Ectopic *scute* induces *Drosophila* ommatidia development without R8 founder photoreceptors

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During development of the *Drosophila* peripheral nervous system, different proneural genes encoding basic helix–loop–helix transcription factors are required for different sensory organs to form. *atonal* (*ato*) is the proneural gene required for chordotonal organs and R8 photoreceptors, whereas the *achaete-scute* complex contains proneural genes for external sensory organs such as the macrochaetae, large sensory bristles. Whereas ectopic *ato* expression induces chordotonal organ formation, ectopic *scute* expression produces external sensory organs but not chordotonal organs in the wing. Proneural genes thus appear to specify the sensory organ type. In the ommatidium, or unit eye, R8 is the first photoreceptor to form and appears to recruit other photoreceptors and support cells. In the *atonal*^{1} (*ato*^{1}) mutant, R8 photoreceptors fail to form, thereby resulting in the complete absence of ommatidia. To our surprise, we found that ectopic *scute* expression in the *ato*^{1} mutant induces the formation of ommatidia, which occasionally sprout ectopic macrochaetae. Remarkably, many *scute*-induced ommatidia lack R8 although they contain outer photoreceptors.

Neural precursors for sensory organs in *Drosophila* are specified by proneural genes, which are first expressed in clusters of equipotent cells, the proneural clusters, and then restricted to the cells singled out from these proneural clusters to become neural precursors (1–7). Different proneural genes, which code for transcriptional regulators with the basic helix–loop–helix motif, are required for the formation of neural precursors for different sensory organs. For example, the proneural gene *atonal* (*ato*) is required for the formation of photoreceptors (4) and chordotonal organs, which are stretch receptors or auditory sensors (3). The proneural genes in the *achaete-scute* complex, on the other hand, are necessary for the formation of external sensory organs, bristles that are sensitive to physical or chemical stimuli (2).

The proneural genes appear to be capable of specifying the type of neural precursor they induce, even though their expression in the proneural clusters depends on positional information, which in principle may specify the type of neural precursors that emerge from these proneural clusters. For example, ectopic expression of *scute* results in the formation of external sensory organs exclusively (3, 8, 10) and cannot rescue the chordotonal organ defect in the *ato*^{1} mutant (8). In the central nervous system, ectopic expression of *achaete* or *scute*, but not *ato*, can restore MP2 precursors of mutants lacking these precursors (11, 12). Only *ato* is capable of promoting ectopic chordotonal organ formation (3, 8, 9); its ectopic expression can even convert external sensory organs into chordotonal organs (9). Like chordotonal organs, photoreceptors normally depend on *ato* for their formation (4). It is thus of interest to determine whether photoreceptors can be induced only by ectopic expression of *ato*.

Drosophila eye morphogenesis begins at the posterior tip of the eye imaginal disk in early third instar larvae (13, 14). The morphogenetic furrow (MF) moves anteriorly during development. Within and posterior to the MF, regular arrays of ommatidia, the unit eyes of the compound eye, are assembled following a precise spatial and temporal order (14). Of the eight photoreceptors (R1–R8) in each ommatidium, R8 is the "founder photoreceptor" that forms first, and its formation depends on the expression of *ato* (4). Other photoreceptors are recruited by R8 sequentially, starting with R2 and R5, followed by R3 and R4, then R1 and R6, and finally R7; formation of the eight photoreceptors precedes the appearance of cone cells (14). Thus, the R1–R7 photoreceptors appear to acquire their cell fates by cell–cell communication processes initiated by R8 (13–16). Mosaic analysis demonstrated that the formation of R8 but not R1–R7 requires *ato* directly. The fact that R1–R7 and R8 all are missing in *ato* loss-of function mutants supports the notion that R8 is the founder photoreceptor required for the subsequent development of R1–R7 (4).

To form ommatidia (4, 17, 18), as in the formation of other sensory organs (1, 2, 6, 7), early expression of a proneural gene in a proneural cluster endows cells in the cluster with the potential to form neural precursor. Subsequent lateral inhibition mediated by cell–cell interaction singles out one cell in the proneural cluster to become neural precursor and maintain proneural gene expression (1, 2). Early expression of the proneural gene *ato* in the developing eye is controlled by two separate regulatory regions of the *ato* gene. Expression of *ato* is first detected in a continuous stripe of cells in the eye disk, and then is quickly resolved into regularly spaced initial clusters of cells (4, 17, 18). This early expression, immediately anterior to (in front of) the MF, is driven by the 3′F:5.8 enhancer region, located 3′ to the *ato* coding sequences (18). Within and posterior to (behind) the MF, *ato* expression is further restricted into intermediate groups and finally into evenly spaced R8 photoreceptors; this later expression pattern requires the 5′ eye enhancer region, located 5′ to the *ato* coding sequences (18). To test whether ectopic expression of *scute* can induce photoreceptors, we used either or both of these two regulatory regions of the *ato* gene to drive *scute* expression. We also used the GAL4-UAS system (19) for ectopic *scute* expression in the eye disk. Unexpectedly, we found that ectopic expression of *scute* induced the formation of photoreceptors in apparently R8-independent manner. This finding suggests the existence of a yet unidentified basic helix–loop–helix gene(s) involved in the formation of R1–R7.

Materials and Methods

DNA Constructs. The *scute* coding sequence was obtained by PCR using plasmid pUC19-*sc* (a gift from Cheng-ting Chien, Aca-

Abbreviations: *ato*, *atonal*; MF, morphogenetic furrow.

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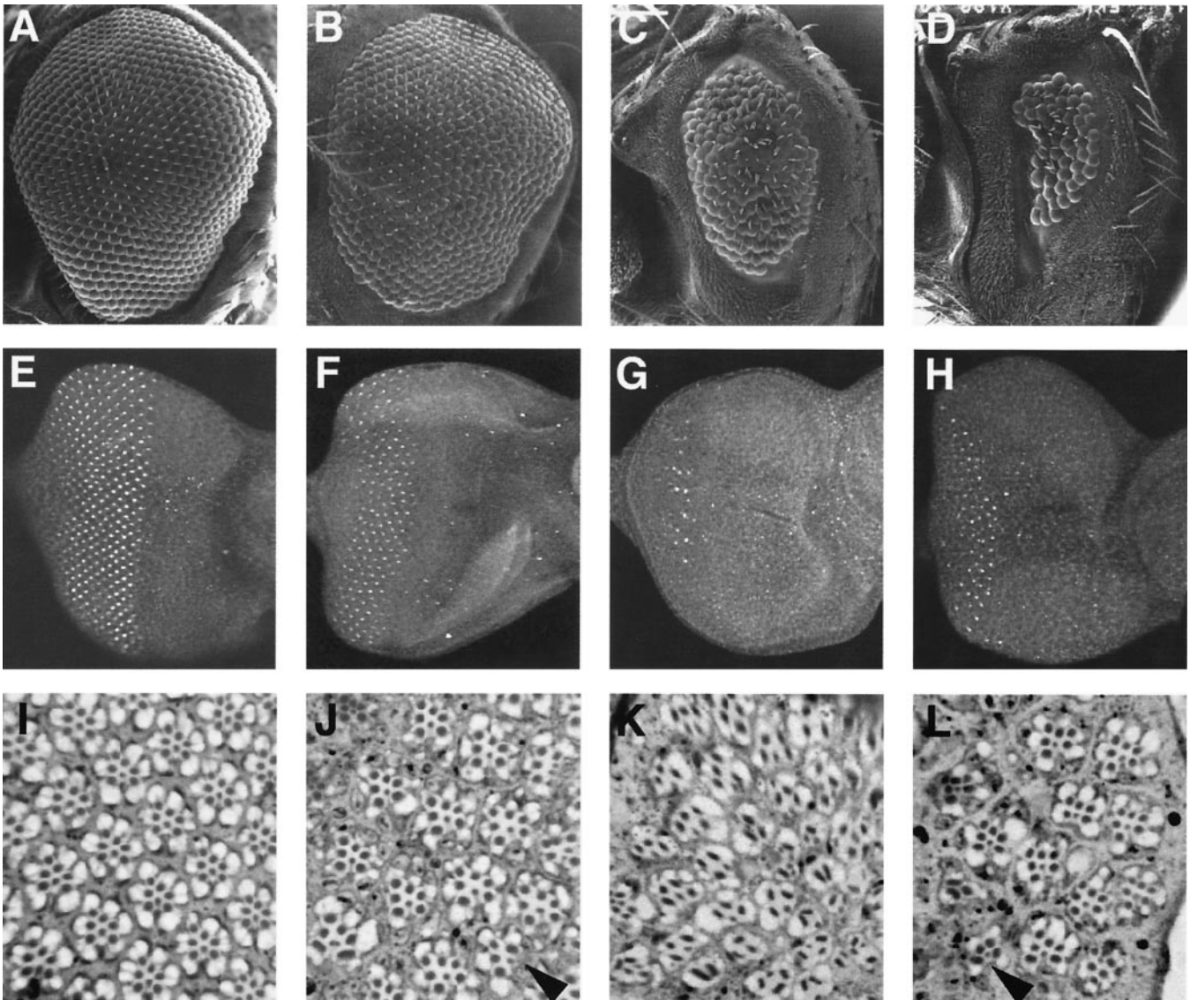

Fig. 1. Ectopic *scute* expression driven by *ato* regulatory regions promotes photoreceptor formation. (*A–D*) Scanning electron microscopy of compound eyes, showing that expression of *scute* either behind or in front of the MF leading furrow, combined with complementary *ato* expression, restores some ommatidia in the *ato*^{1} mutant (*C* and *D*). (*E–H*) Third instar larval eye discs stained with an antibody against Boss, showing much reduced Boss expression if *scute* is expressed behind (*G*) rather than in front of the MF leading edge (*H*). (*I–L*) Tangential sections of compound eyes, revealing ommatidia with reduced number of photoreceptors and no R8 in the *ato*^{1} mutant expressing *ato* in front of and *scute* behind the MF leading edge (*K*). (*A*, *E*, and *I*) Wild type; (*B*, *F*, and *J*) *ato*^{1} mutant expressing *ato* both in front of and behind the MF leading edge (3′F:5.8-*ato*; 5′eye-*ato,ato*^{1}); (*C*, *G*, and *K*) *ato*^{1} mutant expressing *ato* in front of and *scute* behind the MF leading edge (3′F:5.8-*ato*; 5′eye-*sc,ato*^{1}); (*D*, *H*, and *L*) *ato*^{1} mutant expressing *scute* in front of and *ato* behind the MF leading edge (3′F:5.8-*sc*; 5′eye-*ato,ato*^{1}). Arrowheads in *J* and *H* indicate the rare ommatidia with fewer than eight photoreceptors. Posterior is to the left. (Magnifications: *A–D*, ×110; *E–H*, ×225; *I–L*, ×900.)

demia Sinica, Taipei, Taiwan) as the template and the following primers: 5′-CAG TCA TGA AAA ACA ATA AT-3′ and 5′-GAC GGA TCC TTG GGG ATT AAG TCA, which incorporate a *Bsp*HI or a *Bam*HI site, respectively. This fragment then was digested with *Bsp*HI and *Bam*HI. A genomic fragment containing the 1.1-kb basic promoter region of *ato* was obtained by *Sac*II and *Bgl*II double digestion of plasmid pBS.Bm4.2 (a gift from Andy Jarman, University of Edinburgh, Scotland; see ref. 18 for description), followed by gel purification and *Afl*III digestion. The resulting *Sac*II–*Afl*III fragment was ligated to the above *Bsp*HI–*Bam*HI fragment and then inserted into *Sac*II and *Bam*HI sites of the pBluescript to generate pBS.*Sac*II-*sc*. The nucleotide sequence of the *scute* coding region derived from PCR was confirmed by DNA sequencing. The cloning strategies for both pCaSpeR.5′eye enhancer-*sc* (5′eye-*sc*) and pCaSpeR.3′enhancer-*sc* (3′F:5.8-*sc*) rescue constructs are essentially the same as for pCaSpeR.5′eye enhancer-*ato* (5′eye-*ato*) and pCaSpeR.3′enhancer-*ato* (3′F:5.8-*ato*), respectively (18), except that a 2.1-kb *Sac*II–*Sma*I fragment excised from pBS.*Sac*II-*sc* was used in both cases to replace the 2.1-kb *Sac*II–*Sca*I fragment used to make the *ato* rescue constructs.

Scanning Electron Micrography. Fly heads were fixed with 2% glutaraldehyde and 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight, dehydrated with a graded ethanol series, and critical point-dried in CO_2. The samples were sputter-coated with 30 nm of gold palladium and examined with a scanning electron microscope at an accelerating voltage of 5 kV.

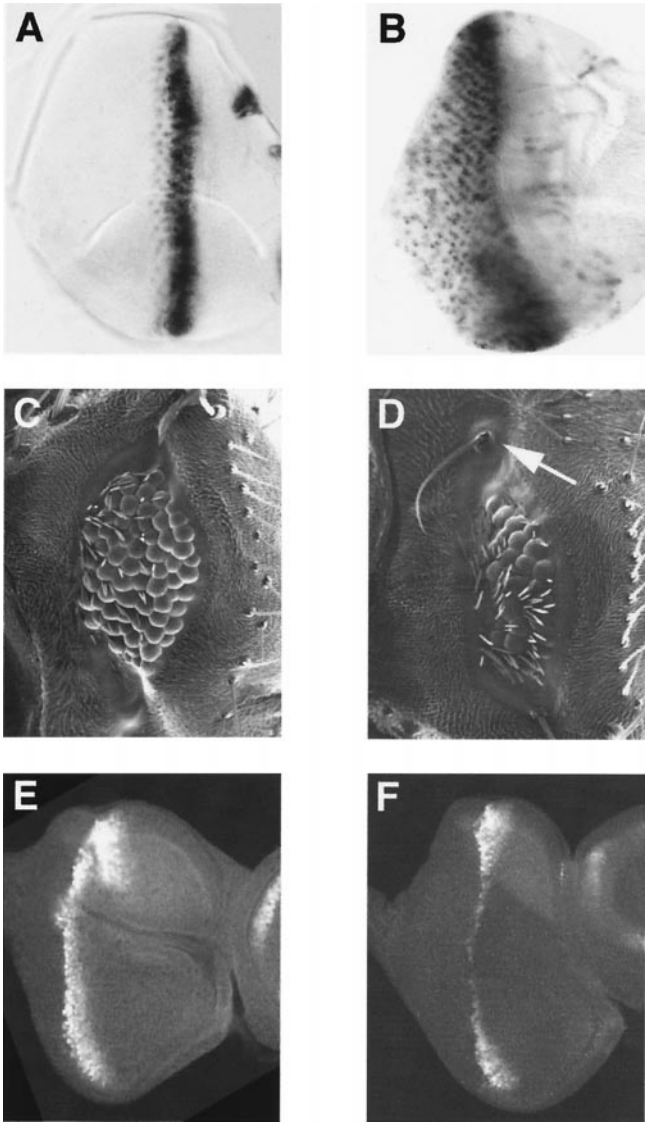

Fig. 2. Ectopic *ato* or *scute* expression driven by the GAL4–7 line rescues ommatidia. (*A*) Third instar larval eye disk hybridized to a digoxigenin-labeled *ato* probe, showing the endogenous *ato* expression pattern. (*B*) Third instar larval eye disk of GAL4–7/UAS-*lacZ* (nuclear) hybridized to a digoxigenin-labeled *lacZ* probe. The GAL4–7 line drives reporter gene expression in many more cells as compared with the endogenous *ato* expression, particularly in the posterior field (*A*). Scanning electron micrographs of compound eyes reveal that ectopic expression of *scute* (UAS-*sc*,*ato*^{1}/GAL4–7,*ato*^{1}) in *ato*^{1} mutant induces ommatidial formation (*D*), but to a lesser extent than expression of *ato* (UAS-*ato*/+; GAL4–7,*ato*^{1}/*ato*^{1}) (*C*). Occasionally, fully developed macrochaete-like sensory organs (arrow in *D* and Fig. 5) are seen in different areas of *scute*-rescued eyes. Third instar eye disk from *ato*^{1} mutant (*E*) and *ato*^{1} mutant expressing *scute* (UAS-*sc*,*ato*^{1}/GAL4–7,*ato*^{1}) (*F*) show similar patterns of staining with an antibody against Atonal. (Magnifications: *A* and *B*, ×225; *C* and *D*, ×145; *E* and *F*, ×200.)

Histology. Fly heads were fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight, dehydrated with a graded ethanol series, and embedded in epoxy. Compound eyes were sectioned tangentially at 2 μm and stained with 1% Toluidine blue and 1% sodium borax.

Isolation of the GAL4–7 Line. The GAL4–7 enhancer trap line was isolated in our lab by Alice Turner and Cheng-ting Chien through mobilizing a previously known enhancer trap line 109C1

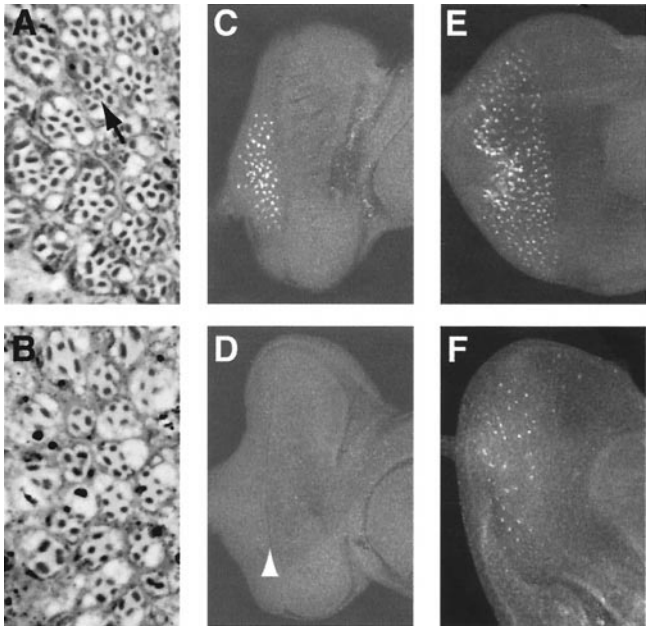

Fig. 3. Ommatidia rescued by *ato* or *scute* exhibit different structure and developmental processes. (*A*, *C*, and *E*) *ato*^{1} mutant expressing *ato* (UAS-*ato*/+; GAL4–7,*ato*^{1}/*ato*^{1}). (*B*, *D*, and *F*) *ato*^{1} mutant expressing *scute* (UAS-*sc*,*ato*^{1}/GAL4–7,*ato*^{1}). (*A* and *B*) Tangential sections of compound eyes. (*A*) Ommatidia in *ato*-rescued eye often contain more than eight photoreceptors. Multiple R8-like cells (arrow in *A*) in one ommatidium frequently are seen. (*B*) Most ommatidia in *scute*-rescued eye have only outer photoreceptors, and the number is lower than normal. (*C–F*) Eye discs from third instar larva (*C* and *D*) or early pupae (*E* and *F*) stained with an anti-Boss antibody. Third instar larval discs of *scute* rescued *ato*^{1} mutant (*D*) express no detectable Boss protein, although the MF has clearly advanced (arrowhead in *D*). Boss is detected only in early pupal discs of *ato*^{1} mutant expressing *scute* (*F*), in many fewer cells than those in the *ato*-rescued discs (*E*). (Magnifications: *A* and *B*, ×700; *C–E*, ×200.)

in an attempt to establish GAL4 lines that can drive UAS-*ato* expression and thereby rescue the loss of ommatidia phenotype in the *ato*^{1} mutant. The P element is inserted on the third chromosome. This line is viable as homozygotes with no discernible phenotype.

Whole-Mount *in Situ* Hybridization. A 0.9-kb Asp-718 fragment from plasmid pBS.84F hs#2 (a gift from Andy Jarman) containing the *ato* coding region and a 3.4-kb *Eco*RI–*Kpn*I fragment from the *lacZ* coding region excised from plasmid pBS.*khc*:*lacZ* (20) were used as template for digoxigenin (Boehringer Mannheim) labeling. The labeling was performed according to the manufacturer. Whole-mount *in situ* hybridization in imaginal discs using digoxigenin-labeled probes was performed essentially as described by Tautz and Pfeifle (21).

Immunohistochemistry. Imaginal discs from late third instar larvae and early pupae were fixed for 10–20 min with 4% formaldehyde in 0.1 M PBS, washed with PBT (PBS + 0.3% Triton X-100) and blocked with 5% normal goat serum in PBT. Primary antibodies used were: rabbit anti-Ato (17), mouse anti-Boss (22), rabbit anti-Prospero (23), mouse anti-Cut (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City). Dichlorotriazinyl aminofluorescein, lissamine rhodamine B sulfonyl chloride (Jackson Immunoresearch), Alexa 488, or Alexa 568 (Molecular Probes)-conjugated secondary antibodies were used. Discs were mounted in Slow Fade mounting medium (Molecular Probes). All comparative experiments between *ato* and *scute* were performed in parallel.

Fly Stocks. All *Drosophila* stocks were raised on standard cornmeal-yeast-agar medium at 25°C. *ato*^{1} is described by Jarman *et al.* (4, 17). UAS-*ato* and UAS-*sc* were established by Jarman *et al.* (3) and Chien *et al.* (8), respectively.

Results

Ectopic Eye Disk Expression of *scute* and/or *ato* Driven by the Regulatory Regions of the *ato* Gene. The eyes of *ato*^{1} mutant are totally devoid of ommatidia. This mutant phenotype can be rescued by expressing *ato* in *ato*^{1} mutant flies via two copies of 5′eye-*ato* and 3′F:5.8-*ato*, because of restoration of R8 photoreceptors (Fig. 1 *B* and *F*) (18). By contrast, two copies of either or both of the *scute* transgenes (5′eye-*sc* and 3′F:5.8-*sc*) did not restore any Boss-expressing R8 photoreceptors or ommatidia in *ato*^{1} mutant (data not shown). To our surprise, *ato*^{1} flies carrying two copies of 3′F:5.8-*sc* and 5′eye-*ato* developed eyes with significant numbers of ommatidia and R8 photoreceptors (Fig. 1 *D* and *H*). Even more remarkably, expression of *ato* in front of and *scute* behind the MF leading edge (two copies of 3′F:5.8-*ato* and 5′eye-*sc*) in *ato*^{1} mutant resulted in a rather robust restoration of the eye without a corresponding restoration of cells expressing the R8 marker Boss, whose expression in wild type is commenced before R7 induction and remains in R8 throughout the period required for *sevenless* activity (22) (Fig. 1 *C* and *G*). Given that either *ato* transgene alone cannot induce ommatidia (18), these observations indicate that *scute* can partially substitute for *ato* in promoting ommatidium development.

The unexpected ability to induce eye formation but not R8 photoreceptors, because of expression of *ato* in front of and *scute* behind the MF leading edge (3′F:5.8-*ato*; 5′eye-*sc*) in *ato*^{1} mutant, is mirrored by an unusual arrangement of photoreceptors in the ommatidia. Many of these ommatidia contain fewer than eight photoreceptors, and most of the remaining photoreceptors resemble outer photoreceptors by their positions and the large size of their rhabdomeres (rhodopsin-bearing stacks of microvilli) (Fig. 1*K*). For comparison, the normal trapezoidal arrangement is found in wild-type eyes (Fig. 1*I*) and eyes of *ato*^{1} mutants rescued by both *ato* transgenes (Fig. 1*J*) or by expression of *scute* in front of and *ato* behind the MF leading edge (3′F:5.8-*sc*; 5′eye-*ato*) (Fig. 1*L*). These results suggest that *scute* is capable of inducing photoreceptor formation; however, the resulting photoreceptors are not R8.

Ectopic Expression of *scute* or *ato* via the GAL4-UAS System and an Enhancer-Trap Line for Early Expression in the Eye Disk. To further examine the ability of *scute* to induce photoreceptor formation independent of the founder photoreceptor R8, we introduced UAS-*scute* (9) into the GAL4–7 line. This enhancer-trap line drives gene expression predominantly in the posterior of the eye disk (Fig. 2*B*), in many more cells than cells that normally express *ato* (Fig. 2*A*). In the hypomorphic *ato*^{1} mutant (17, 18), expression of the mutant Atonal protein is detectable only in a narrow stripe of eye disk cells (Fig. 2*E*) (17) and is incapable of inducing ommatidium development. Expression of UAS-*scute* in the *ato*^{1} mutant, driven by the GAL4–7 line, did not alter the expression pattern of the mutant Atonal (Fig. 2*F*). Nonetheless, this resulted in compound eyes (Fig. 2*D*) with surface morphology similar to the compound eyes induced by expression of UAS-*ato* (3) (Fig. 2*C*). Thus, *scute* is capable of inducing ommatidium development.

Interestingly, whereas many ommatidia induced by UAS-*ato* contain more than the normal number of eight photoreceptors, often including more than one R8 photoreceptor (Fig. 3*A*), ommatidia induced by UAS-*scute* contain only 2–5 photoreceptors, usually lacking the centrally located R8 photoreceptors with small rhabdomeres (Fig. 3*B*).

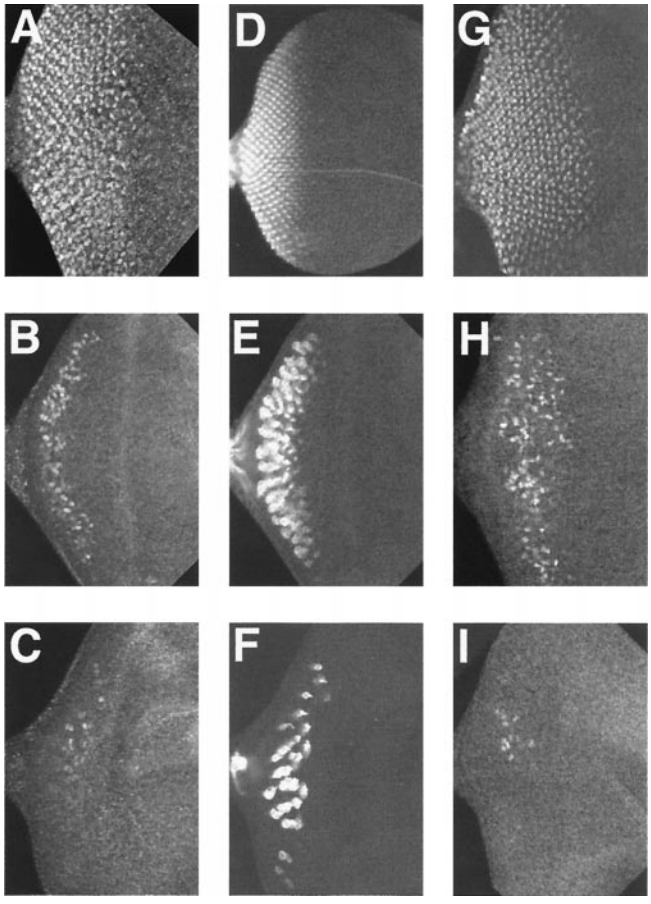

Fig. 4. Expression of *prospero*, 24B10 and *cut* in imaginal discs from *ato*^{1} mutants ectopically expressing *ato* or *scute*. Imaginal discs from third instar larvae of wild type (*A*, *D*, and *G*), *ato*^{1} mutant expressing *ato* (UAS-*ato*/+; GAL4–7,*ato*^{1}/*ato*^{1}) (*B*, *E*, and *H*) or *ato*^{1} mutant expressing *scute* (UAS-*sc*,*ato*^{1}/GAL4–7,*ato*^{1}) (*C*, *F*, and *I*) stained with an anti-Prospero (*A–C*), 24B10 (*D–F*), or an anti-Cut (*G–I*) antibody. All discs are shown with posterior to the left. Both Prospero (*C*) and 24B10 (*F*) are apparent in the UAS-*sc*,*ato*^{1}/GAL4–7,*ato*^{1} discs, albeit in fewer cells as compared with the UAS-*ato*/+; GAL4–7,*ato*^{1}/*ato*^{1} discs (*B* and *E*, respectively). This indicates that some photoreceptor differentiation has occurred in the absence of Boss expression at this stage (Fig. 3*D*). *scute* does not seem to activate *cut* expression directly in this misexpression condition (*I*). Many more cells express the cone cell marker *cut* in *ato* rescued disk (*H*), consistent with stronger ommatidial rescue by *ato*. (Magnification: ×200.)

The R8 Photoreceptors Are Missing in *scute* Rescued Ommatidia Because Their Precursors Fail to Form. In *ato*^{1} third instar larval eye discs expressing *scute* via the GAL4–7 line, there are no cells expressing the R8 marker Boss (22) (Fig. 3*D*). However, these larval eye discs exhibit the advancing MF (arrowhead in Fig. 3*D*). Moreover, they contain cells expressing Cut (Fig. 4*I*), the cone cell marker (24), as well as cells expressing Prospero (Fig. 4*C*), a marker for R7 photoreceptors and cone cells (25). Besides the R7 photoreceptors, other photoreceptors also were present, as indicated by the much greater number of cells stained with the 24B10 mAb (Fig. 4*F*), a general photoreceptor marker (26). These signs of eye development precede the appearance of just a few Boss-expressing cells in the posterior region of the eye disk after puparium formation (Fig. 3*F*). Taken together, these observations suggest that eye development is progressing in the absence of R8 photoreceptors.

Ectopic *scute* Expression Also Induces Ectopic Macrochaetae Formation in the Eye. Normally *scute* functions as a proneural gene for interommatidial bristles in the eye but is not expressed in cells

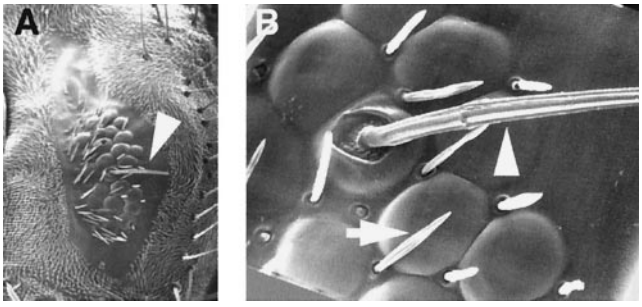

Fig. 5. *scute* induces ectopic external sensory organ formation in the eye. Scanning electron micrographs of a compound eye of *ato*^{1} mutant expressing *scute* (GAL4–7,*ato*^{1}/UAS-*sc*,*ato*^{1}) (*A* and *B*). In addition to photoreceptors, *scute* occasionally induces macrochaete-like external sensory organs (arrowhead in *A* and *B* and arrow in Fig. 2*D*) in various locations in the eye. These ectopic sensory bristles are morphologically distinct from surrounding interommatidial bristles (arrow in *B*). (Magnifications: *A*, ×110; *B*, ×700.)

that form the ommatidia (27). These small bristles develop between ommatidia during the early pupal stage after most photoreceptor cells already have been specified. In *ato*^{1} flies expressing *scute* via the GAL4–7 line, we occasionally observed large bristles in various positions in the eye (arrow in Figs. 2*D* and 5*A*). Some are found in the center of well-developed ommatidia (arrowhead in Fig. 5*B*). These ectopic bristles resemble macrochaetae that are found on the notum of the wild-type fly and are morphologically distinct from the small interommatidial bristles (arrow in Fig. 5*B*). Thus, ectopic expression of *scute* in the eye not only induces ommatidia formation in an unusual, R8-independent manner, but also can induce the formation of macrochaetae-like bristles that normally depend on *scute* although absent from the eye.

Discussion

Formation of the Founder Photoreceptor R8 Can Be Induced by *ato* but Not *scute*. The absence of R8 in *ato*^{1} mutants expressing *scute* via the GAL4–7 line, or expressing *scute* behind the MF leading edge in conjunction with *ato* in front of the MF, demonstrates that *scute* cannot substitute for *ato* in inducing R8 even though it induces ommatidia. Among proneural genes, *scute* is closely related to other genes of the *achaete-scute* complex (about 70% amino acid identity) but significantly different from *ato* (about 45% amino acid identity) (3, 28). The ability of *ato*, but not *scute*, to induce chordotonal organs (3, 8, 9) can be accounted for by the differences in their DNA-binding basic domains (8). Indeed, *scute* induces whereas *ato* represses expression of *cut* (9), a gene that functions as a binary switch to allow a neural precursor to choose between producing an external sensory organ (in the presence of *cut* expression) or a chordotonal organ (in the absence of *cut* expression) (29, 30).

Ommatidia Development Initiated by Ectopic *scute* Without Inducing R8 Photoreceptors. How might ectopic expression of *scute* induce R8-less ommatidia, given that R8 normally is required for the formation of other photoreceptors and support cells of the ommatidia? It is probably not because of the ability of *scute* to induce *cut* expression, because only cone cells in an ommatidium normally express *cut* (25) and ectopic expression of *scute* in the eye causes only a small number of cells in the ommatidia to express Cut (Fig. 4*I*). Presumably, expression of genes that specify the eye primordia, such as the Pax gene *eyeless* (31), directs *scute* to act on a different set of downstream genes than those involved in the formation of sensory bristles. Although it is possible that *scute* induces latent R8 precursors that express none of the known R8 markers but can still recruit the R1–7 photoreceptors, we favor the following possibility. Once R8 is induced by *ato* during normal eye development, it recruits other photoreceptors by inducing the expression of yet unidentified gene(s) that encode basic helix–loop–helix protein, which shares significant sequence similarity with either Scute or both Scute and Atonal. Ectopic *scute* expression in *ato*^{1} mutants mimics the action of this gene(s) and induces the formation of outer photoreceptors, thereby bypassing the normal requirement of R8 founder photoreceptors. It will be very interesting to identify this hypothetical basic helix–loop–helix gene(s) and study its function in eye development.

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