Cholesterol Dietary Intake and Tumour Cell Metabolism Drive Early Epithelial Tumorigenesis

Marine VIALAT , Elissa BAABDATY , Amalia TROUSSON , Ayhan KOCER , Jean-Marc A. LOBACCARO , Silvère BARON , Laurent MOREL , Cyrille de JOUSSINEAU *

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Article

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Marine Vialat [1,2], Elissa Baabdaty [1,2], Amalia Trousson [1,2], Ayhan Kocer [1,2], Jean-Marc Lobaccaro [1,2], Silvère Baron [1,2] and Laurent Morel [1,2] and Cyrille de Joussineau [1,2,*]

[1] Université Clermont Auvergne, CNRS UMR6293, Inserm U1103, GReD, 28 Place Henri Dunant, BP38, F63001 Clermont-Ferrand, France
[2] Groupe Cancer Clermont Auvergne
* Correspondence: cyrille.de_joussineau@uca.fr.

Simple Summary: Cholesterol and cholesterol derivatives accumulate in prostate cancer cells. Studies of the impact of diet and data coming from patients treated with cholesterol lowering drugs indicate that this metabolite plays a role in cancer progression, but do not discriminate its possible role in cancer incidence. The goal of this study is so to determinate whether cholesterol availability impacts tumor formation. Using a drosophila model specifically dedicated to the study of the early epithelial tumorigenesis, we find that basal extrusion, a critical step of tumor formation, directly depends on cholesterol availability through dietary intake and on cholesterol metabolization by the tumor cells. As we find that many genes related to cholesterol metabolism are ill-expressed in primary prostate cancer samples, this work indicates that cholesterol levels and metabolism could play a crucial role in early phases of prostate cancer as well.

Abstract: Epidemiological studies point cholesterol as a possible key factor for both prostate cancer incidence and progression. So, it could represent a targetable metabolite as the most aggressive tumors also appear to be sensitive to therapies designed to decrease hypercholesterolemia, such as statins. However, whether and how cholesterol, through its dietary uptake and its metabolism, could be important for early tumorigenesis, remains unknown. Oncogene clonal induction in *Drosophila melanogaster* accessory gland allows to reproduce tumorigenesis from initiation to early progression, where tumour cells undergo basal extrusion to form extra-epithelial tumors. Here we show that these tumors accumulate lipids, and especially esterified cholesterol, as in human late carcinogenesis. Interestingly, high cholesterol diet displays limited effect on accessory gland tumorigenesis. On the contrary, cell-specific downregulation of cholesterol uptake, intracellular transport or metabolic response, impairs the formation of such tumors. Furthermore, in this context, high cholesterol diet suppresses this impairment. Taken together, these results reveal that during early tumorigenesis, tumour cells strongly increase their uptake and use of dietary cholesterol to specifically promote the step of basal extrusion. Interestingly, expression data from primary prostate cancer tissues indicate an early signature of redirection from cholesterol *de novo* synthesis to uptake. Altogether, these results suggest by which mechanism reduction of dietary cholesterol could lower the risk and slow down the progression of prostate cancer.

Keywords: prostate cancer; cholesterol; early tumorigenesis; basal extrusion

1. Introduction

In the last decades, prostate cancer (PCa) incidence has reached the first rank of men cancers. Within the various factors involved, dietary fat and especially cholesterol has been pointed out as a potential modulator of prostate cancer risk and development [1,2].

In men, the association between cholesterol levels and aggressiveness has largely been reported [3–7] along with the correlation between cholesterol-lowering drugs, i.e. statins, and a decreased

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prostate cancer risk [7–11]. However, conflicting studies and meta-analyses have indicated that there is no clear association between plasma levels of total or fractions of cholesterol, and PCa, [5,12–14]. In the same way, the protective role of statins have recently been debated in regard to cancer progression [15,16], questioning the reality of the connection between plasma cholesterol levels and PCa. Nonetheless, cholesterol metabolism is indeed specifically altered in prostate tumors [17] and, in many preclinical models, cholesterol has been linked to prostate cancer progression notably *via* its role as lipid raft component promoting akt and erk signal transduction [18–21]. There, activation of cholesterol master regulators Liver X Receptors (LXRs) depletes cholesterol from lipid rafts and subsequently induces cancer cell apoptosis [22]. Furthermore, if high-cholesterol diet does not alter murine prostate architecture by itself, the knockout of LXRs in this context induces prostatic intra-epithelial neoplasia [23], suggesting that loss of cholesterol homeostasis could also pave the way for prostate cancer development in case of excessive cholesterol uptake.

We have previously developed an *in vivo* model of epithelial tumorigenesis in the accessory gland of drosophila melanogaster covering the steps from initiation to early progression characterized by tumour formation [24]. Indeed, drosophila represents a strong model for decrypting epithelial cancer-related mechanisms, for example for lung or colon [25,26], and accessory gland itself represents a functional equivalent of a prostatic acinus [27–31]. Furthermore, there is a strong conservation of cholesterol role and metabolism in this insect [32–34]. Also, in mammals, cholesterol is supplied through diet and *de novo* synthesis [35], possibly masking the direct impact of dietary cholesterol on tumorigenesis. Crucially, the cholesterol auxotrophy of the fruit fly makes it only dependent of dietary intake of this metabolite [36]. Together, this renders the drosophila accessory gland a particularly relevant model to assess whether and how cholesterol could play on the early steps of epithelial tumorigenesis.

Herein, we show that, in the accessory gland, oncogene-induced tumours accumulate cholesterol into lipid droplets as in human late carcinogenesis [37]. In this condition, forcing further the activation of cholesterol metabolism through genetic or dietetic approaches has no effect. On the contrary, tumour cell-specific downregulation of cholesterol metabolism decreases the critical step of basal extrusion, limiting the formation of tumours, in a cholesterol dose-dependent manner. Finally, using a cohort of adenocarcinoma, we show that a profound deregulation of cholesterol metabolism occurs already in primary prostate cancer, suggesting that in human also, this deregulation could be important for the early steps of tumorigenesis.

2. Materials and Methods

2.1. 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) . GFP-Egfrλ flies were then crossed with following stocks to realize experiments: UAS-GFP.nls (#4775, control condition, designed in figures as EGFRλ), UAS-LRP1 RNAi (#31151), UAS-LpR2 RNAi (#54461), UAS-Npc1a RNAi (#37504), UAS-Npc1b RNAi (#38296), UAS-CG8112 RNAi (#63035) and UAS-miR DHR96 (#27992, designed as UAS-DHR96 RNAi in figures) from the Bloomington Stock Center.

2.2. 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 2.5 days at 27°C before dissection.

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2.3. High Cholesterol Diet

After the cross, flies were either raised on standard-diet or high-cholesterol-diet (HCD) complemented with 0.2% cholesterol (#3045, Sigma-Aldrich). These diet conditions were also conserved after males' collection at the emergence from pupae.

2.4. Immunohistochemistry and Imaging

Accessory glands were dissected in PBS, fixed for 10 min in 4% formaldehyde, washed and permeabilized for 15 min in PBS containing 0.2% Triton (PBS-T). Glands were blocked for 1 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 h 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 three times with PBS and subsequently mounted in Vectashield (#-1000, Vector Laboratories). Imaging was realised on Leica SP8 confocal microscope, and image stacks were processed either in ImageJ or Imaris softwares.

List of antibodies: Mouse Coracle (1:400, #C566.9 DSHB), NileRed (1ng/mL, Sigma-Aldrich, Saint-Louis, USA), Bodipy (2ng/mL, #D3835 Invitrogen), 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).

2.5. Clones, Cells and Nuclei Size

Clones/tumours, cells and nuclei volumes 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.

2.6. Invasive Tumour Frequency

Tumour frequency was determined as the percentage of flies that displayed at least one tumour on their accessory glands at dissection.

2.7. Statistical Analyses

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 tumour size and nuclei number quantification). Statistical analyses were performed using GraphPad Prism 6: for tumour volume, nuclei number and droplets volume, quantification were compared by Krustal-Wallis test as % of glands with extra-glandular tumours were compared by Chi2 test.

3. Results

3.1. Accessory Gland Tumors Accumulate Cholesterol into Lipid Droplets

Accessory gland represents a perfectly defined epithelial compartment comparable to a single prostatic acinus. It is composed of a monolayer of secretory epithelial cells surrounded by a basement membrane in which a well-organized network of muscle fibers is completely enclosed (revealed by phalloidin staining in Figure 1A,D, yellow). Here, in order to mimic tumour initiation, clonal expression of constitutively active version of EGFR (EGFRλ condition) coupled to GFP was realized in approximatively 1% of the accessory gland cells. As previously described [24], this expression leads to the formation of two kinds of GFP-positive tumour cells (Figure 1). First, epithelial clones composed of slightly hypertrophic cells whose cytoskeleton are disorganized (Figure 1. white arrowheads) and second, GFP-positive tumours that grow outside the epithelial compartment consequently of a phenomenon of basal extrusion (Figure 1. empty arrowheads). These tumors,

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which represent a state of early progression, are composed of cells that bare hallmarks of cancer, such as hypertrophy, hyperplasia, the loss of epithelial markers, and tumours are furthermore associated to neotracheogenesis (a neoangiogensis equivalent) [24]. In this context, we wondered whether this parallel could be extended to abnormal lipid storage. Indeed, we observe by classical Nile Red staining the accumulation of intracellular neutral lipid droplets specifically in tumour cells (Figure 1A,B: magenta; D: Nile Red staining only, grey). To further evaluate the content of these droplets, we used Bodipy, a marker of esterified cholesterol that proves the presence of stored cholesterol into these droplets (Figure 1E,F: magenta; H: Bodipy staining only, grey). So, we concluded that, as in human, formation of tumoursduring progression is accompanied by lipid, and especially esterified cholesterol, accumulation into the tumour cells.

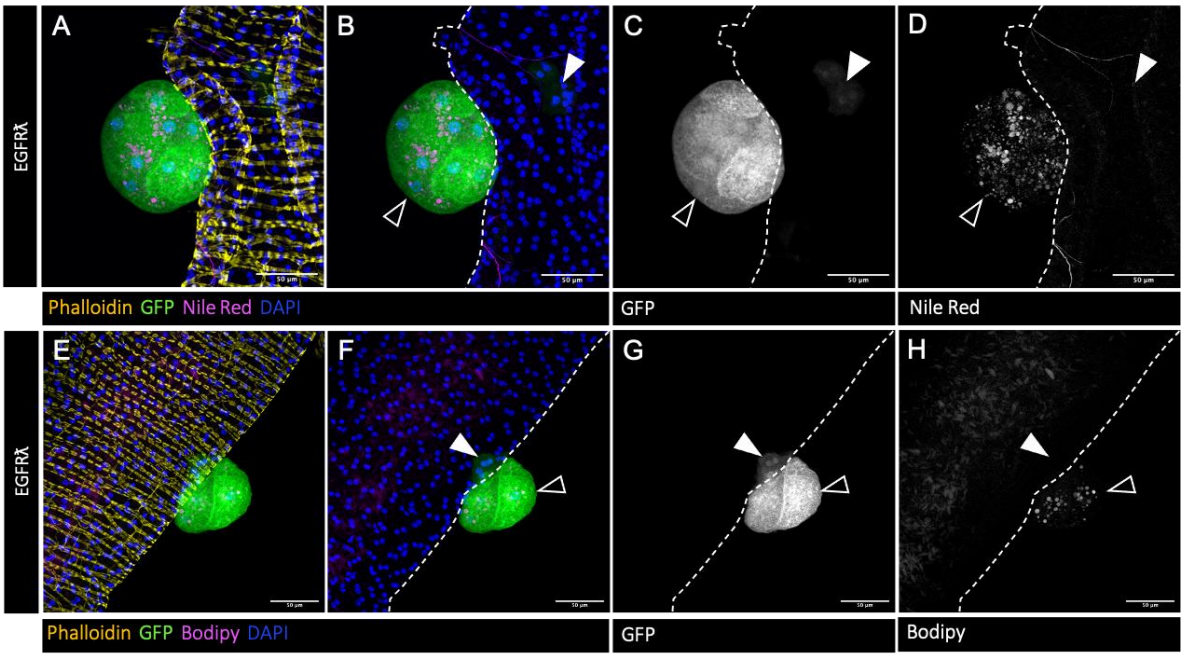

Figure 1. drosophila accessory gland tumour cells accumulate cholesterol esters. A-H : confocal imaging of representative glands initiated for tumorigenesis. Clonal induction of an activated form of EGFR (EGRFl) induces the formation of intraglandular clones (white arrowheads in B-D and F-H) and of tumours outside the gland (empty arrowheads in B-D and F-H). Muscle cells surrounding the glands are revealed by phalloidin staining of F-actin, yellow in A and E). Cell nuclei are revealed by DAPI staining (blue in A-B and E-H). Clonal cells are revealed by GFP co-expression (green in A-B and E-H, grey for single channel in C and G). A-D : Nile Red staining (magenta in A-B and grey in single channel D) shows an accumulation of neutral lipids specifically in tumour cells. E-H : Bodipy staining (magenta in E-F and grey in single channel H) indicates a strong proportion of cholesterol esters among these neutral lipids. Representative images in (A-H) are from three or more experiments. Scale bars: 50µM.

3.2. Cholesterol Metabolism Downregulation Impairs EGFRλ Induced Cholesterol Storage

In order to test whether this excess of cholesterol is important for tumour formation, we then decided to downregulate expression of genes involved in cholesterol uptake (LRP1, LpR2) (Figure 2E-H and I-L, respectively) [38], intracellular trafficking (Npc1a) (Figure 2M-P) [39,40] and storage (Figure 2Q-T) (CG8112 – ortholog of SOAT1/ACAT1) [41] in a clone-specific manner. RNAi against Npc1b (Figure 2A-D), involved in cholesterol import was used as a negative control as its expression is described as restricted to the gut [42]. In all the conditions, except Npc1b RNAi, we observed a strong decrease in lipid droplets volume and number (Figure 2), indicating that tumor-specific cholesterol storage is indeed impaired in the tumours.

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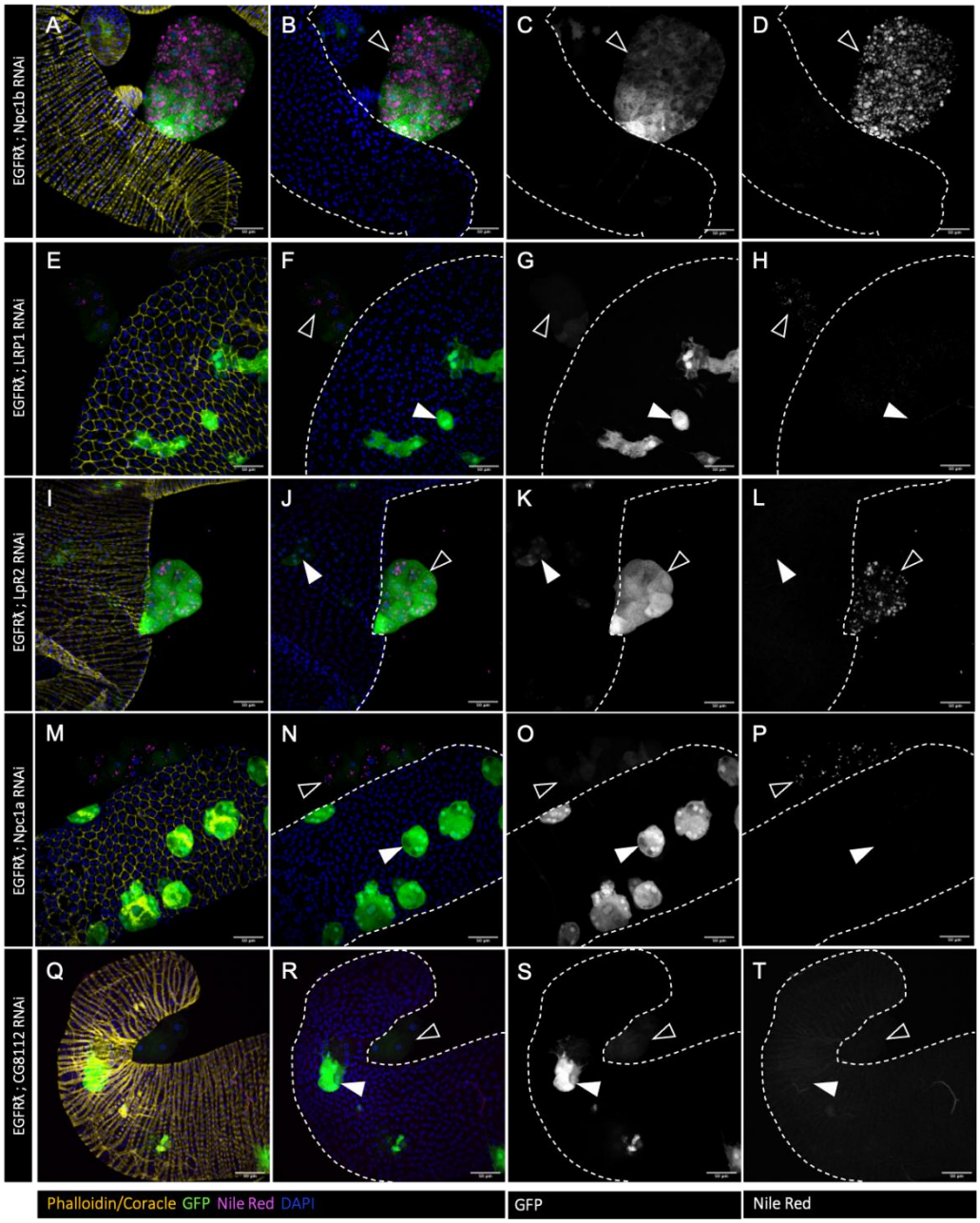

Figure 2. Tumor-specific downregulation of cholesterol metabolism reduces cholesterol esters accumulation. A-T : confocal imaging of representative glands initiated for tumorigenesis. White arrowheads indicate intra-glandular clones as empty arrowheads indicate tumors outside the gland. Muscle cells surrounding the glands are revealed by phalloidin staining in A, I, Q (yellow). Normal epithelial cells are revealed by coracle staining in E and M (yellow). Cell nuclei are revealed by DAPI staining (blue). Clonal cells are revealed by GFP co-expression (green in the first and second columns, grey in third). Neutral lipids accumulation is revealed by Nile Red staining (magenta in the first and second columns, grey in fourth). A-D : Downregulation of ncp1b, which is expressed specifically in intestinal cells, is used as control RNAi. In this condition, a strong accumulation of neutral lipids is seen specifically in the tumor cells (in A, B and D) as for the EGFRl condition. E-T : downregulation of genes implicated in cholesterol uptake (E-L), intracellular trafficking (M-P) or storage (Q-T) strongly impairs lipid accumulation (compare images in right column to D). Representative images in (A-T) from three or more experiments. Scale bars: 50 μm.

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We so concluded that each of these actors (except Npc1b) is necessary for cholesterol accumulation in drosophila accessory gland tumours, showing that all of them represent targetable proteins for regulation of cholesterol abnormal accumulation.

3.3. Hyperactivation of Cell Autonomous Cholesterol Metabolism Specifically Drives Basal Extrusion

We then assessed the percentage of glands baring tumours in the previously described conditions. For all the genotypes, except Npc1b RNAi negative control, we observed a significant decrease in tumour frequency (Figure 3A), showing that not only uptake (LRP1, LpR2), but also intracellular metabolism (Npc1a, CG8112) of cholesterol is necessary for tumour formation itself. This indicates that, in case of cell transformation by oncogene expression, a common cause of initiation, cholesterol metabolism is implicated in early tumorigenesis.

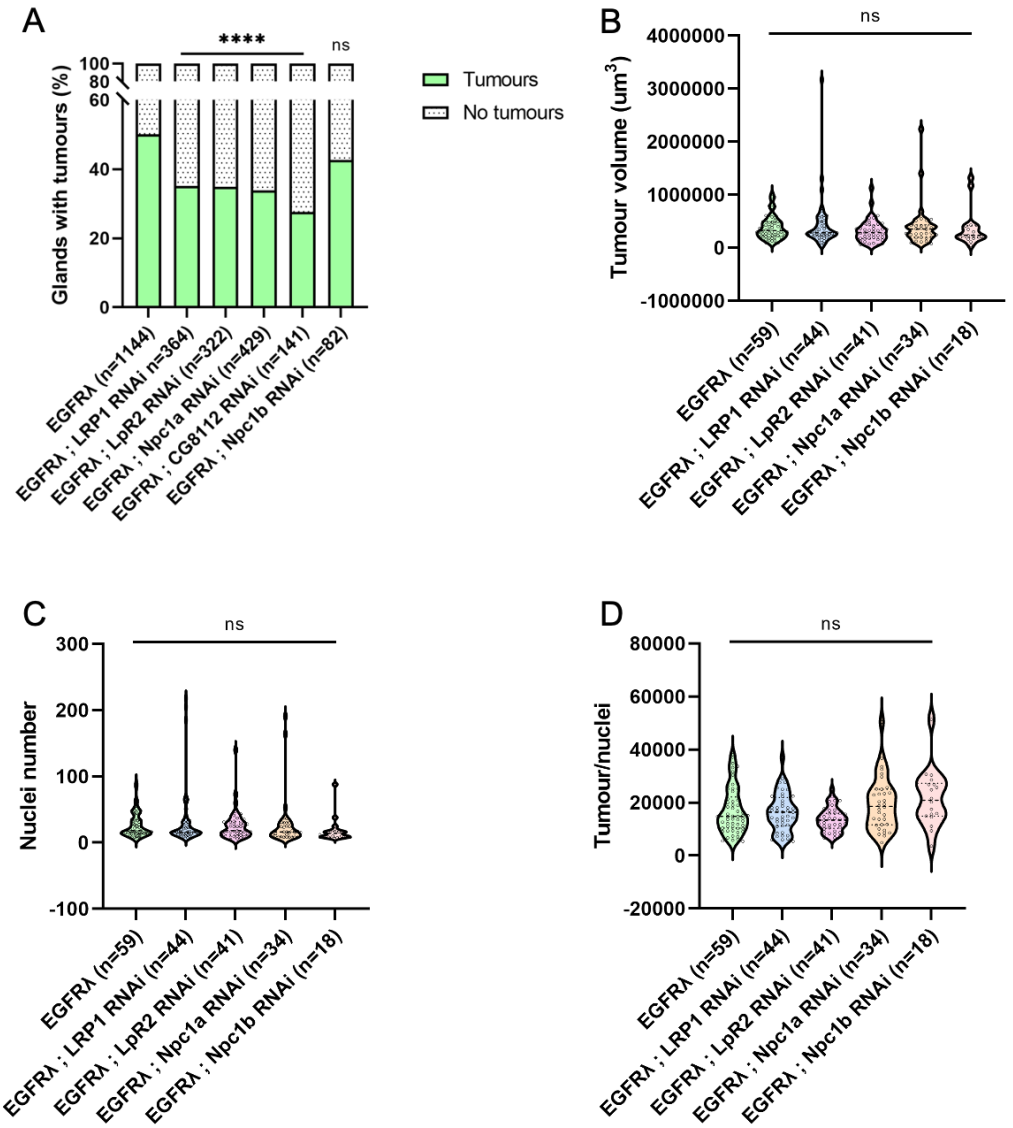

Figure 3. Tumor-specific downregulation of cholesterol metabolism impairs basal extrusion. A : at dissection, presence of tumor(s) was assessed for all considered genotypes outside the glands for several flies (n) in independent experiments (N). As expected, expression of an RNAi against intestinal cell-specific ncp1b does not induce a change in tumor formation compared to EGFRl condition (right and left columns, respectively). On the contrary, downregulation of genes implicated in cholesterol uptake, intracellular trafficking or storage significantly decrease tumor formation. B-D : Tumor size (B), cell number (C) or tumor cell size remain unchanged when cholesterol uptake, intracellular trafficking or storage are down-regulated (see also Figure S1). (A) Chi2-test; EGFRλ:

 doi:10.20944/preprints202405.0400.v1

N=29; LRP1 RNAi: N=9; LpR2 RNAi: N=8; Npc1a RNAi: N=11; CG8112 RNAi: N=5; Npc1b RNAi: N=3 (B-D) Krustal-Wallis tests; EGFRλ: N=11; LRP1 RNAi: N=10; LpR2 RNAi: N=7; Npc1a RNAi: N=8; Npc1b RNAi: N=3. **** p<0.0001; ns: non significant.

In addition, in order to see if cholesterol could be involved in early progression as well, we also characterized tumour volume (Figure 3B), tumour cell number (Figure 3C) and cell volume (Figure 3D) for all considered genotypes. Interestingly, all these parameters remain unaffected, and this phenotype is not altered throughout time (Figure 1SD).

Altogether, these results demonstrate that the observed deregulation of cholesterol metabolism in tumour cells does not impact tumour growth itself, but is necessary for tumour formation. So, we conclude that this apparent hyperactivation of cholesterol metabolism is specifically important for the early step of basal extrusion, when tumour cells actively leave the accessory gland to form tumours outside the epithelial compartment.

3.4. Further Cholesterol Metabolism Deregulation Has No Effect on Early Tumorigenesis

As tumours accumulate high levels of cholesterol, we then wondered whether it was possible to push further this phenotype, and whether this could impact tumour characteristics or formation. In order to do so, we used two different strategies alone or combined. First, we mimicked the so-called western diet, with flies fed either with a standard diet or a diet supplemented with 0.2% of cholesterol (High Cholesterol Diet condition or HCD). Second, in order to maximize tumour cell specific deregulation of cholesterol metabolism, we downregulated the fly cholesterol sensor DHR96 expression [43,44].

Compared to tumour control condition (Nile red staining in Figure 4A-D and quantification in Figure 5A), high cholesteroldiet could slightly increase the accumulation of lipids into the tumours, denoting a limited capacity to exacerbate the abnormal lipid storage induced by the oncogene expression in the tumour cells. Interestingly, neither tumour characteristics (Figure 5B,C) nor basal extrusion (Figure 5D) were significantly affected by high cholesterol diet. We concluded that oncogene transformation is sufficient to obtain an independency to diet for promoting tumour formation. For genetic deregulation of cholesterol metabolism by co-expression of DHR96 RNAi, a limited increase of lipid storage was observed (Figure .4I-L and quantification in Figure 5A), but once more no effect on either tumour characteristics or tumour formation was observed (Figure 5B-D). We concluded that oncogene-driven deregulation of cholesterol metabolism may not exert a maximal effect in term of lipid accumulation, but is definitely on top for tumour promotion. Finally, double deregulation by downregulation of DHR96 in a high cholesterol diet does not result in a higher accumulation of lipid droplets (Figure 4M-P and Figure 5A) and does not affect the studied parameters of tumorigenesis (Figure 5B-D) compared to EGFRl conditions (see also Figure 1. SD).

Overall, these results show that oncogene transformation exerts a profound deregulation of cholesterol homeostasis which in turn promotes basal extrusion, and that further deregulation through diet or targeting cholesterol regulators in the tumour cells cannot potentiate the initial effect due to the oncogene expression.

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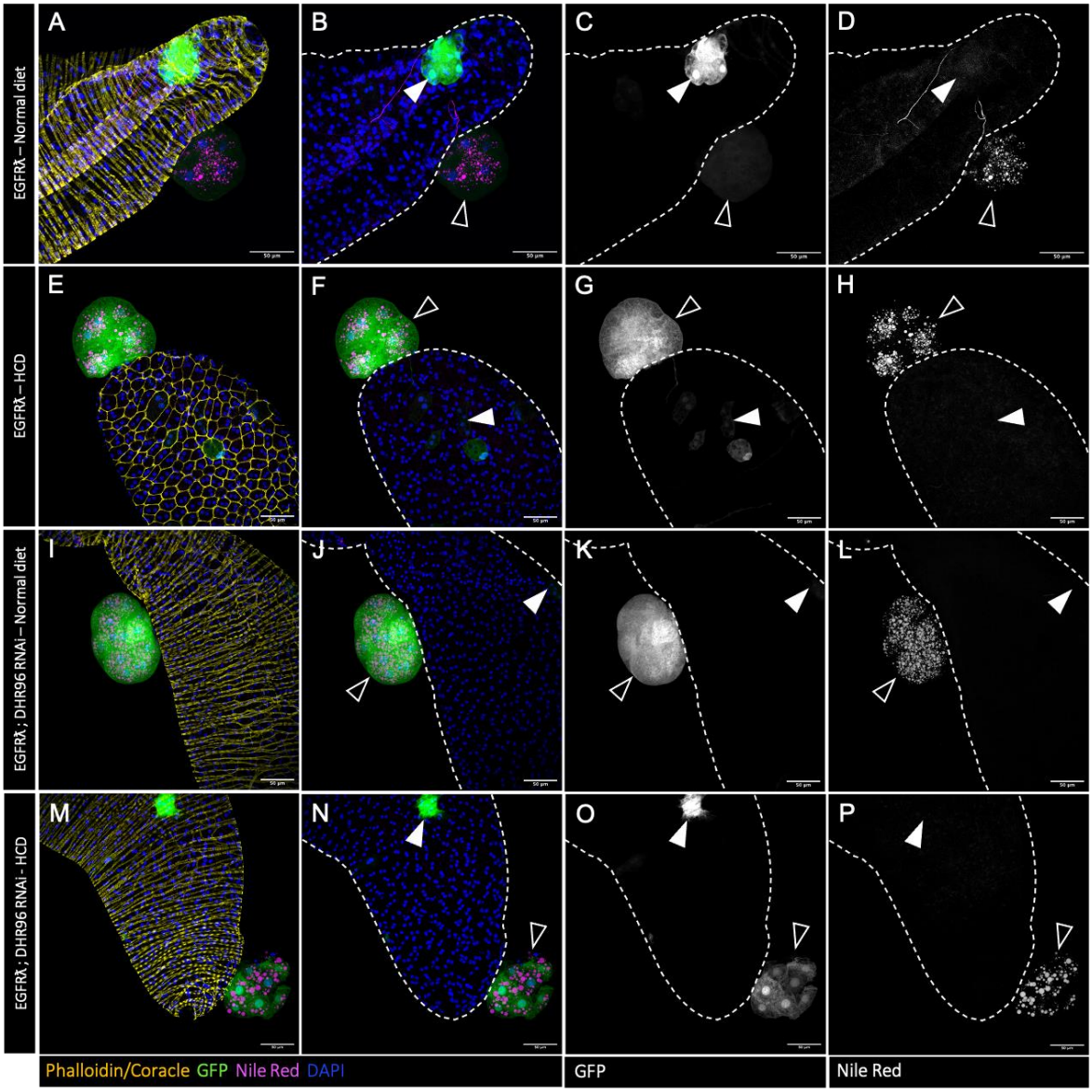

Figure 4. Further cholesterol metabolism deregulation has no effect on tumour phenotype. A-P : confocal imaging of representative glands initiated for tumorigenesis. White arrowheads indicate intra-glandular clones as empty arrowheads indicate tumours outside the gland. Muscle cells surrounding the glands are revealed by phalloidin staining in A, I, M (yellow). Normal epithelial cells are revealed by coracle staining in E (yellow). Cell nuclei are revealed by DAPI staining (blue). Clonal cells are revealed by GFP co-expression (green in the first and second columns, grey in third). Neutral lipids accumulation is revealed by Nile Red staining (magenta in the first and second columns, grey in fourth). A-D :, tumour control condition with normal diet . In this condition, a strong accumulation of neutral lipids is seen specifically in the tumour cells (in A, B and D). E-H : High cholesterol diet (+ 0.2% cholesterol) does not modify tumour phenotype despite slightly increased neutral lipid accumulation (quantification in Figure 5). I-P : Downregulation of master controller of cholesterol homeostasis DHR96 +/- high cholesterol diet (respectively normal diet in I-L and HCD in M-P) induces no evident changes in tumour phenotypes (quantifications in Figure 5). Representative images in (A-P) from three or more experiments. Scale bars: 50 µm.

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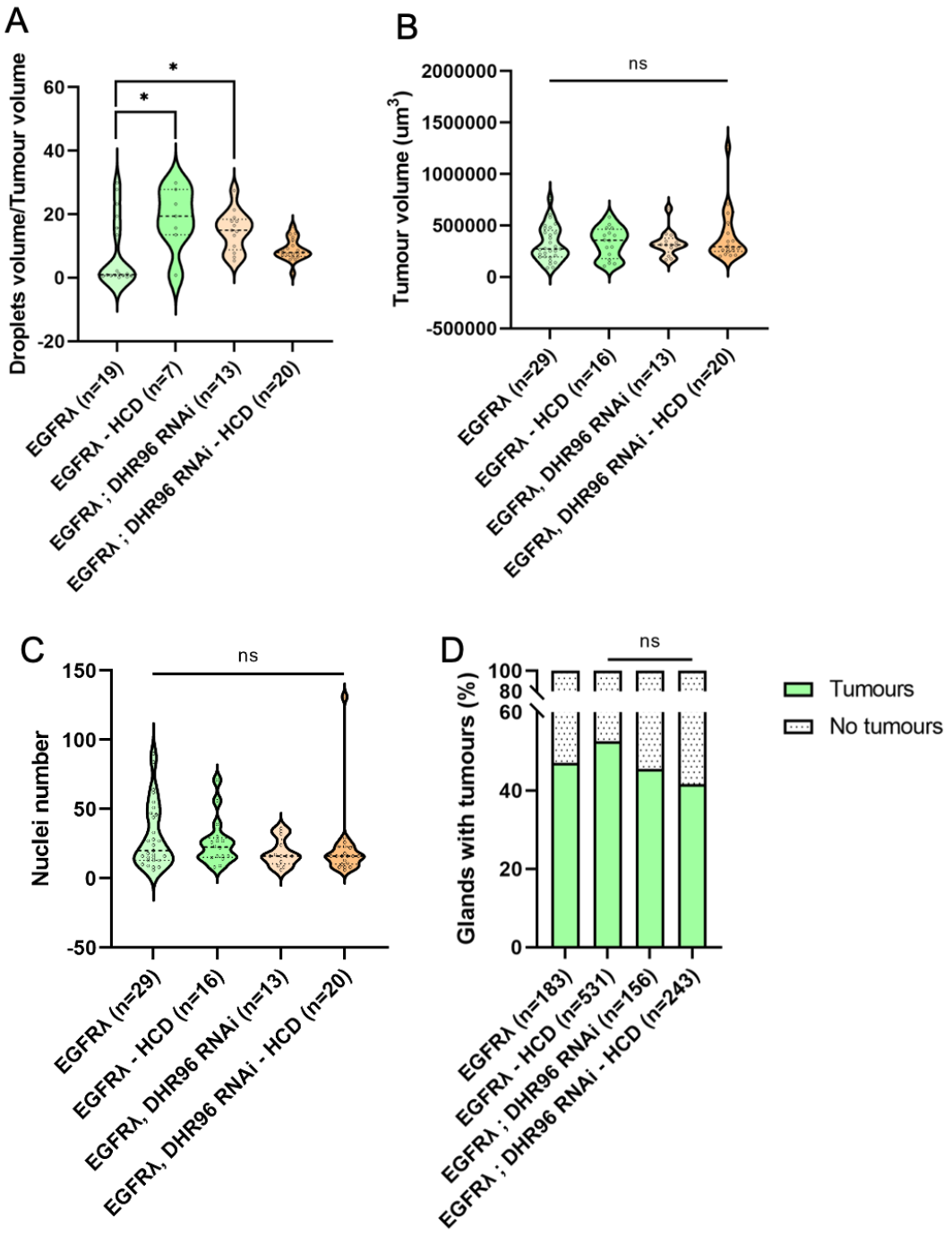

Figure 5. Forcing further the deregulation of cholesterol metabolism has limited effect on tumor phenotype and no effect on basal extrusion. A : high cholesterol diet (HCD) or DHR96 tumor cell-specific downregulation significantly increase the volume of lipid droplets compared to EGFRl condition. B-C : Tumor size (B) and cell number (C) remain unchanged when flies are fed high cholesterol diet and/or for tumor cells tumor cell-specific downregulation of DHR96. D : at dissection, presence of tumor(s) was assessed for all considered genotypes outside the glands for several flies (n) in several independent experiments. Neither HCD nor DHR96 downregulation induce a change in tumor formation compared to EGFRl condition. (A-C) Krustal-Wallis test; EGFRλ: N=6; EGFRλ – HCD: N=6; DHR96 RNAi: N=4; DHR96 RNAi – HCD: N=3 (D) Chi2-test; EGFRλ: N=9; EGFRλ – HCD: N=10; DHR96 RNAi: N=6; DHR96 RNAi – HCD: N=6* $p<0.05$; ns: non significant.

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3.5. High Cholesterol Diet Counteracts Effect of Cholesterol Metabolism Downregulation in Egfrλ Induced Tumorigenesis

As downregulation of cholesterol metabolism reduces basal extrusion, we then wondered if in this case, high cholesterol diet could now impact cholesterol storage and tumour formation. Interestingly, increasing dietary cholesterol does restore Nile red staining in all genotypes (compare Figure 6A-P to Figure 2E-T). Strikingly, it furthermore completely counteracts the reduction in tumour formation that was observed in all the conditions where cholesterol metabolism is genetically impaired (Figure 7A-E). This indicates that dietary intake of cholesterol can finally play a major role in tumour formation in a context of decreased cholesterol access or metabolism by the tumour cells.

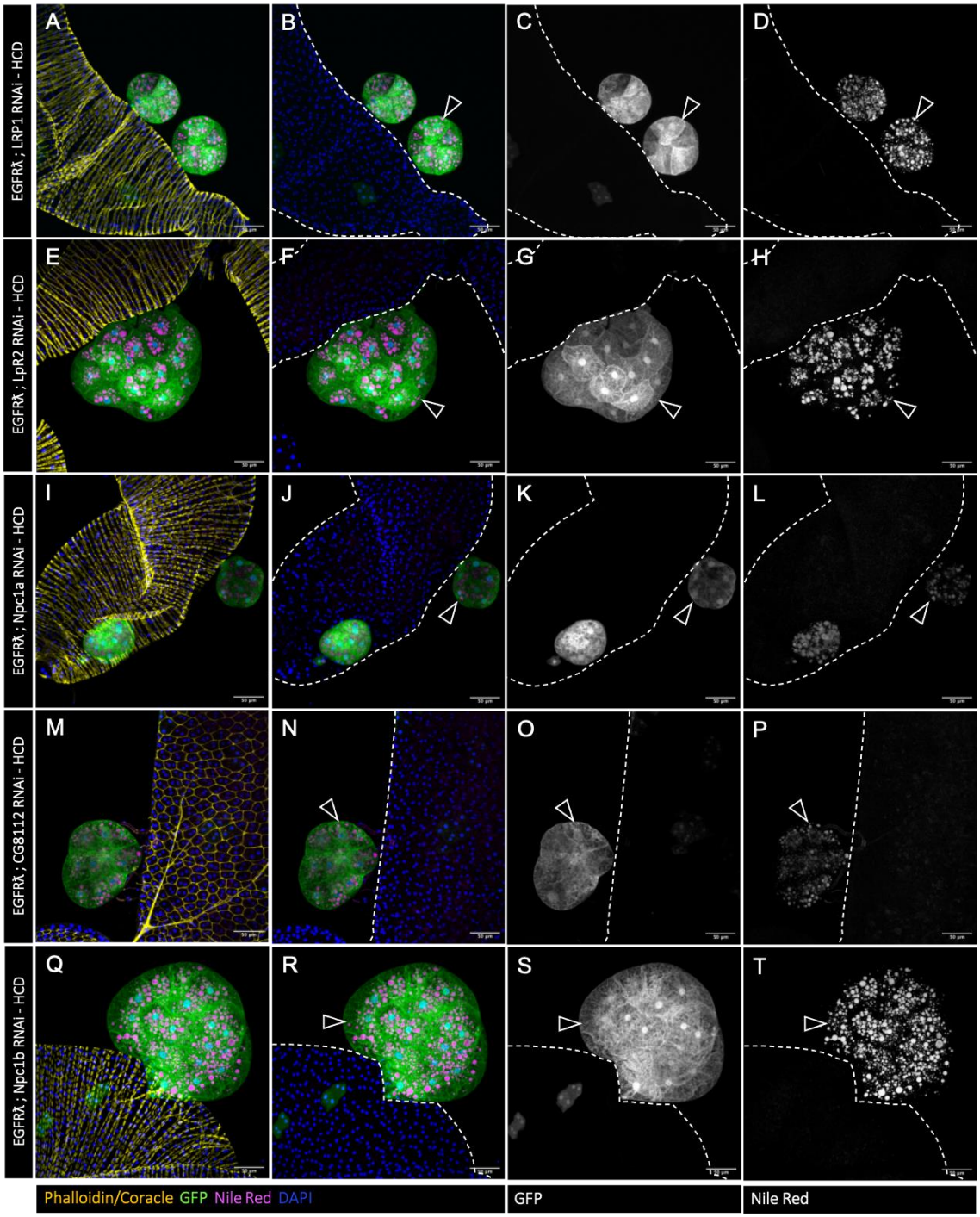

Figure 6. High cholesterol diet restores tumour cell lipid accumulation but has no impact on tumour phenotype. A-T : confocal imaging of representative glands initiated for tumorigenesis. White

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arrowheads indicate intra-glandular clones as empty arrowheads indicate tumours outside the gland. Muscle cells surrounding the glands are revealed by phalloidin staining in A, E, I, Q (yellow). Normal epithelial cells are revealed by coracle staining in M (yellow). Cell nuclei are revealed by DAPI staining (blue). Clonal cells are revealed by GFP co-expression (green in the first and second columns, grey in third). Neutral lipids accumulation is revealed by Nile Red staining (magenta in the first and second columns, grey in fourth). Increasing dietary cholesterol restores lipid accumulation in tumours harboring downregulation of cholesterol import (A-H), intracellular trafficking (I-L) or storage (M-P). Representative images in (A-P) from three or more experiments. Scale bars: 50 μm.

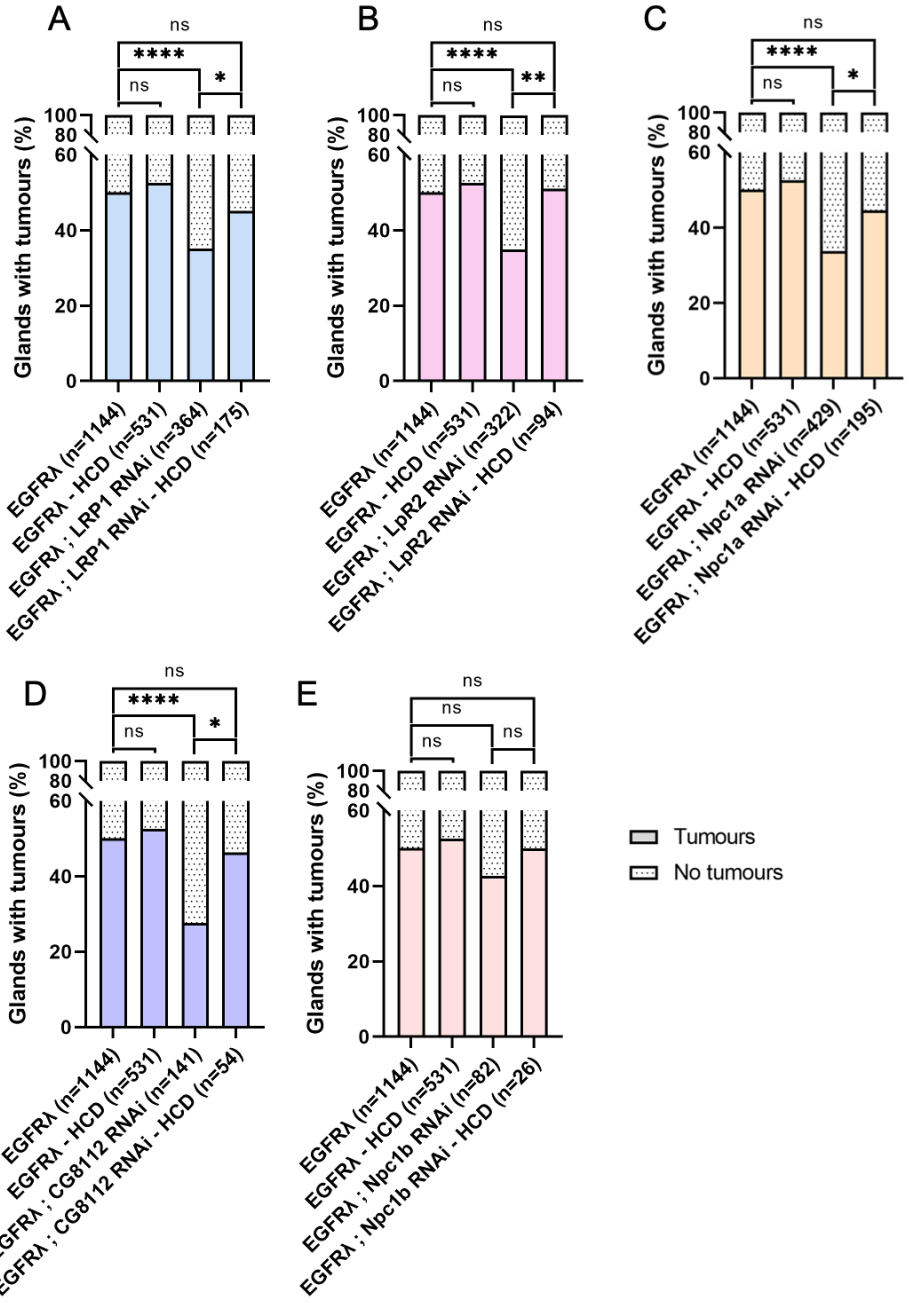

Figure 7. High cholesterol diet counteracts the effect of decreased cholesterol metabolism on basal extrusion. At dissection, presence of tumour(s) was assessed for all considered genotypes outside the glands for several flies (n) in several independent experiments (N). A-D : Increasing dietary cholesterol significantly increases tumour formation in tumours harbouring downregulation of cholesterol import (A-B), intracellular trafficking (C) or storage (D) (compare the two right columns for each graph). By opposition, these high cholesterol diet conditions become indistinguishable from

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EGFRl phenotype independently of diet status (compare right column to the two left columns). E : As expected, a diet enriched in cholesterol has no effect on basal extrusion in a control negative condition, Npc1b RNAi. (A-E) Krustal-Wallis tests; EGFRλ: N=29; EGFRλ – HCD: 10; LRP1 RNAi: N=9; LRP1 RNAi – HCD: N=5; LpR2 RNAi: N=8; LpR2 RNAi – HCD: N=4; Npc1a RNAi: N=11; Npc1a RNAi – HCD: N=6; Npc1b RNAi: N=3; Npc1b RNAi – HCD: N=3; CG8112 RNAi: N=5; CG8112 RNAi – HCD: N=3. *p<0.05; **p<0.01; ****p<0.0001; ns: non significant.

3.6. Genes Coding for Cholesterol Metabolism Are Deregulated in Primary Prostate Cancer

In order to understand when cholesterol metabolism deregulation could be important during prostate carcinogenesis, we then determined from published data the expression of genes associated to this pathway in normal, primary or metastatic samples [45]. First, master regulators of cell cholesterol homeostasis are already deregulated in primary cancer, with a decreased *NR1H2*, expression, coding for LXRb which is associated to the management of excess of cholesterol (Figure 8A). On the contrary, *SREBF1*, which codes for SREBP1 whose role is to increase cholesterol cell levels, remains unchanged even though it tends to be upregulated (Figure 8B). This pleads for an increased uptake and retention of cholesterol. Second, genes involved in cholesterol uptake such as *SCARB1*, coding for the receptor for high density lipoprotein cholesterol(HDL), or LDLR, are either maintained or upregulated in primary cancer (Figure 8C,D). On the contrary, genes implicated in cholesterol *de novo* synthesis such as HMGCr (Figure 8E), HMGCS1 (Figure 8F), *FDFT1* (squalene synthase, Figure 8G) and *SQLE* (Figure 8H) are either maintained or downregulated. This pleads for a role of dietary uptake rather than *de novo*synthesis in the building of higher levels of cholesterol into the tumour cells. Third, *SOAT1*, coding for the enzyme that catalyzes cholesterol esterification, is specifically overexpressed in primary tumours compared to either normal and metastatic samples (Figure 8I). This pleads for an early role for cholesterol accumulation on tumorigenesis.

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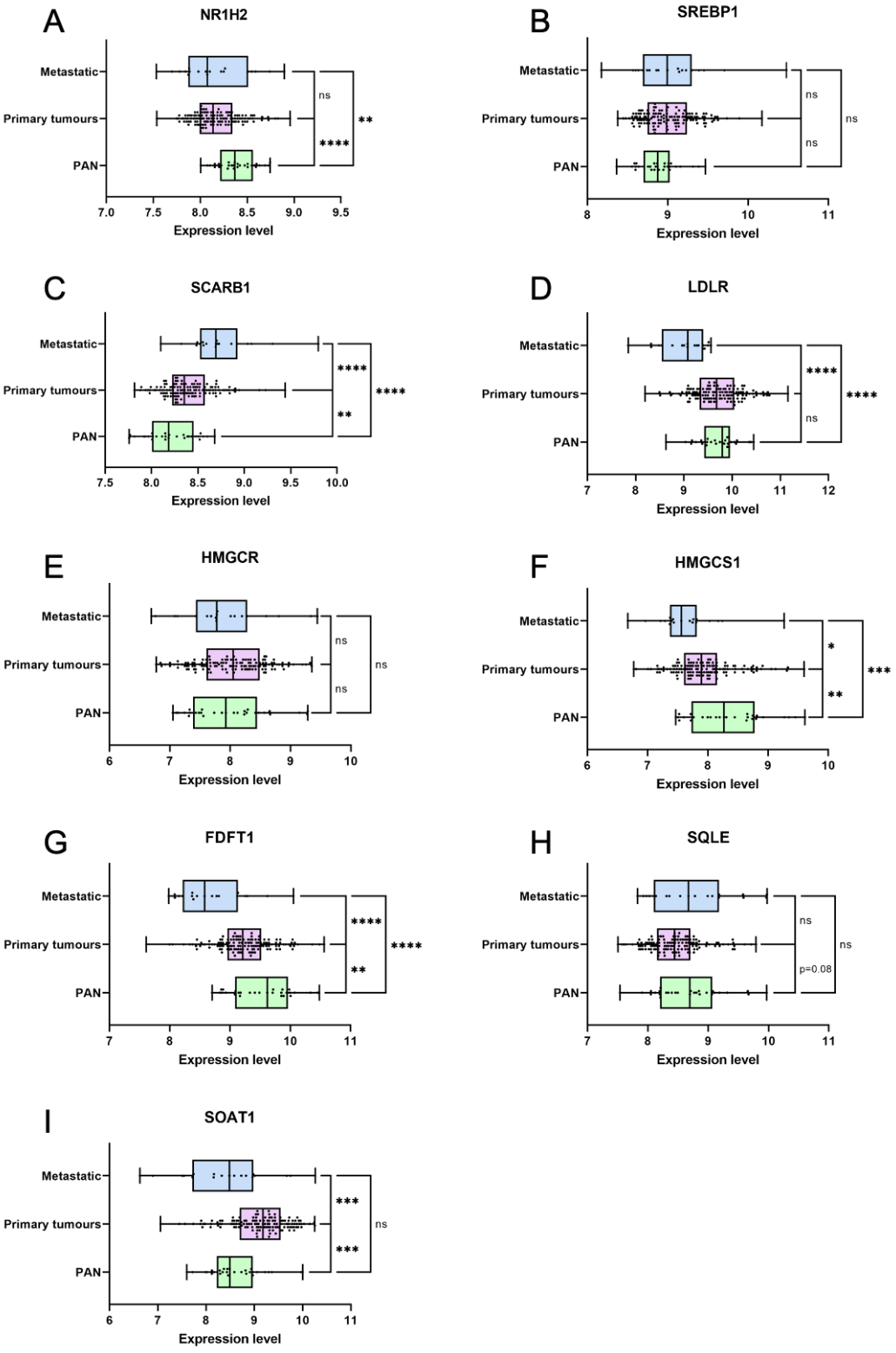

Figure 8. Cholesterol metabolism is deregulated in primary and metastatic prostate cancer. (A-I) Violin-plots showing mRNA expression data for nine genes in normal prostate tissues (PAN, green), primary prostate tumours (Primary tumours, pink) and metastatic prostate tumours (Metastatic, blue). Expression data were first published by Taylor et al. Unpaired t test; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ; ns: non significant. PAN: N=29; Primary tumours: N=131; Metastatic: N=29.

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So, as cholesterol homeostasis is so clearly disturbed in primary cancer already, it could indeed promote the early steps of prostate tumorigenesis in addition to its role in later phases of progression.

Overall, we conclude that targeting cholesterol metabolism in early phases of tumorigenesis could reduce tumour promotion but that this effect is dependent of the level of cholesterol intake.

4. Discussion

Dietary habits have been pointed out as a risk factor in plethora of cancers, and especially western diet characterized by a high content of lipids and sugar and a low level of vegetables. Prostate cancer follows the same trend with some data associating cholesterol blood levels to cancer incidence and aggressiveness [1,2,46]. Experiments with high cholesterol diet have been performed in murine models harboring human prostate cell lines xenografts, indicating that late evolution of cancer to castration resistance, possibly through higher androgen anabolism, correlates with high plasma cholesterol [47,48]. Also, cholesterol anabolism appears increased in CRPC through *SQLE* overexpression, with an impact on lymph node invasion in a xenografted mouse model [49]. However, whether cholesterol metabolism could also be implicated in early tumorigenesis remains unknown. High plasma cholesterol levels have been linked to a risk to develop high grade cancer [5,12], but the role of hypercholesterolemia on cancer incidence, if appearing in some clinical studies, is still not characterized by a meta-analysis approach [13]. Furthermore, relative part of diet versus intra-tumoral production is not resolved. In this context, we have decided to use a drosophila model of early tumorigenesis, in the accessory gland, a functional and structural equivalent of a prostatic acinus previously developed in the team [24]. As insects are auxotroph for cholesterol, by modifying the food content, we can directly evaluate the impact of normal and high diet cholesterol on tumour incidence.

In this context, we have first shown that tumours formed after basal extrusion in the gland accumulate lipids, and especially esterified cholesterol into droplets, a phenomenon to compare with the human pathology [37]. Neither this phenotype nor tumour incidence, volume and cell number are affected by high cholesterol diet. This shows that initiation, here by oncogene expression, is by itself sufficient to render the tumour cells independent of normal to high cholesterol diets. Indeed, lipid accumulation into droplets in tumour cells has never been associated to diet, but rather linked to genetic alterations appearing frequently in the PI3K/Akt pathway during the late stages of cancer progression [37]. However, this pathway is itself implicated in basal extrusion, and could be recruited well before being affected by a genetic alteration by autocrine activation [24] to sustain cholesterol accumulation and promote the early steps of cancer development. Overall, this could explain why, despite clear evidence of the role of western diet on cancer incidence [1,46], role of available cholesterol itself is still not forcefully established through its plasma levels [13].

We also decreased cholesterol metabolism specifically in tumour cells from initiation, by co-expression of RNAi targeting cholesterol import, transport or storage along with the oncogene expression. There, a decrease in lipid accumulation was obtained, and if the average number of tumours was significantly decreased in these conditions, their size and cell composition remained mostly unaffected. This shows that the growth of the tumour itself does not depend much on hyperactivated cholesterol metabolism, but that, on the contrary, formation of tumours outside the accessory gland does depend on it. This itself relies on the capacity for tumour cells to migrate through basement membrane of the epithelial compartment, a phenomenon called epithelial basal extrusion. Basal extrusion is thought to be a funding event in adenocarcinoma formation [50,51], i.e. in a majority of cancers. However, despite its central role in carcinogenesis, this elusive event is largely understudied, due to the paucity of specific tools dedicated to its analysis, and logically to the absence of close-to-normal human samples where this event could be in progress. Overall, the molecular events that are known to be necessary for basal extrusion concern mostly the Ras/MAPK pathway [24,52–54], along with PI3K/Akt pathway co-activation [24]. Furthermore, it has also been shown that T-box transcription factors are necessary for basal extrusion [55], as well as the sphingosine 1-phosphate (S1P) pathway that could play a role as well in cell survival as in cell morphology [52,56]. Here, we so add one component on the control of basal extrusion:

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hyperactivation of cholesterol metabolism. Then, basal extrusion appears more and more as a highly complex and highly controlled step of epithelial tumorigenesis. On the one hand, it provides a view on the richness of pathway deregulation appearing early in the carcinogenesis, and, on the other hand it indicates potential targetable metabolites to limit cancer progression.

Concerning the source of cholesterol in the tumour cells, by the use of an auxotroph model, we showed here that there is no need of intracellular cholesterol anabolism in order to acquire cholesterol accumulation in the tumour cells (Figure 1). This correlates with the expression of genes in cancer patients, where cholesterol uptake genes, as SCARB1 are overexpressed [17] and, in contrast, genes implicated in cholesterol anabolism are downregulated (Figure 8). If tumour cells are more dependent on dietary cholesterol rather than cell production, it could explain why western diet is a good indicator of prostate cancer development [38,39], and why statins, which block *de novo* synthesis of cholesterol, still have an uncertain role on prostate cancer incidence [13].

5. Conclusions

Altogether, this study highlights the pro-tumoral role of dietary cholesterol and of its metabolism *in situ* in a model of prostate cancer, and especially points out at its role in the critical step of basal extrusion leading to the formation of tumours (Figure 9). Considering the strong deregulation of cholesterol metabolism in primary adenocarcinoma, these findings could be indicative of the modus operandum by which high cholesterol metabolism could promote prostate carcinogenesis in human.

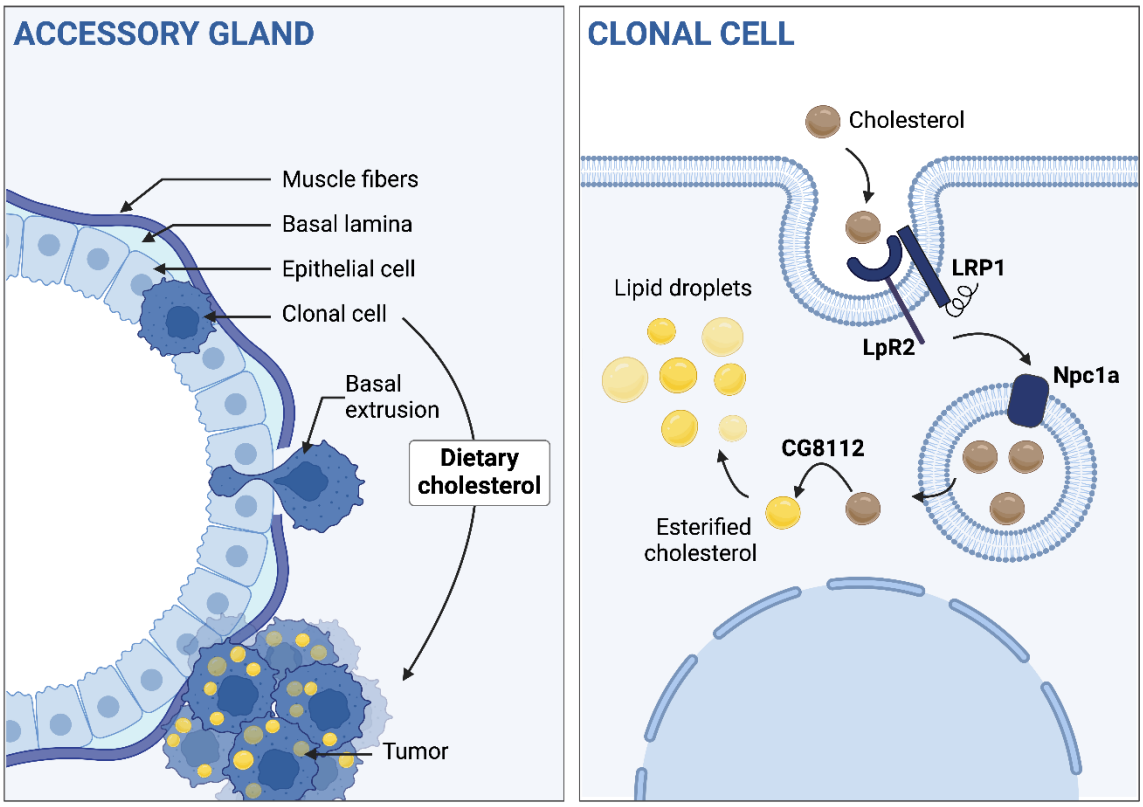

Figure 9. Dietary cholesterol and its metabolism and storage *in situ* is involved in basal extrusion. Left part, from top to bottom: after the oncogenic hit, clonal cells undergo a basal extrusion to form tumours outside the gland and these accumulate cholesterol into droplets. Dietary cholesterol promotes this phenomenon. Right part, from top to bottom: In situ cholesterol intake, trafficking and storage drives the dietary cholesterol effect on tumorigenesis.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: cholesterol dietary intake and/or cholesterol metabolism has no effect on tumour phenotype itself.

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