Abstract:
Steroid signaling underlies developmental processes in animals. Mutations that impair steroidogenesis in the fruit fly Drosophila melanogaster provide tools to dissect steroid hormone action genetically. The widely used temperature-sensitive mutation ecdysoneless1 (ecd1) disrupts production of the steroid hormone ecdysone, and causes developmental and reproductive defects. These defects cannot be satisfactorily interpreted without analysis of the ecd gene. Here, we show that ecd encodes an as yet functionally undescribed protein that is conserved throughout eukaryotes. The ecd1 conditional allele contains an amino acid substitution, whereas three non-conditional larval lethal mutations result in truncated Ecd proteins. Consistent with its role in steroid synthesis, Ecd is expressed in the ecdysone-producing larval ring gland. However, development of ecd-null early larval lethal mutants cannot be advanced by Ecd expression targeted to the ring gland or by hormone feeding. Cell-autonomous ecd function, suggested by these experiments, is evidenced by the inability of ecd– clones to survive within developing imaginal discs. Ecd is also expressed in the ovary, and is required in both the follicle cells and the germline for oocyte development. These defects, induced by the loss of ecd, provide the first direct evidence for a cell-autonomous function of this evolutionarily conserved protein.

Key words: Steroid hormone, Ecdysone, ecdysoneless, Imaginal disc, Oogenesis, Drosophila

Introduction
Steroid hormones play crucial roles in development and reproduction of insects, including the fruit fly Drosophila melanogaster. The insect steroid ecdysone (E), and primarily its active derivative 20-hydroxyecdysone (20E), is responsible for coordination of embryogenesis, larval molting and metamorphosis, the latter involving differentiation of adult structures from precursor imaginal discs (Riddiford, 1993). We will hereafter use the generic name ecdysone to refer to the Drosophila steroid hormone. Blood-circulating ecdysone induces tissue-specific and temporally restricted proliferation, differentiation and programmed cell death. Numerous studies, directed towards understanding how the ubiquitous hormone governs these diverse cellular responses, culminated in detailed dissection of the regulatory cascade downstream of the ecdysone signal (Thummel, 1996).

The major and best-studied source of ecdysone in insect larvae is the prothoracic gland, which in Drosophila consists of the lateral lobes of the ring gland (Dai and Gilbert, 1991). After this part of the ring gland degenerates during metamorphosis, adult ovaries contribute to the whole body steroid titer in Drosophila (Garen et al., 1977; Bownes et al., 1984; Bownes, 1989; Warren et al., 1996). The main role of ecdysone in adult females is to regulate vitellogenesis (Hagedorn, 1985; Bownes et al., 1996). In addition, ecdysone has been implicated in egg chamber maturation during mid-oogenesis (Buszczak et al., 1999). Inactive ecdysone conjugates are maternally deposited to eggs and are mobilized during mid-embryogenesis by the amnioserosa (Bownes et al., 1988; Kozlova and Thummel, 2003).

Recently, several Drosophila genes involved in ecdysone biosynthesis have been cloned. One is dare, a homolog of the human adrenodoxin reductase that is necessary for the reduction of mitochondrial cytochrome
Two other genes, *disembodied (dib)* and *shadow (sad)*, encode Cyp C\(_{22}\)-and C\(_{2}\)-hydroxylases, respectively, which are responsible for the final two hydroxylation steps of ecdysone synthesis (Chavez et al., 2000; Warren et al., 2002). Ecdysone is the final product of the ring gland, which is secreted to the hemolymph and converted to 20E in peripheral tissues. The Cyp C\(_{20}\)-hydroxylase responsible for this conversion is encoded by *shade (shd)* (Petryk et al., 2003). The *dare, dib* and *sad* genes are all expressed in the larval lateral ring gland and in adult ovaries, and their loss-of-function phenotypes can be fully explained as a consequence of ecdysone deficiency. Thus far, only one steroidogenic factor that is not itself an enzyme, *without children (woc)*, has been identified (Wismar et al., 2000; Warren et al., 2001). This gene encodes a zinc finger transcription factor that probably activates expression of the cholesterol 7,8-dehydrogenase that executes the first step of ecdysone biosynthesis. Mutations of *woc* affect a wide range of tissues, suggesting that its transcriptional function is not restricted to regulating expression of the steroidogenic enzyme. No other regulators of the steroidogenic pathway have been identified thus far.

Among steroid-deficient *Drosophila* mutations, ecdysoneless\(^1\) (*ecd\(^1\)*) is used to study ecdysone roles in development. The *ecd\(^1\)* mutation is a recessive, temperature-sensitive allele that reduces whole-body ecdysone titers and causes larval arrest at a restrictive temperature, 29°C (Garen et al., 1977). The effect of *ecd\(^1\)* on ecdysone production is autonomous, because cultured *ecd\(^1\)* mutant ring glands fail to produce ecdysone when upshifted to 29°C (Henrich et al., 1987; Dai et al., 1991; Warren et al., 1996). Ecdysone production is also interrupted in adult ovaries upshifted to the restrictive temperature (Garen et al., 1977; Redfern and Bownes, 1983; Warren et al., 1996). After several days at 29°C, oogenesis pauses at the onset of vitellogenesis (Garen et al., 1977; Redfern and Bownes, 1983). These defects have been interpreted as consequences of the mutationaly induced ecdysone deficiency. However, Redfern and Bownes caution that a range of anomalies in *ecd\(^1\)* adults result from an autonomous *ecd* requirement for cell viability and therefore may not be attributable to ecdysone deficiency (Redfern and Bownes, 1983).

Developmental events disrupted in *ecd\(^1\)* mutants include fat body protein synthesis (Lepesant et al., 1978), progression of the eye-forming morphogenetic furrow (Brennan et al., 1998), salivary gland glue secretion (Biyasheva et al., 2001) and motor neuron outgrowth (Li and Cooper, 2001). These defects have been interpreted as consequences of the mutationally induced ecdysone deficiency. It is difficult to discern which of the phenotypes result from the *ecd\(^1\)* mutation directly, and which are the consequence of low ecdysone titer, without knowing the primary defect in the *ecdysoneless* gene, whose molecular identity remained elusive for over 25 years. We report here that the *ecd* locus encodes a protein whose orthologs in several other species, including humans, have not yet been functionally described. The original *ecd\(^1\)* mutation and three non-conditional lethal alleles have been mapped and assessed for their effects.

We have localized the Ecd protein to both the steroidogenic and non-steroidogenic tissues, and have demonstrated its cell-autonomous roles in imaginal discs and ovaries.

**Materials and methods**

**Drosophila strains**

Flies were cultured on standard cornmeal medium at 25°C unless otherwise specified. The *ecd* mutations examined in this study included the temperature-sensitive *ecd\(^1\)* (Garen et al., 1977) and three non-conditional recessive lethals: EMS-induced alleles *ecd\(^2\)* (*ru ecd\(^e\)* *st e*) (Sliter et al., 1989) and *ecd\(^{h23}\)* (a gift of Dr I. Zhimulev), and *ay*-ray-induced *ecd\(^{24}\)* (*ve R ecd\(^{24}\)* (V.C.H., unpublished). Deficiencies *Df(3L)R+R2* and *Df(3L)Aprt201* were from previous irradiation screens (Sliter et al., 1989; Wang et al., 1994). The *mbf1*-null mutant line (Liu et al., 2003) was used for control in the analyses of mitotic mutant clones.

**Genetic mapping and sequence analysis of ecd**

Deficiencies *Df(3L)R+R2*, in the 62B-D chromosomal region that deletes the *ecd* locus (Sliter et al., 1989), and *Df(3L)Aprt201*, which complements the non-conditional *ecd* alleles, were used to delimit the *ecd* interval by a series of PCR reactions. These were performed on embryos homozygous for either *Df(3L)Aprt201* or *Df(3L)R+R2* with pairs of primers, derived from ten genes (CG17772, CG17771, CG13807, CG5714, CG13806, CG5714, CG13807, CG17771, CG17772, CG13806, CG5714).
CG13805, CG5717, CG13804, CG13803, CG13802) occurring between the right breakpoints of the two deletions according to the BDGP (Berkeley Drosophila Genome Project; Fig. 1). CG5714 was identified as ecd by genetic rescue of the ecd− mutants. Genomic DNA from embryos or larvae homozygous for each of the ecd alleles was amplified with primers flanking the CG5714 gene: 5′-GGTACGAAAGGAGGGAGGAGG-3′ and 5′-GATGAGCAAGATTCCAGGCAGCA-3′. PCR products from three independent reactions were sequenced using the BigDye Terminator Kit (Perkin Elmer), using these and additional internal primers to cover the entire ecd gene in both directions.

**Map of the ecd locus.** The interval of ecd is delimited by the right breakpoints of deletions Df(3L)Aprt201 and Df(3L)R+R2 within the cytological region 62D5-D7 (top). Ten predicted genes (BDGP) located in this interval are shown. The filled boxes represent coding regions and the lines between them denote introns, arrows indicate the orientation of each gene. Five genomic fragments used for the preparation of rescue constructs are indicated by the black lines. CG5714 (underlined), present in the rescuing fragments E5 and S4, is the ecd gene.

**Transformation rescue of ecd mutants**

Five genomic fragments containing the ecd candidate genes were obtained by restriction of the BACR22J16 clone (BDGP) and placed into the pCaSpeR-2 P-element vector (Thummel and Pirrotta, 1992). Clones E5, H13, B2, B13 and S4 (Fig. 1) were used for P-element-mediated germline transformation (Spradling and Rubin, 1982). ecd+/TM6B and ecd^{24}/TM6B females carrying the rescue construct P[w^{+}, RC] on the second chromosome: w; P[w^{+}, RC]; ru ecd^{2} st e/TM6B or w; P[w^{+}, RC]; ve R ecd^{24}/TM6B were mated with males heterozygous for one of the ecd alleles (ecd^{1}, ecd^{2}, ecd^{l(3)23}, ecd^{24} or Df(3L)R+R2) over TM6B to test for genetic rescue of ecd.

**Lethal phase determination**

Each ecd allele was crossed with all other ecd alleles and with the Df(3L)R+R2 deficiency. All lines were balanced with TM3, P[w^{+}, act-GFP]. The flies were allowed to lay eggs on apple juice plates, supplemented with baker's yeast paste at 25°C, or at 29°C in the case of ecd^{l} crosses. Eggs were collected in two-hour periods, and embryos or larvae were identified as ecd homozygotes by the absence of the GFP-marked balancer.
Hormone feeding and titer determination
For the non-conditional ecd^2 and ecd^{ Hind2} mutants, 200 early-second instar larvae of each genotype were placed in vials with a sucrose-yeast medium containing 20-hydroxyecdysone (20E) at concentrations of 1 mg/ml (Garen et al., 1977; Freeman et al., 1999), 250 μg/ml, 50 μg/ml or zero, and animals progressing to the second molt or beyond were counted. The temperature-sensitive ecd^1 mutants were tested for puparium formation as third instar larvae on the same media at 29°C. In all cases the homozygous ecd mutants were compared with their rescued counterparts carrying the S4 construct. Radioimmunoassay of total ecdysteroids was performed in whole-body homogenates as described (Jindra et al., 1994).

Rescue with ectopic Ecd expression
A full-length ecd cDNA (GH14368; BDGP) was subcloned into the pUAST P-element vector (Brand and Perrimon, 1993). Transgenic flies carrying the UAS-ecd construct in the ecd^2 mutant background were crossed with ecd^2 lines carrying transgenic Gal4 drivers to produce UAS-ecd/Gal4; ecd^2/ecd^2. Six drivers were tested for the ability to rescue the ecd^2 lethal phenotype: act-Gal4 (from Dr B. Edgar), ptc-Gal4 (Bloomington stock #2017), sev-Gal4 (from Dr P. Vilmos), en-Gal4 (from Dr Y. Hiromi), Aug21 and Feb36 (Siegmund and Korge, 2001; Andrews et al., 2002). All lines were balanced with TM3, Ser, P[w^+], act-GFP], so that ecd^2 homozygotes could be identified at all developmental stages.

Generation of somatic and germline ecd^− clones
Mutant clones deficient for either Ecd or MBF1 (control) proteins were generated by mitotic recombination using the FLP-FRT technique as described (Xu and Rubin, 1993; Theodosiou and Xu, 1998; Chou and Perrimon, 1996). To induce clones in the developing imaginal discs, w, hs-FLP; P[w^+], ub-GFP]61F FRT 80B females were mated with w; ru ecd^2 FRT 80B/TM3, P[w^+], act-GFP] or with y w; mbl1 FRT 80B males. Their progeny were heat-shocked as larvae for one hour at 38°C, 24-36 hours after egg laying; adult females were heat-shocked for 3 hours at 37°C to generate mutant clones in the ovarian follicle cells. To obtain ecd-null germline clones, females w, hs-FLP; ru ecd^2 FRT^{3L-2A}/TM6B were mated with P[w^+], ovoD1^{3L-2X48} FRT^{3L-2A}/TM3 males. Before reaching the second-to-third instar transition, the progeny was heat-shocked twice for 2 hours at 38°C (Theodosiou and Xu, 1998). Emerged w, hs-FLP/w; ru ecd^2 FRT^{3L-2A}/P[w^+], ovoD1^{3L-2X48} FRT^{3L-2A} females were mated, examined for egg laying, and sacrificed for immunostaining of their ovaries 3-10 days later. Alternatively, germline clones were induced by heat shock for 1 hour at 38°C in adult females, and were analyzed 3-7 days later.

RNA hybridization
Poly(A)^+ RNA was isolated using the QuickPrep mRNA Purification Kit (Amersham) and ecd and mbl1 transcripts were detected on northern blots with full-length cDNA probes as described (Uhlirova et al., 2002). The same ecd probe, and its sense version (for control), was used for in situ hybridization of adult ovaries (Tautz and Pfeifle, 1989; Buszczak et al., 1999); detection was with anti-DIG alkaline phosphatase and the CBIP/NBT substrate (Roche).

ecd-lacZ expression
An ecd-lacZ reporter was constructed by cloning a 1.25 kb ecd upstream genomic region into the pCaSpeR-AUG-βgal vector (Thummel et al., 1988). The same regulatory sequence in the S4 construct was sufficient for the rescue of ecd-null mutants. The ecd-lacZ activity was detected in transgenic animals using a standard X-gal staining procedure.

Ecd antibodies, immunoblot and tissue staining
The central portion of Ecd (amino acids 270-429) was expressed from pET28a (Novagen) as a hexahistidine fusion protein in the BL21-CodonPlus (Stratagene) E. coli strain. The protein was affinity-purified on a Ni-NTA agarose column (Qiagen) under denaturing conditions, then partially re-natured by dialysis and used for rabbit immunization. The collected antiserum was affinity-purified using the entire Ecd protein, produced by the yeast EasySelect Pichia Expression Kit (Invitrogen) and immobilized on the AminoLink Plus Coupling Gel (Pierce). For western blots, embryos or larvae were homogenized in a denaturing sodium dodecylsulphate (SDS)
buffer, and total protein (ca. 10 μg per lane) was analyzed by 10% SDS-PAGE. Blots were probed with the purified anti-Ecd antibody, diluted 1:5000. Detection was with a goat HRP-conjugated anti-rabbit antibody (1:4000) and a chemiluminescent substrate. Whole-mount immunostaining of larvae and adult gonads was performed according to standard procedures, with antibodies diluted as follows: anti-Ecd, 1:1000; anti-MBF1, 1:10,000 (Liu et al., 2003); anti-Orb (4H8 DSHB), 1:30 (Lantz et al., 1994); and anti-FasIII (7G10 DSHB), 1:30 (Patel et al., 1987). Secondary antibodies conjugated with Alexa Fluor 488, Texas-Red (Molecular Probes) and Cy3 (Amersham) were used at a dilution of 1:1000. Images were captured on Axioplan 100 and confocal LSM410 inverted laser scanning microscopes (Zeiss).

**Results**

**Identification of the ecdysoneless gene**

Genetic mapping placed ecd among 10 genes predicted by the Berkeley Drosophila Genome Project to be within region 62D. Four partially overlapping genomic fragments harboring subsets of these 10 genes (Fig. 1) were used for germline transformation. All three obtained transgenic lines carrying the E5 genomic fragment rescued the otherwise lethal ecd genotypes: ecd^2/ecd^2, ecd^2/ecd^{[3]23}, ecd^2/ecd^{[2]24}, ecd^2/Df(3L)R+R2, ecd^2/ecd^2 (29°C) and ecd^2/ecd^{[2]24} (29°C) to adulthood. A shorter construct S4, containing only the CG5714 gene (Fig. 1), rescued the ecd mutants to the same extent as E5. In all cases, a single transgenic copy of the CG5714 gene was sufficient for the complete rescue. These results clearly identify CG5714 as ecdysoneless.

The sequence of the deduced Ecd protein reveals a broad evolutionary conservation. Putative Ecd orthologs have been found in the mosquito Anopheles gambiae (43% overall amino acid identity), humans and mouse (31%), zebrafish (30%), Arabidopsis thaliana (26%) and the fission yeast Schizosaccharomyces pombe (21% identity). The human Ecd ortholog, known as Suppressor of GCR2 (SGT1), is expressed in a wide range of human organs (Sato et al., 1999) and functionally rescues a mutation of GCR2, a transcriptional regulator of glycolytic enzyme genes in the fission yeast (Deminoff and Santangelo, 2001). However, GCR2 is not homologous to SGT1 and thus the normal role of SGT1 in humans is unknown. Interestingly, although several highly conserved motifs are evident among the aligned orthologs (Fig. 2), none of these correspond to any known functional domain. There is a putative ATP/GTP-binding motif (P-loop) near the C terminus of the Drosophila and Anopheles orthologs, as recognized by the PROSITE database (Fig. 2).

**Molecular basis of ecd mutations**

To determine the character of mutations in aberrant ecd alleles, we have sequenced the relevant genomic region from ecd mutants. The temperature sensitive, EMS-induced allele ecd^d contains a substitution of the conserved proline 656 to serine (Fig. 2), resulting from a C to T transition. All the other examined alleles: ecd^e, and the two previously undescribed alleles ecd^{[2]24} and ecd^{[3]23}, produce truncated Ecd peptides (Fig. 3A). The ecd^e allele contains a C to T transition that converts Q_67 to a stop codon. In the γ-ray induced ecd^{[2]24}, a four-base-pair deletion causes a frameshift of four amino acids followed by a stop codon. In ecd^{[3]23}, the premature termination codon results from a C to T transition at Q_650. The extent of the presumed Ecd protein truncations suggests that ecd^e, at least, is a null allele. In agreement with the described mutations, a specific antibody raised against a central portion of the Ecd protein detected a wild-type sized band on western blots from third instar ecd^e larvae (29°C), but not from ecd^e, ecd^{[2]24} or ecd^{[3]23} homozygotes approaching their lethal phases (Fig. 3B; data not shown for ecd^{[2]24}). A truncated Ecd product was found in ecd^{[3]23} homozygotes (Fig. 3B).
Fig. 2. Ecdysoneless is conserved throughout eukaryotes. Alignment of the Drosophila (Dm) Ecd protein (Swiss-Prot Q9W032) with human (Hs) SGT1 (O95905), and its closest relatives from Arabidopsis thaliana (At; Q9LSM5) and Schizosaccharomyces pombe (Sp; Q9US49), using Clustal W. Black shading indicates amino acid identity, and gray shading indicates similarity if present in two or more of the aligned sequences. The putative ATP/GTP-binding site (P-loop) is marked by the horizontal arrow. Asterisks indicate the positions of the premature termination codons in alleles ecd$^2$, ecd$^{24}$ and ecd$^{(3)23}$. The arrowhead indicates the conserved proline 656, which is mutated to serine in ecd$^1$.

Fig. 3. Structure and expression of mutant Ecd forms. (A) Schematic representation of mutant versions of the Ecd protein in the four studied ecd alleles. Numbers are amino acid positions; asterisks denote stop codons. A 4-bp deletion in the γ-ray induced mutant ecd$^{24}$ changes the reading frame, resulting in the indicated amino acid substitutions and a stop codon. (B) Western blot of the wild-type and mutant Ecd forms. A low amount of maternal Ecd is still visible in the first instar, but not in the second instar homozygous ecd$^2$ larvae. The truncated Ecd version is detected by the anti-Ecd antibody in second instar ecd$^{(3)23}$ larvae.
The lethal stage of the *ecd* mutants was examined to establish whether the structural character of the mutations corresponded to their phenotypic effects (Table 1). The single proline-to-serine substitution in *ecd* is consistent with previous (Henrich et al., 1993; Sliter, 1989), and with our own, indications that the mutant gene product retains a residual function. Although most *ecd* homozygotes completed their second molt at 29°C, the majority of the *ecd/Df(3L)R+R2* hemizygotes, and *ecd*/ecd and *ecd*/ecd heteroallelic mutants, died during the second molt, displaying typical molting defects such as double mouth hooks (Fig. 4A). Among the non-conditional mutants, *ecd* homozygotes were the most severely affected (Table 1), and *ecd*/*Df(3L)R+R2* larvae arrested during the first molt with unshed cuticles and double mouth hooks (Fig. 4B,C). This early lethality could be in part caused by the dominant Roughened (R) mutation, or by another unknown mutation, on the *ecd*-bearing chromosome, as animals lacking most or all of the Ecd protein in *ecd* homozygous or heteroallelic combinations arrested during the second instar. The new *ecd* mutation was as severe as *ecd* (Table 1). These results suggest that *ecd*, *ecd* and *ecd* likewise represent *ecd*-null alleles that completely prevent development beyond the second instar.

### Table 1. Lethal phases of *ecd* mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Major lethal stages (% n)</th>
<th>Number of larvae (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ecd</em>/<em>ecd</em></td>
<td>L2 and early-L3 (12); mid-L3 (78)</td>
<td>200</td>
</tr>
<tr>
<td><em>ecd</em>/Df(3L)R+R2</td>
<td>L2-L3 molt (75); L3 (25)</td>
<td>80</td>
</tr>
<tr>
<td><em>ecd</em>/ecd</td>
<td>L2-L3 molt (65); L3 (35)</td>
<td>120</td>
</tr>
<tr>
<td><em>ecd</em>/ecd(1)3</td>
<td>L2-L3 molt (83); L3 (17)</td>
<td>125</td>
</tr>
<tr>
<td><em>ecd</em>/ecd(2)</td>
<td>Prolonged L2</td>
<td>500</td>
</tr>
<tr>
<td><em>ecd</em>/Df(3L)R+R2</td>
<td>Prolonged L2</td>
<td>60</td>
</tr>
<tr>
<td><em>ecd</em>/ecd(3)3</td>
<td>Prolonged L2</td>
<td>75</td>
</tr>
<tr>
<td><em>ecd</em>/ecd(3)3</td>
<td>Prolonged L2</td>
<td>200</td>
</tr>
<tr>
<td><em>ecd</em>/Df(3L)R+R2</td>
<td>Prolonged L2</td>
<td>90</td>
</tr>
<tr>
<td><em>ecd</em>/ecd(4)3</td>
<td>L1 (100)</td>
<td>100</td>
</tr>
<tr>
<td><em>ecd</em>/Df(3L)R+R2</td>
<td>L1 (43); L1-L2 molt (57)</td>
<td>120</td>
</tr>
</tbody>
</table>

*All genotypes containing *ecd* were tested at 29°C.

#### ecd is expressed in steroidogenic as well as non-steroidogenic tissues

Northern blot analysis of whole animals showed a single *ecd* transcript, present throughout development (Fig. 5A). The mRNA was more abundant towards the end of the final larval instar and during metamorphosis; the strongest expression was observed in mature, egg-laying females. In situ hybridization showed that this increase probably resulted from strong *ecd* expression in the ovarian nurse cells (Fig. 6M). The continuous *ecd* expression was confirmed at the protein level using a specific antibody that detected Ecd from early embryogenesis to adulthood (Fig. 5B, Fig. 3B, and data not shown). Ecd was found in unfertilized eggs, showing maternal deposition of the protein (Fig. 5B). A western blot of early larvae homozygous for the *ecd* null allele revealed that low levels of the maternal Ecd protein persisted into the first larval instar (Fig. 3B).
A steroidogenic role of Ecd presumes its presence in the sites of ecdysone synthesis. Staining of late-third instar larvae revealed Ecd expression in the steroidogenic lateral lobes of the ring gland (Fig. 6A, B). However, ring glands of late embryos (not shown), and first or second instar larvae (Fig. 6C), did not show prominent staining. Also the rest of the body displayed only a diffuse signal without a restricted pattern. The ring gland temporal profile was corroborated by using a transgenic β-galactosidase reporter (ecd-lacZ), which was active only in the medial corpora allata region but not in the lateral steroidogenic gland of second instar larvae (Fig. 6D). This construct strongly labeled the whole ring gland in late third instar (Fig. 6E). Except for the medial ring gland, not stained with the antibody (Fig. 6A), the lacZ reporter probably reflected true ecd expression, because it was driven by an ecd upstream genomic region that is sufficient for the rescue of ecd mutants. Besides the ring gland, specific Ecd expression was found in the nervous system (Fig. 6G), in the imaginal discs (Fig. 6H, I) and in developing gonads of third instar larvae (Fig. 6J, K). In all cases the Ecd protein predominantly resided in the cytoplasm.

During metamorphosis the lateral ring gland degenerates. Other organs, such as ovaries, serve as sources of adult ecdysone. In adult ovaries, Ecd protein was expressed in both the somatic follicle cells and the germline nurse cells throughout oogenesis (Fig. 6L). The signal was stronger in the nurse cells of egg chambers staged 8-10, probably because of the deposition of the Ecd protein, as well as mRNA (Fig. 6M) into the oocyte at this time. High levels of Ecd were detected in the apical part of adult testes, where the somatic and germline stem cells are localized and where spermatogenesis begins (Fig. 6N). In summary, Ecd expression was detected in the primary steroidogenic organs – the larval lateral ring gland and the adult ovaries – as well as in the non-steroidogenic central nervous system and imaginal discs.
Hormone feeding or ecd expression in the ring gland cannot rescue non-conditional ecd mutants

The presence of Ecd in the late-third instar ring gland is consistent with the steroid deficiency for which ecd1 was originally identified. The ability to induce puparium formation by feeding the non-pupariating ecd1 larvae at 29°C with 20-hydroxyecdysone (20E) (Garen et al., 1997; Redfern and Bownes, 1983; Berreur et al., 1984), suggested that low steroid levels might be the primary cause of arrest at this stage. To test whether the non-conditional ecd mutants could also be rescued by dietary hormone, we fed homozygous second instar ecd2 and ecd(3)23 larvae with 20E. The feeding of ecd2 larvae at 29°C served as a positive control: 50 μg/ml and 250 μg/ml 20E doses induced pupariation in 26 out of 100, and in 36 out of 100, ecd2 homozygotes, respectively. By contrast, none of 600 ecd2, or 250 ecd(3)23 larvae progressed beyond their lethal phase when fed 20E. These results strongly imply that ecdysone deficiency alone does not account for the second instar lethality of these
mutants. In support of this view, the whole-body ecdysteroid content was not significantly different between ecd²/ecd² (0.61±0.13 pg/animal) and ecd⁺ (0.48±0.08 pg/animal) first instar larvae.

To address the problem of ecd requirement directly, we have targeted ecd expression to the steroidogenic part of the ring gland using transgenic UAS-ecd activated by a Gal4 driver, Feb36 (Siegmund and Korge, 2001; Andrews et al., 2002). As expected from the ability of exogenous 20E to rescue pupariation of ecd¹ homozygotes at 29°C, Ecd expressed under Feb36 allowed formation of defective puparia in around 25% UAS-ecd, ecd²/ecd¹ larvae upshifted to 29°C (n=60). The ectopic Ecd presence in the ring gland, evident during the second instar (Fig. 6F), should therefore restore the impaired hormone synthesis and at least postpone the arrest of ecd-null mutants, if disrupted ecdysone production was the sole cause of their death. However, the Feb36-driven Ecd was insufficient to advance UAS-ecd, ecd²/ecd² larvae even to the second molt. By contrast, the same UAS-ecd construct expressed under a ubiquitous actin-Gal4 driver allowed ecd² homozygotes to reach adulthood (Table 2).

Table 2. Rescue of ecd² homozygotes by Gal4-targeted Ecd expression

<table>
<thead>
<tr>
<th>Gal4 driver</th>
<th>Expression domain in larvae*</th>
<th>Rescue (% n)</th>
<th>Number of larvae (n)</th>
</tr>
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<tbody>
<tr>
<td>actin/+</td>
<td>Ubiquitous</td>
<td>Adult (46)</td>
<td>80</td>
</tr>
<tr>
<td>Feb36/+</td>
<td>Lateral ring gland, salivary glands, fat body, malpighian tubules, tracheae, some midgut cells</td>
<td>None</td>
<td>200</td>
</tr>
<tr>
<td>Aug2/+</td>
<td>Medial ring gland, salivary glands, some midgut cells, malpighian tubules, tracheae</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>patched/+ (ptc/+</td>
<td>Imaginal discs, salivary glands, epidermis, gut, nervous system, fat body</td>
<td>L3 (71)</td>
<td>130</td>
</tr>
<tr>
<td>ptc/ptc</td>
<td>As above</td>
<td>PP (12)</td>
<td>65</td>
</tr>
<tr>
<td>Feb36/ptc</td>
<td>As Feb36 plus patched above</td>
<td>L3 (75)</td>
<td>100</td>
</tr>
<tr>
<td>sevenless/+</td>
<td>Eye imaginal discs</td>
<td>None</td>
<td>120</td>
</tr>
<tr>
<td>engrailed/+</td>
<td>Posterior imaginal discs, part of nervous system</td>
<td>None</td>
<td>180</td>
</tr>
</tbody>
</table>

*Expression patterns of individual drivers were verified using a UAS-lacZ reporter.

The failure to rescue non-conditional ecd mutants with Ecd targeted to the ring gland, or by 20E feeding, correlates with the absence of Ecd from the ring gland before the third instar. Taken together, the data show that ecd is autonomously required in other organs before it is needed for ecdysone synthesis. To identify the tissue-specific requirement, we have expressed Ecd using several other Gal4 drivers (Table 2). Ecd driven by the patched (ptc) promoter provided a partial rescue: a single copy of ptc-Gal4 allowed ecd² homozygotes to molt to the third instar; two copies supported formation of defective but tanned prepupae.

Cell-autonomous function of ecd in imaginal discs

To examine whether ecd plays a cell-autonomous role during development of the adult, we have generated mitotic clones homozygous for the null allele ecd² using the FLP-FRT system. Mutant clones of a non-essential gene, mbf1 (Liu et al., 2003), located as ecd on the 3L chromosome arm, served as a control. For both genes, wild-type sister clones and the heterozygous background were recognizable by the presence of ubiquitin-driven GFP and the mini white⁺ gene markers, placed on the homologous chromosome. When induced early during the first larval instar, large mbf1⁻/⁻ as well as mbf1⁺/+ clones appeared in the adult compound eyes. By contrast, only ecd⁻/+ clones were found with ecd² (Fig. 7A,D). The lack of ecd²/ecd² clones was confirmed by staining of imaginal discs, dissected from late third instar larvae: homozygous mutant clones were only found in mbf1 but not in ecd somatic mosaics (Fig. 7B-F). No defects were observed in the adult eyes, legs, wings or thorax derived from the imaginal discs where ecd²/ecd² clones were induced. As imaginal disc cells normally proliferate throughout larval life (Madhavan and Schneiderman, 1977), we assume that the ecd⁻/⁻ cells were replaced by their ecd⁺ neighbors. The loss of Ecd, however, does not seem to be immediately cell-lethal, because small ecd⁻/+ clones could be seen in eye-antennal imaginal discs when induced at the onset of the third instar (not shown).
Ecd is required for oogenesis

Ecd clearly plays a role in oogenesis, as the restrictive temperature prevents development of egg chambers beyond stage 8 in ecd flies (Audit-Lamour and Busson, 1981). To test whether Ecd is autonomously required in the somatic follicle cells, we induced homozygous ecd and control mbf1 clones in adult females. Ovaries with ecd–/– clones displayed defective egg chambers with extranumerary nurse cells, often double the normal 15 (Fig. 8A,B). Staining with an antibody against Orb, a protein that accumulates in the developing oocyte, confirmed that the aberrant egg chambers resulted from fusions of adjacent cysts, and not by overproliferation of the germline cells (Fig. 8C,D). Fasciclin III (FasIII), normally expressed by one pair of specific follicle cells at each pole of each egg chamber (Fig. 8A′), was detected only at the opposite ends of a fusion between two egg chambers (Fig. 8E). Defective egg chambers that had probably fused from several cysts early in their development showed multiple oocyte precursors (Fig. 8F), as well as FasIII-positive islands of cells (Fig. 8G). None of these defects occurred in ovaries containing large mbf1 mutant clones (not shown). These results show that ecd is required in the follicle cells for normal oogenesis.

To test for a direct role of Ecd in oocyte development, we induced ecd/ecd germline clones using the FLP-FRT system with the ovoD1 dominant female sterile marker. When recombination was induced during the first larval instar, control ovoD1 females laid eggs, whereas females (n=50) carrying the ecd mutation over ovoD1 did not. Their ovaries contained clonal egg chambers that did not stain with the anti-Ecd antibody (Fig. 9A,B) and that arrested prior to vitellogenesis. When recombination was induced in adult females, some of them laid a few eggs (on average 1 per female; n=70) 5-6 days later. Ovaries dissected 3 days after the induction contained mosaic egg chambers, in which some nurse cells lacked the Ecd protein, whereas others strongly stained with anti-Ecd antibody (Fig. 9C,D). Interestingly, only these ecd+/ecd– egg chambers progressed to vitellogenic stages, whereas those entirely devoid of Ecd arrested very early, showing degeneration of the nurse cells. Apparently the ecd+, ovoD1 nurse cells and their adjacent ecd, ovo+ sisters mutually rescued each other, thus allowing further development of the oocyte.
Discussion

The temperature-sensitive *Drosophila* mutation *ecd* has been widely used as an ecdysone-deficient background for developmental studies despite uncertainty about its molecular identity and other possible roles of the *ecdysoneless* gene. The aim of this study is to show that *ecd* encodes a conserved protein, previously not connected with steroid biosynthesis or any other function, and to demonstrate that besides its known steroidogenic role, this protein is required in a cell-autonomous manner independently of the blood-circulating hormone.

We have mapped molecular defects in the original *ecd* (Garen et al., 1977), in *ecd* (Sliter et al., 1989), and in two previously undescribed alleles, *ecd* and *ecd*. The point mutation found in *ecd* is consistent with its hypomorphic nature (Henrich et al., 1993). It converts a proline residue, conserved in all Ecd orthologs identified so far, into serine. This substitution does not cause degradation of the Ecd protein (Fig. 3B), or its subcellular mislocalization in the ring gland at 29°C (not shown). The mutation maps near the C terminus (Fig. 2), which must harbor an important function because a short truncation in *ecd* lacking this region is phenotypically as severe as the *ecd* mutation, removing almost the entire protein (Fig. 3A). Although the non-
conditional ecd<sup>−</sup> mutants die as second instar larvae, temperature shifts of the ecd<sup>+</sup> mutants suggest that Ecd is required during embryogenesis (Kozlova and Thummel, 2003). This early function may be executed by the maternally supplied Ecd protein, which is still detectable in first instar ecd<sup>−</sup> homozygotes (Fig. 3B). As the effects of ecd<sup>2</sup>, ecd<sup>d(3)23</sup> and ecd<sup>24</sup> are not worsened in hemizygous combinations with an ecd<sup>−</sup> deficiency, all of these three mutations are likely to represent ecd-null alleles. A single transgenic ecd<sup>+</sup> copy rescues all ecd<sup>−</sup> mutants to adulthood, showing that the developmental and lethal defects seen in these mutants are fully attributable to the loss of ecd function.

Although the non-conditional ecd<sup>−</sup> mutants often die during the ec dysis to the second instar, displaying phenotypes that might imply defective ec dysone production (Fig. 4B,C), their lethality cannot be a direct consequence of low blood ec dysone for the following reasons. First, ecd<sup>−</sup> animals cannot be advanced to the second molt by 20E feeding, despite the fact that similar doses of 20E are sufficient (1) to avert second instar lethality in mutants for the steroidogenic enzyme Dare (Freeman et al., 1999) and (2) to induce pupariation in ecd<sup>−</sup> larvae at 29°C. Second, as some of the ecd<sup>−</sup> animals die during the transition to the second instar, one would expect that their ec dysone titer would be lower from as early as the first instar. However, we have not found a reduction of ec dysone content in first instar homozygous ecd<sup>2</sup> larvae. Third, although Ecd is abundant in the lateral ring gland during the third instar, no such expression is seen at earlier stages. By contrast, some other steroidogenic genes, such as dib and sad, are strongly expressed in the ring gland beginning at embryogenesis (Chavez et al., 2000; Warren et al., 2002). Finally, development of ecd<sup>2</sup> homozygotes can be completely rescued with ubiquitous Ecd expression but not with Ecd targeted by the Feb36-Gal4 driver to the ring gland and to some other organs (Andrews et al., 2002). As Ecd presence in the ring gland cannot postpone the death of ecd-null mutants, Ecd must be required prior to the initiation of the second molt in some other tissues. One could be the nervous system (Fig. 6G), because patched-driven Ecd promotes further development of the mutants.

A cell-autonomous effect was previously demonstrated for the ecd<sup>+</sup> allele during differentiation of the thorax sensory bristles (Sliter, 1989). Unexpectedly, induction of ecd-null mitotic clones in the primordia of the adult thorax, the wing imaginal discs, did not produce any defective bristles. This was probably because no ecd<sup>−</sup> clones occurred in the adult epidermis. Based on the presence of twin ecd<sup>+</sup> clones in all imaginal discs and in the adult compound eye (Fig. 7), we conclude that the lost ecd<sup>−</sup> clones were replaced by proliferation of the surrounding ecd<sup>+</sup> cells. Redfern and Bownes (Redfern and Bownes, 1983) ascribed many of the defects seen in temperature-upshifted ecd<sup>−</sup> mutants to autonomous cell lethality in the imaginal discs. However, we have detected small clones of ecd<sup>−</sup> cells in imaginal discs upon induction of recombination during early third larval instar, and ecd<sup>−</sup> clones also survived in the adult ovary. Thus, the loss of ecd is not generally cell lethal although it reduces the ability of the mutant cells to proliferate at the normal rate. Our mosaic analyses provide direct evidence for a cell-autonomous, ec dysone-independent function of ecd, which may underlie the previously described defects in adult morphogenesis.

Clones of ecd<sup>−</sup> somatic follicle cells caused profound defects, manifest as fusions of adjacent egg chambers and leading to duplications of the nurse cell set, in some cases with two vitellogenic oocytes present at the opposite poles (Fig. 8D). Similar polarity defects were caused by perturbing the Delta/Notch signaling that specifies the polar follicle cells (PFC), and by perturbing the JAK/STAT pathway through which these cells establish proper separation between egg chambers (Gonzalez-Reyes and St Johnston, 1998; Grammont and Irvine, 2001; McGregor et al., 2002; Torres et al., 2003). It remains to be tested whether the egg chamber fusions in ecd mosaic ovaries might result from a compromised signaling by the PFC. Follicle cells are thought to be the major site of ec dysone production in the ovary (Lagueux et al., 1977; Zhu et al., 1983). However, it is difficult to imagine that the relatively small ecd<sup>−</sup> clones could significantly reduce the ec dysone titer in the female. Therefore we conclude that, as in the case of imaginal discs, the effects of ecd<sup>2</sup> on oogenesis are independent of free-circulating ec dysone.

Germline clones completely lacking ecd function arrest at pre-vitellogenic stages, probably earlier than egg chambers carrying the ovo<sup>B1</sup> mutation, thus showing that ecd is autonomously required for oocyte maturation.
This result is consistent with the phenotype of ecd⁻ mutant ovaries: ecd⁻ females become sterile after a few days at 29°C, with a majority of egg chambers at pre-vitellogenic stages (Audit-Lamour and Busson, 1981).

Interestingly, the steroidalogenic enzyme Dare, and the ecysone response proteins EcR and E75, are similarly required in the nurse cells for egg maturation, as germline clones mutant for these genes arrest as pre-vitellogenic egg chambers as well (Buszczak et al., 1999). This led the authors to propose that ecysone synthesis by the germline is necessary in an autocrine manner for the progression of oocytes to the vitellogenic stage. As normal ecd function is required for autonomous ecysone production by the ovary (Garen et al., 1977), the pre-vitellogenic arrest of the ecd⁻ germline clones is consistent with an autocrine germline function.

By inducing ecd² mutant clones in adult females, we created mosaic egg chambers in which some nurse cells were null for ecd, whereas others carried the ovo⁻Ds² dominant mutation that unconditionally blocks oogenesis. Surprisingly, these mixed-genotype egg chambers continued to mature much beyond the phase of arrest caused by either the ecd² or ovo⁻Ds² mutations acting alone (Fig. 9). This suggests a functional rescue among the cells within the egg chamber. As nurse cells are interconnected by ring canals, we speculate that the ecd⁺ ovo⁻Ds² cells and their ecd⁻, ovo⁺ sisters exchanged materials that complemented them and consequently permitted oocyte development. In the light of the autocrine germline hypothesis (Buszczak et al., 1999), an intriguing possibility is that the product of the ecd⁺ ovo⁻Ds² clones might be ecysone.

Although the ecysoneless gene encodes a protein with highly conserved regions, we have found no data that would describe the function of these regions and thus enlighten the mode of Ecd action. The only published report has implicated the human ortholog of Ecd, which compensates for the loss of an unrelated yeast protein GCR2 in transcriptional regulation (Deminoff and Santangelo, 2001). Our antibody detects Ecd predominantly in the cytoplasm, and thus does not directly support the possibility that Ecd acts at the level of transcription. We have initiated yeast two-hybrid studies to address the mechanism of Ecd action by identifying its protein partners. Until the exact function of Ecd is known, interpretations of results obtained with the ecysone-deficient ecd⁻ mutants should consider its non-steroidalogenic effects.

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