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Loss of canonical sex steroid signaling correlates with prostate cancer progression and induces tumor escape in *Drosophila*

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eLife Assessment

This **valuable** study provides insights into the role of steroid signaling during tumorigenesis in the adult male drosophila accessory gland (functional equivalent of the prostate gland in mammals), hinting at a possible counterintuitive anti-tumoral role of sex hormones during prostate cancer in certain patients. While the Drosophila model provides an elegant way to study the hypothesis derived from the Cancer Atlas analysis, the analyses of public prostate cancer expression data are **incomplete** and critical knowledge on patients' treatment modalities and normalization across different datasets is missing. This work would be of interest to prostate cancer researchers as it suggests that the absence of androgen receptor signaling in humans could constitute a mechanism promoting tumor escape.

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Abstract

In cancer, tumor escape often arises following treatments. This is especially true during deprivation therapies, where this phenomenon has been linked to steroid signaling reactivation despite deprivation. Here, we show that in prostate cancer tissues the canonical androgen pathway itself is deactivated and, in fact, loss of canonical AR signaling tightly correlates with cancer progression. This raises the possibility that loss of canonical sex steroid signaling could promote the progression. We tested this hypothesis a drosophila model of prostate cancer. There, repression of canonical sex steroid ecdysone receptor signaling displays both anti- and protumor effects. On the one hand, it slightly decreases extra-epithelial tumor formation, and increases their propensity for apoptosis. On the other hand, it induces the growth of a new tumor cell population, which growth is normally prevented by autocrine/intracrine ecdysone signaling. Furthermore, this population appears to emerge from epithelial clones within the gland. To do so, tumor cells change the way they migrate out of the epithelium, forming a new layer between the normal epithelium and the basement membrane. This depends on a modification of epithelial basal extrusion that likely relies on the downregulation of Ecdysone target gene *αTub60D*. Thus, in drosophila accessory gland, lack of sex steroid signaling not only coincides with but actually induces tumor escape. Together, these results question the role of sex steroid deprivation on tumor progression, and point to altered basal extrusion as a possible mechanism shaping tumor escape.

Introduction

Epithelial tumorigenesis is a complex, multistep process spanning from hypothetical initiation to metastatic spread. Late stages are better characterized, and have been associated with numerous alterations in molecular mechanisms that appear necessary for tumor progression (reviewed in (Hanahan and Weinberg, 2011)). Among them, specific pathways can act in specific tissues. Typically, the main sex steroids, estrogens and androgens, support growth of adenocarcinoma in reproductive tissues. Accordingly, endocrine therapy (sex steroid deprivation) is widely used in breast and prostate cancers to induce tumor regression.

However, despite strong initial response, tumor escape frequently occurs and even systematically in prostate cancer. Tumor escape is frequently associated with resistance to hormone deprivation. Treatment-resistant cells can adapt through high estrogen or androgen receptor (ER or AR) expression enabling sensitivity to reduced steroid levels, ER/AR mutations or alternative splicing leading to ligand-independent reactivation (Cao et al., 2014; Zundelevich et al., 2020). However, tumor escape also occurs when tumor cells become completely independent of steroid signaling for their progression, following *ER*/*AR* loss of function (Kuukasjärvi et al., 1996). So, it exists more than one path for tumor cells to survive endocrine therapy. Furthermore, cells that escape treatment appear more aggressive, with higher proliferative and metastatic potential (Clarke et al., 1989; Wu et al., 1994).

Despite best efforts, the origin of the cells that escape endocrine therapy remains debated, and the mechanisms allowing them to develop into tumors are still largely unknown. Over the last decades, cancer stem cells (CSCs) have emerged as strong candidates for initiating resistant tumor growth (Rodriguez et al., 2019; Rybak et al., 2015). Detected in many tumor types, these cells are defined by their insensitivity to treatments and their ability to recolonize a tumor that regresses upon treatment (Atashzar et al., 2020). Why these cells are present remains under scrutiny: hypothetically, they could already be present before treatment as a consequence of stochastic tumor heterogeneity, derive from adult stem cells, or transdifferentiate from tumor cells as a consequence of treatment selection. This last hypothesis, implying that deprivation therapy would in part be responsible for tumor progression, is supported by the observation that xenografted tumor cells can escape androgen deprivation in castrated mice (Iwasa et al., 2007; Nagabhushan et al., 1996; Wu et al., 1994; Zhou et al., 2004). However, the cellular and molecular mechanisms by which these cells could emerge and regrow to form a tumor are mostly unknown.

We previously developed a model of epithelial tumorigenesis in the drosophila accessory gland (Rambur et al., 2020). Thanks to its versatility as a genetic model, drosophila has been successfully used in cancer biology to decipher fundamental mechanisms in brain cancer development (Read, 2011; Read et al., 2009) as well as epithelial cancers such as lung (Levine and Cagan, 2016) and colon cancers (Bangi et al., 2016). It has even recently been used as a platform for precision medicine in cancer therapy, demonstrating its direct relevance to patient care (Bangi et al., 2019). Most prostate adenocarcinomas are thought to originate from luminal cells (Humphrey, 2012). The drosophila accessory gland is a functional equivalent of the prostate (Leiblich et al., 2012; Wilson et al., 2017; Xue and Noll, 2002) mainly composed of a monolayer of seminal fluid-secreting cells (Taniguchi et al., 2018; Xue and Noll, 2002) surrounded by a monolayer of stroma-like muscle cells encased in a strong basement membrane (Rambur et al., 2020). It is an established model to study mechanisms of epithelial prostate tumorigenesis (Ito et al., 2014; Molano-Fernández et al., 2022; Rambur et al., 2021, 2020; Vialat et al., 2024; Wilson et al., 2017). Furthermore, accessory gland differentiation and activity are driven by the drosophila sex steroid Ecdysone Receptor (EcR) (Sekar et al., 2023; Sharma et al., 2017), paralleling the role of human Androgen Receptor (AR) in the prostate physiology.

In this study, we have first interrogated prostate cancer patient expression data. We find that in Castration Resistant Prostate Cancer (CRPC), there is no evidence of canonical AR signaling reactivation; in fact, canonical AR signaling is significantly repressed in CRPC compared to

primary tumors. Furthermore, cancer progression tightly correlates with this loss of canonical AR signaling. These data raise the possibility that this loss could participate to tumor resistance. However, due to the existence of non-canonical pathways, of multiple escape mechanisms and of frequent crosstalk with other steroid signaling such as Glucocorticoid Receptor, which in resistance expresses AR target genes (Arora et al., 2013), it appears difficult to study the specific effect of sex steroid canonical pathway repression on cancer progression. To study this effect, we turned to drosophila where only one steroid is known, ecdysone. So, we reproduced endocrine therapy in the drosophila accessory gland by genetically inducing tumor-specific ecdysone signaling downregulation. We show that repression of canonical sex steroid signaling actively reprograms pre-existent tumor cells to create a new tumor cell population that escapes this sex steroid deprivation. This deprivation induces a newly uncovered kind of basal extrusion, *de facto* creating a new niche for tumor development in what we define as the intrabasal compartment. This change notably relies on downregulation of EcR target gene *βTub60D*, which encodes β3 Tubulin (β3Tub). These findings possibly shed new light on the mechanisms of tumor escape.

Results

Canonical Androgen signaling decreases with cancer progression

Canonical Androgen signaling genes are downregulated in CRPC compared with primary cancer

Even though it is commonly accepted that CRPC corresponds to a phase of reactivation of AR signaling, we first assessed how much activity of canonical androgen signaling can be detected depending on the stage of prostate cancer. Because AR is a transcription factor, transcription of target genes was used as a readout across more than 1000 tumor samples from several public datasets (Abida et al., 2019; Beltran et al., 2016; Cancer Genome Atlas Research Network et al., 2015; Kumar et al., 2016; Labrecque et al., 2019; Lapuk et al., 2012; Sharp et al., 2019; Stelloo et al., 2018; Suntsova et al., 2019; "The Genotype-Tissue Expression (GTEx) project.," 2013) and grouped in the Prostate Cancer Atlas (*prostatecanceratlas.org*). This tool was initially introduced by Bolis et al (Bolis et al., 2021). We first observed that *AR* expression significantly increases in primary prostate cancer compared to normal tissue, and increases again in CRPC samples compared to primary cancer (Figure 1A). We then explored the androgen response using expression of several target genes of AR signaling (*ABCC4*, *ALDH1A3*, *AMACR*, *CAMK2*, *FASN*, *KLK2*, *KLK3*, *NKX3-1*, *PART1*, *PCA3*, *PLPP1*, *PMEPA1*, *STEAP1*, *STEAP2*, *STEAP4*). All these genes exhibit the same expression profile. Compared to normal samples, they are all significantly overexpressed in primary samples, as is *AR*. However, in CRPC samples and even though *AR* expression is still increased there, they are all significantly repressed compared to primary samples (Figure 1B, Table 1). In fact, six of these genes (*ABCCC4*, *ALDH1A3*, *KLK2*, *KLK3*, *PART1*, *STEAP4*) are expressed at even lower levels in CRPC than in normal tissues (Figure 1B,Table 1).

Canonical Androgen signaling gene expression negatively correlates with prostate cancer progression

The Prostate Cancer Atlas also correlates changes in transcriptomic profiles with cancer progression (i.e. pseudotime progression, Figure 1C), with a high degree of confidence, allowing retrospective association of specific changes in gene expression with the cancer trajectory.

Logically, plotting individual patients on this trajectory discriminates normal tissue (green, low pseudotime, Median ± Std error = 54.58 ± 2.396), primary cancer (red, intermediate pseudotime = 105.9 ± 0.7952) and CRPC (blue, high pseudotime = 196.5 ± 1.322) among which the most aggressive form -neuroendocrine cancers (NEPC, violet)- clusters at the final tip of the trajectory (Figure 1D). *AR* expression itself peaks in CRPC samples (Figure 1E) but only weakly correlates with progression (Figure 1F, R = 0.11, FDR = 0.0459). To understand why this correlation is so weak, we plotted *AR* expression against pseudotime (Figure 1G). *AR* expression there appears to vary widely in CRPC samples. We therefore subdivided CRPC samples into four groups based on pseudotime, i.e., their state of progression (Figure 1H). The 1st quartile (1ST QT) displays mean

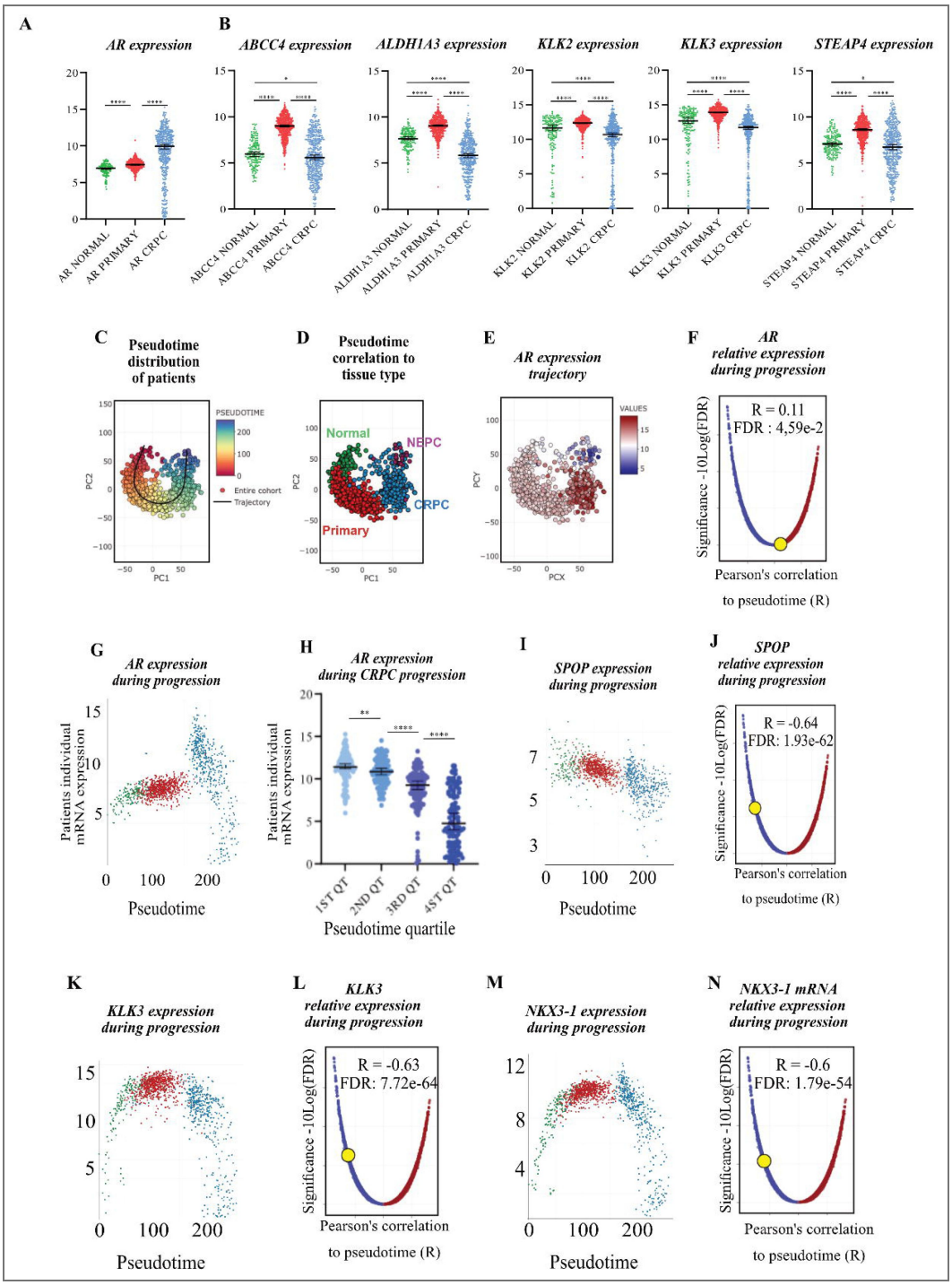

Figure 1. Androgen signaling activity negatively correlates with prostate cancer progression.

(A-B) mRNA expression in normal (green), primary (red) or castration-resistant (CRPC) prostate cancer samples. (A) *AR* expression. (B) *AR* target genes expression. (C) Principal component analysis of sample expression defines a trajectory of disease progression with an associated pseudotime score (extracted from Prostate Cancer Atlas). (D) Pseudotime correlation segregates normal, primary, CRPC and neuroendocrine (NEPC) samples. (E) Individual *AR* expression in the samples along the trajectory. (F) Relative change of expression of AR during progression. X-axis represents Pearson's correlation coefficient between mRNAs and pseudotime as Y-axis displays the associated significance adjusted for false discovery rate (FDR). (G) Individual *AR* expression in the samples depending on pseudotime (X-axis) and sample type (color code). (H) CRPC samples are separated in four quartiles (1ST to 4TH QT) depending on their pseudotime. *AR* expression is then plotted for individual samples. (I-N) Individual expression and relative change of expression of three AR target genes (SPOP, KLK3, NKX3-1) during progression. * p<0.05; ** p<0.01; ****p<0.0001.

	p values			Median ± standard deviation			Mean values ± STD error		
Gene	Normal vs primary	Normal vs CRPC	Primary vs CRPC	Normal	Primary	CRPC	Normal	Primary	CRPC
AR	<0,0001	<0,0001	<0,0001	6.933 ± 0.8056	7.419 ± 0.636	9.929 ± 3.374	6,784 ± 0.06125	7,418 ± 0.0239	9,056 ± 0.1534
ABCC4	<0,0001	0.0367	<0,0001	5.939 ± 1.381	8.991 ± 1.157	5.58 ±2.3	6,06 ± 0.105	8,857 ± 0.04349	5,697 ± 0.1045
ALDH1A3	<0,0001	<0,0001	<0,0001	7.666 ± 1.005	9.068 ± 0.9679	5.825 ± 1.97	7,571 ± 0.07643	8,976 ± 0.03638	5,746 ± 0.08956
AMACR	<0,0001	<0,0001	<0,0001	4.061 ± 1.233	8.642 ± 1.752	7.152 ± 2.431	4,252 ± 0.09375	8,437 ± 0.06586	7,239 ± 0.1105
CAMKK2	<0,0001	<0,0001	<0,0001	6.477 ± 0.6324	7.967± 0.9343	6.828 ± 1.175	6,511 ± 0.04808	7,966 ± 0.03511	6,958 ± 0.0534
FASN	<0,0001	<0,0001	<0,0001	8.332 ± 1.229	9.804 ± 1.181	9.121 ± 1.91	8,539 ± 0.0934	9,742 ± 0.04437	9,112 ± 0.08681
KLK2	<0,0001	<0,0001	<0,0001	11.65 ± 2.982	12.35 ± 0.8887	10.67 ± 3.741	10,7 ± 0.2267	12,21 ± 0.0334	9,458 ± 0.17
KLK3	<0,0001	<0,0001	<0,0001	12.65 ± 3.081	13.87 ± 0.9249	11.69 ± 3.875	11,66 ± 0.2342	13,73 ± 0.03476	10,16 ± 0.1761
NKX3-1	<0,0001	0.2104	<0,0001	8.926 ± 2.264	10.1 ± 0.5656	9.003 ± 2.935	8,021 ± 0.1721	10,05 ± 0.02126	7,975 ± 0.1334
PART1	<0,0001	0.0171	<0,0001	5.019 ± 1.467	6.068 ± 1.04	4.5 ± 2.111	4,744 ± 0.1116	5,956 ± 0.03909	4,267 ± 0 09594
PCA3	<0,0001	0.3735	<0,0001	1.54 ± 1.944	7.868 ± 2.568	2.132 ± 3.014	2,2 ± 0.1402	7,154 ± 0.09653	3,049 ± 0.137
PLPP1	<0,0001	0.1708	<0,0001	8.018 ± 1.084	8.733 ± 0.8458	8.283 ± 1.855	7,94 ± 0.08239	8,702 ± 0.03179	7,903 ± 0.08431
PMEPA1	<0,0001	0.0363	<0,0001	7.931 ± 1.239	9.483 ± 0.6337	8.428 ± 1.874	7,879 ± 0.09418	9,393 ± 0.02382	7,996 ± 0.08516
STEAP1	<0,0001	<0,0001	<0,0001	5.077 ± 1.43	7.043 ± 0.7164	6.638 ± 2.178	5,032 ± 0.1087	6,968 ± 0.02692	6,041 ± 0.09901
STEAP2	<0,0001	0.059	<0,0001	8.058 ± 1.332	9.376 ± 0.8335	8.436 ± 2.767	7,867 ± 0.1013	9,276 ± 0.03132	7,709 ± 0.1258
STEAP4	<0,0001	0.0416	<0,0001	7.036 ± 1.186	8.596 ± 1.178	6.728 ± 2.371	7,045 ± 0.09017	8,486 ± 0.04429	6,581 ± 0.1078

Table 1. Canonical AR Signaling genes are downregulated in CRPC compared with primary cancer.

Based on 173 Normal samples, 708 Primary cancer samples and 484 CRPC samples. In yellow: six of the genes are significantly less expressed in CRPC than in normal samples.

AR expression of 11.41 ± 0.16; 2ND QT is significantly lower at 10.83 ± 0.15 (p = 0.0209). Decrease from quartile to quartile continues for 3RD QT (8.82 ± 0.22, p<0.0001) and 4TH QT (5.27 ± 0.31, p<0.0001). Thus, even though *AR* expression clearly increases in primary cancer and then CRPC, its expression steadily decreases within CRPC when state of progression is considered.

At the same time, canonical androgen signaling appears as the most downregulated pathway along cancer progression -especially in CRPC samples (Bolis et al., 2021)- as shown for *SPOP*, one of the most frequently mutated genes in prostate cancer and a key player in genome integrity (Barbieri et al., 2012; Boysen et al., 2015) (Figure 1I-J, R = -0.64, p = 1.93e-52), *KLK3* -coding for the Prostate Specific Antigen (Figure 1K-L, R = -0.63, p = 7.72e-64)- or *NKX3-1*, which is itself a specific prostate tumor suppressor gene (Bhatia-Gaur et al., 1999) (Figure 1M-N, R = -0.60, p = 1.79e-54). Overall, even though *AR* itself is globally more expressed in CRPC patients, canonical androgen signaling definitely decreases with cancer progression.

CRPC-specific loss of correlation between AR expression and canonical AR signaling

To understand this phenomenon, we plotted expression of *AR* expression against its target genes (here *ALDH1A3*) for normal, primary cancer or CRPC samples (Figure 2A). In normal tissues, *AR* and *ALDH1A3* expression are concentrated in a relatively narrow range, making all samples look alike (Figure 2B). In primary cancer, the increase in *AR* expression (from 6.93 ± 0.81 to 7.42 ± 0.64, p<0.0001) correlates with an increase in *ALDH1A3* expression (from 7.67 ± 1 to 9.07 ± 0.97, p<0.0001), keeping samples close to each other but shifted to a higher expression range compared to normal samples (Figure 2C). However, in CRPC samples, despite a still higher *AR* median expression (from 7.42 ± 0.64 to 9.93 ± 3.37, p<0.0001), individual *AR* expression is dispersed over more than 10 logs (from 0.11 to 13.16). *ALDH1A3* expression also varies hugely across samples, but with a Pearson Coefficient of 0.494, remains weakly correlated with AR expression in individual samples. However, *ALDH1A3* expression does strongly decrease (from 9.07 ± 0.97 to 5.83 ± 1.97, p<0.0001) in CRPC (Figure 2D), indicating that AR does not appear to activate expression of this gene as efficiently as in normal or primary cancer tissues.

We then wondered whether androgen metabolism itself could be disturbed. Interestingly, *SRD5A2*, which encodes the enzyme responsible for the production of the active metabolite of testosterone, DiHydroTestosterone (DHT) (Andersson et al., 1991), is downregulated in primary samples and even more so in CRPC samples (Figure 2E). *SRD5A2* is in fact one the most downregulated genes during cancer progression (Figure 2F, Figure 2G, R=-0,82, p=1.23e-143). However, its progressive loss of expression starts from primary cancer (Figure 2H).

Potentially, the lack of response to the peak of AR expression in early CRPC could result from reduced DHT metabolization that begins in primary tumors. Indeed, DHT levels are lower in untreated cancer tissues than in benign prostatic hyperplasia (Geller et al., 1978). Furthermore, in men suspected of prostate cancer who underwent needle biopsies, a high percentage of cancer-positive cores -associated with poor prognosis- also correlates with lower DHT levels (Miyoshi et al., 2014). Thus, DHT concentration decreases in prostate cancer even before castration and castration resistance, and correlates with poor prognosis. Overall, transcript analysis of major prostate cancer cohorts indicates that canonical Androgen Receptor signaling negatively correlates with cancer progression, even in samples classified as hormone refractory in which androgen signaling is generally considered active.

Tumor escape induced by Ecdysone Receptor downregulation

Considering these results, we asked whether repression of canonical sex steroid signaling could by itself induce cancer progression. To test this hypothesis, we turned to a drosophila model of prostate cancer we previously engineered, in which we can also study in detail the phenomenon of basal extrusion, considered as the founding step of cancer formation.

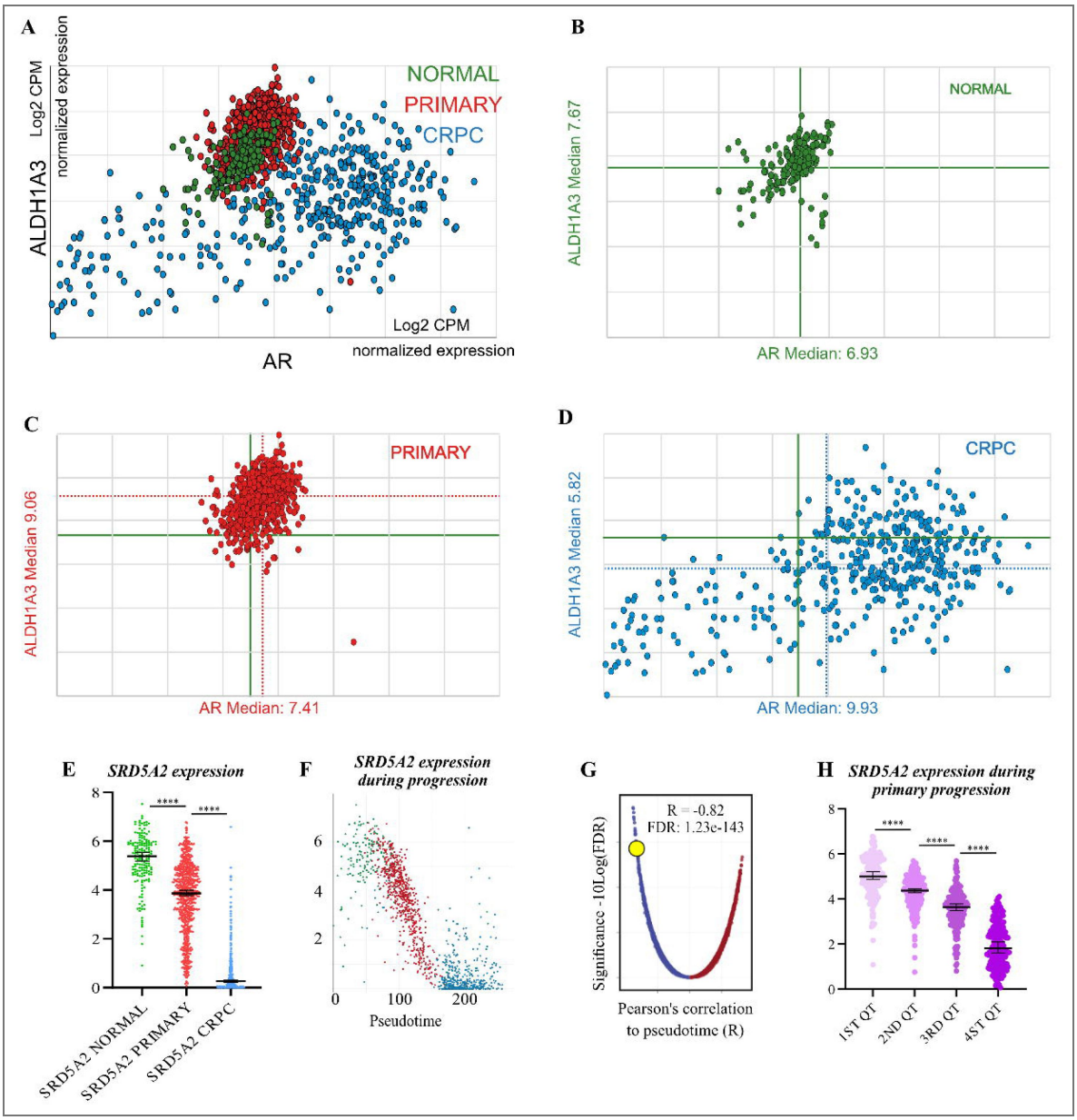

Figure 2. Loss of correlation between AR expression and canonical AR signaling coincides with downregulated expression of *SRD5A2*.

(A) Individual *AR* expression (X-axis) versus ALDHIA3 expression (Y-axis) in the samples depending on sample type (color code). (B-D) Same expression separated by sample type. (E) *SRD5A2* -coding for the DHT-producing enzyme-mRNA expression in normal (green), primary (red) or castration-resistant (CRPC) prostate cancer samples. (F-G) Individual expression and relative change of expression of *SRD5A2* during progression. (H) Primary samples are separated in four quartiles (1ST to 4TH QT) depending on their pseudotime. *SRD5A2* expression is then plotted for individual samples. ****p<0.0001.

Epithelial tumor cells and extra-epithelial tumors in EGFRλ-mediated tumorigenesis

Thanks to genetic tools available in drosophila based on the UAS/Gal4 expression system, it is possible to reproduce tumor initiation in the drosophila accessory gland. For this, clonal expression of a constitutively active form of EGFR -EGFRλ- is realized in ~1% of the main cells of the accessory gland. This oncogene is co-expressed with GFP, allowing tracing of tumor cells. To provide an adequate control for tumorigenesis (EGFRλ CTL), it is furthermore co-expressed with a second nuclear GFP, as every condition must bear the same number of UAS elements to avoid Gal4 titration effect. Under these conditions, tumor initiation displays different outcomes. Some clonal cells undergo proliferation at low rates but do not migrate, and thus remain inside the epithelium (Figure S1A-F, *i.e.* clones, white arrowheads). Among these cells, some display disorganized cell-cell interaction markers, indicating disturbed epithelial differentiation and probable loss of adhesion with neighbor cells (Figure S1A-C, thick empty arrowheads). Due to their mild hyperproliferation, these cells can be considered part of benign tumors. Finally, some clonal cells migrate and develop into extra-epithelial tumors (Figure S1D-F, empty arrowheads), where cells are completely devoid of epithelial markers (Figure S1F), indicating in drosophila a phenomenon of Epithelial-Mesenchymal Transition (EMT).

As demonstrated by their presence outside the muscle sheet that encloses the gland (revealed by F-actin staining in Figure 3A and later), these tumor cells invade through the basement membrane, a behavior known as epithelial basal extrusion that is specific to cancer cells according to the National Cancer Institute. We previously showed that these extra-epithelial tumors harbor phenotypic abnormalities typically associated with tumor development, such as hyperplasia, hypertrophy, loss of epithelial marker expression (Figure S1F, Figure 3) and promotion of angiogenesis-like tracheogenesis. We also previously described that many of them retain a specific feature of accessory gland epithelial cells -twin nuclei-confirming that these cells originate from the accessory gland (Rambur et al., 2020).

Treatment-like effect induced by Ecdysone Receptor downregulation

We further evaluated *EcR* expression during tumorigenesis. By RNA FISH, we show that *EcR* is overexpressed in tumor cells that were able to extrude basally (Figure 3A-B). This phenocopies *AR* overexpression in human prostate cancer and raises questions about the potential role of EcR in accessory gland tumorigenesis. We so co-expressed an RNAi targeting EcR with the oncogene (EGFRλ EcR-KD, Figure 3C-D). At first sight, this co-expression induces growth of epithelial clones and extra-epithelial tumors similar to the control (compare white and empty arrowheads in Figure 3E-H to Figure 3I-L). At 6D, tridimensional reconstruction of image stacks indicates that the tumors display the same volume and the same number of cells in both conditions (Figure 3M, N). Thus, these tumors display similar proliferation rate of ~700% compared with clones expressing GFP only (Figure 3O), which are typically composed of two cells at six days of induction (Figure S3A). We conclude that phenotypic development of extra-epithelial tumors does not rely on ecdysone signaling. We then examined whether extra-epithelial tumor formation itself is modified.

Temporal dynamics of extra-epithelial tumor formation is not affected by EcR RNAi co-expression (Figure 3P): extra-epithelial tumors typically start to appear between the third and fourth days after clonal induction (3D and 4D), peaking at 5D regardless of genotype, indicating that basal extrusion is active only during this short period of time. However, a modest but significant decrease in the total number of extra-epithelial tumors is detected upon EcR downregulation (Figure 3Q). Together, these data show that loss of ecdysone signaling specifically affects the overall quantity of tumor basal extrusion, but has neither an effect on its temporal dynamics nor on tumor growth rate outside the gland.

In human, sex steroid deprivation also induces death of treatment-sensitive tumor cells (Dowsett et al., 2006; Isaacs et al., 1994). 6 days after clone induction (6D), cleaved-caspase 3 staining reveals that, in the control condition, tumor cells do not present signs of apoptosis (Figure 3R-S). However, at the same time point, EcR RNAi co-expression significantly induces apoptosis in extra-

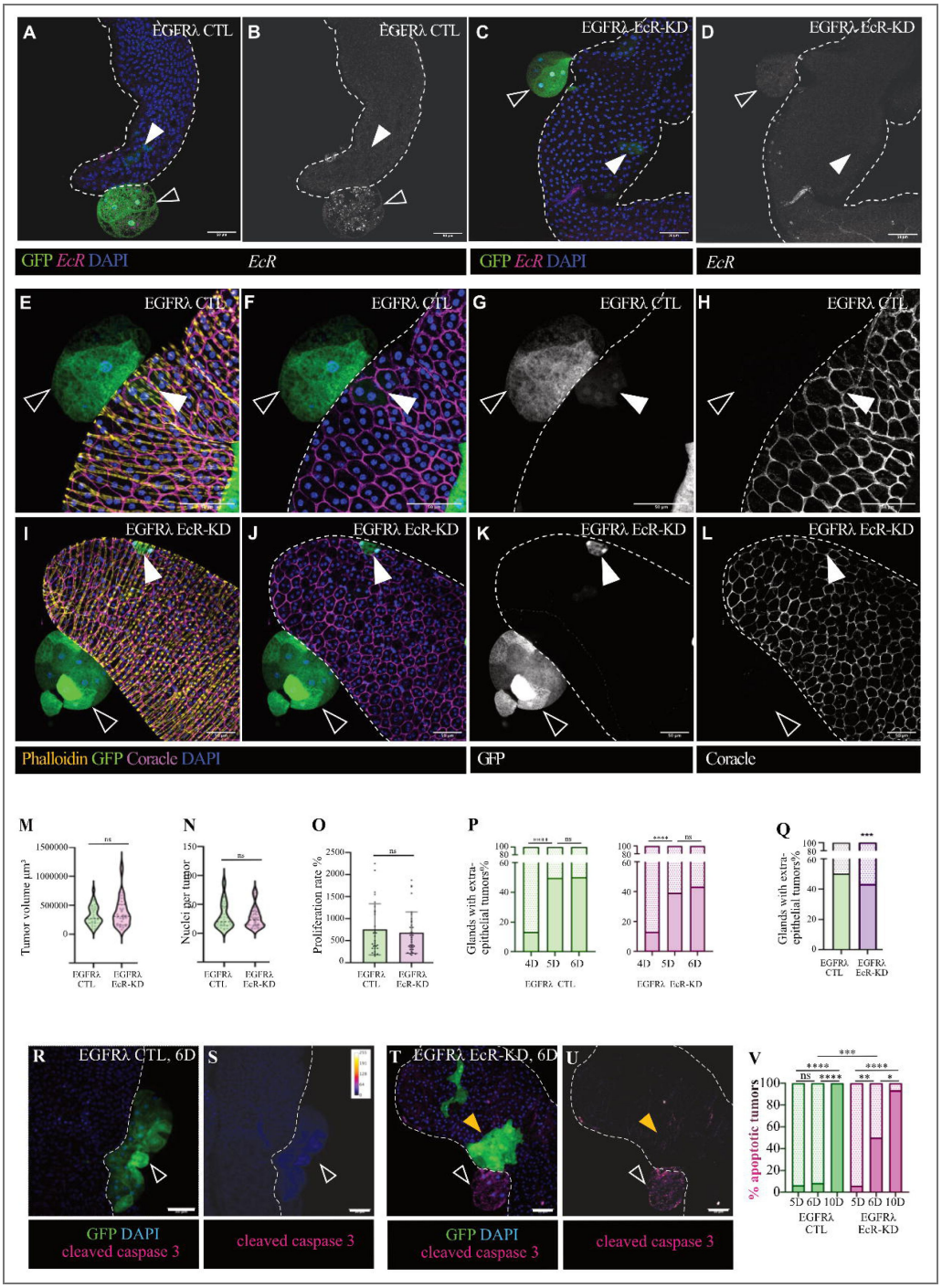

Figure 3. Treatment-like effect induced by Ecdysone Receptor downregulation

(A-L and R-U) Confocal imaging of accessory glands (delimited by white dashes). White arrowheads indicate epithelial clones. Empty arrowheads indicate extra-epithelial tumors. Yellow arrowheads indicate a tumor cell population specific to EGFRλ EcR-KD genotype. (A-D) FISH detection of EcR mRNA in EGFRλ CTL and EGFRλ EcR-KD genotypes. (E-L) Shared phenotypes between the two genotypes. (M-O) Extra-epithelial tumor characteristics defined by 3D-reconstruction. (P) % of glands bearing extra-epithelial tumors 4, 5 or 6 days after clonal induction for the different genotypes. (Q) Comparison of the maximal extra-epithelial tumors percentage (at 6D) between the two genotypes. (R-U) Cleaved caspase 3 staining in the different tumor cell populations. (V) Quantification of the number of apoptotic extra-epithelial tumors at 5, 6 or 10 days after clonal induction for the different genotypes. Scale bars: 50µm. Number of experiments (N) and of individual flies (n) are given in Table 2. Statistical tests are explained in the Methods section; ns: non-significant; * p<0.05; ** p<0.01; *** p<0.001; ****p<0.0001.

epithelial tumors (Figure 3T-U, empty arrowheads in left panels; middle columns in Figure 3V for quantification). This indicates that, in the drosophila accessory gland, some tumor cells rely on sex steroid signaling for their survival, as in the human disease.

Interestingly, at 10 days after induction (10D), most extra-epithelial tumors harbor apoptotic staining, independently of genotype (Figure 3V, right columns). Thus, despite their ability to undergo the complex program of basal extrusion, these cells seem unable to survive for long periods, indicating limited tumor potential.

Together, these results indicate that repression of EcR in accessory gland tumors cells displays a treatment-like effect: extra-epithelial tumor formation is (slightly but) significantly impaired, and cells composing these tumors are sensitive to this deprivation and undergo apoptosis.

Tumor escape induced by Ecdysone Receptor downregulation

Strikingly, in EGFRλ EcR-KD condition, we also detect a new type of tumorigenesis: large tumor masses now grow inside the glands (Figure 3T, Figure 4A-D, yellow arrowheads). Thus, generation of this new phenotype apparently depends solely on lack of ecdysone signaling in tumor cells. Contrary to the cells that remain in the epithelium in the EGFRλ CTL condition, these cells generally express low levels of epithelial markers (Figure 4D), indicating a loss of epithelial differentiation. Notably, unlike extra-epithelial tumors, this new tumor cell population shows no sign of apoptosis regardless of induction time (Figure 4E-H), and tend to invade the whole gland, unlike clone cells in the EGFRλ CTL condition (white arrowheads in Figure 4E-F, yellow arrowheads in Figure 4G-H). Thus, this new population displays an increased proliferative capacity compared with the EGFRλ CTL condition. Together, this indicates that these cells are not only escaping EcR repression but also now thrive over time under this stress.

Markers of higher progression of the new tumor cell population

Contrary to tumor cells in EGFRλ CTL condition, this new cell population displays strong staining for Ser10 Phospho-Histone 3 (pH3), a marker of late phases of the cell cycle prior to cell division (compare white to yellow arrowheads in Figure 4G-H to 4I-J). Furthermore, they exhibit a positive staining for phosphorylated proto-oncogene tyrosine-protein kinase Src (pSrc), a classical marker of aggressiveness (compare white to yellow arrowheads in Figure 4K-L to 4M-N) (Baumgartner et al., 2008). These tumor cells also display many typical defects, such as a clear pleomorphism with either compact or fusiform cells, and strongly disorganized clones (Figure 4Q-T). In the same clone and even within the same cell, nuclei differ markedly in size (anisocaryosis) with frequent hypertrophied nuclei (macrocaryosis). Nuclei shapes are often altered (empty arrowheads in Figure 4Q-R). Clones can grow along multiple axes (double-headed arrowheads in Figure 4S-T) and present a strong fibrillar framework (Figure 4T). Together, these results highlight that this new population has reached a higher step of tumor progression compared to previously described clones and extra-epithelial tumors, which form rounder, well-delimited masses.

Basal extrusion is modified in case of tumor escape

We then asked how these new tumor cells appear inside the glands.

A second kind of basal extrusion unveiled by EcR downregulation

In EGFRλ CTL condition (Figure 5A-D, white arrowheads), as in case of co-repression of Ras/MAPK, PI3K/Akt (Rambur et al., 2020) or cholesterol metabolic pathway (Vialat et al., 2024), tumor clones that appear in the gland remain strictly in the epithelium (orthogonal view in Figure 5C-D, where phalloidin staining (yellow) indicates the position of the muscle layer within the basement membrane). By contrast, in EGFRλ EcR-KD condition, the new population of tumor cells grows between the normal epithelium (delineated by white dashes in the orthogonal views in Figure 5G-H) and the basement membrane (Figure 5E-H, yellow arrowheads). Thus, these cells were able to migrate basally out of the normal epithelium, thereby pushing it into the gland lumen. At the same time, they did not cross the basement membrane. This redefines basal extrusion as two different phenomena with a common start. In this first step, cells extrude basally

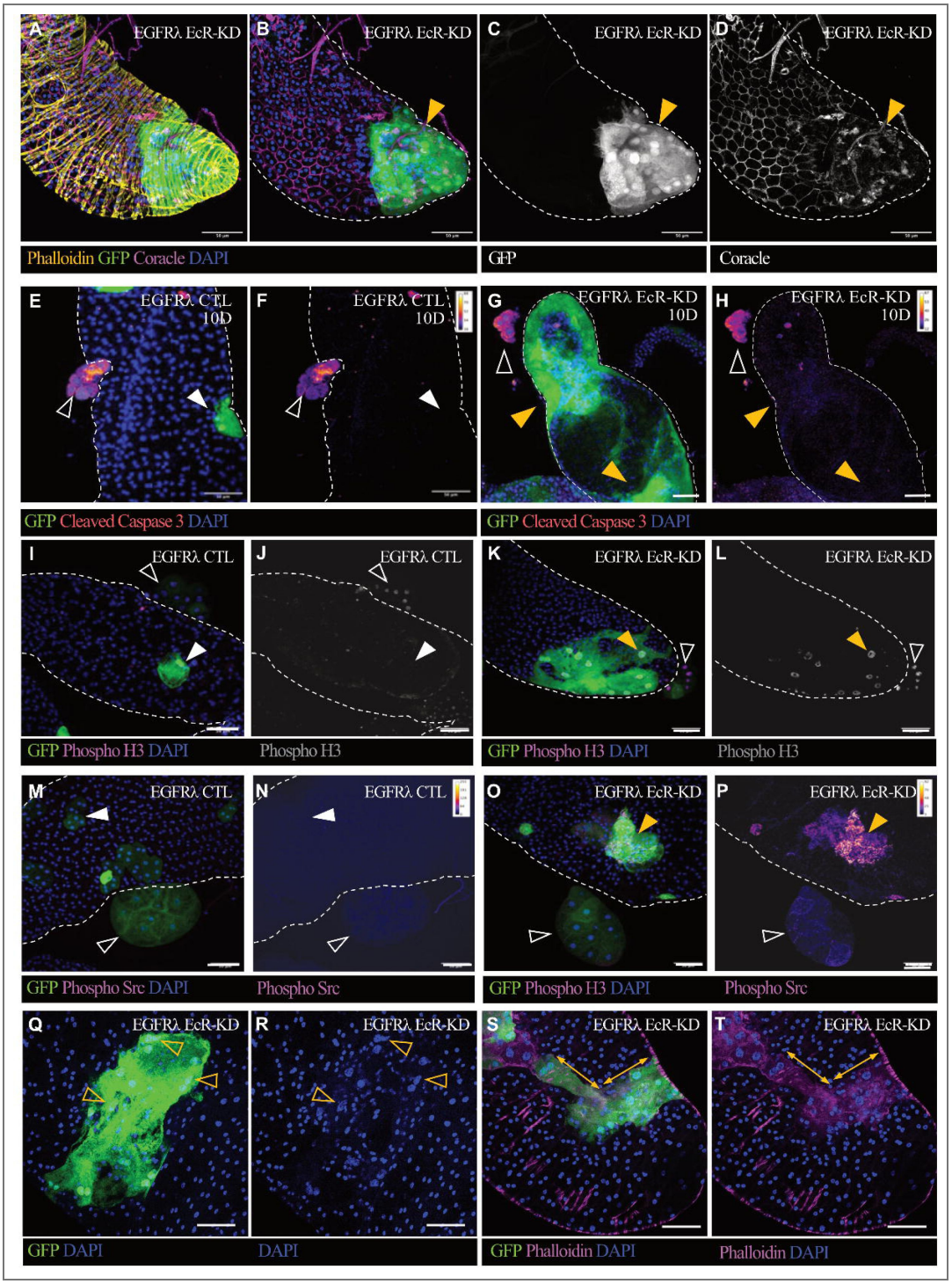

Figure 4. Tumor escape induced by Ecdysone Receptor downregulation.

(A-T) Confocal imaging of accessory glands (delimited by white dashes in some images). White arrowheads indicate epithelial clones. Empty arrowheads indicate extra-epithelial tumors. Yellow arrowheads indicate the new tumor cell population which is specific to EGFRλ EcR-KD genotype. (A-D) Coracle staining indicates epithelial status of the cells. (E-H) Cleaved caspase 3 staining in the different tumor cell populations 10 days after clonal induction for the different genotypes. (I-L) Phospho-Histone 3 staining 6 days after clonal induction for the different genotypes. (M-P) Phospho-Src staining 6 days after clonal induction for the different genotypes. (Q-R) DAPI staining reveals nuclear shapes of cells of the new population (yellow empty arrowheads). (S-T) Multiple axes of growth (double-headed arrows) and F-actin reorganization (phalloidin staining, magenta) in the new tumor cell population. Scale bars: 50μm.

from the epithelium. Then, cells can either cross the basement membrane and form extra-epithelial tumors or, depending on *EcR* repression, grow inside the gland. Blocking ecdysone signaling therefore promotes a new tumor cell fate, in which tumor cells accumulate between the normal epithelium and the basement membrane, forming highly proliferative tumors that escape steroid signaling downregulation in what we define as the intrabasal (IB) compartment.

Intrabasal growth relies on a new, inducible kind of basal extrusion

Given that we concomitantly detected a slight decrease in extra-epithelial tumor formation in the EGFRλ EcR-KD condition (Figure 3Q), we asked whether formation of intrabasal tumors arises from a redirection from full basal extrusion to partial basal extrusion. We show that, in contrast to extra-epithelial tumors, intrabasal tumors continue to appear and grow over time, as more glands display at least one intrabasal tumor at 7D than at 6D (Figure 5I); thus, they apparently do not replace lost extra-epithelial tumors. As a result of this growth, at 7D there are already significantly more glands displaying an intrabasal tumor that the maximal percentage of extra-epithelial tumors achieved in the EGFRλ CTL genotype (61.6% vs 49.6%, $p<0.001$. N=29, n=1117 vs N=3, n=268). This shows that, in EGFRλ EcR-KD condition, formation of intrabasal tumors does not result from mere inhibition of full basal extrusion but from promotion of a different kind of basal extrusion that ultimately generates tumor escape.

We then characterized these intrabasal tumors. At 6D, intrabasal tumors (IB-T) are significantly smaller than extra-epithelial tumors (T) but still significantly larger than epithelial clones of EGFRλ CTL genotype (C) (Figure 5J). This increase in volume mostly results from an increased proliferation rate and, consequently, increased tumor cell number in intrabasal tumors, which at 6D already compares with that of extra-epithelial tumors (Figure 5K-L). Thus, considering the total percentages of extra-epithelial and intrabasal tumors, *EcR* downregulation more than doubled the number of tumor cells during the course of the experiment.

Overall, *EcR* knockdown in tumor cells displays two opposite effects. On the one hand, it induces apoptosis of extra-epithelial tumors and modestly decreases their rate of formation. On the other hand, it strongly induces a new kind of tumorigenesis, which develops and thrives in the newly uncovered intrabasal compartment.

Ecdysone-dependent molecular control of tumor escape and basal extrusion

We then sought to determine the molecular mechanisms linking EcR Signaling to tumorigenesis. Prostate tumor escape is generally linked to resistance to androgen deprivation where AR is able to be activated despite hormone deprivation, through activating mutations in the gene or ectopic sex steroid production by tumors or their surrounding tissues. However, patients data indicate a decreased canonical androgen signaling in CRPC (Figure 1-2). It is therefore important to understand whether the observed escape results from a mechanism of resistance or from a genuine lack of ecdysone signaling. We therefore downregulated multiple targets at different levels of this pathway in a normal or tumoral context (Figure S2).

Loss of ecdysone signaling does not promote tumorigenesis by itself

First, we tested the effect of a loss of ecdysone signaling in a non-tumoral context. As previously shown (Rambur et al., 2020), at 6D, clones expressing GFP are composed of two cells (Figure S3A). Downregulation of EcR expression (EcR RNAi) or impairment of ecdysone synthesis (Shadow RNAi, Sad-KD, see Figure S2) induces the same effect: as in CTL, clones are composed of two cells that appear of normal shape and nuclear number (Compare Figure S3A and S1B and S3C). No tumorigenesis is detected (Figure S3D). The only detected phenotype is a slight decrease in epithelial cell growth without change in proliferation (Figure S3E-G). This hypotrophy could reflect a partial loss of cells' ability to produce seminal fluid, which relies on ecdysone signaling in accessory gland cells.

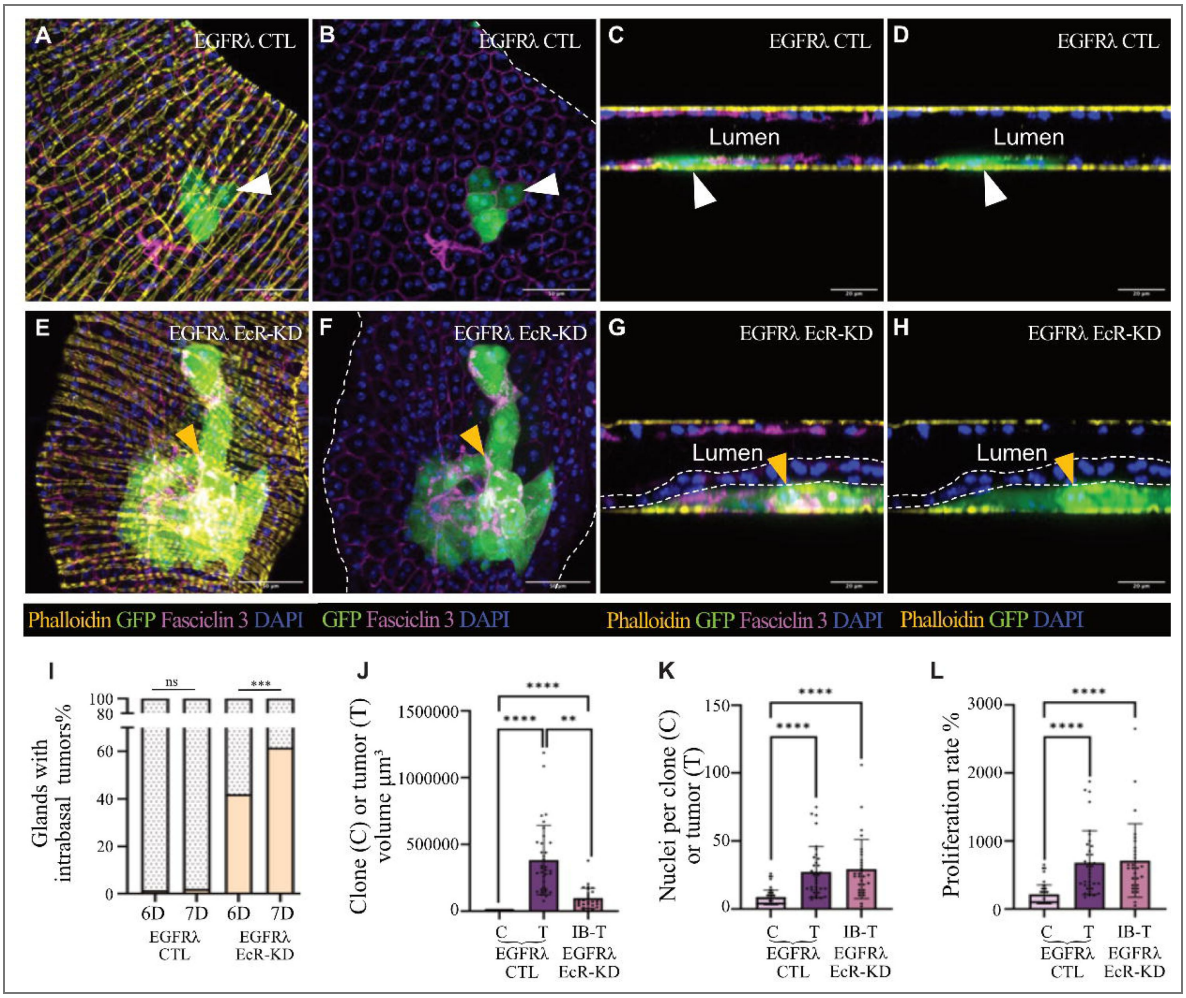

Figure 5. Basal extrusion is modified in case of tumor escape.

(A-T) Confocal imaging of accessory glands. White arrowheads indicate epithelial clones. Empty arrowheads indicate extra-epithelial tumors. Yellow arrowheads indicate the new tumor cell population which is specific to EGFRλ EcR-KD genotype. (A-B, E-F) XYZ imaging. Glands are delimited by white dashes in B and F. (C-D, G-H) XZY orthogonal reconstructions of the glands, showing the lumens and in-depth view of the epithelium. Epithelium pushed into the lumen is delimited by white dashes in G and H, as intrabasal tumor cells lay between this epithelium and the basement membrane (yellow). (I) % of glands bearing intrabasal tumors 6 or 7 days after clonal induction for the different genotypes. (J-L) Comparison of intrabasal vs other tumor characteristics defined by 3D-reconstruction (C: clones; T: extra-epithelial tumors; IB-T: intrabasal tumors). Scale bars: 50µm in XYZ views and 20µm in XZY reconstructions. ns: non-significant; ** p<0.01; *** p<0.001; ****p<0.0001.

Lack of canonical ecdysone signaling drives tumor escape

Paralleling human metabolization of testosterone into active DiHydroTestosterone (DHT) by the SRD5A2 gene product, ecdysone must be metabolized into active 20-hydroxyecdysone by Shade (Shd) (Petryk et al., 2003). As *SRD5A2* dramatically decreases during cancer progression (Figure 2E-H), we downregulated *Shd* in tumor cells. Tumor progression is phenocopied in glands of the EGFRλ Shd-KD genotype compared with the EGFRλ EcR-KD condition, for both the decrease in extra-epithelial tumors and the growth of intrabasal tumors (Figure 6A, magenta columns). By targeting an independent gene, this confirms that lack of EcR signaling leads to tumor resistance in the drosophila accessory gland.

Furthermore, ecdysone signaling relies on recruitment of specific EcR target genes -the early-late transcription factors Hr3 and Hr4- to drive ecdysone responses (Carney et al., 1997; Gauhar et al., 2009). Again, tumor progression in both EGFRλ Hr3-KD and EGFRλ Hr4-KD conditions is strikingly similar to the EGFRλ EcR-KD condition (Figure 6A-B, blue columns, Figure 6C-C', Figure S3), showing that lack of canonical ecdysone signaling leads to tumor escape.

Complete dependence on tumor-produced Ecdysone to alleviate tumor escape

Prostate cancer cells are able to produce steroids (Locke et al., 2008), which contribute to AR reactivation in castration-resistant prostate cancer (CRPC) (Cai et al., 2011). In drosophila, the Halloween genes encode the enzymes that synthetize ecdysone. Phantom (Phm) is the limiting enzyme for this synthesis, whereas Shadow (Sad) is the terminal enzyme producing ecdysone. The EGFRλ Phm-KD and EGFRλ Sad-KD conditions again phenocopy the EGFRλ EcR-KD condition (Figure 6A-B, red columns, Figure 6D-D', Figure S4). This shows -by targeting still two other independent genes and still another process- that, in the drosophila accessory gland, growth of intrabasal tumors results from lack of ecdysone signaling.

Furthermore, this demonstrates that circulating ecdysone cannot compensate for lack of tumor-produced ecdysone, which is therefore the only indispensable resource to prevent tumor escape in the accessory gland. We previously demonstrated that autocrine loops recruiting Ras/MAPK and PI3K/Akt pathways are established to support basal extrusion (Rambur et al., 2020). We show here that an autocrine/intracrine loop is also established to activate ecdysone signaling and sustain the same phenomenon. However, blocking this loop now also induces growth of intrabasal tumors, in contrast to inhibition of Ras/MAPK and PI3K/Akt pathways. Thus, promotion of this mechanism of tumor escape is specifically induced by the loss of ecdysone signaling in these cells.

EcR target gene Tub60D specifically represses intrabasal growth

We then investigated molecular drivers that could promote intrabasal growth. Cytoskeletal reorganization is a major determinant of tumor cell invasion and resistance, and intrabasal tumor cells harbor profound cytoskeletal change. *Beta Tubulin at 60D* (*Tub60D*) encodes β3 Tubulin and is a target of ecdysone signaling (Bruhat et al., 1990). β3 Tubulin accumulates in intra-epithelial clones of EGFRλ CTL condition but is mostly absent in intrabasal tumor cells (compare Figure 6E-F to G-H). Strikingly, downregulation of *Tub60D* (EGFRλ Tub60D-KD condition) is sufficient to induce intrabasal growth (Figure 6I-I', Figure 6J, right columns), but overall has no effect on formation of extra-epithelial tumors (Figure 6J, left columns).

Furthermore, these extra-epithelial tumors remain unchanged compared to the other tested conditions (Figure S6). Thus, in this condition as well, intrabasal growth represents an inducible program that is completely independent of extra-epithelial tumor formation.

Furthermore, in the EGFRλ Tub60D-KD condition, intrabasal tumors do not proliferate more than epithelial clones (Figure 6K). Lack of β3 Tubulin therefore appears as a major determinant of intrabasal growth but does not promote any advantage by itself, even in a tumoral context. This indicates that the ability to achieve escape relies, in the drosophila accessory gland, on ecdysone signaling deprivation and not merely on the ability of tumor cells to reach the intrabasal compartment.

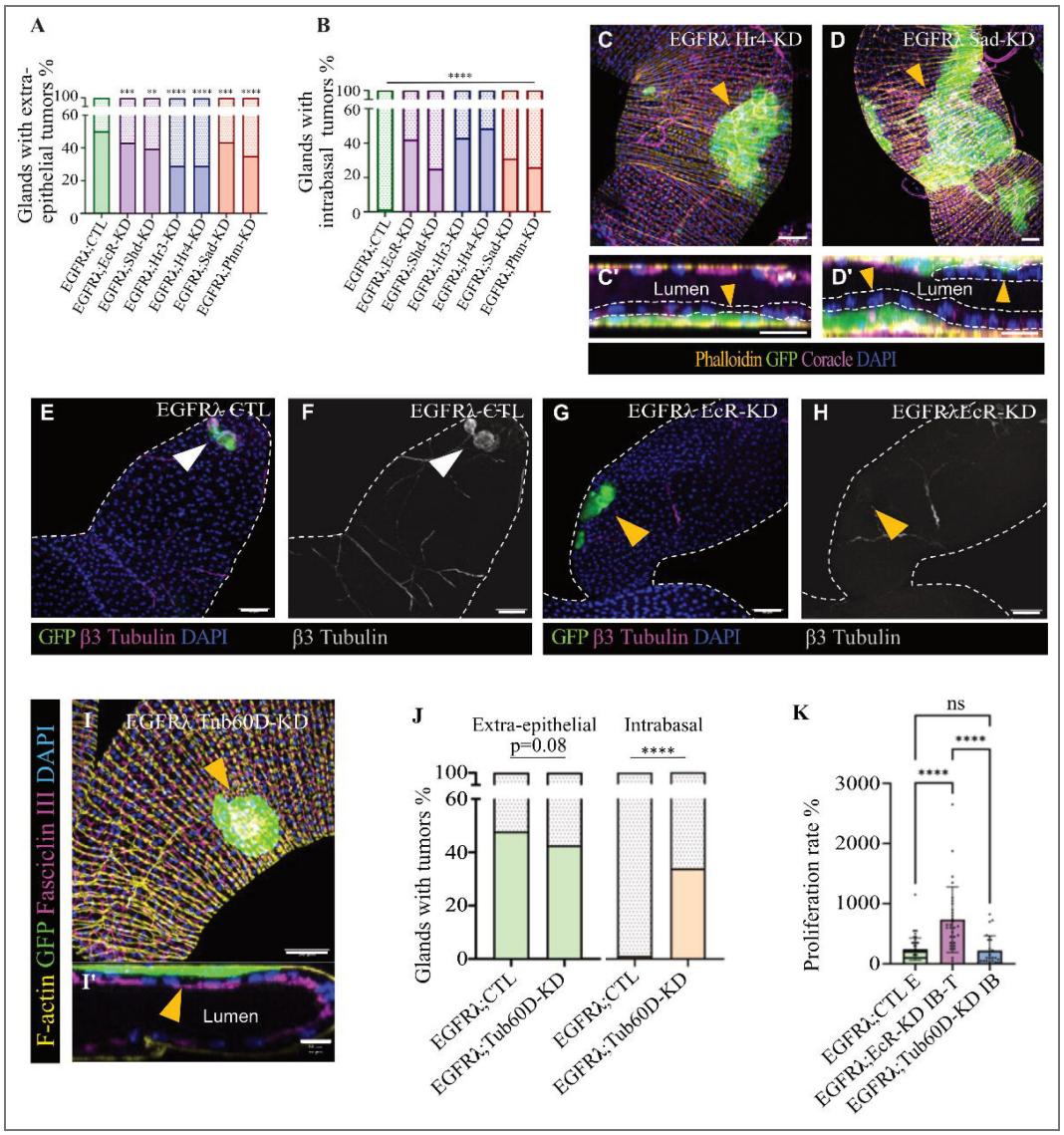

Figure 6. Ecdysone-dependent molecular control of tumor escape and basal extrusion.

(A-B) % of glands bearing extra-epithelial (A) or intrabasal tumors (B) 6 days after clonal induction for different genotypes. (C-D') Confocal imaging of accessory glands. Yellow arrowheads indicate intrabasal tumors. (C, D) XYZ imaging. (C', D') Corresponding XZY orthogonal reconstructions of the glands, showing the lumens and in-depth view of the epithelium. Epithelium pushed into the lumen is delimited by white dashes. (E-H) β3 Tubulin staining 6 days after clonal induction for different genotypes. Glands are delimited by white dashes, white arrowheads indicate tumors clones. (I-I') Confocal imaging of accessory glands. (I) XYZ imaging. (I') Corresponding XZY orthogonal reconstruction of the glands. (J) % of glands bearing extra-epithelial (left) or intrabasal tumors (right) 6 days after clonal induction for different genotypes. (K) Comparison of proliferation rates defined by 3D-reconstruction (E: epithelial clones; IB-T: intrabasal tumors; IB: intrabasal cells in EGFRλ Tub60D-KD genotype). Scale bars: 50µm in XYZ views and 20µm in XZY reconstructions. ** p<0.01; *** p<0.001; ****p<0.0001.

Discussion

The National Cancer Institute defines cancer by their abnormal cells dividing without control and invading nearby tissues. Epithelial cancers represent ~90% of all cancers (Bray et al., 2018). As epithelial cells rest on a strong extracellular matrix on their basal side, epithelial tumor cells must cross this basement membrane to invade nearby tissues. This is why this step of invasion is called basal extrusion. Little is known about basal extrusion when it occurs physiologically during development and when it occurs in the pathophysiological context of tumorigenesis. Basal extrusion appears as a complex process: it can be induced by the clonal expression of different oncogenes (Anton et al., 2018; Rambur et al., 2020; Villeneuve et al., 2019); may occur in balance with apical extrusion (Anton et al., 2018; Marshall et al., 2011; Ventrella et al., 2023); relies on multiple signaling pathways such as Notch (Ventrella et al., 2023), Ras/MAPK and PI3K/Akt (Rambur et al., 2020) or PKC activation (Villeneuve et al., 2019); depends on cholesterol uptake (Vialat et al., 2024) and lipid raft activity (Kajiwara et al., 2022); and can be promoted by extracellular matrix degradation even though, counterintuitively, this does not seem to be a necessary step (Shen et al., 2014). It also depends on profound remodeling of cytoskeletal organization and of cell-cell and cell-matrix interactions (Chountala et al., 2012; Ganier et al., 2018; Melo et al., 2023; Slattum et al., 2009; Villeneuve et al., 2019). Overall, our data indicate that basal extrusion of tumor cells appears furthermore as a two-fate phenomenon that, in drosophila accessory gland, is tightly regulated by ecdysone signaling, as downregulation of this signaling induces growth of intrabasal tumors. Interestingly, clonal expression of an oncogene in mouse epithelia induces formation of dome-like structures where tumor cells accumulate at the same localization, showing that this precise tumor fate also exists in mammals (Shirai et al., 2022). This localization has long been known to favor tumor growth, as exemplified by the widespread use of subcapsular renal xenografting in cancer research (Bogden et al., 1979). Moreover, in a mouse model of intraductal human breast carcinogenesis, Zoeller et al demonstrated that tumor cells adjacent to the basement membrane display extra-resistance to treatments when compared with others, indicating that this specific microenvironment is most conducive to survival upon treatment (Zoeller et al., 2017). An ability for tumor cells to reach this compartment could then confer them a higher chance of survival and progression.

However, we were able to push tumor cell growth in this compartment by manipulating *Tub60D* expression. In this case, no increase in cell proliferation and no apparent cell disorganization are associated with intrabasal growth. Thus, by itself, growth in the intrabasal compartment is not sufficient for tumor progression in the accessory gland.

Conversely, in this context, tumor cell resistance is actively induced by decreased canonical ecdysone signaling. And, in human, we also show that AR signaling decreases with prostate cancer progression. This lack of AR signaling also favors the most aggressive NEPC (Turpin et al., 2023). In breast cancer, ER loss is a common event largely associated with tumor aggressiveness (Kuukasjärvi et al., 1996). In prostate cancer, in the numerous alterations that can affect AR, many mutations found in tumors are loss of function (Hay and McEwan, 2012). Moreover, in human, Androgen Deprivation Therapy (ADT) shows opposite effects depending on cancer stage. For poorly differentiated or metastatic tumors, ADT has largely proven its efficacy by increasing patient survival. However, for localized prostate cancer, some studies argue that ADT displays no beneficial effect and is even associated with worse outcome than active surveillance for patient survival (Lu-Yao et al., 2008; Merglen et al., 2007). In men with hypogonadism, testosterone supplementation therapy has been feared to increase prostate cancer risk. However, reviews of the scientific literature show no increase in prostate cancer incidence with this treatment (Dupree et al., 2014; Michaud et al., 2015).

Finally, in High-Grade Prostate Intraepithelial Neoplasia (HGPIN), low testosterone correlates with an increased risk of prostate cancer development (García-Cruz et al., 2012). This suggests that decreased activity of sex steroid signaling in the early phases of tumorigenesis could be implicated in cancer progression and/or the genesis of tumor cells populations escaping endocrine therapy. In this context, to link changes in basal extrusion, sex steroids and aggressiveness, it is noteworthy

that particularly aggressive forms of ductal carcinoma *in situ* in breast (Baqai and Shousha, 2003) and intraductal cancers in prostate display both a growth initially restricted to the epithelial compartment and defects in sex steroid signaling (Zhao et al., 2018). Thus, lack of sex steroid signaling could potentially induce progression and escape to sex steroid deprivation in some endocrine-dependent forms of cancer.

Overall, we show *in vivo* that epithelial basal extrusion is a complex phenomenon: cells can extrude basally from the epithelium but still remain in the epithelial compartment. We show that repression of sex steroid signaling in an oncogenic context induces a specific change in tumor cell fate, in which these cells grow in a newly unveiled intrabasal compartment. We show that, for tumor cells of the drosophila accessory gland, this represents a key event in the genesis of tumors escaping sex steroid deprivation. Epithelial basal extrusion could therefore be a key event in tumor escape and progression, depending on activity of specific pathways such as sex steroid signaling.

Materials and Methods

Fly stocks and experimental crosses

y,w,HS:flp122/+;Act:FRTstopFRTGal4, UAS:GFP/CyO flies allowed conditional clonal expression of GFP. Combination with UAS:EgfrλTop (#59843) allowed conditional clonal co-expression of GFP and Egfrλtop (EGFRλ flies). EGFRλ flies were then crossed with following stocks to realize experiments: UAS-GFP.nls (#4775, control condition), UAS-EcR RNAi (#50712), UAS-Shd RNAi (#67456), UAS-Hr3 RNAi (#27253), UAS-Hr4 RNAi (#54803), UAS-Sad RNAi (#67352), UAS-Phm RNAi (#65017), UAS-Tub60D RNAi (#64856) from the Bloomington Stock Center.

Conditional expression induction

Briefly, flippase (flp)/FRT system was activated by a 12 min heat-shock induction at 37°C during the pupal stage, to create an average of 4–6 clones per accessory gland (≈1% of total number of epithelial cells). Flies were then kept at 27 °C until the end of pupal stage. Males were collected at emergence from pupae 3 to 3.5 days after heat shock and kept for another 1 to 5 days at 27 °C before dissection.

Immunohistochemistry and imaging

Accessory glands were dissected in PBS, fixed for 10 min in 4% paraformaldehyde, washed and permeabilized for 15 min in PBS containing 0.2% Triton (PBS-T). Glands were blocked for one hour with 0.5% of BSA in PBS-T then incubated overnight at 4°C with primary antibodies diluted in the same blocking solution. After three washes in PBS-T, glands were incubated in secondary antibody diluted 1:1000 in blocking solution for 1 hour at room temperature with DAPI (DiAminidoPhenylIndol, D8417, Sigma) 1:1000 (DNA staining) and/or Alexa633-phalloidin (A22284, Life Technology) 1:5000 (to reveal F-actin). Glands were then washed twice in PBS-T, once in PBS and subsequently mounted in Vectashield (#-1000, Vector Laboratories). Imaging was realized on Leica SP8 confocal microscope, and image stacks were processed either in ImageJ or Imaris software. All stainings were repeated a minimum of 3 times on independent experiments.

List of antibodies: Mouse Coracle (1:300, #C566.9 DSHB), mouse Fasciclin III (1:400, #7G10 DSHB), goat GFP (1:1000, #5450 Abcam), rabbit pSrc (1:500, #44-660G Invitrogen), rabbit pH3 (1:500, #06-570, Merck Millipore), rabbit cleaved-caspase 3 (1:400, #9661S, Cell Signaling), secondary antibodies coupled to different fluorophores 488 (1:1000, A11055 Invitrogen), Cy3 or Cy5 (1:1000, 711-165-152, 715-165-151, 715-175-150, Jackson Immunology).

Clones sizes, cells and nuclei size and numbers, proliferation rate

Clones/tumors sizes, cells and nuclei volumes and numbers were determined from 3D reconstruction and automatic quantification in Imaris software. For each clone/tumor, average cell size was determined by ratio between clone size and number of nuclei in the considered clone. Proliferation rate was calculated compared to number of cells in a clone expressing GFP only (as

in Figure S3A). A typical clone is composed of 2 cells, and so 2 cells was taken as 100% proliferation. See Table 2 for the number of experiments and total numbers of individual flies that were processed for each experiment.

Extra-epithelial or intrabasal tumor frequency

Extra-epithelial tumor frequency was determined as the percentage of flies that displayed at least one tumor on their accessory glands at dissection. For intrabasal tumors, individual glands were considered. See Table 2 for the number of experiments and total numbers of individual flies that were processed for each experiment.

RNA FISH

Single-molecule RNA fluorescence in situ hybridization (smRNA-FISH) for transcripts of EcR from the accessory gland was performed using Stellaris probes (Biosearch Technologies). Probe sequences are listed in table S1. Accessory gland were dissected in PBS and fixed in 4% formaldehyde, 0.3% Triton X-100, 1x PBS for 20 min at room temperature, rinsed and permeabilized in 70% ethanol at 4°C overnight. Glands were rehydrated in smRNA FISH wash buffer (10% formamide in 2x SSC) for 10 min, resuspended in 50µL hybridization buffer (10% dextran sulphate, 10% formamide in 2xSSC) supplemented with 1.5µL of smRNA FISH probes. Hybridization was performed at 37°C overnight. Glands were then washed twice with smRNA FISH wash buffer at 37°C for 30 min and twice with 2xSSC solution. Then, DNA was stained with DAPI (1/500 dilution in 2x SSC) at room temperature for 20 min. Accessory glands were then mounted in 30µL Vectashield prior to imaging. The resulting images were processed using FIJI/ImageJ.

Statistical analyses and human data

All experiments were repeated independently a minimum of three times (N: number of independent experiments) on numerous glands (n: number of pairs of glands or number of imaged and quantified glands for tumor size and nuclei number quantification; see Table 2). Statistical analyses were performed using GraphPad Prism 6 for tumor size and nuclei number quantification were compared by Krustal-Wallis test; % of glands with extra-glandular tumors were compared by Chi2 test; gene expression in qPCR was compared by two-tailed impaired T-test. Human data processing was done using the tools developed by Theurillat et al. and available at *prostatecanceratlas.org*. Expression data and pseudotime progression were individually retrieved for each considered gene using the Explore/bulk RNA function, and were grouped by stage (NORMAL, 173 samples, PRIMARY, 708 samples, mCRPC, 484 samples). Statistical analyses were then performed using GraphPad Prism 6 and quantifications were compared by Mann-Whitney test.

In all figures, significance is described as follows : * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure panel	N = Number of independent experiments	n = total number of individuals
Fig. 1 and 2	N = 13 independent cohorts	n = 1140
Fig. 3M-O	EGFRλ CTL: 6; EGFRλ EcR-KD: 8	EGFRλ CTL: 29; EGFRλ EcR-KD: 35
Fig. 3P	for respective column: 4, 3, 30, 4, 3, 36	for respective column: 60, 131, 1144, 217, 123, 1525
Fig. 3Q	EGFRλ CTL: 30; EGFRλ EcR-KD: 36	EGFRλ CTL: 1144 EGFRλ EcR-KD: 1525
Fig. 3V	3	for respective column : 32, 36, 7, 17, 22, 25
Fig. 5I	3	for respective column: 203, 196, 217, 268
Fig. 5J-L	3	for respective column of each panel: 53, 35, 30
Fig. 6A	For respective column, 30, 36, 7, 9, 8, 33, 12.	For respective column: 1144, 1525, 228, 287, 207, 1618, 637.
Fig. 6B	N = 3 for all columns	For respective column: 203, 217, 52, 126, 62, 252, 167.
Fig. 6J	Left panel: 30, 14; right panel: 3 or more	Left panel: 1576, 354; right panel: 248, 130
Fig. 6K	3 or more	for respective column: 51, 30, 25

Table 2. Numbers of independent experiments (N) and individuals (n) used in the different experiments.

Supplementary figures

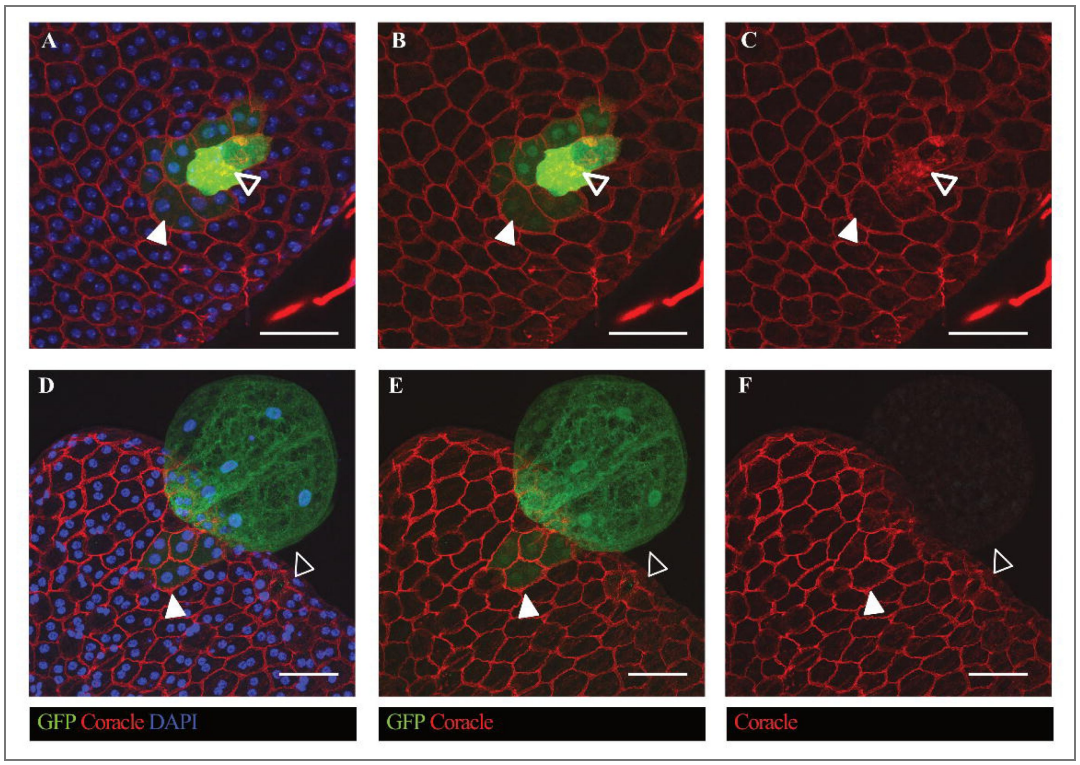

Figure S1. EGFRλ clonal expression induces tumorigenesis in the drosophila accessory gland White arrowheads: mildly hyperproliferative, hypertrophic cells are well integrated into the epithelium with undisturbed cell-cell interaction (A-F, Coracle staining). Thick empty arrowheads: some of these epithelial tumor cells express higher levels of GFP (A-B), reflecting modified gene expression, and higher levels of epithelial markers. However, these markers do not properly localize at the membrane (C), indicating a loss of cohesion with neighbor cells. Empty arrowheads: some tumor cells are able to undergo epithelial basal extrusion to form extra-epithelial tumors. These cells are devoid of epithelial markers, indicating a change in their fate (F). Scale bars: 50 micrometers.

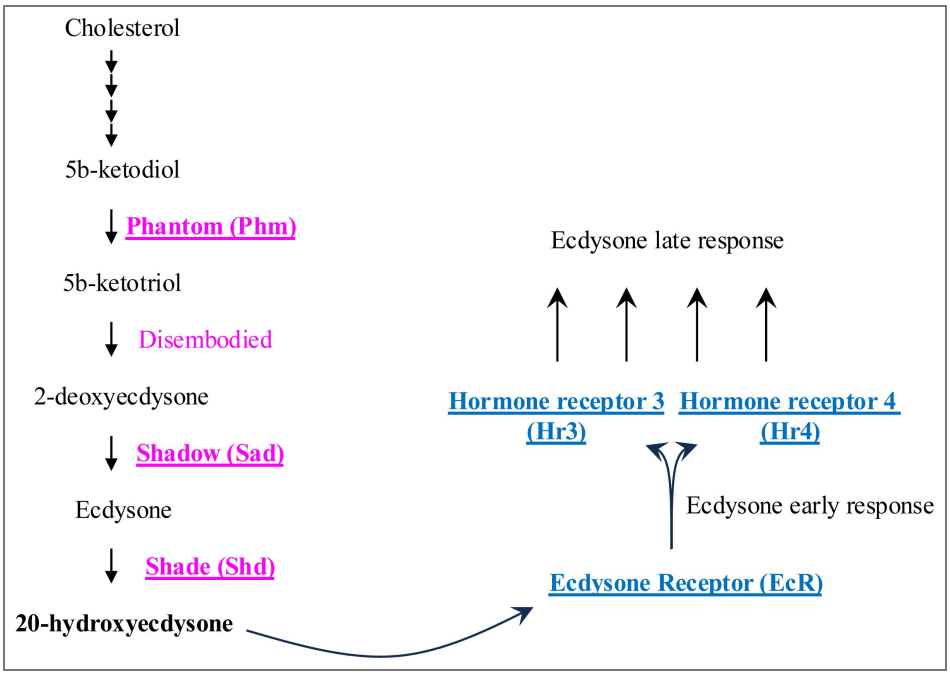

Figure S2. Schematic representation of the late steps of ecdysone synthesis and canonical Ecdysone Signaling.

Six different genes (underlined) were independently targeted by RNAi to block tumor cell production of ecdysone (Phm, Sad), metabolization of active 20-hydroxyecdysone (Shd), or EcR Signaling (EcR, Hr3, Hr4). Results are shown in Figure 6.

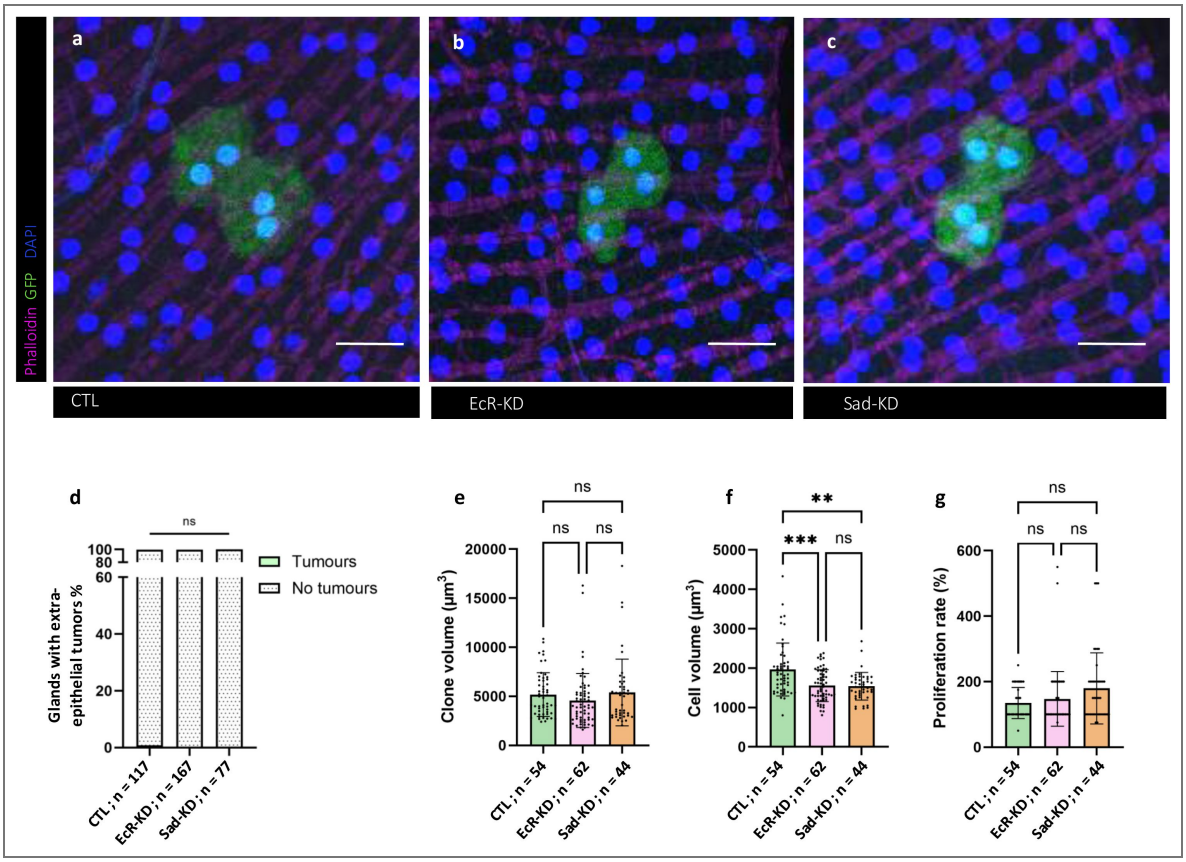

Figure S3. Ecdsyone deprivation does not promote tumorigenesis by itself.

(a-c) In the absence of expression of an oncogene (CTL), clonal induction of NLS-GFP (a) EcR RNAi (b) or Sad RNAi (c) brings no evident phenotype to the clonal cells. (d) It does not promote tumorigenesis. (e-g) In the absence of average clone volume (e) or difference in proliferation rate compared to control (g), the only noticeable phenotype is a slightly decreased cell size in clones expressing EcR or Sad RNAi (f). Chi2 test; **P < 0.01; ***P < 0.001. Representative images in (a-c) from three or more experiments. Scale bars: 20 µm.

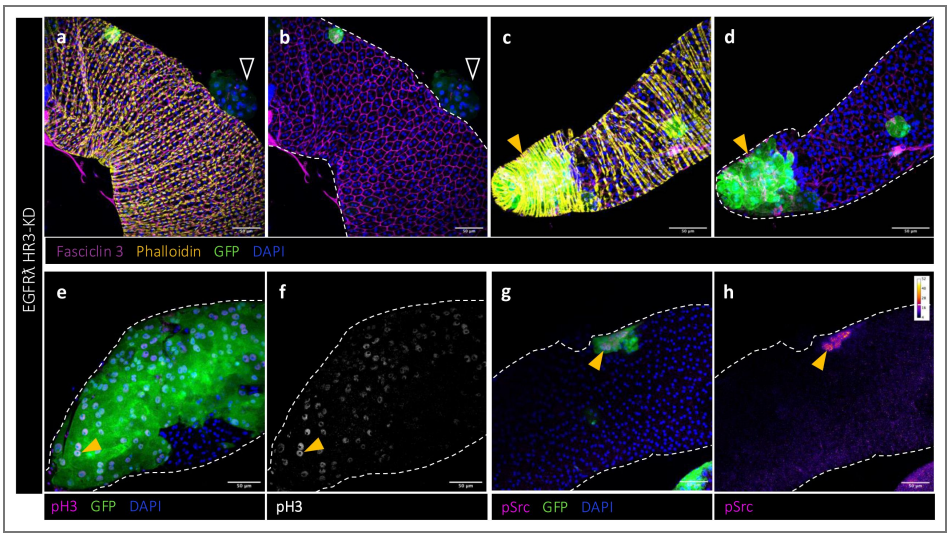

Figure S4. Downregulation of canonical Ecdysone Signaling induces intrabasal tumorigenesis.

(a-d) Co-expression of Hr3 RNAi suppresses only partially extra-glandular tumors (empty arrowheads in (a-b)) but leads to the appearance of highly proliferative, intrabasal tumor cells into the glands (yellow arrowheads in (c-d)). (e-h) This new population displays a strong staining for classical aggressiveness markers (yellow arrowheads, pH3 staining in (e-f) and pSrc staining in (g-h)). Representative images from three or more experiments. Scale bars: 50 μM.

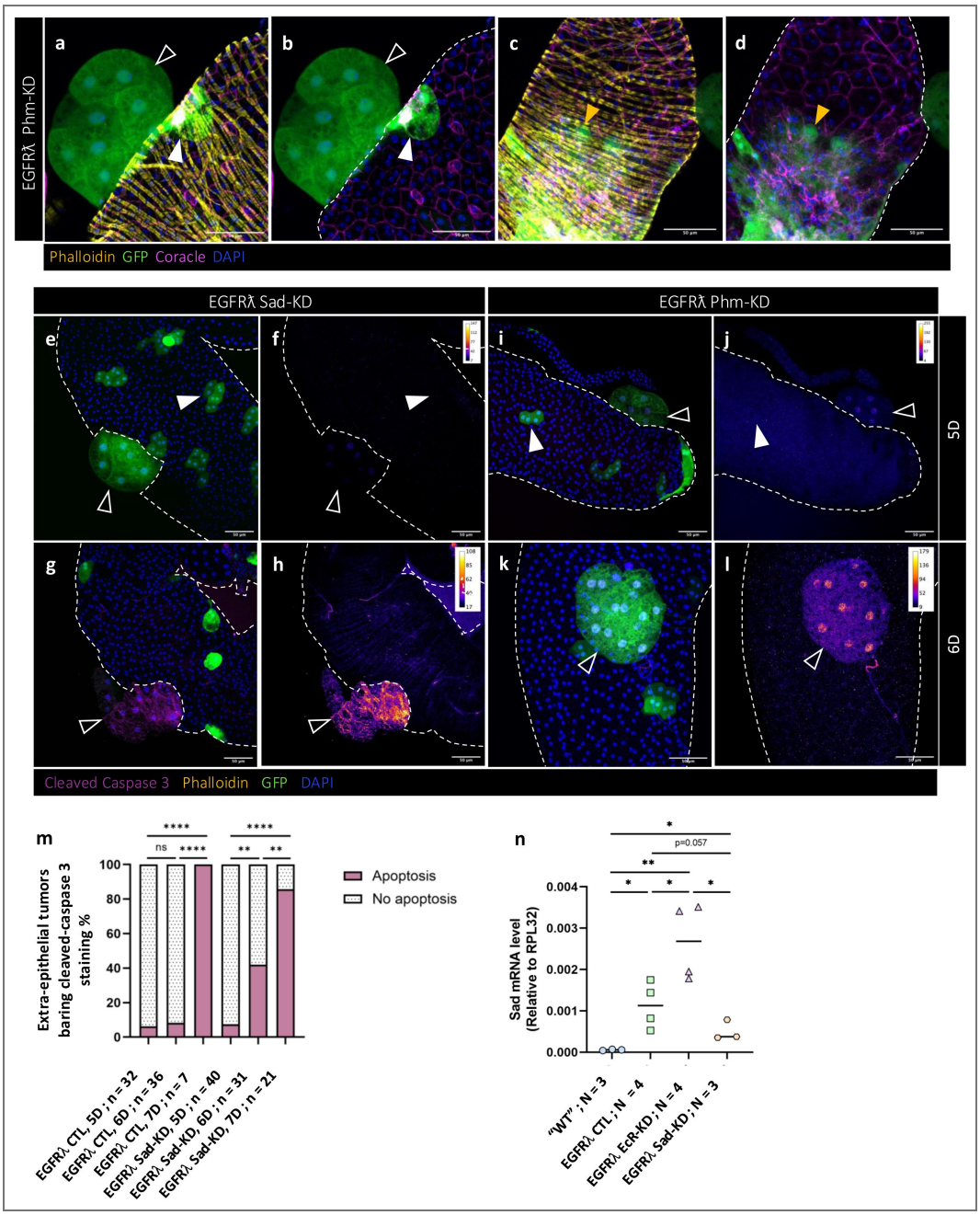

Figure S5. Loss of autocrine Ecdysone Signaling induces the same paradoxical response as loss of other members of the same pathway.

(a-d) Co-expression of Phm RNAi suppresses only partially the formation of extra-epithelial tumors (empty arrowheads in (a-b)) but leads to the appearance of highly proliferative intrabasal tumors (yellow arrowheads in (c-d)). (e-l) Co-expression of Sad (RNAi (e-h) or Phm RNAi (i-l) induces early apoptosis of extra-epithelial tumors (at 6D, cleaved-caspase 3 staining), mimicking initial effect of steroid deprivation on human tumors (empty arrowheads). (m) Quantification of this early apoptosis phenomenon appearing at 6D for the coexpression of Sad RNAi. (n) qPCR quantification of whole glands expression of Sad for the given genotypes. "WT": glands baring the EGFRλ genetic background, but unable to produce clones, i.e. "wild type" glands. Compared to these, oncogene induction (column 2) induces a significant overexpression of Sad mRNA; co-expression of EcR RNAi (column 3) still significantly increases Sad mRNA expression. Compared to this condition, co-expression of Sad RNAi (column 4) significantly decreases Sad mRNA expression, as expected. Representative images in (a-l) from three or more experiments. Scale bars: 50 μM.

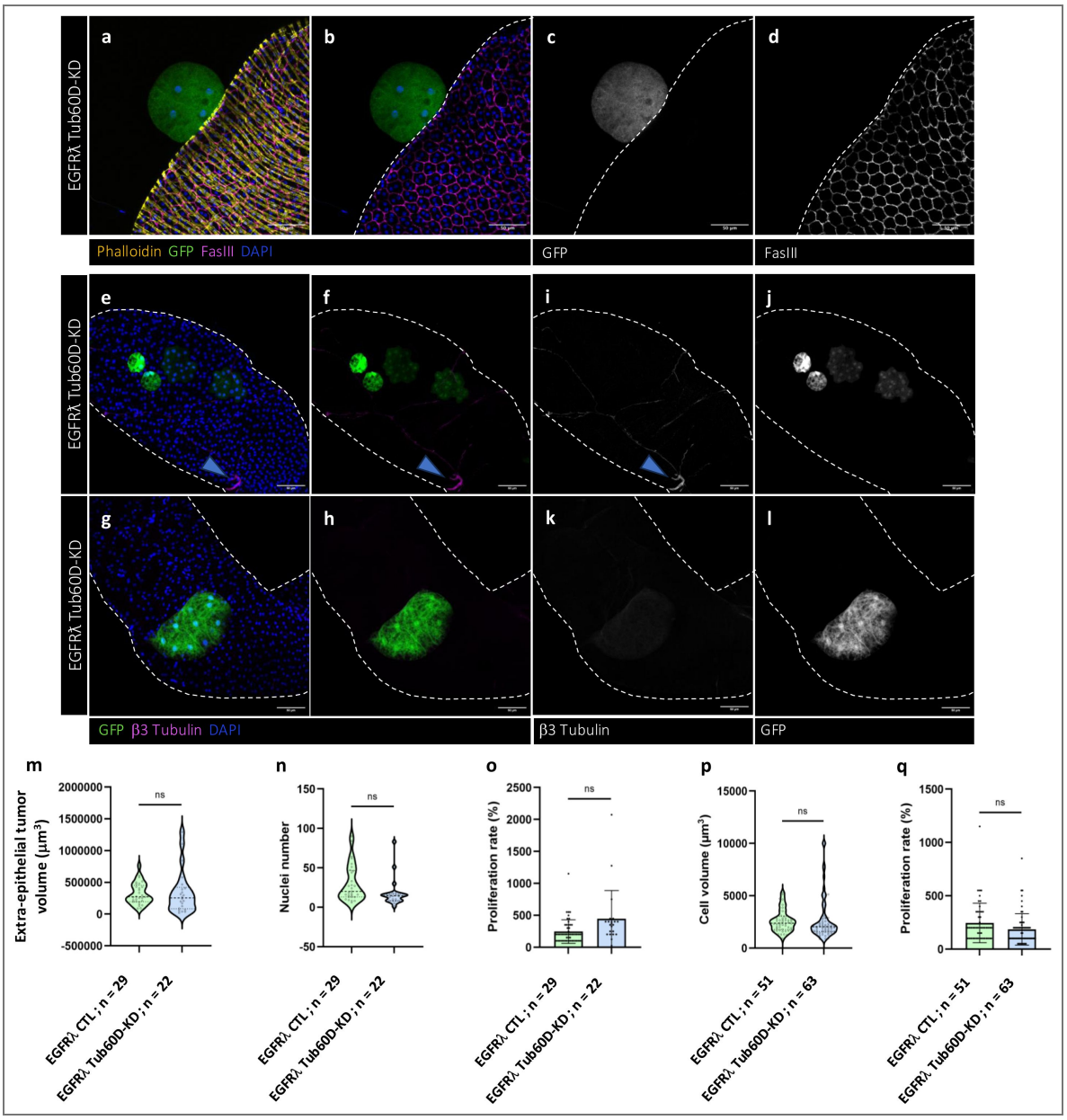

Figure S6. Efficient downregulation of Tub60D has little effect on epithelial tumor cells.

(a-d) Co-expression of Tub60D RNAi does not affect the phenotype of extra-epithelial tumors. (e-l) Downregulation of Tub60D efficiently suppresses the accumulation of β3 Tubulin in clonal cells (e-h) and tumor cells (i-l). Remaining staining can be seen in other cell types such as tracheal cells (blue arrowheads in (e-i). (m-r) Characteristics of the different types of tumor cells co-expressing Tub60D RNAi. (m-q) Extraepithelial tumors co-expressing Tub60D RNAi display the same size (m), cell number (n) and proliferation rate (o) as EGFRλ CTL tumors. (pq) Intra-epithelial clones co-expressing Tub60D RNAi are composed of cells of same size (p) and display the same proliferation rate (q) as as EGFRλ CTL intra-epithelial clones. Chi^2 test; ns non significant. Representative images in (a-l) from three or more experiments. Scale bars: 50 µM.

Data availability

All data generated during this study are included in the manuscript and supporting files.

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Peer reviews

Reviewer #1 (Public review):

Summary:

In this article, Vialat and his colleagues examine the early stages - which remain largely unknown - of the tumor escape process, particularly the basal extrusion of tumor cells following endocrine therapy for prostate cancer.

They first used the "Prostate Cancer Atlas" database, which provides access to a vast amount of transcriptomic data, to perform high-throughput analyses. Interestingly, analyzing a series of Androgen Receptor (AR) target genes in castration-resistant prostate cancers, they concluded that the loss of the canonical AR signaling pathway may contribute to tumor resistance.

Using a well-established model in Drosophila, they then replicated in vivo an endocrine therapy targeting the accessory gland by genetically inhibiting the expression of ecdysone, the only sex steroid present in Drosophila. These experiments induced basal extrusion similar to the mechanism observed in tumor escape in humans.

These results suggest that the deprivation of sex steroids may play an important role in tumor progression.

However, although the data from the "Prostate Cancer Atlas" constitutes a powerful tool that serves as the basis for this new concept, clinical validation using carefully selected human tumor samples would help strengthen the authors' conclusions.

Strengths:

(1) The Prostate Cancer Atlas is a comprehensive collection of clinical data derived from RNA sequencing and serves as a powerful tool for conducting high-throughput analyses in this paper.

(2) The Drosophila model used in this article is well established and has already been the subject of publications by the team. In addition to being an in vivo model, Drosophila offers a threefold advantage for this study: i) the presence of an accessory gland, similar to the prostate, which allows for the simulation of tumor formation and, in particular, extrusion mechanisms; ii) its regulation by a single sex steroid, ecdysone; iii) the genetic ability to modulate or inactivate ecdysone expression, which allows for a parallel to be drawn with hormonal deprivation in humans.

(3) This study presents interesting and original findings. The data are, for the most part, of high quality.

Weaknesses:

(1) The Prostate Cancer Atlas, which is an essential tool in this study, was described only briefly - if at all - in both the introduction and the "Materials and Methods" section. The selection criteria used to distinguish CRPC or NEPC from adenocarcinoma in the Atlas or as determined by the authors, as well as the analytical methods, were not specified. It is therefore difficult to be convinced by the results, particularly those presented in Figures 1 and 2.

(2) Although the hypothesis put forward by the authors - that the deprivation of sex hormones contributes to tumor progression - is strongly supported by the Drosophila model and by the in silico analysis of transcriptomic data from the Atlas, this concept still needs to be clinically validated by analyzing a series of prostate cancer samples, either through transcriptomic analysis or by tracking gene expression in histological sections.

(3) With regard to the cells responsible for tumor escape, stem cells have been described as "candidates for the initiating resistant tumor growth" (lanes 50-55), but it is also essential to address the recent concept of "persistent cells". Indeed, these cells have been primarily associated with their tolerance to treatment (chemotherapy) and are referred to as "drug-tolerant cells". However, persistent cells could also correspond to cells that evade hormone therapy in the case of prostate cancer. This possibility should be discussed in the article.

https://doi.org/10.7554/eLife.111674.1.sa2

Reviewer #2 (Public review):

Summary:

In this study, Vialat and collaborators study the role of steroid hormone signalling on the development of prostate cancer (patients) and of accessory gland tumours in Drosophila, a tissue functionally equivalent to the prostate. Mining publicly available prostate cancer expression data and using gene expression signatures, they uncover that androgen signalling is actually down-regulated in castration resistant prostate cancers (CRPC) compared to "primary" cancers, leading the authors to wonder whether down-regulation of canonical androgen signalling could represent an important event increasing tumour aggressiveness. They then take advantage of their recently published tumour model in the accessory gland of Drosophila adult males, in which cells are primed for tumorigenesis by the constitutive activation of the EGFR receptor, to test directly this hypothesis. They show that the genetic invalidation of ecdysone reception and signalling increases the aggressiveness of the "pre-cancerous" lesions, and that ecdysone-insensitive tumours present higher proliferation and initiate basal extrusion.

Strengths:

The authors bring original observations on the role of ecdysone signalling to prevent male accessory gland tumour development in Drosophila

Weaknesses:

(1) The link between the human data mining and Drosophila model is not straightforward.

(2) Important information, in particular clinical information, is missing in the presentation of the cancer patients' data, making it complicated to grasp the solidity of the claims.

(3) Data-mining insights should be validated by orthogonal approaches.

(4) Ecdysone signalling activity should be monitored.

While the two parts of the study both investigate the role of steroid signalling on tumour growth, the link remains slightly artificial. I think starting with Drosophila and then opening with some patient data would be better suited to the level of proof reached here, implying that the anti-tumoral role of steroids observed experimentally in the fly might be conserved based on data mining in patients, rather than trying to prove in the fly the hints gained from public data mining. Indeed, there are many important differences between the mammalian prostate and the fly accessory gland, as well as between sex hormone androgen signalling and developmental timing ecdysone signalling.

The prostate cancer data mining and re-evaluation brings some interesting observations that appear to challenge the androgen driver, contrary to the vast amount of literature. Indeed, the authors observe an apparent decrease in androgen signalling in the more advanced states of the disease, in particular CRPC. In order to better evaluate its clinical relevance, more background on the tumours analysed should be provided.

What treatments were received by the patients? Hormonotherapy? LH/RH analogues? +/- anti-androgens? Are these treatments still given when CRPC emerge and tissues were banked? Metastatic disease? Are these only primary tumours in situ? Are there metastases included in the analyses?

Frequently, castration resistance is associated with alternatively spliced variants of the AR (AR-V7) that become constitutive and could bind to new AR-sensitive enhancers, even in the absence of androgen. Is the splice variant status of patients known, or could it be inferred from the expression data? Would there be different responses according to AR-V7 status?

Regarding the signature used. Why not monitor PSMA, one of the major prostate cancer markers, which is regulated by AR?

Finally, to consolidate the surprising observation that AR signalling is repressed in CRPCs, the authors should back these in silico predictions with orthogonal approaches such as histochemistry on patients' TMA or tissues from mouse models, monitoring AR activity.

Regarding the fly experiments, the observation that ecdysone signalling depletion cooperates with EGFR-lambda activation to generate big overgrowths that delaminate basally without passing through the muscular sheet is interesting. However, several important controls need to be provided in order to support the claims:

a) The authors should use an ecdysone reporter (ERE-LacZ, ERE-GFP...) to monitor and show that Ecdysone signalling is indeed lower in the tumours after genetic manipulations, or that it is higher in EGFR-lambda small clones.

b) EcR is normally a repressor, which is turned into an activator in the presence of 20-hydroxyecdysone. The removal of EcR could lead to de-repression of genes and thus slightly activate the pathway. Monitoring ecdysone signalling activity is thus critical.

c) The authors should also monitor the expression of Phantom, Shadow, Shade, and EcR in the different accessory glands (wild-type, EGFR-lambda, EGFR-lambda & EcR-RNAi). It is extremely surprising that systemic ecdysone has so little role since Phantom, Shadow, or Shade RNAi appear as potent as EcR-RNAi. This quantification has actually been performed for Sad in Figure S5, which is not even mentioned in the text. It should be done for Phtm.

A UAS-yellow-RNAi (or similarly irrelevant RNAi) rather than UAS-GFP should be used as a control for the EcR, Phtm, Sad, Shd, and Tub RNAi. Indeed, loading the RNAi machinery could have some unexpected effects not controlled by the UAS-GFP.

The authors should not use the term "sex steroid" when referring to ecdysone. It is a steroid hormone important for developmental timing and rate of growth, but is not a sex hormone, as sex is cell autonomously genetically determined in the fly.

https://doi.org/10.7554/eLife.111674.1.sa1

Author response:

> ***eLife Assessment***
>
> *This valuable study provides insights into the role of steroid signaling during tumorigenesis in the adult male drosophila accessory gland (functional equivalent of the*

> *prostate gland in mammals), hinting at a possible counterintuitive anti-tumoral role of sex hormones during prostate cancer in certain patients. While the Drosophila model provides an elegant way to study the hypothesis derived from the Cancer Atlas analysis, the analyses of public prostate cancer expression data are incomplete and critical knowledge on patients' treatment modalities and normalization across different datasets is missing. This work would be of interest to prostate cancer researchers as it suggests that the absence of androgen receptor signaling in humans could constitute a mechanism promoting tumor escape.*

We thank the reviewers for the time they have spent on the manuscript, the production of a public review and their useful recommendations. As a general goal for the corrected version, we will try to provide more insight on the data (especially the human data), and more controls, to strengthen our conclusions. We are aware of the lack of a definitive proof of the role of the apparent decrease in AR signaling on tumour progression, but hope that this manuscript will encourage medical scientists to test/challenge its counterintuitive results in large cohorts of tissues and mouse/human models.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *In this article, Vialat and his colleagues examine the early stages - which remain largely unknown - of the tumor escape process, particularly the basal extrusion of tumor cells following endocrine therapy for prostate cancer.*
>
> *They first used the "Prostate Cancer Atlas" database, which provides access to a vast amount of transcriptomic data, to perform high-throughput analyses. Interestingly, analyzing a series of Androgen Receptor (AR) target genes in castration-resistant prostate cancers, they concluded that the loss of the canonical AR signaling pathway may contribute to tumor resistance.*
>
> *Using a well-established model in Drosophila, they then replicated in vivo an endocrine therapy targeting the accessory gland by genetically inhibiting the expression of ecdysone, the only sex steroid present in Drosophila. These experiments induced basal extrusion similar to the mechanism observed in tumor escape in humans.*
>
> *These results suggest that the deprivation of sex steroids may play an important role in tumor progression.*
>
> *However, although the data from the "Prostate Cancer Atlas" constitutes a powerful tool that serves as the basis for this new concept, clinical validation using carefully selected human tumor samples would help strengthen the authors' conclusions.*
>
> *Strengths:*
>
> *(1) The Prostate Cancer Atlas is a comprehensive collection of clinical data derived from RNA sequencing and serves as a powerful tool for conducting high-throughput analyses in this paper.*

> *(2) The Drosophila model used in this article is well established and has already been the subject of publications by the team. In addition to being an in vivo model, Drosophila offers a threefold advantage for this study: i) the presence of an accessory gland, similar to the prostate, which allows for the simulation of tumor formation and, in particular, extrusion mechanisms; ii) its regulation by a single sex steroid, ecdysone; iii) the genetic ability to modulate or inactivate ecdysone expression, which allows for a parallel to be drawn with hormonal deprivation in humans.*
>
> *(3) This study presents interesting and original findings. The data are, for the most part, of high quality.*
>
> *Weaknesses:*
>
> *(1) The Prostate Cancer Atlas, which is an essential tool in this study, was described only briefly - if at all - in both the introduction and the "Materials and Methods" section. The selection criteria used to distinguish CRPC or NEPC from adenocarcinoma in the Atlas or as determined by the authors, as well as the analytical methods, were not specified. It is therefore difficult to be convinced by the results, particularly those presented in Figures 1 and 2.*

As the tool has been published in different articles, we chose to limit its description. However, we agree that explanation are necessary, that will be added in the new version. First, we initially used here just basic categories, in order to avoid any possible bias; so mCRPC includes rare DNPC and NECP patients. We will also put the data with true ARPC, with essentially the same results.

For human data, we also expect to use transcriptomic data from an independent cohort to check whether the same loss of AR signaling occurs during progression. Furthermore, we consider to add the data showing that decrease in canonical AR signaling also (logically with the previous results) correlates with castration status or ADT exposure (these info are available on ProstateCancerAtlas). Interestingly, and this can be put in supplementary data, prostatecanceratlas detects changes in EMT genes or proliferation genes that are coherent with what is known about cancer progression, indicating that the apparent decrease in AR signaling should correspond to a real phenomenon.

> *(2) Although the hypothesis put forward by the authors - that the deprivation of sex hormones contributes to tumor progression - is strongly supported by the Drosophila model and by the in silico analysis of transcriptomic data from the Atlas, this concept still needs to be clinically validated by analyzing a series of prostate cancer samples, either through transcriptomic analysis or by tracking gene expression in histological sections.*

There are many indirect evidences that loss of AR signaling induces tumor progression in mouse (as stated in the intro or the discussion of the manuscript). However, as suggested in the introduction of the letter, we believe that medical scientists are the most qualified to prove that sex steroid deprivation indeed induces tumor progression in human. We will add in any case data to at least reinforce this puzzling finding of a decrease in AR canonical signaling during progression.

> *(3) With regard to the cells responsible for tumor escape, stem cells have been described as "candidates for the initiating resistant tumor growth" (lanes 50-55), but it is also essential to address the recent concept of "persistent cells". Indeed, these cells have been primarily associated with their tolerance to treatment (chemotherapy) and are referred to as "drug-tolerant cells". However, persistent cells could also correspond to cells that evade hormone therapy in the case of prostate cancer. This possibility should be discussed in the article.*

This is an interesting suggestion, which can be discussed: on the one hand, intrabasal cells may not have accumulated mutations to survive the loss of EcR signaling, as would do persistent cells. On the other hand, they strongly proliferate, and show no sign of senescence, behaving more like resistant cells. So, it does not look to us that we induced the appearance of persistent cells in the *Drosophila* accessory gland, except if these cells are quickly reactivating to give rise to intrabasal cells.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *In this study, Vialat and collaborators study the role of steroid hormone signalling on the development of prostate cancer (patients) and of accessory gland tumours in Drosophila, a tissue functionally equivalent to the prostate. Mining publicly available prostate cancer expression data and using gene expression signatures, they uncover that androgen signalling is actually down-regulated in castration resistant prostate cancers (CRPC) compared to "primary" cancers, leading the authors to wonder whether down-regulation of canonical androgen signalling could represent an important event increasing tumour aggressiveness. They then take advantage of their recently published tumour model in the accessory gland of Drosophila adult males, in which cells are primed for tumorigenesis by the constitutive activation of the EGFR receptor, to test directly this hypothesis. They show that the genetic invalidation of ecdysone reception and signalling increases the aggressiveness of the "pre-cancerous" lesions, and that ecdysone-insensitive tumours present higher proliferation and initiate basal extrusion.*
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> *Strengths:*
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> *The authors bring original observations on the role of ecdysone signalling to prevent male accessory gland tumour development in Drosophila*
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> *(3) Data-mining insights should be validated by orthogonal approaches.*
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> *While the two parts of the study both investigate the role of steroid signalling on tumour growth, the link remains slightly artificial. I think starting with Drosophila and then opening with some patient data would be better suited to the level of proof reached here, implying that the anti-tumoral role of steroids observed experimentally in the fly might be conserved based on data mining in patients, rather than trying to prove in the fly the hints gained from public data mining. Indeed, there are many important differences between the mammalian prostate and the fly accessory gland, as well as between sex hormone androgen signalling and developmental timing ecdysone signalling.*

This is an interesting suggestion. Actually, we first wrote the manuscript by starting with *Drosophila* data and then going to patients data, and previous reviewers said that this was not possible to directly go from *Drosophila* to human. So, we suppose that the real way to solve this will be by the validation or refutation of the data by other teams in different models.

> *The prostate cancer data mining and re-evaluation brings some interesting observations that appear to challenge the androgen driver, contrary to the vast amount of literature. Indeed, the authors observe an apparent decrease in androgen signalling in the more advanced states of the disease, in particular CRPC. In order to better evaluate its clinical relevance, more background on the tumours analysed should be provided.*

This point is also important to Reviewer 1, and will be implemented.

> *What treatments were received by the patients? Hormonotherapy? LH/RH analogues? +/- anti-androgens? Are these treatments still given when CRPC emerge and tissues were banked? Metastatic disease? Are these only primary tumours in situ? Are there metastases included in the analyses?*

Most CRPC come from metastatic sites. Most of the CRPC were treated by ADT. We chose to have an approach that included all the samples; but we will provide insight, whenever available, on these absolutely relevant questions.

> *Frequently, castration resistance is associated with alternatively spliced variants of the AR (AR-V7) that become constitutive and could bind to new AR-sensitive enhancers, even in the absence of androgen. Is the splice variant status of patients known, or could it be inferred from the expression data? Would there be different responses according to AR-V7 status?*

As a first approach, from the cohorts that were used, it seems that in the PCA patients, there are around or less than 15% of patients harboring the AR-V7 driver. It could be of interest to test their behavior regarding the same set of genes, and it will be done if we can identify the patients.

> *Regarding the signature used. Why not monitor PSMA, one of the major prostate cancer markers, which is regulated by AR?*

It will be done. PSMA behaves as the others, even though the drop between primary samples and ARPC samples is very limited and just statistically significant.

> *Finally, to consolidate the surprising observation that AR signalling is repressed in CRPCs, the authors should back these in silico predictions with orthogonal approaches such as histochemistry on patients' TMA or tissues from mouse models, monitoring AR activity.*

As said previously, we believe that this specific work will be better done by medical scientists.

> *Regarding the fly experiments, the observation that ecdysone signalling depletion cooperates with EGFR-lambda activation to generate big overgrowths that delaminate basally without passing through the muscular sheet is interesting. However, several important controls need to be provided in order to support the claims:*

Considering the comments regarding the fly experiments, we agree that, if experiences are taken individually, controls are lacking. However, we have to explain our strategy and why the results taken in their entirety have a significance. In our model of epithelial tumorigenesis, we have started to explore the EcR pathway after years of work on other pathways. At the first experiment (with the EcR RNAi line), we were struck by the intrabasal phenotype that did not occurred in our previous experiments, and especially for the 14 RNAi lines that we published in two independent articles on Ras/MAPK, Pi3K/Akt pathways and cholesterol metabolism. As justly said in the review, many unexpected effects can happen, so we decided to explore the role of five other genes of the same pathway to be sure of the reproducibility of the phenotype when we block the EcR pathway. The odds of having the same specific phenotype for 6 lines of the EcR pathway when there is always another phenotype for 14 lines targeting other pathways can be calculated: p = 0.00000494. So, the

best control we offer, and it is largely significant, is the repetition of the experiments intended to downregulate the EcR pathway, that produce the same phenotypes independently of the target.

Furthermore, all lines we used were previously tested, validated and most of the time published in other scientific works. This is essential to us, as one complexity of doing rare clones in an otherwise normal tissue is that decreasing an mRNA in less than 5% of the cells of course difficultly leads to a detectable drop in overall expression in the whole gland. This is also the reason why we always tried pairs of fly lines to block the receptor activity itself (RNAi EcR, RNAi Shd), the receptor's downstream targets (RNAi HR3, RNAi HR4), and the production of ecdysone (RNAi Sad, RNAi Phtm). As we validated RNAi Sad, we can try anyway to validate at least another RNAi of another category. Furthermore, we did use a RNAi White control: it behaves in the same way as the GFP control. We will put the results comparing the two lines in supplementary data.

> *(a) The authors should use an ecdysone reporter (ERE-LacZ, ERE-GFP...) to monitor and show that Ecdysone signalling is indeed lower in the tumours after genetic manipulations, or that it is higher in EGFR-lambda small clones.*

This would be of interest to validate that the 6 lines are behaving in the same way (at least, they give similar phenotypes). However, in the adult accessory gland, ERE activity is largely lower than during development (DOI: 10.1016/j.jinsphys.2011.03.027), and to be able to decrease it, authors had to express notoriously strong dominant-negative EcR-DN. We can try the experiment but are really not persuaded that we will be able to see a drop of activity with only a decrease of expression of the gene. If we can think of another solution that could be more efficient, we will try it as the idea is of course interesting.

> *(b) EcR is normally a repressor, which is turned into an activator in the presence of 20-hydroxyecdysone. The removal of EcR could lead to de-repression of genes and thus slightly activate the pathway. Monitoring ecdysone signalling activity is thus critical.*

Actually, there are different EcR isoforms. EcR-B1 is generally considered as the main activator of the pathway, as EcR-A is a repressor of the pathway. The EcR RNAi line which was used does not target a specific isoform.

> *(c) The authors should also monitor the expression of Phantom, Shadow, Shade, and EcR in the different accessory glands (wild-type, EGFR-lambda, EGFR-lambda & EcR-RNAi). It is extremely surprising that systemic ecdysone has so little role since Phantom, Shadow, or Shade RNAi appear as potent as EcR-RNAi. This quantification has actually been performed for Sad in Figure S5, which is not even mentioned in the text. It should be done for Phtm.*

The levels of ecdysone are tenths of times lower in adult compared to the peaks during embryogenesis or metamorphosis. And one source of production is the epithelial cells of the accessory glands themselves. Considering that EcR is expressed in all the cells of the accessory gland (epithelial cells and muscle cells), it seems plausible that there is only a very little amount of ecdysone that can in fact be available for the other epithelial cells.

> *A UAS-yellow-RNAi (or similarly irrelevant RNAi) rather than UAS-GFP should be used as a control for the EcR, Phtm, Sad, Shd, and Tub RNAi. Indeed, loading the RNAi machinery could have some unexpected effects not controlled by the UAS-GFP.*

The NLSGFP line we used here is the one we already published twice (and we compared it to RNAi lines), and this is the reason why we used this already validated control. However, we tested a RNAi White line, and it behaves in the same way.

> *The authors should not use the term "sex steroid" when referring to ecdysone. It is a steroid hormone important for developmental timing and rate of growth, but is not a sex hormone, as sex is cell autonomously genetically determined in the fly.*

In human, sex hormones control sexual differentiation (up to adult characteristics) and sexual reproduction. In *Drosophila*, ecdysone controls sexual reproduction in both sexes and sexual differentiation at least in the female (DOI: 10.1007/s004270050186). From these results, we do not think that saying it is a sex steroid (not a sex hormone) is ill suited. We intend to precise what we put in this term in the introduction to avoid overinterpretation from our part.

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