Steroidal sex hormones including oestrogen, progesterone and testosterone regulate the growth and physiology of reproductive organs during puberty, the oestrous cycle and pregnancy. Consequently, these hormones also promote tumorigenesis in the breast, uterus and prostate. Although sex-specific differences in physiology and disease predisposition extend to nearly all organs, the functions of sex-specific steroids in non-sex organs remain relatively poorly explored and controversial. Drosophila uses one major steroid hormone, 20-hydroxy-ecdysone (ecdysone, also known as 20HE) and its derivatives. Similar to vertebrate steroids, 20HE is synthesized by cytochrome P450 enzymes from cholesterol. The ecdysone receptor comprises a ligand-binding EcR subunit and a DNA-binding Usp subunit—orthologues of human farnesoid X and liver X receptors (FXR and LXR) and retinoid X receptor (RXR), respectively. In juvenile insects, 20HE regulates developmental transitions including metamorphosis and sexual maturation. In adult Drosophila, 20HE is made by the ovaries after mating, resulting in higher levels in females than in males. It acts in the adult nervous and reproductive systems and affects metabolism and lifespan, but a role in the gut has not been described.

Drosophila intestinal stem cells (ISCs) are more proliferative in females than in males, and females are more prone to age-dependent gut dysplasia and intestinal tumours. These sex-specific traits could be due to ISC-autonomous and/or systemic factors. Consistent with the former, stress-dependent ISC divisions, which are more frequent in females than in males, are reduced if the ISCs are masculinized by repressing the sex-determination genes sxl or tra (Extended Data Fig. 1a, b). Mated females support more ISC division than virgin flies (Extended Data Fig. 1a–c), which suggests hormonal influences. Because mated females have higher titres of ecdysteroid than virgins or males, we tested whether 20HE might affect ISC proliferation. Indeed, feeding virgin females 5 mM 20HE strongly induced ISC divisions. This effect was independent of ISC sex identity (Fig. 1a, Extended Data Fig. 1d), and also occurred in mated females and males (Fig. 1b–d, Extended Data Fig. 1a). Using reporters of receptor activity, we confirmed that exogenous 20HE promotes EcR–Usp signalling in midgut ISCs, transient progenitors known as enteroblasts (EBs) and differentiated absorptive enterocytes (ECs) (Extended Data Fig. 1f–j).

Unlike stress caused by detergents, 20HE treatment induced two successive waves of ISC division (Fig. 1d, Extended Data Fig. 1e). Using RNA interference (RNAi) under the control of conditional cell-type-specific Gal4 drivers, we found that the first wave (at 6 h after 20HE feeding) required EcR only in ISCs (Fig. 1e), but that later divisions (at 16 h) also depended partially on EcR in EBs (Fig. 1e, Extended Data Fig. 2a–f). Neither wave of division required EcR in ECs, enteroeocnic or neural cells (Extended Data Fig. 2g–i). Isoform-specific tests revealed that EcR-A was much more important than EcR-B for the 20HE-induced division of ISCs (Extended Data Fig. 2k–m). 20HE-induced divisions were reversible (Extended Data Fig. 2j), which suggests a lack of toxicity. EcR activity was not induced by enteric infection (Extended Data Fig. 1f–h), and EcR was dispensable for infection-induced gut regeneration (Fig. 1h, Extended Data Fig. 2a, k, l, n–q), which indicates a distinct role for EcRin the gut. Loss of Usp, however, did block infection-induced ISC divisions, which suggests that Usp has EcR-independent functions (Fig. 1h, Extended Data Fig. 2a, n, p).

Next we asked whether ISC activation by 20HE involves the Upd–Jak–Stat or Egfr–ERK signalling pathways, which are known to

Sexual dimorphism arises from genetic differences between male and female cells, and from systemic hormonal differences. How sex hormones affect non-reproductive organs is poorly understood, yet highly relevant to health given the sex-biased incidence of many diseases. Here we report that steroid signalling in Drosophila from the ovaries to the gut promotes growth of the intestine specifically in mated females, and enhances their reproductive output. The active ovaries of the fly produce the steroid hormone ecdysone, which stimulates the division and expansion of intestinal stem cells in two distinct proliferative phases via the steroid receptors EcR and Usp and their downstream targets Broad, Eip75B and Hr3. Although ecdysone-dependent growth of the female gut augments fecundity, the more active and more numerous intestinal stem cells also increase female susceptibility to age-dependent gut dysplasia and tumorigenesis, thus potentially reducing lifespan. This work highlights the trade-offs in fitness traits that occur when inter-organ signalling alters stem-cell behaviour to optimize organ size.
activate ISCs after stress. Six hours of 20HE feeding induced the Egfr ligands spi and knn and their activating protease rho, but not the upd2 or upd3 cytokines or Stat signalling (Extended Data Fig. 3a, b). Exposure to 20HE for 16 h, however, moderately induced upd2, upd3 and Stat activity (Extended Data Fig. 3c–h). The induction of upd2, upd3 and rho required EcR in ISCs and EBs (that is, ‘progenitors’), although not in ECs (Extended Data Fig. 3c–e). The Egfr effector ERK was also mildly activated by 16 h of 20HE exposure, mostly in progenitors but occasionally in ECs (Extended Data Fig. 3i). ERK activation required upd2 (Extended Data Fig. 3i), which suggests a signalling relay. Importantly, the induction of all of these targets (upd2, upd3, Socs36E, rho, spi and knn) by 20HE was suppressed by blocking ISC mitoses with RNAi molecules that target string (also known as stg or cdc25) or Egfr (Extended Data Fig. 3f). This suggests that the observed increases in Jak–Stat and Egfr–ERK signalling are responses to epithelial stress from the early ISC divisions. In further tests, we found that Upd2 from EBs and ECs contributed strongly to ISC divisions 16 h after 20HE feeding, but only weakly to the early divisions at 6 h (Fig. 1g, Extended Data Fig. 3j–l). Egfr and Rho, however, were always required (Fig. 1f, Extended Data Fig. 3m). We conclude that ISC divisions are initially activated ISC-autonomously via EcR, and require Egfr and Rho, whereas later divisions depend in part on cytokines produced by EBs and ECs, perhaps in response to stress from the first mitoses. The relationship of EcR to Egfr signalling warrants further investigation.

Because mated females produce more ecdysone than virgins or males, we tested whether 20HE might account for sex-specific differences in the gut. Consistent with this, long-term exposure of males to 20HE phenocopied the female condition, increasing ISC mitoses, stress responsiveness, epithelial turnover and midgut size (Fig. 1i–k, Extended Data Fig. 4a–o). Genetically feminizing the male ISCs did not give these effects (Fig. 1l), which suggests that 20HE acts independently of genetic sex determination. Forced expression of the ISC mitogen13 sSpI also failed to enlarge male midguts (Fig. 1j), which indicates that 20HE affects more than just the ISC mitotic rate. Long-term 20HE feeding also endowed ISCs in virgin females with proliferative characteristics similar to those seen after mating (Extended Data Fig. 4d).

By contrast, RNAi lines that antagonized 20HE signalling in ISCs and EBs decreased gut size in mated females and suppressed mitoses in response to detergent stress (Fig. 2c, d, Extended Data Fig. 4e–g). Thus, sexually dimorphic proliferative traits of ISCs are determined in part by 20HE signalling.

Similar to human oestrogen and progesterone, ecdysone promotes behavioural and metabolic changes that enhance female reproductive fitness. Dose–response assays showed that 1 mM 20HE fed to virgin females activated EcR targets and ISC mitoses to similar levels to mating (Fig. 2i, Extended Data Fig. 5a). Hence, we tested whether endogenous, mating-induced 20HE activates ISCs. Indeed, mating induced a large, transient increase in ISC division and enduring gut enlargement (Fig. 2a–d, Extended Data Fig. 5b–h, k). This was independent of genetic sexual identity (Fig. 2e, Extended Data Fig. 5i). As with exogenously fed 20HE, these effects initially required EcR only in ISCs, although EcR in EBs contributed later (Fig. 2f, g, Extended Data Fig. 5j–e). Similar to exogenous 20HE, mating also increased expression of upd2 and rho (Extended Data Fig. 5i), which suggests that these are normal physiological responses.

**Fig. 1 | Ecdysone induces ISC proliferation and gut growth.** a. Midgut mitotic counts of esg-Gal4 ts>UAS-tri RNAi virgin females after overnight infection with *Pseudomonas entomophila* (P.e.), and feeding with 5 mM 20HE or 1% SDS for 16 h. The esg-Gal4 ts driver (esg-Gal4 tub-Gal80*) activates UAS target gene expression specifically in ISC and EBs (‘progenitor’ cells). b. ISC lineage-tracing using esgF0ts, which drives UAS target gene expression in progenitor cells and their newborn progeny (ECs or EBs) after a temperature shift. c. Midgut mitotic counts of esg-Gal4 ts>UspRNAi flies fed 5 mM 20HE for 16 h. d. Midgut mitotic counts from a200 controls fed 5 mM 20HE for different durations. e. ISC mitoses in midguts expressing EcR RNAi in ISCs and EBs (left) or in EBs (right) after 6 or 16 h of 5 mM 20HE feeding. f. Mitotic counts in midguts expressing rho RNAi or Egfr RNAi in ISCs and EBs 6 h after 20HE feeding. g. Mitog...
To confirm the source of endogenous ecdysone, we used ovary-specific \textit{Gal4} drivers to express RNAi transgenes that target the ecdysone synthesis enzymes Dib or Spory. This suppressed mating-induced ISC divisions and midgut growth, both of which could be restored by exogenous 20HE (Fig. 2h, j, Extended Data Fig. 5n–p). \textit{spo} mutants also failed to resize the midgut after mating (Fig. 2i, Extended Data Fig. 5m), confirming these results. To learn how the gut grows in mated females, we investigated the effects on cell size and number. Depleting EcR in ECs did not reduce EC size (Extended Data Fig. 5q), but mating caused a large 20HE- and EcR-dependent increase in female ISC numbers (Fig. 2k, Extended Data Fig. 5r–t). This expansion of the stem-cell pool could cause an increase in the total number of midgut cells. These results indicate that mating-dependent ISC division, ISC expansion and gut growth
are driven by 20HE signalling from the ovaries to progenitor cells in the gut.

Gut growth after mating is expected to increase the absorption of nutrients by the intestine and the supply of nutrients to other organs. Because egg production is limited by nutrient availability to the ovaries, it is essential to understand how ecdysone activates ISCs, we tested two known EcR targets: the transcription factor Broad, and the nuclear receptor Eip75B, a homologue of human PPARγ and REV-ERBβ. Eip75B and broad (br) mRNA were induced in midguts by 20HE or mating (Fig. 2l, Extended Data Fig. 8a), and progenitor-cell-specific depletion of either factor suppressed 20HE-induced mitoses (Fig. 2m, Extended Data Fig. 8b–e). ISC clonal growth, however, required Eip75B but not br (Extended Data Figs. 2b, c, 8e, f), highlighting that Eip75B is a more essential effector. Overexpression of Eip75B was sufficient to promote ISC division and gut epithelial turnover (Fig. 2n, Extended Data Fig. 8g), whereas Eip75B loss impaired both ISC mitoses and maintenance (Extended Data Fig. 5s, 8c, f, h, i). Progenitor-specific loss of Eip75B also blocked gut growth after mating (Fig. 2c), and compromised egg production (Fig. 2p, Extended Data Fig. 6b–d), phenocopying the effects of EcR loss. Eip75B binds DNA to repress target genes, and also binds the nuclear receptor Hr3 to inhibit Hr3-mediated transcriptional activation28. Consistent with this mechanism, overexpression of Eip75B or 20HE feeding suppressed an Hr3 activity reporter, and Hr3 overexpression suppressed ISC proliferation (Extended Data Fig. 8j–l). Moreover, depletion of Hr3 counteracted losses in ISC proliferation caused by Eip75B depletion (Extended Data Fig. 2o, 8m, n), which indicates that Eip75B has other targets. Further tests revealed that Eip75B and Hr3 mediate 20HE-independent ISC responses to stress. Enteric infection strongly induced levels of Eip75B mRNA (Extended Data Fig. 8a) and
suppressed Hr3 activity (Extended Data Fig. 8j). Removing Eip75B or broad from ISCs by mutation (Extended Data Figs. 2b, c, 8e) or by RNAi (Extended Data Figs. 8b, c, h, i, 9a–d) blocked infection-induced ISC mitoses, as did overexpression of Hr3 (Extended Data Fig. 9e). Eip75B was also required for ISC mitoses in response to the oxidative stress agent paraquat (Extended Data Fig. 8h, i). Furthermore, we obtained evidence consistent with previous work\textsuperscript{2} that the action of Eip75B is modulated by haem (a Eip75B ligand) and nitric oxide (Fig. 2m, Extended Data Fig. 9f, g). Functions for haem and nitric oxide in the fly gut are unknown, but potentially interesting. We conclude that Eip75B, Broad and Hr3 integrate several inputs in addition to 20HE to control ISC proliferation (Extended Data Fig. 9h).

As females age, they experience progressive gut dysplasia in which ISCs overproliferate and mis-differentiate, leading to high microbiota loads (dysbiosis), barrier breakdown and decreased lifespan\textsuperscript{20}. Age-dependent intestinal dysplasia is more pronounced in females than in males\textsuperscript{11}, and can be identified by increases in mitoses and mis-differentiated cells doubly positive for ISC and EC markers. Supressing Ecr, Usp or Eip75B in midgut progenitors significantly reduced both parameters of dysplasia in aged flies (Fig. 3a–c, Extended Data Fig. 10a). Similarly, suppressing ec dysplasia synthesis enzymes (Dib, Spo) in the ovaries, or ubiquitously, also curtailed age-dependent gut dysplasia (Fig. 3d, Extended Data Fig. 10b, c). This effect could be reversed by 20HE supplementation. These results indicate that age-dependent gut dysplasia is potentiated by ovary-derived ec dysplasia, explaining the sex bias of this condition.

Female Drosophila are known to be more susceptible than males to genetically induced ISC-derived tumours. We found that isc/eb-specific RNAi targeting Notch, a receptor required for EC differentiation, drove tumour induction in 100% of mated females but was far less tumorigenic in virgin females or males (Fig. 3e–g, Extended Data Fig. 10d, e). Three results indicate that this tumour predisposition is modulated by 20HE. First, in contrast to mated females, virgins were extremely resistant to Notch\textsuperscript{9} and ec dysplasia induction (Fig. 3e–h). Second, targeting 20HE signalling in ISCs with a dominant-negative Ecr-A (EcR\textsuperscript{ADN}) inhibited tumour growth in mated females (Fig. 3e, g, Extended Data Fig. 10d). Third, supplementing males or virgin females with 20HE increased tumour initiation and growth (Fig. 3g, h, Extended Data Fig. 10f).

Gut dysplasia, tumorigenesis and egg production can all shorten lifespan\textsuperscript{2,20,21}, which suggests that the effects of ec dysplasia on the gut might adversely affect longevity. In fact, earlier reports showed that EcR mutants live longer\textsuperscript{9}, and proposed that reproduction can shorten lifespan by damaging the soma\textsuperscript{6}. Our own lifespan assays, although subject to the same caveats as previous work\textsuperscript{20} (Supplementary Discussion), support this view: suppression of EcR in midgut progenitors extended lifespan in females but not males (Extended Data Fig. 10g–i). In evolutionary terms, the disadvantage of a slightly shorter lifespan due to sex-specific hormonal signalling is probably insignificant relative to the reproductive fitness advantage conferred by increased egg production. This may be especially true in the wild, where gut dysplasia-dependent mortality is probably counteracted by nutrient deprivation\textsuperscript{22}. Similarities in the reproductive biology of Drosophila\textsuperscript{23,24} and mammals\textsuperscript{25} suggest that these inter-organ relationships have relevance to human biology. The mitogenic effects of insect ec dysplasia parallel those of oestrogen and testosterone as drivers of breast, uterine and prostate growth and tumorigenesis. Yet how these steroids affect the human intestine remains poorly explored. Adaptive growth of the intestine is well documented in pregnant and lactating mammals\textsuperscript{26,27}, and might depend on oestrogen and/or progesterone. Laboratory tests with rodents and human cells, as well as some studies with human participants, have linked oestrogen, testosterone and their receptors to gastrointestinal cancers\textsuperscript{28,29}, but epidemiological studies provide conflicting evidence regarding this association\textsuperscript{30–32} (Supplementary Discussion). The contributions of sex steroids to intestinal physiology deserve more detailed study.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-2462-y.

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**Methods**

**Drosophila stocks and cultures**

*Drosophila melanogaster* were raised on standard media and maintained in incubators with controlled temperature and humidity on a 12 h light/dark cycle. Flies were transferred to fresh vials every 2 days. Male and female *Drosophila* were raised mated for all experiments, unless otherwise indicated. To generate controls, *w*¹¹¹⁸ (VDRC 60000) flies were typically outcrossed to the appropriate *Gal4* driver line. To generate controls for experiments using VDRC ‘KK’ RNAi lines, the stock *y w¹¹¹⁸; P[Plattpy]/+; w¹¹¹⁸* (VDRC 60100) was outcrossed to the appropriate *Gal4* driver line. Full genotypes of all stocks used, and for each figure panel, are listed in Supplementary Tables 1 and 2.

**Drosophila husbandry**

For transgene expression using the *Gal4/Gal80* system, experimental crosses were maintained at 18 °C (permissive temperature for *Gal80*) in standard medium. Animals of the desired sex and genotype were collected within 48 h of eclosion and aged for an average of 5 days before shifting to 29 °C (restrictive temperature for *Gal80*) to induce UAS transgene expression. Adult midguts were dissected after different periods of time as indicated in each figure. The esg-Flip Out system (esgfO*)¹⁹ and the MARCM system²⁰ were used to generate ISC-derived clones. Flies were aged for 3–6 days after eclosion before clonal induction by temperature shift to 29 °C for esgfO clones or heat shock for MARCM clones. Further details on transgene expression times are indicated in the figure legends. MARCM 80B flies were heat-shocked for 45–60 min in a 37 °C water bath, and then aged for 12 days at 29 °C before overnight treatment with vehicle, 5 mM 20HE or *Psuedomonas entomophila*.

**Mating experiments**

At least 10–15 virgin females for each genotype were collected at 18 °C as they emerged. They were aged for approximately 5 days and then shifted to 29 °C until the time points indicated in each figure. At the start of mating, females were transferred to fresh vials and allowed to mate with equal numbers of adult 3–7 days old wild type *w¹¹¹⁸* males, devoid of any transgenes, at 25 °C, for optimal fecundity. Time when males were introduced to females in the same vial is denoted as *t*₀. If indicated as mated once, then after 18–20 h, the males were removed and the females were flipped into fresh vials every 45–60 h until the indicated time in the respective figures. Otherwise, males were left together with the females for the following time points: 24 h, 37–40 h, 46–48 h or 72–74 h.

**GAL4-LBD ‘ligand sensor’ system**

Adult flies with bipartite detection system consisting of the LBD of the *Drosophila* nuclear receptor fused to the DNA-binding domain of yeast GAL4, along with a GAL4 UAS-controlled GFP reporter gene were used as previously described¹⁰,¹³. Flies were raised and maintained at 25 °C. For visualization of ligand sensor patterns, 5–7-day-old mated females were starved for 2–4 h, heat-treated for 30 min in a 37 °C water bath only once for Ecr, Usp and Hr3 reporters, and allowed to recover at room temperature for 15 min. Then, flies were transferred to vials containing a fresh feeding vial (see ‘Feeding experiments’) and kept at 25 °C for 16–18 h until dissection.

**In vivo 10XSTAT92E-GFP reporter system**

Adult mated female flies of the genetic background 10XSTAT92E-GFP that have 10 Stat92E-binding sites driving GFP expression were aged for 5–7 days and treated for 6 h with 5 mM 20HE and for 16–18 h with 5 mM 20HE or *P. entomophila* infection.

**In vivo upd3-lacZ reporter**

Adult mated female flies of the genetic background Upd3.1 LacZ/TM6B were aged for 5–7 days and treated for 16–18 h with 5 mM 20HE or *P. entomophila* infection.

**Overnight feeding experiments**

For all experiments except 20HE or SDS feeding (as indicated in the figures), flies were fed for 16–20 h, then dissected to remove the intestines, which were analysed using immunofluorescence and confocal imaging. For timed 20HE feeding, flies were collected as early as 4 h and as late as 22 h after continuous 20HE exposure. We observed a window of strong mitotic response at 6 h and again at 16–18 h that persisted to 22 h after exposing the flies to the 20HE feeding solution.

For 20HE removal experiments, flies were fed overnight for 16–18 h with 5 mM 20HE, and then transferred to a fresh vial for another overnight treatment after which the midguts were dissected and stained. 20HE feeding: 10–15 adult male, mated female or virgin female flies were used for the ecdsyone feeding experiments, as indicated. 20HE was dissolved in 100% ethanol, water was added to make a 25 mM stock solution in 10% ethanol, and stocks were stored at -20 °C. A final concentration of 0.25–10 mM ecdysone or 2% ethanol (as control) was used for the feeding experiments as indicated. Then, 200 μl of 5% sucrose solution, 5 mg ml⁻¹ dry yeast and 5 mM 20HE (Sigma-Aldrich H5142) mix was deposited on top of a standard food vial to which flies were transferred. If the experiment required *P. entomophila* infection, then 400 μl of the same yeast/sucrose mix (described above) was deposited on filter-paper discs (Whatmann) to which flies were being transferred. The sucrose yeast mix with 2% ethanol was used as vehicle treatment.

Detergent treatment: flies were left to feed on yeast sucrose solution (described above) with 0.1% or 1% SDS for 18–20 h or at the times indicated.

*Enteric* *P. entomophila* infection: a 25 ml pre-culture was started the first day by inoculating *P. entomophila* bacteria from glycerol stocks (stored at -80 °C) in Rifampicin-supplemented Luria Broth (LB; final antibiotic concentration: 100 μg ml⁻¹). The pre-culture was grown overnight at 29 °C, shaking at 130 rpm. The next day, the pre-culture was diluted in 175 ml Rifampicin-supplemented LB and the culture was again grown overnight at 29 °C, shaking at 130 rpm. After the growth of the bacterial culture reached optical density of approximately 0.5, the culture was spun down at 2,500g for 25 min at 4 °C and the pellet was re-suspended in 3 ml of 5% sucrose plus 150 μl yeast. Before infection, flies were starved for 2 h (optional step), and then placed in vials with 500 μl of this *P. entomophila* solution or 5% sucrose with yeast as the control vehicle.

Other treatments in Fig. 3 and Extended Data Figs. 8, 9 include feeding with 2.5 mM paraquat, Nω-nitro-L-arginine methyl ester hydrochloride (Sigma-Aldrich, N7571) (200 mM l-NAME stock solution in distilled water; final 10 mM concentration was used), (±)-N-nitroso-N-acetylpenicillamine (Sigma-Aldrich, N3398) (500 mM SNAP stock solution in 10% ethanol and 10 mM SNAP final solution was used), hemin (Frontier Scientific, H651-9) (2 mM stock solution dissolved in 0.1 M NaOH, pH adjusted to 7 with sodium phosphate buffer and 0.5 mM final solution was immediately used) and their corresponding vehicle. Treatments were diluted in 400 IL total volume of 5% sucrose and 5 mg ml⁻¹ yeast then added vials containing a fresh feeding paper.

**Long-term ecdysone feeding**

At least 10–15 adult male and/or female flies were transferred to standard fresh food vials (2.5 cm diameter) containing circa 3 ml of food. To prepare ecdysone treated food, the food in the vial was scraped on the surface and 200 μl 1 mM 20HE, 22 mg ml⁻¹ yeast in 5% sucrose solution was added. After 15 min, this solution diffused into the food. Flies were added to these vials and flipped into fresh 20HE containing vials every 48 h for 14 days unless otherwise indicated. As vehicle, vials with fly media containing 200 μl 0.43% ethanol in sucrose/yeast solution were used. Flies were dissected to remove the intestines, which were analysed using immunofluorescence and confocal imaging. For the flies raised on low nutrient food, flies were fed with 1 mM 20HE.
5 mg ml⁻¹ yeast in 5% sucrose solution that was deposited on filter-paper discs (Whatmann) and exchanged every 24–30 h. For *P. entomophila* infection after long-term 20HE feeding in Extended Data Fig. 4c, we discontinued feeding the flies on ecdysone-containing food for one day before the flies were fed with the *P. entomophila* bacterial solution.

**Fecundity assays**

Fig. 2p. Extended Data Fig. 6b: 10–15 virgin females for each genotype/replicate were collected at 18 °C as they eclosed, and pooled in one vial. For each genotype, 3–4 replicates were performed for every experiment. Virgins were aged one day and then shifted to 29 °C to activate Gal4. Females were then transferred to fresh cages and allowed to mate with equal numbers of *u*<sup>IBa</sup> males. Females were housed in groups of 7–10 with equal number of males for this experiment. Standard *Drosophila* media was poured in 5 cm plates and stored at 4 °C. Flies in each egg collection cage were flipped onto fresh food plates every 24–48 h for the indicated number of days, and the number of eggs/replicate were scored and averaged over the number of flies in each cage. Three to four independent experiments were performed, all results were pooled, and are shown in Fig. 2p and Extended Data Fig. 6b. Raw egg counts, processed cumulative sums, averages, and *P* values for each experiment are in the Source Data.

Extended Data Figure 6d: virgins were aged for 8 days and shifted to 29 °C to activate Gal4 first before mating to equal number of males. Females were housed in groups of 7–10 with equal number of males for this experiment. Flies in each egg collection cage were flipped onto fresh food plates every 24–48 h for the indicated number of days, the number of eggs/replicate were scored and averaged over the number of flies in each cage. Cages with dead flies were excluded from the analysis. Raw egg counts, processed cumulative sums, averages, and *P* values are available in the online source data.

Extended Data Figure 6c, d: virgins were aged for one day and then shifted to 29 °C to activate Gal4. Females were then transferred to fresh vials and allowed to mate with equal numbers of *u*<sup>IBa</sup> males. All subjects were housed overnight in the same vial to ensure mating success and numbers of eggs were counted and averaged for the number of females/vial. Next day, every female and male pair was separated and individual females or vials were followed up for 14 days. Vials were exchanged every 24–48 h in this experiment and the total number of eggs laid every 2 days was counted for every female vial. Vials with dead flies were excluded from the analysis. Raw egg counts, processed cumulative sums/averages and *P* values are in the online Source Data. A 2- or 3-day sum was calculated from the average number of eggs or flies laid every day, and then an average sum of eggs laid per fly per 3 days across the replicates was plotted with error bars ± confidence intervals. Alternatively, the average or individual cumulative numbers of eggs were summed up and mean values were plotted ± standard deviation. To test statistical significance for each day, two-sample unequal variance *t*-test were performed, with a two-tailed distribution assuming unequal variance for test genotype relative to control at every time point. Individual *P* values are in the online Source Data. Alternatively, for Fig. 2p, general linear models with binomial errors were used to examine the effect of the genotype on the average cumulative number of eggs.

**spo mutant rescue experiment**

Males of either deficiency backgrounds BM#7584 or BM#24411 were crossed to heterozygous *spo* mutant virgins and allowed to lay eggs on apple plates for several days before the experiment. Two deficiency genotypes were used to increase the likelihood to getting rescued homozygous *spo* mutant flies. On the day of the experiment, the parents were left to lay eggs for 4 h then, were removed. The eggs were allowed to age 4–6 h at 25°C then, were all pooled in a sieve and de-chorionated by bleach. After washing in PBS-T, the de-chorionated embryos were incubated in PBS-T supplemented with 100 μM 20E for 3 h. The embryos were covered with Halocarbon 27 oil and incubated at 18 °C overnight. Over the next 2 days, homozygous *spo* embryos were selected under a fluorescent stereoscope by the lack of GFP expression in the hatched larvae. The phenotypically correct larvae were collected in fresh food vials at the density of 40–60 larvae per vial and allowed to develop at 25 °C until eclosion and selection of virgin or mated homozygous *spo* mutant flies.

**Lifespan assays**

Males and females of the genotype 5961GS EcR A DN were allowed to mate for 48 h and were then isolated in groups of 25 flies of the same sex per vial. For RU486 food supplementation, 100 μl of a 5 mg ml⁻¹ solution of RU486 or vehicle (ethanol 80%) was deposited on top of a food vial and dried for at least 4–6 h, resulting in a 0.2 mg ml⁻¹ concentration of RU486 in the food accessible to flies. Flies were flipped every 48 h into a fresh vial. Dead flies were visually identified (flies not moving, not responding to mechanical stimulation and lying on their side or back were deemed dead), and the number of dead flies was recorded. Oasis software was used for data analysis. A log-rank non-parametric test was performed by the software and the *P* values were derived from pairwise comparison with Bonferroni correction as displayed in Extended Data Fig. 10g, h.

**Immunohistochemistry and microscopy**

*Drosophila* adult midguts were dissected in 1× PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. For all immunostainings except anti-dpErk, samples were washed with 0.015 Triton-X in PBS three times at room temperature, then permeabilized with 0.15% Triton-X in PBS for 15 min at room temperature with shaking. Then, samples were re-washed and blocked in PBS with 2.5% BSA, 10% normal goat serum and 0.1% Tween-20 (blocking solution) for at least 1 h at room temperature. Midguts were incubated with primary antibody at 4 °C overnight at the following dilutions: chicken anti-GFP (Life Technologies/Molecular Probes, 1:500); rabbit anti-phospho-histone 3 (Merck Millipore 1:1,000); mouse anti-phospho-histone 3 (Cell Signaling, 1:1,000); guinea pig anti-GFP (Teleman Lab, 1:1,000); chicken anti-β-galactosidase (Abcam, 1:1,000).

For the dpERK detection, samples were fixed in 4% paraformaldehyde, dehydrated for 5 min in 50%, 75%, 85% and 100% methanol, and rehydrated for 5 min in 50%, 25% and 12.5% methanol in PBS (0.1% Triton X-100 in 1× PBS). After washing in 1× PBST, midguts were blocked in PBS with 2.5% BSA, 10% normal goat serum and 0.1% Tween-20 (blocking solution) for at least 1 h at room temperature then incubated with rabbit phospho-p44/42 MAPK (Erk1/2) (Th202/Tyr204) 9101 (Cell Signaling, 1:400) at 4 °C overnight.

After washing, all samples were incubated with secondary antibodies (Alexa 488, 568 or 633, Invitrogen) for more than 2 h at room temperature at a dilution of 1:1,000. All antibody incubations were performed in blocking solution. DNA was stained with 0.5 μg ml⁻¹ DAPI (Sigma).

For the plasma membrane cell stain: Freshly dissected midguts were stained with CellMask deep red plasma membrane stain, ThermoFisher in 1× PBS at a concentration of 1:1,000 then fixed in 4% formaldehyde and stained with 1× PBS/DAPI according to the manufacturer’s instructions.

Ovary staining: one-day-old mated females have been placed on active yeast paste for 4–5 days at 29 °C. Ovaries were dissected in PBS, transferred in PBS containing 8% paraformaldehyde and fixed for 10 min at room temperature with mixing. After washes in PBS with 0.15% Triton, ovaries were blocked for 1 h in 0.15% PBST containing 2.5% BSA. The following primary antibodies were incubated at 4 °C overnight in blocking buffer: chicken anti-GFP 1:500, mouse anti-coracle 1:500 (DSHB, C566-9). Ovaries were then washed five times for 5 min in 0.15% PBST and incubated for 1 h 30 min with the following secondary antibodies and dyes in blocking buffer at room temperature: goat anti-chicken488 1:1000, goat anti-mouse568 1:1,000, Hoechst 1:1,000.
phalloidin633 1:10,000. After two washes for 10 min in 0.15% PBST, ovaries were mounted on slides with Vectashield. Images have been acquired using a Leica Sp8 confocal microscope and the figures made using Fiji with the ScentiFig plugin.

Imaging: midguts were mounted on glass slides in Vectashield (Linaris). All midgut images were acquired on a Leica TCS SP5II inverted confocal microscope, equipped with HCX Plan APO 20×/1.30 glycerol-immersion (for quantifications) or 40×/1.30 oil-immersion objectives (for representative images/quantifications), using Leica Application Suite (LAS) AF software and processed with Fiji/ImageJ software33. Representative images are shown. GFP, in green (native GFP for all genotypes except for the reporter midguts and Su(H)+ cells marked with Su(H)ydriver that were also stained with GFP for better visualization of the signal); DNA: DAPI, in blue. To display images in the figure panels, a Z-stack of defined steps for control and test genotypes in a single field was acquired in the R4 region (a region which is bounded by the apex of the midgut tube’s most distal 180° turn) as previously described41. Images represent maximal intensity projections of the acquired Z-stacks. Scale bars are 100 μm in all images, unless otherwise indicated.

Quantifications and statistics
ISC proliferation: mitotic indices were determined by manually counting all PH3-positive cells in entire midguts using Leica DM5000B or Zeiss Axiovert fluorescence microscopes through a 40× objective. Statistical analysis of all the mitotic counts was performed using two-tailed Mann–Whitney test. All dot plot graphs indicating mitoses are showing mean ± s.d. Exact P values are provided in the Supplementary Information. Data were from at least three independent experiments.

Quantification of the GFP+/delta+ cells: Z-stacks of both epithelial sides in R4a/b region were imaged at steps of 5.0 μm at 40× then the total number of GFP+ or delta+ cells were analysed after limiting the particle size to 10–250 μm, circularity 0.00–1.00 and excluding holes after maximal Z-projection have been applied.

Quantification of the delta+ and Su(H)+ cells: Z-stacks of both epithelial sides in the R4a/b region were imaged by confocal Zeiss LSM 780 Spinning Disc. The total number of DAPI+, Su(H)+ and delta+ cells were automatically segmented and counted using a custom ImageJ/FIJI macro (Supplementary Data 6). Su(H)+ and delta+ cells were manually recounted and verified and the numbers of each cell type were recorded to derive the percentage cell type to total cell number/stack.

Quantification of cell size: midguts were mounted as previously described and Z-stacks of both epithelial sides in the R4a/b were imaged at steps 5.0 μm at 40× then a custom ImageJ/FIJI macro (Supplementary Data I) was created to segment the cytoplasm in reference to DAPI nuclear stain and intracellular distances. Area of the cells in micrometre-squared were outputted to Microsoft Excel and a mixed effects two-way ANOVA statistical model was computed to calculate the significance between the different conditions.

Quantification of clonal size: Z-stacks of both epithelial sides in the R4a/b were imaged at steps 5.0 μm at 40× then a custom ImageJ/FIJI macro (Supplementary Data 2) was used to semi-automatically segment and determine the location and size of the GFP+ clones then the sizes in micrometre-squared were outputted to Microsoft Excel and a mixed effects two-way ANOVA statistical model was computed to calculate the significance between the different conditions.

Quantification of the GFP+ areas: for analysis of the mating effects, Z-stacks of both epithelial sides in R4a/b region were imaged at steps 5.0 μm either at 40× or at 20×. For analysis, the quantification of the area occupied by GFP+ cells was performed automatically using a custom ImageJ/FIJI macro (Supplementary Data 3). The macro created maximum Z-projection of image stacks, median and Gaussian filtering, automatic thresholding and measurement of GFP+ and gut occupying area. The measurements were exported to Microsoft Excel and the GFP+/gut area ratio was derived from these values for at least 10 midguts for most experiments.

Quantification of the GFP+ area/DAPI+ cells: for analysis of the tumour effects (Extended Data Fig. 10e), a fixed median filter was created for each stack, a fixed Gaussian blur value was applied; then the median was thresholded for DAPI+ cells and GFP+ cells; then areas for both were calculated and a ratio was derived. An ImageJ/FIJI macro was used (Supplementary Data 3).

Data are displayed in scatter plots with the mean ± s.d. for each series of experiments. Data shown are representative of at least two or three independent repeated experiments with similar results. Statistical significance was calculated either by two-tailed Mann–Whitney test without a multiple comparison test. Results were considered to be significantly different at P < 0.05. All calculations were performed using the Prism 7.0 software (GraphPad Software).

Gut measurements: after immunofluorescence staining and before mounting, midguts were put on a glass slide and imaged using a Leica M205 FA Stereo Microscope or Stereo Discovery.V8, unmounted guts were imaged at a defined magnification and these images were exported to Fiji for further analysis. Custom ImageJ/FIJI macros (Supplementary Data 4, 5) were used to threshold each image then measure the area of each midgut. With the distance mapping technique, the midgut length was derived. For the width measurements, a line was drawn. Before quantifying any midgut dimensions, the genotype of each sample was concealed. Samples were randomly analysed then the genotype was revealed only after completing analysis. For statistical analyses of gut sizes, normality test was performed with Shapiro–Wilk normality test and the gut sizes showed normal Gaussian distribution. Thus, statistical significance of gut size measurements was calculated by ordinary ANOVA test, followed by Bonferroni’s multiple comparisons test. Data are displayed in scatter plots with the mean ± s.d. Data were plotted from at least three independent repeated experiments with similar results. All ImageJ/FIJI macros are available as supplementary online source material (Supplementary Data 1-6), or upon request from the authors.

Sample sizes, randomization and blinding
No statistical methods were used to predetermine sample sizes, but typically between 5 and 20 flies were used per replicate per genotype in each experiment. Exact n values for each experiment are in the online Source Data. When selecting animals for an experiment, the parental genotype was not concealed because it was required to select pertinent progeny. Animals were first selected by genotype and then randomly chosen for experimental analysis. For measurements of mitoses/gut, gut sizes and tumour frequencies, the genotype of each sample was concealed during analysis. Samples were then randomly scored and genotypes were revealed only after completing the analysis.

RT–qPCR
Approximately 10–12 female intestines per genotype were dissected and RNA isolated using the RNAeasy kit (QIAGEN). Then, 750 ng of total RNA was used for cDNA synthesis reactions using the Quantitect reverse transcription kit (QIAGEN). RT–qPCR was performed on a Light Cycler 480 II (Roche) using SYBR Green I (Roche). Experiments were performed in at least biological triplicates. Relative fold differences in expression level of target genes were calculated as ratios to the mean of the reference genes rp49 and tubulin using the ΔΔCt method. A series of tenfold dilutions of an external standard was used in each run to produce a standard curve. Primer sequences are listed in Supplementary Table 3.

ΔΔCt method: ΔΔCt (or log2-transformed fold change) is the difference in threshold cycles for the test and control sample normalized to the threshold cycles for the reference gene.

ΔΔCt = Ct (test) − Ct (control)
ΔCt (test) or ΔCt (control) = Ct target gene − Ct reference gene
All data are presented as mean log2-transformed fold change with s.d.
Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
Source data are provided with this paper.

Code availability
Code for all FIJI macros used in this study is available for download via the Supplementary Information. These macros are available as Supplementary Data 1–6.

Acknowledgements
We thank J. Zhou, I. Miguel-Aliaga, C. Thummel, P. Patel and L. O’Brien for stocks and discussions. We thank O. Salem of McMaster Immunology Research Centre for Fig. 3i and M. AbdelMoety of American University of the Middle East for the chemical structure in Fig. 3i. This work was supported by ERC AdG 268515, DFG SFB873 and NIH GM124434 to B.A.E., and by the Helmholtz Zukunftsthema ‘Aging and Metabolic Programming’ (AMPro) to A.A.T.

Author contributions
S.M.H.A. performed and analysed all experiments except Extended Data Figs. 8m, 9a–d (G.O.P.-S.) and Extended Data Fig. 6a, e–q (C.P.). J.A.M. contributed to Extended Data Fig. 8a, n and Fig 2o. The conception and design of experiments was by S.M.H.A., B.A.E., J.A.M. and A.A.T. Image-processing methods were designed by D.K. S.M.H.A., A.A.T. and B.A.E. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information is available for this paper at https://doi.org/10.1038/s41586-020-2462-y.

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Peer review information
Nature thanks Henri Jasper, Marc Tatar and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | See next page for caption.
20HE feeding promotes sexually dimorphic ISC mitotic activity. a, Male ISCs do not divide strongly in response to infection elicited by pathogenic bacteria, but divide to a similar extent as mated female ISCs in response to 20HE feeding, quantified by counting the number of dividing ISCs per midgut using pH3 staining (also termed the mitotic index) in males and mated females after 16–18 h treatment with 5 mM 20HE or pathogenic *P. e.* infection. Males are fully and equally competent to respond to 20HE treatment as mated females. b, Mating boosts the mitotic divisions of ISCs. Feeding 0.1% SDS for 16 h to virgin females induces ISCs mitoses and this is inhibited by masculinizing ISC clones using *sxl* or *tra* RNAi. Mating increases the ISC mitotic responses to SDS feeding and restores the ability to masculinized ISCs to divide to stress. c, Mating induces basal ISC mitoses in both female (control) ISCs and in masculinized ISC clones with *tra* or *sxl* depletion. d, 20HE feeding leads to the proliferation and expansion of both control ISCs and ISCs of *tra* or *sxl* masculinized progenitors. Representative images are shown 16 h after 5 mM 20HE feeding. This experiment was repeated three times with similar results. Quantification is shown in Fig. 1a. e, Quantification of ISC division at different time points (6, 9 and 12 h) after feeding 0.1% SDS to mated females. f–j, Males or mated females of the genotypes Gal4.DBD-Usp.LBD>GFP (Gal4-Usp>GFP) (f) or Gal4.DBD-EcR.LBD>GFP (Gal4-EcR>GFP) (g–j) were heat-shocked for 30 min to induce expression of the ligand sensor system, and then either infected with *P. e.* or fed with 5 mM 20HE or vehicle and dissected 18–20 h later. These GFP ligand traps express GFP under the control of heat-inducible promoter and mark cells with active 20HE signalling. When fed with vehicle, both Gal4-EcR>GFP and Gal4-Usp>GFP flies were expressed in a few cells in the R4 region posterior midgut (image shown) and in many more in the anterior midgut (image not shown). White arrows indicate cells that are doubly positive for delta or *Su(H)* lacZ markers. Feeding of 5 mM 20HE caused a strong increase in GFP expression in the posterior midgut, indicating an upregulation in the activity of both reporters. GFP was expressed in many delta+ cells (g, h) and much fewer *Su(H)*+ cells (i, j) of both males and females after 5 mM 20HE feeding. Most of the remaining positive cells are enterocytes. After 20 h of *P. e.* infection, the GFP signal disappears from males and females guts, indicating that EcR is not involved in infection-induced stress response (g, h). However, the Usp reporter was still active in many gut cells as a consequence of *P. e.* infection (f). The Usp reporter was also positive in many cell doublets and bigger cells of the midgut. These reporter data suggest that EcR and Usp are both activated by exogenous 20HE feeding, but they act differently in response to infection. Representative images are shown. This experiment was repeated five times with similar results. For all panels, control flies express UAS-GFP instead of the transgene. The period of RNAi induction is indicated. Results in dot plots are from at least three independent biological replicates. Data are mean and s.d. *n* ≥ 10 are plotted for each genotype in each scatter plot. **P ≤ 0.01, ***P ≤ 0.001, ****P < 0.0001, Mann–Whitney test with two-tailed distribution. Exact *n* numbers and *P* values are in the Source Data. Scale bars, 50 μm (f) or 100 μm (d, g–j). The overnight standard period of feeding the flies was 16–20 h.
Extended Data Fig. 2 | See next page for caption.
mitoses were scored and midguts with EcR depletion in the CNS did not exhibit the adult central nervous system. Sixteen hours after 5 mM 20HE feeding, ISCs depletion was induced using elav-Gal4 tub-Gal80ts system is not required for intestinal 20HE-stem-cell induced mitoses. EcR in enteroendocrine cell-specific depletion of EcR in ESs using the enteroendocrine cell-specific pros VI-Gal4 tub-Gal80ts driver indicate that EcR in enteroendocrine cells is dispensable to the 20HE induced ISC mitoses. Results shown are for two different RNAi lines. 1, 20HE only transiently induces ISC mitoses, quantified by mitotic indices of male and female wild-type flies subjected to two-day of the indicated treatment regimes. ISC proliferation is restored to basal levels after 5 mM 20HE was withdrawn, which suggests that the actions of 20HE are not detrimental. Male and female flies were fed vehicle or 20HE in different successions such that flies were exposed for 20 h to the first treatment, then for another 24 h to the second treatment. ISC mitoses returned to basal levels after 16–20 h treatment with 20HE then vehicle.

Extended Data Fig. 2 | The second mitotic wave of 20HE requires EcR–Usp in progenitors whereas EcR is dispensable to ISCs in their response to P.e. infection. a, Representative images of samples from Fig. 1c, h. Both EcR and Usp are required in progenitors for the mitoses induced 16 h after 20HE feeding, whereas only Usp is cell-autonomously required by the ISCs for P.e.-induced mitoses. Shown are images of progenitor accumulation in mated females after 20HE feeding or P.e. infection. b, ISCs depleted of EcR or its downstream target Eip75B are unable to form clones in response to 20HE feeding. Eip75B-null mutant clones also fail to regenerate the epithelium after P.e. infection. EcR-depleted or Eip75B-null mutant clones were generated by MARCM and analysed 12 days after clonal induction followed by 5 mM 20HE feeding or P.e. infection for 16–18 h. Vehicle-fed control clones were multicellular and spread throughout the epithelium, whereas EcR-depleted clones were considerably smaller, mostly between two and four cells, and rarely up to ten small cells per clone. Eip75B-null mutant clones remained mostly single ISC clones. After 16 h of 20HE feeding, the epithelium is populated with newly formed cells within the control clones; however, both EcR- and Eip75B-depleted clones remained unable to divide, indicating the ISC cell-autonomous requirement of EcR and Eip75B for ISC mitoses both basally and in response to exogenously fed 20HE. Similarly, after P.e. infection, GFP + cells expanded in control clones, whereas Eip75B-null mutant clones were considerably smaller.

c, Quantification of data in b by a macro designed to assess clonal sizes/maximum Z projection (Methods, Supplementary Data 2). d, Both EcR and Usp are required in gut progenitor cells for the 20HE-induced mitotic response as shown by the reduced ISC mitotic activity 16 h after feeding 5 mM 20HE to flies with progenitor-specific depletion of EcR or Usp in males and mated females. Results shown are for a second RNAi line to complement the results in Fig. 1c. e, EcR or Usp depletion in ISCs abolishes ISC mitoses 16 h after feeding 5 mM 20HE to males and mated females. Results shown are for two different RNAi lines. f, EcR is required in EBs for the second wave of ISC mitoses induced 16 h after feeding 5 mM 20HE to males and mated females. Results shown are for two different RNAi lines. This experiment indicates that in contrast to the first wave (Fig. 1e), EcR is required non-cell-autonomously in EBs for 20HE induced ISC divisions. g, EcR is non-autonomously required in ECs for maximal induction of ISC mitoses in response to 20HE. The Myo1A-Gal4 driver (Myo1A-Gal4 tub-Gal80ts) activates UAS target gene expression specifically in ECs. Results shown are for two different RNAi lines for both males and females, and for a dominant-negative isoform of EcR (EcR-AΔNMD) in females. h, EcR in the nervous system is not required for intestinal 20HE-stem-cell induced mitoses. EcR depletion was induced using elav-Gal4 tub-Gal80ts, a pan-neuronal driver for the adult central nervous system. Sixteen hours after 5 mM 20HE feeding, ISCs mitoses were scored and midguts with EcR depletion in the CNS did not exhibit a change in their division rates in comparison to control females. i, EcR in enteroendocrine cells has a minimal role in 20HE-induced ISC mitoses of the midgut. Slightly compromised mitotic indexes in 20HE-fed mated females after enteroendocrine cell-specific depletion of EcR in ESs using the enteroendocrine cell-specific pros VI-Gal4 tub-Gal80ts driver indicate that EcR in enteroendocrine cells is dispensable to the 20HE induced ISC mitoses. 20HE signals mostly through isoform EcR-A to mediate ISC proliferation. Progenitor-specific expression of EcR AΔNMD and EcR BΔNMD shows that EcR A, more than EcR B, is required in ISCs for their mitotic response 16–20 h after feeding of 20HE. Knockdown of either EcR A or EcR B had an effect on the P.e.-induced ISC mitoses. m, EcR isoform A is much more important than isoform B for driving the intestinal hyperplasia, as shown in images of posterior midguts of mated females expressing different EcR dominant-negative isoforms. Left, images of clonal expansion under basal conditions at 5 days after induction of expression of different EcR dominant-negative isoforms in mated female midguts. Right, ISC mitotic counts. n–q, EcR in ISCs or other differentiated cells is not required for the P.e.-induced mitotic response of ISCs, whereas Usp is cell-autonomously required by ISCs to proliferate in response to P.e. infection. Quantification of the mitotic indexes of ISCs after P.e. infection in mated females in which EcR or Usp was depleted: constitutively in all cells using the tub-Gal4 driver (m), in EBs (n), in ISCs (p) or in ECs (q). Collectively, these experiments indicate a functional bifurcation of EcR and Usp, in which Usp is essential in ISCs for the P.e.-induced ISC response. RNAi was induced in progenitors of mated females for 8 days before 16–20 h of P.e. infection or 20HE feeding. For all panels, control flies express UAS-GFP instead of the transgene. The period of RNAi induction is indicated. Results in dot plots are from three independent biological replicates. Data are mean ± s.d. n ≥ 10 are plotted for each genotype in each scatter plot. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P < 0.0001, Mann–Whitney test with two-tailed distribution. Exact n numbers and P values are in the Source Data. Representative images are shown from experiments that were repeated three times. Scale bars, 100 µm. The overnight standard period of feeding the flies was 16–20 h.
Extended Data Fig. 3 | See next page for caption.
induced whereas unpaired cytokines upd2, upd3, and Jak-Stat target Socs36E are not induced 6 h after 20HE feeding. mRNA levels of Egf ligands signalling and requires Egfr signalling in the midgut progenitors. Extended Data Fig. 3 | The second mitotic wave of 20HE regulates Jak–Stat signalling and requires Egfr signalling in the midgut progenitors.

a. Components of Egf signalling but not the Jak–Stat pathway are transcriptionally induced 6 h after 20HE feeding. mRNA levels of Egf ligands such as keren, spitz and their cleaving protease rho are transcriptionally induced whereas unpaired cytokines upd2, upd3, and Jak-Stat target Socs36E are not induced 6 h after 20HE (light green bars) relative to vehicle-fed control females (dark pink bars). By contrast, P.e. infection causes a strong induction of Jak-Stat signalling components upd2, upd3, Socs36E as well as a milder upregulation of Egf signalling components keren, ein and rho (light pink bars). Mated female midguts of wildtype flies were fed with vehicle, P.e. or 5 mM 20HE for 6 h then expression levels in guts were determined by RT–qPCR. Expression is indicated as mean fold change relative to vehicle-treated midguts ± s.d. (n = 3). b. Left, representative images of three categories of activity for the phenotypes of STAT92E-QFP reporters on chromosome II or III. The frequency of phenotype was quantified (right and in g) in reference to phenotypes observed in the R4 region. Dark green text/bars denote no activation of the reporter. Bright green text/bars denote a mild activation pattern. Purple text/bars denote the strongest activation pattern. 5–7-day-old mated females were used for the experiment. Right, under homeostatic conditions, the reporter expresses GFP only in ISCs (dark green bar). At 6 h after 20HE feeding, GFP is localized in midgut progenitors all over the gut (bright green bar). 18% of the guts that express the reporter on chromosome II show a slight accumulation of GFP in other cells after 20HE feeding, but the GFP signal was not as strong as in the category ‘GFP in many cells’. c–e, Ec9 is required in midgut progenitors (c) and EBs (d) but not ECs (e) for transcriptional induction of rho, upd2 and upd3 during the second mitotic wave in response to 20HE feeding. By contrast, induction of spitz and keren are unchanged relative to 20HE fed controls. qRT–PCR was performed on midguts from mated females 8 days after RNAi induction at 29 °C followed by feeding with vehicle or 5 mM 20HE for 16 h. Expression is indicated as mean fold change relative to vehicle-treated midguts ± s.d. (n ≥ 3). f, ISCs need to proliferate in order for rho, upd2 and upd3 to be induced during the second mitotic wave after 20HE feeding. Egf and Jak–Stat signalling are transcriptionally induced 16 h after 20HE feeding. Control midguts have a transcriptional induction of rho, upd2 and Socs36E and to a lesser extent upd3 mRNA levels (vehicle denoted as purple versus control 20HE-fed denoted as pink bars). Cell cycle arrest via string depletion or reduced Egfr signalling in midgut progenitors halts the upregulation of 20HE-induced rho, upd2, Socs36E and upd3. These data suggest that ISC division is cell autonomously controlled and this event is an initial requirement for the non-cell autonomous induction of promitotic factors to promote later ISC divisions. mRNA induction of spitz and keren is slightly decreased in string-depleted progenitors but are slightly higher in Egfr-depleted progenitors relative to 20HE fed controls. Mated female midguts of wild-type flies, string or Egfr-depleted progenitors for 8 days at 29 °C were fed with vehicle or 5 mM 20HE for 16 h then expression levels were determined by RT–qPCR. Expression is indicated as mean fold change relative to vehicle-treated midguts ± s.d. (n ≥ 3). g, 20HE feeding induces activity of a Jak–Stat reporter more mildly than P.e. infection. Frequency of phenotype occurrence is analysed based on the categories of activity in b. Under homeostatic conditions, the reporter expresses GFP only in ISCs (dark green bar). Sixteen hours after 20HE feeding, most midguts of the reporter on chromosome II have GFP localized in many midgut cells including polyploid ECs (purple bar). However, most midguts of the reporter on chromosome III have GFP localized in the midgut progenitors (bright green bar). By contrast, P.e.-infected midguts of the reporters on either chromosome showed a strong uniform activation pattern in all midgut cells of the R4 region. 5–7-day-old mated females were used for the experiment. h. The upd3-lacZ reporter is not activated by 20HE feeding. Images of the R4 region of the midgut showing basal expression of the upd3 reporter in vehicle-fed flies relative to strong activation of the reporter after P.e. infection. By contrast, 16 h of 20HE feeding did not appreciably activate the upd3 reporter. These data indicate that 20HE does not primarily activate upd3 to promote ISC mitoses in the midgut. 5–7-day-old mated females were used for the experiment. All images were acquired at the same settings and the intensities of activation are accurately represented. i, Left, representative images of Erk activity, assayed as dpErk showing the most prevalent phenotype for each condition. Right, quantifications of the prevalence of each phenotype are shown. Under non-stressed conditions, dpErk is present either in very few ECs per gut, or in progenitor cells and very few ECs. After enteric infection, there is a strong upregulation of dpErk mainly in ECs. Although 20HE feeding also induces dpErk in midguts, the pattern is distinct from the one caused by enteric infection. After 20HE feeding, dpErk in mainly visible in progenitors and young ECs, and the signal is often localized to small patches of cells. By contrast, P.e. infection induces strong dpErk broadly throughout the gut. dpErk is absent in non-stressed upd2 or upd2,3 mutants. Enteric infection induces dpErk also in upd2 or upd2,3 mutants, albeit to a lower level than wild-type flies. By contrast, upd2 or upd2,3 mutants show very little or no dpErk after 20HE feeding. 5–8-day-old mated females were used for the experiment. j, Upd2, Egfr and rho are required in gut progenitors for the second wave of mitoses induced by 20HE as shown by the diminished ISC mitoses 16 h after feeding 5 mM 20HE to mated females with progenitor-specific depletion of Upd2, Upd2+Upd3, Egfr or rho. k, Upd2 and rho are required in EBs for the second wave of mitoses induced by 20HE as shown by the diminished ISC mitoses 16 h after feeding 5 mM 20HE to mated females with EB-specific depletion of Upd2, Upd2 and Upd3 or rho. Results shown are for two different RNAi lines for Upd2. l, Upd2 but not Upd3 or rho is required in ECs for the second wave of mitoses induced by 20HE as shown by the diminished ISC mitoses 16 h after feeding 5 mM 20HE to mated females with enterocyte-specific depletion of Upd2, Upd2 and Upd3 or rho. The remaining scatter plots. Data are mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Mann–Whitney test with two-tailed distribution. Exact n numbers and P values are in the Source Data. Representative images are shown from experiments that were repeated at least three. Scale bars, 100 μm. The overnight standard period of feeding the flies was 16–20 h.
Extended Data Fig. 4 | Long-term 20HE feeding promotes sexually dimorphic ISC division and gut growth. a, 1 mM 20HE feeding does not obviously increase epithelial turnover in females. Representative images are shown and are relevant to Fig. 1i. b, 20HE feeding causes male-specific midgut growth also on a low-protein diet, quantified by counting mitotic indexes of males and females raised on 20HE-laced low yeast sucrose solution or sucrose-yeast solution as vehicle. 20HE- or vehicle-fed female ISCs did not differ in their mitotic counts. However, 20HE-fed males had a strong increase in their mitotic index compared to vehicle-fed males. c, 20HE feeding enhances ISC mitotic activity in P.e.-infected males, altering their behaviour to resemble P.e.-induced ISC division in females, assayed by mitotic counts of males and females. Flies were raised on 20HE or vehicle-supplemented food for 12 days then the treatment was withdrawn overnight followed by P.e. infection for 20 h. Male ISCs that were 20HE-fed were able to respond to P.e. infection at similar rates to the age-controlled females fed on 20HE or vehicle. d, 20HE-fed virgins undergo epithelial turnover much faster than age-controlled virgins, which have infrequently dividing ISCs. Flies were raised on 20HE or vehicle-supplemented food for 12 days then the treatment was withdrawn overnight followed by P.e. infection for 20 h. Male ISCs that were 20HE-fed were able to respond to P.e. infection at similar rates to the age-controlled females fed on 20HE or vehicle. e, Eip75 and EcR are required in midgut progenitors to maintain proper midgut size, quantified as midgut areas in images of guts from mated females with progenitor-specific depletion of EcR or Eip75B aged for 42 days. f, Quantification of midgut lengths of control males, 20HE-fed males, control virgin females, or virgin females depleted of ecdysone via ovary-specific knockdown of dib RNAi, shows the plasticity of male and female midgut growth to 20HE levels. 20HE-fed males have increased midgut length in contrast to dib RNAi female virgins, with decreased 20HE levels and notably shorter guts. In both cases, there was a one-third gain or loss in midgut length in comparison to a control male or virgin female, respectively. g, Ecdysone signalling via EcR and Eip75B is required in ISC clones of mated females for maximal proliferation in response to SDS. ISC mitotic counts of virgin females are minimal under basal conditions. After SDS feeding, control ISC clones divide to regenerate the epithelium but EcR- or Eip75B-depleted ISC clones are significantly impaired in their ability to divide. RNAi was induced in ISC clones for 8 days before 16–18 h of 0.1% SDS feeding. For all panels, control flies express UAS-GFP instead of the transgene. The period of RNAi induction is indicated. Results in dot plots are from three independent biological replicates. N ≥ 10 are plotted for each genotype in the remaining scatter plots. Data are mean ± s.d. **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, Mann–Whitney test with two-tailed distribution (all panels except f) or ordinary ANOVA test followed by Bonferroni’s multiple comparisons test (f). Exact n numbers and P values are in the online Source Data. Long-term 20HE feeding indicates that 1 mM 20HE was fed to the flies for 12 (c) or 14 days (a, b, d). Representative images are shown from experiments that were repeated three independent times. Scale bars, 100 μm.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Mating requires ecdysteroidogenic enzymes from the early ovarian follicles and escort cells to induce ISC divisions in the gut through EcR–Usp, which causes increased stem-cell number and subsequent gut growth. a, 20HE induces ISC mitoses in a dose-dependent manner in ISCcs of virgin females. Virgin females were fed with different doses of 20HE and their mitotic indexes were assessed after 16–18 h of feeding. At 0.25–1.00 mM 20HE, ISCcs divide similar to basal levels in mated females. At 2 mM 20HE, ISCcs mildly divide (3–4 times higher than divisions induced by 1 mM 20HE). At 5 mM 20HE, ISCcs divide at 10–11 times higher that divisions induced by 1 mM 20HE. b, The increase in width of the R4 region in response to mating in females requires EcR and Eip75B in progenitors. c, EcR is required in intestinal progenitors for their accumulation upon mating, shown by quantification of the GFP+ labelled areas of progenitors in the midgut after progenitor-specific depletion of EcR ± mating at early and later time points after mating. d, EcR-depleted ISC clones are unable to divide in response to mating, as quantified by the GFP+ clonal area in EcR-depleted ISC-derived clones and age-matched control clones. ISC-derived clones in control females have GFP+-labelled ISCcs and all their subsequent progeny stably express GFP as well. e, Usp is required in progenitors for the mating-induced midgut growth as shown by quantification of midgut areas in females with Usp-depleted progenitors ± mating. f, EcR is cell-autonomously required in ISCcs for mating-induced midgut growth, shown by quantification of midgut areas in females with EcR-depleted ISCcs ± mating. After the first mating, control female midgut initially grows and midgut growth persists in flies that are raised repeatedly mated. This midgut growth requires EcR functions in ISCcs. g, Ecdysone signalling via EcR, Usp and Eip75B are required in midgut progenitors for the mating-induced mitotic response, as shown by the reduced ISC mitoses upon 48 h mating in female midguts with progenitor-specific depletion of EcR, Usp or Eip75B. Virgins were left to mate for 48 h before dissection, then mitotic counts were assessed. Results shown are for a second RNAi line to complement the results in Fig. 2. h, EcR is cell-autonomously required in ISCcs for mating-induced ISC mitoses shown by mitotic counts of midgut in females with EcR-depleted ISCcs ± 72 h after mating. Results shown are for a second independent RNAi to complement Fig. 2f. i, Masculinized traΔ53x progenitors undergo mating-induced expansion of GFP+ progenitors similar to controls, indicating that the mating effects on progenitors are independent of the sex determination pathway, quantified as GFP+ area of progenitors in the R4 region. Virgins typically have GFP-marked single cells (ISCcs) or few pairs (ISC-EB). Shortly after mating, the ISC cells divide and the resulting progeny are transiently marked with GFP, but then turn off GFP expression as they differentiate. j, EcR is not required in EBs for mating-induced ISC mitoses. 48 h to 72 h after mating, ISCcs of EcR-depleted EBs midguts divide at similar rates to control midguts indicating that EcR in EBs is dispensable to mating-induced ISC mitoses. Results shown are for two different RNAi lines. k, Representative confocal image of ISC-expressing progenitors using eg3' in females 5 days after mating. Flies were raised as virgins and were aged for 8 days (similar to conditions in Fig. 2b), and then mated for 5 days. Females were always mated to males with no genetic manipulations. Equal number of males and females were allowed to mate (a ratio of 1:1). Image is acquired in the R4 region. This suggests that the strong mitotic effect of mating is transient. Scale bars, 100 μm. l, Rho and upd2 are transcriptionally upregulated in female midguts 24 h (green) or 72 h (orange) after mating relative to virgins (pink). Lower 7 day-old control virgin flies were mated for 24 or 72 h, then mRNA expression levels were determined by RT–qPCR. Expression is indicated as mean fold change relative to vehicle-treated midguts ± s.d. (n = 4). m, Representative images of whole-body spo mutants that are either heterozygous and hence viable with no growth or egg-laying defects (top) or sterile, homozygous spo mutants rescued to adulthood with by a pulse of 20HE given to dechorionated embryos (bottom). Images are complementary to Fig. 2i. Scale bars, 1 mm. n, RNAi-mediated depletion of spo in ovaries blunts ISC mitoses in response to mating. The traffic jam (tj-Gal4) driver that is expressed in somatic gonadal cells was used for spo depletion. Flies were raised as virgins then mated for 72 h. o, spoAX RNAi depletes the spo gene efficiently. Constitutive driver tubGFP was used to deplete spo in mated females for 8 days, and then mRNA expression levels were determined by RT–qPCR. Expression is indicated as mean fold change relative to vehicle-treated midguts ± s.d. (n = 4). p, Ovary-derived ecdysone is required for the proper size of the midgut, shown by quantification of midgut areas in mated female midguts depleted of 20HE-synthesizing enzyme Dib in the ovary. The CS87q driver, which is expressed in escort cells and immature follicle cells of the ovary, is used to induce ecdysteroidogenic enzymes depletion. Decreased midgut area in mated females with reduced 20HE levels is completely rescued by raising females on exogenous 1 mM 20HE. DibRNAi was previously validated31. q, Depletion of EcR in midgut ECs does not significantly decrease their size 8 days after mating. Cells of the midgut were stained with CellMask, a plasma membrane stain, and a custom macro (Supplementary Data 1) was used to segment the cells according to size. Shown is a frequency distribution of the different cell sizes. EcR-depleted ECs have a bigger proportion of cells sized 75–175 μm2 than control midguts. However, the differences in distribution of the cell sizes are statistically non-significant. Data are from n ≤ 5 stacks of midguts taken at the R4 region. r, Basal levels of EcR signalling are required to maintain the optimal number of progenitors in the midgut as shown by quantification of GFP+ progenitors in mated females expressing dominant-negative EcR A in comparison to the control. s, Basal levels of Eip75B are required for maintenance of ISCcs in non-stressed flies, quantified by the number of GFP+ progenitors in mated females after progenitors-specific depletion of Eip75B. A small reduction of progenitor numbers (−25%) suggests that Eip75B is not critical for ISC survival. Note that y axis does not go to zero. t, Control midguts display an increase of delta− cells at several time points following mating shown by quantification of delta− (red) and Su(H)+ (green) cells. At 24 h after mating, most delta− cells remain singlets, similar to virgins. At 40 h after mating, most delta− cells expand to doublets to triplets (Fig. 2k). At 7 days after the first mating most delta− cells are again singlets; however, their numbers are irreversibly increased relative to virgins. Females were mated to males with no genetic manipulations. Equal number of males and females were allowed to mate (a ratio of 1:1) and females were allowed to mate for 18–20 h after which males were removed, except for the condition ‘raised mated for 7 days’, in which males were always in the vial with the females. Images are acquired in the R4 region. This suggests that mating induces an initial symmetric increase in the number of ISCcs that is irreversible. Representative images for other conditions and quantifications are shown in Fig. 2k. Each dot represents a gut, and the percentage of delta− cells is calculated from absolute number of positive cells relative to total DAPI+ cells. Scale bars, 100 μm. For all panels, control flies express UAS-GFP instead of the transgene. The period of RNAi induction is indicated. Results in dot plots are from three independent biological replicates. n ≥ 10 are plotted for each genotype in the remaining scatter plots. Data are mean ± s.d. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, ordinary ANOVA test followed by Bonferroni’s multiple comparisons test (gut measurements in b, e, f, p) or Mann–Whitney test with two-tailed distribution (all other panels). Exact n values and P values are in the online Source Data. Representative images are shown from experiments that were repeated three times.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Ovaries of the esg-Gal4\(^{ts}\), esg\(^{ts}\) Su(H)-Gal80 midgut drivers have GFP expression in their germaria in a subset of escort cells. 

**a.** Top, most midgut drivers express GFP in ovary germaria. The frequency of germaria expressing GFP is displayed in the bar graph. Some ovaries with the esg\(^{ts}\) driver have no GFP in their germaria while almost all ovaries of the esg\(^{ts}\) Su(H)gal80 driver express GFP. Bottom, the number of GFP\(^{+}\) cells per germarium for both midgut drivers esg\(^{ts}\) or esg\(^{ts}\) Su(H)-Gal80, which are expressed in midgut progenitors and ISCs respectively. Further examination of esg\(^{ts}\) driver shows that it is expressed in approximately 4 escort cells, whereas the esg\(^{ts}\) Su(H)-Gal80 driver shows expression in around 14 escort cells. The number of germaria analysed is indicated. Control germaria typically have 45–70 escort cells. 

**b.** Mated females with EcR- or Eip75B-depleted midguts have reduced reproductive output. This graph is related to Fig. 2p. Average eggs per fly per 3 days are plotted instead of the cumulative sums. Flies that died during the experiment were excluded in the analysis. c. Mated females with EcR- or Eip75B-depleted midguts have reduced reproductive output. Flies with control, EcR- or Eip75B-depleted midgut progenitors were raised as virgins for 8 days and then allowed to mate to males with no genetic manipulations at a ratio of 1:1. Average eggs per fly every 3 days are plotted from stage 2 to 9 (e, h) and germaria (f, g) isolated from esg-Gal4\(^{ts}\) flies and stained for GFP (green), coracle (red) and DNA (DAPI, grey). No GFP signal was detected in follicles from stage 2 to 9 (e, h) or in later stages (not shown). However, 96% of germaria showed GFP in a subset of cells in the anterior region I (f, g). The GFP-expressing cells were located in between the germline cysts and exhibited a triangular shape indicating that they were the escort cells. i–l, All germaria from esg\(^{ts}\) Su(H)-Gal80, UAS-GFP flies express GFP in escort cells (a, j, k, l) and no GFP expression was detected from stage 2 to 9 (i, j) or in later stages (not shown). m–q, We detected expression of the Switch GS5961-Gal4 driver within ovaries in the posterior follicular cells from stage 8 of oogenesis. Confocal sections of follicles isolated from GS5961-UAS-GFP flies kept on yeast paste only (RU\(^{-}\)) or yeast paste supplemented with RU486 (RU\(^{+}\)) for 4 days and stained for GFP (green), actin (phalloidin, grey) or DNA (DAPI, grey). In the absence of RU486 induction, no GFP was detected in the ovary (m, n). After RU486 feeding, no expression was detected in germaria or follicles before stage 7 (p, q). At stage 7, a subset of the most posterior follicular cells started to express weakly the GFP, this expression was then stronger and spreading to more follicular cells in a posterior to anterior gradient during stage 8 of oogenesis (q, most posterior follicle) and maintained later on in most of the posterior follicular cells that cover the oocyte (o, stage 10). All pictures are presented with the anterior on the left and the posterior on the right.
Extended Data Fig. 7 | JH receptors are required for ISC divisions, and exogenously fed JH inhibits ISC mitoses in response to other pro-mitotic stimuli. a, JH receptors Met and Gce are required for exogenously fed 20HE to induce ISC mitosis. Virgin females were fed with 1.5 mM methoprene, 5 mM 20HE, or 20HE and methoprene in combination, and their mitotic indexes were assessed after 16–18 h of feeding. Knockdown of Met or Gce in progenitors blunted the proliferative response to all three fed stimuli. Virgins were aged for 8 days at permissive temperature then fed with the different hormone regimes for 16–18 h. b, c, Met and Gce receptors are required in midgut progenitors of mated females for P.e.-induced ISC mitoses. Mated females of indicated genotypes were aged for 8 days at permissive temperature then fed with P.e. for 18–20 h. b, ISC mitotic counts. c, Images of progenitor accumulation after P.e. feeding to mated females. d, Methoprene induces ISC mitoses in ISCs of virgin females. Virgin females were fed with active JH III ligand (JH), JH agonist methoprene (M), 2 mM or 5 mM 20HE, or the two compounds in combination, and their mitotic indexes were assessed after feeding for 16–18 h (left) or 72 h (right side). After 16–18 h of feeding, the average number of ISC mitoses per midgut was as follows. Vehicle fed: 3.8, 1 mM JH: 6.6, 1.5 mM methoprene: 8, 2 mM 20HE: 14, 5 mM 20HE: 41. A combination feeding of 1.5 mM methoprene with either 2 mM 20HE or 5 mM 20HE blunts mean ISC mitoses to 3.6 or 2.3, respectively. Combination feeding of 1 mM JH with 5 mM 20HE suppresses ISC mitoses to 11.5. After 72 h of feeding, the average number of ISC mitoses per midgut was as follows. Vehicle control: 5.5, 1.5 mM methoprene: 9.5, 5 mM 20HE: 13.5 mitoses, 5 mM 20HE + 1 mM JH: 10.9, 5 mM 20HE + 1.5 mM methoprene: 10. These results indicate that 16 h of 2–5 mM 20HE act as a strong promitotic signal to ISCs of virgin females, but after 72 h the mean 20HE-induced mitoses drop towards basal levels. 1.5 mM methoprene causes a mild but persistent increase in ISC mitoses over 72 h. Overnight combination feeding of 20HE and 1.5 mM methoprene or 1 mM JH strongly suppressed 20HE-induced mitoses. e, Methoprene does not promote ISC mitoses in mated females. Mated females were fed with 1 mM or 5 mM active JH III ligand (“JH”), JH agonist methoprene (“M”), 1 mM or 5 mM 20HE, or 20HE and JH in combination and their mitotic indexes were assessed 16-18 h after feeding. Feeding of 1 mM or 5 mM JH, 1 mM 20HE, 1.5 mM or 5 mM methoprene do not induce mitoses in mated females. 5 mM 20HE feeding induces a boost of ISC mitoses that were suppressed by combination feeding with 1 mM JH. f, Exogenous JH feeding inhibits ISC mitoses when combined with other promitotic stimuli. Mated females were heat-shocked for 30 min, infected with P.e. for 18–20 h or fed with 20HE, either alone or in combination with 1 mM JH feeding for 16–18 h, and mitotic indexes were scored. In each case, feeding 1 mM JH suppresses the mitotic response of the stimulus. g, Ovarian ecdysteroidogenic enzymes are required for methoprene-induced mitoses of the midgut. 1.5 mM methoprene causes ISC mitoses in control midguts (mean of 6.5 mitoses relative to 2 mitoses in vehicle-fed flies). In animals in which the ecdysteroidogenic enzyme Dib is depleted in ovaries, methoprene failed to significantly induce ISC proliferation (mean of 3.6 mitoses relative to mean of 5.5 basal mitoses in dibRNAi vehicle-fed flies). Virgins were aged for 8 days at permissive temperature then fed with the different hormone regimes for 3 days. For all panels, control flies express UAS-GFP instead of the transgene. The period of RNAi induction is indicated. Results in dot plots are from three independent biological replicates. n ≥ 10 are plotted for each genotype in the remaining scatter plots. Data are mean ± s.d. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P < 0.0001, Mann–Whitney test with two-tailed distribution. Exact n numbers and P values are in the online Source Data. Representative images are shown from experiments that were repeated three independent times.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Eip75B is a downstream ecdysone-inducible effector required to stimulate ISC proliferation, through Hr3 repression. a, 20HE feeding or P.e. infection transcriptionally upregulate the ecdysone-inducible targets Eip75B and Broad. 5–7-day-old mated females were fed with 20HE or infected with P.e. for 6 h, then mRNA levels were determined by RT–qPCR on RNA from whole midguts. Expression is indicated as mean fold change relative to vehicle-treated midguts ± s.d. (n = 4). b, Broad and Eip75B are required by adult Drosophila midgut progenitors for P.e. or 20HE-induced ISC mitoses. Increased mitoses were observed after P.e infection or 20HE feeding in control mated flies, which were significantly blunted after Broad or Eip75B depletion in midgut progenitors. c, d, Eip75B is only cell-autonomously required in ISCs (c), but not EBs (d) for P.e. or 20HE-induced ISC mitoses. Flies were fed with 20HE or P.e. for 16–20 h. Results are shown for two independent RNAi lines. e, Eip75B-null mutant clones are strongly impaired in their ability to divide and regenerate the epithelium. Eip75B-null mutant clones were generated by MARCM and analysed 6 days after P.e. infection. This experiment was done with a different recombinant mutant stock than that used in Extended Data Fig. 2. f, Eip75B^{exs} blocks renewal of the midgut epithelium; hr^{exs} does not. Representative images from ISC clones of ageing epithelia with reduced levels of Eip75B or Broad. Broad depletion does not affect ISC clonal growth, whereas Eip75B depletion blocks any ISC growth and most cells remain singlets. g, Eip75B overexpression in ISC-derived esg^{F0}\textsuperscript{ts} clones is pro-proliferative as shown by representative images of ISC clones in the epithelium of mated females. h, i, Eip75B is required by ISCs to divide in response to 20HE, haem, paraquat and enteric infection. h, Representative images of Eip75B-depleted ISC clones in response to the different stresses. Clonal growth to any stress stimulus is impaired. i, Quatification of mitotic counts. Results for P.e.-induced mitoses are shown for two independent Eip75B^{exs} lines. j, Representative images of the heatshock-inducible Hr3 reporter (hs-Gal4, DBD-Hr3.LBD>GFP). Conditions of low Eip75B activity result in high Hr3 reporter expression and high Eip75B activity is reflected by low Hr3 reporter expression. Of note, owing to its transcriptional repressive activity, the Eip75B reporter cannot be used to monitor its activity\textsuperscript{30}. Under basal conditions, midguts express high levels of Hr3 reporter. Hr3 activity is repressed by 20HE or haem feeding, P.e. infection (stimuli that require Eip75B) or co-expression of Eip75B. Nitric oxide (NO) inhibits Eip75B binding to Hr3\textsuperscript{37}. SNAP is a nitric oxide donor compound that modulates nitric oxide availability and is used to regulate Eip75B activity. However, increased nitric oxide levels through SNAP feeding relieved the repressive actions of P.e. and Eip75B on GFP expression. This indicates that in ISCs, Eip75B inhibits Hr3 and nitric oxide blocks this suppressive effect. Right, mitotic counts are shown for vehicle-fed, haem-fed, P.e., or P.e.+SNAP-fed mated females after 30 min heatshock (to induce the Hr3-GFP reporter) and 18–20 h of feeding. k, l, Hr3 overexpression strongly impairs epithelial renewal as the flies age, depicted by quantifications of mitotic indexes in k, l, Representative images of GFP-marked Hr3-overexpressing ISC clones showing impaired clonal growth in midguts of mated females. m, Hr3 depletion permits ISCs to divide in response to P.e. infection as shown by mitotic counts of Hr3-depleted ISC clones in mated females, which respond to P.e. infection at similar rates to control midguts. n, Repression of ISC mitoses in Eip75B-depleted esg^{F0}\textsuperscript{ts} clones is rescued by Hr3^{exs} as shown by mitotic counts of ageing or P.e.-infected guts with Eip75B, Hr3 depletion or both. This experiment shows that Hr3 is epistatic to Eip75B. For all panels, control flies express UAS-GFP instead of the transgene. The period of RNAi induction is indicated. The overnight standard period of feeding the flies was 18–20 h. Results in dot plots are from three independent biological replicates. n ≥ 10 are plotted for each genotype in the scatter plots. Data are mean ± s.d. ****P < 0.0001, Mann–Whitney test with two-tailed distribution. Exact n numbers and P values are in the online Source Data. Representative images are shown from experiments that were repeated three times. Scale bars, 100 μm.
Extended Data Fig. 9 | Nitric oxide modulates the interaction of Eip75B and Hr3 to regulate ISC division. 

**a**–**d**, Eip75B is not required in other midgut cell types besides progenitors for *P. e*. infection to induce ISC proliferation. Eip75B was depleted in progenitors using *esg-gal4* (two independent RNAi lines are shown to complement results in Fig. 2) (**a**), visceral muscle using *how-Gal4* (**b**), ECs using *Myo1A-gal4* (**c**), or enteroendocrine cells using *prosV1-gal4* (**d**). 

**e**, Overexpression of Hr3 in ISC-derived clones impedes the mitotic ability of ISCs to divide in response to *P. e*. infection. 

**f**, **g**, Inhibition of nitric oxide (NO) rescues the ISC mitotic activity of Hr3-overexpressing progenitors. 

**f**, ISC mitotic counts. 

**g**, Representative images of progenitor-specific overexpression of GFP with or without Hr3 followed by *P. e*. infection alone or in combination with the NO inhibitor l-NAME. NO represses the ability of Eip75B to interact with Hr3 hence, allowing transcriptional regulation of Hr3 targets. 

Treatment with l-NAME rescued the ISC ability to divide and progenitors expanded to fill the epithelium similar to the control mated females after infection (compare to results in Extended Data Fig. 8j). 

**h**, Model summarizing the regulation of Eip75B, Hr3 and Broad. 

**i**, Model summarizing the crosstalk between the gut and the ovary. For all panels, control flies express UAS-GFP instead of the transgene. The period of RNAi induction is indicated. The overnight standard period of feeding the flies was 18–20 h. Results in dot plots are from three independent biological replicates. *n* ≥ 10 are plotted for each genotype in the scatter plots. Data are mean ± s.d. **** *P* < 0.0001, Mann–Whitney test with two-tailed distribution. Exact *n* numbers and *P* values are in the online Source Data. Representative images are shown from experiments that were repeated three times. Scale bars, 100 μm.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Ovary-derived 20HE promotes intestinal dysplasia through EcR, Usp and Eip75B, which may affect Drosophila lifespan. a. The number of mitotic cells in midguts increases with age, and this is inhibited by RNAi-mediated knockdown of EcR or Usp in ISC clones (esgFOts). Mitotic counts are shown at 19, 23 and 27 days after eclosion in non-stressed female guts. b. Basal 20HE levels promote age-dependent intestinal dysplasia. Mitotic indexes are shown in aged mated female midguts from flies ubiquitously expressing dbEx at two different ages after RNAi induction. c. Ovary ecdysone is required for ISC mitoses in non-stressed animals. Young and old mated females with spo knockdown in their ovaries have reduced ISC mitoses compared to controls. This was rescued by feeding the flies 1 mM 20HE. A second independent RNAi for spo is shown to complement data in Fig. 2. d. Representative images for the three classes of tumour phenotypes used to score mated female tumours in Fig. 3. e. 20HE feeding potentiates the tumour growth in N RNAi males. Left, representative images with which males have been scored in Fig. 3. Males exhibiting big tumour clusters of at least 30 neighbouring cells along the gut were classified strong. By contrast, guts with one or two tumour clusters with less than ten neighbouring cells were classified mild. Right, quantifications are derived by calculating the ratio between the GFP+ area and DAPI+ area. Tumour induction was commenced a few days before 20HE feeding. f. 20HE feeding potentiates the tumour initiation in virgin females with N RNAi. Representative images are shown for the quantifications presented in Fig. 3. Guts with no tumour clusters and just doublets of progenitor cells were classified as mild. Guts with tumour clusters of fewer than 10 neighbouring cells were classified as moderate, and guts with tumour clusters of at least 30 neighbouring cells were classified as strong. g–i. Progeny of the GS5961-Gal4 UAS-EcR F645A genotype were mated for 48 h. The populations followed up were segregated based on their sex (males (g) and females (h)) and separated into groups of 25 flies per vial. Approximately half of the flies were fed 0.2 mg ml−1 RU486 to induce dominant-negative EcR expression in progenitors and the other half were fed vehicle. RU486 or vehicle (ethanol) was deposited on the food vials 4–6 h before flipping the flies into the vials at 48-h intervals. Dead flies were visually identified and recorded. Lifespan assays were performed in two replicates and for each replicate the percentage survival was plotted as a function of days elapsed after the start of the assay. Statistical analysis was performed using log-rank test. χ² represents chi-squared value and the Pvalues were provided from pairwise comparison with Bonferroni correction. l. Experimental details and the percentage mortality of the male or female replicates. Exact n numbers and Pvalues are in the online Source Data.
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- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

  Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Image data were collected using LAS AF software (version 2.7.3) for Leica DM and SP5 microscopes. RT-qPCR data were collected using Roche Light Cycler 480 (software version 1.5) and StepOnePlus Real-Time PCR System Applied Biosystems (StepOne software version 2.0). Life span data was analyzed using Oasis (version 2).

Data analysis

Data were collated using Microsoft Excel (v16.33). Data was then entered into GraphPad Prism (v8.3.1), which was used to derive p values, standard deviations, to perform indicated statistical tests, and to generate the graphs displayed in all figures. Image files were analyzed in FIJI image analysis software (version 2.0.0-rc-69/1.52p), using custom macros as indicated. All custom macros are available for download as supplementary files or from the authors.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The source data for all figures is available via the online Supplementary Information.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample sizes were determined based on practical considerations (e.g. number of animals available, processing time required) and expected variations between animals for a given sample. Based on these considerations, sample sizes were generally set at >20 animals per genotype/condition, which allowed modest standard deviations and highly significant p values (p<0.0001) to be obtained.

Data exclusions

No data were excluded.

Replication

All experiments were replicated three or more times. In many cases, data from each of the three replicates were pooled for display. In some cases, data from one or two representative replicates are displayed.

Randomization

Data were not randomized. Each sample was defined by a specific genotype and condition, and all animals from any specific genotype/condition were analyzed and included in the dataset.

Blinding

Many samples were scored manually by observation with a microscope. These include experiments in which PH3+ cells/gut were counted. These samples, as well as those in which tumor quantifications or gut sizes (area, width, length) were measured or scored blinded.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants
- Clinical data

Methods

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

Antibodies used

Primary antibodies: Chicken anti-GFP (Life Technologies/Molecular probes, 1:500); rabbit anti-phospho-Histone 3 (Merck Millipore 1:1000); mouse anti-phospho-Histone 3 (Cell Signaling, 1:1000); guinea pig anti-GFP (Teleman Lab, 1:1000); Chicken anti-beta-galactosidase (Abcam, 1:1000); Rabbit phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) #9101 (Cell Signaling, 1:400). Secondary antibodies: (Alexa 488, 568 or 633, Invitrogen).

Validation

All antibodies were validated on Drosophila tissue samples known to have positive and negative signals based on genotype and condition.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Drosophila melanogaster were used for all experiments. Sexes and ages are noted in the Methods section, with sex and age details for each experiment listed in the Figure Legends and Figures. The precise genotypes and sources for each genetic strain used for each experiment are listed in Supplementary Tables S1 and S2.

Wild animals

This study did not use wild animals. All Drosophila stocks were obtained from public stock centers or other research labs, as listed in Supplementary Table S2.

Field-collected samples

This study did not use field-collected samples.
Ethics oversight

These studies were performed at the German Cancer Research Center (DKFZ, Heidelberg), which provides ethics guidance for laboratory animal studies according to German law. However, no ethical guidance was required for our work with Drosophila melanogaster, an invertebrate insect.

Note that full information on the approval of the study protocol must also be provided in the manuscript.