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Ligand-independent requirements of steroid receptors EcR and USP for cell survival

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The active form of the *Drosophila* steroid hormone ecdysone, 20-hydroxyecdysone (20E), binds the heterodimer EcR/USP nuclear receptor to regulate target genes that elicit proliferation, cell death and differentiation during insect development. Although the 20E effects are relatively well known, the physiological relevance of its receptors remains poorly understood. We show here that the prothoracic gland (PG), the major steroid-producing organ of insect larvae, requires EcR and USP to survive in a critical period previous to metamorphosis, and that this requirement is 20E-independent. The cell death induced by the downregulation of these receptors involves the activation of the JNK-encoding *basket* gene and it can be rescued by upregulating EcR isoforms which are unable to respond to 20E. Also, while PG cell death prevents ecdysone production, blocking hormone synthesis or secretion in normal PG does not lead to cell death, demonstrating further the ecdysone-independent nature of the receptor-deprivation cell death. In contrast to PG cells, wing disc or salivary glands cells do not require these receptors for survival, revealing their cell and developmental time specificity. Exploring the potential use of this feature of steroid receptors in cancer, we assayed tumor overgrowth induced by altered *yorkie* signaling. This overgrowth is suppressed by EcR downregulation in PG, but not in wing disc, cells. The mechanism of all these cell death features is based on the transcriptional regulation of *reaper*. These novel and context-dependent functional properties for EcR and USP receptors may help to understand the heterogeneous responses to steroid-based therapies in human pathologies.
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Steroid hormones control multiple biological processes and, consequently, their faulty regulation underlies many pathologies.[1,2] The main *Drosophila* steroid hormone, ecdysone, is synthesized in the larval prothoracic gland (PG) using dietary sterols and cytochrome P450 enzymes.[3] Following its pulsated secretion into the hemolymph, peripheral tissues convert ecdysone into biologically active 20-hydroxyecdysone (20E) to control larval molting and metamorphosis.[4]

The 20E signal is transduced by heterodimers of two nuclear receptors, ecdysone receptor (EcR) and Ultraspiracle (USP).[5] USP exhibits a single form throughout development. By contrast, *EcR* encodes three protein isoforms (EcRA, EcRB1 and EcRB2). These show common DNA- and hormone-binding domains but different N-termini although all of them can heterodimerize USP.[6,7] Each EcR isoform is hypothesized to have specific functions based on their distinct spatial and temporal patterns and N-termini.[8–10] EcR and USP DNA-binding domains recognize ecdysone response sequences which are short palindromes.[11] Like its vertebrate counterparts,[12] the ligand-EcR/USP complex activates transcription, whereas the unliganded receptor act as a repressor.[13–15] The *in vivo* validation of repressor activities for unliganded complexes, however, is mostly indirect. For example, EcR or USP loss triggers precocious, meaning prior to 20E surge, cell differentiation through the expression of *Broad Z1* in eye disc[13] or wing sensory neurons.[14] Therefore, it is assumed that *Broad Z1* was repressed by the unliganded EcR/USP. In the activator configuration, the 20E/receptor complex activates characteristic early genes including *Broad complex (BrC)*, *E74* and *E75*. When the corresponding proteins, which are also transcription factors, reach a critical threshold, they repress their own promoters and activate the so called late genes.[16,17]

In humans, estrogen stimulates cell proliferation and survival through activation of the estrogen receptor but, it can also induce tumor regression of hormone-dependent breast cancer in women who have received anti-hormone treatments.[18,19] The COSMIC data base shows an intriguing upregulation of YAP and TAZ factors in endocrine cancers (thyroid, breast, ovary and prostate).[20,21] The *Drosophila* homologue of YAP and TAZ is Yorkie whose overactivation also results in overgrowth.[22] Cell death during metamorphosis is considered a suitable model to investigate the basic mechanisms of tumor growth regulation by mammalian steroids. However, the puzzling diversity of steroid effects on

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Abbreviations: 20E, 20-hydroxyecdysone; AEL, After egg laying; Atg, Autophagy-related gene; bsk, Basket; Br-C, Broad complex; C3, Caspase-3; COSMIC, Catalogue of Somatic Mutations in Cancer; dIAP, Drosophila inhibitor of apoptosis-1; EcR, Ecdysone receptor; EcRA, Isoform A of the ecdysone receptor; EcRB1, Isoform B1 of the ecdysone receptor; EcRB2, Isoform B2 of the ecdysone receptor; en, Engrailed; FLP, Flipase enzyme; FRT, Flp recombinase target; Gal80^{ts}, Gal80 temperature sensitive; Hid, Head involution defective; JNK, Jun-kinase; lgl, Lethal giant larvae; Phm, Phantom; PG, Prothoracic gland; puc, Puckered; ppl, Pump less; RNAi, RNA interference; RT-PCR, Real time polymerase chain reaction; TAZ, Transcriptional coactivator with PDZ-binding motif; USP, Ultraspiracle; wg, Wingless; YAP, Yes-associated protein; yki, Yorkie

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tumor growth, including the lack of effect, challenges the insect approach. In this context, it seems justified to explore cell death mechanisms that could be differentially controlled by steroid receptors *versus* their canonical hormone ligand, and to assay their effect on Yorkie-dependent tumor outgrowths.

Results

EcR and USP are required in the PG for metamorphosis. After tool validation (Supplementary Data and Supplementary Figure S1), we analyzed the phenotypes due to depletion of EcRs in the PG by using the *phantom* (*phm-Gal4*) driver. The symbols↓or↑indicate downregulation or upregulation, respectively. The condition EcRA↓ allows normal development until L3 stage which is lengthened 48 h (Figure 1a). The same effect occurs in 60% of the EcRB1↓ larvae. In the remaining 40% of EcRB1↓ and 100% of EcR↓ or USP↓ larvae, development is halted at L3 stage where they remain over 25 days without entering metamorphosis (Figure 1a) and doubling their size by day 5 past due pupariation time (Figure 1b).

At day 5 post due pupariation time, the fat body of EcR↓ and USP↓ larvae presents 30% fewer cells and larger than normal lipid vesicles (Supplementary Figure S3A). Larvae become progressively inactive and develop a brown pigmentation,

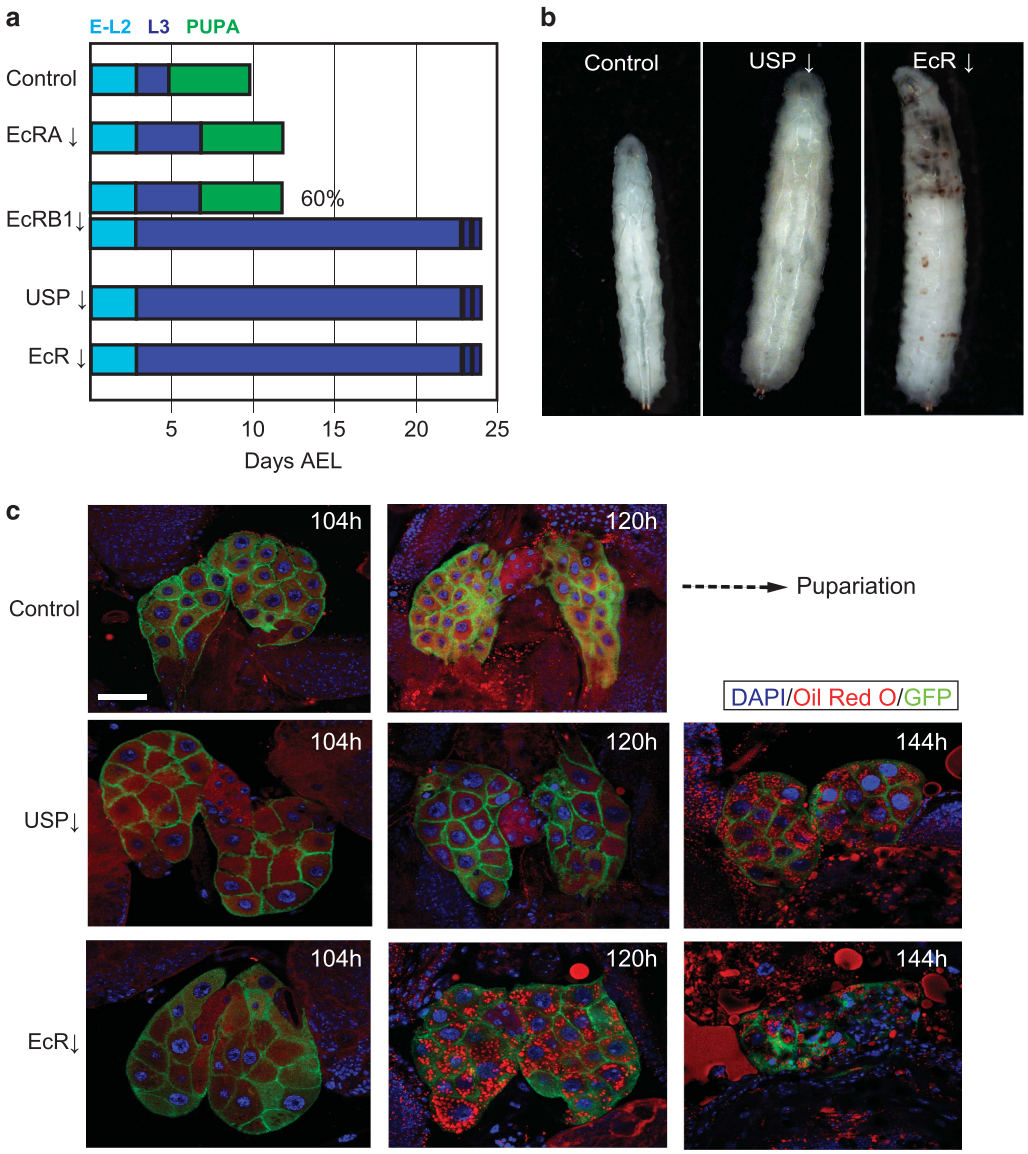

Figure 1 Metamorphosis requires the expression of EcR and USP in the PG. (**a**) Staged development of genotypes analyzed. *phm-Gal4* driven expression of EcR-A^{RNAi} (EcRA↓) prolongs the duration of L3; while that of B1 isoform (EcRB1↓) lets 60% of larvae to pupariate and reach adulthood although with some delay, while about 40% of them remain as L3. When all EcR isoforms (EcR↓) or USP (USP↓) are downregulated, 100% of larvae never undergo metamorphosis. (**b**) Representative examples of control late L3 compared with experimental larvae (EcR↓ and USP↓) 5 days past due pupariation time. Notice the larger size of mutant larvae. (**c**) Larval PGs stained with OilRed-O to reveal lipid content. Control larvae at 104 and 120 h AEL show a homogenous and diffuse signal. Under USP↓ conditions, lipids accumulate as droplets at 144 h AEL (24 h delayed with respect to normal). Phenotype is stronger in EcR↓ as droplets appear at 120 h AEL, just before the due pupariation time. Genotypes: Control = *phm-Gal4>UASCD8-GFP; UAS-LacZ*. EcR↓ = *phm-Gal4>UASCD8-GFP; UAS-EcR*^{RNAi}. USP↓ = *phm-Gal4>UAS-CD8-GFP; UAS-usp*^{RNAi}. Bar in **c** = 20 μm. Images in **b** are at the same magnification

more prominent in the case of EcR↓ than in USP↓ (Figure 1b). Although brain size did not appear affected, larval neuromuscular junctions are enlarged with extended branching and double number of synapses (Supplementary Figures S3B and C). This neuronal effect following EcR inactivation in the PG is akin to that previously reported,[23] albeit, in that report, metamorphosis was delayed, rather than prevented, and the method used was the downregulation of the *torso* receptor. Moreover, the imaginal discs of larvae with EcR↓ and USP↓ in the PG show increased cell death and reduced size with abnormal localization of Wingless (Supplementary Figure S3D).

In addition to these systemic effects, the PG was examined in detail. Whereas cell number remained normal, EcR↓ and USP↓ PGs stained with Oil Red O show lipid droplets abnormally accumulated in the cytoplasm. The accumulation occurred 24 h sooner in EcR↓ animals than in USP↓, but in both cases, the effect increased by 144 h AEL, 24 h after due pupariation time (Figure 1c).

EcR and USP are required for ecdysone synthesis in PG cells. To study the role of EcR/USP in ecdysone synthesis, we measured the expression of ecdysone-synthesizing genes by qRT-PCR. Expression of *disembodied*, *shadow* and *phantom* increased significantly between early and late L3 stages in controls. However, this was abolished in experimental larvae (Figure 2a and Supplementary Figures S4A and C). The modest increase of *torso* expression in the control was also absent in experimental larvae (Figure 2a and Supplementary Figure S4D).

To determine whether the failure to pupariate was due to altered levels of ecdysteroids, we measured them. As expected, ecdysteroids increased between early and late control L3 corresponding to the ecdysone synthesis peak previous to pupariation, but that increase was not observed in EcR↓ or USP↓ larva (Figure 2b). When ecdysone production is compromised, metamorphosis can be rescued by feeding 20E to mutant larvae. Surprisingly, this procedure failed in our EcR↓ and USP↓ larvae, although it succeeded to rescue *phm-Gal4>UAS-phm^{RNAi}* larvae (data not shown).

Inspection of *phm-Gal4* EcR↓ and USP↓ larvae showed a weak GFP reporter signal in the trachea that increased with age (Supplementary Figure S5). This signal prompted a closer analysis of the GFP expression domain in *phm-Gal4>UAS-GFP* control larvae. The reporter was already detected in controls albeit at a much lower intensity. Thus, the trachea

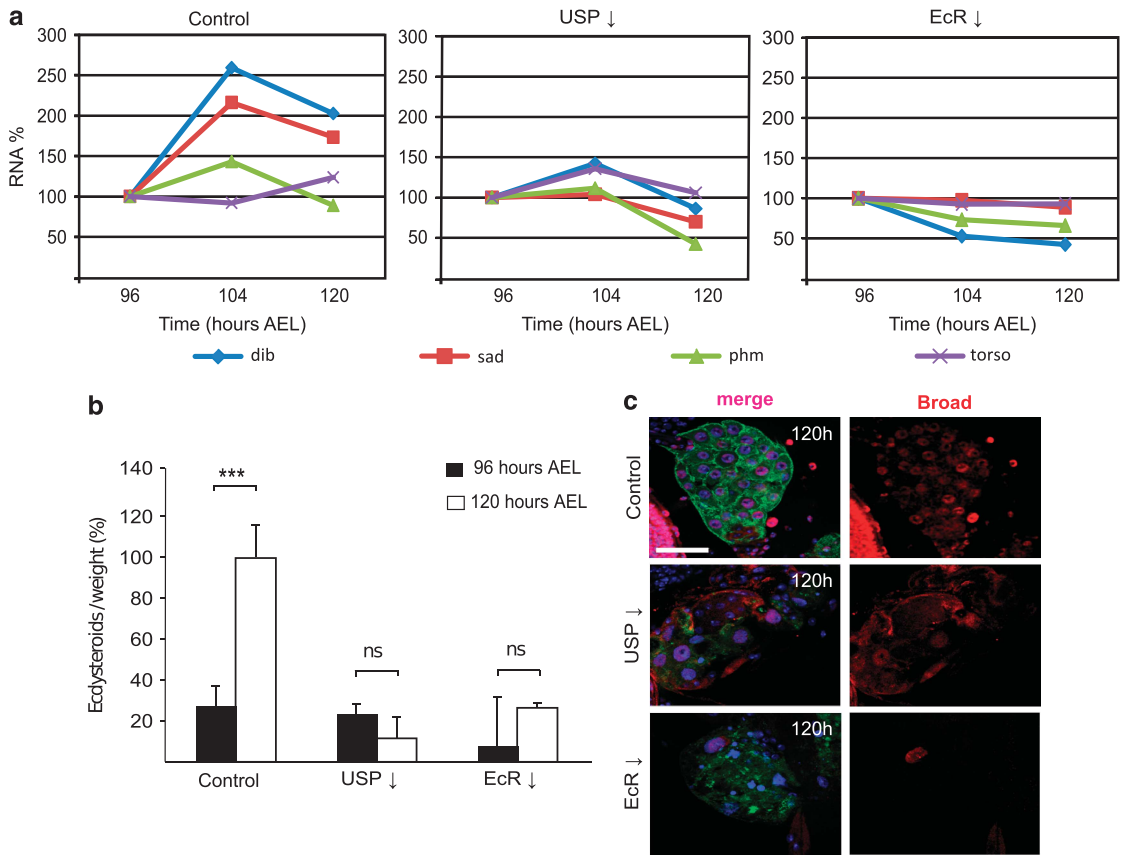

Figure 2 EcR and USP are required for ecdysone synthesis. (**a**) Transcriptional profiles for three ecdysone-synthesizing genes (*dib, disembodied; sad, shadow* and *phm, phantom*) and the PTTH receptor *torso*. The qRT-PCR data from whole larvae were obtained at 96, 104 and 120 h AEL, and are shown as a percentage of the value at 96 h AEL. In the control, gene expression peaks just before puparium formation, while in USP↓ or EcR↓ larvae, this peak does not occur. Triplicate measurements from experiments repeated three times. Graphs containing error bars and significance for each gene are shown in Supplementary Figures S4 A-D. (**b**) Ecdysteroid (E and 20E) levels in control and mutant larvae at different time points. Data (pg/mg) are normalized to the 120 h time point. The peak of ecdysteroids that triggers pupariation in control larvae is not detected in larvae expressing EcR↓ or USP↓ in the PG. (**c**) *Broad* expression (red) in control PG is substantially abolished by USP↓ or EcR↓ at 120 h. Genotypes: Control = *phm-Gal4>UAS-CD8-GFP; UAS-LacZ*. EcR↓ = *phm-Gal4>UAS-CD8-GFP; UAS-EcR^{RNAi}*. USP↓ = *phm-Gal4>UAS-CD8-GFP; UAS-usp^{RNAi}*. Bar = 20 μm

should be included jointly with the wing disc (see below) as a low intensity expression domain of *phm-Gal4*. Likely, the overdue experimental larvae increase *phm-Gal4* expression in these low intensity domains. The cumulative expression will increase also the depletion of 20E receptors in target tissues rendering them unable to respond to the fed 20E and, consequently, larvae do not enter metamorphosis.

As further evidence that EcR↓ and USP↓ PGs do not produce ecdysone signaling, we monitored *Br-C* expression. This family of zinc finger transcription factors is upregulated in all cell types as an early response to the ecdysone surge of late L3.[24] The data show that Br-C is not detected in experimental PGs (Figure 2c). Thus, we conclude that the characteristic function of PG cells, pro-hormone synthesis, requires EcR and USP.

EcR and USP, but not ecdysone, deprivation affects PG cell survival. The functional defects of PG cells prompted an analysis of cell survival, either causative of, or resulting from, EcR/USP downregulation. Activated caspase-3 (C3) served as cell death indicator. Control larvae do not show immuno-C3 staining. However, EcR↓ and USP↓ glands show consistent signs of cell death which become massive by 144 h (Figure 3a). Many C3-positive cells also show fragmented nuclear bodies revealed by DAPI. The TUNEL assay confirmed the apoptosis-like process of PG cell death under EcR↓ or USP↓ conditions (Figure 3b).

As ecdysone synthesis failure and cell death coincide in time, we questioned whether ecdysone deprivation could cause cell death. To that end, we analyzed PGs where *phantom*, one of the ecdysone-synthesizing genes, is knocked down. In this experiment, the *phantom*↓ condition was switched-on in L2, using the *Gal80^{ts}* repressor, in order to bypass the vital requirement of this gene in embryogenesis. As expected, these larvae showed extremely low ecdysteroid levels (Supplementary Figure S6) and did not pupariate. However, opposite to EcR↓ or USP↓, *phantom*-depleted PG cells did not show C3 activation (Figures 3c and d). Actually, cells showed a notable increase in size. Next, we questioned whether ecdysone could be required for PG cell survival through an autocrine mechanism. To that end, we inactivated *Kish* and *Gryzun*, two genes required for vesicle trafficking and secretion.[25] In both cases, PG cells did not show signs of apoptosis although, as expected, larvae did not pupariate (Figures 3e and f). We conclude that the lack of ecdysone does not cause PG cell death. More likely, the activation of a cell death program may cause halting of ecdysone synthesis.

EcR- and USP-depleted PGs degenerate through a mechanism different from autophagy. Wild-type PG degenerates during metamorphosis in a process that shows electron microscopy features characteristic of autophagy, rather than apoptosis.[26] To ascertain the mechanism of PG degeneration in the absence of EcR or USP, we considered autophagy. The *Atg8-cherry* marker appears to indicate autophagy triggering because the reporter signal accumulated in cytoplasmic dots, presumably autophagosomes (Figure 4a). However, downregulation assays of autophagy genes,[27] including *Atg1, Atg4, Atg5* and *Atg7* were unable to rescue EcR/USP-dependent cell death in the PG or the failed pupariation phenotype (Figure 4b). To consider alternative cell death mechanisms, we screened through genetic modifiers. The *dIAP1*↑ and *reaper*↓ conditions were most effective, along with constitutively active *ras*↑ and *p35*↑ (a caspase inhibitor from baculovirus). Notably, *dIAP1*↑ was a more effective suppressor of EcR↓ than of USP↓ (Figure 4b). This differential effect could reflect the distinct set of genes targeted by EcR *versus* USP. On the other hand, *sickle*↓ and *hid*↓ (apoptosis effectors) failed to rescue the L3 arrest phenotype. In addition, upregulating the receptor that triggers ecdysone synthesis, Torso,[28] or upregulating the early effectors of ecdysone signaling, *Br-C* genes,[24] also failed to bypass the deleterious EcR or USP downregulation (Figure 4b). This is consistent with the lack of ecdysteroid synthesis in experimental larvae shown by direct measurements above (Figure 2b).

Interestingly, mutated EcR isoforms that do not bind ecdysone, EcRB1^{W650A}, or cannot activate target gene transcription, EcRB1^{F645A},[7] rescue to some extent the metamorphosis blockade (Figure 4b). This is additional evidence that EcR/USP-dependent effects in the PG are ecdysone-independent. Further, downregulating *Eip93F*, which is a cell death signal induced by the pupal ecdysone pulse once metamorphosis is underway and that regulates *reaper* and other apoptosis genes during salivary gland cell death,[29] failed to rescue the phenotype under study (Figure 4b). Thus, *Eip93F* can also be excluded from the EcR/USP-dependent cell death mechanism.

Beyond the use of RNAi lines to elicit the USP/EcR↓ conditions, we tested also FRT/FLP clones of mutant allele *usp^{5}* (Figures 4c and d). PG cells show smaller than normal and fragmented nuclei (Figure 4c) but the co-expression of EcRA rescues that phenotype (Figure 4d). Finally, the down-regulation of EcR or USP increases *reaper* transcription (Figure 4e, left panel), and a concomitant decrease in *dIAP1*, a prosurvival gene (Supplementary Figure S4E). Together, these data support the cell survival requirement of EcR and USP in the PG and suggest that their deprivation causes cell death through a mechanism that is unrelated to the autophagy that occurs during pupal metamorphosis, but that is similar to apoptosis.[30] In this context, then, it is likely that the observed activation of *Atg8-cherry*, which is not followed by the involvement of autophagy genes, could reflect a stress-dependent event as previously described.[31]

EcR and USP promote PG cell survival by repressing the JNK pathway. The Jun Kinase (JNK) pathway activates apoptosis in many *Drosophila* cells where the enzyme is encoded by *basket (bsk)*.[32,33] We explored the JNK signaling in the PG cell death induced by EcR or USP depletion. To that end, we monitored the expression of its effector *puckered (puc)* as reported by *puc-LacZ*. While control PG does not show noticeable *puc-LacZ* expression, the EcR↓ condition does (Figures 5a and b). The *puc-LacZ* signal was validated by direct *bsk* overexpression in the PG[34] (Figure 5c). Driving *bsk* causes PG cell death with caspase-3 activation (Figure 5d) and, as expected, failure to enter metamorphosis. Interestingly, the co-expression of EcRB1^{W650A} suppresses the bsk/JNK effect (Figure 5e) and allows larvae to pupariate and reach adulthood. This suppression includes the

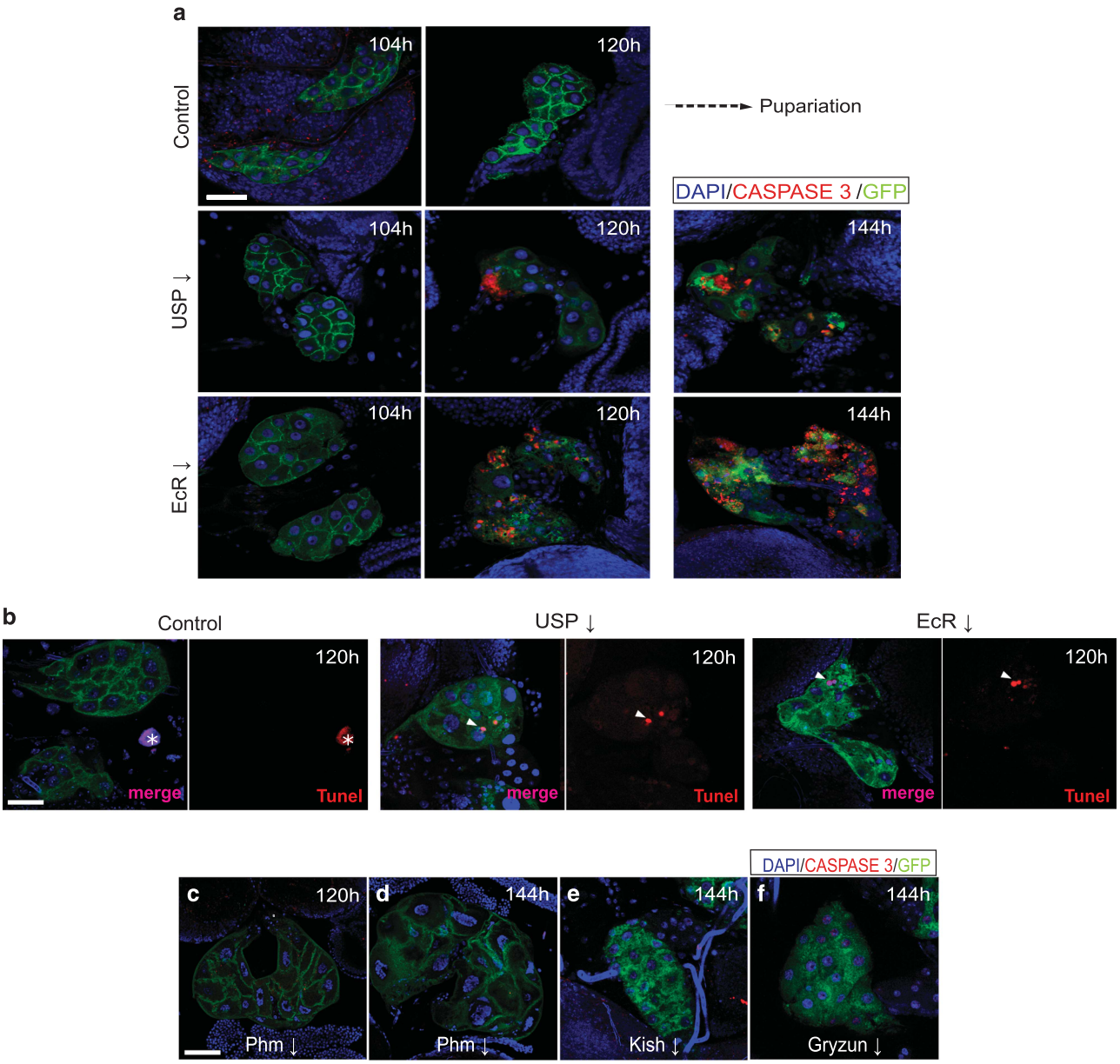

Figure 3 Survival of PG cells requires EcR and USP, but not ecdysone. (**a**) In control PG, cells do not show activated caspase-3 (C3) (red). However, under EcR↓ or USP↓ conditions, many cells become C3-positive by the normal pupation time (120 h), and their number increases by 144 h. (**b**) TUNEL assay (arrow heads) detects apoptosis in EcR↓ and USP↓, but not in control PG cells at 120 h. The asterisk marks a TUNEL-positive cell outside the PG and serves as internal control of the assay. (**c**) Downregulation of *phantom* in L2 PG prevents cells from initiating metamorphosis and synthesize ecdysone (see Supplementary Figure S6). However, instead of activating apoptosis, cells greatly enlarge by 120 h (**c**) and more so by 144 h (**d**). (**e, f**) Blocking ecdysone secretion by downregulating Kish (**e**) or Gryzun (**f**) does not elicit caspase-3-dependent apoptosis although larvae do not enter into metamorphosis. Genotypes: Control = *phm-Gal4 > UAS-CD8-GFP; UAS-LacZ.* EcR↓ = *phm-Gal4 > UAS-CD8-GFP; UAS-EcR^{RNAi}.* USP↓ = *phm-Gal4 > UAS-CD8-GFP; UAS-usp^{RNAi}.* Phm↓ = *phm-Gal4 > UAS-CD8-GFP; Gal80^{ts}/UAS-phm^{RNAi}.* Kish↓ = *phm-Gal4 > UAS-CD8-GFP; UAS-kish^{RNAi}.* Gryzun↓ = *phm-Gal4 > UAS-CD8-GFP; UAS-gry^{RNAi}.* Bar = 20 μm

transcriptional normalization of *reaper* (Figure 5f), an apoptosis hallmark gene whose transcription is directly regulated by EcR/USP.[35] As expected, *dIAP1* transcription is reduced by bsk/JNK↑ and normalized to some extent by EcRB1^{W650A} (Supplementary Figure S4F). These data favor an apoptosis-type mechanism for the EcR/USP-dependent cell death in the PG. It is plausible that EcR/USP depletion triggers JNK activation through *puc* and, simultaneously, relieves repression of *reaper*. This relief would further activate JNK which, in turn, would induce *reaper* expression. On the other hand, the suppression of apoptotic features by EcRB1^{W650A} overexpression further supports the ligand-independent nature of the EcR requirement for cell viability.

Imaginal disc and salivary gland cells do not require EcR/USP for cell survival. To test whether the effects of EcR or USP depletion in the PG are common to other cell

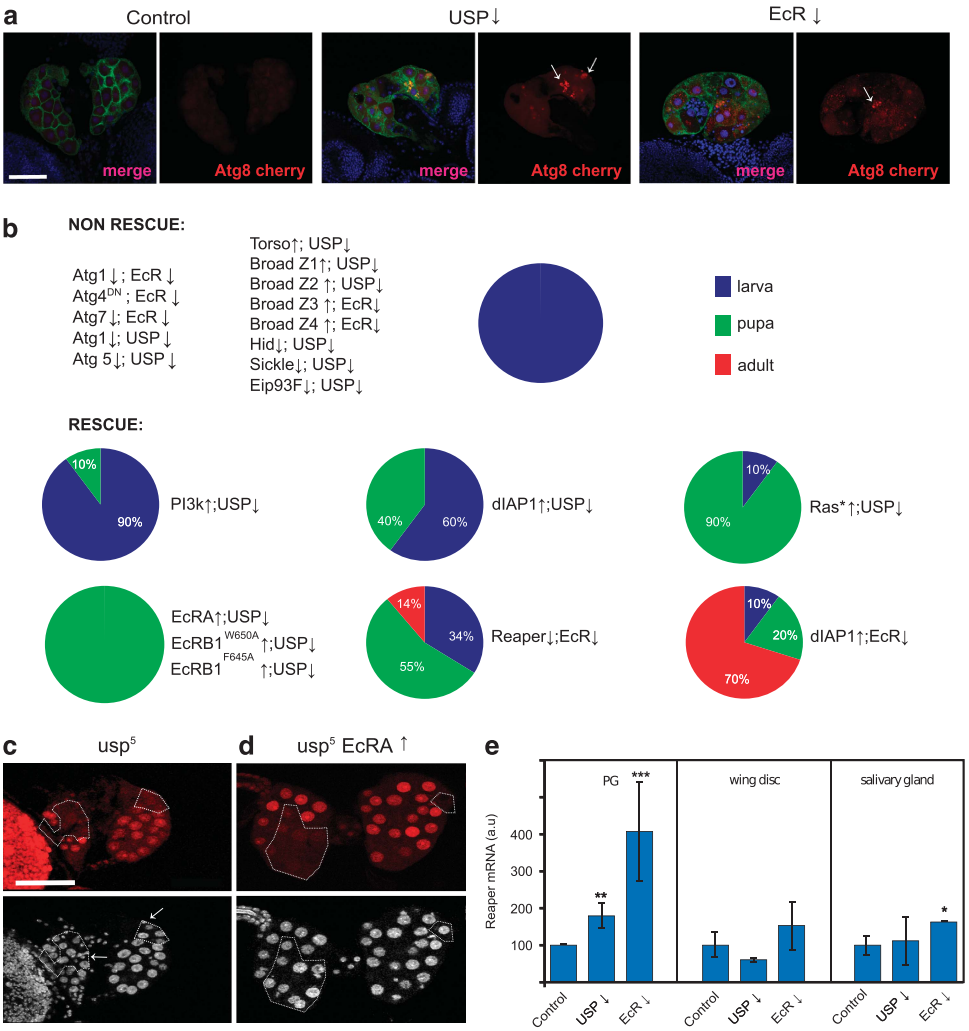

Figure 4 EcR and USP phenotypes are rescued by downregulating apoptosis genes. (**a**) The cherry-reported autophagy marker Atg8 accumulates (mainly in autolysosomes) of PG cells when USP or EcR are knocked down (arrows in right panels) compared with the background signal from control PG (left panel). This could suggest that autophagy has been initiated in the absence of EcR or USP. (**b**) Attempts to rescue the metamorphosis phenotypes due to USP↓ or EcR↓. Circles indicate developmental stages (larva = blue, pupa = green, adult = red) and numbers indicate the relative proportion of each stage ($n > 100$). Note that apoptosis genes (e.g.: *dIAP1* or *reaper*) rescue the mutant phenotypes to various extents. Autophagy genes (Atg), however, do not. Also, regular targets of ecdysone signaling (e.g.: *Broad* or *Eip93F*) are unable to rescue. (**c**, **d**) FLP/FRT clones (absence of red signal, dotted lines) in the PG. *usp*^{5}clones (**c**) show small cells with fragmented nuclei (arrows), while the simultaneous upregulation of EcRA (**d**) rescues that phenotype. Only five-stack maximum projections are shown for clarity. (**e**) *Reaper* expression levels by qRT-PCR in dissected Ring glands (*phm-Gal4*), wing discs and salivary glands (*rotund-Gal4*) at 120 h. Only in the PG, there is a strong upregulation of *reaper* in the absence of EcR or USP which illustrates the differential cell system response to USP↓ or EcR↓. Genotypes in **c** and **d** = *usp*^{5} *w UAS-mCD8-GFP Ptp4E*^{LL4} *FRT19A/ Ubi-mRFP*^{nls}, *w, hsFLP, FRT19A; phm-Gal4 > UAS-GFP/+* or *UAS-EcRA*, respectively

types, we analyzed the wing pouch and salivary glands using the *rotund-Gal4* driver combined with the *elav-Gal80* repressor. Suppressing the driver in the nervous system was needed to allow larval viability. Following C3 immunostaining, no increase of apoptosis was detected in either cell system (Figures 6a and h). Also, *bsk*↑-induced apoptosis in the wing pouch was not rescued by upregulating EcR isoforms, including the one that does not bind ecdysone, in contrast to PGs (Figures 5g and j). These data are consistent with the lack of *reaper* upregulation in wing discs and salivary glands in EcR↓ and USP↓ animals (Figure 4e, right panels). The absence of cell survival effects was also confirmed in the leg disc (*en-Gal4*) and Malpighian tubuli (*ppl-Gal4*) by the C3 criterion (Supplementary Figure S7). Also, depletion of EcR or USP in the wing pouch did not affect cell proliferation as revealed by the phospho-histone H3 criterion (Supplementary Figure S8).

In the salivary glands, cell size was strongly reduced in USP↓ and EcR↓ conditions (Figures 6e and i). This phenotype was not rescued by upregulating any EcR isoform, in particular EcRB1^{W650A}, indicating that it could be ecdysone-dependent. A minor increment of *reaper* expression was detected with EcR↓ (Figure 4e), suggesting that depletion of this receptor, but not USP, could have some deleterious effect on salivary gland cells although still compatible with viability. Thus, we conclude that EcR and USP are required for cell survival in a cell type-specific manner.

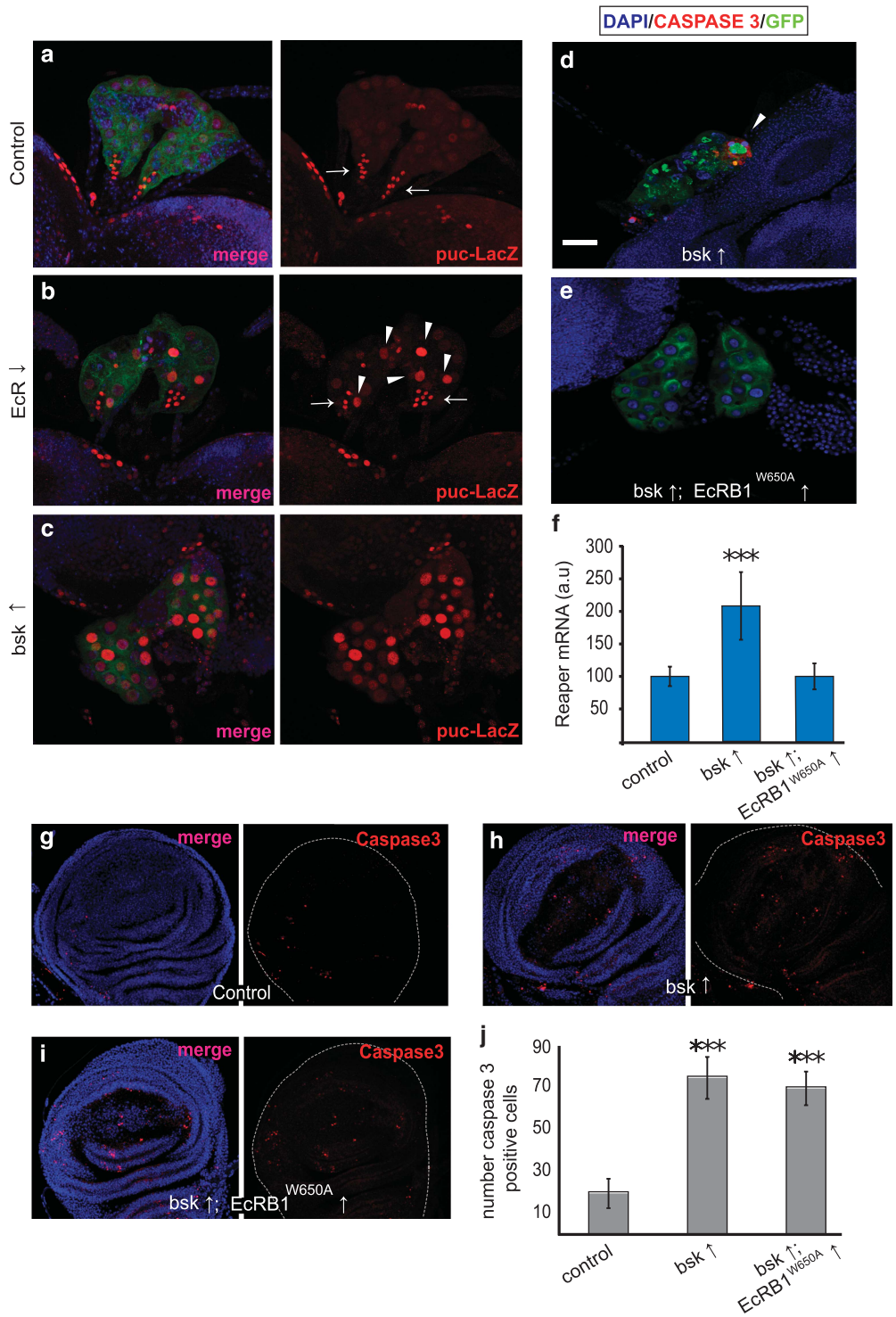

Figure 5 EcR-related cell death in the PG activates Basket/JNK. (**a**) Control PG cells (120 h) do not activate the *bsk*-encoded JNK as monitored by the *LacZ*-reported expression of *puckered (puc)*. Notice the *puc-LacZ* expression (red) in some cells of the brain and Ring Gland (arrows), but not in those of the PG. (**b**) Following the driven EcR downregulation at 96 h, however, *puc-LacZ* becomes upregulated (arrow heads) by 120 h. (**c**) As expected, the direct upregulation of *bsk* in the PG at 96 h reflects into the expression increase of *puc-LacZ* as visualized at 120 h. (**d–f**) *bsk* overexpression in PG induces apoptosis by 144 h (**d**), as indicated by activated caspase-3 (arrow head), and larvae do not pupate, akin to EcR↓ or USP↓ conditions (see Figure 1), but the phenotype is rescued by EcR-B1^{W650A}↑(**e**) which allows larvae to pupariate and yield normal adults. This suppression includes the transcriptional normalization of *reaper* expression (**f**) as measured by qRT-PCR in dissected Ring glands. (**g–i**) Caspase-3 monitored apoptosis (red) in the wing disks (*rotund-Gal4*) of control (**g**) or *bsk* (**h**) larvae are not rescued by EcR-B1^{W650A}↑ (**i**). (**j**) Quantification of caspase-3-positive wing disc cells in the genotypes of (**g–i**). Genotypes in (**a–c**) = *Gal-80ts/UAS-bsk* or *UAS-EcR^{RNAi}; phm-Gal4> UAS-GFP/puc^{E69}-LacZ*. (**d–f**) = *UAS-bsk* or *UAS-bsk, UAS-EcR-B1^{W650A}/+; phm-Gal4> UAS-GFP/+*. (**g–j**) = *rn-Gal4> UAS-bsk* or *UAS-bsk, UAS-EcR-B1^{W650A}*. Bar in **d** = 20 μm

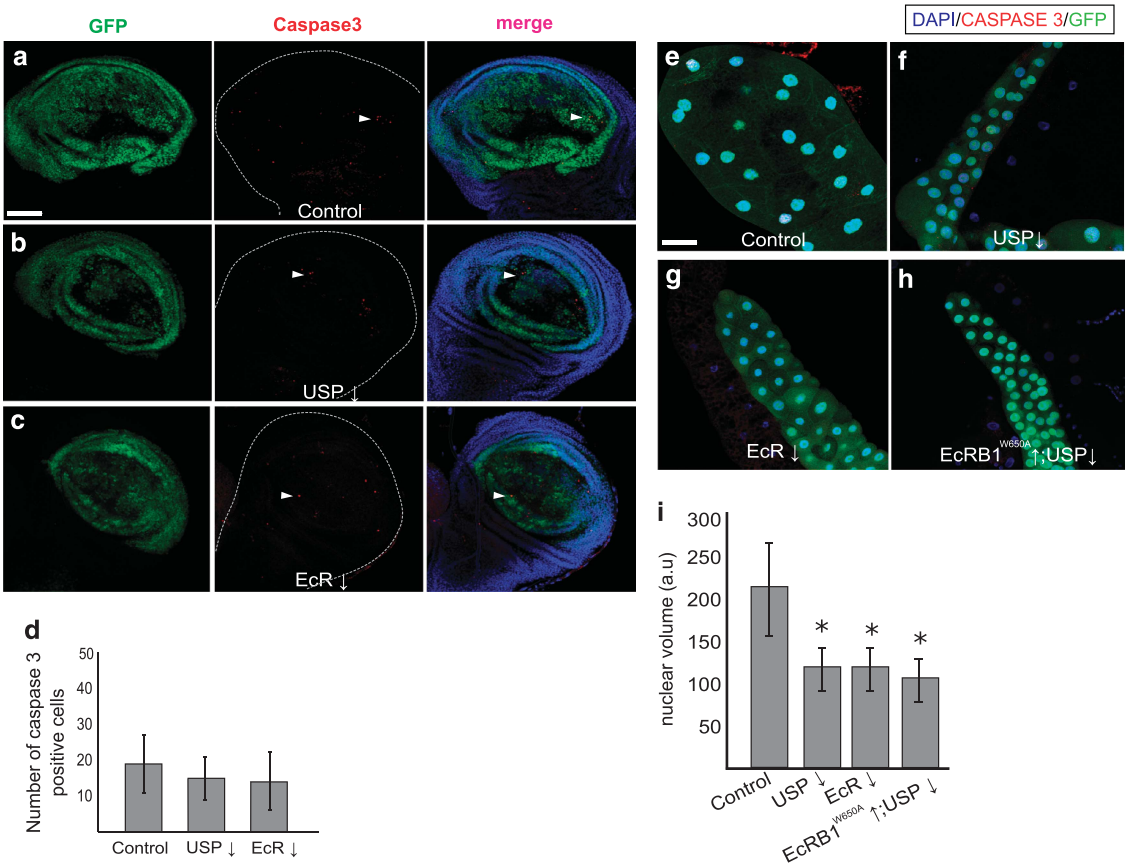

Figure 6 Cell type-specific responses to EcR or USP downregulation. Staining for activated caspase-3 (red, arrow head) in *rotund-Gal4* (green) wing discs from control (**a**), USP↓ (**b**) or EcR↓ (**c**) genotypes. (**d**) Quantification of cell death showed no difference for all genotypes. (**e–h**) Experimental salivary glands (*rotund-Gal4*) showing caspase-3 in control (**e**), USP↓ (**f**), EcR↓ (**g**) or EcR-B1^{W650A}↑ USP↓ (**h**) genotypes. No evidence of apoptosis is detected. However, nuclear size is reduced in USP↓ or EcR↓ and the phenotype is not rescued by EcR-B1^{W650A}↑ (**h**). (**i**) Salivary gland nuclear volumes from **e–h** genotypes. Genotypes = *rn-Gal4*> *UAS-usp^{RNAi}* (**b, f**) or *UAS-EcR^{RNAi}* (**c, g**) or *UAS-usp^{RNAi}, UAS-EcR-B1^{W650A}* (**h**). Bar in **a** and **e** = 20 μm

EcR and USP can repress tumor growth. Anti-hormonal treatment is often used against human cancers that overexpress steroid receptors. However, development of resistance and heterogeneous responses to steroid treatments over prolonged therapy are not uncommon.[36,37] We reasoned that the ligand-independent cell survival effects of EcR and USP in the PG of *Drosophila* could eventually help to understand that heterogeneity of responses to therapies. In that context, we explored the effects of EcR and USP deprivation in *yorkie*-elicited PG tumors. A constitutively active form of *yki, yki*,* (see Materials and Methods) was driven to the PG yielding a substantial overgrowth (Figures 7a and b). The overgrowth was largely suppressed by EcR↓ (Figure 7c) and about 10% of larvae entered metamorphosis. Larvae that did not pupariate showed activated C3 staining over time (168 h AEL) (Figure 7d). The *yki**-induced overgrowth during L3 could be restrained also by *bsk*-induced cell death, albeit, in this case, no larvae entered metamorphosis and overdue PGs (168 h AEL) showed abundant C3 activity (Figures 7e and f). Thus, EcR↓ rather than bsk↑, seems a better strategy against this type of tumors.

Although seldom mentioned, the *phm-Gal4* expression domain includes a low intensity, small number of wing disc cells corresponding to the adult wing hinge (Figure 7g). We took advantage of this domain to analyze *yki** and *EcR* effects in the wing disc to test the cell type specificity of EcR requirement in the context of tumor overgrowth. *yki** caused overgrowth by 120 h AEL (Figure 7h) that, contrary to the PG, neither EcR↓ (Figure 7i) nor bsk↑ (Figure 7j) conditions could counteract. Past the due pupariation time (168 h AEL), the *yki**-induced overgrowth became massive and, again, neither of the two experimental conditions repressed it (Figures 7k and l). The overgrown domain of wing discs expressed *wingless* (*wg*) albeit in an aberrant pattern (Supplementary Figure S9).

We addressed the mechanism of EcR↓-dependent cell death in the context of *yki**-dependent tumor condition in the PG. As *yki* is a *reaper* repressor,[38] we monitored by qRT-PCR the expression levels of *reaper* in dissected Ring glands (Figure 7m). The data show a significant transcription increase by either of the two methods of inducing cell death, bsk↑ or EcR↓, even when overexpressing *yki**. To discard that the partial suppression of tumor overgrowths could result from using several UAS target constructs that may have diluted the Gal4 driver, we generated genotypes with an extra *UAS-cherry* construct. Under these conditions, no weakening of *bsk* or *yki** phenotypes in the PG were evident (Supplementary Figure S10).

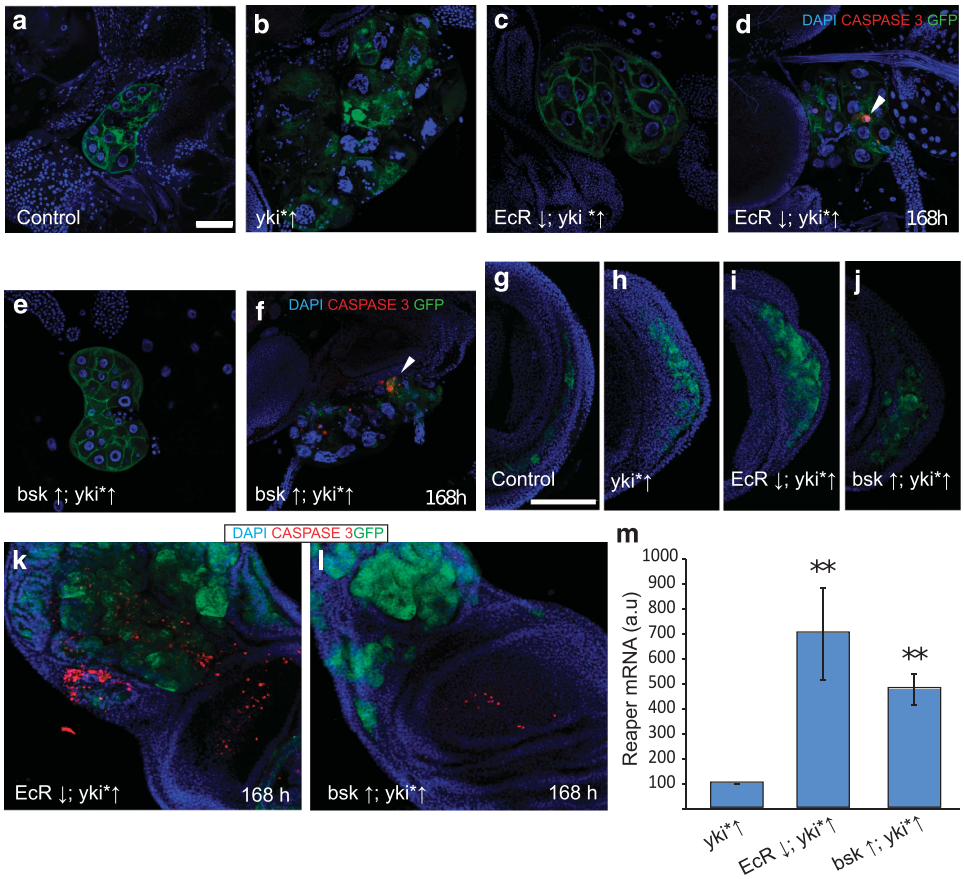

Figure 7 EcR counteracts tumor overgrowth in PG, but not wing disc, cells. (**a**–**f**) Normal and experimental PG at two developmental times, 120 h (**a**–**c**, **e**) and 168 h (**d, f**). Caspase-3-positive cells are in red (arrow head). (**g**–**l**) Normal and experimental wing discs at the same developmental times. Note that caspase-3-positive cells are present in the wing tissue but not in the tumor overgrowth. *Yki** and *bsk* overexpression causes cell death (red) within the overgrowth. However, this cell death does not counteract the proliferation rate in the tumor, and the wing disc develops a very large hyperplasia by several days after the due pupariation time (**l**). (**m**) qRT-PCR data of *reaper* expression in dissected Ring glands of the tumor-inducing genotypes. Note the strong upregulation of *reaper*. Genotypes: *phm-Gal4 > UAS-CD8-GFP; UAS-LacZ* (control) (**a, g**), *UAS-yki*^{S111A,S168A,S250A}(*yki**) (**b, h**), *UAS-EcR*^{RNAi}(**c, d, i, k**) or *UAS-bsk* (**e, f, j, l**). Bar in **a** and **g** = 20 μm

Finally, in an attempt to explore other tumor-eliciting conditions, we generated FLP/FRT somatic clones for the double condition *Ras*^{V12}*/lgl*^{RNAi} and tested the effect of USP↓. The simultaneous attenuation of *lethal-giant-larvae (lgl)* and the constitutive activation of *Ras (Ras*^{V12}) yield enlarged PG cells, albeit not tumorous overgrowths (Supplementary Figure S11A). However, USP↓ did rescue this phenotype (Supplementary Figure S11B and C) which validates the USP/EcR effects on overgrowths of a different origin.

Discussion

The data show a novel requirement for steroid receptors EcR and USP for cell survival that is ligand-independent and cell type-specific. PG cells depend on these receptors and their deprivation causes apoptosis-like death that involves *reaper, bsk/JNK* and *dIAP*. This death process seems unrelated to autophagy which characterizes the ecdysone-triggered cell degradation of other larval tissues at normal metamorphosis. The case further illustrates the diversity of pathways, often sharing signals, which can lead to cell death. Recently, evidences have been gathered indicating that sensitivity to apoptosis is acquired by tissues at two specific steps during development, embryogenesis and pupariation.[39] The data on EcR and USP coincide with the pupariation time requirement and provide a mechanism for this sensitivity.

On the basis of *phm-Gal4* and *phm-Gal4/Gal80*^{ts} driving systems, two types of experiments have been carried out: chronic and L2 switch-on (*Gal-80*^{ts}), respectively. The EcR↓ and USP↓ conditions were tested in both experiments yielding the same result, arrest in late L3 before pupariation. This, plus the lack of detectable ecdysteroid biosynthesis, frames the temporal requirement of EcR and USP for cell survival just prior to metamorphosis at 120 h AEL. The *phm-Gal4* driver is expressed in early embryo.[40] However, the two receptors do not appear to cause deleterious effects, further supporting the pre-metamorphosis time requirement for cell survival. By contrast, experiments with *bsk* and *Br-C* had to be carried out in the L2 switch-on mode to bypass their early lethality.

From this study, it seems clear that liganded and unliganded EcRs must either regulate different gene repertoires or operate in different modes on the same gene repertoire. As a conceptual frame, two types of ecdysone response sequences in the target genes have been proposed:[14]

inductive ones, where the liganded receptor strongly activates transcription; and permissive ones, in which the unliganded receptor represses transcription until the time when the 20E binding relieves repression. The important difference between the inductive and permissive response elements is that, in the absence of receptor – as it is the case in this study – there will be no activation of the gene controlled by the inductive response element, whereas in the case of the permissive element, the repression is absent and the gene is activated precociously.

It was also suggested that EcR and USP monomers separately act as repressors.[7,15] The observation that EcR↑ rescues USP↓, suggests that the normal repression by EcR/USP heterodimers can be efficiently carried out by EcR alone, either acting as a monomer or as a homodimer. Alternatively, in the absence of USP, EcR could heterodimerize with a third, unknown, receptor. That idea was suggested for USP in the eye disc morphogenetic furrow progression because EcR is not required in that system.[41] However, the suggestion seemed to conflict with data in *Manduca*[42] and *Drosophila*[43] showing that morphogenetic furrow progression requires ecdysone. The issue was provisionally settled by proposing a novel hormone transduction pathway involving an uncharacterized receptor. The EcR-independent function of USP in the eye would occur via heterodimerization of USP with one of the numerous orphan nuclear receptors identified in *Drosophila.*[44] Actually, it has been shown that USP transiently interacts with FoxO in the PG, as a nutritional-dependent mechanism to control ecdysone biosynthesis before reaching critical weight (i.e., at 82 h AEL).[45] These proposals would be in line with another study showing that silencing USP in the salivary gland does not block glue protein synthesis but it requires EcR.[46] Yet, the binding of ecdysone to a receptor, other than the heterodimer EcR/USP, remained to be proven.

The quantitative data on *reaper* expression provide an *in vivo* assay to these proposals. In the PG, but not in the wing disc, *reaper* becomes de-repressed when EcR or USP are depleted. This transcriptional change is ligand-independent because ecdysone synthesis is abolished in the PG under these conditions, and it can be re-established by EcR alone. Although formally possible, the involvement of a third, unknown, co-repressor would require its strict tissue specificity. The *Drosophila* homolog of Alien, COUP-TF1, is a co-repressor reported to bind EcR but not USP.[47,48] Out of the current transcriptional cofactors known to bind EcR and USP, histone methyltransferases TRR,[49] dSet2,[50] dG9a,[51] nucleosome remodeling factor NURF[52] and Taiman,[53] all of them act as co-activators, rather than co-repressors. In any event, assuming co-repressors, in the light of the current data, the search for them should be conducted in the PG. Noticeably, the magnitude of *reaper* de-repression is different in EcR↓ *versus* USP↓, and in prothoracic *versus* salivary glands. Thus, either the repressing activity of EcR is stronger than that of USP or, alternatively, additional, still unknown, components of the gene repression complex will be required to account for this quantitative feature on *reaper* expression control.

Anti-estrogen treatments are often considered in several types of human cancers. However, development of resistance,[36,37] sometimes due to mutations in their receptors,[54] or undesirable secondary effects[55] including bone mass loss[56] represent serious handicaps. The EcR requirement for cell survival shown here in flies is operative in tumor overgrowths, at least those elicited by deregulated *yki* or *Ras* signaling which are conserved in humans.[57] Notably, the *yki** condition, although leading to aberrant overgrowth, does not imply a loss of cell identity as tested for the EcR requirement or, at least, does not imply the *de novo* acquisition of a requirement for EcR. Taken together, the data reported here suggest that targeting steroid receptors may be an efficient strategy against tumor overgrowth providing that the affected cell type requires that particular receptor for survival. This is in contrast to current strategies that focus on modulating systemic steroid levels. A prior characterization of receptor requirement in the affected tissue may provide more consistent results.

Materials and Methods

Fly strains. The following fly stocks were obtained from the Bloomington *Drosophila* Stock Center (Fly Base http://flybase.bio.indiana.edu) except where indicated: *usp*^{5} (#44383), *UAS-bsk* (#9310), *UAS-Atg8-mCherry* (#37750), *Df(3 L) H99, kni*$^{ri\text{-}1}$*p*^{p} */ TM3,Sb* (#1576, a deficiency for *reaper* and *hid*), *UAS-Ras*^{V12} *(#4847), UAS-p35 (#5072), UAS-dIAP1(#6657), tub-Gal80*^{ts} *(#7108), UAS-EcRA-RNAi* (#9328), *UAS-EcRB1-RNAi* (#9329), *UAS-EcRB1*^{W650A} (#6872), *UAS-EcRB1*^{F645A} (#6869), *UAS-yki*^{S111A,S168A,S250A}*(#28817), puckered*^{E69}*-LacZ*,[32] *ppl-Gal4*,[58] *phm-Gal4UAS-CD8-GFP* (gift from Mike O'Connor, University of Minnesota), *UAS-Torso* (gift from Jordi Casanova, Instituto de Recerca Biomédica), *UAS-β-FTZ-f1* (gift from Rosa Barrio, CIC-Biogune, Center for Cooperative Research in Biosciences), *UAS-Broad Z1-Z4* isoforms (gift from Lynn Riddiford, Howard Hughes Medical Institute), *UAS-Atg4*^{C98A} (gift from Dolores Ganfornina, University of Valladolid). RNAi lines used were obtained from Vienna Drosophila RNAi Center (VDRC) (https://stockcenter.vdrc.at): *UAS-usp-RNAi* (#16893GD), *UAS-rpr-RNAi* (#12045GD), *UAS-Hid-RNAi* (#7912GD), *UAS-Sickle-RNAi* (#102512KK), *UAS-Atg1-RNAi* (#16133GD), *UAS-Atg5-RNAi* (#104461KK), *UAS-Atg7-RNAi* (#45558GD), *UAS-EcR-RNAi* (#37058GD), *UAS-ptth-RNAi* (#102043KK) and *UAS-EcR-RNAi* (#9327). The genotype *elav-Gal80; rotund-Gal4 UAS-GFP*^{nls}was used to drive expression to the salivary gland and imaginal discs without interference from the nervous system expression of the driver

Inmunohistochemistry. Larvae were fixed with 4% paraformaldehyde in PBS for 20 min, and then washed with PBS. GFP was directly visualized. Anti-Broad core and anti-EcR antibodies were from the Developmental Studies Hybridoma Bank (University of Iowa) while anti-caspase-3 was from Cell Signaling (Danvers, MA, USA) and anti-phosphohistone H3 (Ser10) was from Millipore (Billerica, MA, USA). Primary antibodies were incubated overnight in PBS containing: 0.1% Triton-X100 5% normal goat serum and 5% bovine serum albumin. After washing with PBS, they were incubated with Alexa 488-, 568- or 594-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). For mounting, Vectashield (Vector Laboratories, Burlingame, CA, USA) containing DAPI was used. Images were acquired using a Leica TSC SP5 confocal microscope (Leica, Wetzlar, Germany).

Quantitative RT-PCR. For quantitative RT-PCR assays from whole larvae, RNA from at least five larvae per genotype was extracted using Trizol (Invitrogen). In case of organ-specific qRT-PCR, 20 Ring glands or 10 salivary glands or 20 imaginal wing discs were dissected from each genotype and RNA was extracted using RNeasy micro Kit (QIAGEN, Hilden, Germany). One to 5 μg RNA were used for reverse transcription, RT, performed with Supercript II (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was carried out using Taqman MGB probes (Applied Biosystems, Waltham, MA, USA) for the different genes analyzed. RNA polymerase II (RNApolII) was used as a housekeeping gene control. Data were captured on a 7500 Real Time PCR System (Applied Biosystems) and analyzed using relative expression to RNApolII and plotted as a percentage of the respective control larva.

Oil Red O staining. Ring glands were fixed in 4% paraformaldehyde for 20 min, washed twice in PBS and incubated in an Oil Red O (Sigma-Aldrich,

St Louis, MO, USA) solution at 0.06% for 30 min. Samples were washed twice with PBS before mounting in Vectashield (Vector Laboratories).

Larval staging and 20E feeding. The corresponding crosses were allowed to lay eggs in 2 h batches over agar plates with yeast paste, and this was considered as point 0 h after egg laying (AEL). Twenty four hours later, all hatched larvae were collected in a new plate and kept at 25°C up to the indicated time for harvesting. For 20E feeding: *UAS-EcR^{RNAi}* or *UAS-USP^{RNAi}* flies were crossed with *phm-Gal4* to knockdown expression in the PG. Experimental and control animals (*phm-Gal4>UAS-LacZ*) were collected at 116 h AEL and placed in groups of 10 individuals in plates supplemented with 20E (Sigma-Aldrich) dissolved in ethanol at 1 mg/ml and mixed with yeast. Control larvae were fed with yeast mixed with ethanol.

Ecdysteroid titers. Fifteen to 20 staged larvae 3 were weighed and preserved in 1 ml of methanol. Prior to the assay, samples were homogenized and centrifuged (10 min at 18 000 × *g*) twice and the resulting methanol supernatants were dried. Samples were resuspended in 50 μl of enzyme immunoassay buffer (0.4 M NaCl, 1 mM EDTA, 0.1% bovine serum albumin in 0.1 M phosphate buffer). Ecdysteroid levels were quantified by ELISA as described.[59] 20E (Sigma-Aldrich) and 20E-acetylcholinesterase (Cayman Chemical, Ann Arbor, MI, USA) were used as the standard and enzymatic tracer, respectively. The antiserum (Cayman Chemical) was used at a dilution of 1 : 50 000. Absorbance was read at 450 nm using a MultiscanPlus II Spectrophotometer (Labsystems, Ramat-Gan, Israel). The ecdysteroid antiserum has the same affinity for ecdysone and 20E,[59] but because the standard curve was obtained with the latter, results are expressed as 20E equivalents.

Statistics. All numerical data are presented as average ± S.E.M. Statistical significance was calculated using Student's two-tailed *t*-test (unpaired two samples for means) after application of the Kolmogorov–Smirnov method to verify the normality of data distribution. Significance was denoted as $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Differentiation website (http://www.nature.com/cdd)