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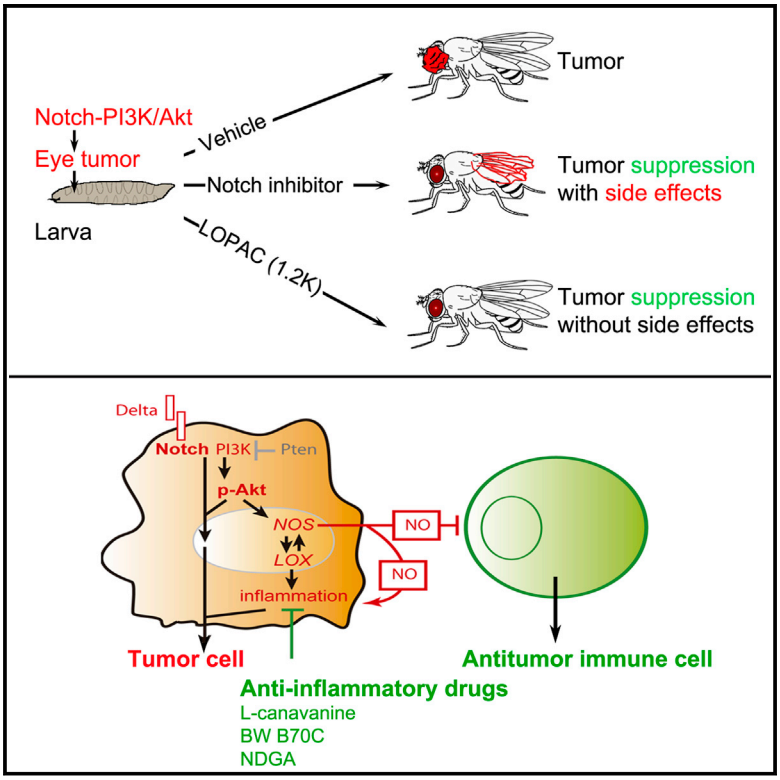
PI3K/Akt Cooperates with Oncogenic Notch by Inducing Nitric Oxide-Dependent Inflammation

Graphical Abstract



Highlights

- An effective drug screening platform in flies for targeting oncogenic cooperation
- Specific anti-inflammatory drugs block Notch-PI3K/Akt oncogenesis
- Activated PI3K/Akt induces inflammation and immunosuppression via NO synthase
- NO synthase and immunosuppression fuel tumorigenesis by activated Notch

Authors

Santiago Nahuel Villegas, Rita Gombos, Lucia García-López, ..., Esther Ballesta-Illán, József Mihály, Maria Dominguez

Correspondence

svillegas@umh.es (S.N.V.), m.dominguez@umh.es (M.D.)

In Brief

Villegas et al. devised a high-throughput screen for compounds targeting oncogene cooperation without side effects. The screen revealed that a nitric oxide- and LOX-dependent inflammatory environment induced by activated PI3K/Akt facilitates Notch-driven cancer promotion.

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PI3K/Akt Cooperates with Oncogenic Notch by Inducing Nitric Oxide-Dependent Inflammation

Santiago Nahuel Villegas,[1,*] Rita Gombos,[2] Lucia García-López,[1] Irene Gutiérrez-Pérez,[1] Jesús García-Castillo,[1,3] Diana Marcela Vallejo,[1] Vanina Gabriela Da Ros,[1,4] Esther Ballesta-Illán,[1] József Mihály,[2] and Maria Dominguez[1,5,*]

[1]Instituto de Neurociencias, Consejo Superior de Investigaciones Científicas-Universidad Miguel Hernández (CSIC-UMH), Avda. Ramón y Cajal s/n, 03550 Sant Joan d'Alacant, Alicante, Spain

[2]Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences, MTA-SZBK NAP B Axon Growth and Regeneration Group, Temesvári krt. 62, H-6726 Szeged, Hungary

[3]Present address: Instituto Murciano de Investigación Biosanitaria (IMIB), 30120 Murcia, Spain

[4]Present address: Instituto de Biología y Medicina Experimental (IBYME-CONICET), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina

[5]Lead Contact

*Correspondence: svillegas@umh.es (S.N.V.), m.dominguez@umh.es (M.D.)

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SUMMARY

The PI3K/Akt signaling pathway, Notch, and other oncogenes cooperate in the induction of aggressive cancers. Elucidating how the PI3K/Akt pathway facilitates tumorigenesis by other oncogenes may offer opportunities to develop drugs with fewer side effects than those currently available. Here, using an unbiased *in vivo* chemical genetic screen in *Drosophila*, we identified compounds that inhibit the activity of proinflammatory enzymes nitric oxide synthase (NOS) and lipoxygenase (LOX) as selective suppressors of Notch-PI3K/Akt cooperative oncogenesis. Tumor silencing of *NOS* and *LOX* signaling mirrored the antitumor effect of the hit compounds, demonstrating their participation in Notch-PI3K/Akt-induced tumorigenesis. Oncogenic PI3K/Akt signaling triggered inflammation and immunosuppression via aberrant *NOS* expression. Accordingly, activated Notch tumorigenesis was fueled by hampering the immune response or by *NOS* overexpression to mimic a protumorigenic environment. Our lead compound, the LOX inhibitor BW B70C, also selectively killed human leukemic cells by dampening the NOTCH1-PI3K/AKT-eNOS axis.

INTRODUCTION

Tumorigenesis requires cooperative action among two or more signaling pathways or genes, but the basis of cooperation often remains undefined. Concurrent activation of Notch and phosphatidylinositol 3-kinase (PI3K)/Pten/Akt pathways can trigger tumorigenesis in flies and mice (Palomero et al., 2007; Piovan et al., 2013; Hales et al., 2014; Kwon et al., 2016). This oncogenic combination is also prevalent in aggressive cancers in humans (Eliasz et al., 2010; Kwon et al., 2016; Muellner et al., 2011), such as pediatric T cell acute lymphoblastic leukemia (T-ALL) (Palomero et al., 2007; Gutierrez et al., 2009). Although Notch and PI3K/Akt inhibitors effectively kill cancer cells, only their combination can bypass single-agent pathway inhibitor resistance (Hales et al., 2014). Unfortunately, these pathways have many physiological functions (Bray, 2016; Engelman, 2009; Fruman and Rommel, 2014; Kopan and Ilagan, 2009), so the systemic inhibition of Notch or PI3K/Akt results in severe and lasting side effects (Akinleye et al., 2013; Ntziachristos et al., 2014). Therefore, to minimize side effects, drugs that dampen oncogenic interactions more selectively are needed.

The fruit fly *Drosophila* is a suitable genetic model for exploring the molecular mechanisms of cancer (Bangi, 2013; Pagliarini and Xu, 2003; Ferres-Marco et al., 2006; Vidal and Cagan, 2006; Palomero et al., 2007) and for developing drugs using phenotype-based screening approaches (Dar et al., 2012; Gladstone and Su, 2011; Gonzalez, 2013; Markstein et al., 2014; Willoughby et al., 2013; Bangi et al., 2016). Here, using a *Drosophila* cancer model (Palomero et al., 2007) to screen the Library of Pharmacologically Active Compounds (LOPAC^{1280}), we have identified compounds capable of suppressing Notch-PI3K/Akt cooperative tumorigenesis. Notch inhibitors impeded the development of these tumors, but this was accompanied by high animal mortality and notched wings—two effects characteristic of Notch deficiency. However, we found many other compounds capable of blocking tumor formation by this oncogene cooperation without side effects. These include the anti-inflammatory drug BW B70C (our top hit compound, which suppressed tumorigenesis with the lower dose), a lipoxygenase (LOX) inhibitor, and drugs inhibiting nitric oxide (NO) production.

NO is generated by nitric oxide synthase (NOS) and is a key signaling molecule in inflammation, immune response, and cancer (Fukumura et al., 2006). Arachidonate metabolites produced by LOX enzymes are also primary mediators of inflammation (Dennis and Norris, 2015) and cancer (Chen et al., 2009, 2014; Wang and Dubois, 2010; Greene et al., 2011; Steinhilber et al., 2010). Inflammation is an important contributing factor to solid cancer associated with infection and autoimmunity (Coussens and Werb, 2002) and with certain oncogenes (e.g., Myc and Ras) (Mantovani et al., 2008). Therefore, it is particularly important to understand the interplay between these inflammatory mediators and Notch-PI3K/Akt cooperative oncogenesis.

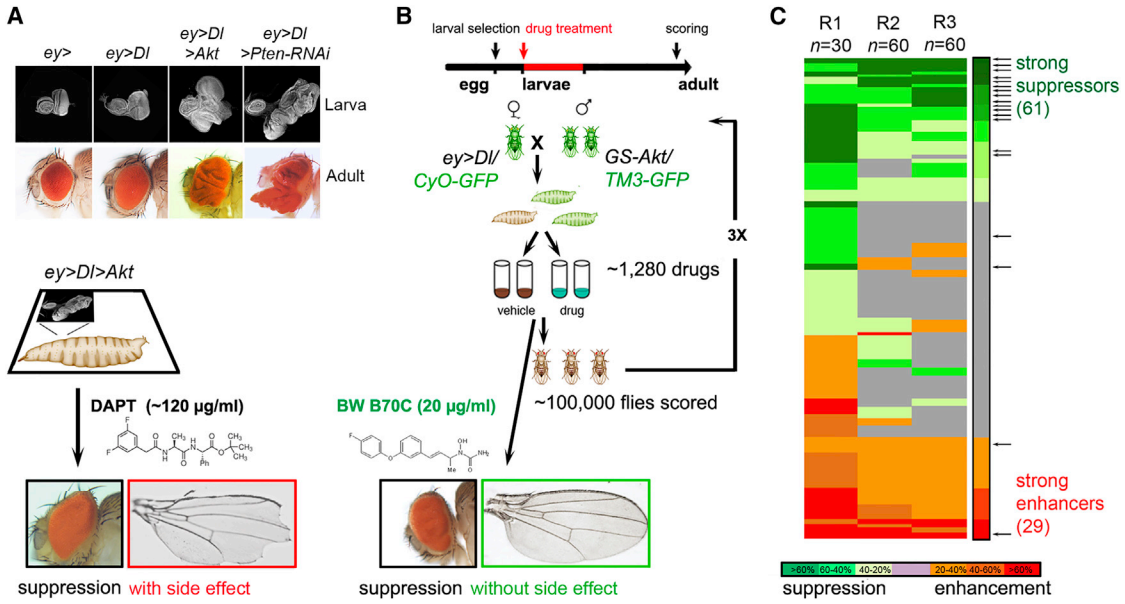

Figure 1. Drug Screen Selectively Targeting Notch-PI3K/Akt Cooperative Oncogenesis

(A) Larval eye imaginal discs (upper row) and adult eyes (lower row) of the control and two tumor models, involving co-overexpression of *Dl* and either *Akt* or *Pten-RNAi* (*BL25967*) using *ey-Gal4* (*ey* >). Below: example of the adult resulting from GSI (DAPT)-treated, tumor-bearing larva. The side effect (notched wings) mimics genetic Notch pathway inhibition.

(B) Schematic of the screen design. Tumor-bearing larvae (non-GFP) were treated with compounds (100 µg/mL in the food) or vehicle. Below: representative adult fly *ey* > *Dl* > *Akt* treated with the top hit compound BW B70C during the larval stage.

(C) Heatmap of the screen results (right column, mean effect). Green, suppression; red, enhancement; gray, no significant change. Arrows point to anticancer drugs in the LOPAC^{1280}. n, number of larvae per drug per round (R).

In vertebrates, the expression of inflammatory markers such as reactive oxygen species, NO, and macrophage infiltration are hallmarks of inflammation in cancer (Colotta et al., 2009; Mantovani et al., 2008). In *Drosophila*, inflammation contributes to adult gut tumorigenesis (Petkau et al., 2017), and both LOX (Miller et al., 1994; Merchant et al., 2008; Stanley, 2006) and NO (Nappi et al., 2000) pathways participate in general inflammatory responses to infection and/or epithelial tissue repair (Wood and Martin, 2017). However, whether *Drosophila* NOS and LOX have a role in tumorigenesis was unknown. To address this, we genetically validated the contribution of the NOS and LOX pathways and inflammation in Notch-PI3K/Akt-driven tumorigenesis. Furthermore, we provide proof-of-concept evidence that BW B70C blocks tumorigenesis in human T-ALL cells by dampening a conserved NOTCH1-PI3K/AKT-eNOS axis.

RESULTS

Unbiased Drug Screen for Targeting Notch-PI3K/Akt Oncogenic Cooperation

We devised a phenotype-based chemical screen to identify agents that block Notch-PI3K/Akt oncogenic cooperation without harming normal cells. We used our *Drosophila* eye cancer model, which captures the molecular features of Notch-PI3K/Akt cooperative oncogenesis (Figures 1A and S1A) (Palomero et al., 2007). The Notch ligand *Delta* (*Dl*) is co-expressed with *Akt* or with an RNAi transgene to silence *Pten*, a PI3K-negative regulator, using the eye-specific promoter *eyeless* (*ey*)-*Gal4*. The cooperative action of these pathways is what causes the development of eye tumors, and the activation of either pathway alone is not sufficient to promote tumorigenesis (Figure 1A) (Ferres-Marco et al., 2006; Palomero et al., 2007). The *ey* > *Dl* > *Akt* and *ey* > *Dl* > *Pten-RNAi* models yield a similar robust eye tumor phenotype (tumor incidence, 70%) (Figures 1A and S1A), allowing the identification of compounds that suppress or further enhance the tumor phenotype. Systemic inhibition of Notch using the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) not only blocks tumorigenesis but also interferes with normal growth, resulting in smaller notched wings and lethality (Figures 1A and S1B). Systemic inhibition of PI3K/Akt signaling using LY294002 or wortmannin also resulted in high lethality (Figure S1B), indicating toxic side effects comparable to those seen in mice and humans (Muellner et al., 2011).

We screened the LOPAC^{1280} library of 1,280 small molecules, including a set of U.S. Food and Drug Administration (FDA)-approved anticancer drugs as internal controls. An annotated list of the known targets of the LOPAC^{1280} drugs is readily available, enabling the transformation of phenotypic screening results into a target-based drug discovery approach (Jones and Bunnage, 2017). We administered each drug in food during the larval period at a concentration of 100 µg/mL in three double-blind rounds (Rs) and then assessed the impact on tumorigenesis and normal tissue growth in adults (Figure 1B). This allowed us to directly evaluate responses and side effects. Antitumor response was calculated as the ratio of non-tumor eyes to tumor eyes in treated flies, normalized to the vehicle control

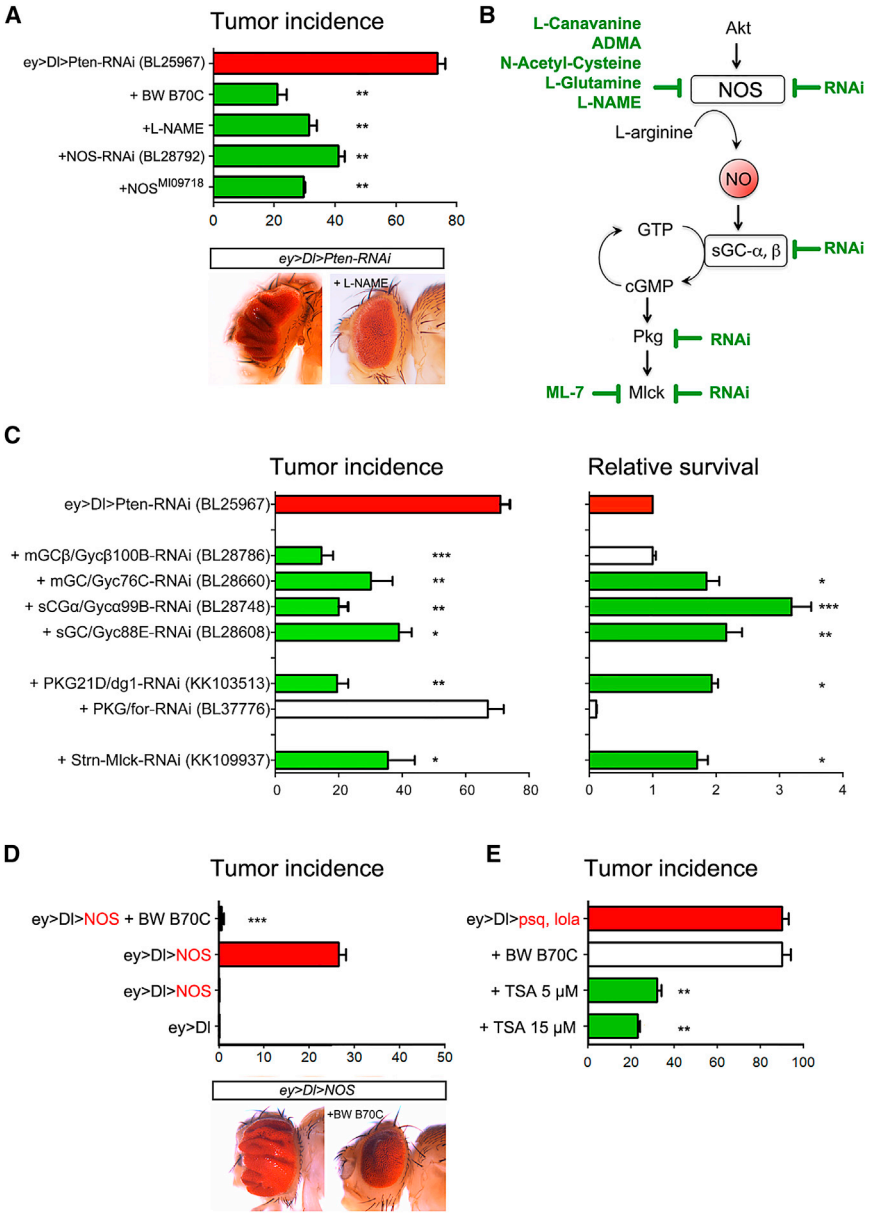

Figure 2. NOS Facilitates Notch-Induced Tumorigenesis

(A) Tumor incidence (as a percentage) in control flies and after pharmacological or genetic inhibition or activation of *NOS*. Below: representative images of control and L-NAME-treated eyes.

(B) Schematic of NO pathway and antitumorigenic drugs identified in our screen and RNAi-based validation.

(C) Tumor incidence (as a percentage, left graph) and normalized survival (right graph) in RNAi-silenced flies (n = 50–100 eyes/genotype).

(D) Tumor incidence (as a percentage) in flies co-expressing *Dl* and *NOS*. Below: representative images of control and BW B70C-treated animals.

(E) Tumor incidence (as a percentage) in Notch-pipsqueak (psq) lola (eyeful cancer) flies with or without trichostatin A (TSA) or BW B70C treatment.

Mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (one-way ANOVA followed by Bonferroni's multiple comparisons test).

group (Figure S1C). Compounds that showed a lethal effect in R1 (n = 30 larvae/drug) were re-tested at lower doses (20 μg/mL).

After R1, any compound causing a response greater than 20% was re-screened (198 suppressor and 276 enhancer compounds) (Figure S1D) using a larger number of animals (n = 60 larvae/drug/R). This significantly reduced the number of false positives and increased reproducibility (>80%) between R2 and R3 (Figure 1C). After screening approximately 100,000 tumor-bearing flies, we found 90 compounds (Figure 1C) that strongly (>60% response) suppressed (61) or enhanced (29) tumorigenesis (see representative eyes and wings in Figures 1A and 1B to compare responses and side effects of DAPT and BW B70C) (Tables S1 and S2). All positive hits were counter-screened in larvae with single oncogene overexpression; none of them rescued single *Dl*- or Akt-induced phenotypes (data not shown), indicating that the identified drugs target the cooperative action of Notch and Akt.

Our screen identified 15 of the 21 known anticancer compounds included in the library (Figure 1C; Table S3) as strong (13) and moderate (2) suppressors of tumorigenesis. Of the remaining 6, 2 were strong enhancers, 2 were lethal, and 2 had no effect. We were able to single out these anticancer drugs, some of which are approved by the FDA for the treatment of leukemia and solid cancers, thus confirming the validity of our screen. These results show a strong positive correlation with the response observed in human cells.

RNAi-Based Validation of Drug Screen Results

The remaining 48 strong suppressors (excluding the 13 known anticancer drugs) are previously unappreciated modulators of Notch-PI3K/Akt-driven tumorigenesis. Because most compounds have a known human molecular target, we validated these results genetically by examining whether tumor-specific RNAi downregulation of candidate target genes (Figures S2A and S3A) mimicked the action of the corresponding compounds. We targeted 92 RNAi lines corresponding to 77 ortholog genes of the annotated and predicted molecular targets of the hit compounds (Table S4). We reasoned that an antineoplastic effect would also rescue tumor-associated lethality. *PI3K-RNAi* was used as a blind positive control, and effects were assessed in adult flies. As a result, we confirmed that 64% of the compounds act through conserved targets rather than indirect side effects (Figures S2B and S2C). This indicates that despite the evolutionary distance of *Drosophila* from humans, we can use our

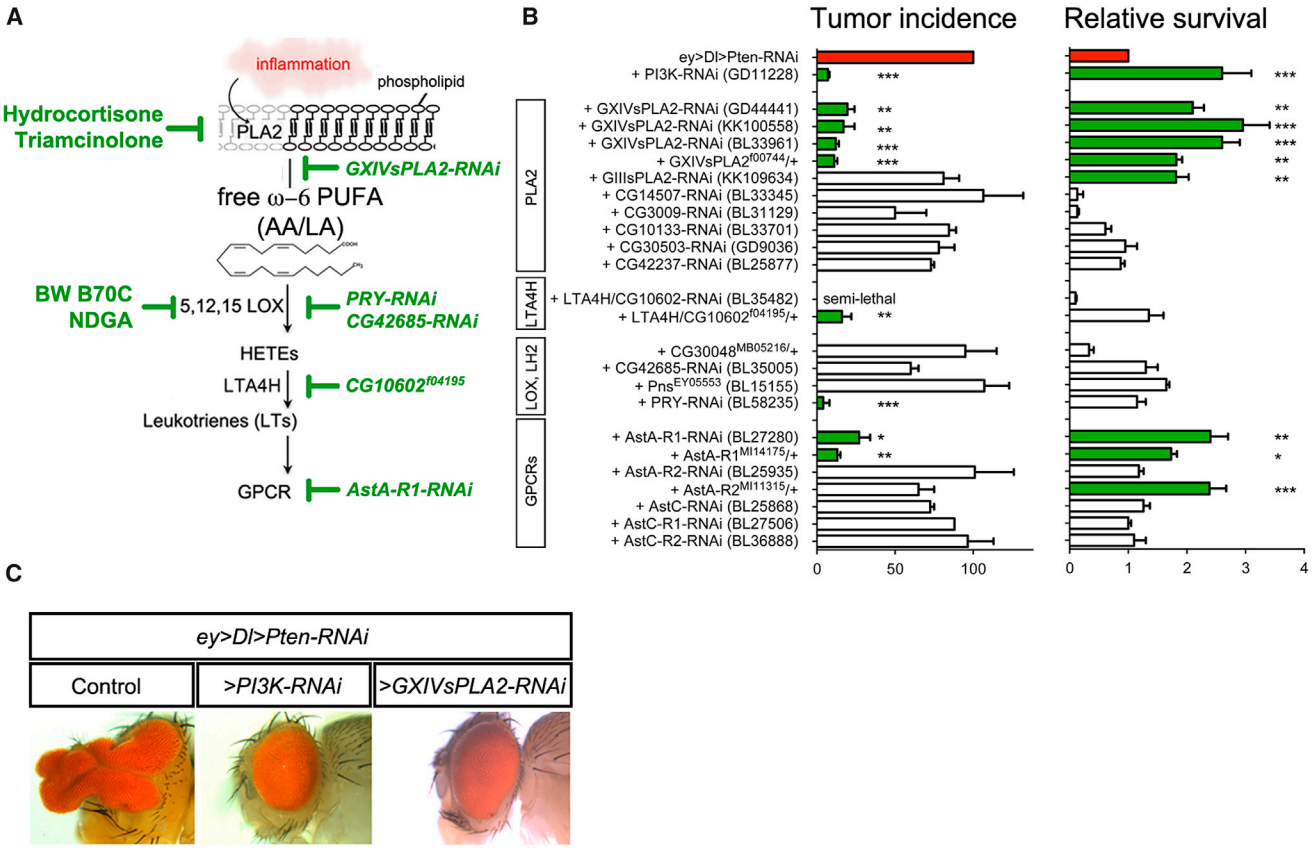

Figure 3. Genetic Targeting of LOX Signaling Blocks Notch-PI3K/Akt Cooperative Oncogenesis

(A) Schematic LOX signaling pathway. Left labels: antitumorigenic drugs identified in our screen and RNAi-based silenced genes. Right labels: homologous *Drosophila* genes. In response to inflammatory stimuli, PLA2 releases arachidonic acid (AA) and/or linoleic acid (LA) from the membrane phospholipids, which are converted to a variety of bioactive lipids via LOX enzymes.

(B) Tumor incidence (left) and normalized survival to adulthood (right) of control and *ey > Dl > Pten-RNAi* flies after depleting the indicated genes via RNAi or mutation. *PI3K92E-RNAi* is the internal positive control. n = 50–100 eyes/genotype.

(C) Example eyes of *ey > Dl > Pten-RNAi* without or with depleted *PI3K92E* or *GXIVsPLA2* via RNAi.

Mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA followed by Bonferroni's multiple comparisons test).

Drosophila-based strategy to identify anticancer drugs, as well as their clinically relevant targets.

PI3K/Akt Fuels Notch-Driven Tumorigenesis through NOS

A survey of the hit compounds classified as strong to moderate suppressors revealed the presence of numerous anti-inflammatory agents targeting the NO/NOS and LOX signaling pathways (Table S1), including BW B70C and nordihydroguaiaretic acid (NDGA), each of which inhibits 5- and 12/15-LOX enzymes (Payne et al., 1991; Hussey and Tisdale, 1996; Rudhard et al., 2015). BW B70C drew considerable attention because it blocked tumorigenesis at a very low dose (20 μg/mL) (Figure 1B; Table S1), especially compared with DAPT (Figures 1A and S1A).

We first investigated how NO signaling contributes to Notch-PI3K/Akt-induced tumorigenesis. Using the *NOS* reporter $NOS^{MI09718}$ (Venken et al., 2011), we observed aberrant expression of *NOS* within the tumor eye tissue (Figure S3B), an action induced by *Pten* depletion (Figures S3A and S3C). Treatment of *ey > Dl > Pten-RNAi* larvae with N(G)-nitro-L-arginine methyl ester (L-NAME), a selective NOS inhibitor with documented activity in *Drosophila* (Mukherjee et al., 2011), significantly suppressed tumor growth (Figure 2A). Similarly, genetic silencing of the single *Drosophila NOS* gene (*ey > Dl > Pten-RNAi > NOS-RNAi*) or a *NOS* endogenous mutation (*ey > Dl > Pten-RNAi;* $NOS^{MI09718/+}$) selectively suppressed tumorigenesis (Figures 2A and S2C).

Moreover, targeting the NO canonical pathway within tumor cells by RNAi silencing of genes encoding soluble guanylyl cyclases (*sGC-α* and *sGC-β*), cyclic guanosine monophosphate (cGMP)-*PKG21D*, and its target, myosin light-chain kinase (*Mlck*), suppressed tumorigenesis (Figures 2B and 2C). These results validate another of the top hit compounds that we identified in our screen: ML-7, an inhibitor of Mlck (Figures 2B and 2C). Altogether, we found that *NOS* was aberrantly expressed in tumor cells and that tumor cell-specific knockdown of NO signaling suppressed tumorigenesis. These results highlight the importance of the NO-sGC/cGMP/PKG (cGMP-dependent protein kinase G) pathway in Notch-PI3K/Akt-driven tumorigenesis.

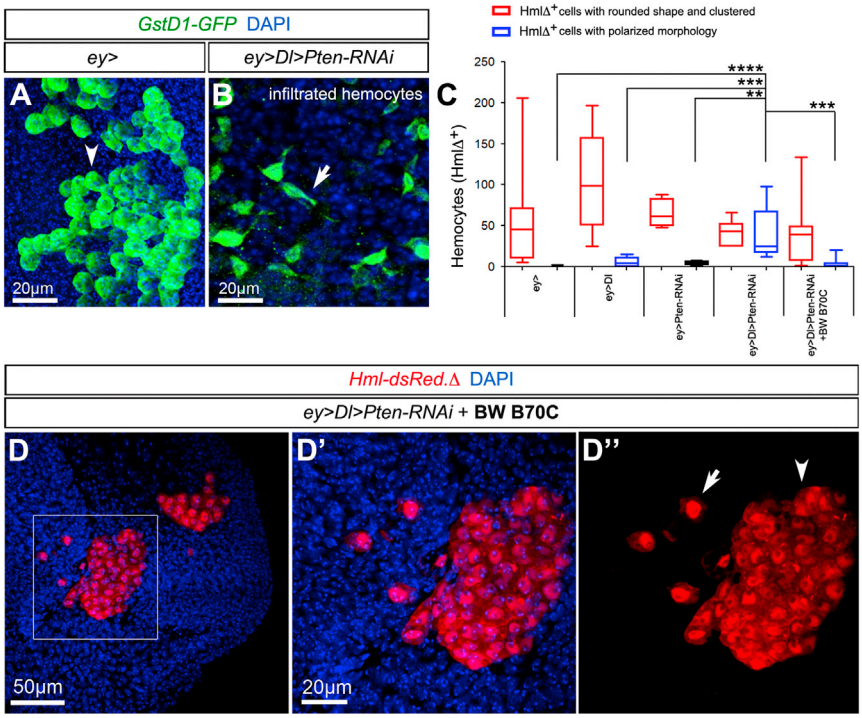

Figure 4. Tumor-Associated Hemocytes and Response to LOX Inhibitor

(A) Hemocytes (arrowhead) in control eye discs (*ey* >) are rounded and form clusters attached to the disc epithelium.

(B) Representative hemocytes in a neoplastic tumor disc with a migratory spindle shape (arrow).

(C) Hemocyte counts in the indicated genotypes (n = 14 eye discs/genotype). Mean ± SD. **p < 0.01, ***p < 0.001, ****p < 0.0001 (one-way ANOVA followed by Bonferroni's multiple comparisons test).

(D) Hemocytes in a Notch-PI3K/Akt eye disc treated with BW B70C (20 μg/mL, 63.2 μM). Right: magnifications of the outlined area. Arrow and arrowhead point to round (pancake-like) and clustered hemocytes, respectively.

Tissue and tumor resident hemocytes are labeled with GstD1-GFP (green, A and B), *Hml-dsRed.Δ* (red, D), and DAPI (blue). For co-localization of GstD1-GFP with the pan-hemocyte marker *Hml-dsRed.Δ*, see Figure S4.

Overexpression of *NOS*, together with overexpression of *Dl*, induced tumorigenesis in the absence of further hyperactivation of PI3K/Akt (*ey* > *Dl* > *NOS*) (Figure 2D). Eye-specific silencing or overexpression of the *NOS* gene alone is inconsequential for eye growth (Cáceres et al., 2011; Jaszczak et al., 2015). BW B70C treatment blocked Notch-NOS-driven tumorigenesis (Figure 2D), suggesting that this process involves an axis with LOX/NOS interdependency. Conversely, tumors induced by the cooperation of Notch with the epigenetic regulators Pisqueak and Lola (Ferres-Marco et al., 2006) were not sensitive to BW B70C, even though they could be suppressed using the epigenetic drug trichostatin A (Figure 2E). Hence, BW B70C does not generally suppress Notch-driven tumorigenesis but dampens a tumor formation process orchestrated by inflammatory NOS.

LOX Pathway Inhibition Blocks Notch-PI3K/Akt-Driven Tumorigenesis

LOX enzymatic activity and LOX-derived lipids have been detected in *Drosophila* extracts and other insects, but the *LOX* gene or genes remained undefined (Pagés et al., 1986; Tan et al., 2016). We therefore searched for *Drosophila* LOX pathway homologs that could be suitable for further validation of our screen results.

Leukotriene A4 hydrolase (LTA4H) catalyzes the production of leukotriene B4 (LTB4), a major lipid product of LOX enzymes that is highly expressed in some cancers (Steinhilber et al., 2010). The *Drosophila* gene *CG10602* encodes an LTA4H homolog (Figure 3A). Halving its gene dosage (*ey* > *Dl* > *Pten-RNAi* > *CG10602*$^{f04195/+}$) markedly suppressed tumorigenesis and rescued tumor-associated lethality (Figures 3B and S3A). Leukotrienes act through G protein-coupled receptors (Wang and Dubois, 2010), and we silenced the allatostatin receptors, the structural orthologs of leukotriene receptors in *Drosophila* (Figure 3A; Table S4). Inactivation of *AstA-R1* suppressed tumorigenesis, whereas silencing *AstA-R2*, *AstC-R1*, and *AstC-R2* did not affect it (Figure 3B).

The most upstream step in LOX-mediated production of proinflammatory lipid metabolites is the release of arachidonic acid from the plasma membrane, mediated by phospholipase A2 (PLA2) (Dennis and Norris, 2015) (Figure 3A). Five suppressor drugs identified in our screen target this step (Figure 3A; Table S1). We tested the seven predicted *Drosophila PLA2* genes (Renault et al., 2002) and found that tumor-specific RNAi silencing of *GXIVsPLA2*, as well as halving its gene dosage (*GXIVsPLA2*$^{f00744/+}$), strongly suppressed tumorigenesis (Figures 3B and 3C), mirroring the antitumor effect of the identified drugs. This confirmed that LOX-generated lipids are required for Notch-PI3K/Akt-driven tumors.

Protumorigenic Immune Inflammation Underlies Notch-PI3K/Akt Cooperation

The participation of the NO/NOS and LOX pathways in Notch-PI3K/Akt-promoted tumorigenesis hints at an unanticipated connection between inflammation and this oncogenic cooperation. Work in vertebrates has implicated macrophage infiltration and expression of inflammatory markers such as NO as key hallmarks of inflammation in solid cancer (Colotta et al., 2009; Mantovani et al., 2008), and immune cells that infiltrate tumors facilitate tumor growth or survival (Grivennikov et al., 2010). In *Drosophila*, macrophage-like hemocytes (Lemaitre and Hoffmann, 2007) have been implicated in the immune response against epithelial tumors (Pastor-Pareja et al., 2008; Cordero et al., 2010).

We examined the hemocytes associated with these tumors using the hemocyte-specific marker *Hml-dsRed.Δ* (Makhijani

et al., 2011) and the oxidative stress reporter *GstD1-GFP*, which we found is expressed in hemocytes (Figure S4A). Wild-type and hyperplastic eye disc-associated hemocytes typically form aggregates with a rounded morphology (Figures 4A, S4B, and S4C) and are attached to the basal membrane (Cordero et al., 2010). We observed that hemocytes within Notch-PI3K/Akt discs were dispersed and became polarized (spindle shaped) (Figures 4B, S4D, and S4E), infiltrating the tumor epithelium (Figures 4C, S4F, and S4G). This suggests that hemocytes change their morphology in response to signals from tumor cells. Consistent with this idea, these morphological changes were suppressed in mutant discs treated with BW B70C (Figures 4C and 4D), suggesting that NOS/LOX activity shapes the inflammatory response in Notch-PI3K/Akt tumors. Altogether, these data link inflammation to tumorigenesis driven by these oncogenes.

Genetic Depletion of Prophenoloxidase in Immune Cells Fuels Notch-Mediated Tumorigenesis

A salient feature of cancer-related inflammation is immunosuppression (Coussens and Werb, 2002; Mellman et al., 2011). In *Drosophila*, melanization—a process mediated by the enzyme phenoloxidase (PO) encoded by the prophenoloxidase (PPO) genes—is a critical innate immune response to tumor cells (Minakhina and Steward, 2006). Platelet-like crystal cells, another class of hemocytes present in larval stages, are the site of *PPO* gene synthesis (Binggeli et al., 2014). We examined *PPO* expression and function to further investigate the participation of inflammation and immunosuppression in Notch-PI3K/Akt tumorigenesis. Larvae with single Notch pathway overactivation (*ey > Dl*) showed robust stimulation of *PPO1* and *PPO2* expression in immune cells (Figure 5A). Conversely, tumor-bearing (*ey > Dl > Pten-RNAi*) and single PI3K/Akt (*ey > Pten-RNAi*) larvae did not show this response (Figure 5A), suggesting that activated PI3K/Akt signaling dampens a secreted signal required in crystal cells to activate the immune response. To ascertain the role of immune cell-derived PPO/PO in single *Dl*-induced overgrowth, we created a genetic immunosuppressed condition using a triple *PPO1, PPO1, PPO3* knockout (Binggeli et al., 2014). Halving *PPO* gene dosage resulted in 55% of the emerging adults bearing full-blown tumors (*ey > Dl*, $PPO1^{-/+}$, $PPO2^{-/+}$, $PPO3^{-/+}$) (Figure 5B), equal to the effect of *NOS* overexpression (Figure 2D). Reducing *PPO* in Notch-PI3K/Akt larvae with already-low *PPO* levels did not enhance tumorigenesis. Furthermore, we found that aberrant *NOS* expression was sufficient to dampen *PPO* expression (Figure 5C) and the immune response triggered by the PO-activating cascade manifested as a strong reduction of melanized crystal cell response after heat stress (Neyen et al., 2015) (Figures 5D and 5E) (see Supplemental Experimental Procedures). Altogether, these observations indicate that immunosuppression is driven by aberrant NOS promoted by activated PI3K/Akt in the tumor cells, which explains how activated PI3K/Akt unleashes the oncogenic potential of Notch.

Validation in Human T Cell Acute Lymphoblastic Leukemic Cells

We validated the antitumor effect of BW B70C in well-established human T-ALL cell models that depend on NOTCH1 and PI3K/AKT signaling (Palomero et al., 2007). We observed that BW B70C treatment killed T-ALL cells (Palomero et al., 2007) that were resistant to Notch inhibitors (*PTEN*-negative, γ-secretase inhibitor [GSI]-resistant T-ALL cell lines RPMI8402, CCRF-CEM, P12-ICHIKAWA, JURKAT, and MOLT-3), as well as *PTEN*-positive, GSI-sensitive T-ALL lines (CUTLL1, ALL-SIL, and DND-41) (Figure 5F). BW B70C treatment had little or no toxicity against normal T lymphocytes (peripheral blood mononucleated cells [PBMCs]) derived from healthy donors (Figure 5F). Moreover, paralleling the results obtained in *Drosophila* tumors, we found that one of the three *NOS* genes, endothelial NOS (*eNOS*), was aberrantly enriched in AKT/NOTCH1-driven T-ALL cells (Figure 5G). Healthy PBMCs did not show *eNOS* expression (Figure 5H). Finally, we found that BW B70C selectively killed T-ALL cells associated with suppression of the aberrant *eNOS* in leukemic cells (Figure 5H).

DISCUSSION

Several Notch and PI3K/Akt inhibitors with potent antineoplastic activity are available, but their progress toward clinical use is hindered by side effects associated with the inhibition of physiological signaling and by drug resistance (Andersson and Lendahl, 2014; Chia et al., 2015; Fruman and Rommel, 2014). The characterization of the targets and mechanisms downstream of Notch-PI3K/Akt in tumorigenesis that are distinct from their targets in normal cells is crucial for identifying cancer vulnerabilities that could be exploited therapeutically. Using an *in vivo* drug screen in *Drosophila* we have identified pharmacologically active compounds that block Notch-PI3K/Akt-driven tumors in flies and validated the top hit compound in human T-ALL cells with NOTCH1 and PI3K/AKT mutations. In addition, BW B70C and compounds inhibiting specific inflammatory pathways were found to elicit potent and selective antitumorigenic responses in Notch-PI3K/Akt tumors by blocking a hitherto unsuspected NOS/LOX axis. Our screen identified 15 of the 21 well-known anticancer compounds included as internal controls; some of them have anti-inflammatory properties (Table S3), but most act mainly by blocking cell proliferation non-specifically through DNA damage.

Genetic studies further highlighted a strong requirement for tumor-specific inflammation driven by LOX- and NOS-dependent Notch-PI3K/Akt cooperation. Human LOX signaling (Chen et al., 2009; Hussey and Tisdale, 1996) and NO signaling (Fukumura et al., 2006; Lim et al., 2008) have been linked to specific cancers as both tumor suppressors and tumor enhancers. Here we linked these inflammatory pathways to tumor initiation by Notch-PI3K/Akt cooperation. The oncogenes Ret, Myc, and Ras can trigger an intrinsic inflammatory response that creates a protumorigenic microenvironment (Mantovani et al., 2008), which accelerates cancer development (Grivennikov et al., 2010). We found that activated PI3K/Akt signaling triggers inflammation and immunosuppression via aberrant *NOS* expression. Overexpressing *NOS* or diminishing the endogenous immune response is sufficient to facilitate tumor initiation via the activated Notch pathway, supporting the notion that inflammation is a key mechanism to unleash the oncogenic potential of

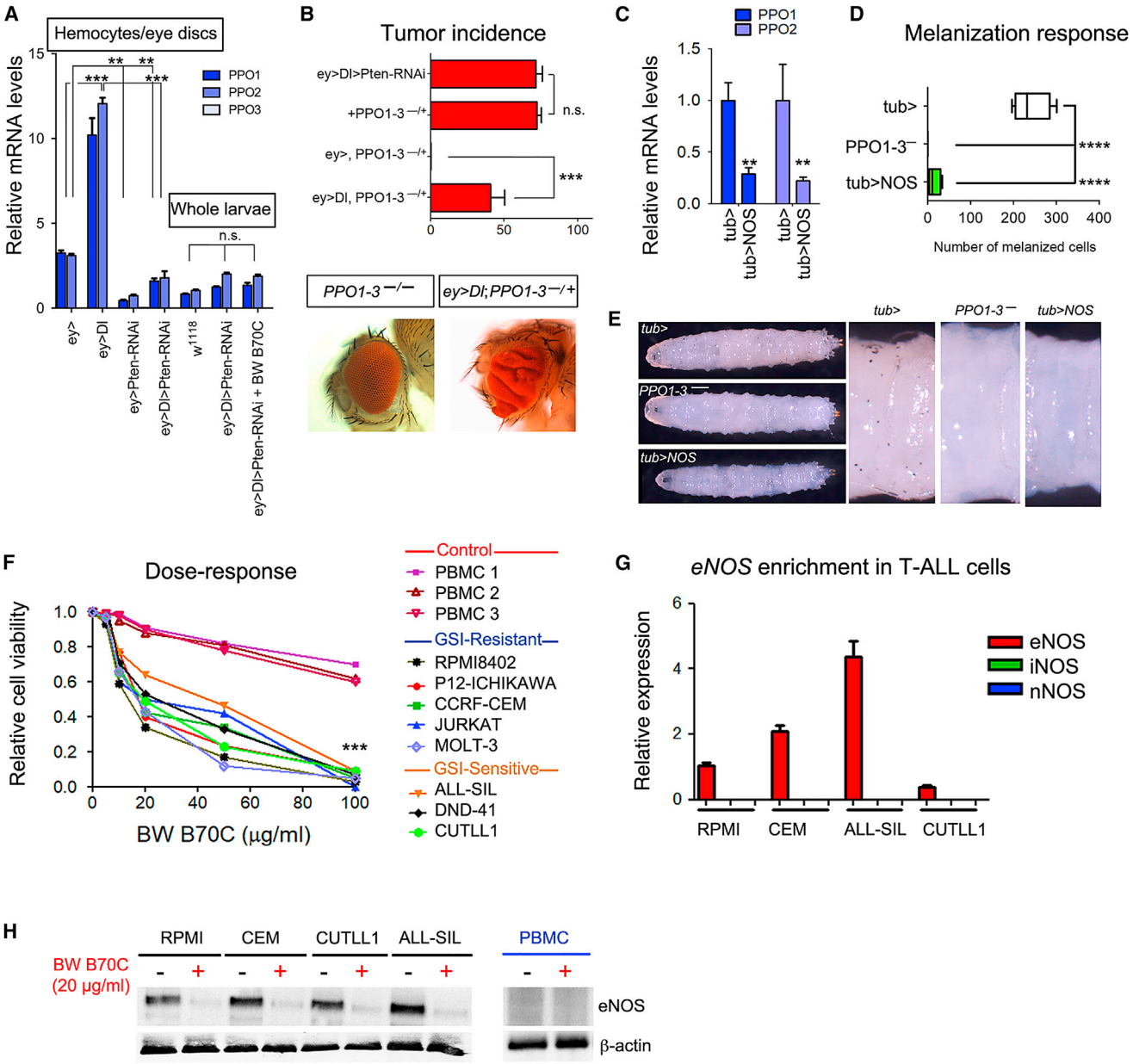

Figure 5. Immunosuppression Releases Notch Oncogenic Potential

(A) *PPO* gene expression in immune cells attached to eye discs (n = 30/genotype) and whole larvae (n = 5/genotype). *PPO3* was undetected in these assays. Experiments were performed in triplicate.

(B) Relative tumor incidence (as a percentage) in *ey > Dl; PPO1–PPO2*$^{-/+}$ (n = 50–100 eyes). Below: representative eyes.

(C) *PPO1* and *PPO2* expression in control and *tub > NOS* larvae.

(D and E) Melanized crystal cell counts (D) and images (E, right, magnifications) of larvae with crystal cell-mediated PPO/PO activity (black cells) response to heat shock. Negative control was *PPO1–3*$^{-}$.(F) BW B70C treatment in a panel of T-ALL cell lines and healthy PBMCs. Data represent three independent experiments and are expressed as mean ± SD. Student's t test for each T-ALL cell line response was ***p < 0.001. Mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA followed by Bonferroni's multiple comparisons test in B and Student's t test in D).

(G) qRT-PCR analysis of the three *NOS* genes in T-ALL cells (relative to *GADPH*). Graph shows pooled data from three independent experiments and represents mean ± SD.

(H) Representative western blots of three independent analyses showing eNOS levels in PBMCs and T-ALL cells treated with BW B70C (20 µg/mL, 48 hr) or DMSO (vehicle).

Notch. LOX/NOS inhibition did not harm normal cells, which suggests that these pathways represent promising, safe, drug-gable targets for human cancers.

Validation of the anti-inflammatory drug BW B70C in a panel of human T-ALL cells dependent on NOTCH1 and PI3K/AKT yet resistant to Notch inhibitors (Palomero et al., 2007) further

highlights the considerable value of unbiased chemical screens in *Drosophila* when it comes to deciphering targets and potential therapeutic approaches relevant to human cancers.

EXPERIMENTAL PROCEDURES

Drosophila Husbandry

The list of RNAi transgenes used is in Table S4. Other fly stocks used were w^{1118}, *ey-Gal4*, *UAS-Dl, CyO twist-GFP, CyO tub-Gal80, Pten-RNAi* (BL25967), *UAS-NOS* (BL56830 and BL56823), $GXIVsPLA2^{f00744}$, $CG10602^{f04195}$, $Pns^{EY05553}$, $AstA\text{-}R1^{MI14175}$ (y^1 *w*; Mi{MIC}AstA-*$R1^{MI14175}$), $NOS^{MI09718}$ (y^1 *w*; Mi{MIC}* $Nos^{MI09718}$), and $PKG/dg2^{MI02855}$ (y^1 *w*; Mi{MIC}dg2*^{MI0285}), all from the Bloomington Drosophila Stock Center; *PI3K92E-RNAi* (*GD11228*, *v38985*) from the Vienna Drosophila RNAi Center; *GS(2)1D233C* (*dAkt1*) (Palomero et al., 2007); $GS(2)88A8^{lola\ pipsqueak}$ (the eyeful cancer strain) (Ferres-Marco et al., 2006); $PPO^{\Delta 1-2,3}$ (a gift from B. Lemaitre); GstD1-GFP (a gift from D. Bohmann); and *Hml-dsRed.Δ* (FBtp0069700) (a gift from K. Brueckner). Flies were reared and maintained in standard fly food at 27°C on a 12-hr light/dark cycle.

Statistical Methods

All statistical analyses were performed in GraphPad Prism 6. qPCR data and melanized crystal cell counts were analyzed using unpaired Student's t tests. For tumor incidence and hemocyte counts, p values were calculated using one-way ANOVA followed by Bonferroni's multiple comparison tests.

All research and human cell procedures were conducted in strict compliance with the European Community Council Directives and Spanish legislation. The protocols were approved by the Universidad Miguel Hernández (2017/VSC/PEA/00154) at the Institute of Neuroscience.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.049.

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AUTHOR CONTRIBUTIONS

S.N.V., R.G., I.G.-P., J.G.-C., D.M.V., and V.G.D.R. performed the drug screen; S.N.V. and L.G.-L. performed the functional experiments; E.B.-I. provided technical support; J.M. contributed to supervision of the *Drosophila* drug screening; M.D. contributed by providing the general concept, study design, and supervision; and S.N.V. and M.D. performed data analyses and interpretation and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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