Sequential Ras/MAPK and PI3K/AKT/mTOR pathways recruitment drives basal extrusion in the prostate-like gland of Drosophila

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One of the most important but less understood step of epithelial tumourigenesis occurs when cells acquire the ability to leave their epithelial compartment. This phenomenon, described as basal epithelial cell extrusion (basal extrusion), represents the first step of tumour invasion. However, due to lack of adequate in vivo model, implication of emblematic signalling pathways such as Ras/Mitogen-Activated Protein Kinase (MAPK) and phosphoinositide 3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signalling pathways, is scarcely described in this phenomenon. We have developed a unique model of basal extrusion in the Drosophila accessory gland. There, we demonstrate that both Ras/MAPK and PI3K/AKT/mTOR pathways are necessary for basal extrusion. Furthermore, as in prostate cancer, we show that these pathways are co-activated. This occurs through set up of Epi-dermal Growth Factor Receptor (EGFR) and Insulin Receptor (InR) dependent autocrine loops, a phenomenon that, considering human data, could be relevant for prostate cancer.

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In this study, we describe this unique model of basal extrusion and tumour formation in the accessory gland that recapitulates most aspects of cancer development. We demonstrate that both Ras/MAPK and PI3K/Akt/TOR pathways are overactivated in the produced tumours, and that these pathways cooperate to induce basal extrusion and subsequent tumour formation. Furthermore, we describe the mechanism that allows the coactivation of these pathways, which relies on the sequential recruitment of a double autocrine feedback loop dependent on Epidermal (EGF/Spitz) and Insulin-like (IGF/Ih6) Growth Factors and their respective receptors. Finally, using publicly available data of prostate cancer samples and migration assay in human pre-tumoural prostate epithelial cell line, we assess the possible role of these findings in the actual human pathology.

**Results**

**Accessory gland recapitulates prostate microenvironment.** To realise our study, we first determined whether accessory gland represents a good model to study basal epithelial cell extrusion. Prostate compartment is mostly composed of acini of epithelial cells adjacent to a basement membrane and surrounded by a fibromuscular stroma. It is already known that a layer of muscle fibres completely encloses the accessory gland. Phalloïdin staining reveals this well-organised muscle sheet that forms a continuous layer at the surface of the gland (Fig. 1a). Using higher magnification, we further show that contiguous muscle fibres are linked together by actin bridges, a phenomenon that could explain how mostly unchanged distance between the muscle fibres could be conserved in spite of natural contraction of the fibres (Fig. 1b). Furthermore, we show the presence of a basement membrane along the epithelium, where collagen IV (Viking) and Perlecain can be detected (Fig. 1c–f). This basement membrane encloses the muscle layer (arrow in Fig. 1g, h) which must reinforce the stability of the accessory gland. We conclude that indeed, accessory gland recapitulates classical epithelial microenvironment as its epithelial cells are adjacent to a basement membrane and closely linked to a stromal-like compartment of muscle cells.

**Oncogene induces epithelial tumourigenesis.** We then set up experimental conditions to induce epithelial tumourigenesis. Tumourigenesis initiation is thought to start by alteration of one single gene in one single cell. Thanks to genetic tools available in *Drosophila*, we were able to precisely mimic this step by clonal expression of known oncogene RasV12 in a controlled number of cells of the epithelium. Experiments were designed to induce an average of ten clones per gland. Due to time of clonal induction, before last cell division of accessory gland epithelial cells, expression of GFP only (control, Fig. 2a) typically produces clones of two cells that are of same shape and size than non-expressing neighbouring cells (Fig. 2b, c). In contrast, co-expression of GFP and RasV12 was repeatedly correlated to the presence of green masses composed of numerous cells, which clearly appear outside the actual glands. This revealed that RasV12 expression induced clonal epithelial cell hyperproliferation and suggested that some of the clones were able to develop into tumours (Fig. 2d, e).

Then, we verified that these masses are actual tumours of epithelial origin. Importantly, these masses are composed of binucleated cells, a specific feature that confirms that they originate from accessory gland epithelial compartment (Fig. 3a). However, these cells are mostly devoid of cell membrane staining for all the epithelial markers we tested: Fasciclin III, Coracle and E-cadherin (Fig. 3b–g). As Fasciclin III and E-Cadherin are adhesion molecules, their absence suggests that clonal cells lost physical contact with surrounding epithelial cells, a phenomenon
associated with neoplastic growth in human27 and tumour growth in Drosophila28. Loss of epithelial identity also indicates that RasV12 expressing cells are able to initiate epithelium to mesenchyme transition (EMT), which is considered as a key tumourigenic event in the prostate29. Src, a non-receptor kinase, is associated with EMT and cell invasion in cancer30, and notably in metastatic progression of prostate adenocarcinoma31. Its role in tumourigenesis is assessed as well in Drosophila epithelium as in mouse prostate epithelium32,33. Phosphorylated Src is present at the membrane of RasV12 expressing cells, defining them as tumour cells (Fig. 3h). Src activation is also correlated to neo-angiogenesis in cancer35. RasV12 clonal expression is correlated to the presence of overgrown ectopic tracheas (Fig. 3i). These tracheas can be characterised by the use of two different antibodies directed against Gasp and Matrix Metalloprotease 1 (MMP1) (Fig. 3i–l). Gasp, which is implicated in the opening of tracheas during their growth36, is strongly expressed in low diameter tracheas at the surface of tumours (arrow in Fig. 3k), but at weaker level in the larger tracheas (Fig. 3j). This shows that these ectopic tracheas are almost entirely mature, i.e. functional, but also still growing, a phenomenon that does not normally occur in adult flies. MMP1 is detected at high levels in the tracheas (Fig. 3i, l), as in embryo and larva in which it is involved in tracheal development37,38. This MMP1 staining furthermore reveals that an arborecence of tracheas grows inside the tumours from the tracheas that are normally situated at the surface of the accessory glands (arrow in Fig. 3l). This shows that not only tracheas are growing actively, but also in tight coordination with tumours. We conclude that RasV12 clonal expression induces neo-tracheogenesis in adult accessory gland.

Then, we wanted to definitively confirm that we are in the presence of a phenomenon of basal extrusion. First, we noticed that the presence of tumours is linked to defects in the muscle layer, reflecting the probable migration of the clonal epithelial cells through this muscle sheet (arrow in Fig. 4a), especially as tumours frequently stay attached to the normal epithelium (arrow in Fig. 4b). Then, we revealed in the same experiments tumours in red (RFP), basement membrane (viking-GFP in green39) and the stromal compartment of muscle cells (phalloidin staining in magenta) (Fig. 4c–j). Tumours appear outside both the epithelial and the stromal-like compartment of muscle cells (yellow arrows in c–f, i, j). Surface of the tumours display no evident basement membrane (yellow arrows in Fig. 4c–h) and only basement membrane within these masses corresponds to tracheas that grew inside the tumours (white arrows in Fig. 4c–h).

Together, our results demonstrate that expression of a known oncogene in clones of accessory gland epithelial cells induces epithelial cell extrusion and early invasion resulting in the formation of adenocarcinoma-like tumours. Due to the shape and structure of accessory gland, it is also particularly easy to visualise the tumours directly at the dissection, allowing easy quantification of this basal extrusion phenomenon.

Invasive tumours display RTK-dependent pathways coactivation. Our aim was then to determine if accessory glands tumours were able to reproduce in Drosophila this specific feature of prostate adenocarcinoma which is the co-deregulation of Ras/ MAPK and PI3K/AKT/mTOR pathways23,35. To test the activity of the two considered pathways, the phosphorylation of two downstream targets, ERK (Rolled) and 4E-BP, was assessed. In response to RasV12 expression, P-ERK is logically detected in tumours (Fig. 5a) which indicates canonical Ras/MAPK pathway activation. In the tumours, cells display up to 40 times their normal size (see Fig. 3a for membrane staining and Fig. 5c for quantification), showing that their growth is abnormally activated. Correlatively, they display oversized nuclei (Fig. 5d), indicating a probable phenomenon of endoreplication. In Drosophila, cell size is tightly controlled by TOR activity47,40. Indeed, 4E-BP phosphorylation is detected in tumour cells (Fig. 5b), indicating a co-recruitment of the PI3K/AKT/TOR pathway in these cells.

RTK-dependent pathways coactivation drives basal extrusion. We then wanted to determine whether the activated pathways were implicated in the process of invasion itself. So, we quantified invasive tumour frequency for different combinations of activation/inactivation of the two considered pathways. Expression of
ERK RNAi in the RasV12 expressing cells significantly decreases invasive tumour frequency (i.e. clones that left the epithelium compartment to form external masses) (Fig. 6a), showing that RasV12 oncogenic activity depends on the canonical Ras/MAPK pathway. Similarly, expression of PI3K RNAi or TOR RNAi in the RasV12 expressing cells significantly decreases invasive tumour frequency (Fig. 6b, c), showing that RasV12 oncogenic activity depends also on the PI3K/ AKT/TOR pathway in the accessory gland.

PI3K/AKT/TOR pathway can be directly activated either by phosphatase and tensin homolog (pten) deletion or by expression of a myristoylated form of Akt (myr-Akt). The use of one or the other of these genetic modifications does not induce tumour development, but only mild cell hypertrophy is observed (Fig. 6d, e). Furthermore, myr-Akt expressing clones display 4E-BP phosphorylation (Fig. 6f, g), but no ERK phosphorylation (Fig. 6h, i), showing that PI3K/ AKT/TOR overactivation does not induce Ras/MAPK pathway. Thus, it indicates that a failure to co-recruit the two pathways correlates with a lack of basal extrusion in the accessory gland.

From our data, we conclude that both the canonical Ras/ MAPK pathway and the PI3K/ AKT/TOR pathway are necessary for cells to evade the epithelial compartment, and that lack of recruitment of one or the other pathway impairs the basal extrusion and subsequent tumour formation.

**Tumour invasion depends on an EGF/EGFR autocrine loop.**

Then, we decided to determine how the pathways are recruited during tumorigenesis in the accessory gland. Considering that, in prostate cancer, a majority of primary tumours display no activating mutation in the considered pathways, we searched for an alternative mechanism. Classical activation of the PI3K/ AKT/mTOR and the Ras/MAPK pathways is thought to mostly rely on growth factors. Interestingly, RasV12 clones overexpress EGF/Spitz, an EGF/TGFα homolog and ligand of Drosophila EGFR41 (Fig. 7a, b), EGFR is upstream of both Ras/MAPK and PI3K/ AKT/TOR pathways in mammals42, but could be more specific to Ras/MAPK pathway in Drosophila43. Strikingly, suppression of EGF/Spitz by RNAi expression in RasV12 clones strongly reduces invasive tumour frequency (Fig. 7c), showing that expression of this ligand sustains RasV12-driven basal extrusion in the accessory gland. Moreover, if clonal overexpression of a secreted form of EGF/Spitz (Spitzsc)44 in epithelial cells mostly induces a high rate of mortality during pupal stage, the remaining adult flies can display tumours exhibiting the same hallmarks of cell overgrowth and loss of epithelial markers as in RasV12 induced tumours (Fig. 7d). These results agree with the existence of an EGFR-dependent autocrine feedback loop that is necessary for RasV12 oncogenic transformation. Logically, co-expression of an EGFR RNAi in RasV12 clones strongly decreases invasive properties of developed tumour (Fig. 7e), confirming the role of EGFR in RasV12-driven tumourigenesis. Furthermore, expression of a constitutively active form of EGFR, EGFRα, leads to the formation of tumours that display the same hallmarks as in RasV12 induced tumours (Fig. 7f), showing that overactivated EGFR can induce basal extrusion of epithelial cell in the accessory gland. Finally, co-expression of a Ras RNAi in EGFRα clones strongly decreases their invasive properties (Fig. 7g), confirming the role of Ras/MAPK signalling pathway in the EGFR-dependent tumourigenic process. Interestingly, observation of epithelial clones co-expressing EGFR RNAi and RasV12 reveals that they can be composed of many cells (observe the extension of the clone in Fig. 8a), suggesting that EGFR recruitment is less required for cell division than for cell extrusion.

We conclude that upon RasV12 dependent initial activation, Ras/MAPK pathway must be re-activated by an EGF/EGFR autocrine loop to induce cell invasive capacities, definitively showing that Ras/MAPK pathway is necessary for basal extrusion independently of the mechanism of oncogenic initiation.
Tumour invasion depends on an IGF/InR autocrine loop. We then wanted to determine how PI3K/AKT/TOR pathway is recruited. At first, we observed that P-4E-BP staining is never detected in RasV12/EGFR RNAi clones, and cells stay smaller in this condition compared to RasV12 expressing cells (Fig. 8a, b), indicating a lack of recruitment of the PI3K/AKT/TOR pathway in these clones and suggesting that EGFR could be implicated in the recruitment of this pathway. However, we also observed that clonal expression of EGFR\textsubscript{λ} induces PI3K/AKT/TOR pathway recruitment, as for RasV12 expression, but this recruitment and associated cellular hypertrophy occurs only in external cell masses (Fig. 8c, d). This suggests that PI3K/AKT/TOR pathway is not recruited directly by EGFR, but via a molecular intermediate. In Drosophila, PI3K/AKT/TOR pathway is mainly recruited by the Insulin Receptor (InR), and seven ligands are known to activate InR. We then searched for their expression. Both RasV12 and EGFR\textsubscript{λ} clonal expression in the accessory gland induces mRNA overexpression of one ligand, IGF/Ilp6 (left columns in Fig. 8e, f). Indeed, we decided to determine the potential role of IGF/Ilp6 overexpression in Ras\textsuperscript{V12}-driven tumourigenesis. Strikingly, downregulation of IGF/Ilp6 (Ilp6 RNAi) in Ras\textsuperscript{V12} clones significantly decreases invasive tumour frequency (Fig. 8g). Logically, co-expression of a dominant-negative form of InR (InR\textsubscript{dn}) in Ras\textsuperscript{V12} clones also decreases invasive tumour frequency (Fig. 8h), showing that this receptor is also implicated in Ras\textsuperscript{V12} oncogenic activity, and then defining a second autocrine loop that participates to accessory gland invasive tumour formation.

Next, we decided to precise which molecular actors are necessary for this IGF/Ilp6 overexpression. First, we co-expressed Ras RNAi with EGFR\textsubscript{λ}. It completely abolishes IGF/Ilp6 mRNA overexpression compared to EGFR\textsubscript{λ} expression alone, demonstrating that IGF/Ilp6 overexpression depends also on ERK activation (right column in Fig. 8e). We conclude that IGF/Ilp6 mRNA overexpression exclusively depends on the activation of the classical EGFR/Ras/MAPK pathway.

EGF is specifically overexpressed in primary prostate cancer. Finally, to determine whether our findings could be relevant to prostate cancer, we decided to examine EGF and IGFs expression in human samples. We selected data where mRNA levels were available in normal, primary and metastatic samples. In Ras\textsuperscript{V12} clones also decreases invasive tumour frequency (Fig. 8h), showing that this receptor is also implicated in Ras\textsuperscript{V12} oncogenic activity, and then defining a second autocrine loop that participates to accessory gland invasive tumour formation.
determined whether these mRNA could reflect alterations that are hallmarks of tumour progression. Typically, advanced prostate cancers are associated to loss of PTEN expression. In the used cohort, PTEN mRNA expression tends to decrease in primary tumours when compared to normal tissue \((p = 0.07)\) and significantly decreases in metastatic samples compared to normal tissue \((p = 0.00047)\) and primary tumours \((p = 0.002)\) (Fig. 9a).

Thus, PTEN expression is, as expected, inversely correlated with tumour grade. We then considered the expression of EGF, IGF1 and IGF2 (Fig. 9b–d). EGF mRNA expression presents a bell-shaped pattern (Fig. 9b): it is significantly increased in primary tumours compared to both normal \(p = 2 \times 10^{-5}\) and metastatic \(p = 0.0089\) tissues which are expressing very similar levels of EGF \(p = 0.92\). Furthermore, this expression increases even in primary tumours with the lowest available Gleason score \(p = 0.0012\). In contrast, IGF1 expression does not significantly change between the considered conditions (Fig. 9c), and IGF2 expression tends to decrease in primary tumours compared to normal tissue \(p = 0.05\) and significantly increases in metastatic samples compared to primary tumours \(p = 0.0036\) (Fig. 9d). Together, these results indicate that EGF mRNA is specifically overexpressed during early carcinogenesis, suggesting that EGF could play a role in this particular stage of cancer.

Then, we decided to check the role of the EGFR signalling pathway in P69, a cell line derived from immortalisation of human normal prostate epithelial cells. Using transwell chamber assay, we show that supraphysiological doses of EGF, or lower doses in a context of EGFR overexpression, significantly increase P69 cells migration (Fig. 9e). Furthermore, expression of

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**Fig. 4 Oncogene expression induces basal epithelial cell extrusion in the accessory gland.** a, b GFP-Ras\(^{V12}\) and (c–f) RFP-Ras\(^{V12}\) expressing clones are able to migrate from the epithelial compartment. a Alexa633-phalloidin (Phalloidin) staining reveals disorganisation of the muscle fibres at the site of extrusion of GFP-Ras\(^{V12}\) expressing clones (white arrow). b Zoomed image of (a) reveals that the tumour is still anchored to the epithelium (white arrow). c, j Alexa633-phalloidin (Phalloidin) staining reveals the muscle fibres (c–f, i, j) as Viking-GFP staining reveals basement membranes (c–h). (d, f, h, j): optical cross-sections of top panels respectively. c, e, g, i). Staining reveals that RFP-Ras\(^{V12}\) tumour develops outside the epithelial compartment, as it is not enclosed by a basement membrane or a muscle layer (yellow arrows). Basement membrane staining reveals trabecular branches inside the tumour (white arrows). DAPI (blue) reveals nuclei in (a–j). Representative images from two experiments. Scale bars: 50 μm.

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**Fig. 5 Ras/MAPK and PI3K/AKT/TOR pathways are concomitantly activated in Ras\(^{V12}\) expressing tumours.** GFP-Ras\(^{V12}\) expressing tumours are encircled by dotted lines. Fasciclin III (FasIII) staining reveals normal epithelial cells. a GFP-Ras\(^{V12}\) expressing tumours display phosphorylation of ERK, a downstream target of the Ras/MAPK pathway. b GFP-Ras\(^{V12}\) expressing tumours display phosphorylation of 4E-BP, a downstream target of the PI3K/AKT/mTOR pathway. c, d Comparison of cell (c) and nuclei (d) volumes of tumour cells with wild-type cells (WT) (** Unpaired t test; \(p < 0.0001\)) reveals strong hypertrophy of GFP-Ras\(^{V12}\) expressing cells (Ras\(^{V12}\)). Data are represented as mean values±SEM (cell volume: \(n = 12\) and \(n = 14\) accessory glands Control and Ras\(^{V12}\) analysed respectively, from seven experiments; nuclei volume: \(n = 11\) and \(n = 14\) accessory glands Control and Ras\(^{V12}\) analysed respectively, from seven experiments). Representative images in (a, b) from three or more experiments. DAPI (blue) reveals nuclei in (a, b). Scale bars: 50 μm.
a constitutively active form of EGFR (EGFR-L858R48) induces similar migration of P69 cells in the absence of EGF, and this migration is impaired by treatment with specific inhibitors of either KRAS or PI3K (Fig. 9f). We conclude that, as demonstrated in Drosophila, migration of human pre-tumoural prostate epithelial cells depends on EGFR signalling and downstream activation of both Ras/MAPK and PI3K/Akt/mTOR pathways.

So, collectively, our data show that accessory gland tumourigenesis, and more precisely basal extrusion, relies on a double autocrine loop: the first one depends on EGF/Spitz and its receptor EGFR to amplify Ras/MAPK pathway activation; the second one depends on Ras/MAPK-dependent production of IGF/IIP6 to recruit the PI3K/AKT/TOR pathway via InR activation. And this coactivation is necessary in a cell-autonomous manner for the epithelial cells to be able to leave the epithelial compartment to form adenocarcinoma-like tumours (Fig. 10).

**Discussion**

More than 95% of prostate cancers are of epithelial origin and are thought to mainly arise from a single cell. To faithfully reproduce what is thought to happen in the earliest stages of tumour formation in patients, we have decided to produce a single genetic alteration in few clones of randomly selected and mostly
differentiated cells. Furthermore, we show that accessory gland epithelium is adjacent to a basement membrane, is surrounded by a stromal-like sheet of muscle fibres, and that oncogene-induced epithelial cells are able to cross both layers to form external tumours (Figs. 1 and 4). This recapitulates the phenomenon of basal epithelial cell extrusion, which is thought to be central to cell invasion\textsuperscript{11,50}. Basal extrusion has been described in cell culture\textsuperscript{50}, in \textit{Drosophila} imaginal disc\textsuperscript{51}, in zebrafish embryo\textsuperscript{13} and once in mouse\textsuperscript{14}. However, to the best of our knowledge, implication of Ras/MAPK and PI3K/AKT/mTOR pathways has never been assessed in this phenomenon, despite the fact that these pathways are among the most deregulated in cancers\textsuperscript{52,53}, and especially in epithelial cancers\textsuperscript{54–57} such as prostate adenocarcinoma\textsuperscript{58}. Here, we show in a new model of accessory gland tumourigenesis that both pathways are implicated in basal extrusion, indicating that this step demands a particular state of activation for the cell that undergoes this basal extrusion. Furthermore, this finding correlates with the fact that the two considered pathways are already frequently co-deregulated in

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\caption{Ras\textsuperscript{V12}-driven tumour formation depends on the setting of an EGF/EGFR autocrine activation loop. a, b Immunostaining reveals that EGF/Spitz (Spitz) is expressed in GFP-Ras\textsuperscript{V12} expressing clones. c Invasive tumour frequency of GFP-Ras\textsuperscript{V12} expressing clones (Ras\textsuperscript{V12}) is impaired by downregulation of EGF/Spitz (Spitz RNAi) (Chi\textsuperscript{2} test; \( P < 0.0001 \)). d Clonal expression of a secreted form of EGF/Spitz (Spitz) leads to tumour formation. e Invasive tumour frequency of GFP-Ras\textsuperscript{V12} expressing clones (Ras\textsuperscript{V12}) is impaired by downregulation of EGFR (EGFR RNAi) (Chi\textsuperscript{2} test; \( P < 0.0001 \)). f Clonal expression of a constitutively active form of EGFR (EGFR\textsubscript{\lambda}) induces the formation of tumours. g Invasive tumour frequency of GFP-EGFR\textsubscript{\lambda} expressing clones (EGFR\textsubscript{\lambda}) is strongly impaired by downregulation of Ras (Ras RNAi) (Chi\textsuperscript{2} test; \( P < 0.0001 \)). Data are represented as mean values±SEM. Representative images in (a, b, d, f) from three or more experiments. ****\( P < 0.0001 \). Scale bars: 50 \( \mu \text{m} \).
\end{figure}
primary tumours. From our experiments, where oncogene expression is restricted to few cells and intra-tumoural inhibition of the pathways decreases invasion, we can infer that the mechanisms of basal cell extrusion are cell autonomous, as previously shown in cell lines. Indeed, we show that this cell-autonomous mechanism relies on the production of two growth factors, and subsequent activation of two autocrine loops (Fig. 10). Role of autocrine loops has been hypothesized in late tumourigenesis, as higher levels of growth factors have been found in tumoural tissues, and has been studied in cell models where inhibition of these loops decreases tumourigenic features such as migration or proliferation capacity as their activation have been linked to transformation of various epithelial cells (reviewed in ref.). However, to the best of our knowledge, role of autocrine loops has never been demonstrated for basal extrusion in vivo. If these loops seem implicated in tumour late progression, so could they be more important for early human tumour development. In fact, many strategies have been attempted to treat cancer patients especially by blocking EGFR/EGFR autocrine loop. However, for advanced prostate cancer, these strategies have shown poor results, as well for monotherapies as for combined treatments with classical anti-prostate cancer agents. It could be logical if autocrine loops are less implicated in late stages of cancer but more in the capacity for tumour cells to leave the epithelial compartment. In later stages, higher rates of activating mutations in the Ras/MAPK and PI3K/AKT/mTOR pathways could suppress the need for RTK-driven activation. In contrast, in early tumourigenesis, as fewer genetic alterations are present, activation of signalling pathways must rely on different mechanisms. As we show in the accessory gland, this recruitment could be efficiently done in tumour cells by autocrine production of growth factors, autocrine activation of their RTK and subsequent activation of the pathways necessary for the tumour development. In a human cohort of prostate cancer samples, we found that EGF is more expressed in primary tumours than either in normal tissue or in metastases. This could correlate with an early requirement for such growth factor in the formation of adenocarcinoma. Contrary to our observations in Drosophila, no early overexpression of IGFs can be detected in human samples. However, in human, EGF is able to recruit both Ras/MAPK and PI3K/AKT/mTOR pathway. The role of autocrine loops is well studied in human samples. However, in human, EGFR is able to recruit both Ras/MAPK and PI3K/AKT/mTOR pathway. The role of autocrine loops is well studied in human samples. However, in human, EGFR is able to recruit both Ras/MAPK and PI3K/AKT/mTOR pathway. The role of autocrine loops is well studied in human samples. However, in human, EGFR is able to recruit both Ras/MAPK and PI3K/AKT/mTOR pathway. The role of autocrine loops is well studied in human samples. However, in human, EGFR is able to recruit both Ras/MAPK and PI3K/AKT/mTOR pathway.
and EGF overexpression could drive their activation and act in the same way as EGF/Spitz and IGF/Ipf in Drosophila.

To study early phases of tumourigenesis remains difficult in vivo, especially for epithelial cells that can develop into benign tumours still in the epithelial compartment such as benign prostatic intraepithelial neoplasia, or into adenocarcinoma that are characterised by an expansion out of the epithelial compartment. The model we developed in the Drosophila accessory gland represents a unique in vivo model to explore basal extrusion and early invasion. We were able to show that two major pathways of cancer progression are implicated in this basal extrusion, and to demonstrate that these two pathways are co-recruited by autocrine loops (Fig. 10). Further investigation will be necessary to test whether other pathways implicated in late tumourigenesis are important in this phenomenon. Furthermore, it will also be important to determine which genes are activated or inhibited by these pathways and which mechanisms are recruited to promote the actual extrusion.

Methods
Fly stocks and experimental crosses. y,w,HSfly122/+;Act:FRStopFRTag14, UAS:GFP-CyO flies allowed conditional clonal expression of GFP. When combined with UAS:RasV12 (4847) or UAS:EGfRTaptor (59894), they allowed conditional clonal co-expression of Rap and RasV12 (GFP-RasV12/ flies) or EgrfTaptor (GFP-Egrf flies). GFP-RasV12 and GFP-Egrf flies were then crossed with following stocks to realise experiments: UAS-GFP.p (4775), UAS-TorRNAi (35578), UAS-SprRNAi (34645), UAS-Pi3KRNAi (61182), UAS-MyrTomato (32221), UAS-RolledRNAi (34855), UAS-EGfRTaptor (59894), UAS-RasRNAi (106642) UAS-Indk4voA8 DN (8252), UAS-IpfRNAi (33684), UAS-Spr (58436) from the Bloomington Stock Center and UAS-EgrfRNAi (43267) from Vienna Drosophila Resource Center. y,w, HSfly122/+;Act:FRStopFRTag14, UAS:GFP-CyO/UAS-myr-AKT/TM3Sb were also used to induce AKT/PTOR pathway activation (GFP-myr-AKT flies). Other stocks used: HSfly122/+;FRT64A,Ptenfl/TM6B, Ubi:nlsRFP.

Conditional expression induction. Briefly, condition of use and act of the flipper (flip)/ FRT system was determined to produce an average of 4–6 clones per accessory gland (% of total number of epithelial cells). Flipper-dependent recombination was induced during pupal stage by 12 min (for GFP-RasV12 and GFP-EGFRA flies) or 20 min (for GFP-MyrAkt flies) heat-shock at 37 °C. Flies were then kept at 25 °C until the end of pupal stage. Males were collected at emergence from pupae 3 to 5 days after heat-shock and kept for another 3 days at 25 °C before dissection.

Immunohistochemistry and imaging. Accessory glands were dissected in 1X PBS or 1X PBS containing phosphate inhibitors (orthovanadate 1 mM, β-glycerophosphate 20 mM and NaF 1 mM) for the detection of phosphorylated proteins. They were fixed for 10 min in 4% paraformaldehyde, washed once with 1X PBS and three times with 1X PBS containing 0.2% Triton (PBS-T) for permeabilization. The samples were blocked for 10 min with 0.5% of bovine serum albumin in PBS-T and incubated overnight at 4 °C in primary antibodies diluted in the same blocking solution. The tissues were then washed twice for 5 min with PBS-T and incubated for 1 h at room temperature in secondary antibodies diluted 1:1000 in blocking solution. Added with secondary antibodies, DAPI (D-Amino-phenylindol, D8417, Sigma) 1:1000 was used to stain DNA and 1:5000 Alexa568-phallolidin (A12380, Life Technology) or Alexa635-phalloidin (A22284, Life Technology) were used to reveal F-actin. Following two washes in PBS-T, the samples were then mounted in Vectorshield (+ 1,000, Vector Laboratories) and visualised using a Leica SP5 or SP8 confocal microscope. Image stacks were processed in ImageJ or Imaris software.

We used the following antibodies: Mouse Coracle (1:300, #CS666 DHSB), mouse Fascleratin IIII (1:400, #7610 DSCH), rat E-Cadherin (1:1000, #14672 DSCH), rabbit P-E-P (1:200, 28555 Cell Signalling), rabbit P-ERK (1:500, 43705 Cell Signalling), goat GFP (1:1000, #5450 Abcam), mouse GASP (1/5, 2A12 DSHB), mouse MPM1 (1/100, 14A3D2 DSHB; 1:100 #36AE4 #3BBD1 #5HB71 DSHB), rat Spitz (1:1000, DSCH), rabbit P-Src (1:500, #44-660G Invitrogen), secondary antibodies coupled to different fluorophores 488 (1:1000, A11022 Invitrogen), Cy3 or Cy5 (1:1000, 711-165-152, 711-175-152, 712-165-153, 712-175-153, 715-165-150, 715-165-151, 715-175-150, 715-175-151, Jackson Immunology).

RNA extraction, cDNA synthesis and Real-time Quantitative PCR. RNAs were isolated from accessory glands dissection, by Trizol-extracted total accessory glands (Invitrogen). Reverse transcription was performed by using SuperScript IV Reverse Transcriptase kit (ThermoFisher Scientific). Quantitative PCR was performed using the LC480. rp32 was used for the normalisation.

Primers for qRT-PCR analysis were. Dilp65: forward: 5′-TGCCTAGCTTGGGACGTGTCGTG-3′; reverse: 5′-GGAAAATACATCGCCAAAGGCCGACCAC-3′
Rp32: forward: 5′-TTGGCTCTGTTGTCGCGCA-GACG-3′; reverse: 5′-ATCGA TTCGACGTGGCCGGAT-3′

Cells and nuclei size. Nuclear and nucleus volume were determined from 3D reconstruction with Imaris software. Cells area was determined with direct measure of contour cells in Image J software.

Invasive tumour frequency. During dissection, direct observation under binocular microscope allowed to visualise the presence of tumours at the surface of the accessory glands. Tumour frequency was determined as the percentage of flies that displayed at least one tumour on their accessory glands. Following preliminary tests, it is important to note that we designed the experiments to obtain a similar number of UAS in all the flies tested to avoid C4H titration effect; to avoid a bias in clonal induction, for each repeat of each experiment, all procedures were done in the same time for all the genotypes compared in the experiment (as visualised by identical number of dots for each condition in the different panels).

Cell culture, transfection and migration assay. Pre-tumoral human prostate epithelial cells (P69) were cultured in RPMI-1640 medium (Invitrogen, USA) with penicillin/streptomycin (100 mg/ml) (Invitrogen, USA), l-glutamine (2 mM) (Invitrogen, USA) and supplemented with 10% of foetal bovine serum (Eurobio, France). All cells were grown at 37 °C in a humidified chamber with 5% CO2.

Transient plasmid DNA incorporation was performed on P69 cells. pGFP (supercoiled, pEGFPR and pEGFP-LB5) vectors were obtained from Addgene and transfection were performed with JetPEI® transfection reagent (Polyplus transfection, France), according to manufacturer’s protocol. After transfection and serum starvation overnight, cell migration ability was challenged in transwell chambers of 8-mm pores (Corning, USA), followed by addition in the lower chamber of recombinant human-EGF (Bisco, USA) at 1 or 50 ng/ml in serum-free RPM1 medium or in some experiments in the upper chamber with P3K inhibitor LY294002 (2 µM) (Cell signalling technology) or KRAS inhibitor SAH-SOS1A (10 µM) (EMD Millipore, USA) in serum-free RPM1 medium.

RNA-seq data. We retrieved processed RNA-seq data from the website http://chibio.mskcc.org/cancergenomics/prostate/data/.

We only considered already treated and normalised log2 expression data.

Violin Plots. Violin plot were made using R package ggplot2” applying “geom-violin()” function. “geomboxplot()” function was used to add boxplot inside violin.

Statistical analyses. All experiments were done a minimum of four times (independent experiments, values represented as dots in the graphs) on numerous glands, or cells for Figs. 2b and 5c, d (total numbers indicated in the figures as n). Statistical analyses (n = 4 or more) were performed using GraphPad Prism 5 or 6. Data are shown as means±SEM (±SD for Fig. 9f–g). For cells and nuclei volumes the two-way Mann–Whitney test (for temporal Gaussian data) or the two-sided unpaired t test (for Gaussian data). For the invasive tumour frequency, Chi² test was used. Unpaired t test was used to compare human mRNA levels and Two-
talled Mann–Whitney test was used for Drosophila mRNA levels. P69 migration tests were analysed with unpaired t test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The human prostate tissues data referenced during the study are available in a public repository from the http://cbio.mskcc.org/cancergenomics/prostate/data/ website. Raw data corresponding to nucleus and cell size, tumour counts, qPCR, human mRNA, eukaryotic cell migration are available as source data. All the other data (imaging) supporting the findings of this study are available within the article from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file.

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Author contributions
The project was conceived by A.R., C.L.C., C.B., I.M. and C.J. and developed by A.R., C.L.C., C.B., V.M., A.T., J.M.A.L., S.B., I.M. and C.J. A.R. and C.J contributed most Figs., with contributions of C.L.C. for Figs. 1-3, 5-6 and 8, of J.B. for Fig 9e-f, of M.V. for Fig 4c-i, of Y.R. for Fig 9a-d. A.R. and C.J. wrote and prepared the paper. C.L.C., C.B., J.B., V.M., A.T., J.M.A.L., S.B. and I.M. edited the paper.

Competing interests
The authors declare no competing interests.

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