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ORIGINAL ARTICLE

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The transmembrane protein, Tincar, is involved in the development of the compound eye in *Drosophila melanogaster*

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Abstract We previously cloned and characterized the *Drosophila* gene, *tincar* (*tinc*), which encodes a novel protein with eight putative transmembrane domains. Here, we have studied the expression pattern and functions of *tinc* during developmental processes. *tinc* mRNA is expressed in the central and peripheral nervous systems, and midgut during embryogenesis. In the third-instar larval eye disc, *tinc* mRNA is strongly expressed in all the differentiating ommatidial cells within and in the vicinity of the morphogenetic furrow. Loss-of-function analysis using the RNA-interference method revealed severe defects of eye morphogenesis during the late developmental stages. Our results suggested that *tinc* may have an indispensable role in the normal differentiation of ommatidial cells.

Keywords *tincar* · Transmembrane protein · *Drosophila melanogaster* · Ommatidial cells · Differentiation

Introduction

The *Drosophila* compound eye is a powerful experimental system for analyzing the functions of various genes during developmental processes. The adult *Drosophila* eye is composed of about 750 simple units called ommatidia. Each ommatidum contains eight photoreceptor cells (R1–R8) and 16 accessory cells (Wolff and Ready 1993). The accessory cells consist of four lens-secreting cone cells, three types of pigment cells, and bristle group cells. The compound eye develops from a monolayer epithelium, the eye imaginal disc. During the third-instar larval stage, cellular differentiation of ommatidial cells begins in the region posterior to the morphogenetic furrow (MF), an apical constriction of the disc epithelium (reviewed by Wolff and Ready 1993). The sequence of cell fate decision of these ommatidial cells is fixed (Tomlinson and Ready 1987a). Thus, numerous genes involved in cell fate decisions have been identified by taking advantage of this system (reviewed by Nagaraj et al. 2002; Voas and Rebay 2004).

After the cell fates are specified, the cellular architecture of ommatidial cells changes dynamically during the later developmental stages (reviewed by Wolff and Ready 1993; Ready 2002). The ommatidial cells undergo various characteristic shape changes. Recruited cone cells expand to cover the apical surfaces of the photoreceptor cells. Under the cone cells, photoreceptor cells organize themselves into a new arrangement that prefigures the adult trapezoid pattern. Furthermore, the primary pigment cell pairs expand to surround the cone-cell quartet. Additionally, the apical surfaces of the photoreceptor cells undergo explosive growth to form the rhabdomeres, which are columnar stacks of photosensitive membrane microvilli. Until now, few genes involved in the later stages of ommatidial development have been identified, and unknown molecules may be involved in these processes.

Tincar (Tinc) was primarily isolated as a molecule that was selectively expressed in subsets of developing cardioblasts forming the dorsal vessel, the primitive heart of *Drosophila* (Hirota et al. 2002). Tinc is a novel multipass transmembrane protein showing no homology to any known functional motifs, and is evolutionarily conserved in the nematode (*Caenorhabditis elegans*) and mosquito (*Anopheles gambiae*). In this work, to gain insight into the

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role of *tinc* during normal development, we conducted a detailed examination of the expression pattern of *tinc* mRNA in embryonic and larval tissues, and found strong expression of the mRNA in the eye discs. We performed a loss-of-function analysis of *tinc* using the RNA-interference (RNAi) technique, and also gene overexpression experiments during development of the compound eye. Our results suggest that *tinc* may be indispensable for the differentiation of ommatidial cells during the later developmental stages of the eyes.

Materials and methods

Fly cultures

Fly crosses and embryo collections were performed using standard procedures. The embryos were individually staged according to the staging system proposed by Campos-Ortega and Hartenstein (1997). Glass-binding site (*GBS*)-*GAL4* flies, which express GAL4 in all cells posterior to the morphogenetic furrow of the eye discs, similar to the Glass protein, were kindly gifted by Y. Hiromi (National Institute of Genetics, Japan). *ey-GAL4* was obtained from the Bloomington *Drosophila* Stock Center. *Canton-S* was used as the wild-type strain.

In situ hybridization, antibody staining, and scanning electron microscopy

In situ hybridization of the embryos was performed as described previously (Hirota et al. 2002). Mouse anti-ELAV (9F10) and anti-β-galactosidase (4a-10) antibodies were obtained from the Developmental Studies Hybridoma Bank. Microscopic analyses were performed on a Zeiss Axioplan2, and the images were processed using Adobe Photoshop 7.0 software. Scanning electron microscopy and semi-thin sectioning of adult eyes were performed as described previously (Tomlinson and Ready 1987b). Cobalt sulfide staining of the pupal retinas was performed as described previously (Wolff and Ready 1991a).

Reverse transcription-polymerase chain reaction

Total RNA was isolated from larval and pupal tissues using TRIzol (Invitrogen). Approximately 7 μg total RNA from each tissue was subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis using a standard protocol with pairs of gene-specific oligonucleotides for *tinc* (5′-GGTGTTGGCCTCTTCGATGG-3′ and 5′-GCCAATAGATGTCCTCCTGG-3′) and *DGPDH* (5′-CCACTGCCGAGGAGGTCAACTA-3′ and 5′-GCTCAG GGTGATTGCGTATGCA-3′).

Generation of the UAS-IR *tinc* and UAS-*tinc* transgenes

We cloned a 3.0-kb *Eco*RI-*Xho*I DNA fragment containing the complete coding sequence for *tinc* (GenBank accession number AF509933) into a pUAST vector (Brand and Perrimon 1993), to obtain UAS-*tinc*. The RNAi transgenic fly lines of *tinc* were obtained using the inducible RNAi method. A 500-bp *tinc* cDNA fragment (bp 52–551 of the coding sequence) was amplified by PCR, and inserted as an inverted repeat (IR) into a modified pUAST transformation vector, pUAST-R57 (Pili-Floury et al. 2004). pUAST-R57 possesses an IR formation site consisting of paired *Kpn*I-*Cpo*I and *Xba*I-*Sfi*I restriction sites, and carries a 282-bp genome fragment of the *DrosophilaRet* oncogene, containing introns 5 and 6 between the two IR fragments which enhances the RNAi effect (Kalidas and Smith 2002). The IR was constructed in a head-to-head orientation using a combination of tag sequences on the PCR primers and the restriction sites on the vector. The primers used were 5′-aaggcctacatggccggaccgagcctcaactccagcctctgc-3′ and 5′-aatctagaggtaccgtccaaagggctcgaagggtg-3′. Transformation of *Drosophila* embryos was carried out using standard protocols.

Cell culture and transfections

The cell culture and transfection methods have been described previously (Hirota et al. 2002). pUAST-HA-*tinc* and pWAGAL4 have also been described previously (Hirota et al. 2002).

Western blotting

Transfected S2 cells were homogenized in 1× Laemmli sample buffer. After being boiled for 5 min, the lysates were spun at 10,000×*g* for 5 min. Samples were fractionated by electrophoresis on an SDS-10% polyacrylamide gel and transferred to an Immobilon PVDF membrane (Millipore). Immunodetection was performed using an anti-HA antibody (Roche) at a dilution of 1:20, and an alkaline phosphatase-conjugated secondary antibody (Sigma) at a dilution of 1:400.

Results and discussion

Expression pattern of *tinc* during development

To determine the expression pattern of the *tinc* mRNA during the developmental process, we performed whole-mount in situ hybridization in wild-type embryos and larvae. *tinc* mRNA was first detected at stage 5 of embryonic development, in the dorsal region (Fig. 1a). At stage 6, *tinc* mRNA expression was detected in stripes (Fig. 1b), suggesting that *tinc* may be involved in the early patterning of the embryo. During gastrulation, *tinc* mRNA accumulation

was detected in the ventral region and ventral nerve cord (Fig. 1c, d, d′, e, e′). Additionally, many neurons in the external sensilla and chordotonal organ also expressed *tinc* mRNA (Fig. 1e′, f′, g). At stage 16, *tinc* mRNA was expressed on the surface of the midgut and in subsets of cardioblasts (Fig. 1f; Hirota et al. 2002). In the third-instar larval tissues, *tinc* mRNA expression was observed in the eye and antennal discs (Fig. 1h). In the antennal discs, *tinc* mRNA was expressed in the second antennal segments (A2). In the eye discs, the strongest expression of *tinc* mRNA was detected in the ocelli, and in all the cells within and in the vicinity of the MF (Fig. 1i). No *tinc* mRNA

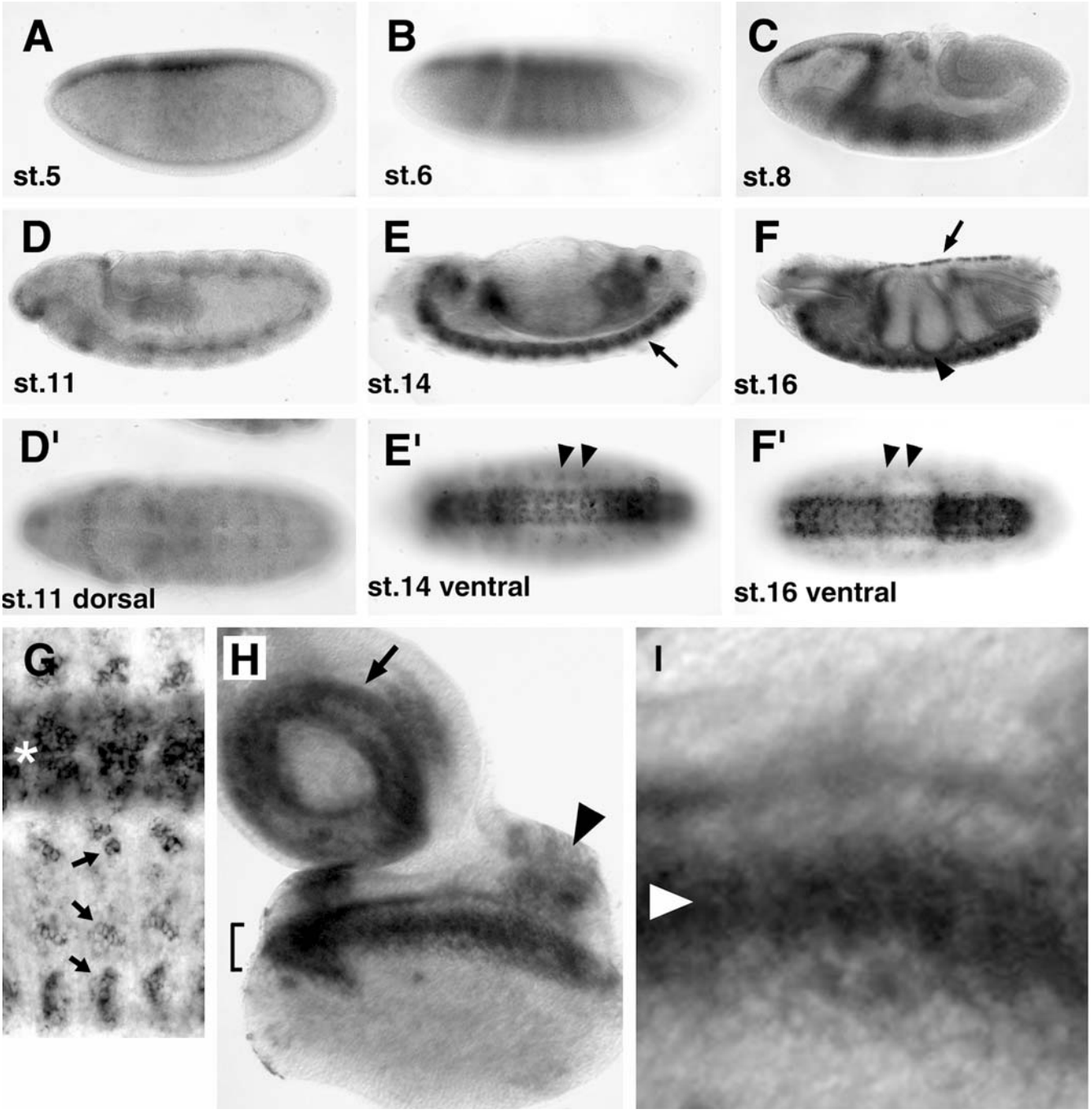

Fig. 1 Expression pattern of *tinc* mRNA during normal development. Anterior is to the *left* in **a–g**. **a–f** Lateral view of developing embryos at different stages. A different view of the embryos shown in **d–f** is presented in **d′–f′**. **a** Stage 5. **b** Stage 6. **c** Stage 8. **d, d′** Stage 11. **e, e′** Stage 14. **f, f′** Stage 16. **g** Ventral view of a stage-16 embryo at high magnification. From stage 14, *tinc* mRNA accumulation is noted in the ventral nerve cord (*arrow* in **e**, *asterisk* in **g**) and also in the neurons of the peripheral nervous system (*arrowheads* in **e′** and **f′**, *arrows* in **g**). At stage 16, *tinc* mRNA is detected in cardioblasts (*arrow* in **f**) and on the surface of the midgut (*arrowhead* in **f**). **h** *tinc* mRNA expression in a ring in the second antennal segment (*arrow*), in the ocelli (*arrowhead*), and in the differentiating ommatidial cells (*bracket*). **i** Higher magnification view of **h**. *Arrowhead* indicates morphogenetic furrow

expression was detected in other larval tissues. To reveal *tinc* expression in ommatidial cells after pupation, we examined the *tinc* mRNA level in larval eye discs and pupal eyes using semiquantitative RT–PCR. *tinc* mRNA was detected in the pupal eyes at early and middle stages of development as well as in the larval eye discs (Fig. 2). This spatiotemporally regulated expression pattern suggests that *tinc* may be closely involved in the development and/or functions of multiple organs.

Effect of altered *tinc* expression on eye development

Since the *Drosophila* compound eye is highly organized, with a regular array of ommatidia and therefore easy to analyze, we focused on the role of *tinc* during eye development. To examine whether altered expression of *tinc* might influence eye development, we performed a loss-of-function analysis using the GAL4/UAS system-mediated (Brand and Perrimon 1993) RNA-interference (RNAi) technique (Kennerdell and Carthew 1998, 2000; see Materials and methods), and also a wild-type *tinc*-gene overexpression experiment. To confirm the RNAi effects in vitro, we co-transfected S2 cells with HA-tagged *tinc* and control plasmids, with or without the inverted repeat (IR) DNA construct made from the 5′-terminal region of *tinc* (UAS-IR *tinc*). Western blotting showed that UAS-IR *tinc* efficiently down-regulated Tinc protein expression, but not the expression of the control protein (Fig. 3a), indicating that the UAS-IR *tinc* construct specifically repressed the expression of Tinc. We created transgenic UAS-IR *tinc* flies using this construct and crossed them with *GBS-GAL4*. Adult flies with both UAS-IR *tinc* and *GBS-GAL4* (see Materials and methods), which became active in all differentiating ommatidial cells posterior to the MF, had abnormal eyes with disorganized and fused ommatidia (Fig. 3c, g), in contrast to the normal eyes of the *GBS-GAL4*/+ control flies (Fig. 3b, f). Inner tangential sections showed abnormality of the photoreceptor cells, wherein almost all of the photoreceptor cells were collapsed or were split (Fig. 3k, p). To confirm that the phenotypes of UAS-IR *tinc* with *GBS-GAL4* were caused by the loss of *tinc* function, a rescue experiment was performed by introducing full-length *tinc* cDNA as a transgene; the transgene

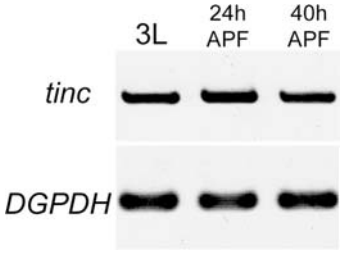

Fig. 2 RT-PCR analysis of *tinc* mRNA expression during eye development. An RT-PCR analysis was performed on mRNA prepared from the eye discs of third instar larvae (*3L*) and pupal eye discs obtained at 24 h after puparium formation (*24 h APF*) and 40 h APF (*40 h APF*) using specific primers for *tinc*. The amplification of *DGPDH* mRNA was used as an internal control

almost completely rescued the disorganization of the ommatidial array (Fig. 3d, h) and morphology of the photoreceptor cells (Fig. 3m, q), indicating that *tinc* may be essential for normal eye development. Next, we conducted an overexpression experiment in which *tinc* was overexpressed in all differentiating ommatidial cells posterior to the MF, using the *GBS-GAL4* driver. Overexpression of *tinc* also resulted in disorganized eyes with fused ommatidia (Fig. 3e, i) and collapsed photoreceptor cells (Fig. 3n, r). Taken together, these results suggest that precise control of Tinc protein expression is essential for normal morphogenesis of the eye.

Defects of differentiation of ommatidial cells in the *tinc*-RNAi animals

To investigate the role of Tinc in the developing eye in greater detail, we analyzed the larval eye discs and pupal eyes of *tinc*-RNAi animals using immunohistochemistry and cobalt sulfide staining. The cell fate of the photoreceptor cell is known to be determined at the third-instar larval stage. The eight photoreceptor cells develop in a fixed sequence: R8, R2/R5, R3/R4, R1/6, and R7 (Tomlinson and Ready 1987b). The cell type of each photoreceptor can be identified based on its position. To examine whether the differentiation of the photoreceptor cells might be affected in the *tinc*-RNAi animals, third-instar larval eye discs were stained for ELAV (Fig. 4a, b), a neuronal marker (Robinow and White 1991). The pattern of ELAV staining in the *tinc*-RNAi eye discs was indistinguishable from that in the wild-type eye discs: ommatidia in rows 4–5 posterior to the MF contained five photoreceptor cells (five-cell preclusters), composed of R8/R2/R5/R3/R4 (insets in Fig. 4a, b), and ommatidia in the eighth row posterior to the MF contained eight photoreceptor cells (eight-cell cluster), composed of R8/R2/R5/R3/R4/R1/R6/R7 (data not shown). To confirm that *tinc*-RNAi expressed during the earlier stages of eye development did not affect the differentiation of the photoreceptor cells, we performed *tinc*-RNAi experiments using an *eyeless(ey)-GAL4* driver, which drives GAL4 expression in cells anterior to the MF. The *ey-GAL4*/UAS-IR *tinc* eye discs showed a normal pattern of ELAV staining (Fig. 4c). Thus, we found that the abnormalities occurred in the later developmental stages. In the wild-type eye, 40 h after puparium formation (40 h APF), the eight ELAV-positive photoreceptor cells are arranged in a circle within an ommatidium (Fig. 5a). In contrast, in the *tinc*-RNAi eye, the patterning of the photoreceptor cells was irregular (Fig. 5b). Some ommatidia were found to be fused toadjacent ones, and the spatial patterning of the photoreceptor cells was disturbed. Ommatidia contain pigment and cone cells above the photoreceptor cells (Fig. 5d). Cobalt sulfide staining revealed that the pigment and cone cells were also arranged in a disorganized pattern in the *tinc*-RNAi eye as compared to the wild-type eye (Fig. 5e). Some cone cells and primary pigment cells showed deformed shapes (Fig. 5e). These phenotypes were almost completely

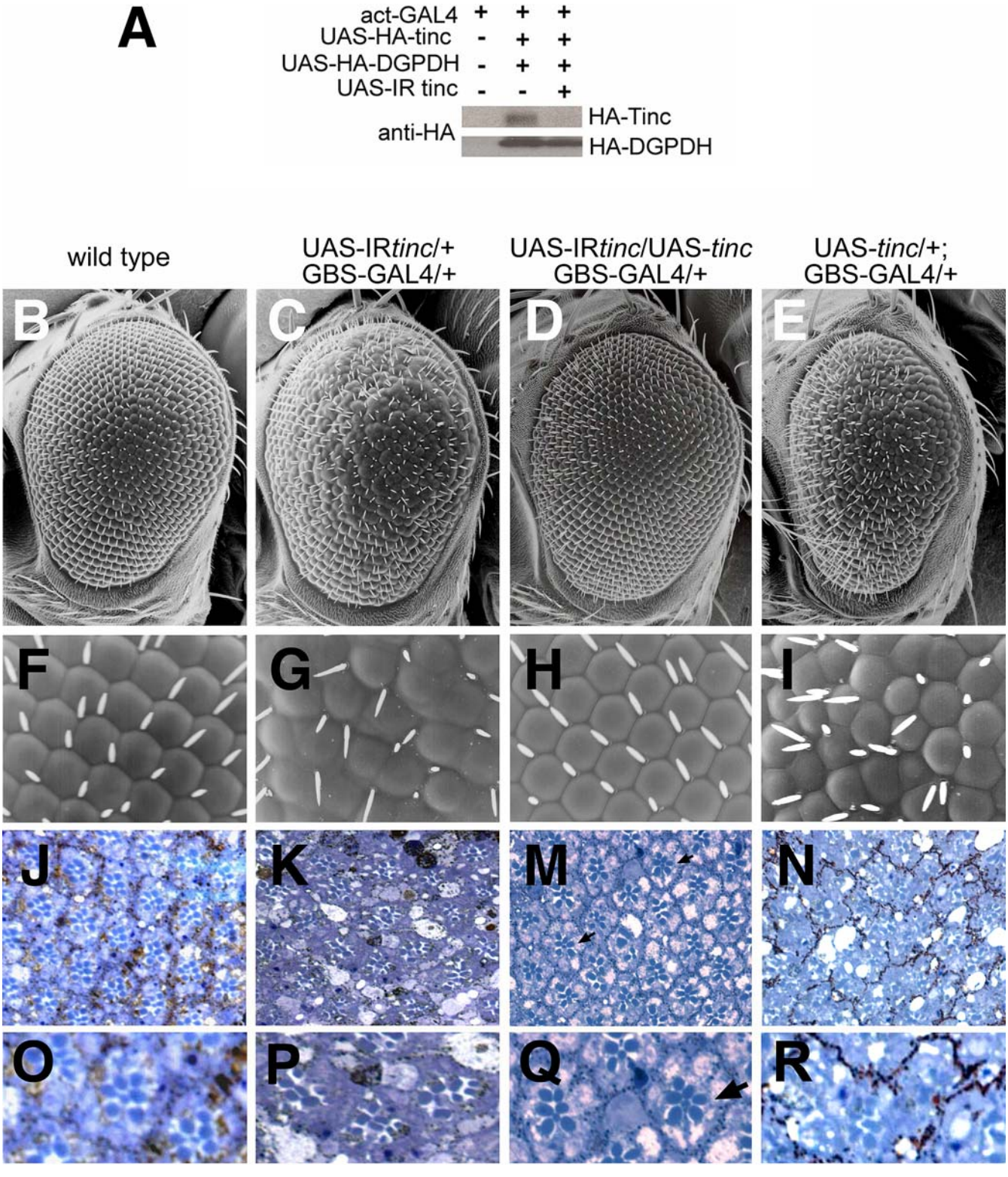

Fig. 3 *tinc*-RNAi experiments. **a** The *tinc*-RNAi DNA construct specifically downregulated expression of the Tinc protein in S2 cells. **b–n** The effects of *tinc*-RNAi and of the overexpression of *tinc* during eye development using the GBS-GAL4 driver. **b–e** Scanning electron photomicrographs of the compound eyes. **f–i** Higher magnification view of **b–e**. **j–n** Tangential eye sections of the same genotypes as those shown *below*: **b, f, j** *GBS-GAL4*/+ (control), **c, g, k** UAS-IR *tinc*/+;*GBS-GAL4*/+, **d, h, m** UAS-IR *tinc*/UAS-*tinc*; *GBS-GAL4*/+, **e, i, n** UAS-*tinc*;*GBS-GAL4*/+. **o–r** Higher magnification view of **j–n**. *Arrows* in **m** and **q** indicate rescued ommatidia, which are indistinguishable from the control

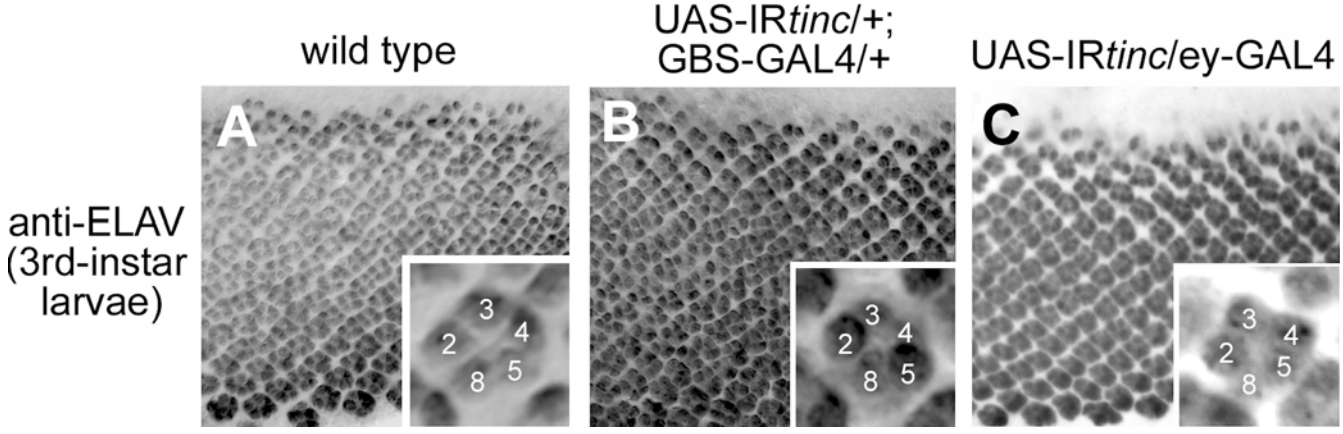

Fig. 4 Differentiation of photoreceptor cells in the *tinc*-RNAi eye discs. Eye discs from third-instar larvae stained with anti-ELAV antibodies (**a–c**). **a** *GBS-GAL4*/+, **b** UAS-IR *tinc*/+;*GBS-GAL4*/+, **c** UAS-IR *tinc*/*ey-GAL4*. *Insets* show images of a single ommatidial cluster at the five-cell precluster stage. *Numbers in insets* indicate the presumptive photoreceptor cells

rescued by the introduction of the *tinc* transgene (Fig. 5c, f). Taken together, these results suggest that *tinc* may be closely involved in the maturation of ommatidial cells during the late larval and/or early pupal stages, but not in the earlier stages of differentiation.

Which developmental processes were affected in the *tinc*-RNAi animals? Anti-Elav staining of the third-instar larvae indicated that the differentiation of the photoreceptor cells was normal at this stage (Fig. 4b). Abnormalities of ommatidial cells observed at the pupal stage (Fig. 5b, e) suggest that Tinc might have an essential role in the later developmental processes. Notably, the most striking defects at the pupal stage were ommatidial fusions (Fig. 5e) that appeared to be characterized by a lack of pigment cells. This phenotype can be explained as follows. Pigment cells are recruited from a group of undifferentiated cells formed by synchronous rounds of mitosis, referred to as the second mitotic wave, that occur immediately posterior to the MF (Wolff and Ready 1991b). Surplus pigment cells are eliminated by the programmed cell death that occurs during

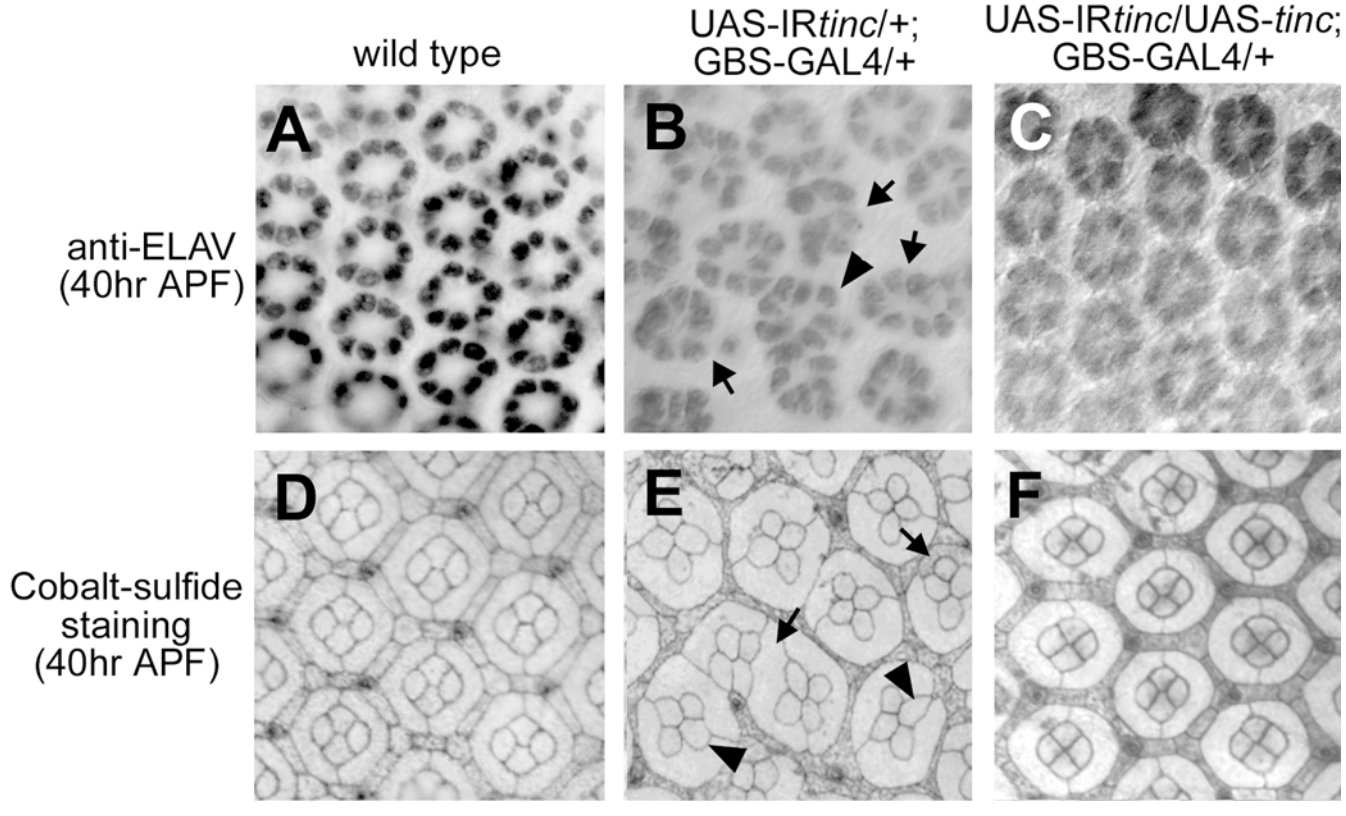

Fig. 5 Effects of *tinc*-RNAi on the differentiation of ommatidial cells. Pupal eye discs at 40 h APF (after puparium formation) were stained with anti-ELAV antibodies (**a–c**) and analyzed by cobalt sulfide staining (**d–f**). **a, d** *GBS-GAL4*/+, **b, e** UAS-IR *tinc*/+; *GBS-GAL4*/+, **c, f** UAS-IR *tinc*/UAS-*tinc*; *GBS-GAL4*/+. *Arrows* and *arrowhead* in **b** indicate the disorganized photoreceptor cells and fused ommatidia, respectively. *Arrows* and *arrowhead* in **e** indicate pigment cells and cone cells with abnormal shapes, respectively

the pupal stage (Wolff and Ready 1991a). Therefore, reduced cell division in the second mitotic wave or increased pigment cell death may occur in *tinc*-RNAi eyes. Another characteristic phenotype observed in the *tinc*-RNAi pupal eyes is the abnormal arrangement of the photoreceptor cells (Fig. 5b) and deformed accessory cells (Fig. 5e), suggesting that Tinc might be involved in the mechanisms that control the cell shape changes required for accurate arrangement of the ommatidial cells. Several mutations yielding a similar phenotype have been described previously. Elongation of the cone and pigment cells was disrupted in mutants of *myopheroid*, which encodes a subunit of integrin receptor complexes (Longley and Ready 1995), indicating that Integrin may regulate ommatidial cell shape changes through the cell-extracellular matrix contacts. The morphological changes of the ommatidial cells are also affected by mutations in the genes encoding cytoskeltal proteins, including *karst*, which encodes the membrane skeleton component, spectrin (Thomas et al. 1998), and *glued*, which encodes the cytoplasmic dynein-binding protein, dynactin (Fan and Ready 1997). Furthermore, collapsed or split rhabdomeres were observed in *tinc*-RNAi eyes (Fig. 3k, p), suggesting that Tinc might be involved in rhabdomere morphogenesis. Recently, Moesin, a member of the Ezrin-Radixin-Moesin (ERM) family of proteins, has been reported to be essential for proper organization of the membrane skeleton of the rhabdomere (Karagiosis and Ready 2004). It has been suggested that Moesin is involved in linking the microvillar cytoplasmic ends to the underlying actin cytoskelton (Karagiosis and Ready 2004). The nonmuscle myosin-II/F-actin system is assembled at the rhabdomeres and is involved in the alignment of the rhabdomeres (Baumann 2004). The phenotypic similarity among these mutants suggests that the transmembrane protein Tinc may be involved in these later processes during morphogenesis of the ommatidial cells, controlled by the extracellular matrix and cytoskeletal proteins. Genetic and biochemical experiments to study the relationships between *tinc* and the genes described above may reveal novel mechanisms regulating the later stages of ommatidial development.

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