Notch signals modulate lgl mediated tumorigenesis by the activation of JNK signaling

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Abstract

Objectives: Oncogenic potential of Notch signaling and its cooperation with other factors to affect proliferation are widely established. Notch exhibits a cooperative effect with loss of a cell polarity gene, scribble to induce neoplastic overgrowth. Oncogenic Ras also show cooperative effect with loss of cell polarity genes such as scribble (scrib), lethal giant larvae (lgl) and discs large to induce neoplastic overgrowth and invasion. Our study aims at assessing the cooperation of activated Notch with loss of function of lgl in tumor overgrowth, and the mode of JNK signaling activation in this context.

Results: In the present study, we use Drosophila as an in vivo model to show the synergy between activated Notch (Nact) and loss of function of lgl (lgl-IR) in tumor progression. Coexpression of Nact and lgl-IR results in massive tumor overgrowth and displays hallmarks of cancer, such as MMP1 upregulation and loss of epithelial integrity. We further show activation of JNK signaling and upregulation of its receptor, Grindelwald in Nact/lgl-IR tumor. In contrast to previously described Notchact/scrib-/- tumor, our experiments in Nact/lgl-IR tumor showed the presence of dying cells along with tumorous overgrowth.

Keywords: Notch, lgl, Drosophila, Tumor overgrowth, JNK signaling, Cell death

Introduction

In the past decade, a keen interest has been shown to explore the oncogenic cooperation with loss of cell polarity in tumor progression and malignancy. Studies in Drosophila have revealed that the oncogenic form of Ras cooperates with loss of tumor suppressors, namely scrib, lgl and dlg to cause tumor cell invasion [1, 2]. The oncogenic form of Notch has also shown to cooperate with scrib-/- to induce neoplastic overgrowth [2]. The loss-of-function mutation of Scribble complex genes (scrib, lgl and dlg) results in disruption of epithelial integrity followed by neoplastic tissue hyperproliferation [3–5]. However, the tumor formation caused by loss of scrib, lgl and dlg has been found to be restricted by the compensatory JNK mediated apoptosis [2, 6–8]. Among the Scrib complex genes, lgl was the first neoplastic tumor suppressor gene described in Drosophila [9]. The phenotypes of lgl mutant tissues show close similarity with that of the human epithelial cancers [10–12]. Although it has been shown that Notch cooperates with scrib-/- to induce neoplastic growth, it is still unknown whether Notch works in the same way with loss-of-function of lgl also. Recently, Lgl has been shown to regulate Notch signaling via endocytosis [13]. However, it gives no substantial evidence on coupling of lgl-Notch effect on tumorigenesis. In the present study, we checked the effect of a tumor suppressor gene mutation, lgl, in activated Notch background, and found that lgl downregulation synergizes with activated Notch to induce overgrowth and migratory behavior. Here, we show that Nact/lgl-IR tissues display the hallmarks of tumor overgrowth. Moreover, our study revealed that the effect of Nact/lgl-IR tumor is mediated by the activation of JNK signaling through the upregulation of its receptor, Grindelwald.

Main text

Methods

Detailed description of methods used in this study is provided in Additional file 1.
Results

Oncogenic Notch synergizes with Igl-IR to promote tissue overgrowth

Coexpression of both Igl-IR and Notch\textsuperscript{act} in the Drosophila eye imaginal discs using ey-GAL4 dramatically induced overgrowth (Fig. 1d, d′) as compared to that of only Notch\textsuperscript{act} overexpressed (Fig. 1b, b′) or only Igl-IR overexpressed (Fig. 1c, c′) eye discs. To further describe the phenotype of Notch\textsuperscript{act}/Igl-IR tumor, expression of Matrix metalloproteinase 1 (MMP1) was monitored. MMPs are enzymes with clear association to tumor cell invasion and cancer progression [14, 15]. Coexpression of Notch\textsuperscript{act} and Igl-IR resulted in massive upregulation of MMP1 expression throughout the entire eye disc (Fig. 1d) as compared to that of only Notch\textsuperscript{act} or only Igl-IR (Fig. 1b′, c′). Further, we extended our observation into the brain since ey-GAL is mildly expressed in the brain also. Except endogenous expression, no MMP1 activation was observed in the ey-GAL driven Igl-IR (Fig. 1g) and Notch\textsuperscript{act} larval brain (Fig. 1f). In case of Notch\textsuperscript{act}/Igl-IR larval brain, excessive amount of GFP marked cells with enhanced MMP1 expression was observed in the optic lobes (Fig. 1h, h′). The increment in GFP and MMP1 expression was also found in the ventral nerve cord (VNC) of Notch\textsuperscript{act}/Igl-IR larval brain (Fig. 1h, h′ marked with arrows). This indicates that the weak expression of ey-GAL in VNC is also inducing MMP1 expression in Notch\textsuperscript{act}/Igl-IR tissue. When we quantified the amount of GFP in upper region of VNC, a significant increment in the amount of GFP in Notch\textsuperscript{act}/Igl-IR was found as compared to that of the controls (Additional file 2: Figure S1a). We also quantified the presence of MMP1 in the VNC of Notch\textsuperscript{act}/Igl-IR (Additional file 2: Figure S1b), which clearly shows a significant increase as compared to that of the controls. Moreover, transcript levels of mmp1 in the cephalic complex were also found to be upregulated in Notch\textsuperscript{act}/Igl-IR tumor as compared to that of the controls (Additional file 2: Figure S1c).

In order to examine the cytoskeleton network and cell–cell adhesion, we marked the tissues with phalloidin and adherens junction marker proteins, Armadillo (Arm) and Cadherin (DE-Cad). The F-actin network marked by phalloidin revealed a defective actin cytoskeleton network in Notch\textsuperscript{act}/Igl-IR tumor tissues compared to that of controls (Additional file 3: Figure S2). In the same way, the localization of DE-Cad and Arm were also deregulated in Notch\textsuperscript{act}/Igl-IR tumorous eye discs (Additional file 4: Figure S3a–d, e–h). We, next, determined if neuronal differentiation was defective in Notch\textsuperscript{act}/Igl-IR tumor using a neuronal marker, Elav that marks the differentiated neurons in eye disc and brain. Remarkably, coexpression of Notch\textsuperscript{act} and Igl-IR led to severe loss of Elav positive cells in the eye disc and abnormal expression of Elav in the optic lobes indicating an impaired neuronal differentiation (Additional file 4: Figure S3i–l, m–p).

In parallel, we also used dominant-negative version of Notch to see the effect of depletion of Notch signaling on Igl-IR tumors. Previously, expression of mam\textsuperscript{DN} in Igl\textsuperscript{−} tissues partially rescued the Igl\textsuperscript{−} mosaic adult eye phenotype [13]. Our analysis also found that reduction of Notch signaling partially rescued the phenotypes of Igl loss-of-function flies (Additional file 5: Figure S4). Thus, our analysis support the notion that the Igl loss-of-function wing phenotype is dependent on elevated Notch signaling, consistent with the previous study [13].

Involvement of JNK pathway in Notch\textsuperscript{act}/Igl-IR tumor

Previous studies in Drosophila have revealed that oncogenic Ras along with loss of Igl or scrib or dlg induces JNK signaling, which is crucial for tumor invasion [7, 16]. This prompted us to check the expression of Puckered (puc), a transcriptional target of JNK signaling and widely used to check the activation of JNK signaling. An enhancer trap allele, puc-LacZ [17] was used to monitor the activation of JNK signaling. Coexpression of both Notch\textsuperscript{act} and Igl-IR resulted in intense upregulation of puc throughout the wing disc (Fig. 2d), indicating the activation of JNK signaling in Notch\textsuperscript{act}/Igl-IR tumor. We also observed a significant
increase in size of the wing disc in N<sup>act</sup> and lgl-IR coexpressed condition compared to that of the wild-type, only N<sup>act</sup>, and only lgl-IR wing discs (Fig. 2i).

To check the mode of activation of JNK signaling, we examined the transcript level expression of ligand eiger (egr), and its receptor wengen (wgn), in N<sup>act</sup>/lgl-IR tumor.
whether blocking JNK could affect the

activation or senescence [21]. When we checked the status of cell death in N\textsuperscript{act}/\textit{Igl}-IR tumor, we observed a significant amount of acridine orange (Compare Fig. 3d with a–c) and caspase positive cells (Compare Fig. 3i with f–h) indicating severe cell death. Since loss of \textit{Igl} in a tissue known to induce cell competition to remove the unfit cells [22], dying cells in N\textsuperscript{act}/\textit{Igl}-IR tissue could be an indication of cell competition. To check the effect of cell death on overgrowth and MMP1 expression, we blocked cell death by expressing a caspase inhibitor, p35 (Fig. 3e, j). It was found that blocking cell death in N\textsuperscript{act}/\textit{Igl}-IR overexpressed condition did not obstruct MMP1 expression (Fig. 3o). Coexpression of p35 with N\textsuperscript{act}/\textit{Igl}-IR resulted in an increased wing disc size as compared to N\textsuperscript{act}/\textit{Igl}-IR overexpressed wing disc (Fig. 3r). As the caspase inhibitor, p35 is known to block cell death [23], the increase in the tissue size is expected since blocking cell death in N\textsuperscript{act}/\textit{Igl}-IR tumor allowed more cells to overgrow that, in turn, increased the disc size.

Discussion

In the present study, we unveil a cooperation of Notch with RNAi-mediated downregulation of a polarity cum tumor suppressor gene, \textit{Igl} to promote tumor overgrowth. Our data, presented here, illustrate that coexpression of N\textsuperscript{act} and \textit{Igl}-IR in Drosophila eye disc results in overgrowth, loss of positional clues and upregulation of MMP1 expression, which is less prevalent in only N\textsuperscript{act} overexpression or only \textit{Igl}-IR overexpression. Earlier the loss of polarity gene \textit{scribble} found to cooperate with Notch signaling to promote neoplastic overgrowth [2]. Another two independent studies of similar context show that oncogenic Ras cooperates with loss of cell polarity genes (\textit{Igl, scrib, dlg}) to induce metastasis and secondary tumor formation at distant sites [7, 14]. Interestingly, we found that Notch synergizes with loss of \textit{Igl} to promote tumorous overgrowth and elevated expression of MMP1, and inhibiting Notch signaling rescues the defects caused by loss of \textit{Igl}. It indicates the potential function of Notch signaling
Our data also show distorted epithelial integrity in Nact/lgl-IR tumor that point towards epithelial to mesenchymal transition, where tightly joined epithelial cells with regularly spaced cell–cell junctions convert to mesenchymal cells which are of irregular shape without tight intracellular adhesion [24].
Further, we found upregulation of JNK signaling and its receptor Grindelwald in N\textsuperscript{act}/lgl-IR tumor. Two previous studies have shown that Notch cooperates with two different proteins to induce proliferation and metastasis by the activation of JNK signaling in ligand-dependent and -independent manner [25, 26]. In case of N\textsuperscript{act}/lgl-IR tumor, we showed that the transcript levels of egr (ligand) and wgn (receptor) were not upregulated, whereas a significant upregulation of grind transcripts in the N\textsuperscript{act}/lgl-IR tumor was observed. Earlier the active form of Grindelwald has shown to activate JNK signaling in vivo [18]. Thus, in case of N\textsuperscript{act}/lgl-IR tumor, JNK signaling might get activated through Grindelwald. Previously, it has been shown that JNK signaling can initiate tumor initiation and growth in Eiger-independent manner also [27].

Another most important hallmark of almost all types of cancer is the ability to evade apoptosis that, in turn, helps tumor cell population to increase in number [21]. In other similar tumor models such as Ras\textsuperscript{v12}/dlg\textsuperscript{−/−}, dying cells of dlg\textsuperscript{−/−} clones evade apoptosis in presence of oncogenic Ras, where JNK signaling switches its role from proapoptotic to progrowth [7]. In contrast, Ras/lgl\textsuperscript{−/−} from proapoptotic to progrowth [7]. In contrast, Ras/lgl\textsuperscript{−/−} from proapoptotic to progrowth [7]. In contrast, Ras/lgl\textsuperscript{−/−} from proapoptotic to progrowth [7]. In contrast, Ras/lgl\textsuperscript{−/−} from proapoptotic to progrowth [7].

Additional file 1: Materials and methods.

Additional file 2: Figure S1. Quantification of GFP and MMP1 in the VNC of N\textsuperscript{act}/lgl-IR tumor (a). GFP quantification in VNC shows a four-fold increment in the amount of GFP positive cells in N\textsuperscript{act}/lgl-IR as compared to that of the wild-type, only N\textsuperscript{act} and lgl-IR overexpressed tissues. b. MMP1 quantification in VNC shows around four-fold increase in N\textsuperscript{act}/lgl-IR, whereas only N\textsuperscript{act} and lgl-IR overexpressed tissues show almost same level of MMP1 in VNC as of wild-type. c. Real-Time PCR analysis shows significant increase in mmp1 transcripts in the cephalic complex of N\textsuperscript{act}/lgl-IR as compared to that of wild-type, only N\textsuperscript{act} or only lgl-IR tissues. Data was normalized to eps17. Analysis of data was done using One-way ANOVA with Tukey’s multiple comparison test; data represents mean ± SEM (**p < 0.001 and ns p > 0.05).

Additional file 3: Figure S2. N\textsuperscript{act}/lgl-IR tumor leads to distorted actin cytoskeleton. Coexpression of N\textsuperscript{act} and lgl-IR causes distorted actin cytoskeleton organization (d) compared to that of wild-type (a), only N\textsuperscript{act} overexpressed (b) and only lgl-IR overexpressed condition (c). F-actin was marked using phallolidin. Scale bars: 10 µm (a-d).

Additional file 4: Figure S3. N\textsuperscript{act}/lgl-IR shows hallmarks of migratory tumor. Fluorescent micrographs of eye imaginal discs and larval brains are shown. a, a′ Endogenous Cadherin and (e, e′) Armadillo localize to the adherens junctions and marks the photoreceptors in the ey-GAL4/+ eye imaginal discs. Morphogenetic furrow in a and e is marked with an arrow. Overexpression of N\textsuperscript{act} leads to overgrown discs and the localization pattern of Cadherin (b, b′) and Armadillo (f, f′) have been modified. Overexpression of lgl-IR results in distorted localization of Cadherin (c, c′) and Armadillo (g, g′). Coexpression of N\textsuperscript{act} and lgl-IR in eye imaginal disc causes complete deformation of Cadherin (d, d′) and Armadillo (h, h′) localization pattern. Images a′-d′, e′-h′ are higher magnification of the square region from a-d, e-h. i Expression of Elav, a marker for differentiated neurons in wild-type eye discs is shown. j Overexpression of N\textsuperscript{act} in eye disc shows increased expression of Elav, probably due to overproliferation of the disc. k lgl-IR over-expressed eye disc shows comparatively less Elav-positive cells. I Interestingly, N\textsuperscript{act} and lgl-IR coexpressed eye disc shows hardly any Elav-positive cells. Images f, j, k, and l are merged images of GFP along with i, j, k, and l, respectively. Elav expression in the brains of N\textsuperscript{act} (n) and lgl-IR (o) driven by ey-GAL4 is found to be similar to that of the wild-type brain (m) p Coexpression of N\textsuperscript{act} and lgl-IR resulted in an abnormal expression pattern of Elav, where clump like distribution is found in the optic lobes (marked with arrow). Images m′, o′, and p′ are merged images of GFP along with m, n, and o, respectively. Scale bars: 50 µm (a-d, e-h, i-l, f′-p′), 5 µm (a′-d′, e′-h′) and 100 µm (m-p, m′-p′). All eye discs are oriented with dorsal to the left and anterior to the top. Ventral view of the brains is shown.

Additional file 5: Figure S4. Lowering the dose of Notch partially rescues lgl-IR-induced MMP1 expression and restores the adult wing. a MMP1 expression in wild-type wing disc is shown. b Overexpression of only Notch-DN did not induce expression of MMP1. c Overexpression of lgl-IR induces MMP1 expression in the wing disc. d Coexpression of Notch-DN in lgl-IR background partially rescues the expression of MMP1 caused by lgl-IR overexpression. a′, b′, c′ and d′ are merges of DAPI along with a, b, c, and d, respectively. Moreover, Coexpression of Notch-DN with lgl-IR resulted in reduced wing disc size as compared to that of only overexpression of lgl-IR (f). e GFP marked vestigial domain in wing disc is shown. e′ is the merge image of DAPI along with (e). f Overexpression of Notch-DN resulted in held out wings with wing nicking phenotype. g Overexpression of lgl-IR using vg-GAL4 led to necrotic lesions followed by deformation of adult wings. h Coexpression of Notch-DN with lgl-IR partially restored deformed adult wings. j Phenotype penetrance in adult flies is shown for each genotype; the phenotype observed in Notch-DN show 100% penetrance and around 70% lgl-IR flies showed deformed wings. In case of Notch-DN, lgl-IR flies, around 60% flies showed the depicted phenotype and, the rest of the flies showed less developed wings but

Limitations

- The present study is not the first one to show the cooperation between Notch and loss of cell polarity genes. Activated Notch is known to cooperate with another cell polarity gene, scribble, to induce neoplastic overgrowth.
- In the present study, experiments were performed using RNAi line of lgl, but not with the lgl loss-of-function mutants.
they were not of the lgl-IR category. Analysis of data was done using One-way ANOVA with Tukey’s multiple comparison test; data represents mean ± SEM. (**p < 0.01 and ns > 0.05). All wing discs are oriented with dorsal to the top and posterior to the right. Scale bar: 50 µm (a, d, a’-d’).

Additional file 6: Figure S5. Inhibition of JNK pathway suppresses the N\textsuperscript{L62}/+;lgl-IR tumor growth and MMP1 expression. Fluorescent micrographs of wing imaginal discs are shown. a Overexpression of both N\textsuperscript{L62} and lgl-IR in wing imaginal disc using vg-GAL4 resulted in massive upregulation of MMP1. b Coexpression of bsk\textsuperscript{IR} in the background of N\textsuperscript{L62} and lgl-IR resulted in the suppression of MMP1 expression. a”–b” is the merge images of a–a’ and b–b’. The N\textsuperscript{L62}/lgl-IR wing disc size was significantly reduced; when bsk\textsuperscript{IR} was expressed in the background. Analysis of data was done using Unpaired t test with Welch’s correction; data represents mean ± SEM (**p < 0.01). All wing discs are oriented with dorsal to the top and posterior to the left. Scale bar: 50 µm (a–a’, b–b’).

Abbreviations
scrib: scribble; lgl: lethal giant larvae; dlg: discs large; Nact: activated Notch; Abd-B/10/806/2010 and BT/PR14080/BRB/10/805/2010) and UGC-UPE, BHU to AM and MM. MSP was supported by the fellowship from JNMF while AS and BRB/10/806/2010). Not applicable.

Ethics approval and consent to participate Not applicable.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
Data available on request from the corresponding author.

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Not applicable.

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References
1. Pagliarini AR, Xu T. A genetic screen in Drosophila for metastatic behavior. Science. 2003;302:1227–31.
2. Brumby AM, Richardson HE. scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. EMBO J. 2003;22:5769–79.
3. Gateff E, Schneiderman HA. Developmental studies of a new mutant of Drosophila melanogaster: lethal malignant brain tumor (l(2)lgl 4). Am Zool. 1967;7:760.
4. Stewart M, Murphy C, Fristrom JW. The recovery and preliminary characterization of X chromosome mutants affecting imaginal discs of Drosophila melanogaster. Dev Bio. 1972;192:77–83.
5. Bider D, Perrimon N. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature. 2000;403:676–80.
6. Uhlírova M, Jasper H, Bohmann D. Non-cell-autonomous induction of tissue overgrowth by JNK/Ras cooperation in a Drosophila tumor model. Proc Natl Acad Sci USA. 2005;102:13123–8.
7. Igaki T, Pagliarini RA, Xu T. Loss of cell polarity drives tumor growth and invasion through JNK activation in Drosophila. Curr Biol. 2006;16:1139–46.
8. Froldi F, Ziosi M, Garioa F, Pession A, Grzeschik AN, Bellostia P, et al. Thie- rthal giant larve tumour suppressor mutation requires dflyc onc-Baz to promote clonal malignancy. BMC Biol. 2010;8:1–16.
9. Gateff E. The genetics and epigenetics of neoplasms in Drosophila. Biol Rev. 1997;7:123–68.
10. Froldi F, Ziosi M, Obiga M, Casanova J. Juregemonic properties of Drosophila epithelial cells mutant for lethal giant larvae. Dev Dyn. 2016;245:834–43.
11. Parsons LM, Portella M, Grzeschik NA, Richardson HE. Lgl regulates Notch signaling via endocytosis, independently of the apical aPKC-Par6-Baz polarity complex. Curr Biol. 2014;24:2073–84.
12. Uhlírova M, Bohmann D. JNK and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila. EMBO J. 2006;25:5294–304.
13. McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol Med Today. 2000;6:149–56.
14. Leong GR, Goulding KR, Amin N, Richardson HE, Brumby AE. scribble mutants promote aPKC and JNK-dependent epithelial neo-plasia independently of Crumbs. BMC Biol. 2009. https://doi.org/10.1186/1741-7007-7-72.
15. Shiklover J, Mithnavecki K, Levy-Adam F, Kurant E. JNK pathway activation regulates by DIAP1. EMBO J. 2000;19:598–611.
16. Andersen SD, Colombani J, Palmerini V, Chakrabandhu K, Boone E, Rothlisberger M, et al. The Drosophila TNF receptor Grindelwald couples loss of cell polarity and neoplastic overgrowth. Nature. 2015;522:482–6.
17. Moreno E, Yan M, Basler K. Evolution of TNF signaling mechanisms: JNK dependent apoptosis triggered by Eiger, the Drosophila homologue of the TNF superfamily. Curr Biol. 2002;12:1263–8.
18. Cordero BJ, Macagno PJ, Stefanatos KR, Strathdee EK, Cagan LR, Vidal M. Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter. Dev Cell. 2010;8:999–1011.
19. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
20. Menéndez J, Pérez-Garajo A, Calleja M, Morata G, Sollazzo M, Fontana E, Froldi F, Ziosi M, Grzeschik AN, Bellosta P, et al. Therie-
27. Muzzopappa M, Murcia L, Milan M. Feedback amplification loop drives malignant growth in epithelial tissues. Proc Natl Acad Sci USA. 2017;114:E7291–300.
28. Kulshammer E, Uhlirova M. The actin cross-linker filamin/cheerio mediates tumor malignancy downstream of JNK signaling. J Cell Sci. 2013;126:927–38.

29. Doggett K, Turkel N, Willoughby LF, Ellul J, Murray MJ, Richardson HE, et al. BTB-zinc finger oncogenes are required for Ras and Notch-driven tumorigenesis in Drosophila. PLoS ONE. 2015;10.e0132987.