Unexpected Role of the Steroid-Deficiency Protein Ecdysoneless in Pre-mRNA Splicing

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Abstract

The steroid hormone ecdysone coordinates insect growth and development, directing the major postembryonic transition of forms, metamorphosis. The steroid-deficient ecdysoneless⁴ (ecd⁴) strain of Drosophila melanogaster has long served to assess the impact of ecdysone on gene regulation, morphogenesis, or reproduction. However, ecd also exerts cell-autonomous effects independently of the hormone, and mammalian Ecd homologs have been implicated in cell cycle regulation and cancer. Why the Drosophila ecd⁴ mutants lack ecdysone has not been resolved. Here, we show that in Drosophila cells, Ecd directly interacts with core components of the US snRNP spliceosomal complex, including the conserved Prp8 protein. In accord with a function in pre-mRNA splicing, Ecd and Prp8 are cell-autonomously required for survival of proliferating cells within the larval imaginal discs. In the steroidogenic prothoracic gland, loss of Ecd or Prp8 prevents splicing of a large intron from CYP307A2/spookier (spok) pre-mRNA, thus eliminating this essential ecdysone-biosynthetic enzyme and blocking the entry to metamorphosis. Human Ecd (hEcd) can substitute for its missing fly ortholog. When expressed in the Ecd-deficient prothoracic gland, hEcd re-establishes spok pre-mRNA splicing and protein expression, restoring ecdysone synthesis and normal development. Our work identifies Ecd as a novel pre-mRNA splicing factor whose function has been conserved in its human counterpart. Whether the role of mammalian Ecd in cancer involves pre-mRNA splicing remains to be discovered.

Introduction

The insect steroid hormones, ecdysteroids, regulate growth, stimulate molting, and orchestrate tissues to undergo complex morphogenetic changes during metamorphosis [1–3]. Temporal control of ecdysteroid synthesis by the larval prothoracic gland (PG) is therefore critical (for recent reviews see [4,5]). The biosynthetic pathway commences by converting cholesterol to 7-dehydrocholesterol by a Rieske oxygenase Neverland (Nvd) [6]. A short-chain dehydrogenase/reductase encoded by shroud (sro) [7] and cytochrome P450 (CYP) enzymes encoded by spook (spo)/spookier (spok), phantom (phm), disembodied (dib), and shadow (sad) then catalyze the subsequent steps to produce ecdysone (E) [8,9]. Once released from the PG, E becomes hydroxylated in peripheral tissues by another CYP, Shade (Shd), to yield the main active hormone, 20-hydroxyecdysone (20E) [10]. Reflecting the necessity of 20E for early cuticle formation, Drosophila melanogaster loss-of-function mutants that are available for sro [7], spo, phm, dib, sad, and shd [8] die as embryos.

Understandingly, Drosophila mutants that display reduced steroid titers during larval development provide invaluable experimental tools. Among these, the ecdysoneless⁴ (ecd⁴) mutants are homozygous viable at 22°C, but exposure to 29°C reduces their ecdysteroid title and causes a developmental arrest [11,12]. The ecd⁴ allele has been widely used since its discovery in 1977 [11] to test effects of ecdysteroid signaling on a number of processes from morphogenesis to reproduction to behavior. Yet why these mutants lack the hormone has not been determined. Our original identification of the ecd gene [13] has revealed homology from fission yeast to humans but none that would illuminate the mode of Ecd action.

Mammalian Ecd (also known as SGT1 and hEcd in humans) has been shown to stimulate cell proliferation by interacting with the Retinoblastoma (Rb) proteins [14]. Conditional deletion of the Ecd gene from mouse embryonic fibroblasts stalls these cells at the G1-S phase transition, suggesting that Ecd normally lifts the inhibitory effect of Rb on E2F-dependent cell cycle progression [14]. High hEcd expression has been correlated with malignancy of human breast [15] and pancreatic [16] tumors. In a mouse model, Ecd has been suggested to promote tumorigenesis via enhancing glucose import and glycolysis in the pancreatic tumor cells [16]. These observations illustrate the emerging importance of Ecd, but an underlying mechanism for Ecd action is still lacking.

A systematic mapping of Drosophila protein-protein interactions [17] has uncovered contacts between Ecd and proteins responsible for pre-mRNA splicing. Among these are members of the U5 small nuclear ribonucleoprotein particle (snRNP) complex,
Author Summary
Steroid hormones perform pivotal roles in animal development, sexual maturation, reproduction, and physiology. Also insects possess a hormonal steroid, commonly known as ecdysone, that was originally found to promote ec dyses in growing larvae and their metamorphosis to adults. Since the discovery of ecdysone-inducible puffs on the polytene chromosomes in the 1960’s, genetics of the Drosophila fruit flies has substantially advanced our understanding of steroid hormone impact on gene regulation during development. In the present study, we have solved an old puzzle of the “ecdysoneless” mutant that has traditionally provided investigators with a steroid-deficient animal model. Unexpectedly, we find that the Ecdysone-less protein (Ecd) does not primarily regulate ecdysone biosynthesis but that a critical steroidogenic enzyme requires Ecd for splicing of its precursor mRNA (pre-mRNA). Ecd physically contacts the immensely complex pre-mRNA splicing machinery. Outside the ecdysone-producing gland, Ecd is essential for survival of dividing cells within developing tissues. Despite vast evolutionary distance, a human homolog of Ecd can functionally substitute for its counterpart in the fly. A conserved role of Ecd in pre-mRNA splicing might underlie a recently described involvement of mammalian Ecd in cell cycle progression and its contribution to malignancy of certain tumor types.

including orthology of the RNA helicase Br2r, the GTPase Snu114, the Aar2 protein, and the highly conserved spliceosome core component, Prp8 [18]. Another global proteomic study [19] has detected a corresponding interaction between human Prp8 and hEcd. The spliceosome is a most elaborate machinery and pre-mRNA splicing is extensively coupled with transcription as well as with posttranscriptional mRNA surveillance and degradation of incorrectly spliced transcripts [20]. This network involves hundreds of proteins whose individual functions are often inferred from work on yeast, or still remain unknown [21–23]. Genetic studies in Drosophila have shown essential roles of several spliceosome components such as Prp8, Prp38, Prp31, or BCAS2 for tissue growth, cell proliferation or cell viability [24–27]. Proteomic dissection of spliceosomal complexes has revealed a large overlap in protein composition between Drosophila and humans while suggesting novel and/or fly-specific components [28].

With the hypothesis that Ecd might be a new player in pre-mRNA splicing, we have verified that Ecd interacts with a complex containing Drosophila Prp8, Aar2, Br2r, and Snu114 orthologs. Consistently with a vital role in pre-mRNA splicing, we show that Ecd is required by dividing imaginal disc cells for survival, even when their apoptosis is blocked or cell growth enhanced. In the larval PG, loss of Ecd compromises pre-mRNA splicing and abolishes protein expression of the essential steroidogenic enzyme Spok (CYP307A2), thus accounting for the systemic steroid deficiency in ecd mutants. Remarkably, human Ecd can functionally substitute for its fly ortholog.

Results
Ecd binds proteins within the spliceosomal U5 snRNP complex
The Drosophila Ecd protein has been recently associated with the U5 snRNP complex [17]. In order to verify the proteome-wide data, we expressed Myc epitope-tagged Ecd in Drosophila S2 cells and using mass spectrometry we examined material that co-precipitated with Myc::Ecd (Figure 1A). This analysis identified three proteins of the U5 snRNP complex that were previously shown to associate with Ecd [17], namely orthologs of the budding yeast (Saccharomyces cerevisiae) Prp8p, Snu114p, and Br22p that are encoded by D. melanogaster genes CG8877/prp8, CG4849/clau2, and CG5931/CG4849 respectively. Our mass spectrometry did not detect another Ecd interactor, identified as a product of the CG12320 gene [17] that is homologous to S. cerevisiae Aar2p. For clarity, we will refer to the Drosophila proteins as Prp8, Snu114, Br22, and Aar2.

To test the individual protein-protein interactions, we cloned the above Drosophila genes and performed co-immunoprecipitation with pairs of their epitope-tagged products expressed in S2 cells. First, we verified interactions between the fly counterparts of the known spliceosomal components. As expected, binding occurred between Prp8 and Snu114, Prp8 and Br22, and Prp8 and Aar2 (Figure 1B). Of these four proteins, Prp8 and Aar2 formed complexes with Ecd, whereas binding between Ecd and either Br22 or Snu114 was not detected (Figure 1C). It was of interest to examine interactions of mutated Ecd versions that occur in Drosophila. We chose the temperature-sensitive ecd2 allele that carries a single Pro-656 to serine substitution and the non-conditonal lethal, ecd(3)23, where the Ecd polypeptide is prematurely terminated after Ser-649, thus lacking the C-terminal 35 amino acids [13]. Both the Ecd(3)23 (tested at a non-permissive temperature of 30 uC) and Ecd(ecd2) mutant proteins retained the capacity to bind Prp8 and Aar2 (Figure 1C). This indicates that the steroid-deficiency and lethality phenotypes in Drosophila result from a failure of the mutant Ecd protein to perform functions other than binding Prp8 and Aar2. The above results suggest that Ecd interacts with at least two well-established members of the U5 snRNP complex, of which Prp8 is evolutionarily the best-conserved core protein of the spliceosome [18].

Subcellular localization of the spliceosomal proteins and Ecd
In S. cerevisiae, a part of the U5 snRNP complex that contains Prp8p, Snu114p, and Aar2p assembles in the cytoplasm before being imported to the nucleus, where Br22p replaces Aar2p [29,30]. Localization of Drosophila Prp8, Snu114, Aar2 or Br22 has not been previously reported. Using the epitope-tagged proteins in transfected S2 cells, we detected Prp8, Snu114, and Aar2 predominantly in the cytoplasm, whereas Br22 occurred in the nucleus (Figure 2A). Expression of Flag::Prp8 in transgenic Drosophila larvae revealed that Prp8 also resided in the cytoplasm of the PG (Figure S1A).

The Ecd protein was previously detected in the cytoplasm of Drosophila PG cells [13]. We confirmed this result with a new antibody raised against the N-terminal part of Ecd for the endogenous protein (Figure S1B, S1C) and for Ecd overexpressed in the PG using the phi-Gal4 driver (Figure S1D). This antibody also detected a clear cytoplasmic signal in the wing imaginal discs (Figure S2). The cytoplasmic localization of hEcd in human cells was shown to depend on leptomycin B-sensitive nuclear export [31]. In transfected S2 cells, GFP-tagged Ecd co-localized in the cytoplasm with Prp8 (Figure 2A). Only when the cells were treated with leptomycin B, some of the GFP::Ecd fusion protein was retained in the nucleus (Figure 2B). Therefore, together with the spliceosomal proteins with which Ecd interacts, Ecd primarily resides in the cytoplasm. Like its human counterpart, Ecd is subject to active nuclear export.
Cell-autonomous requirement for Ecd and Prp8 in imaginal discs

If Ecd plays a role in pre-mRNA splicing as suggested by its protein interactions, its loss would be expected to disturb vital cellular functions as has been found for several Drosophila splicingomosomal proteins including Prp8 [24–27]. To track the fate of Ecd-deficient imaginal cells in vivo, we employed the MARCM technique [32] using the non-conditional ecd(3)23 allele that behaves as a genetic null [13]. In contrast to control, no mitotic clones homozygous for this mutation were found in eye/antennal or wing imaginal discs of third-instar larvae (Figure 3A, 3B, 3E, 3F). Rare and extremely small ecd(3)23 clones could be detected when cell death was prevented in these clones with the anti-apoptotic protein p35, or when their cellular growth was enhanced with activated RasV12 (Figure 3C, 3D). To achieve a milder loss-of-function effect, we generated transgenic flies for RNAi-mediated silencing of ecd. Observation of wing discs two days after heat-dependent clonal induction of ecd dsRNA revealed frequent ecdRNAi clones. These gradually disappeared over the next 24 hours (Figure 3G, 3H), indicating that the cells autonomously required Ecd to survive.

To see the effect of Ecd knockdown on morphogenesis, we induced ecd RNAi in a restricted region along the anterior-posterior boundary of the wing imaginal disc using the dpp-Gal4 driver. Figure S2 shows depletion of the Ecd protein from the dpp-expressing cells. The loss of Ecd disrupted the regular pattern of the dpp expression domain, and the affected cells underwent apoptosis as assessed by active Caspase 3 staining (Figure 4A, 4B). Consequently, the adult wings of dpp>ecdRNAi flies displayed reduced size of specific intervein regions and loss of the anterior crossvein (Figure 4E, 4F). The same anomalies were induced by RNAi against the components of the U5 snRNP complex, Prp8 (Figure 4C, 4G) and Brr2 (Figure S3A, S3B). Overexpression of Ecd in the Ppc-deficient cells did not suppress the ecd RNAi phenotype (Figure S3C, S3D), suggesting that Ecd plays a unique role that cannot be substituted by surplus of its partner protein. Strikingly, apoptosis of imaginal disc cells expressing ecd dsRNA as well as the adult wing defect could be averted by supplementing the human ortholog, hEcd (Figure 4D, 4H).

Loss of Ecd disrupts splicing of spok pre-mRNA and eliminates its protein product

To address the putative role of Ecd in ecdysone biosynthesis, we removed the Ecd protein specifically from the PG by triggering ecd RNAi with the phm-Gal4 driver [33] (Figure S1C). Affected larvae reached the third instar but were unable to pupate. Unlike in the imaginal discs, depletion of Ecd was not cell-lethal in the polyploid PG, although it reduced the size of PG cells and nuclei (Figure 5A, 5B). Using available antibodies, we could therefore examine expression of steroidogenic enzymes, namely CYP307A2/Spok and CYP306A1/Phm, in late-third instar phm>ecdRNAi larvae. The strong Spok staining in control PGs was completely lost in the Ecd-deficient gland, whereas the Phm signal appeared only partly reduced (Figure 5A, 5B). While both enzymes are indispensable for E biosynthesis, Spok acts upstream of Phm in the larval PG to mediate the essential conversion of 7-dehydrocholesterol to ketodiol [33,34]. Thus, PGs deprived of Phm cannot synthesize ecdysone even in the presence of Phm. As was the case in the wing imaginal discs, expressing the human Ecd ortholog in the PG compensated for the depletion of the endogenous Ecd protein. hEcd improved morphology of the gland (Figure 5G) and restored expression of Spok (Figure 5C).

Similarly to ecd RNAi, knockdown of the splicingomosomal proteins Prp6 (Figure 5D) and Brr2 (Figure S4A, S4B) also reduced size of the PG and eliminated the Spok protein while only partially reducing Phm levels. These results suggested that expression of Spok, but not of Phm, was highly sensitive to perturbation of pre-mRNA splicing. Interestingly, the phm gene contains two small introns, whereas spok lies within the heterochromatin and its single intron separates the two coding exons by nearly 30 kilobases (Flybase, http://flybase.org/) (Figure 6A).

To examine whether the absence of Ecd affects spok pre-mRNA splicing, we determined the pre-mRNA:mRNA ratio, a standard measure of intron retention or splicing defect (e.g., [35–37]). In phm>ecdRNAi larvae and in larvae homozygous for the temperature-sensitive ecd allele, mRNA levels were measured using quantitative reverse-transcription (qRT) PCR with primers positioned in two separate exons, and pre-mRNA was amplified with primer pairs spanning exon-intron boundaries or primers within the intron (Figure 6A). All RNA samples were free of residual genomic DNA and qRT-PCR data were normalized to rp49 mRNA levels that did not change appreciably between control and ecd-deficient larvae (Figure S5). Because spok and phm are expressed specifically in the PG during larval development, we were able to use RNA from entire larvae.

As assessed with all three pre-mRNA-specific primer sets, spok pre-mRNA:mRNA ratio strongly increased in both phm>ecdRNAi larvae and in ecd mutants upshifted to 29°C (Figure 6B). The relative enrichment of unspliced to spliced spok transcript was markedly stronger upon PG-specific knockdown of ecd than in the hypomorphic mutants. Unexpectedly, loss of ecd also reduced levels of spok pre-mRNA, again more substantially in phm>ecdRNAi larvae (Figure 6C), suggesting that in addition to splicing, either spok transcription or stability of its pre-mRNA might have been affected. Consistently with the absence of the Spok protein (Figure 5B’), ecd RNAi diminished spok mRNA (Figure 6D). Levels of phm pre-mRNA and mRNA were also lowered in PGs of ecd-deficient animals relative to controls (Figure 6C, 6D). However, in contrast to spok there was no significant increase of the pre-mRNA:mRNA ratio and therefore no appreciable intron retention in the case of phm (Figure 6B). These data suggested that Ecd was required for splicing of the large intron from the spok pre-mRNA. For comparison, we examined the effect of Prp8 knockdown. As expected, spok mRNA disappeared in phm>prp8RNAi larvae due to compromised splicing (Figure 6E). However, because the absolute amount of unspliced spok transcript accumulated upon prp8 RNAi, the pre-mRNA:mRNA ratio rose dramatically more than in the case of ecd RNAi (Figure 6E).

Finally, we tested whether human Ecd restored Spok expression at the level of pre-mRNA splicing. In a separate set of experiments, we confirmed that the loss of spok mRNA was...
accompanied by intron retention (increased pre-mRNA:mRNA ratio, Figure 6F). Importantly, expression of hEcd in the PG of phm ecdRNAi larvae restored levels of spok mRNA, pre-mRNA, and of their normal proportion (Figure 6F), suggesting that the human protein could substitute for Ecd in splicing of spok pre-mRNA.

Systemic effect of PG-specific loss of Ecd

Because the PG produces a circulating hormone, knockdown of Ecd would be expected to affect the entire organism even when restricted to the gland. In agreement with the absence of the Spok/CYP307A2 protein (Figure 5B”), third-instar phm>ecdRNAi larvae on day 6 after egg laying (AEL) showed reduced ecdysteroid titer (Figure S6A). Compared to controls, they were retarded in growth (Figure 7A–C). Without interruption, they continued to feed for another 10 or more days, producing large “permanent” larvae (Figure 7D). This phenotype corresponded with effects of lacking ecdysteroid surge and inadequate expression of steroidogenic genes including spok and phm [38,39]. Although feeding phm>ecdRNAi larvae on day 5 AEL with 20E was not sufficient for pupation, it induced wandering behavior (Figure S6B). Importantly,

Figure 2. Subcellular localization of Ecd and the US snRNP proteins. (A) Br2 was enriched in cell nuclei while Ecd, Prp8, Snu114, and Aar2 were detected in the cytoplasm. The Flag and Myc epitope-tagged proteins were expressed in S2 cells; mRFP::Prp8 and GFP::Ecd (bottom row) were co-transfected. (B) Inhibition of nuclear export with leptomycin B resulted in nuclear retention of the GFP::Ecd protein. Nuclei are stained with DAPI. All images are single confocal sections. Scale bars, 5 μm. doi:10.1371/journal.pgen.1004287.g002
substituting hEcd for the depleted fly protein in the PG increased ecdysteroid titer (Figure S6A) and restored normal growth and development of phm\(>\)ecdRNAi animals to adults (Figure 7A, 7E, 7F).

The PG-specific knockdown of Ecd was accompanied by lowered mRNA levels of at least two 20E-response genes, the Ecdysone receptor (EcR) and E74, as assessed in whole phm\(>\)ecdRNAi larvae (Figure 7G). A similar reduction in EcR and E74 mRNAs occurred in ecd\(^{0}\) mutant larvae at 29°C (Figure 7G). However, ecd\(^{0}\) mutants could have lost these transcripts for two different, mutually non-exclusive reasons: (1) due to the lack of circulating ecdysteroids, and/or (2) because Ecd may be required for splicing of the EcR and E74 pre-mRNAs, both of which contain multiple large introns. Indeed, we found a major rise in the pre-mRNA:mRNA ratio for EcR (but not for E74; data not shown) in ecd\(^{0}\) mutants, and a much less pronounced increase of this ratio in phm\(>\)ecdRNAi larvae (Figure 7H). These data suggest that EcR pre-mRNA splicing was sensitive to loss of ecd function in tissues throughout the body. In support of this notion, we observed that dietary 20E did not induce EcR mRNA expression in ecd\(^{0}\) mutants at 29°C nearly as efficiently as under the permissive temperature or in phm\(>\)ecdRNAi larvae (Figure S6C), where ecd function in the peripheral tissues was unaffected.

Discussion

Ecd, originally discovered through the “ecdysoneless” Drosophila ecd\(^{0}\) mutant [11], has become known as a product of the CG5714 gene [13]. The steroid deficiency results from an autonomous failure of the larval PG [12,40] and of the adult ovary [11,40,41]. However, some effects of the ecd\(^{0}\) allele are autonomous to the imaginal discs and other tissues, and therefore not attributable to the lack of the hormone [41,42]. This notion has been confirmed upon molecular definition of ecd-null mutations whose effects could not be remedied with exogenous 20E [13]. As no causal relationship between Ecd and E biosynthesis could be established, Ecd has been omitted from the current list of ecdysteroidogenic factors [4,5,8,9]. Nonetheless, the ecd\(^{0}\) background is still in use to demonstrate specific effects of 20E (e.g., [43]).

Our present work shows that Ecd indeed does not primarily regulate steroidogenesis. Although abolished expression of the essential steroidogenic enzyme Spok in Ecd-deficient larvae inevitably causes ecysyne deficiency, Ecd should not be regarded as a regulator of the E biosynthetic pathway. Rather, the absence of Spok in the PG reflects a more general role of Ecd within the complex network of pre-mRNA splicing. This function is required in the PG as well as in some other Drosophila organs, and is likely to be common to flies and mammals.

A role for Ecd in pre-mRNA splicing

A proteome-wide study [17] has uncovered interactions of Drosophila Ecd with multiple U5 snRNP-associated spliceosomal proteins. We likewise found a complex of Ecd with Ppr8, Snu114, and Br2 using mass spectrometry, and interactions of Ecd with Ppr8 and Aar2 by immunoprecipitation. We detected Brr2 in cell nuclei, whereas Ecd co-localized with Ppr8, Snu114, and Aar2 in the cytoplasm of Drosophila cells. In contrast, S. cerevisiae Ppr8p resides in the yeast nuclei owing to its nuclear localization signal [29]. Although Ppr8 is remarkably well conserved [18], this particular sequence shows poor homology with the fly protein. Nuclear import of Drosophila Ppr8 might therefore rely on another mechanism. Nonetheless, our results conform to a current model from S. cerevisiae, where Ppr8p, Snu114p, and Aar2p preassemble in the cytoplasm, then upon nuclear import Aar2p is replaced by Brr2p as the U5 snRNP complex matures [29,30]. Nuclear-cytoplasmic shuttling of Ecd, which also occurs in human cells [31], corresponds with such translocation.
Phenotypes caused by disrupted pre-mRNA splicing match those inflicted by loss of Ecd. Cell death of Drosophila imaginal disc clones lacking spliceosomal proteins Prp38, MFAP1, and BCAS2 has been reported [24,25]. Our data show that knockdown of the Prp8 and Brr2 proteins caused apoptosis of imaginal disc cells and recapitulated a specific ecd RNAi wing phenotype. Removal of Prp8 or Brr2 from the PG eliminated the Spok protein, confirming that its expression was sensitive to deficiency in pre-mRNA splicing factors. Similar to Prp8 knockdown, spok pre-mRNA:mRNA ratio strongly increased in ecd or phm RNAi larvae, thus evidencing a role of Ecd in spok pre-mRNA splicing. However, removal of Ecd also affected transcription as judged from reduced levels of spok or phm pre-mRNAs. It is important to note here that pre-mRNA splicing is not an isolated event but that it is intimately coupled with transcriptional elongation [44,45] as well as with 3′-end processing, quality control, and nuclear export or degradation of the RNA product (reviewed in [20]). Indeed, there are indications that hEcd may be involved in transcriptional regulation in mammalian cells [31].

An intriguing question is why would dysfunction of Ecd impact splicing of spok but not phm pre-mRNA in the steroid-producing gland? Surprisingly, even the relatively few and simple intron-containing genes in S. cerevisiae have been shown to respond differently to mutations in individual splicing core components [36]. In multicellular organisms, spliceosomal proteins such as Prp8 are not ubiquitously expressed and not equally required to splice every pre-mRNA [46]. Instead, many perform exquisitely tissue-specific, gene-specific, and even exon-specific functions. An RNAi screen in Drosophila cells has uncovered the necessity of several core spliceosomal proteins including Brr2 for inclusion of particular exons in different, alternatively spliced transcripts [47]. A striking example of how remarkably specific physiological functions may be affected by faulty pre-mRNA splicing is the human disease retinitis pigmentosa (RP), where degeneration of photoreceptor cells leads to blindness. Autosomal-dominant forms of RP are linked to mutations in Prp8, Prp31, Prp3, and PAP1 homologs that contribute to the U5/U4/U6 tri-snRNP complex [46,48]. One plausible explanation for the sensitivity of the photoreceptor neurons to defective splicing is insufficient production of mRNAs that are highly expressed in these cells [48]. It has been argued that ubiquitously expressed pre-mRNAs carry universal splicing signals in order to be correctly spliced in any cell type, whereas transcripts with restricted expression patterns might rely on less robust, tissue-specific splicing signals that are
more prone to fail in the absence of individual splicing factors [46].
Although the mechanism of Ecd action remains to be determined,
we presume that spok pre-mRNA might be particularly sensitive to
the absence of Ecd, Prp8 or Brr2 for similar reasons.

Figure 5. Ecd and Prp8 are required for expression of Spok in the PG. (A–D) Relative to control PG dissected 6 days AEL (A’, A’), expression
of the Spok protein (B’) was undetected while the Phm signal (B”) was weakened in the PG of phm>ecdRNAi (B) and phm>prp8RNAi (D’, D”) larvae.
Note the moderate reduction in size of PG cells and nuclei in phm>ecdRNAi (B) compared to a more severe PG deterioration in phm>prp8RNAi larvae (D).
Expression of hEcd restored Spok and Phm expression (C’, C”) and improved the morphology of the Ecd-deficient PG (C). Cell membranes are
decorated with CD8::GFP; DAPI stains the nuclei. Panels show single confocal sections. Scale bars, 20 μm. See Figure S1 for RNAi-mediated depletion
of Ecd.
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Ecdysone-dependent versus organ-autonomous
consequences of Ecd depletion

In addition to abolishing the expression of Spok, loss of ecd function partially reduced mRNA and pre-mRNA levels of phm (Figure 6C, 6D) and of two other genes (nvd and dib; data not
shown) that are likewise required for E biosynthesis. However, the
lower expression of phm, nvd and dib was not accompanied by
increased pre-mRNA:mRNA ratios, indicating that pre-mRNA
splicing of these genes did not depend on Ecd. These results might
suggest that Ecd exerts some effect on transcription or transcript
stability. Alternatively, Ecd might be required for pre-mRNA
splicing of a factor acting upstream of the steroidogenic gene
expression. One such candidate is EcR, which has recently been
shown to mediate a positive-feedback of 20E on the expression of
phm, dib, sro and sad, but not of spok [49]. Elevated pre-
mRNA:mRNA ratio indicated that splicing of EcR pre-mRNA
was compromised in ecd” mutants (Figure 7H), and the EcR
protein was markedly reduced in the PGs of phm>ecdRNAi larvae
(Figure S6D). Therefore, whereas the absence of the Spok protein
primarily resulted from disrupted splicing, the partial reduction of
nvd, phm and dib expression might be attributable to the lack of EcR
in the PG of phm>ecdRNAi larvae.

A failure of the PG to produce E inevitably provokes systemic
developmental defects in peripheral organs. However, an issue arises
with the ecd” mutants as to whether any observed tissue-specific
phenotype may be ascribed to the lacking hormone. Because Ecd is
required cell-autonomously, effects of ecdysteroid deficiency and
those caused by loss of any cell-autonomous Ecd function cannot be
discriminated in ecd” mutant background. For example, low
expression of 20E-response genes such as EcR in ecd” larvae likely
results from a combined impact of disrupted E synthesis in the PG
and compromised EcR pre-mRNA splicing in the peripheral tissues.
The problem of 20E-dependent and 20E-independent effects of
ecd” mutation may be reflected by a recent transcriptome
analysis, revealing that of about a thousand genes affected in
ecd” background, only a minority were regulated by the 20E receptor,
EcR [43].

Based on the elimination of ecd” mutant clones from the
imaginal discs, we conclude that cells lacking Ecd cannot be
sustained within proliferating tissue context. This was the case even when the mutant clones were protected from apoptosis with p35, or enhanced for growth through Ras V12. Small ecd(3)23 or ecdRNAi clones were replaced by surrounding cells without phenotypic consequences for the adult. In contrast, ecdRNAi delivered to larger areas was lethal or caused visible defects, such as the aberrant wings in dpp>ecdRNAi flies. Rarely emerging MS1096>ecdRNAi flies had vestigial wings (Figure S7A) similar to those induced by depletion of the BCAS2 spliceosomal protein under the same Gal4 driver [25]. Interestingly, imaginal disc cells were not affected by triggering ecdRNAi after their proliferative phase was completed, as in the eyes of GMR>ecdRNAi flies (Figure

Figure 6. Loss of Ecd interferes with splicing of spok pre-mRNA. (A) Schematic of Drosophila spok and phm gene loci. Open and colored boxes represent untranslated and translated exons, respectively; black arrows point in the direction of transcription. Colored arrowheads mark the positions of primer pairs used to discriminate between pre-mRNA and mRNA species by qRT-PCR: yellow, in exons separated by an intron; red, within an intron; green and blue, spanning exon-intron boundaries of the first (EI1) and second (EI2) spok exon, respectively; black arrows point in the direction of transcription. Colored arrowheads mark the positions of primer pairs used to discriminate between pre-mRNA and mRNA species by qRT-PCR: yellow, in exons separated by an intron; red, within an intron; green and blue, spanning exon-intron boundaries of the first (EI1) and second (EI2) spok exon, respectively. (B) Pre-mRNA:mRNA ratios, determined by qRT-PCR with primers depicted in (A), were significantly elevated for spok but not for phm in third-instar phm->ecdRNAi larvae and in ecd1 mutants at 29°C. (C, D) spok and phm pre-mRNA (C) and mRNA (D) levels decreased in phm->ecdRNAi and ecd1 larvae. Levels of α-tubulin 84B mRNA did not change upon ecd RNAi and expression of ecd itself was unaffected by the ecd1 mutation (D). (E) RNAi knockdown of prp8 in the PG diminished spok mRNA, whereas unspliced transcripts accumulated, causing the pre-mRNA:mRNA ratio to rise dramatically. (F) Expression of hEcd in the Ecd-deficient PG restored normal spok transcription and pre-mRNA splicing as judged from restored levels of pre-mRNA, mRNA, and their ratio. In all experiments, qRT-PCR was performed with total RNA from whole larvae 6 days AEL, and levels of rp49 transcripts were used for normalization. The pre-mRNA:mRNA ratios in (B, E, F) were calculated from the normalized qRT-PCR data by dividing values obtained with intron primer sets (A, red triangles) or primers spanning exon-intron boundaries (green or blue triangles) with values obtained using mRNA-specific primers (yellow triangles). Data are mean ± S.E.M; n≥4; *p<0.05, **p<0.01, and ***p<0.001. doi:10.1371/journal.pgen.1004287.g006
S7B; *GMR-Gal4* is a strong but late-acting driver expressed predominantly in the post-mitotic cells posterior to the eye morphogenetic furrow [50]. Intriguingly, deletion of Ecd in mouse embryonic fibroblasts has been shown to cause a proliferative block and to reduce expression of several cell-cycle regulators such as CyclinE1 (CycE1) downstream of the transcription factor E2F [14]. However, we were unable to advance proliferation of *ecdRNAi* clones in imaginal discs by expressing *Drosophila* CycE [51] or combinations of either E2F with its partner DP [52] or CycD with Cdk4 [53] (data not shown). Therefore, the lack of Ecd in proliferating tissues cannot be compensated by gain of individual factors that stimulate cellular growth or cell cycle progression. These results suggest that proliferating cells are particularly sensitive to loss of Ecd. Such sensitivity corresponds to the fact that splicing factors prevailed among genes positively screened as being required for cell division in a human cell line [54].

### Evolving Ecd function?

Unlike some of the core spliceosomal factors, no Ecd-like protein has been found in the budding yeast, *S. cerevisiae*, in which only 5% of the genes contain introns. This contrasts with 43% of intron-containing genes in the fission yeast, *Schizosaccharomyces pombe* [55], where the spliceosomal protein composition is more akin to humans than to the budding yeast [56]. Interestingly, *S. pombe* has an Ecd ortholog, Sgt1p, which resides in the cell nuclei and is essential for the yeast growth [57]. Conditional *Sgt1* mutation alters expression of genes involved in virtually all cellular processes including metabolism, and impairs growth on glucose.

Figure 7. PG-specific loss of Ecd results in systemic effects. (A–E) Growth of *phm>ecdRNAi* larvae was retarded. Although reaching the third instar, *phm>ecdRNAi* larvae were smaller on day 6 AEL (A, C) compared to controls (B) or larvae supplemented with hEcd (*phm>ecdRNAi hEcd*) (E). Deficiency of Ecd in the PG prevented metamorphosis, generating permanent, eventually lethal larvae (D). (F) Expression of hEcd in the Ecd-deficient PG restored development of normally sized adults. (G) Expression of *EcR* and *E74* (as assessed by qRT-PCR with primer sets detecting all alternatively spliced mRNA isoforms of each gene, see Table S1) was lowered to a similar degree in *phm>ecdRNAi* and in *ecd1* larvae at 29°C. (H) The *EcR* pre-mRNA:mRNA ratio increased much more dramatically in *ecd1* mutants at 29°C than in *phm>ecdRNAi* larvae. Data are mean ± S.E.M; n≥4; **p<0.01, and ***p<0.001.

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ecdRNAi

UAS-GFP/CyO; FRT2A tubGal80/TM6B females were crossed to generate UAS-ecdRNAi

site-directed mutagenesis by CCT to TCT codon transition. To (not specified otherwise, crosses were carried out at 25

w1118 established with standard P Flag::hEcd [58]. New transgenic lines carrying UAS

11066), was cloned into the pET28b plasmid (Novagen), and the antigen

Drosophila gentec) against bacterially expressed TM6B

ecd1 37

Recombination was induced by exposing progeny to heat shock at

males at 20

y HS FRT2A/TM6B, and control

C for 30 min, followed by incubation for 48 and 72 h at 25

plasmid pWIZ vector (DRC, 110666), phm-Gal4, UAS-CyDs::GFP [33], and dpp-Gal4, UAS-GFP [58]. New transgenic lines carrying UAS-ecd, UAS-ecdRNAi, UAS-Flag::Ecd, UAS-Flag::p63, and UAS-Flag::Sgt1 constructs were established with standard P-element germline transformation. If not specified otherwise, crosses were carried out at 25°C. Crossing all Gal4 driver lines to U118 background provided controls for each experiment. Mutant clones within eye/antennal imaginal discs were generated using the MARCM (mosaic analysis with a repressible cell marker) method [32] with eyGal4, act>y>Gal4, UAS-GFP/CyO; FRT2A tubGal80/TM6B flies as described [59]. To induce ecdRNAi “flip-out” clones, hsFLP; act>y>Gal4, UAS-GFP/CyO [60] females were crossed to UAS-ecdRNAi males at 20°C. Recombination was induced by exposing progeny to heat shock at 37°C for 30 min, followed by incubation for 48 and 72 h at 25°C prior to dissection. Temperature-sensitive ecd and control U118 larvae were grown at 22°C until day 4 AEL, then placed to 37°C for 45 min and to 29°C until dissection on day 6 AEL.

Plasmids and Ecd antibody production

Coding Drosophila melanogaster DNA sequences of ppp8 (CG8877, sma114 (CG4849), am2 (CG12320), bre (CG5931), ed (CG5714, ecd3523) (amino acids 1-650 of Ecd), and human Ecd (hg1, NP_069196.1) were amplified from respective cDNAs using the Phusion polymerase (New England Biolabs). Schizosaccharomyces pombe Sgt1 (SPAC1002.11c) was amplified from genomic DNA. See Table S1 for all PCR primers. The fragments were cloned into pTFW, pTMW or pTGW vectors enabling expression of proteins with N-terminal Flag, Myc, or GFP tags, respectively (T. Murphy, Carnegie Institution of Washington), using the Gateway cloning system (Invitrogen). The ed cDNA was mutated using site-directed mutagenesis by CCT to TCT codon transition. To generate UAS-ecdRNAi, a 497-bp cDNA fragment from the ed gene was amplified and cloned as inverted repeat into the pWIZ vector [61]. A polyclonal rat anti-EcdNocm antibody was raised (Eurogentec) against bacterially expressed Drosophila Ecd polypeptide (amino acids 204-458). The corresponding ed cDNA fragment was cloned into the pET28b plasmid (Novagen), and the antigen was purified using a hexahistidine tag.

Mass spectrometry and protein identification

Protein extracts from S2 cells containing Myc::Ecd or the empty pTMW vector (for control) were resolved on SDS-PAGE. Upon silver staining (SilverQuestTM, Invitrogen), bands of interest were cut out. In-gel tryptic digestion with 12.5 ng/µl porcine trypsin (Promega) in 10 mM NH4HCO3 and further extractions were performed as described [62]. Collected extracts were concentrated by vacuum centrifugation and desalted using STAGE Tip C18 spin columns (Proxeon, Thermo Scientific) [63]. Eluted peptides were vacuum-concentrated and resuspended to a final volume of 20 µl in 0.5% acetic acid, of which 10 µl were used for analysis. Reversed-phase liquid chromatography (LC) coupled to nano-flow electrospray tandem mass spectrometry (MS) were carried out using an EASY nLC II nano-LC (Proxeon, Thermo Scientific) with a C18 column (internal diameter 75 µm) coupled to a LTQ Orbitrap mass spectrometer (Thermo Scientific). Peptide separation was performed at a flow rate of 250 nL/min over 60 min (10–40% acetonitrile; buffer A: 0.1% formic acid in water; buffer B: 0.1% formic acid in acetonitrile). Survey full scan MS spectra (m/z 350 to 2000) of intact peptides were acquired in the Orbitrap at a resolution of 30,000 using m/z 445.12003 as a lock mass. The mass spectrometer acquired spectra in data dependent mode and automatically switched between MS and MS/MS acquisition. Signals with unknown charge state and +1 were excluded from fragmentation. The ten most intense peaks (threshold 500) were isolated and automatically fragmented in the linear ion trap using collision induced dissociation (CID).

The search algorithm Mascot [64], implemented in the Proteinscape software (Bruker), was used for peptide and protein identification. MS/MS data were searched using the canonical and isoform sequence database of the Drosophila melanogaster complete proteome, provided by the UniProt Consortium. Oxidation of methionine residues was used as a variable modification and carbamidomethylation of cysteine residues as a fixed modification. For Orbitrap data, 10 ppm mass tolerance was
allowed for intact peptide masses and 0.8 Da for CID fragment ions detected in the linear ion trap. Peptides were filtered for Mascot score ≤20. Protein identifications were based on at least two peptides.

Cell and tissue immunostaining

*Drosophila* S2 cells grown on cover slips, dissected ring glands and imaginal discs were processed as described [65] and stained overnight at 4°C (tissues) or 2 h at room temperature (S2 cells) with the following antibodies: rat anti-Ecd*Ne expres*(1:500, this study), guinea pig anti-Spok (1:1000) and rabbit anti-Phm (1:300) [33], rabbit anti-cleaved Caspase-3 (1:500, ASP175, Cell Signaling, #9661), rabbit anti-pH3 (1:100, Cell Signaling, #9701), mouse anti-Flag M2 (1:300, Sigma Aldrich), rabbit anti-c-Myc (1:500, sc-7899, Santa Cruz), and mouse anti-Lamin (1:500, ADL67.10), mouse anti-EcR (1:200, DDA2.7), and mouse anti-Fascin III (1:300); the latter three from the Developmental Studies Hybridoma Bank (DSHB, Iowa). After washing, samples were incubated with corresponding secondary antibodies coupled to Cy3 or Cy5 (Jackson ImmunoResearch), counterstained with DAPI (0.5 μg/ml, Invitrogen) to visualize nuclei, and mounted in Dabco:Mowiol (Sigma-Aldrich).

Image acquisition and processing

Confocal single sections and stacks were acquired at room temperature with Olympus FV1000 confocal microscope. Maximum projections were generated using Fluoview 2.1c Software (Olympus) and ImageJ [66]. Final image processing including panel assembly, brightness and contrast adjustment were done in Photoshop CS3.1 (Adobe Systems, Inc.). To allow comparison among genotypes, images were taken and processed with the same settings. Adult wings were mounted in Hoyer’s medium. Images of larvae, flies, adult wings, and Z-stacks of adult eyes were taken using Leica M165 FC fluorescent stereomicroscope equipped with a DFC490 CCD camera. Images were processed using a Multifocus module of the LAS 3.7.0 software (Leica).

qRT-PCR

Total RNA was isolated from eight third-instar larvae with IsolRNA Lysis Reagent (5 Prime). cDNA was synthesized from 2 μg of RNA treated with DNase I (Ambion) using random primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed in triplicates with the SYBR green mix (Bio-Rad, Hercules, CA) using the CFX96 (Bio-Rad, Hercules, CA) or the 7900HT (Applied Biosystems) real-time PCR systems. qRT-PCR primers (Table S1) were designed to anneal at 62°C. Primers were initially tested by qPCR on serially diluted DNA templates and those deviating from typical standard curves were redesigned. Data were normalized to *Gapdh* levels and fold changes were calculated using the ΔΔCT method [67] or the Relative standard curve method [68]. At least four biological replicates were analyzed per each experiment.

Hormone feeding and ecdysteroid measurement

For hormone feeding, 40 larvae aged 5 days AEL were transferred per vial on food containing 100 μg/ml of 20E. Control food contained solvent only (6% w/w ethanol). Total RNA was isolated after 4 h (for *ecd*Δ) or one day of feeding (6 days AEL for *phm-Gal4* experiments); wandering behavior was photographed on day 7 AEL. To measure ecdysteroid titer, larvae 6 days AEL (15 animals per assay) were homogenized in 500 μl of methanol and centrifuged at 20,000 g. The pellets were re-extracted with 300 μl methanol and supernatants pooled and vacuum dried. The dried extracts were processed using the ELISA immunoassay system (Cayman Chemical) as described [69]. Ecdysteroid concentration was calculated from an eight-point standard curve of serially diluted 20E.

Statistical analyses

An unpaired two-tailed Student’s *t*-test with unequal variation and one-way analysis of variance (ANOVA) with a post hoc Newman-Keuls Multiple Comparison Test were used to determine statistical significance for changes in gene expression in all qRT-PCR experiments, larval size, and ecdysteroid measurements.

Supporting Information

Figure S1 Localization of Prp8 and Ecd, and RNAi-mediated depletion of Ecd in PG cells. Flag:Prp8 expressed under the *phm-Gal4* driver localizes to the PG cytoplasm (A). Endogenous (B) or overexpressed (D) Ecd protein primarily resides in the cytoplasm of PG cells. *phm-Gal4* driven RNAi silencing of ecd results in depletion of the endogenous Ecd protein specifically in the PG part of the ring gland. Ecd was visualized with an antibody against the N-terminal half of the *Drosophila* Ecd protein (*EcdNe expres*; this study). Membrane-targeted CD8::GFP marks the PG cells, DAPI stains the nuclei, and anti-Lamin staining outlines the nuclear envelope. Panels show single confocal sections. (A–D’): are magnified views of the PG cells. Scale bars, 20 μm (A–D), 5 μm (A’–D’).

Figure S2 Efficiency of Ecd RNAi knockdown in wing imaginal discs. Compared to control (A), *dpp-Gal4* driven expression of *UAS-ecdRNAi* (*dpp>*ecdRNAi*) (B) results in marked depletion of endogenous Ecd protein in the cells along the anterior-posterior boundary (arrowheads) of third-instar wing discs. The *dpp* expression domain is visualized by co-expression of *UAS-GFP*. Note the cytoplasmic localization of Ecd. DAPI stains cell nuclei. Scale bars, 50 μm.

Figure S3 Brr2 is required for survival of imaginal cells; ectopic Prp8 does not rescue defects caused by *ecd* RNAi. (A) RNAi knockdown of Brr2 under the *dpp-Gal4* driver causes massive cell death of wing imaginal disc cells as visualized by staining with anti-cleaved Caspase 3 antibody (A’, arrows), and results in morphological anomalies of adult wings (B), namely loss of anterior crossvein (arrowhead) and reduced size of intervein region (double arrow). These defects phenocopy depletion of Ecd or Prp8 (compare with Figure 4B, 4C, 4F, 4G) but cannot be suppressed by supplementing *ecdRNAi* cells with extra Prp8 protein (*dpp>*ecdRNAi* *prp8*) (C, D). Scale bars, 100 μm (A, C), 20 μm (A’, C’), and 1 mm (B, D).

Figure S4 Expression of Spok and Phm proteins in the PGs subjected to RNAi against Brr2, a component of the U5 snRNP complex. (A) Staining of a control PG dissected 6 days AEL with anti-Spok (A’) and anti-Phm (A”) antibodies. (B) PG-specific RNAi targeting of Brr2 abolished expression of Spok (B’) and reduced levels of Phm (B”) proteins. Brr2 knockdown also altered PG morphology and size. Panels show single confocal sections. Scale bars, 20 μm.

Figure S5 (A) DNAse-treated RNA samples used for cDNA synthesis were free of contaminating genomic DNA as determined by end-point PCR (34 cycles). Primer sets specific to an *EcR* intron.
and to exons of the \( \gamma f g \) gene amplified bands only in cDNA and genomic DNA but not in RNA samples from larvae of the indicated genotypes. Note the increased size of the \( \gamma f g \) PCR product in genomic DNA due inclusion of an intron positioned between the primers. (B) A representative example of amplification curves obtained by qRT-PCR on \( \gamma f g \) and control cDNA samples with the \( \gamma f g \) primer set shows that \( \gamma f g \) mRNA level was not significantly altered by loss of \( e c d \) function and therefore was suitable for normalization of qRT-PCR data. The green line marks the amplification threshold (Ct value). (C) Expression of z-

**Figure S6** Systemic effects of Ecd deficiency. (A) Ecdysteroid content was reduced in whole \( phm>ecdRNAi \) larvae on day 6 AEL compared to controls (\( w^{1118}; \) \( phm-Gal4/ +\) ), and it was increased above control levels over by over-expression of hEcd (\( phm>eodRNAi \) \( hEcd\)). (B) After two days of feeding 20E, \( phm>ecdRNAi \) larvae (second from left), but not their solvent-treated siblings (far left), displayed wandering behavior on day 7 AEL. At that time, 20E-treated and untreated controls (\( w^{1118}; \) \( phm-Gal4/ +\) ) began to pupariate. (C) Expression of \( E74 \) and \( E74 \) genes (as assessed by qRT-PCR with primer sets detecting all alternatively spliced mRNA isoforms of each gene, see Table S1) were significantly higher in third-instar \( ecd \) larvae reared at 20°C than in their siblings at the restrictive temperature (29°C) after 4 h of exposure to 20E. Levels of spliced \( E74 \) and \( E74 \) mRNAs (all isoforms) were induced on day 6 AEL in \( phm>ecdRNAi \) larvae fed for 24 h on 20E-containing diet relative to controls of the same age. Note that the induction was weaker in \( ecd \) mutants under 29°C. (D) EcR protein (detected with an antibody against all EcR isoforms) was markedly diminished upon depletion of Ecd (\( phm>ecdRNAi \)) from the PG (dissected 6 days AEL). Panels show single confocal sections. Scale bars, 20 μm.

**Table S1** List of primers used for expression constructs and qRT-PCR.

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**Author Contributions**

Conceived and designed the experiments: AKC MJ MU. Performed the experiments: AKC MJ MU. Analyzed the data: AKC MJ MU. Contributed reagents/materials/analysis tools: AKC MU TL. Wrote the paper: AKC MJ MU.

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