT signalling crucially contributes to early anterior patterning. We posit that this signalling cascade is involved in achieving accurate levels of expression of individual pair-rule and gap gene domains in early embryonic patterning.
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Introduction

The Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway is an evolutionarily conserved signalling system that plays essential roles in numerous biological processes in vertebrates and invertebrates (for review: Arbouzova and Zeidler, 2006; Denef and Schupbach, 2003). Insect oogenesis is one such example, where the JAK-STAT pathway regulates the differentiation of various cell types (Assa-Kunik et al., 2007; McGregor et al., 2002; Silver et al., 2005; Xi et al., 2003).

During early stages of *Drosophila* oogenesis, three follicle cell populations can be distinguished: (1) polar cells, which serve as key signalling centres, (2) stalk cells, which form interfollicular stalks that connect neighbouring egg chambers, and (3) main-body follicle cells, which build an epithelium overlying the germline cyst (for review: Bastock and St Johnston, 2008; Horne-Badovinac and Bilder, 2005; Spradling, 1993). Polar and stalk cells appear to differentiate from a common group of precursor cells in a stepwise manner (Tworoger et al., 1999). Notch as well as JAK-STAT signalling acts in subdividing the stalk/polar follicle cell primordium (Assa-Kunik et al., 2007; Xi et al., 2003): Polar cell fate requires the activation of Notch by the ligand Delta, which is produced by germline cells (Assa-Kunik et al., 2007; Lopez-Schier and St Johnston, 2001). These polar cells then express the JAK-STAT ligand *unpaired* (*upd*), which activates the JAK-STAT signalling pathway in neighbouring polar/stalk precursors. This induces the remaining precursors to differentiate as stalk cells (McGregor et al., 2002). The stalk cells intercalate to form the stalk and separate the egg chamber from the germarium. Accordingly, inactivation of JAK-STAT signalling results in the loss of the stalk and expansion of the polar follicle cell population, which results in fused egg chambers (compound follicles) (McGregor et al., 2002; Xi et al., 2003). Ectopic expression of Upd, on the other hand, results in the loss of polar cells and in the formation of supernumerary stalk-like cells (McGregor et al., 2002).

Oogenesis in *Drosophila* and in the beetle *Tribolium* follows two different modes of oogenesis (out of three to be found among insects), the polytrophic-meroistic (*Drosophila*) and telotrophic-meroistic (*Tribolium*) oogenesis (Trauner and Buning, 2007). Both have in common that germline cells can differentiate either in oocytes or in nurse cells. In polytrophic-meroistic ovaries, represented by *Drosophila melanogaster*, each germ cell cluster matures as one unit encased by somatic follicle cells. Exactly one cell of each cluster develops into an

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doi:10.1016/j.ydbio.2010.10.020

oocyte, while all others differentiate into nurse cells. In contrast, in telotrophic-meroistic ovaries as represented by the red flour beetle *Tribolium castaneum*, oocytes and nurse cells of a germ cell cluster separate such that each follicle contains only one germ cell, the oocyte. This oocyte remains connected to the tropharium – a syncytium of nurse cells – by a nutritive cord (Trauner and Buning, 2007). Although telotrophic ovary organogenesis has been studied at the morphological level in *Tribolium* (Trauner and Buning, 2007), virtually nothing is known about the molecular mechanisms underlying telotrophic oogenesis. In recent years *Tribolium castaneum* has developed into an advanced insect model system second only to *Drosophila melanogaster* (Richards et al., 2008), such that it is now possible to carry out functional studies of this mode of oogenesis. In order to gain insight into the patterning mechanisms underlying telotrophic oogenesis, we analysed the roles of JAK-STAT signalling including the *Tribolium* Suppressor of Cytokine Signalling (SOCS) orthologues for their functions in egg chamber formation and axes polarisation.

Also the mode of embryogenesis differs between *Drosophila* (long-germ mode) and *Tribolium* (short-germ mode). Therefore, we investigated the functions of JAK-STAT signalling throughout metamerisation of this beetle. In *Tribolium*—as in most other insects—only the anteriormost segments are patterned in the blastoderm stage, while all following segments are formed one by one from a posterior growth zone. While growth zone formation in *Tribolium* depends on the localised activity of the Torso-pathway (Schoppmeier and Schroder, 2005), it still is under debate, how blastodermal and growth zone patterning is achieved (Brown et al., 2001a; Bucher and Klingler, 2004; Cerny et al., 2005, 2008; Schoppmeier et al., 2009; Schoppmeier and Schroder, 2005; Schroder, 2003). It has been shown that early patterning in *Tribolium* does not involve *bicoid* and that gap genes have drastic different functions in *Tribolium* when compared to *Drosophila* (Brown et al., 2001a; Bucher and Klingler, 2004; Cerny et al., 2005, 2008; Kotkamp et al., 2010; Marques-Souza et al., 2008; Stauber et al., 1999, 2002). Also the regulation of pair-rule genes differs from *Drosophila*, as pair-rule interactions in the growth zone suggest that a segmentation clock is active (Choe and Brown, 2007; Choe et al., 2006). In contrast, at the level of segment-polarity genes studied so far, both expression and function appear to be conserved to a great extent (Bolognesi et al., 2008; Nagy and Carroll, 1994; Ober and Jockusch, 2006).

While we found conserved as well as divergent requirements for JAK-STAT signalling in patterning the telotrophic egg chamber, this signalling cascade apparently acts in the framework of the segment-polarity network during short-germ segmentation. In addition, we demonstrate that JAK-STAT signalling crucially contributes to early anterior (head) patterning, a function that we found to be related to regulatory input on gap and pair-rule gene domains.

Material and methods

Isolation of genes

The *Drosophila* JAK-STAT pathway components include the transmembrane receptor *domeless* (*dome*), one JAK (*hopscotch*, *hop*), one STAT (*Stat92E)*, three Unpaired ligands (*upd 1 to 3*), and three SOCS-like genes that are thought to inhibit STAT function (Arbouzova and Zeidler, 2006). We used the *Drosophila* JAK-STAT components as query sequences in tBLASTn searches of the *Tribolium* genome (Richards et al., 2008) and identified a single *domeless* gene (*Tc-dome*, XP_001807060), one JAK (*Tc-hop*, XP_968564), one STAT (*Tc-STAT*, XP_969477), and four *Tribolium* SOCS paralogs, which we named *Tc-Socs-2* (XP_972490), *Tc-Socs-6* (TC003320), *Tc-Socs-16D* (XP_973720*)*, and *Tc-Socs-36E* (TC003596) according to their closest *Drosophila* or vertebrate orthologs, respectively. We were not able, however, to identify any *unpaired* orthologs; this is most likely due to rapid sequence evolution of this gene family (Boulay et al., 2003).

Candidate genes were amplified from cDNA, cloned into pBluescript KS vector and sequenced to confirm their identity. Fragments of 900–1200 bp were used as template for dsRNA synthesis and digoxigenin labelled antisense RNA probes.

Immunohistochemistry and whole mount in situ hybridization

Female gonads were dissected as previously described (Trauner and Buning, 2007). Ovaries were fixed with 5% formaldehyde/PBS on ice for 60 min and washed in PBT (PBS with 0.1% Triton-X 100 and 3% BSA). To visualise the morphology, Hoechst 33258 and TRITC labelled Phalloidin, which labels the f-actin cytoskeleton were used.

For whole mount in situ hybridization, *Tribolium* ovaries were post-fixated for 15 min in 5% formaldehyde/PBT and rinsed four times in PBT followed by a proteinase K treatment (5 mg/ml) for 1 h on ice and an additional post-fix step (5% formaldehyde/PBS). Ovaries are again rinsed in PBT and pre-hybridised for 1 h at 65 °C (50% formamide, 5× SSC, 2% SDS). Hybridisation is performed overnight at 65 °C (50% formamide, 5× SSC, 2% SDS, 2% BBR (Boehringer Blocking reagent), 250 μg/ml tRNA, 100 μg/ml heparin) with 1–5 μl of a digoxigenin labelled riboprobe. After several washing steps, ovaries are rinsed with MAPT buffer (100 mM Maleic Acid, 150 mM NaCl, pH to 7.5 with NaOH, 0.1% Tween 20) and blocked for at least 2 h (BBR 0.5% in MABT). The alkaline phosphatase conjugated anti-dig antibody (Roche) is added at a concentration 1:2000 and incubated overnight at 4 °C. To remove unbound antibodies, ovaries are washed several times in MAPT buffer (for at least 2 h). Staining is performed in a standard AP buffer using FastRed as substrate (Roche). A detailed protocol is available from the authors.

To visualise the morphology after in situ hybridization, ovaries were counterstained by Hoechst 33258 (5 μg/ml) and an anti-α-tubulin antibody (mouse monoclonal, 1:1000; Sigma). Mitotically active cells were labelled by an anti-phospho-histone H3 antibody (rabbit polyclonal, 1:200; Upstate). The following secondary antibodies were used: Cy2 or Cy3 conjugated goat anti-rabbit (Jackson Immuno-Research, 1:50); Cy2 or Cy3-conjugated sheep anti-mouse (1:200, Sigma). All incubations were done at 4 °C overnight. Ovary images were captured on a Zeiss ApoTome.

Embryo fixation and embryo whole mount in situ hybridizations were essentially carried out as described before (Schoppmeier and Schroder, 2005).

RNAi and cuticle preparations

Pupal and adult RNA interference was essentially performed as described previously (Bucher et al., 2002; van der Zee et al., 2006). First instar offspring larvae were cleared in lactic acid/10% ethanol overnight at 60 °C. After washing once with lactic acid, cuticles were transferred to a drop of lactic acid on a slide. Cuticle autofluorescence images were captured on a Zeiss Axiophot and maximum projection images were generated from image stacks.

Results

Formation of interfollicular stalks

While in *Drosophila* several subpopulations of follicle cells can be distinguished by their specific cell morphologies (Horne-Badovinac and Bilder, 2005; Spradling, 1993) follicle cells in *Tribolium* form a rather uniform epithelium surrounding the developing oocytes (Fig. 1). In early pre-vitellogenic stages, the nuclei of the follicle cells are interlaced, which gives the epithelium a somewhat disorganised appearance (Figs. 1 C and D). During vitellogenic growth of the oocyte, follicle cell nuclei form a uniform epithelium (Fig. 1 A; compare stages III to IV follicle). In addition, interfollicular stalk cells become morphologically distinguishable (Figs. 1 E–J; stalk cells are defined as those

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