Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(6): 124
https://doi.org/10.31083/j.fbl2806124

Original Research

Exposure to Therapeutic BTK Inhibitors Induces Phenocopying of *Btk29A* Mutants in the Fruit Fly *Drosophila melanogaster*

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Academic Editor: Elena Levantini
Submitted: 19 April 2023 Revised: 22 May 2023 Accepted: 1 June 2023 Published: 27 June 2023

Abstract

Background: Bruton's tyrosine kinase (BTK) is a non-receptor type tyrosine kinase originally identified as the genetic signature responsible for X-linked agammaglobulinemia (XLA) when mutated. Its functional form is required for B lymphocyte maturation in both humans and mice, whereas loss-of-function causes a different form of developmental defect in the fruit fly, *Drosophila melanogaster*. **Methods**: Ibrutinib and other therapeutic inhibitors of BTK have been extensively used to successfully treat various leukemias and lymphomas. *Btk29A* type 2 is the ortholog of BTK in the fruit fly. We show that feeding wild-type flies an ibrutinib-containing diet induces phenocopying of *Btk29A* mutants, i.e., failure in the fusion of left and right halves of the dorsal cuticles, partial loss of wing tissues and dysregulation of germ cell production. **Results**: We have previously reported that *Btk29A* phosphorylates *Drosophila* Arm (β-catenin), and ibrutinib reduces phosphorylation at Tyrosine142 of endogenously expressed β-catenin in Cos7 cells transfected with *Btk29A* type 2 cDNA. **Conclusions**: Thus, *Drosophila* is suitable for screens of novel BTK inhibitor candidates and offers a unique *in vivo* system in which the mode of action of BTK inhibitors can be examined at the molecular, cellular, and organismal levels.

Keywords: non-receptor tyrosine kinase; beta-catenin; germ cell proliferation; morphogenesis; drug screens; disease model

1. Introduction

Bruton tyrosine kinase (BTK) was originally identified as a non-receptor tyrosine kinase whose deficiency is responsible for X-linked agammaglobulinemia (XLA) in humans [1–3]. Patients with XLA suffer from severe antibody deficiency and recurrent infections due to a differentiation defect causing the absence of mature B lymphocytes [4–6]. Conversely, inhibition of BTK has been shown to ameliorate various forms of leukemia and lymphoma [7,8], with the premier example being chronic lymphocytic leukemia (CLL) [9–11]. Ibrutinib is the first such inhibitor of BTK to be clinically applied [7,8,10,11]. However, there remain uncertainties as to how it alters signaling mediated by BTK *in vivo*, ultimately affecting development, physiology, and behavior at the organismal level. In addressing these questions, a genetically tractable *in vivo* animal model would be most useful.

Drosophila melanogaster (hereinafter *Drosophila*) is an outstanding model organism due to its amenability to experimental manipulations at the molecular, cellular, and organismal levels. The *Btk29A* type 2 isoform encoded by the gene *Btk29A* is the sole ortholog of human BTK in *Drosophila*: the other isoform, *Btk29A* type 1, has a unique N-terminus and distinct expression and is thus structurally and functionally separable from *Btk29A* type 2 [12]. *Btk29A* type 2 has been shown to play an essential role in dorsal closure in postembryonic development [13], a morphogenetic process to suture the left and right halves of the exoskeleton. *Btk29A* type 2 is also pivotal for stem cell niche functions, e.g., germ cell proliferation and differentiation, as well as follicle precursor migration in the ovary at the adult stage [13–18]. In the ovary, the *Btk29A* type 2 protein was shown to bind to β-catenin and to phosphorylate specific tyrosine residues of β-catenin to modulate Wnt4 signaling, thereby regulating germ cell development [19,20]. Furthermore, *Btk29A* type 2 was shown to mediate behavioral habituation in adult flies, which was abrogated by ibrutinib [18]. Thus, *Drosophila* will offer an ideal platform to evaluate the actions of ibrutinib on the BTK ortholog. Here we show that dietary ibrutinib recapitulates the effects of the *Btk29A* mutant on the morphogenesis of external structures and germ cell development, likely through its interference with β-catenin–*Btk29A* interactions.

2. Materials and Methods

2.1 Flies

Flies were raised on cornmeal-agar-yeast medium at 25 °C. The Canton-Special (CS) strain was used to test the drug effects. The *Btk29A*^{ficP} allele was isolated in our laboratory. The *c587-Gal4* line was a generous gift from D. Drummond-Barbosa. The *UAS-RNAi* and *UAS-Dicer2* strains were purchased from the Vienna *Drosophila* RNAi Center and Bloomington Stock Center, respectively.

2.2 Characterization of External Structures

Wings: Wings were removed from thoraces for observation and image acquisition using an Axio microscope (ZEISS, Oberkochen, Germany).

Thoraces: Dorsal thoraces were photographed under an M205FA microscope (Leica, Wetzlar, Germany). The distance between the left and right counterparts of homologous innermost bristles was measured by counting the number of intervening pixels on the computer screen with the aid of Photoshop software. Half of the value of the inter-bristle distance thus measured was defined as the distance between the bristle and the midline and used for comparisons among tested fly groups. Some of the flies fed an ibrutinib-containing diet during the larval stage showed disrupted dorsal structures. The proportion of flies exhibiting the dorsal open phenotype was calculated to quantify the ibrutinib action on the dorsal structure formation.

2.3 Histology

For the antibody staining, ovaries were dissected in Phosphate-Buffered Saline (PBS) and immersed in 4% paraformaldehyde in PBS for 40 minutes. The ovaries were then washed three times in PBS-T (PBS supplemented with 0.1% Triton-X), and the reaction was blocked for 1.5 h in PBS supplemented with 0.1% Triton and 0.5% Bovine Serum Albumin (BSA). Then, the tissue was incubated with a primary antibody for 3 h at room temperature or at 4 °C overnight. The primary antibodies used in this study were mouse monoclonal 1B1 (1:10; Developmental Studies Hybridoma Bank) and rat anti-Vasa (1:10; Developmental Studies Hybridoma Bank). The fluorescence-conjugated secondary antibodies were from Molecular Probes and were used at a 1:200 dilution. All samples were mounted in 80% glycerol.

2.4 Transfections and Western Blotting

Cos7 cells (American Type Culture Collection (ATCC), Washington, DC, USA) were seeded overnight at 50% confluence in 6-well plates. Mycoplasma testing has been done for the cell lines used. Transfections were carried out by using polyethylenimine (PEI) (Polyscience, Warrington, PA, USA). Cells at 48 h post-transfection were harvested, washed twice with PBS in Eppendorf tubes, and re-suspended in 1 mL PBS. Cells were collected by low-speed centrifugation of 1250 rpm (500 g) at 4 °C. The collected cells were re-suspended in 100 µL of lysis–Radioimmunoprecipitation assay (RIPA) buffer containing cocktails of protease inhibitor and phosphatase inhibitors, and the sample was then boiled at 65 °C for 2–3 min, vortexed, and centrifuged. The protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with the respective antibody. The anti-pY142 (ECM Biosciences, Versailles, KY, USA) antibody was diluted 1:1000, the anti-HA (Sigma-Aldrich, Burlington, MA, USA) antibody was diluted 1:2000, and the anti-β-catenin (BD Biosciences, Franklin Lakes, NJ, USA) antibody was diluted 1:2000.

Pharmacological inhibition was achieved with ibrutinib (PCI-32765; Pharmacyclics, Sunnyvale, CA, USA). The ibrutinib kept at –20 °C was dissolved in dimethyl sulfoxide (DMSO) to produce a 10 mM solution, which was added to PBS to attain a final concentration of 10 µM for application onto cells. For each experiment, fresh dilutions were prepared. At 36–48 h post-transfection, cells were starved under serum-free conditions for 4 h, and inhibitors were added during the last 2 h period of starvation.

The band intensity was quantified with the aid of the ImageJ (version 1.53t, NIH, Bethesda, MD, USA) software run on a MacIntosh computer, and obtained data were transferred to Microsoft Excel (version 16, Microsoft, Redmond, WA, USA) and presented as column histograms. The bars represent the means and standard errors of two independent experiments. Data analysis was carried out using statistically available software Graph Prism software (version 6 for MacOS, Boston, MA, USA). Comparisons between groups were made using one-way analysis of variance (ANOVA). p values < 0.05 were considered significant. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., statistically non-significant.

2.5 Bruton's Tyrosine Kinase Inhibitor Feeding

Fly food was softened by heating and then kept for 3 min to cool it down to ~40 °C before mixing with concentrations of BTK inhibitors, followed by cooling again to room temperature. DMSO (the final concentration: 0.38%) as a control was similarly added to food following the same protocol. Parental flies were transferred to food containing DMSO or a BTK inhibitor for 5 days so that their offspring would develop in food containing DMSO or the BTK inhibitor. Adult offspring were transferred 5 days after emergence to analyze their phenotypes. To determine the concentrations of ibrutinib for oral administrations in phenotypic analyses, female flies were allowed to lay eggs on the culture medium (2.6 mL) supplemented with either 200, 100, 75, 50, 25, 20, 15, or 10 µL of the 50 µM drug solution and the offspring were subjected to preliminary analysis: eggs deposited on the medium containing 200 or 100 µL of the drug solution failed in hatching; larvae were unable to complete development in the medium with 75 or 50 µL of the drug solution; adults emerged when grown on the

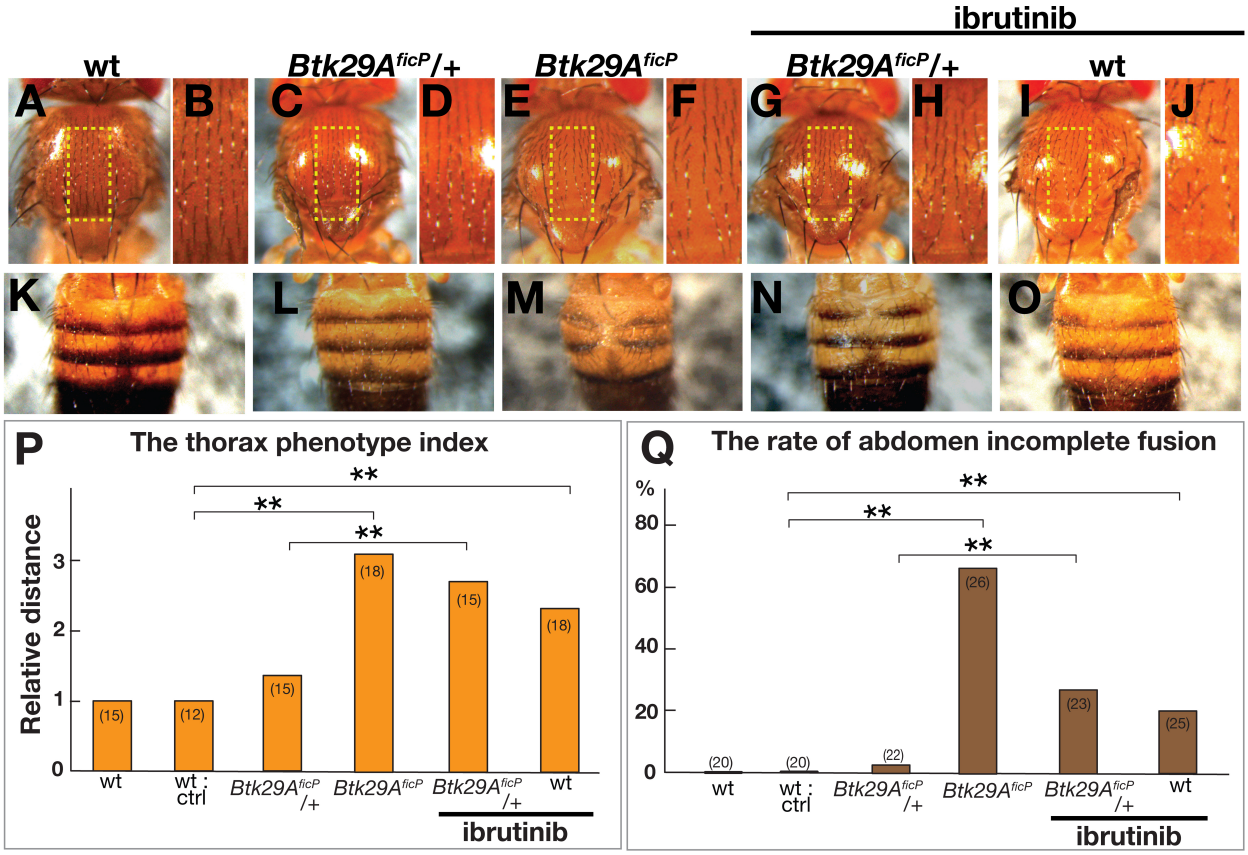

Fig. 1. Dorsal nota and tergites of *Drosophila melanogaster*. (A–O) Dorsal nota (A–J) and tergites (K–O) of male wild-type (A, B, K and I, J, O), *Btk29A*^{ficP}/+ (C, D, L and G, H, N), or *Btk29A*^{ficP} (E, F, M) flies without (A–F and K–M) or with the administration of 10 µL of 50 µM ibrutinib onto 2.6 mL fly culture media (G–J and N and O), throughout the life cycle. The boxed region in (A, C, E, G, and I) is enlarged in (B, D, F, H, and J), respectively. (P and Q) Quantitative analysis of suture levels in the dorsal notum and tergite. (P) The distances were measured for a bilateral bristle pair that were positioned closest to the midline. Shown are the distances relative to the mean inter-bristle distance of wild-type flies (wt) for indicated fly groups. The numbers of flies examined are indicated in parentheses for each fly group in (P) and (Q). The data for untreated wild-type flies are indicated as wt, whereas the data for flies treated with solvent DMSO only are indicated as wt: ctrl (these designations were also used in the other figures). The statistical significance of differences was evaluated by Student's *t*-test: $^{**}p < 0.01$ (P) or by Fisher's exact test: $^{**}p < 0.01$ (Q).

media containing 15 or 10 µL of the drug solution. Based on this observation, we applied 10 µL of 50 µM ibrutinib onto fly media for subsequent phenotypic analyses. Larvae consume only small portions of the drug-supplemented medium during the entire larval stage, which spans about 7 days, whereas 400–500 mg ibrutinib is administered daily to adult human patients.

3. Results

Visual inspection of the notum of adult flies suggested that feeding ibrutinib throughout the larval stage results in the widening of the distance between left and right homologous bristles along the midline (Fig. 1A–D,G–J), a phenotype also observed in *Btk29A*^{ficP} mutants (Fig. 1E,F,P). Measurements of the distance between the midline and innermost bristles in the control and test groups of flies demonstrated that feeding ibrutinib significantly widens this distance (Fig. 1P, **Supplementary Fig. 1**), suggesting a defect in dorsal closure in the ibrutinib-fed flies. Failures in dorsal closure were also manifested as an incomplete fusion of bilateral tergites in *Btk29A*^{ficP} mutants (Fig. 1K–M), which was phenocopied by feeding an ibrutinib-containing diet to flies of control genotypes (Fig. 1N,O,Q). It remains an open question as to whether flies with a severe thorax phenotype concordantly have a severe dorsal phenotype. In addition, we noted that a substantial proportion of ibrutinib-fed flies display distorted wing structures, particularly a loss of certain regions near the wing margin (Fig. 2C *cf.* Fig. 2A,B). Similar wing phenotypes were observed with an equivalent frequency in *Btk29A*^{ficP} mutant flies that were not treated with ibrutinib (Fig. 2C,E).

We then examined, in wild-type flies, the possible effects of ibrutinib feeding on oogenesis, during which *Btk29A* mutations are known to induce overproduction of

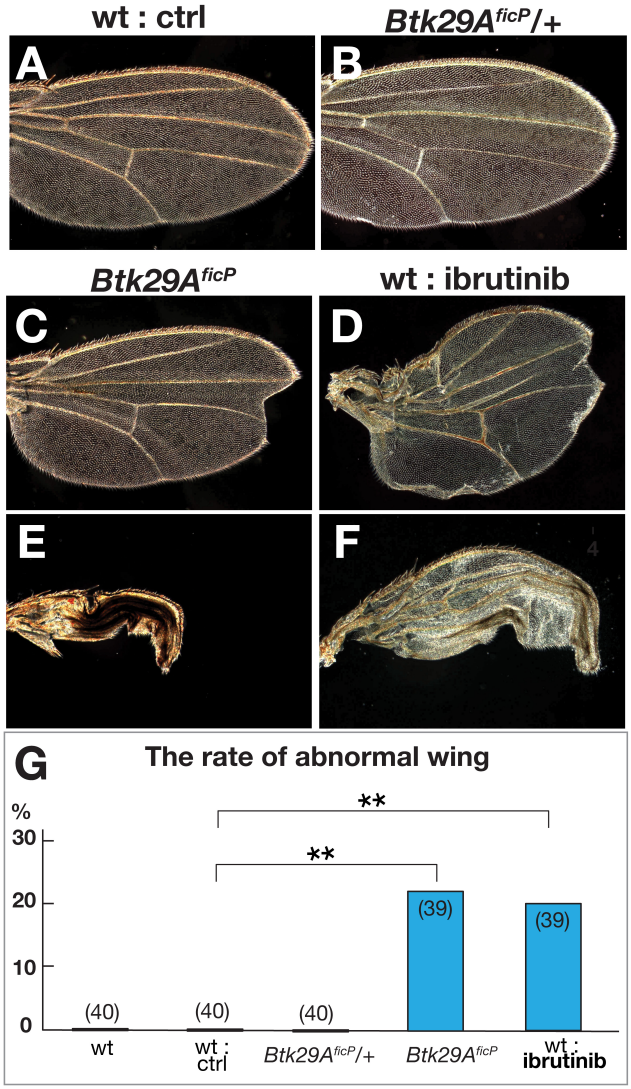

Fig. 2. Wing phenotypes in flies deficient in functional *Btk29A* type 2. (A–F) Wings of wild-type (A, D, and F) flies without (A) or with ibrutinib (D and F) and *Btk29A*^{ficP}/+ (B) and *Btk29A*^{ficP} (C and E) flies without ibrutinib throughout the life cycle. (G) The proportion of flies (%) with wing defects is indicated in each panel. **Statistically significant at $p < 0.01$ by Fisher's exact test.

cystoblasts (see below), which are directly derived from germ stem cells (GSCs); a cystoblast differentiates into cystocytes that transform into an oocyte and nurse cells after four rounds of divisions [21,22]. This process of germ cell proliferation takes place in the anterior tip of an ovariole called the germarium, which contains one to three GSCs, several cystoblasts, and tens of cystocytes in wild-type flies [21]. Feeding ibrutinib caused underproduction of cystoblasts (Fig. 3E,F,K and L *cf.* Fig. 3A,G), as identified by the presence of spectrosomes (recognized as round structures rich in actin), often leading to a complete lack of cystoblasts in a substantial proportion of germaria (Fig. 3M). This is in sharp contrast to the germaria of *Btk29A*^{ficP} mutants, where supernumerary cystoblasts were usually observed (Fig. 3B,H,M). However, when *Btk29A* was knocked down with *Btk29A* RNAi in escort cells that support the maintenance of GSCs in the germarium, some ovaries of the manipulated flies carried an empty germarium (Fig. 3D,J,M). We infer that GSCs failed to reproduce themselves as the result of a strong bias toward differentiating into cystoblasts, resulting ultimately in the empty germarium. Conversely, when a GSC was maintained even upon accelerated cystoblast differentiation, the number of cystoblasts would increase. Because the *Btk29A*^{ficP} mutation removes the type 2 *Btk29A* isoform, sparing the type 1 isoform, it might be that the type 1 isoform partially compensated for the lack of type 2 so that the GSC was maintained and produced more cystoblasts. In contrast, the *UAS-Btk29A* RNAi transgene likely knocked down both type 1 and type 2 isoforms, leading to contrasting outcomes, i.e., loss of the GSCs and cystoblasts.

Finally, we examined whether ibrutinib affects molecular interactions between *Btk29A* and β-catenin. As an experimental system, we chose the primate cell line Cos7 rather than flies because the former express no endogenous Btk, simplifying the interpretation of the assay result, while the latter has two *Btk29A* isoforms under complex control by other kinases, which complicates the analysis of *Btk29A*-β-catenin interactions. Indeed, our previous experiment demonstrated that fly *Btk29A* phosphorylates endogenous β-catenin at its Y142 when transfected into Cos7 cells [19]. *Btk29A* carrying an HA tag was transfected into Cos7 cells, and the cell lysates were subjected to immunoblotting with an anti-pY142 β-catenin antibody. The tyrosine residue at position 142 (Y142) is one of the major *Btk29A*-dependent phosphorylation sites of β-catenin *in vivo* [19]. The results showed that incubation of cells with 10 µM ibrutinib reduced the amount of a Y142-phosphorylated population of endogenously expressed β-catenin (Fig. 4A), and this reduction was statistically significant (Fig. 4B). We suggest that ibrutinib interferes with *Btk29A*–β-catenin interactions, thereby manifesting defects in oogenesis and other morphogenetic processes *in vivo*. It remains to be examined whether the phosphorylation of Armadillo Y150 homologous to β-catenin Y142 is inhibited by ibrutinib in the fly ovary.

4. Discussion

In the present work, we showed that ibrutinib faithfully reproduced some of the *Btk29A* mutant phenotypes in *Drosophila* when orally administered during the larval and adult stages, an observation consistent with the view that BTK and its ortholog are the major targets for ibrutinib in both humans and flies. It is also reminded that ibrutinib does not distinguish between the two *Btk29A* isoforms because they share the same kinase domain. Our results also point to the possibility that *Drosophila*, with its genetic tractability and rich collections of molecular resources, may provide an ideal *in vivo* experimental system for elucidating the mode of action of therapeutic drugs that

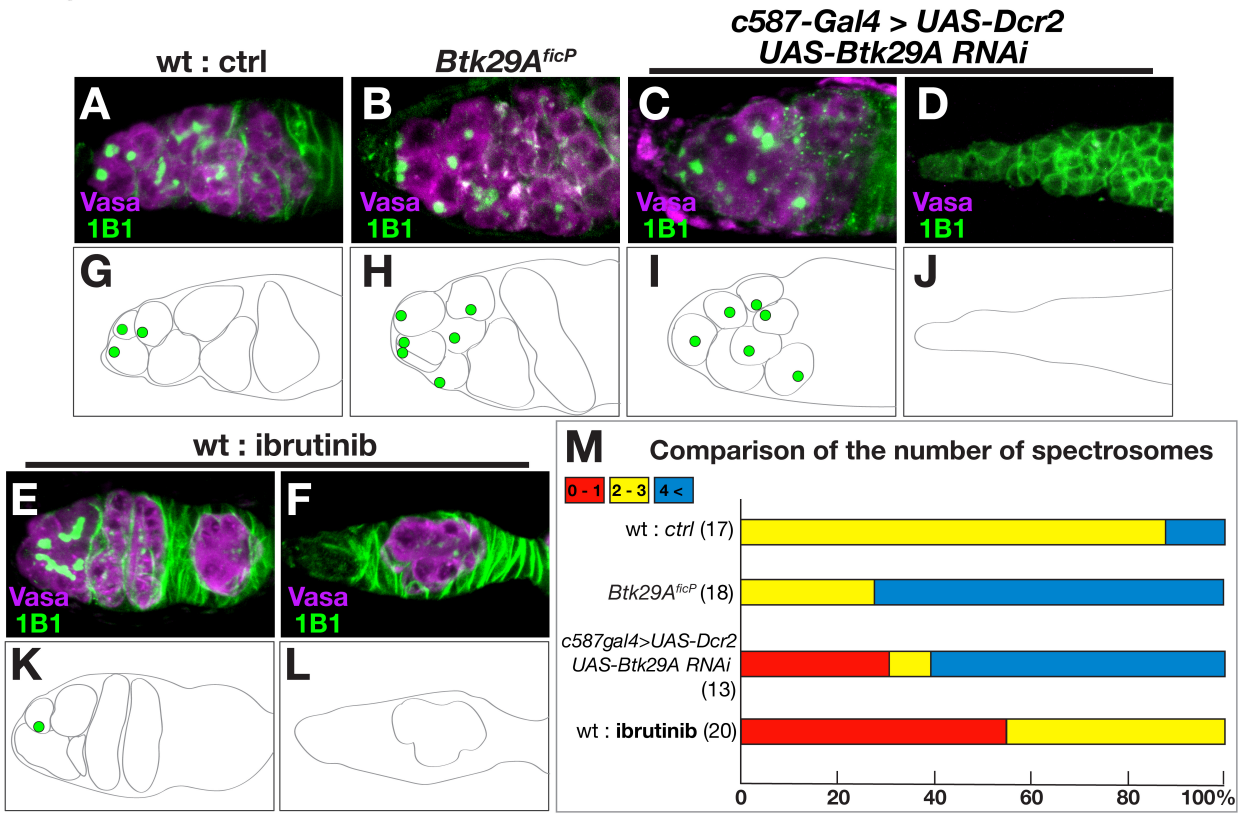

Fig. 3. *Btk29A* deficiency impairs germ cell production in the ovary. (A–F) Enlarged views of the germarium at the anterior tip of the ovary, which contains the niche where germ stem cells (GSCs) divide into cystoblasts while reproducing GSCs themselves. (G–L) Schematic drawings of the germarium are shown below the photographs to highlight germline cells containing spectrosomes. Compared with the wild-type germarium (A and G), the *Btk29A*^{ficP} mutant germarium (B and H) contains excessive cystoblast-like cells immunopositive to monoclonal antibody 1B1, which labels the spectrosome (green), a round structure characteristic of GSCs and cystoblasts. The anti-Vasa antibody was used to counterstain all germline cells (magenta). The majority of wild-type females fed on diets containing ibrutinib (E and K) carried germaria containing a reduced number of cystoblasts. Some wild-type females treated with ibrutinib (F and L) had germaria devoid of any germline cells, indicative of GSC loss. (M) The results of quantitative analysis of the numbers of cystoblasts contained in the fly germaria of different groups are shown. The proportions of germaria containing different numbers of spectrosome-containing cells are shown; red: 0–1 cells; yellow: 2–3 cells; blue: >4 cells (see the **Supplementary Tables** for details. **Statistically significant at $p < 0.01$ by Fisher's exact test).

target BTK. *Drosophila* will be particularly effective when used to screen chemicals that potentially act on BTK for the development of novel therapeutic drugs to control CLL because a large number of flies can be easily obtained for rounds of assays, and due to their short life cycle, life-long chronic effects of compounds can be evaluated in only a few weeks. Visible phenotypes such as a collapse in the dorsal closure and wing formation and other external structures [23,24] will help to promptly reveal the effects of compounds without dissection of tissues or any other experimental handling, making it possible to enrich promising candidate molecules within a short period of time.

While our phenotypic and biochemical data presented in this and other works suggest functional homology between human BTK and fly *Btk29A* [25,26], one caveat is that we have no direct evidence that ibrutinib exerts its effects on BTK/Btk29A through the same mechanism in humans and flies. In inhibiting human BTK, irreversible inhibitors, including ibrutinib, bind to the ATP pocket, in which Cystine481 plays an important role in inhibitor–BTK interactions [11,27]. However, fly *Btk29A* harbors a cystine-to-serine replacement at the corresponding position. Irreversible inhibitors, including ibrutinib, were found to be less effective for patients with CLL carrying the cystine-to-serine substitution in BTK. It remains to be clarified how ibrutinib modulates the *Btk29A* functions in flies. It might be envisaged that fly *Btk29A* has a higher order structure, distinct from that of human BTK, which compensates for the effect of cystine-to-serine replacement that reduces the binding of ibrutinib and other irreversible inhibitors.

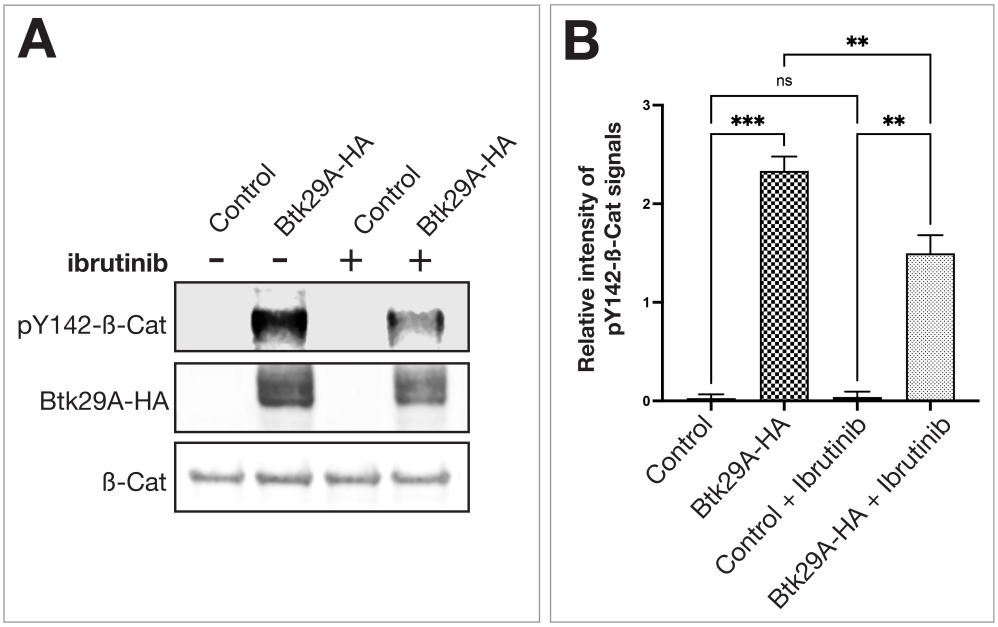

Fig. 4. Ibrutinib reduces phosphorylation of Y142 β-catenin in Cos7 cells. (A) Western blotting of Cos7 cell lysates probed with the anti-pY142 β-catenin antibody (upper panel), the anti-HA antibody that reacts with *Btk29A* type 2::HA (middle panel), or anti-β-catenin antibody (lower panel) in the absence (-) or presence (+) of ibrutinib (shown above the panels). Lysates were immunoprecipitated with an anti-β-catenin antibody and probed with anti- anti-pY142 β-catenin antibody. Cos7 cells were transfected with an empty vector (indicated as Control) or *Btk29A* type 2 tagged with HA, while β-catenin was endogenously expressed. (B) Quantification of western blot results. The signal intensity of bands was evaluated by band-area densitometry measurements and normalized by the amount of endogenous anti-β-catenin. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., statistically non-significant.

5. Conclusions

Feeding wild-type flies an ibrutinib-containing diet induces phenocopying of *Btk29A* mutants. Thus, *Drosophila* is suitable for screens of novel BTK inhibitor candidates and offers a unique *in vivo* system in which the mode of action of BTK inhibitors can be examined at the molecular, cellular, and organismal levels.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

NHK—Investigation, writing, reviewing, editing; CIES, BFN, YE, RZ—Conceptualization, reviewing; DY—Writing, reviewing, editing, conceptualization, and interpretation of data for the work. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

We thank Yoshimi Takamura for secretarial assistance. The authors are grateful to the Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VDRC) for providing fly strains.

Funding

This work was supported in part by a MEXT Grant-in-Aid to DY (21H04790), a Grant-in-Aid for JSPS Fellows to NHK, and grants from the Center for Innovative Medicine to CIES, the Swedish Cancer Society (22 2361 Pj 01 H) to CIES, the Stockholm County Council (ALF-project) to CIES, and The Swedish Cancer Society (201044 PjF) to YE.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2806124.

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