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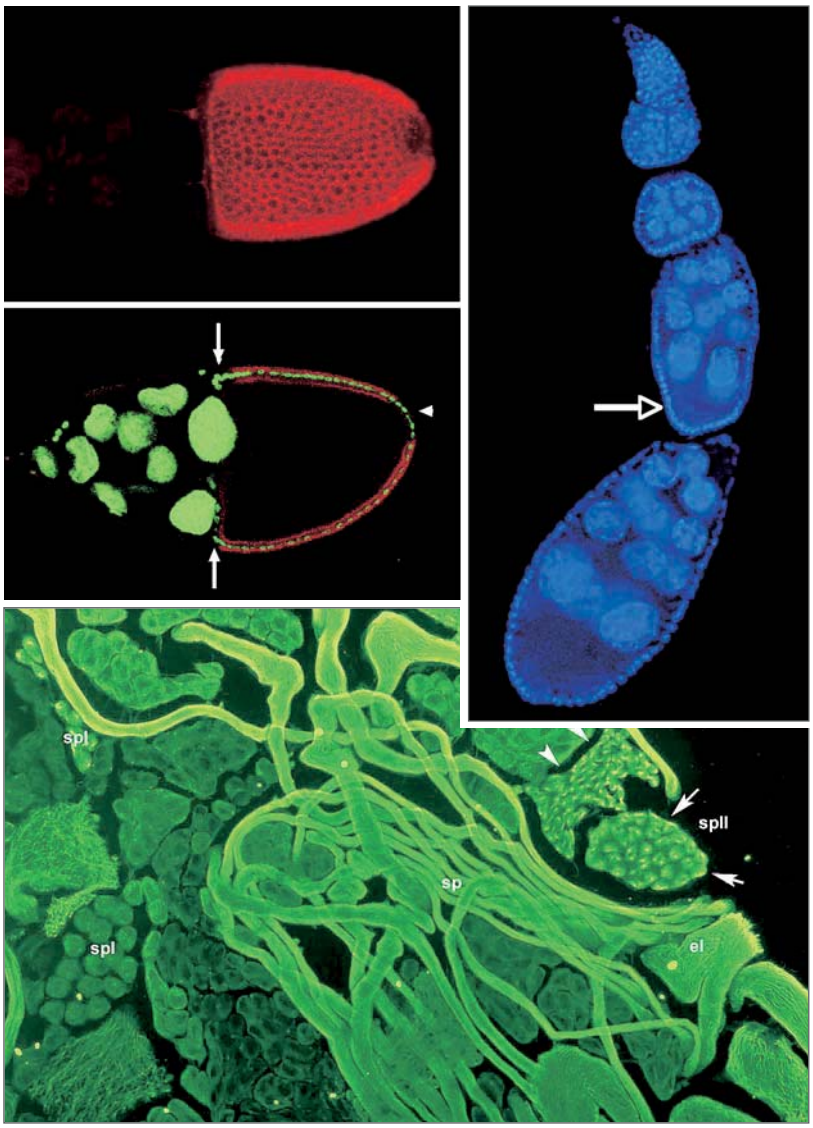
Tavole Rotonde sui maggiori problemi
riguardanti l'Entomologia Agraria in Italia
Sotto gli auspici del MIPAAF

XVII.
GENETICA MOLECOLARE DELLO SVILUPPO DEGLI INSETTI



ACCADEMIA NAZIONALE
ITALIANA DI ENTOMOLOGIA
Firenze 2008

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Rendiconti Anno LVI - 2008

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PRESENTAZIONE

> «Dobbiam fare le maraviglie come i Naturalisti tentato non abbiano intorno quello soggetto (lo sviluppo) alcune sperienze nella famiglia degli insetti; giacché abbiam luogo di presumere che non sarebbono senza esito.»
>
> *C. Bonnet (1773)*
> *«Contemplazione della Natura»*

Nel corso delle riunioni del 2008 questa Accademia ha discusso alcuni aspetti della genetica molecolare nello sviluppo degli insetti. In questo campo di studio, l'applicazione delle tecniche di manipolazione genetica, sviluppate in un insetto modello, il moscerino della frutta Drosophila melanogaster*, associate alla grande mole di conoscenze disponibili sulla embriologia comparata e sulla filogenesi degli insetti, stanno permettendo di fare rapidi progressi in campi fino ad ora inesplorati ad un livello molecolare (Carla Malva). Un numero relativamente piccolo di queste vie di segnalazione controlla una straordinaria diversità di risposte e decisioni chiave che, nelle cascate di eventi che regolano lo sviluppo di un organismo, portano alla acquisizione di destini cellulari diversi. I componenti principali di queste cascate di trasduzione di segnali, sono evolutivamente conservati dagli insetti all'uomo.*

Come primo esempio Carla Malva ha usato il suo stesso lavoro sperimentale. Nel corso di questi ultimi studi, soprattutto in quelli relativi a Toxoneuron nigriceps *e al* polydnavirus *ad esso associato, svolti in collaborazione con i gruppi diretti da E. Tremblay e F. Pennacchio, è stato scoperto che il parassitoide, per alterare molti processi biologici vitali del suo ospite, quali ad esempio lo sviluppo e la risposta immunitaria, usa molecole molto simili a quelle descritte in* Drosophila *ed anche nei mammiferi. Fra esse, le proteine cactus-IκB, inibitori dei fattori di trascrizione della famiglia NFκB, sono simili a quelle che in* Drosophila *controllano una serie di processi biologici fondamentali. Esse competono con le molecole endogene dell'insetto ospite, in quanto simili, ma, non avendo più la loro attività biologica, alterano vie di trasduzione del segnale importanti per la sopravvivenza dell'ospite. Da questi studi emerge che non soltanto sono conservate le vie di segnalazione che regolano le risposte cellulari mediate dai fattori di trascrizione, dagli insetti ai mammiferi, ma anche le strategie utilizzate dai patogeni e parassiti per interferire con l'attivazione di questi fattori nei loro ospiti. Infatti proteine simili agli inibitori cactus-IκB del TnBV sono state trovate nel genoma di altri* polydnavirus*, ma anche in altri virus dei vertebrati che mostrano efficienti strategie di soppressione della risposta immunitaria.*

Un altro interessantissimo esempio di conservazione evolutiva di molecole implicate nei processi di sviluppo è stato presentato e discusso da Miodrag Grbic del Department of Biology, University of Western Ontario, Canada. La maggior parte degli studi sono stati compiuti sull'insetto parassitoide poliembrionico Copidosoma floridanum *che parassitizza il nottuide* Trichoplusia ni*, e forma, da un singolo uovo, fino a 2000 embrioni che daranno origine ad individui differenziati in caste.*

Lo sviluppo embrionale del C. floridanum *differisce da quello degli altri insetti. Il* Copidosoma *depone uova piccole e senza vitello, circondate da un sottile corion. Il gene* vasa *è stato isolato in* Copidosoma *e si è osservato che è trascritto nelle cellule nutrici dell'ovario. È quindi veramente stupefacente che la presenza o assenza di una proteina possa decidere il destino riproduttivo e, come conseguenza, un diverso destino di casta. La formazione del guscio dell'uovo richiede la sintesi coordinata di differenti componenti proteiche nelle cellule follicolari della camera ovarica.*

Giuliano Callaini, del Dipartimento di Biologia Evolutiva dell'Università di Siena, si interessa da tempo dei processi di duplicazione dei centrioli e dei centrosomi nella divisione meiotica durante la spermatogenesi. La funzione delle differenti componenti dei centrosomi viene studiata in condizioni in cui si producono fusi con centrosomi multipli o fusi monopolari. In conclusione, lo studio della gametogenesi in Drosophila *permette di dissezionare processi biologici fondamentali quali la regolazione della espressione genica tessuto-specifica e la regolazione del ciclo cellulare, come è stato illustrato da Giuseppe Gargiulo del Dipartimento di Biologia Evoluzionistica Sperimentale dell'Università di Bologna.*

Per finire, riallacciandosi alla frase del Bonnet del 1773 riportata all'inizio, appare evidente che se studiando la D. melanogaster *si sono scoperti meccanismi di regolazione dello sviluppo così efficienti e precisi, immaginiamo quali meraviglie potranno scaturire dallo studio, ad un livello molecolare e con le sofisticate metodologie oggi disponibili. L'oggetto delle discussioni avvenute nel 2008 in Accademia ha fornito un basilare contributo in questo ambito di conoscenze.*

BACCIO BACCETTI
Presidente dell'Accademia Italiana di Entomologia

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GENETICA MOLECOLARE DELLO SVILUPPO DEGLI INSETTI

CARLA MALVA (*)

(*) *Istituto di Genetica e Biofisica, via Pietro Castellino 111, Napoli; e-mail: malva@igb.cnr.it*
Tematica oggetto delle Sedute pubbliche dell'Accademia tenute nell'Anno accademico 2008.

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Come primo esempio ho usato il mio stesso lavoro sperimentale in cui, dallo studio dello sviluppo e dell'oogenesi della *D. melanogaster*, sono approdata alla analisi delle basi molecolari delle interazioni fisiologiche fra alcuni braconidi parassitoidi ed i loro ospiti. Nel corso di questi ultimi studi, soprattutto in quelli relativi a *Toxoneuron nigriceps* e al polydnavirus ad esso associato (TnBV), svolti in collaborazione con i gruppi diretti da E. Tremblay e F. Pennacchio, abbiamo scoperto che il parassitoide, per alterare molti processi biologici vitali del suo ospite, quali ad esempio lo sviluppo e la risposta immunitaria, usa molecole molto simili a quelle descritte in *Drosophila* ed anche nei mammiferi. Fra esse, le proteine cactus-IκB, inibitori dei fattori di trascrizione della famiglia NFκB, sono simili a quelle che in Drosophila controllano una serie di processi biologici fondamentali, tra cui lo stabilirsi dell'asse dorso-ventrale dell'embrione e la risposta antimicrobica, ma sono troncate e mancano di alcuni siti attivi nella regolazione della risposta da essi controllata. Esse competono con le molecole endogene dell'insetto ospite, in quanto simili, ma, non avendo più la loro attività biologica, alterano vie di trasduzione del segnale importanti per la sopravvivenza dell'ospite. Da questi studi emerge che non soltanto sono conservate le vie di segnalazione che regolano le risposte cellulari mediate dai fattori di trascrizione NFκB, dagli insetti ai mammiferi, ma anche le strategie utilizzate dai patogeni e parassiti per interferire con l'attivazione di questi fattori nei loro ospiti. Infatti proteine simili agli inibitori cactus-IκB del TnBV sono state trovate nel genoma di altri polydnavirus, ma anche in altri virus dei vertebrati che mostrano efficienti strategie di soppressione della risposta immunitaria.

Un altro interessantissimo esempio di conservazione evolutiva di molecole implicate nei processi di sviluppo è stato presentato e discusso da Miodrag Grbic del Department of Biology, University of Western Ontario, Canada. Grbic studia le strategie e modalità di embriogenesi in alcuni parassitoidi nei quali si osserva una delle più interessanti e peculiari soluzioni adattative sviluppate da parassiti animali: la poliembrionia. La maggior parte degli studi sono stati compiuti sull'insetto parassitoide poliembrionico *Copidosoma floridanum* che parassitizza il nottuide *Trichoplusia ni*, e forma, da un singolo uovo, fino a 2000 embrioni che daranno origine ad individui differenziati in caste.

Lo sviluppo embrionale del *C. floridanum* differisce drammaticamente da quello degli altri insetti. Il *Copidosoma* depone uova piccole e senza vitello, circondate da un sottile corion. La prima divisione dell'uovo è totale e dà luogo alla formazione di due blastomeri, la seconda crea un blastomero piccolo e altri tre uguali tra di loro e di grandezza maggiore. La cellula piccola è diversa dalle altre cellule e non condivide con esse lo stesso destino cellulare. Questo si è potuto dimostrare studiando l' espressione del gene *vasa* identificato prima in *D. melanogaster* e poi in altri insetti. La proteina codificata da questo gene è una RNA elicasi e in Drosophila è implicata nella specificazione della linea germinale. Il gene *vasa* è stato isolato in *Copidosoma* e si è osservato che è trascritto nelle cellule nutrici dell'ovario. La proteina materna Vasa in *Copidosoma* si localizza in modo asimmetrico nell'uovo e durante l'embriogenesi risulta invariabilmente localizzata solo nel blastomero piccolo e successivamente nel gruppo delle cellule figlie che da esso derivano, suggerendo che queste cellule rappresentano una specifica linea cellulare, la linea germinale, da cui si formeranno le gonadi.

È quindi veramente stupefacente che la presenza o assenza di una proteina possa decidere il destino riproduttivo e, come conseguenza, un diverso destino di casta.

È molto importante che nel corso dello sviluppo controlli stringenti modulino trascrizione, traduzione e processi post-traduzionali, localizzazione asimmetrica e segregazione delle molecole, per garantire la presenza di specifici messaggeri e proteine, sia con funzione di regolazione che strutturale, nel posto giusto ed al momento giusto. Un esempio di regolazione spaziale e temporale molto preciso è rappresentato dal controllo della espressione dei geni che codificano per le proteine della Membrana Vitellina (VM), che, insieme al corion, costituisce il guscio dell'uovo di *Drosophila melanogaster*. Questo tema è stato presentato e discusso da G. Gargiulo, del Dipartimento di Biologia Evoluzionistica Sperimentale, Università di Bologna.

La formazione del guscio dell'uovo richiede la sintesi coordinata di differenti componenti proteiche nelle cellule follicolari della camera ovarica. Le proteine sono successivamente secrete nello spazio extracellulare situato tra le cellule follicolari e la superficie dell'uovo, dove possono andare incontro a tagli proteolitici e a trasporto selettivo. Infine esse si aggregano in complessi sopramolecolari insolubili attraverso la formazione di legami covalenti che coinvolgono specifici domini proteici. I geni che codificano per le varie componenti della membrana vitellina sono espressi coordinatamente verso la fine dell'oogenesi tra lo stadio 8 e lo stadio 10, con una specifica sequenza temporale correlata alle funzioni svolte dalle singole proteine.

Mentre quasi tutti i geni VM sono espressi in tutto l'epitelio follicolare, il gene chiamato *VM32E*, che è l'ultimo in ordine temporale ad essere trascritto, ha una peculiare localizzazione del messaggero che si trova solo nelle cellule follicolari colonnari laterali e mai in quelle anteriori e posteriori.

Solo dopo che il messaggero è stato tradotto e la proteina secreta, questa si muove ai poli dell'uovo ed assume una distribuzione uniforme. Quindi, per motivi ancora sconosciuti, è necessario che la proteina *VM32E* sia presente ai due poli solo alla fine del processo di sintesi delle altre proteine VM e appena prima dell'assemblaggio irreversibile del guscio. Questo peculiare profilo di espressione spaziale e temporale si ottiene attraverso l'attività di varie proteine di controllo della trascrizione, che agiscono su specifiche sequenze bersaglio, presenti nel promotore del gene *VM32E*.

Quindi la proteina *VM32E* sembra svolgere un ruolo molto peculiare non solo nella formazione e costruzione del guscio dell'uovo, ma anche nei processi di localizzazione ed intrappolamento di segnali materni che, secreti durante l'oogenesi, saranno attivati con la fertilizzazione e saranno determinanti per la formazione degli assi di simmetria dell'embrione ed il suo corretto sviluppo.

In *Drosophila* non solo l'oogenesi ma anche la spermatogenesi rappresenta un ottimo modello sperimentale che permette di dissezionare processi biologici fondamentali, primo tra tutti la regolazione del ciclo cellulare. Giuliano Callaini, del Dipartimento di Biologia Evolutiva dell'Università di Siena, si interessa da tempo dei processi di duplicazione dei centrioli e dei centrosomi nella divisione meiotica durante la spermatogenesi, particolarmente adatta allo studio dei processi di formazione del fuso e della citodieresi. La meiosi nei maschi di *D. melanogaster* manca, infatti, di «checkpoints». Questo consente alle cellule di proseguire nel ciclo cellulare anche se compaiono difetti di organizzazione del fuso, dei centrosomi o dell'allineamento dei cromosomi. Quindi si può studiare il ruolo di specifici geni regolatori attraverso l'effetto che specifiche mutazioni a loro carico producono sulla duplicazione e segregazione dei centrosomi. La funzione delle differenti componenti dei centrosomi si studia in condizioni in cui si producono fusi con centrosomi multipli o fusi monopolari. Con esperimenti di immunolocalizzazione si possono inoltre localizzare questi differenti componenti nei vari distretti del fuso e nelle varie fasi delle divisioni cellulari, avendo così informazioni utili per la loro funzione.

In conclusione, lo studio della gametogenesi in Drosophila permette di dissezionare processi biologici fondamentali quali la regolazione della espressione genica tessuto-specifica e la regolazione del ciclo cellulare, qui brevemente riassunti e approfonditamente discussi nelle riunioni accademiche 2008, ma anche il mantenimento e rinnovo delle cellule staminali, la comunicazione intercellulare tra linea germinale e linea somatica, l'acquisizione di destini cellulari diversi, la generazione ed il mantenimento della polarità negli epiteli, i meccanismi di migrazione cellulare e di conversione di cellule epiteliali in cellule migratorie invasive, l'apoptosi, il traffico nucleo-citoplasmatico, il trasporto e la localizzazione asimmetrica di RNA e proteine.

Per finire, riallacciandomi alla frase premonitrice del Bonnet del 1773 riportata all'inizio, se studiando la *D. melanogaster* si sono scoperti meccanismi di regolazione dello sviluppo così efficienti e precisi, immaginiamo quali meraviglie potranno scaturire dallo studio, ad un livello molecolare e con le sofisticate metodologie oggi disponibili, della incredibile diversità di stili di vita, modalità riproduttive, processi di sviluppo e forme degli insetti. Questo futuro è già cominciato e ogni giorno si chiariscono nuovi meccanismi e vengono prodotti tanti nuovi dati: quanto discusso nel 2008 in Accademia fornisce un interessante contributo in questo ambito di conoscenza.

Atti Accademia Nazionale
Italiana di Entomologia
Anno LVI, 2008: 37-41

FROM *D. MELANOGASTER* TO INSECT PARASITOIDS: DIFFERENT PROCESSES, CONSERVED MOLECULAR MECHANISMS

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Lettura tenuta nella Seduta pubblica dell'Accademia - Firenze, 23 febbraio 2008.

From D. melanogaster *to insect parasitoids: different processes, conserved molecular mechanisms*

Several parasitic wasps, when ovipositing in host insects, inject factors that disrupt their physiology, development and immune reaction. Among these factors, the polydnaviruses (PDVs), obligate symbionts of the parasitoids, play a pivotal role in successful parasitism. We have shown that the genome of the polydnavirus associated with *Toxoneuron nigriceps* (TnBV) codes for truncated forms of Cactus/IκB-like proteins. In *Drosophila,* Cactus regulates the nuclear import of various NF-κB/Rel proteins, which are transcription factors controlling embryonic dorso-ventral patterning and antimicrobial response. Insect immune response shares many components with the mammalian innate immunity, including the intracellular signaling pathways that activate NF-κB. The viral cactus-like proteins may act as dominant inhibitors leading to the cytoplasmic retention of NF-κB/Rel proteins, thus providing a molecular mechanism accounting for the impairment of insect immune response induced by parasitoids.

We discuss the role of Cactus/IκB molecules as an example of the reiterated use of intracellular signaling pathways conserved during evolution.

KEY WORDS: *Drosophila melanogaster*, developmental genetics, insect parasitoids, polydnaviruses, immune response.

THE *CACTUS* GENE IN *DROSOPHILA MELANOGASTER*

a) establishment of Dorsal-Ventral axis

Antero-Posterior (A/P) and Dorsal-Ventral (D/V) axis formation of the *Drosophila* embryo is established during oogenesis and requires reciprocal cell signaling between germline (oocyte and nurse cells) and somatic follicle cells in the egg chamber (SPRADLING, 1993; FULLER and SPRADLING, 2007). The first visible asymmetry during the egg chamber development is the positioning of the oocyte posteriorly in the germline cyst (Fig. 1) and this early event plays a key role in the establishment of both major body axes. The posterior localization of the oocyte relative to the nurse cells in the egg chamber allows a reciprocal cross-talk between the oocyte and the few follicle cells that contact it. The key signaling pathway is composed of the ligand Gurken (Grk), the transforming growth factor α (TGFα) homologue, present in the oocyte, and its receptor, the epidermal growth factor receptor homologue (Egfr-top), expressed by all somatic follicle cells (CLIFFORD and SCHUPBACH, 1992; ROTH *et al.,* 1995; GONZALEZ-REYES *et al.,* 1995). A large number of factors regulate or amplify the signal generated by this ligand/receptor interaction, leading the follicle cells adjacent to the oocyte to adopt a posterior fate. Signaling from the follicle cells back to the oocyte induces the reorganization of the cortical cytoskeleton and the polarization of the microtubule network whose plus ends become directed toward the posterior pole. As a consequence, *grk* mRNA, associated with the oocyte nucleus, moves towards the future dorsal-anterior corner of the oocyte, where the newly translated Grk protein acts as dorsalizing signal (Fig. 2a). At the same time, the microtubule network is also involved in the intracellular transport of a large number of RNA and protein molecules establishing the A-P axis (Fig. 2b, c).

The induction of D-V polarity in the follicular epithelium triggers differential gene activity in the dorsal and ventral sides which, in turns, shapes the egg shell, chorion and vitelline membrane, constructed by the follicle cells around the oocyte (see GARGIULO in this volume). This polarity is visibly marked by the chorion appendages, located in a dorsolateral position of the egg.

The differential expression of genes in the ventral part of the follicular epithelium also produces extraembryonic signals secreted only in the ventral regions of the perivitelline space. These signals, once activated, promote the inductive process that establishes the D-V polarity of the embryo (MOUSSIAN and ROTH, 2005).

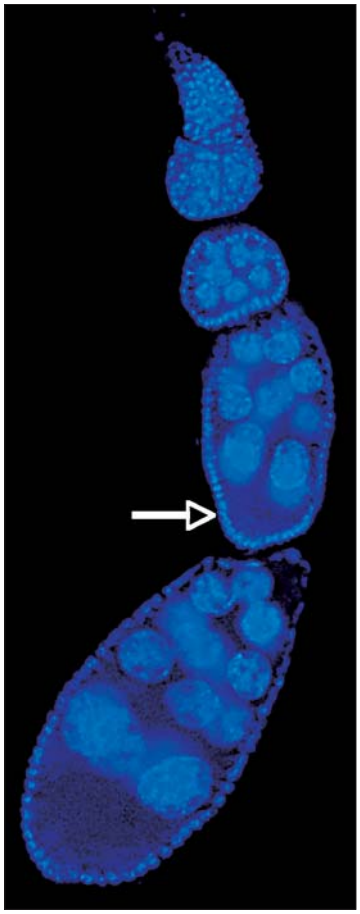


Figure 1
Wild type *D. melanogaster* ovariole stained with DAPI to mark nuclei. Egg chambers from wild type females were stained with DAPI and analyzed by conventional epifluorescence. Arrow indicates the posterior position of the oocyte in the egg chamber.

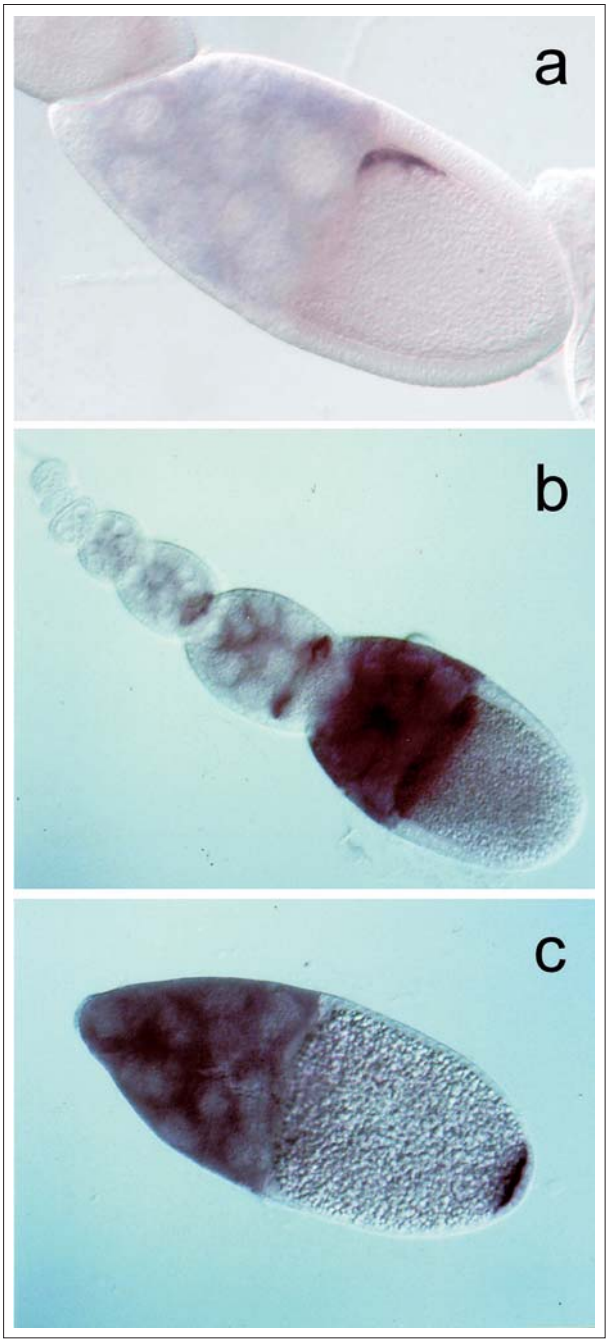


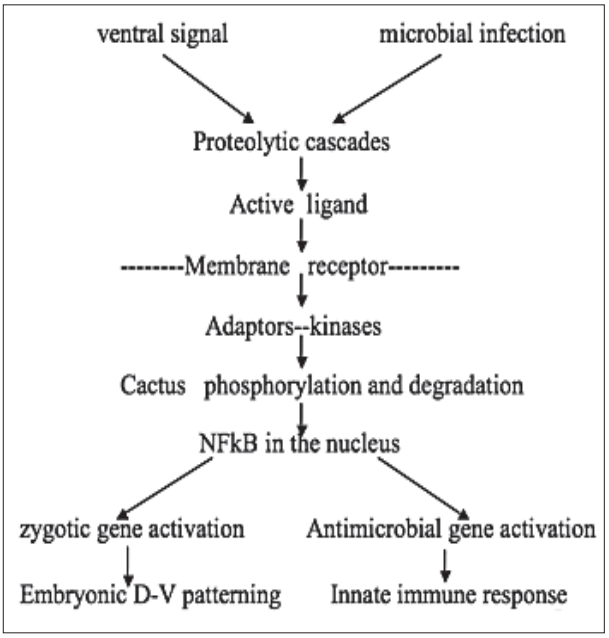
Figure 2
In situ hybridization experiments on wild type *D. melanogaster* egg chambers showing the mRNA localization of *grk* (a), linked to the oocyte nucleus at the dorsal anterior corner, *bicoid* (b), localized anteriorly, and *oskar* (c) in the posterior part of the growing oocyte.

The first step of this process consists in the ventral activation of a protease cascade culminating in the production of an active ligand for a membrane receptor called *Toll*. Ventral *Toll* activation is transduced into the embryo by adaptor proteins and kinases leading to phosphorylation and degradation of the protein Cactus (Cact), which is the inhibitor of the NF-κB transcription factor called Dorsal (Dl). (Scheme I). Cactus belongs to the family of IκB proteins, characterized by the presence of multiple ankyrin domains able to bind one or more NF-κB/rel family members (ROTH *et al*., 1991, MOUSSIAN and ROTH, 2005). Upon Cactus phosphorylation and degradation, Dorsal is released, becaming able to enter the nucleus and activate zygotic genes required for ventral cell fate specification.

b) Immune response

The NFκB signaling system has been conserved to operate on divergent genes in many different species (GHOSH *et al*., 1998; SILVERMAN and MANIATIS, 2001). In *Drosophila*, beside embryonic D-V patterning, the IκB protein Cactus, interacting with a set of NF-κB related transcription factors belonging to the Rel family, regulates multiple cellular responses, including the antimicrobial defences operated by the innate immune system (HOFFMANN and REICHHART, 2002;). The *Drosophila* genome encodes three Rel proteins, sharing a Rel-homology domain, which consists of a conserved region of 300-amino-acid (aa) that is responsible for dimerization and DNA binding.

These proteins are Dorsal, described above in D-V patterning of the embryo, Dorsal-related



Scheme 1
A simplified scheme of the NFκB pathways regulating the Dorso-Ventral embryonic patterning and the adult antimicrobial response.

Immunity Factor (DIF) and Relish (WU and ANDERSON, 1998). Of these three Rel proteins, DIF is the main transactivator of a large number of genes encoding Drosomycin and other antifungal and anti-bacterial peptides directed against Gram-positive pathogens. The Relish protein is upregulated upon bacterial infection and is mainly involved in the defensive reactions against Gram-negative bacteria leading to the transcription of genes coding for the antibacterial peptides Diptericin, Cecropin, Drosocin and Attacin. Relish is not inhibited by Cactus, but carries by itself ankyrin repeat domains located in the C terminal region (HOFFMANN, 2003).

This short summary obviously represents a huge oversimplification of the complex process of immune response in insects, which involves cross talks between the different pathways, concomitant activation of multiple pathways and multiple responses (FERRANDON *et al.,* 2007).

THE CACTUS-LIKE ANK GENES OF PARASITOIDS

In parallel with our study of *Drosophila* development, several years ago we begun a constructive collaboration with the groups directed by E. Tremblay and F. Pennacchio on the insect parasitoid *Toxoneuron nigriceps* (Viereck) (Hymenoptera, Braconidae) and its associated polydnavirus (TnBV).

Several parasitic wasps, when ovipositing in host insects, inject factors that disrupt their physiology, development and immune reaction. Among these factors, the polydnaviruses (PDVs), obligate symbionts of the parasitoids, play a pivotal role in successful parasitism. Polydnaviruses are integrated as proviruses in the genome of the parasitoids and vertically transmitted through the germline. The viral particles contain circular DNA segments of different sizes, which excise and replicate in the epithelium of the ovarian calyx and are injected in the host's body at oviposition. The viruses enter various target tissues, where they express a set of genes redirecting host physiology, thus allowing parasitoid development (for a review see PENNACCHIO and STRAND, 2006).

To contribute to the study of the molecular bases of host-parasitoid interactions in insects, the strategy we pursued was based on the sequencing of the TnBV genome and on the characterisation of the viral genes expressed in the host. We found that, among other genes (FALABELLA *et al.*, 2003, MALVA *et al.*, 2004, PROVOST *et al.*, 2004), the TnBV genome contained three genes, located in two DNA circles of 10.5 and 4.7 kb (Fig. 3), coding for proteins showing an average 30%

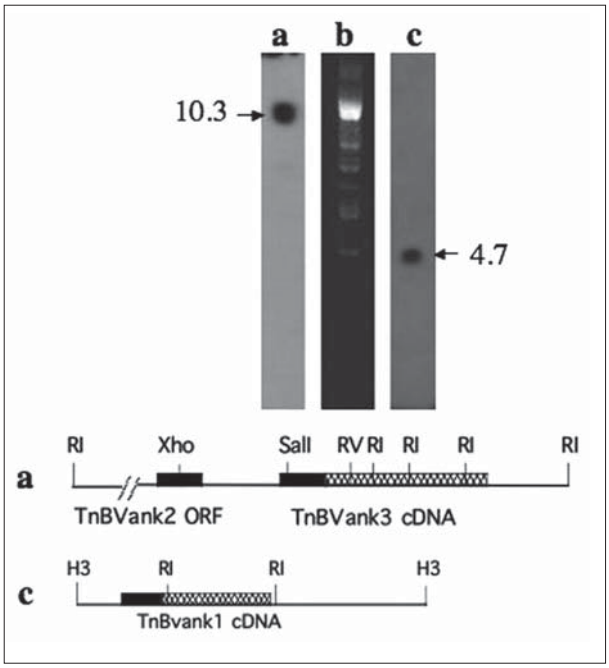


Figure 3
Mapping of *ank* genes on the *Tn*BV genome. Southern blot experiments on undigested *Tn*BV DNA using as probes *Tn*BVank3 (a) and *Tn*BVank1 (c) cDNAs. In (b) the ethidium bromide stained gel of undigested *Tn*BV DNA is shown to indicate the correspondence between the hybridization signals and the DNA circles of the *Tn*BV genome.
Below the gels are indicated the linear maps of two circles of the TnBV genome, circle 10.3 of 10,292 bp and circle 4.7 of 4,734 bp, where, by sequence analysis, the *Tn*BVank genes have been identified. Circle 10.3 contained both *Tn*BVank2 and *Tn*BVank3 genes while circle 4.7 contained *Tn*BVank2. The Southern blot experiments identified a band of 10.3 kb with the *Tn*BVank3 probe (a) and of 4.7 kb with the *Tn*BVank1 cDNA, which experimentally confirmed the sequence data.

identity with Cactus and other IκB proteins from several species. We called these genes TnBVank1, 2 and 3 (FALABELLA et al, 2007). As described above, in *Drosophila,* Cactus regulates multiple cellular responses activated by the nuclear import of various NF-κB/Rel proteins, which control embryonic D-V patterning and antimicrobial response. This central role of Cactus/IκB proteins in the regulation of key signaling pathways prompted us to characterise the structure of the viral genes and their expression pattern in parasitized hosts. In Fig. 4 the structure of the predicted TnBVank proteins is schematically reported and compared to that of *Drosophila* Cactus and mammalian IκBα and IκBβ. All viral proteins are small, ranging between 146 and 194 aa, and consist of 3 ankyrin repeats. Notably, unlike other IκB homologue proteins, the viral proteins do not contain the conserved *N*-terminal motif and the relative serine residues recognized by the IκB kinase (IKK), which triggers Cactus/IκB phosphorylation and degradation. Furthermore, none of the viral proteins contains the C-terminal PEST domain, present in the other Cactus/IκB proteins, correlated with rapid protein turnover.

The transcription profile of the three TnBV genes showed that two genes, TnBVank1 and TnBVank3, are expressed very early after parasitization in the haemocytes of *H. virescens* larvae and, at much lower level, in the thorax, which contains abundant fat body, known to be targeted by TnBV. Thus, the transcription profiles are consistent with a possible role of TnBVank genes in the suppression of the host immune response shortly after parasitization.

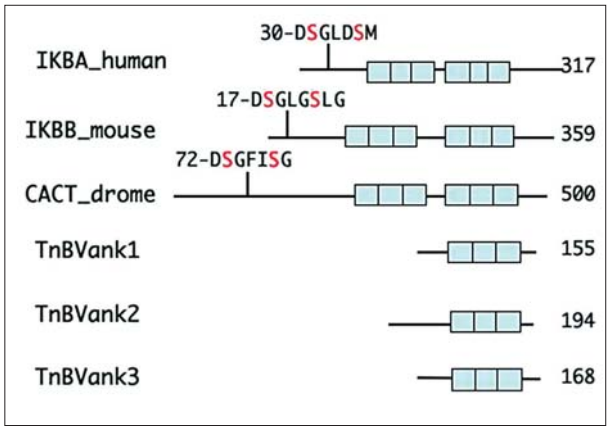


Figure 4
Structure of the putative TnBV ank proteins. Schematic representation of the proteins encoded by TnBVank genes, compared with *Drosophila* Cactus, human IκBa and mouse IκBb. The small boxes correspond to the ankyrin repeats and the short amino acid sequences reported are the IKK target region. Numbers on the right indicate the length of each protein.

The predicted sequence of the viral truncated Cactus-like proteins suggests that they might bind to NF-κB/Rel transactivators, competing with endogenous host IκB proteins. Since they cannot be phosphorylated, they would behave as dominant inhibitors, retaining the transactivators in the cytoplasm and disrupting a number of cellular pathways in parasitized hosts.

We tested this model with different approaches and we observed that NF-κB/Rel cellular pathways are disrupted in parasitized *H. virescens* larvae, thus providing a molecular mechanism accounting for the parasitoid-induced impairment of host immune defences (FALABELLA *et al*., 2007).

Given the conservation of the Cactus/IκB structure and function, we decided to test if the viral cactus-like genes act out of the parasitized host context. We used the powerful *Drosophila* transformation system that allows conditional and tissue specific expression of any gene of interest (BRAND and PERRIMON, 1993). We produced *Drosophila* transgenic lines carrying the TnBVank1 gene and intend to investigate if the expression of this viral gene in different tissues may affect *Drosophila* development and-or immune response.

In conclusion we described a mechanism for the impairment of NF-κB/Rel functions, mediated by the TnBV-encoded IκB-like proteins, which plays a central role in the cyto-pathological effects induced in the host during parasitization. This conclusion is strongly supported by the fact that similar genes have been described in other bracoviruses and also in ichnoviruses (KROEMER and WEBB, 2005). In addition, some viruses of vertebrates (REVILLA *et al*., 1998) appear to use similar strategies to manipulate the NF-κB pathways and disrupt the immune response of infected organisms.

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RIASSUNTO

DALLA DROSOPHILA MELANOGASTER *AGLI INSETTI PARASSITOIDI: PROCESSI DIVERSI, MECCANISMI MOLECOLARI CONSERVATI.*

Molti insetti parassitoidi, all'atto di depositare l'uovo nel loro insetto ospite, iniettano in esso fattori che ne alterano la fisiologia, lo sviluppo e la risposta immunitaria. Tra questi fattori i polydnavirus (PDV) sono simbionti obbligati dei parassitoidi e sono indispensabili per il successo della parassitizzazione. Noi abbiamo dimostrato che il genoma del polydnavirus associato al *Toxoneuron nigriceps* (TnBV) codifica per forme troncate di proteine appartenenti alla famiglia Cactus/IκB. In *Drosophila,* Cactus regola l'importo nucleare di varie proteine appartenenti alla famiglia NFκB/Rel, che sono fattori di trascrizione che controllano una serie di processi biologici fondamentali, tra cui lo stabilirsi dell'asse dorso-ventrale dell'embrione e la risposta antimicrobica. La risposta immunitaria degli insetti ha molte tappe in comune con la immunità innata dei mammiferi, compresa la via intracellulare di trasduzione del segnale che attiva NFκB. Le proteine virali ank, simili a proteine cactus-IκB troncate, potrebbero funzionare come inibitori dominanti che, mantenendo intrappolate nel citoplasma proteine della famiglia NF-κB/Rel dell'ospite, ne alterano la risposta immunitaria. Il ruolo delle proteine Cactus/IκB sarà discusso come un esempio dell'uso reiterato di vie di trasduzione di segnali molto efficienti e conservate nel corso dell'evoluzione.

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EVOLUTION OF POLYEMBRYONIC DEVELOPMENT IN PARASITIC WASPS

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Evolution of polyembryonic development in parasitic wasps

Major developmental innovations have been associated with adaptive radiations that have allowed particular groups of organisms to occupy empty ecospace. However, an understanding of the evolutionary forces and molecular mechanisms behind developmental novelties still remains tenuous. A little studied adaptive radiation in insects from the developmental perspective is the evolution of parasitism. The parasitic lifestyle has allowed parasitic insects to occupy a novel ecological niche where they have evolved a plethora of life history strategies and modes of embryogenesis, developing on or within the body of the host. One of the most striking adaptations to development within the body of the host includes polyembryonic development, where certain wasps form clonally up to 2000 embryos from a single egg. Taking advantage of well-established insect phylogeny, techniques developed in a model insect, the fruit fly, and a wealth of knowledge in comparative insect embryology, we are starting to tease apart the evolutionary events that have led to this novel mode of development in insects.

KEY WORDS: embryogenesis, evolution of parasitism, molecular mechanisms.

INTRODUCTION

Polyembryonic development represents the formation of multiple embryos from a single zygote. The accidental form of polyembryonic development, where an individual egg occasionally forms multiple embryos, has been described in almost all animal groups studied to date (OLSEN 1962; STANSFIE, 1968; KAUFMAN, 1982; LAALE, 1984; ASHWORT *et al.,* 1998). This accidental form of polyembryony suggests that eggs of otherwise monoembryonic species have the regulative capacity to generate multiple embryos. On the other hand, obligatory polyembryonic development, where a single zygote of certain species invariably produces multiple embryos, is a relatively rare event in metazoans, but quite frequent in plants (SHAANKER and GANESHAIAH, 1996; CARMAN, 1997). In metazoans, obligatory forms of polyembryonic development are present in both vertebrates and invertebrates. Species exhibiting polyembryonic development are scattered in multiple phyla including Cnidaria, Platyhelmintes, Arthropoda, Bryozoa, Echinodermata and Chordata (reviewed in CRAIG *et al.,* 1997). It should be noted that in certain groups, the source of clones is not the embryo but the larva, as in all described cases of polyembryony in the phila Cnidaria and Echinodermata, and in Cestodea and Trematoda (Platyhelmintes) and Crustacea (Arthropoda) (NOBLE *et al.,* 1989; SHOSTAK, 1993; GLENNER and HOEG, 1995; JAECKLE, 1994).

The focus of this review is obligatory polyembryony in insects that arises by embryonic cloning. The term *polyembryony* denotes both the developmental process, and the form of reproduction. Developmental processes include complex cellular and molecular events whereby multiple embryos form clonally from a single zygote. In addition, polyembryony refers to a unique form of reproduction in which a single egg results in multiple progeny, maximizing the reproductive capacity of the species and increasing its fitness. Along with its ecological and reproductive ramifications, study of the phenomenon of polyembryony in insects has the potential for addressing one of crucial questions in the evolution of development: How do developmental novelties arise? Polyembryony in insects represents a developmental novelty whereby both precursor structure and evolutionary processes are basically unknown (type A novelty *sensu* WILKINS, 2001). In general, true developmental novelties are rare and often their evolution is not easily tractable. However, the combination of a relatively well-established insect phylogeny, embryological studies of insect polyembryony that span more than a century (MARCHAL, 1898), and techniques and concepts established in a closely-related model Arthropod, *Drosophila melanogaster*, demonstrate a promising system that could provide clues as to how complex developmental novelties are formed.

MULTIPLE EVENTS OF INDEPENDENT EVOLUTION OF POLYEMBRYONIC DEVELOPMENT IN WASPS

Hymenoptera (wasps) represents a holometabolous insect order that consists of two suborders. Suborder Symphita includes basal plant-eating groups, and Apocrita, an advanced group of parasitic species (Figure 1). Hymenoptera posses polytrophic meroistic ovaries (BUNING, 1994) and basal groups produce yolky eggs which undergo long germband embryogenesis (SPEICHER, 1936; FLEIG and SANDER, 1986, reviewed in SANDER 1976). Apocrita (parasitic wasps plus ants and bees) represents a monophyletic assemblage which includes ectoparasitic species (laying the egg on the surface of the host), endoparasitic species (ovipositing within the body of the host), and free-living pollinators including eusocial species (WHITFIELD, 1998). Basal species in all parasitic groups whose life histories are known appear to be ectoparasitic. They lay large yolky eggs, and undergoing long germband development, such as described in the honeybee (FLEIG, 1990; BINNER and SANDER, 1997) and the endoparasitic basal braconid *Bracon hebetor* (GRBIC and STRAND, 1998). This suggests that the basal state of embryonic development in parasitic wasps includes canonical long germband development associated with meroistic polytrophic oogenesis, where critical determinants are transcribed in nurse cells and transported to the oocyte in a manner described in *Drosophila*. However, many parasitic lineages contain parasitic species that have evolved a derived form of development within the body of the host (endoparisites). This switch in life history strategy subjects them to a different selection regime compared to other terrestrial insects. The evolution of endoparasitism appears to be crucial for further evolutionary innovations, such as polyembryony. Polyembryony evolved independently four times in wasps: in Braconidae, Encyrtidae, Dryinidae and Platygasteridae (IVANOVA-KAZAS, 1972). The association of endoparasitc lifestyle with evolution of polyembryony is strengthened by the fact that the only other case of polyembryony in insects is displayed by endoparasitic Strepsiptera (NOSKIEWICZ and POLUSZYNSKI, 1935).

POLYEMBRYONIC EMBRYOGENESIS: EMBRYOLOGICAL INNOVATIONS

Independent evolution of polyembryony evokes several important questions. First, what is qualitatively novel in polyembryonic development relative to canonical insect embryogenesis? Second, which

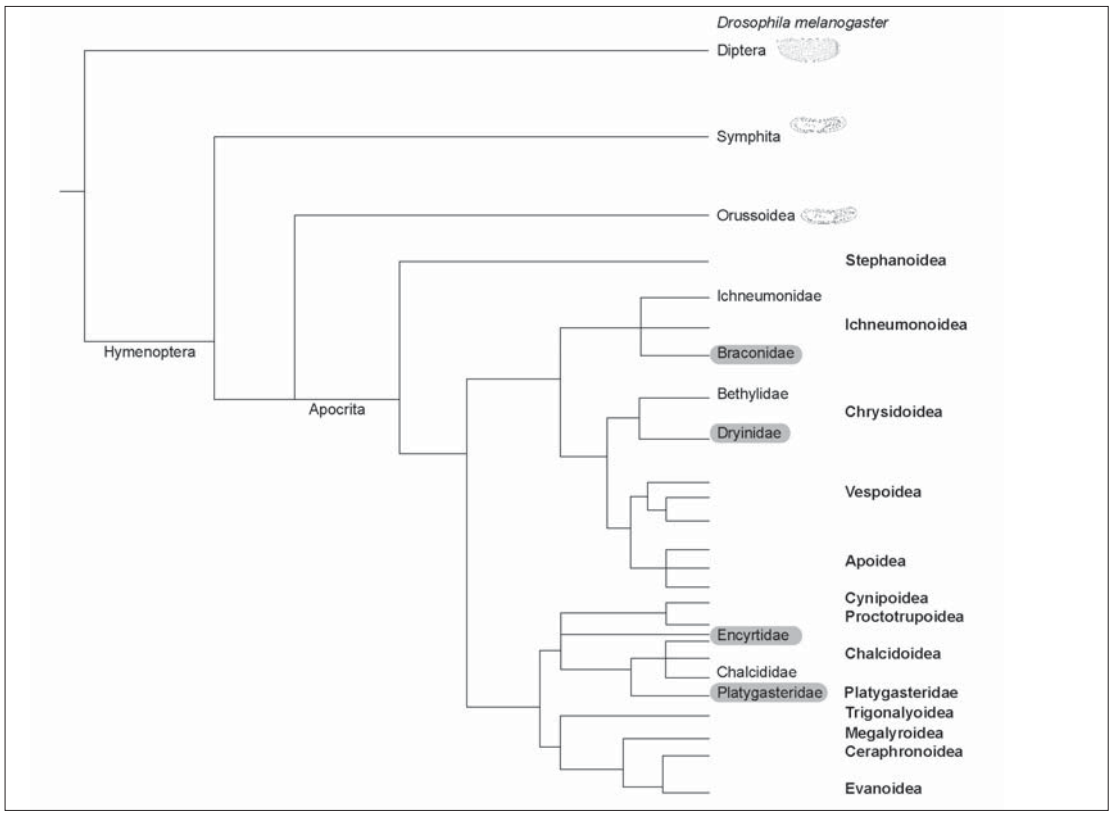

Figure 1
Phylogeny of Hymenoptera (modified from Whitfield 1998). Families that display polyembryonic development are highlighted by gray shading. Drawing of the egg illustrates long germband development of more primitive basal Hymenoptera.

elements of the regulatory mechanisms were modified to result in a novel, obligatory form of embryo cloning? Finally, understanding such independently evolved, but similar novelties could inform us about evolutionary constraints and plasticity. For example, are there multiple pathways in the evolution of certain features, or are similar evolutionary innovations based on a common program?

Thus far, our model insect for polyembryonic development has been the polyembryonic encyrtid *Copidosoma floridanum* (SILVESTRI, 1906; GRBIC *et al.,* 1996; GRBIC *et al.,* 1998, ZHUROV *et al.,* 2004). This wasp parasitizes noctuid moths and produces up to 2000 embryos from a single egg. However, a poor understanding of encyrtid phylogeny and a lack of knowledge of closest monoembryonic ancestors led us to initiate studies on another independently-evolved polyembryonic wasp, the braconid *Macrocentrus grandii*. A better understanding of the phylogeny of braconids could help us to determine the closest monoembryonic relatives, and to generate a hypothesis about transitory forms that may have led to polyembryonic development. In addition, studies of multiple forms of polyembryony could uncover common features and possible variations in polyembryonic development.

POLYEMBRYONY IN *COPIDOSOMA*: A CHALLENGE FOR THE *DROSOPHILA* PARADIGM OF DEVELOPMENT

Copidosoma floridanum is a parasitic wasp that parasitizes the eggs of the host, the moth *Trichoplusia ni* (Fig. 2). After parasitization, the host emerges and undergoes five host instars. During the process of host development *Copidosoma* undergoes embryonic development within the host body surrounded by the nutritive insect blood (heamolymph). As a result of embryonic proliferation, up to two thousand larvae are formed synchronously during the fifth host instar. These larvae pupate and emerge as adult wasps.

The embryonic development of this endoparasitic insect differs dramatically from the development of other insects. First, *Copidosoma* oviposits tiny yolkless eggs (50mm in size, a size similar to mouse eggs) that are surrounded by a thin chorion. The first cleavage of the egg is total, and leads to the formation of two posterior blastomeres (which will give rise to the embryo proper) and an anteriorly localized polyploid cell (Fig. 2 gray) that results from the fusion of polar nuclei (GRBIC *et al.,* 1998). This cell will form the polyploid syncytial extraembryonic membrane (gray). The second embryo cleavage creates one small blastomere and three equal-size blastomeres. The

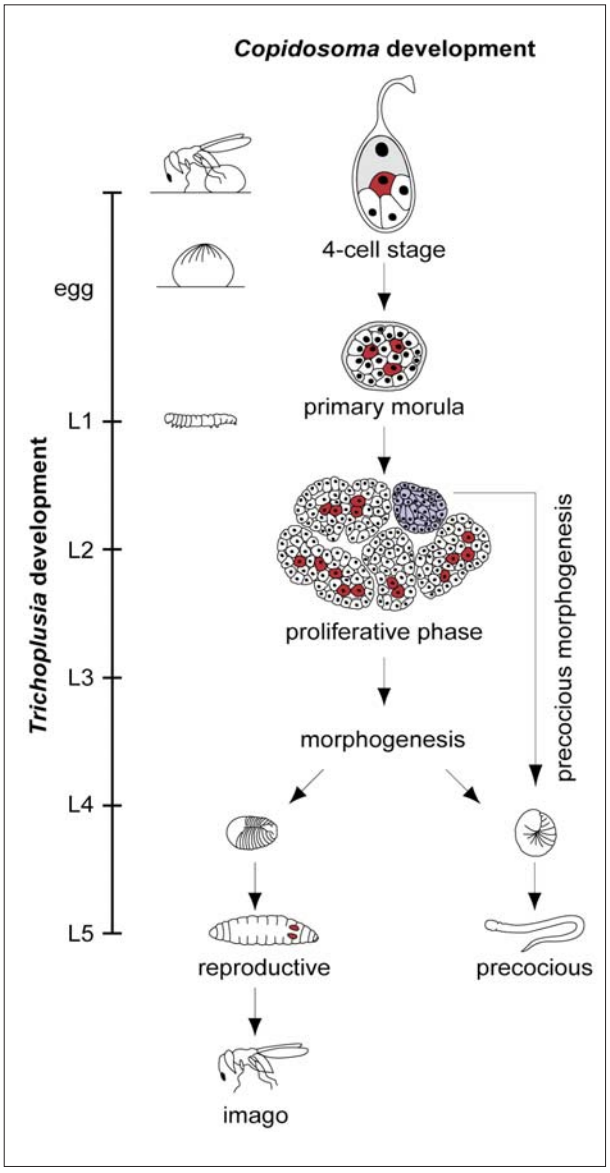

Figure 2
Embryonic development of *Copidosoma floridanum* and its host *Trichoplusia ni.* A) Embryonic development of *Copidosoma*. Polar body and polar body-derived extraembryonic membrane – grey; germ line (PGC cells) – red; proliferating morulae without PGC (progenitors of the precocious embryos) blue; L1-L5 larval instars of *T. ni.*

small cell (Fig 2 red) is different from the other cells as it retains an injected fluorescent tracer, and is thus dye-uncoupled from other cells (GRBIC *et al.,* 1996). Embryonic blastomeres then undergo cleavages and become enveloped by the syncytial extraembryonic membrane, and embryos emerge from the chorion into the host haemolymph and form primary morula. The primary morula implants in the host tissue and initiates the proliferative phase of development that increases cellular mass many fold (GRBIC *et al.,* 1998). In monoembryonic animals, developmental progression from that point in embryogenesis would include a transition from morula-stage

embryo to gastrulation and segmentation leading to a completely segmented animal. In contrast, «insertion» of the proliferative phase in the canonical monoembryonic developmental program represents the developmental novelty responsible for clonal production of thousand embryos in *Copidosoma*. The proliferative phase is initiated by the split of the primary morula and creation of the polymorula, which consists of many proliferative morulae. Each proliferative morula at this stage consists of hundreds of round, apparently non-differentiated cells (Fig. 2), surrounded by the extraembryonic membrane (GRBIC *et al.,* 1998). These packages of cells become subdivided by the ingressing extraembryonic membrane into progressively smaller clusters of cells. When the number of cells per cluster reaches about 20-30 at the fourth host instar larva, these cells undergo a change in cell shape from round to fibroblastic. The establishment of cell contacts results in cell compaction and simultaneous *de novo* formation of 2000 embryonic primordia (GRBIC *et al.,* 1998). Following compaction, each embryo undergoes gastrulation and segmentation to form larva. Thus, polyembryonic embryogenesis in *Copidosoma* shows similarities to mammalian embryonic development, including early separation of embryonic and extraembryonic lineages, morula morphology, implantation, compaction and most importantly the net increase of the embryonic mass that is unique to mammals (DAVIDSON, 1990; GURDON, 1992). These evolutionary changes apparently represent convergent evolution driven by the similar developmental environment: placental development in mammals and nutritive host environment in *Copidosoma*. Obligatory polyembryony evolved in mammals (armadillo) (FERNANDEZ, 1909) and insects (parasitic wasps), but while in mammals polyembryony is conceptually compatible with the regulative development of the mouse embryo, polyembryony in insects is in sharp contrast with maternal pre-patterning of the *Drosophila* embryo.

MATERNAL PRE-PATTERNING IN *COPIDOSOMA*: SPECIFICATION OF THE GERM-LINE

The germ line is one of first developmental fates specified in many organisms (SAFFMAN and LASKO, 1999). The RNA helicase *vasa* is the ubiquitous germ line marker in metazoans involved in the specification of the primordial germ cell (PGC) lineage. PGCs represent the first cells that give rise exclusively to germ cells by clonal mitotic divisions (NIEUWKOOP and SUTASURYA, 1979). PGCs are progenitors of germ line stem cells (GSCs) that undergo self-renewal, differentiate into gametes, and ultimately produce all of the cell types in future offspring.

Isolation of the *Copidosoma vasa* mRNA (*Cfvas*) homologue and examination of its pattern of expression showed that *Cfvas* is transcribed in nurse cells in *Copidosoma* ovaries (ZHUROV *et al.,* 2004). Vasa protein localizes in the structure called oosome (ZHUROV *et al.,* 2004), which was proposed to be homologous to the *Drosophila* germ line pole plasma (nouage). Thus, in *Copidosoma* at least one asymmetrically localized maternal determinant is deposited in the forming egg. After oviposition Vasa protein is invariably localized into the dye-uncoupled small cell, showing that this early cellular asymmetry is also paralleled by a molecular asymmetry. In the primary morula this asymmetry is perpetuated in several cells that express Vasa protein (Fig 2, red). Following the initiation of the proliferative phase Vasa-positive cells are scattered in individual proliferating morulae (Figure 2). During the process of division, the daughter cells all inherit Vasa protein, suggesting that they represent cell lineage. Following the entrance into the morphogenetic phase each reproductive embryonic primordium receives two Vasa-positive cells. These cells remain localized at the posterior and give rise to the embryonic gonads (Fig. 2). Thus, maternal cellular asymmetry marked by the expression of Vasa protein perpetuates throughout the proliferative phase and becomes continuous with the germ line, suggesting that *Copidosoma* specifies the PGC maternally.

DEVELOPMENT OF THE PRECOCIOUS EMBRYOS: DIFFERENTIAL DISTRIBUTION OF PGC SPECIFIES THE REPRODUCTIVE POTENTIAL

While it was known that the reproductive larvae give rise to adults and have a reproductive function, the reproductive potential of the precocious larvae in *Copidosoma* was uncertain. They do not molt and become consumed by their reproductive sibling. This poses the question of whether they have a reproductive potential that is simply not realized due to their premature death or they entirely lack potential for the reproduction. In social hymenoptera, workers are sterile in contrast to fertile queens. However, this sterility is often conditional. Both queens and workers have germ line progenitor cells, but in queens the reproductive apparatus become hypertrophic while in workers ovarioles degenerate (SCHMIDT CAPELLA and HARTFELDER, 2002). However, in some cases workers can restore their reproductive potential and

become reproductives (NIJHOUT, 1999). During the proliferative phase in *Copidosoma* it was noticed that some proliferative morulae do not contain Vasa-positive cells (Fig. 2 blue). These morulae undergo differentiation and give rise into the precocious embryos that do not inherit PGCs. Thus, the mechanism based on segregation of PGC lineage in reproductives, and the failure of the precocious embryos to inherit PGCs represents novel cell-sorting mechanism that specifies the caste fate. This mechanism specifies in all-or-none fashion a different reproductive capacity in genetically identical embryos.

FUNCTION OF PGCS IN *COPIDOSOMA*: CASTE FATE AND PROLIFERATION

In contrast to *Drosophila*, where PGCs undergo migration through the embryo to reach their position in future gonads (UNDERWOOD *et al.,* 1980), *Copidosoam* PGCs undergo a complex journey which includes cell parceling during the proliferative stage, differential segregation to two castes and final localization at the embryonic gonads (ZHUROV *et al.,* 2004). Clearly PGCs must be involved in the formation of the germ-line, but their complex ontogeny poses the question of whether they have other functions in polyembryonic development? Besides Vasa, these cells likely contain many other determinants that may have a role in embryo germ cell specification, proliferation or caste fate. One possibility is that these cells have a cell-autonomous function in specifying the germ-line as in *Drosophila*. This scenario predicts that the removal of PGC progenitor cell will result in formation of the reproductive larvae without gonads. Alternatively, this cell could have non-cell autonomous function(s) so that the germ line specification is coupled with other developmental processes. Laser ablation of Vasa-positive cell at the four cell stage (red Fig 1) has revealed that it has multiple functions (ZHUROV *et al.,* 2004, DONNELL *et al.,* 2004) As a consequence of ablation *Copidosoma* reproductive embryos did not proliferate as detected by lack of formation of the reproductive embryos (ZHUROV *et al.,* 2004). However, the precocious larvae development was not affected, resulting in normal numbers. Laser ablation of the Vasa-positive cell reduced 95% of polyembryonic proliferation. In contrast, ablation of any of the large blastomeres at the same stage (Fig. 2 white cells) restores the development and proliferation of reproductive embryos (ZHUROV *et al.,* 2004). This suggests that the PGC progenitor has a dual function: it regulates proliferation and the reproductive caste fate.

TRANSITORY STEPS PRECEDING POLYEMBRYONIC DEVELOPMENT

In order to address the question of evolution of the polyembryonic development it is necessary to turn to the system that preceded the evolution of polyembryonic development and to look at the development in the closest monoembryonic ancestor. The putative ancestor has to be an endoparasitic wasp. Second, it should undergo total egg cleavage. Finally, it should emerge from the chorion into the host hemocoel, and should utilize the polar body-derived cell to form the extraembryonic membrane surrounding the embryo.

The braconid endoparasite *Aphidius ervi* exhibits the predicted features of the hypothesized ancestor of polyembryonic wasps. This wasp lays tiny transparent eggs that undergoe total cleavage . Its embryo emerges from the egg shell into the host hemocoel and remains enveloped by the polar body-derived extraembryonic membrane. Following the emergence from the chorion, morphogenesis is initiated by the formation of an embryonic primordium that consists of a solid ball of cells, similar to the *Copidosoma* embryonic primordium. The embryo of *Aphidius* initially forms just the anterior structures of the embryo. The rest of the trunk is formed by sequential proliferation , exhibiting characteristic short germband development. Since basal braconids display long germband development, *Aphidius* development represents secondarily derived short germband embryogenesis.

The embryogenesis of polyembryonic parasitic braconid *Macrocentrus grandii* wasps could help us to understand how polyembryony evolved in a defined phylogenetic context of braconids. Eggs of this species is also transparent, surrounded by tiny chorion and in contrast to those of their basal, ectoparasitic relatives, contain almost no yolk. Initial cleavage events in these tiny eggs differ from the canonical type of insect syncytial cleavage. Both wasps undergo total (holoblastic) cleavage in which nuclear division is immediately followed by cytoplasmic division, forming individual cells (blastomeres). This novel type of early cleavage appears to be common also in polyembryonic platygasterids (IVANOVA-KAZAS, 1972), and its general presence in all polyembryonic species suggests that it represents a prerequisite for the evolution of polyembryonic development.

Following early cleavages, *Macrocentrus* embryos emerge from the tiny chorion into the host haemocoel and enter the proliferative phase of development. In this phase, the number of cells increases and cells become subdivided by the extraembryon-

ic membrane into several independent spatial domains. The proliferative stage in *Macrocentrus* results in a smaller rate of proliferation than in *Copidosoma* to form ultimately up to 20 embryos. Finally, initiation of the morphogenetic phase results in the formation of the embryonic primordium. In both species embryonic primordia are formed from the very beginning as cellularized structures. However, in *Copidosoma* the embryonic primordium is solid, without the blastocoel (GRBIC *et al.,* 1996), while the *Macrocentrus* primordium consists of single layer of cells that surrounds the hollow space of the blastocoel. These species also differ in the type of germband. *Copidosoma* embryogenesis was hard to classify. It more resembled long germband development by its proportional growth and expression of molecular markers (GRBIC *et al.,* 1996). On the other hand, *Macrocentrus* embryogenesis is clearly of a short germband type. The initial primordium consists of anterior structures and the remaining trunk is generated by posterior growth.

The comparison of development in two independently evolved polyembryonic species and their putative monoembryonic ancestor suggests that evolution of polyembryony is compatible with meroistic ovarial apparatus present in basal monoembryonic wasps. On the other hand, innovations that are conserved in both polyembryonic species include a novel type of cleavage, and the proliferative phase responsible for creation of multiple embryos. It appears that in both polyembryonic wasps the proliferative phase has been simply «inserted» into the monoembryonic developmental program without any consequences for the later phases of development. Even though the proliferative phase seems to be similar in specific embryological events but different in the amount of proliferation, the late morphogenetic phase displays two completely different trajectories. In *Copidosoma* three-dimensional tissue specification proceeds from the morphogenesis of a solid ball of cells, resembling the long germband type of embryogenesis (GRBIC *et al.,* 1996). In contrast, the *Macrocentrus* primordium forms a single cell layer, and extension of the embryo trunk represents a form of short germband development, as described in primitive insects. Even though short germband development is considered to be a primitive remnant of insect ancestors, its secondarly-derived development in *Macrocentrus* indicates that the evolutionary trajectory can be inverted: short germband development can evolve from a long germband ancestor.

Collectively, descriptions of embryogenesis in these wasps illustrate the surprising level of plasticity and modularity of developmental programs. First, meroistic polytrophic ovaries that synthesize determinants for syncytial cleavage and long germband development in *Drosophila* are compatible with specification of determinants for polyembryonic development. Second, innovations in the cleavage type and proliferative phase which should theoretically scramble *Drosophila* localized maternal determinants and diffusion-based action of the transcription factors are perfectly compatible with *de novo* formation of thousands of embryonic axes many days after oviposition. On the other hand, these multiple independent evolutionary events of polyembryony suggest that evolution of such a complex developmental program could have a relatively simple genetic basis that includes changes in very few genes.

SCENARIOS FOR EVOLUTION OF POLYEMBRYONY

An analysis of multiple independent events of polyembryony in wasps within the phylogenetic framework suggests that it consists of a complex and stepwise processes. The ancestral type of development in all polyembryonic lineages included an ectoparasitic life history strategy and a large yolky egg, exhibiting long germband embryogenesis. With the evolution of endoparasitism, wasp embryos gained the advantage of exploiting the nutritive environment of the host not only for larval feeding, but also for embryo development. This shift resulted in several changes in egg architecture. First, the chorion which consists of elaborate structures in ectoparasites and other terrestrial insects protecting them from dessication, decreased in its complexity once the embryo evolved emergence from the chorion into the host nutritive haemolymph. In addition, because host nutrients were utilized for embryo development it was not necessary to stockpile a large amount of yolk in the eggs. Consequently, endoparasitic egg size decreased. In smaller eggs evolution favoured a new type of cleavage: total cleavage, immediately forming individual cells.

It is unique that in many endoparasitic wasps polar nuclei do not degenerate as in other terrestrial insects (TREMBLAY and CALVERT, 1972). Instead, they participate in the formation of extraembryonic membranes that completely surround the embryo. It appears that this structure evolved many new functions in contrast to the extraembryonic membranes in terrestrial insects. In many endoparasitic wasps, at the completion of morphogenesis the extraembryonic membrane fragments into individual polyploid cells called teratocytes. In

some endoparasitic wasps teratocytes circulate in the host hemolymph and synthesize proteins which are secreted, altering host physiology in support of endoparasitic development (RANA *et al.,* 2002). However, in the polyembryonic embryogenesis of *Copidosoma,* the extraembryonic membrane is involved in the proliferative phase of development, separating proliferative cells into spatial domains. It never fragments to form the teratocytes and continues to surround both embryos and larvae. Even though endoparasitic embryos can take advantage of the host nutritive environment, they must first evolve a defense against the host immune system. Findings by CORLEY and STRAND (2003) that the extraembryonic membrane in *Copidosoma* protects the larvae from the host immune system may provide a clue as to the primary reason for the evolution of this structure. In addition, it has been proposed that the polar cell-derived extraembryonic membrane plays a role in the uptake of nutrients from the host haemolymph (KOSCIELSKI and KOSCIELSKA, 1985). Analyzing the expression pattern of genes in the proliferative phase of development, it was determined that all cells of the extraembryonic membrane in *Copidosoma* express alkaline phosphatase mRNA (TERZIN and GRBIC, unpublished). This enzyme is involved in nutrient absorption and transport mechanisms in insects and vertebrates (EGUCHI, 1995), suggesting that the extraembryonic membrane actively absorbs nutrients from the host heamolymph. Thus, the primary role of the extraembryonic membrane initially was probably to protect the emerged embryo of monoembryonic endoparasites against the host immune system, and to absorb nutrients. Later on, the existing structure was likely co-opted to the proliferative phase of embryogenesis in polyembryonic insects to organize proliferative growth.

Evolution of small egg size, total cleavage, and novel, multifunctional extraembryonic membranes were the prerequisites for the evolution of the novel proliferative stage. This stage represents the true developmental innovation (Type A) because it was derived from novel structures (the extraembryonic membrane) and a cleavage type that does not have a known precursor in ancestral, ectoparasitic, insects. It is hard to conceptualize the evolution of a novel stage that disrupts one of the crucial paradigms of *Drosophila* development, maternal specification of the embryonic axis, while at the same time creating *de novo* 2000 independent embryonic axes! If the syncytial environment of the *Drosophila* pre-blastoderm embryo has created complications in understanding how pattern formation proceeds in the cellular milleu of short and intermediate germband insects (WILKINS, 2001), then polyembryonic development represents a real challenge for the *Drosophila* paradigm. One of first prerequisites for such an event appears to be the uncoupling of posterior patterning and germ cell specification. The second step should include the initiation of the proliferation mechanisms to generate at least 40,000 cells necessary for initiation of 2000 embryonic primordia (GRBIC *at al.,* 1998). There are several relatively simple possible means how to initiate proliferation. In the monoembryonic ancestor cleavages must generate enough cells for the formation of the single embryonic primordium. At this point proliferation has to stop and become coupled with axial patterning. Thus, a simple change in the regulatory region of the mitogenic signal could extend the period of proliferation necessary for polyembryonic development. Another avenue generating the same effect would be to produce a mutation in the putative suppressor of proliferation that terminates early proliferation and regulates entry into the blastoderm stage of the monoembryonic ancestor. Both of these changes are relatively simple and could involve existing genes without requiring new gene recruitment (WILKINS, 2001). In a likewise manner, removal of the mitogenic signal by a similar mechanism at the completion of proliferation could regulate the exit from the proliferative stage.

It is hard to conceptualize how is the proliferative stage integrated with *de novo* establishment of embryonic axes. All 2000 embryo axes appear to form independently with random axial orientation relative to each other (GRBIC *et al.,* 1996). This favours an independent specification of the axial polarity within each embryo rather than a global mechanism specifying simultaneous polarity in 2000 embryos.

CONCLUDING REMARKS

Evolution of developmental novelties is a complex phenomenon that requires understanding of both the ecological processes and developmental mechanisms responsible for its creation. Analysis of the evolution of polyembryonic development within the phylogenetic context, and studies of multiple independent events of polyembryony have been important stepping stones toward beginning to understand the processes and mechanisms shaping the evolution of this novel form of development. As stated by WILKINS (2001), there is no general analytical method that can be applied to all developmental novelties. However, clues derived from a

broader phylogenetic context suggesting the polarity of change and an examination of possible ancestral states are essential in constructing testable hypotheses.

RIASSUNTO

EVOLUZIONE DELLO SVILUPPO POLIEMBRIONICO DELLE VESPE PARASSITE

La comparsa di nuovi ed innovativi meccanismi di sviluppo embrionale è stata associata a radiazioni adattative che hanno permesso a gruppi particolari di organismi di occupare ecosistemi vuoti. Tuttavia, le forze evolutive ed i meccanismi molecolari responsabili dell'origine di queste nuove modalità di sviluppo sono poco studiati o ancora del tutto sconosciuti. L'evoluzione del parassitismo negli insetti, ad esempio, è un meccanismo adattativo molto interessante ma poco approfondito nel campo della embriogenesi. Gli insetti parassitoidi, sviluppandosi su o dentro il corpo dell'ospite, hanno evoluto una pletora di strategie di vita e modalità di sviluppo embrionale. Uno dei più affascinanti adattamenti allo sviluppo che avviene all'interno del corpo dell'ospite è rappresentato dalla poliembrionia presente in alcune vespe in cui, da un singolo uovo, si formano clonalmente fino a 2000 embrioni. Traendo vantaggio dalle tecniche sviluppate in un insetto modello, la *Drosophila melanogaster*, e da una grande quantità di conoscenze sulla embriologia comparata degli insetti, stiamo cercando di affrontare gli eventi evolutivi che hanno portato a questa nuova modalità di sviluppo negli insetti.

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CONTROL OF VITELLINE MEMBRANE GENE EXPRESSION DURING *D. MELANOGASTER* OOGENESIS

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Control of vitelline membrane gene expression during D. melanogaster *oogenesis*

The formation of extracellular structures is a complex process that requires time-coordinate synthesis, cleavage and transport of various proteins, and finally, cross-linking mediated by particular functional domains. Exactly how the precise features of such biological structures are constructed remains a fascinating problem. We approach this question by studying the eggshell assembly in *Drosophila melanogaster*.

Although much is known about the induction and refinement of the signaling pathways involved in the formation of the anterior eggshell structures, little is known about the regulation and the function of the genes encoding eggshell structural proteins.

This review will summarize the knowledge on vitelline membrane gene expression, focusing on our results on the expression pattern and the regulatory elements controlling transcription of a member of this gene family, the *VM32E* gene. Compared with the other vitelline membrane genes, this gene shows a peculiar expression pattern that suggests interesting perspectives of VM32E protein function in eggshell assembly.

KEY WORDS: *Drosophila melanogaster*, eggshell, vitelline membrane genes, gene expression.

OVERVIEW OF *DROSOPHILA* EGGSHELL

The *Drosophila* eggshell is an extracellular structure functional to the different roles it absolves, from egg fertilization to the hatching of the larva at the end of embryogenesis. This multilayered extracellular matrix, that forms between the oocyte and the overlying follicle cells, is made up of the following layers (Fig.1A): a vitelline membrane, which is the innermost layer, the wax layer, the crystalline innermost chorionic layer, the tripartite endochorion (inner part, pillars, outer part), and the exochorion which is the outer layer of the eggshell (MARGARITIS *et al.,* 1980; reviewed by CAVALIERE *et al*., 2008). The formation of these extracellular structures is a complex process that requires time-coordinated synthesis, cleavage and transport of various proteins and, finally, cross-linking at specific functional domains (ANDRENACCI *et al*., 2001; MANOGARAN & WARING, 2004). The eggshell proteins are synthesized and secreted by the follicle cells surrounding the oocyte during stages 8-14 of egg chamber development (reviewed by CAVALIERE *et al*., 2008). From stages 8 to 10, the synthesis of vitelline membrane components is predominant. Subsequently, during stages 11-14, the chorion proteins are synthesized and secreted. The chorion becomes insoluble during stage 14 as a result of a peroxidase-type enzyme activity that cross-links two or three tyrosine residues of the chorion proteins (PETRI *et al*., 1976). The vitelline membrane proteins remain soluble till stage 14 and become insoluble only in laid eggs (reviewed by WARING, 2000). Like most insect eggs, several regional specializations are apparent on the surface of *Drosophila* eggshell. Its anterior end is characterized by specialized eggshell structures such as the micropyle that allows sperm entry, the operculum that forms the exit hatch for the developed embryo, and two dorsal respiratory appendages (Fig. 1B) (reviewed by CAVALIERE *et al*., 2008). This eggshell specialization depends on sequential and in some cases combined activities of the known major signaling pathways during the middle and late stages of oogenesis. The epidermal growth factor receptor (Egfr) signaling induces dorsal follicle cells fates (reviewed by RAY & SCHÜPBACH, 1996; PERRIMON & PERKINS, 1997; reviewed by DOBENS & RAFTERY, 2000), leading to the definition of two separate populations of dorsal follicle cells (WASSERMAN & FREEMAN, 1998; PERI *et al*., 1999) that will guide the production of the two dorsal appendages. The patterning of the eggshell along the AP axis also requires transforming growth factor-beta (Tgf-β) family member, Decapentaplegic

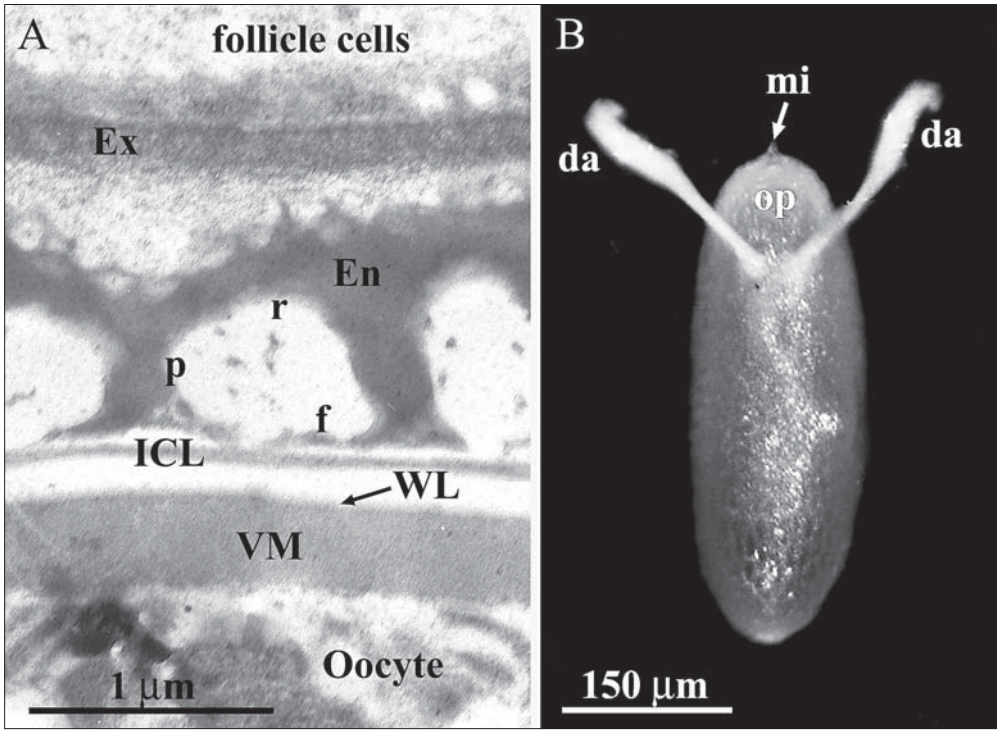

Fig. 1
Eggshell morphology. (A) Stage 14 egg chamber showing the building up of the different eggshell layers. The inner layers include the vitelline membrane (VM), the wax layer (WL), and the inner chorion layer (ICL). The outer layers include the endochorion (En), where a thin fenestrated floor (f) is separated from an outer roof (r) by vertical pillar (p) creating cavities as indicated, and the exochorion (Ex). (B) Dorsal view of *Drosophila melanogaster* egg illustrating the specialized regions of the anterior shell. The prominent structures indicated are the micropyle (mi), the operculum area (op), and the dorsal appendages (da).

(Dpp) (PADGETT *et al.*, 1987; TWOMBLY *et al.*, 1996; DENG & BOWNES, 1997; PERI & ROTH, 2000). The size and placement of the operculum and dorsal appendages are quite sensitive to altered levels of Dpp signal (TWOMBLY *et al.*, 1996).

The Ecdysone signaling pathway also appears involved on eggshell production by controlling chorion gene transcription and amplification (CHERBAS *et al.*, 2003; ORO *et al.*, 1992; HACKNEY *et al.*, 2007). Ecdysone activity is mediated by a heterodimer of two nuclear receptors, Ecdysone receptor (EcR) and Ultraspiracle (Usp) (YAO *et al.*, 1992; THOMAS *et al.*, 1993; YAO *et al.*, 1993). *EcR* encodes three protein isoforms: EcR-A, EcR-B1 and EcR-B2, which have common DNA- and ligand-binding domains but differ in their N-terminal region. The EcR/Usp heterodimer controls gene transcription by binding to specific sequences named Ecdysone Response Elements (EcREs) (RIDDIHOUGH & PELHAM, 1987; CHERBAS *et al.*, 1990).

EXPRESSION OF VITELLINE MEMBRANE GENES

Four *Drosophila* vitelline membrane protein genes have been characterized so far: *VM26A.1*, *VM34C*, *VM26A.2* and *VM32E* (HIGGINS *et al.*, 1984; MINDRINOS *et al.*, 1985; BURKE *et al.*, 1987; POPODI *et al.*, 1988; GIGLIOTTI *et al.*, 1989).

Although the vitelline membrane protein genes *VM26A.1*, *VM34C*, *VM26A.2* are expressed in all follicle cells surrounding the oocyte, from stage 8 to stage 10 of oogenesis, the *VM32E* gene is active only at stage 10 of egg chamber development (BURKE *et al.*, 1987; POPODI *et al.*, 1988; GIGLIOTTI *et al.*, 1989), and it is differently expressed in the distinct domains of the follicular epithelium (GARGIULO *et al.*, 1991). Within the main body follicle cells, the expression of *VM32E* is temporally regulated. The expression starts at stage 10A and it appears to spread from the ventral to the dorsal follicle cell domain (Fig. 2A). By stage 10B the gene is transcribed in all main-body follicle cells, but is absent in the anterior follicle cells (centripetal cells) and posterior follicle cells (Fig. 2B, arrow and arrowhead respectively). Differently, at stage 10B of egg chamber development, the other vitelline membrane genes are expressed in all follicle cells surrounding the oocyte. In Fig. 2C is shown the expression pattern of the *VM26A.2* gene, which encodes one of the most abundant vitelline membrane structural proteins (POPODI *et al.*, 1988; PASCUCCI *et al.*, 1996).

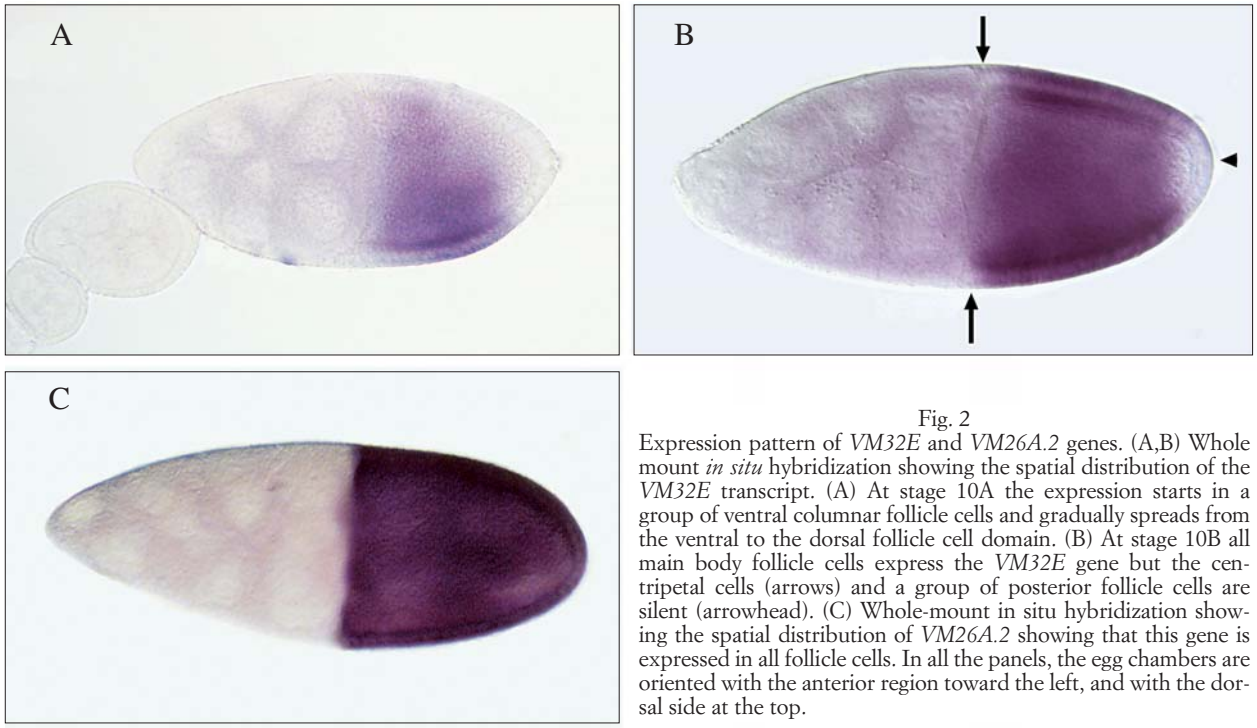

Fig. 2
Expression pattern of *VM32E* and *VM26A.2* genes. (A,B) Whole mount *in situ* hybridization showing the spatial distribution of the *VM32E* transcript. (A) At stage 10A the expression starts in a group of ventral columnar follicle cells and gradually spreads from the ventral to the dorsal follicle cell domain. (B) At stage 10B all main body follicle cells express the *VM32E* gene but the centripetal cells (arrows) and a group of posterior follicle cells are silent (arrowhead). (C) Whole-mount in situ hybridization showing the spatial distribution of *VM26A.2* showing that this gene is expressed in all follicle cells. In all the panels, the egg chambers are oriented with the anterior region toward the left, and with the dorsal side at the top.

VM32E PROTEIN SYNTHESIS AND DISTRIBUTION

In order to analyze the *in situ* distribution of the VM32E protein during egg chamber development we have raised a polyclonal antibody against the C-terminal VM32E peptide EELRGLGQGI QGQQY (anti-CVM32E), specific to this protein (ANDRENACCI *et al.*, 2000). Our analysis of the localization and assembly of the VM32E protein demonstrated several unique features of this protein that add more detail on the complex process of eggshell morphogenesis. The VM32E protein synthesis reflects the *VM32E* gene transcription pattern (Fig. 3A). At stage 10B this protein is synthesized in the columnar follicle cells, except the most anterior and posterior ones (Fig. 3C arrows and arrowhead respectively), and secreted in the extracellular space between the follicle cells and the oocyte surface. Furthermore we have found that once secreted this protein moves to the poles and by stage 11 it appears uniformly distributed in the vitelline membrane. The VM32E protein distribution was also studied at the ultrastructural level by immunogold electron microscopy on egg chamber thin sections (ANDRENACCI *et al.*, 2001). At the late stage 10B the immunogold particles strongly labeled the vitelline membrane. At stage 12 (Fig. 3B) immunogold particles were detected in the vitelline membrane and in the forming endochorion pillars. Finally, at stage 14 gold particles labeled both the vitelline membrane and the endochorion. The VM32E protein is therefore an integral component of both the vitelline and endochorion layers.

SPATIAL AND TEMPORAL REGULATION OF *VM32E* GENE EXPRESSION

The complex temporal and spatial expression pattern of *VM32E* is under the control of the Dpp and Egfr signaling pathways. The *VM32E* gene is not expressed in the centripetal migrating follicle cells, where the Decapentaplegic (Dpp) pathway is active in patterning the anterior eggshell structures. By analyzing the expression of *VM32E* gene in genetic backgrounds altering the Dpp pathway we have demostrated that in the centripetal cells the *VM32E* gene is negatively regulated by the Dpp signaling (BERNARDI *et al.*, 2006). The temporal and spatial *VM32E* gene expression pattern, in which the gene is initially active only in the ventral follicle cells, is under the control of the Egfr signaling which display a gradient of its activity along the dorsoventral axis. We have shown that in follicle cell clones expressing a constitutively active form of the Egfr the *VM32E* gene is down-regulated, while the loss of the Egfr activity up-regulates *VM32E* expression (BERNARDI *et al.*, 2007). More recently we have demonstrated that ecdysone signaling plays a crucial role in activating the expression of the *VM32E* gene in the main body follicle

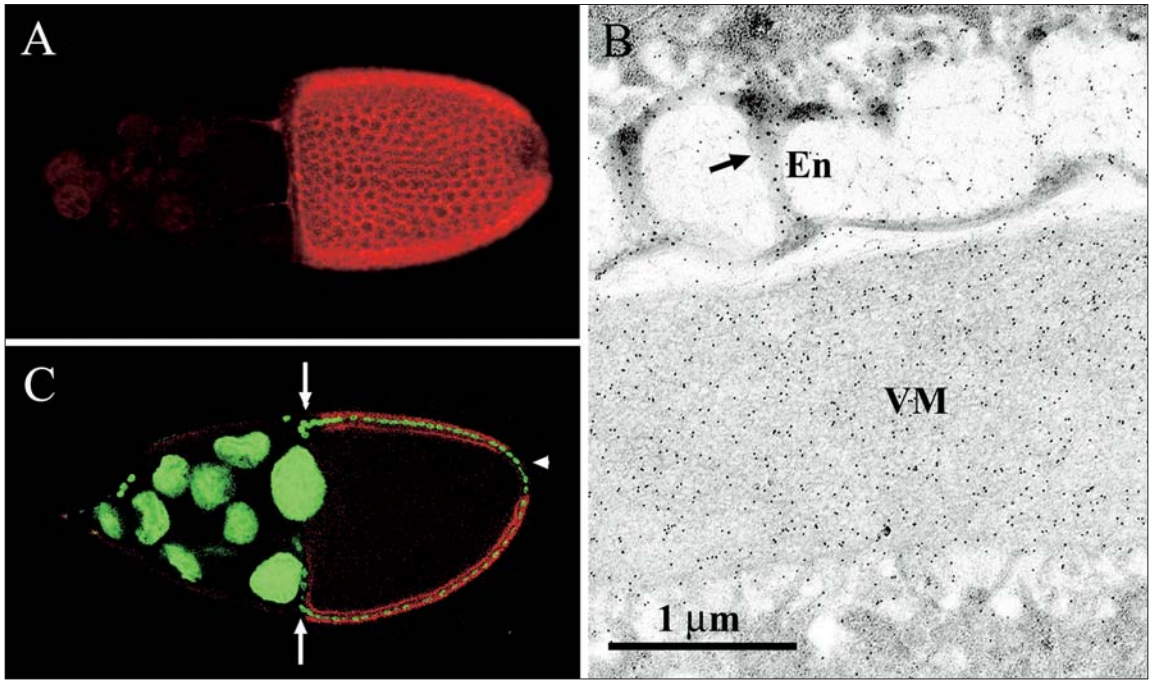

Fig. 3
VM32E distribution in egg chambers and in the forming eggshell layers. (A) Whole-mount egg chambers stained with anti-CVM32E antibody showing that the VM32E protein is not synthesized in the anterior and posterior follicle cells. (C) Confocal image showing the cross section view of a stage 10B egg chamber expressing the fused *His2avDGFP* protein (Clarkson and Saint, 1999) that marks all the nuclei (green). VM32E protein (red) is not detected in the centripetal follicle cells (arrows) and in a small group of posterior follicle cells (arrowhead). In the (A) and (C) panels, the egg chambers are oriented with the anterior region toward the left, and with the dorsal side at the top. (B) Immunoelectron microscopy detection of VM32E protein using anti-CVM32E antibody showing the multilayered distribution of VM32E protein. As shown by the localization of immunoglod particles at stage 12 the protein is localized in the vitelline membrane (VM) and in the pillars (arrow) of the forming endochorion layer (En).

cells. This control is mediated by Ecr-B1 isoform and Usp receptors (BERNARDI *et al.*, 2009).

Interestingly the Dpp, Egfr and Ecdysone signaling pathways do not appear to play a general role in controlling the expression of the other members of the vitelline membrane gene family. These findings highlight the distinguishing characteristics of *VM32E* gene among the other vitelline membrane genes.

In an effort to elucidate the cis-acting regulatory signals that dictate the developmental expression of the *VM32E* gene, we have performed an extensive analysis of its promoter. Results from germ line transformation experiments using different fragments of the *VM32E* promoter fused to the *lacZ* reporter gene have shown that the spatial and temporal expression pattern of *VM32E* is achieved by the combined activity of positive and negative controlling elements (Fig. 4A). Within the minimal *VM32E* promoter (-348/-39) we defined the *cis*-acting regulatory regions dictating *VM32E* gene expression in the different follicle domains (CAVALIERE *et al.*, 1997; ANDRENACCI *et al.*, 2000). The 3' most segment of the minimal promoter (proximal element; -112/-39) interacts with two other *cis*-regulatory regions (-253/-113 and -348/-254) and is absolutely required for their output. In transgenic flies, the -112/-39 region by itself drives the expression of the *lacZ* reporter gene only in the centripetal cells that normally do not transcribe the *VM32E* gene (Fig. 4B). When this upstream element is joined with the -253/-113 region, the *lacZ* reporter gene is expressed in the ventral follicle cells (Fig. 4B). The -253/-113 fragment besides containing the positive element(s) that specify *VM32E* gene expression in the ventral domain contains the *cis*-acting element(s) repressing the gene activity in the centripetal follicle cells through the Dpp signaling pathway (BERNARDI *et al.*, 2006). In addition, the -253/-39 region contains the response element(s) to the Egfr pathway that modulates the level of *VM32E* gene expression (BERNARDI *et al.*, 2007). Within the -348/-39 minimal promoter, the -348/-254 region appears essential to drive the dorsal expression of the gene and to enhance the ventral expression. When the -348/-254 region is joined with the -112/-39 segment, the expression is higher than the one shown by the -348/-39 promoter and involves also centripetal follicle cells, due by the absence of the -253/-113 region (ANDRENACCI *et al.*, 2000). When analyzed by itself the -348/-254 region drives expression only in the border cells, suggesting that, in these cells, native *VM32E* gene is switched off by negative

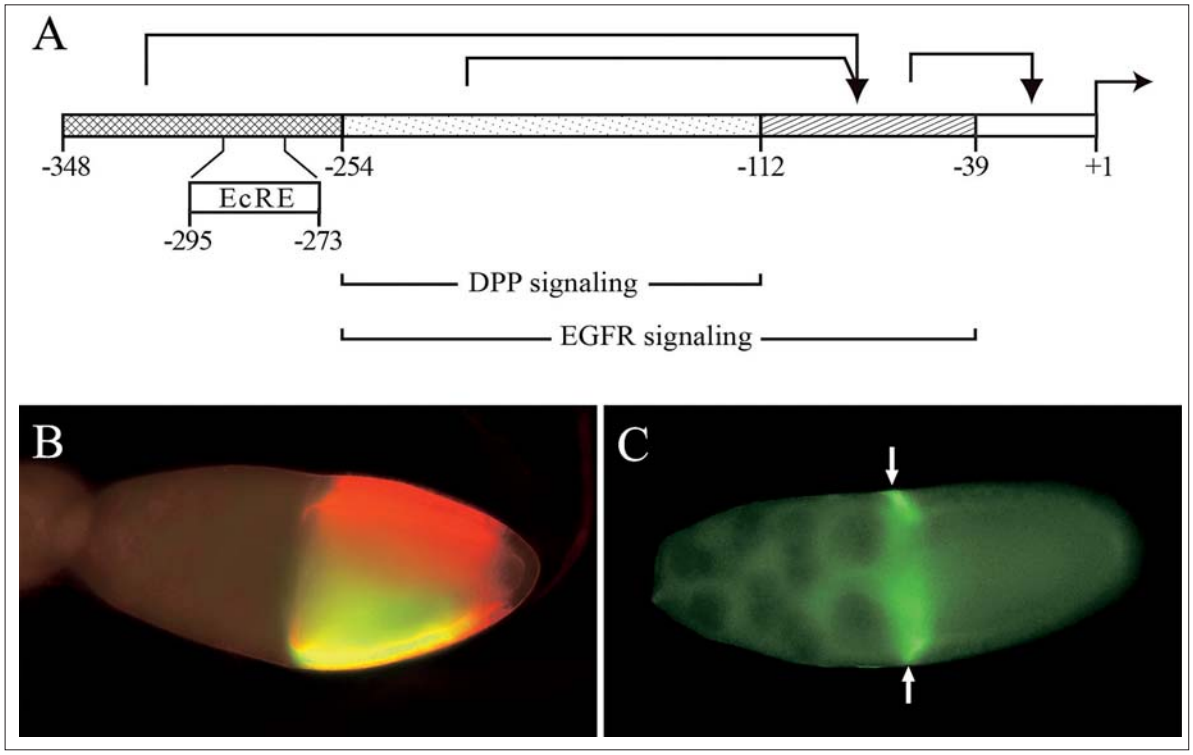

Figure 4
Schematic representation of the interactions among the *cis*-regulatory elements (patterned boxes) of the *VM32E* promoter. The -348/-254 and the -253/-113 elements interact with the -112/-39 fragment to control the *VM32E* expression (continuous arrows) in the different follicle cell populations. The -348/-254 region contains an EcRE site (-295/-273) marked by a box. *cis*-acting regions responsive to Dpp and Egfr signaling are delimited by brackets.
(B) Stage 10B egg chamber showing the VM32E protein expression pattern of the native gene (red) and the ventral *lacZ* expression pattern (green) driven by the -253/-39 *VM32E* promoter region. (C) Stage 10B egg chamber showing the *lacZ* expression (green) in the centripetal follicle cells (indicated by arrows) driven by the –112/-39 *VM32E* promoter region. In (B) and (C) panels, the egg chambers are oriented with the anterior region toward the left. In the panel (B) the dorsal side is at the top.

control element(s) which could be located within the -112/-39. Within the -348/-254 region we found a putative EcRE sequence to which the Ecr/Usp transcription complex could bind and activate gene expression (BERNARDI *et al.*, 2009).

The precise temporal and spatial control of the *VM32E* eggshell structural gene, mediated by Dpp, Egfr and Ecdysone signalings, suggests a crucial role of VM32E protein in the complex process of eggshell assembly. Many aspects of eggshell biogenesis are still to be elucidated. Besides its structural contribution to eggshell formation, it has been suggested that the vitelline membrane may perform an important function for the localization of maternal signals involved in embryonic patterning (reviewed by CAVALIERE *et al.*, 2008). The vitelline membrane proteins appear not functionally redundant suggesting that each structural component may have a specific role in the eggshell assembly (ANDRENACCI et al., 2001). The peculiar temporal and spatial regulation of *VM32E* gene activity may suggest that the VM32E protein could be involved in the process that determines the proper localization of maternal cues within the vitelline membrane.

RIASSUNTO

ESPRESSIONE GENICA DI CONTROLLO DELLA MEMBRANA VITELLINA DURANTE L'OOGENESI DI D. MELANOGASTER

La formazione di strutture extracellulari è un processo complesso e in parte ancora ignoto che ha inizio all'interno della cellula con la sintesi coordinata delle sue differenti componenti proteiche. Le proteine sono successivamente secrete nello spazio extracellulare dove possono andare incontro a tagli proteolitici e a trasporto selettivo. Infine esse sono assemblate in complessi sopramolecolari stabili attraverso la formazione di legami covalenti che coinvolgono specifici domini proteici. I meccanismi che stanno alla base della formazione di tali strutture rappresentano un quesito biologico affascinante e il guscio dell'uovo di *Drosophila melanogaster* rappresenta un ottimo sistema per indagare questi fenomeni. Numerosi studi hanno permesso di identificare il ruolo di varie vie di segnalazione nella formazione delle strutture anteriori del guscio. Meno noti sono invece i meccanismi alla base del controllo trascrizionale dei geni che codificano per le componenti della membrana vitellina, così come le funzioni svolte dalle singole proteine nell'assemblaggio del guscio.

Questa review riassume le conoscenze attuali riguardanti l'espressione dei principali geni della membrana vitellina e in particolare sono descritti i risultati da noi ottenuti sui meccanismi che regolano l'espressione del gene *VM32E*, uno dei membri di questa famiglia genica.

L'analisi di tale gene è interessante in quanto esso presenta un profilo di espressione peculiare che suggerisce interessanti prospettive svolte dalla proteina nella formazione del guscio dell'uovo.

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DROSOPHILA SPERMATOGENESIS: A SYSTEM MODEL TO CELL CYCLE ANALYSIS

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Drosophila *spermatogenesis: a system model to cell cycle analysis*

Drosophila melanogaster represents a powerful tool to examine cell division mechanisms and to decipher centriole/centrosome duplication processes. Male meiosis is particularly suitable to study the regulation of cell cycle progression and the process of spindle assembly and cytokinesis. Male meiosis lacks, indeed, working cell-cycle checkpoints. Thus, germ cells can progress through the cell cycle despite the occurrence of abnormalities in spindle assembly, chromosome alignment, centrosome structure. Overriding canonical blocks in the presence of spindle-DNA damages leads to unusual conditions in which eventual problems are enlarged, becoming more evident. *Drosophila* genetics also offers the opportunity to study the role of specific regulators in centriole duplication/assembly, such as SAK/PLK4 and SAS-6. Making transgenic flies for these genes gives useful information to understand how centriole assembly and duplication are controlled during the cell cycle. Further studies in *Drosophila* may establish common developmental and cellular pathways underlying the phenotype of several human diseases.

KEY WORD: *Drosophila*, spermatogenesis, cell cycle, centrosomes, centrolies.

ORGANIZATION OF THE *DROSOPHILA* TESTIS

Drosophila testis consists of an elongated coiled tube filled of germ cells at different developmental stages (Fig. 1), that have their origin in a small distinct apical region where somatic and stem cells form a distinct niche. In the male gonad of *Drosophila* two stem cell lines, germline (GSCs) and somatic cyst progenitor (CPCs) cells, lie at the apical tip of the testis, anchored by thin cytoplasmic extensions to a cluster of non-dividing cells, the hub cells (HARDY *et al*., 1979). Each GSCs has been proposed to be flanked in the niche by a pair of SSCs, to form a complex of three cells that are thought to divide in synchrony. The three daughter cells remaining in contact with the hub cells retain stem cell fate, while the other three cells associate into a cyst. Within these cysts, the cytoplasmic extensions of two squamous cyst cells form a thin, microtubule-rich shell that surrounds the gonioblast, that undergoes four rounds of mitotic divisions with incomplete cytokinesis.

An interesting feature of the male meiosis in *Drosophila* is the reduction of centriole number during transition from meiosis I to meiosis II. Spermatocytes I have two centrosomes containing a centriole pair each. These centrioles behave throughout each centrosome during the meiotic cell cycle as somatic cell centrioles, and each daughter spermatocyte II inherits two centrosomes containing only one centriole. However, unlikely somatic cells, the centrioles of spermatocytes II do not reproduce. So, the spermatocytes II progress trough meiosis with centrosomes containing only one centriole. This represents an interesting exception to the general rule: these cells are able to undergo meiosis and cytokinesis with non-canonical centrosomes.

Sperm formation in *Drosophila* occurs by two distinct steps: spermatogenesis and spermiogenesis. During spermatogenesis a germ line stem cell divides asymmetrically to produce a primary spermatogonium, that after four incomplete mitoses gives rise to a progeny of 16 primary spermatocytes. Spermatocytes are connected by cytoplasmic bridges and reside in a common cyst delimitated by two large and flat somatic cyst cells. After a growth period in which the spermatocytes increase several times their volume, they undergo two rounds of meiotic divisions to form cysts of 64 interconnected spermatids (FULLER 1993).
Spermiogenesis is a complex process that involves a series of elaborate transformations of the spermatid components. These dramatic transformations from an immotile cell to a motile one

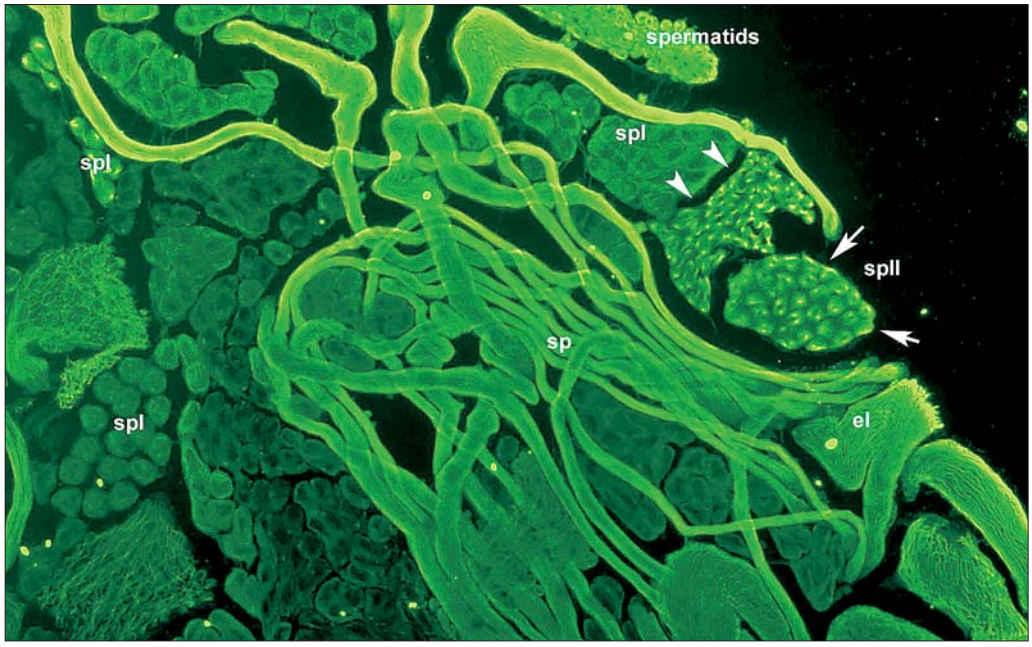

Fig. 1
Preparation of a pupal testis for immunofluorescence microscopy localization of microtubules. spI, primary spermatocytes; spII secondary spermatocytes during prophase (arrows) or telophase (arrowheads); spermatids, el, elongationg spermatids, sp, sperm.

include chromatin condensation, cell and axoneme elongation, mitochondrial rearrangement, and sperm individualization (FULLER, 1993). After elongation the syncytial spermatids must be individualized to become mature sperm. Cytoplasmic bridges are broken and the excess cytoplasmic material within the flagella is sequestered, whereas mitochondrial derivatives are remodeled. This process is mediated by 64 distinct conical complexes, or individualization complexes (ICs) (TOKUYASU *et al.*, 1972), mainly composed of actin (FABRIZIO *et al.*, 1998), that translocate along the length of the spermatid bundles from the nuclei to the tail ends.

DROSOPHILA MALE MEIOSIS

One approach to obtain insight into the mechanisms of spindle assembly during cell division is the isolation and characterization of genes involved in specific controls of this process. Meiosis in *Drosophila* males offers the opportunity for a detailed analysis of the process of spindle assembly and cell division through the combined use of genetics and cell biology. The phenotype of some *Drosophila* mutants that uncouple centrosome organization from spindle bipolarity should therefore be helpful in unraveling the contributions of centrosomes to spindle organization (GLOVER *et al.*, 1995; GONZALEZ *et al.*, 1998; ROTHWELL and SULLIVAN, 2000). Due to the relatively large size of primary and secondary spermatocytes, meiotic spindles and centrioles are very prominent and can be readily detected by staining with several antibodies (Fig. 2). Moreover, several studies demonstrated that meiotic mechanisms have been broadly conserved and that substantial similarities between meiotic regulation in flies and vertebrates exist (MAINES and WASSERMAN, 1998). Products of *Drosophila* genes such as twine (ALPHEY *et al.*, 1992), *boule* (EBERHART *et al.*, 1996) and *polo* (LLAMAZARES *et al.,* 1991) have counterparts in the vertebrate germ line. Moreover, the cytology of male meiosis has been thoroughly described in *Drosophila* (CENCI *et al.,* 1994) and many mutants that disrupt specific aspects of this process have been isolated (see CASTRILLON *et al.*, 1993). Mutations in the genes *polo* (SUNKEL and GLOVER, 1988), *aurora* (GLOVER *et al.*, 1995) and *slimb* (WOJCIK *et al.*, 2000) impair centrosome segregation or duplication leading to the formation of spindles with multiple centrosomes. Monopolar spindles organized by either unreplicated centrosomes or multiple centrosomes which have failed to segregate are common to many mitotic mutants in *Drosophila*. Mutational analysis also suggested that a component of the *Drosophila* mitotic centrosome, centrosomin (*cnn*), is required for spindle

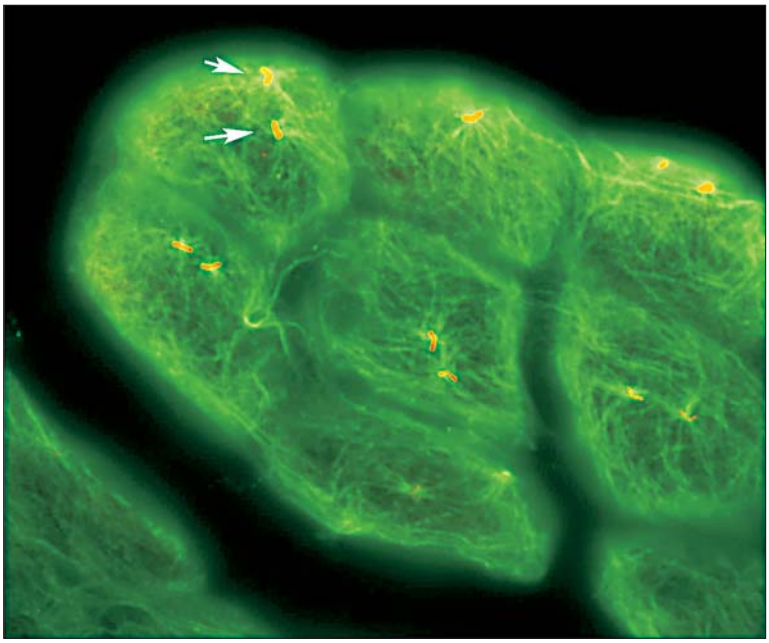

Fig. 2
Detail of a cyst of primary spermatocytes stained for tubulin (green) and γ-tubulin (red). Note that each germ cells contains two pairs of orthogonal rod-like centrioles (arrows).

organization during male meiosis. Mutations in the *abnormal spindle* locus (*asp*) result in abnormal spindle morphology leading to a metaphase arrest in third-instar larval neuroblasts. Asp localizes to the spindle poles from prophase to anaphase of the syncytial divisions and asp mitotic spindles are bipolar but have disorganized broad poles at which γ-tubulin has a highly abnormal clump-like distribution (AVIDES and GLOVER, 1999). However, despite these studies have provided insight into the function of some centrosomal components, the role of the centrosome in spindle organization and dynamics still deserves particular attention.

These observations suggest that *asp* may regulate the stability or other dynamic properties of the spindle microtubules at the centrosome (RIPARBELLI *et al.,* 2002). In late anaphase-telophase of male meiosis Asp protein localises to the spindle mid-zone, but unlike other centrosomal antigens such as Pavarotti-KLP and Polo kinase that become associated with the very central region of the spindle midzone, the majority of Asp protein decorates the very terminal regions of mid-zone microtubules, which at this stage are positioned between the telophase nuclei and the centre of the spindle. These appear to be the minus ends of microtubules that have been released from the centrosomes which at this stage nucleate independent asters of microtubules. This association of Asp with the spindle mid zone appears to be required for the correct structures of the late central spindle and in turn for cytokinesis. Cytokinesis defects are not seen in other tissues of *asp* mutants which generally cause cell division to arrest at metaphase. This could reflects either the apparent lack of a requirement for *asp* function to organize the poles of the meiotic spindle, or perhaps more likely that the spindle integrity checkpoint delays M-phase progression less effectively in male meiosis that it does in mitosis. The central spindle plays an essential role during cytokinesis and there is a cooperative interaction between this structure and the acto-myosin contractile ring: whenever one of the structures is disrupted the other fails to assemble and function. In keeping with this we have found that many cells within *asp* mutant cysts have abnormal central spindles lacking the characteristic interdigitating microtubules. Moreover, molecules that participate in forming parts of the contractile ring, Pavarotti-KLP, the septin Peanut, Polo kinase and Actin, do not localise properly in *asp* mutant spermatocytes. Interestingly, human hortologues of Asp and centrosomin (cnn) are also mutated in primary microcephaly, suggesting that further studies in *Drosophila* may establish common developmental and cellular pathways underlying the human primary microcephaly phenotype.

Centrosome and spindle assembly

An essential feature of cell division is the fidelity with which the chromosomes are segregated, because errors in chromosome inheritance can lead to severe congenital malformations or contribute to tumour progression. At the onset of each M phase, the assembly and appropriate organization of the microtubule cytoskeleton in a bipolar array is necessary to distribute to the daughter cells replicated chromosomes (during mitosis or meiosis II) or homologous chromosomes (during meiosis I). The role of the centrosome, in spindle assembly and function was suggested as far back as the late 1800s, when centrosomes were first identified.

The centrosome is the primary microtubule-organizing centre (MTOC) of eukaryotic cell and as such plays a fundamental role in the organization of the cytoplasm. During interphase, the centrosome organizes a microtubule network that is involved in fundamental cellular functions such as intracellular trafficking, cell motility, cell adhesion, and cell polarity. During cell division, two centrosomes are needed to correct organize the bipolar spindle required to accurately segregate chromosomes and play main roles in its orientation with respect to cortical cues, and the late events of cytokinesis. Since each daughter cell receives only one centrosome upon cytokinesis, the centrosome, like DNA, must duplicate once prior to next mitosis. In proliferating cells, the centrosome duplicates just before, or at, the onset of S phase. Thus, at any given time in the cell cycle, cells have either one unduplicated or two duplicated centrosomes. In addition to its function in microtubule organization, the centrosome serves as a scaffold for anchoring several regulatory proteins. Among these are cell-cycle regulators whose association with the centrosome is required for several cell-cycle transitions, including G1 to S-phase, G2 to mitosis and metaphase to anaphase.

The centrosomes exert a strong influence on the spindle architecture and dictate the number of the poles formed (Sluder and Nordberg,. 2004; Nigg, 2002). Any deviation from normal centrosome numbers can result in the formation of monopolar or multipolar spindles, with direct consequences on the proper chromosome segregation, condition often associated with aneuploidy, a hallmark of cancer cells (Nigg, 2006). Accordingly, abnormalities in the spindle pole organization function occur in many cancers and can be associated with genomic instability because extra and irregular centrosomes can give rise to aberrant cell division (Nigg, 2006).

The centriole: the master of centrosome organization

The centrosome typically consists of two components: a core structure consisting of a pair of centrioles, which serve as centrosomal organizer; and a surrounding electron-dense matrix called pericentriolar material (PCM). PCM serves as a framework to anchor microtubules and contains gamma-tubulin ring complexes, which nucleate microtubules, although other proteins also appear to be involved in this process (Gergely and Basto, 2008).

The structure of the centrioles is conserved from ancient eukaryotes to mammals. They are barrel-shaped organelles, 100-250 nm in diameter and 100-500 nm in length, with ninefold symmetric array of single, doublet or triplet microtubules. Centriolar characteristics determine most properties, such as stability, dynamicity and polarity of the centrosome (Acilan and Saunsers, 2008). Studies using antibodies to polyglutamylated tubulin to ablate centrioles confirmed that centrosome size and organization of PCM depend on centriolar integrity (Bornens, 2002). It has, also, been demonstrated how the acquisition of the pericentriolar material is mechanistically linked to centriole duplication, thus determining final centrosome size (Bobinnec *et al.*, 1998). Thus, single centrioles, and even small centrioles, can recruit the PCM, but centriole loss leads in general to PCM dispersal. The ability of centrioles to duplicate is therefore central to the reproductive capacity and function of the centrosome.

In addition, the centrioles have another distinct function as basal bodies to seed the growth of flagella and to template the assembly either of a primary cilium or of a beating cilia during ciliogenesis in specialized cells. Recent studies have demonstrated that cilia have crucial roles in an increasing number of cellular and developmental processes, establishing a link between dysfunctional cilia and several genetic diseases (for review see Delattre *et al.,* 2006; Sluder *et al.*, 1989; Davis *et al.*, 2006). For example, inability of centrioles to properly migrate prior to ciliary assembly has recently been linked to Meckel-Gruber syndrome (Dawe *et al.*, 2007a). Additionally, proper orientation of cilia via centriole positioning towards the posterior of embryonic node cells is critical for establishing left-right asymmetry during mammalian development (Eley *et al.,* 2005). However, how centrioles and basal bodies are assembled and how their numbers are controlled within cells constitute long-standing unresolved questions.

At the onset of the centrosome duplication cycle, the two tightly apposed centrioles, mother and daughter, split slightly from one another. Mother centrioles are so-called because they were assembled in a previous cell cycle to the daughter centriole. At the G1/S transition new centrioles growth orthogonally from each of the two pre-existing centrioles. The new centrioles continue to elongate during late G2 and remain strictly orthogonal (engaged) until early M phase (DAWE *et al*., 2007b). Upon entry into mitosis the two centrosomes separate from each and participate in mitotic spindle organization. Centriole pairs become disengaged at the transition from metaphase to anaphase, losing their orthogonal configuration. According to a recently proposed model (NONAKA *et al.,* 2005), the engagement of new duplicated centrioles blocks their further duplication, whereas their disengagement at the end of mitosis represents a licensing mechanism for ensuring that centrioles duplicate only once in every cell cycle. Separase, a protease involved in severing sister chromatid cohesion during mitosis and meiosis, has been recently implicated in this process (KURIYAMA and BORISY 1981). However, it remains to be seen whether the overexpression of separase induces ectopic centriole duplication. Interestingly, the overexpression of SAK/PLK-4 and SAS-6 in human tissue cultured cells and *Drosophila* embryos leads to centriole amplification (TSOU and STEARNS, 2006), also in the absence of pre-existing centrioles (HABEDANCK *et al.,* 2005; BETTENCOURT-DIAS *et al.,* 2005; PEEL *et al,* 2007), suggesting that these proteins could be a potential players in the licensing mechanism for the regulation of centriole duplication. HsSAS-6 has been, indeed, shown to be a positive regulator of procentriole formation during the centrosome duplication cycle of HeLa cells (RODRIGUES-MARTINS *et al.,* 2007a).

CENTRIOLE ASSEMBLY

The general mechanisms that orchestrate centriole assembly remain poorly understood, although substantial progress has recently been made in invertebrates. Studies in *Caenorhabditis elegans* have identified five proteins as being essential for centriole formation (BETTENCOURT-DIAS and GLOVER, 2007, for a review). In particular, the SPD-2 protein acts upstream in centriole assembly pathway by recruiting the ZYG-1 kinase to the site of daughter centriole formation. This process then leads to the recruitment of SAS-5 and SAS-6, two coiled-coil proteins needed for central tube assembly, a structure onto which singlet microtubules are subsequently assembled in a SAS-4-dependent manner (RODRIGUES-MARTINS *et al.,* 2007b). Homologs of nematode SAS-4 and SAS-6 were also required for centriole biogenesis in *Drosophila* (HABEDANCK *et al.,* 2005; PEEL *et al.,* 2007). It has also been proposed that the Polo kinase family member SAK/PLK-4, that is essential for centriole duplication in both human cells and *Drosophila* (TSOU and STEARNS, 2006) could be related to ZYG-1. Downregulation and overexpression of SAK is associated with cancer in humans, mice and flies. The regulation of SAK is thus crucial in the control of centriole number and tumorigenesis.

The characteristic orthogonal disposition of the centrioles within the centrosome has led to the suggestion that centriole duplication in proliferating cells requires the presence of a pre-existing centriole acting as "template" for the formation of a daughter. However, the findings in some biological systems, that centrioles can be formed de novo, without pre-existing template, have blurred this dogma. Studies of multi-ciliated epithelial cells (VLADAR and STEARNS, 2007), early mouse embryos (CALARCO-GILLAM *et al.,* 1983), *Chlamydomonas* (MARSHALL *et al.,* 2001) and parthenogenetic insects (RIPARBELLI and CALLAINI, 2003; RIPARBELLI *et al.,* 2005) clearly show that centrioles can be assembled de novo. Strikingly, de novo assembly can also occur in vertebrate somatic cells when the centrosomes are destroyed or removed (LA TERRA *et al.*, 2005). Moreover, the premise that each maternal centriole serves as a template for the formation of one and only one daughter centriole during each cell division cycle does not readily explain the rapid induction of aberrant centriole numbers detected under certain experimental conditions.

SAK/PLK4, an upstream regulator of canonical centriole biogenesis, can promote centrosome amplification in *Drosophila* embryos and unfertilised eggs. In embryos this is initiated on the gonomeric spindle in a process that appears to be templated from the sperm basal body. Because centrioles are eliminated from the oocyte during oogenesis, their generation in unfertilised eggs expressing SAK/PLK4 must represent their *de novo* formation. Both SAK/PLK4 promoted templated and *de novo* centrosome amplification require the activity of DSAS-6 and DSAS-4, two conserved molecules required for centriole duplication. This suggests the existence of a universal self-organizing process of centriole assembly.

Recent studies in *C. elegans* have highlighted a

group of molecules necessary for centriole assembly (BETTENCOURT-DIAS and GLOVER, 2007). ZYG-1 kinase recruits a complex of two coiled-coil proteins, SAS-6 and SAS-5, which are necessary to form the *C. elegans* centriolar tube, a scaffold in centriole formation (LEIDEL *et al.,* 2005; DELATTRE *et al.,* 2006; PELLETIER *et al.,* 2006). This complex also recruits SAS-4 which is important for the assembly of the centriolar microtubules that decorate that tube. *Drosophila* SAS-6 is necessary for centriole assembly and cohesion. Overexpression of DSAS-6 in syncitial embryos led to the *de novo* formation of multiple microtubule organizing centers (MTOCs). Strikingly, the center of these MTOCs did not contain centrioles, as described previously for SAK/PLK4 overexpression (RODRIGUES-MARTINS *et al*., 2007b). Instead, tube-like structures were present, supporting the idea that centriolar assembly starts with the formation of a tube-like scaffold, dependent on DSAS-6. In *DSAS-6* loss of function mutants, centrioles failed to close and to elongate the structure along all axes of the 9-fold symmetry suggesting modularity in centriole assembly.

Overexpression of SAK/PLK4 is sufficient to induce amplification of centrioles of normal ultrastructure in *Drosophila* embryos and their *de novo* formation in unfertilised eggs. Both these processes require the activity of DSAS-6 and DSAS-4, two regulators of canonical duplication. Centriole biogenesis is a template-free self-assembly process that is locally triggered and regulated by molecules that ordinarily associate with the existing centriole. This suggests that the role of the mother centriole is not one of a bona-fide «template» but a platform for a set of regulatory molecules, hence catalyzing and regulating daughter centriole assembly.

Centrosome amplification is normally inhibited by degradation of SAK and SAK is targeted for degradation by the SCF/Slimb ubiquitin ligase. This complex physically interacts with SAK, and in its absence, SAK levels accumulate, leading to the striking formation of multiple daughter centrioles surrounding each mother. This interaction is mediated via a conserved Slimb binding motif in SAK. Mutations in this degron impair the interaction with Slimb, stabilise SAK levels and lead to centrosome amplification (CUNHA-FERREIRA *et al.,* 2009). This regulation is likely to be conserved, because knockout or depletion of the orthologue of Slimb, ß-Trcp1 in mice, also shows centrosome amplification (GUARDAVACCARO *et al.,* 2003). Since the SCF/ß-Trcp complex plays an important role in cell cycle progression, these results lead to new understanding on the control of centrosome number and how it may go awry in human disease. *Drosophila* eggs and embryos should, therefore, provide an ideal experimental system for the further analyses of these phenomena and how it may go awry in cancer. The fact that both canonical and *de novo* centriole formation depend on SAK/PLK4, DSAS-6 and DSAS-4 suggests these as good candidates to target to control aberrant centriole formation in human disease.

RIASSUNTO

LA SPERMATOGENESI DI DROSOPHILA*: UN SISTEMA MODELLO PER LO STUDIO DEL CICLO CELLULARE*

Drosophila melanogaster rappresenta un ottimo sistema per esaminare in dettaglio i meccanismi della divisione cellulare e per capire il processo di duplicazione dei centrioli o dei centrosomi. La meiosi maschile è particolarmente adatta allo studio della regolazione del ciclo cellulare e del processo di formazione del fuso e citodieresi. La meiosi maschile manca, infatti, di checkpoints. Questo consente alle cellule di proseguire nel ciclo cellulare nonostante possano comparire difetti di organizzazione del fuso, dei centrosomi o allineamento incompleto dei cromosomi. Il superamento dei consueti blocchi in presenza di difetti nell'assemblaggio del fuso o di danni al DNA, porta ad una particolare condizione in cui i problemi sono ingigantiti, diventando così evidenti e più facilmente studiabili. La genetica di *Drosophila* offre anche l'opportunità di studiare il ruolo di specifici regolatori coinvolti nei processi di assemblaggio e duplicazione dei centrioli, come SAK/PLK4 e SAS-6. La produzione di mosche transgeniche per questi geni fornisce utili informazioni per capire come l'assemblaggio e la duplicazione dei centrioli sono regolati durante il ciclo cellulare.

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