A new BCR-ABL1 *Drosophila* model as a powerful tool to elucidate the pathogenesis and progression of chronic myeloid leukemia

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 doi:10.3324/haematol.2018.198267

Received: May 21, 2018.

Accepted: November 8, 2018.

Pre-published: November 8, 2018.

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Supplemental data

Methods

Generation of *BCR-ABL1 Kinase Dead* (*BCR-ABL1^{KD}*) transgenic flies.

The human *BCR-ABL1 Kinase Dead* (*BCR-ABL1^{KD}*) cDNA was obtained through site-directed mutagenesis. The following mutagenic primers containing the desired mutation (Val-Met-Thr instead of Val-Lys-Thr) in the P-loop of the BCR-ABL1 tyrosine kinase domain were used: fwd GTGGCC**GTGATGACC**TTGAAGGAGG and rev TCCTTCAA**GGTCATCAC**GGCCACCG. 25ng of plasmid DNA containing the *BCR-ABL1* cDNA was PCR amplified using the Expand Long Template PCR System (Roche Applied Science, Penzberg, Upper Bavaria, Germany) according to the manufacturer's instructions. The non-mutated parental methylated DNA template was digested for 2 hours with *DpnI* and the plasmid carrying the desired mutation was finally prepared using the Qiafilter^{TM} Plasmid Maxi Kit (Qiagen, Venlo, Netherlands). Plasmid DNA was injected into *Drosophila* embryos (Trans-FlyER, Startup Company, Ferrara, Italy) and 3 independent transgenic lines were obtained.

Drosophila stocks

The following fly stocks, enhancer trap lines, deficiencies and mutants were obtained from the Bloomington Stock Center (Department of Biology, Indiana University, Bloomington, IN, USA) and they are described at FlyBase (flybase.bio.indiana.edu): Oregon R (adopted as wild type strain), *gmrGal4* (P(Gal4-*gmr*)-3rdchr.)[1], *sevenlessGal4*[2], *STAT92E^{06346}* (stock# 11681) *UAS-Abl* (#8567), *UAS-Abl^{K417N}* (#8566), *Abl[1]* (#8566), *Df (3L)st-f13 (fax deficiency-Df*, #2993), *Df(3R)T-32 (pros Df,* #3003), *Df(3L)81k19 (dab Df, #*2998*), Df(2R)P34 (ena Df, #*757*), fax[M7]* (#8786), *pros[17]* (#5458), *Dab EY10190* (#16974), *ena[23]* (#8571). RNA interference (RNAi) lines for *Abl* (#2897), *Dab* (#14008 and #13005) and *ena* (#43056 and #106484) were obtained from VDRC (Vienna *Drosophila* RNAi Center, Wien, Austria). *domelessGal4* (from Noselli S.[3]) and *STAT^{DN}*

(from Betz A.[4]) fly stocks were kindly provided by A. Giangrande. In order to generate a sensitive genetic background in flies and to express transgenes in a tissue specific pattern, flies carrying the *BCR-ABL1* transgene were crossed with flies expressing the yeast transcriptional activator Gal4 in the eye imaginal disc (*sevGal4* and *gmrGal4*). Flies were cultured in standard medium and grown at 25°C, if not otherwise specified. To study the eye phenotypes, images of adult eyes were captured using a stereomicroscope equipped with a photo-camera (Olympus, Olympus Italia srl, Segrate MI, Italy, or SMZ1500, Nikon Instruments, Amsterdam, Netherlands). Oregon R wild type strain was used as control if not otherwise specified.

Protein extracts, immuonoprecipitation, immunoblotting.

Protein extraction buffers **Fly Heads:** Tris-HCl pH 7.4 10mM, NaCl 150mM, EDTA 5mM, EGTA 5mM, Glycerol 10%, DTT 5mM, Urea 4M, supplemented with protease (CLAP cocktail containing Chymostatin 25mg/ml, Leupeptin 25mg/ml, Antipain 25mg/ml and Pepstatin A 25mg/ml, use 1:1000) and phosphatase (Sodium Orthovanadate 1mM) inhibitors. **Cell lines:** Tris-HCl pH 8 50mM, NaCl 150mM, Np40 1%, DOC 0,5%, SDS 0,5% supplemented with protease and phosphatase inhibitors. Lysates were incubated on ice for 30 minutes followed by centrifugation at 18000 g for 15 minutes at 4°C. Proteins (50 μg) were denatured in 4X SDS Laemli's buffer by boiling for 5 min, resolved on a SDS 8% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Bioscience, GE Healthcare, Waukesha, WI). The membrane was blocked for 1 h at room temperature with 5% BSA (Bovine Serum Albumin; Sigma-Aldrich Corp., St. Louis, MO) in 1X Tris-buffered saline (TBS) and then probed overnight at 4°C with primary antibodies in 1% BSA 1X TBS-tween buffer. Detection was performed using anti-mouse IgG HRP-conjugated secondary antibodies (anti-mouse sc-2005, anti-rabbit sc-2004, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 4°C. For Immunoprecipitation, 1 mg of total protein extract was incubated for 3h at 4°C with 4 μg of anti-Enabled supernatant and subsequently for 45 min at 4°C with Protein A Sepharose (Amersham Bioscience, GE Healthcare, Waukesha,

WI, USA). Proteins signal intensities were measured using a Java software (Image J). Unless noted differently, each experiment was repeated three times on biologically independent samples.

Fluorescent Immunolabeling analysis

Immunolabeled imaginal discs or cells from patients were analyzed, respectively, with Nikon A1R confocal laser-scanning microscope, equipped with a Nikon PlanApo 40× lens and captured using NIS Elements AR 3.10 software (Nikon) or with fluorescence microscope (DM2000 LED, Leica Microsystems, Wetzlar, Germany) and captured using x100 oil immersion objective. Fluorescent signal from cells was measured by Image Processing and analyzed with Graphpad Prism 5.

Genetic analysis

Melanotic nodule phenotype. The constitutive expression of the oncoprotein BCR-ABL1 under the control of the *domelessGal4 (domeGal4)* induces a fully penetrant lethality. This early lethality phase is due to *domeGal4* activity in essential tissues other than the lymph gland. To overcome this problem and analyze the effect of BCR-ABL1 overexpression in the hematopoietic precursor cells of the lymph gland Medullary Zone, we ubiquitously expressed under the control of the *tubulin* gene promoter a temperature-sensitive mutant of Gal80 (*tubulin-Gal80^{TS}*) that represses Gal4 activity by binding to the Gal4 transcriptional activation domain. Gal80^{TS} is active at 18°C and inactive at 29°, releasing the Gal4 activity from repression (temporal and regional gene expression targeting - TARGET system[5-7]). *domelessGal4; UAS-BCR-ABL1 3M; tubulin-Gal80^{TS}* synchronized larvae were kept at 18°C until the indicated instar then they were exposed to 29°C to inactivate the Gal80^{TS} protein and release Gal4 transactivation activity.

Patients and cell line

After informed consent 103 samples from 95 CML patients and 20 healthy donors were collected and included in the study. Eight patients were analyzed during follow up. K562 cell line (ATCC, Manassas, VA) was used for transfection and proliferation assays.

K562 transfection

30 µg of pReceiver-M07-Dab1 Vector containing the whole *Dab1* coding sequence (GeneCopoeia™, Rockville, MD, USA) was electroporated in K562 cell line using the GenePulser™ electroporation apparatus (Bio-Rad Laboratories) under the following conditions: 1 pulse at 300Volt and 75mF capacitance. Electroporation with 30 µg pEGFP alone was performed as control.

Proliferation assay

Transfected cells were seeded at $10x10^4$ concentration in RPMI with 10% FBS for 18 hours and then starved. After 12 hours, 10% FBS was added and finally 6 hours later 1 µCi/ml ^{3}H-Thymidine was added (GE Healthcare). After 24 hours of incubation, the ^{3}H-Thymidine was removed; cells were then washed with PBS and 5% trichloric acetic acid and resuspended with NaOH. The amount of incorporated ^{3}H-Thymidine was detected using a β-counter. Experiments were performed in triplicate.

Statistical analysis

The statistical significance of difference between distributions of the adult eye phenotypic classes considered in the different experimental sets has been evaluated applying the Mann-Whitney test. The difference of the melanotic nodules phenotype between the compared different genotypes were validated by applying the two-tailed Student's t-test to data from at least three independent experiments and calculating the p-value using the software GraphPad Prism 5. The expression data of Dab1 and Dab2, and the Dab1 proliferation data, were analyzed with the Student's t-test, the p-value calculated using the software GraphPad Prism 7.

Bleeding, preparation of hemolymph samples and estimate of the circulating hemocytes.

Three groups of ten late L3 instar larvae conditionally expressing BCRABL1 under the control of the *domeGal4* driver or larvae carrying only the *domeGal4* construct as negative control, were bled into 30 μl of PBS1X, labeled for 20 min at room temperature with Cy3 conjugated Phalloidin (20μg/ml, Sigma) that binds filamentous actin. The nuclei were labeled 5 min at room temperature with HOECHST 33342 (10μM Sigma) that is able to enter into unfixed alive cells. The bled circulating hemocytes were mounted on a slide under a 20x20 mm coverslip adding 5μl of Fluormount. In order to estimate the number of circulating hemocytes, cells co-labeled by Phalloidin/HOECST were counted in three random fields of three slides representing biological replicas. The average number of hemocytes per field between the slides was calculated. The data were analyzed with the Student's t-test and the p-value calculated using the software GraphPad Prism 7.

Supplemental Figures

Figure S1

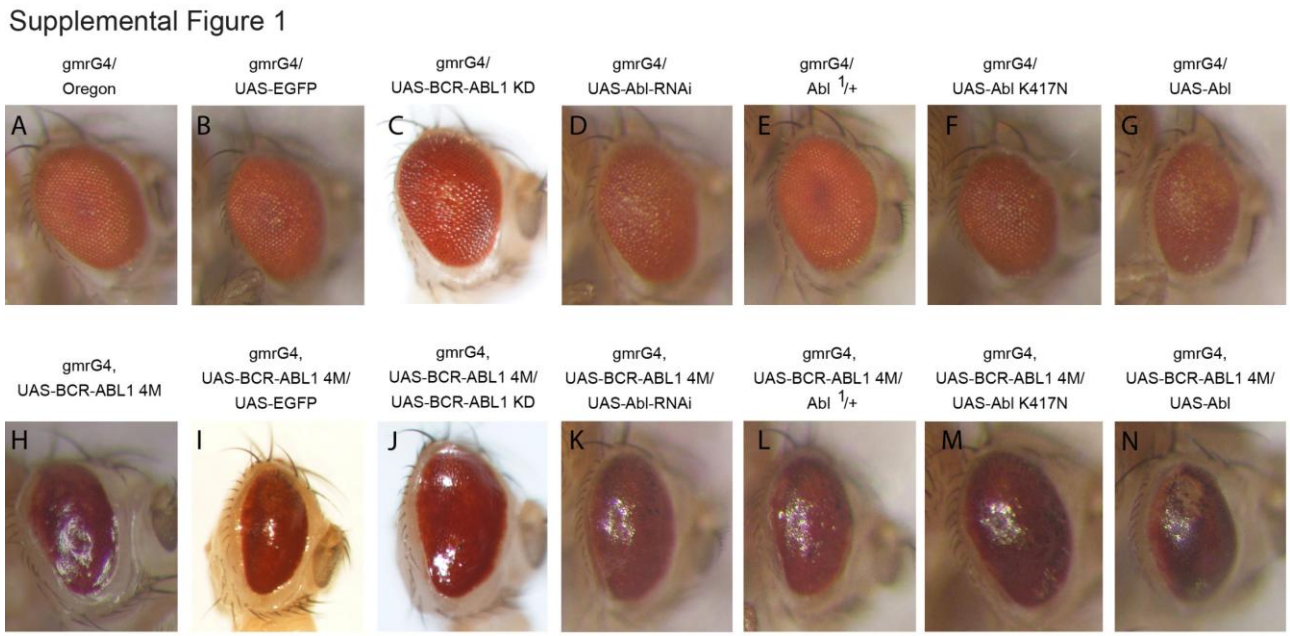

Figure S1. Adult eyes representative of the most frequent phenotypic class in the analysis of animals carrying the indicated genotypes and concerning the data included in Figure 2B.

Figure S2

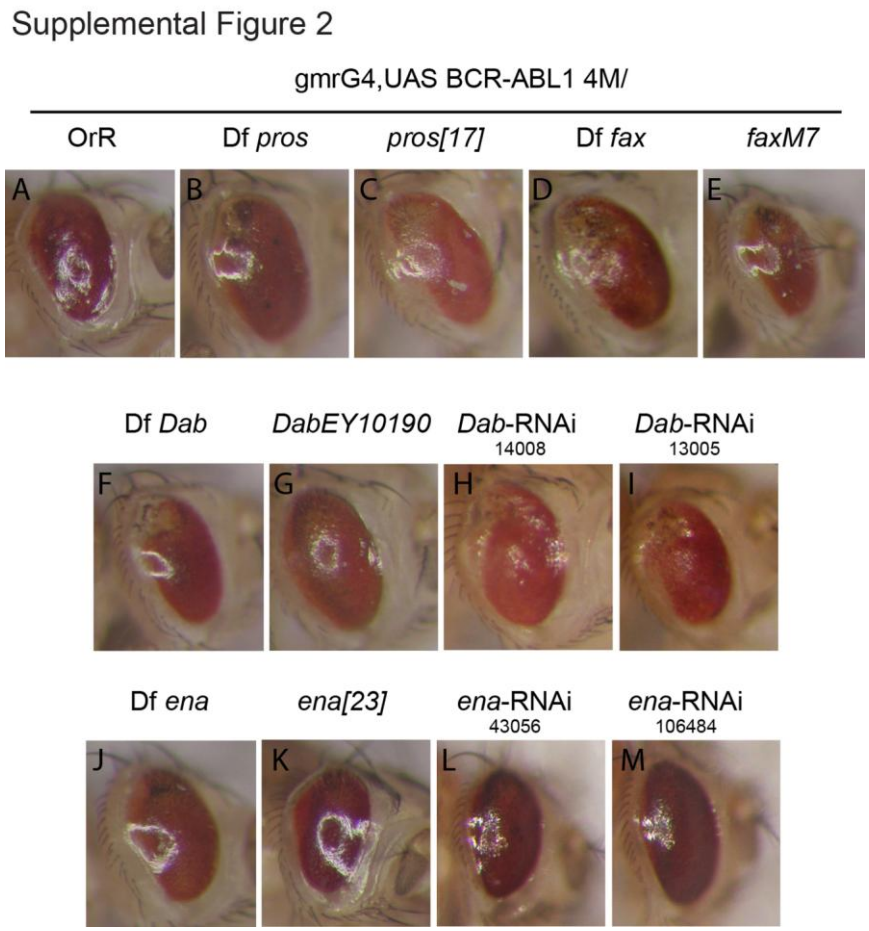

Figure S2. Adult eyes representative of the most frequent phenotypic class in the analysis of animals carrying the indicated genotypes and concerning the data included in Figure 2C-F, Figure 3A and Figure 5A.

Figure S3

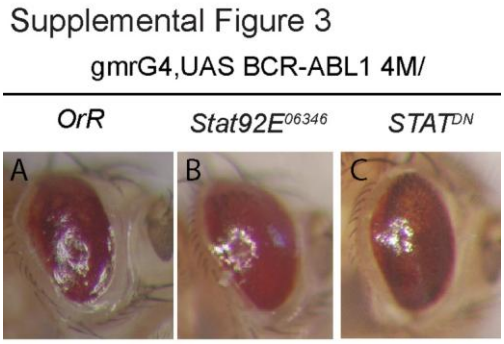

Figure S3. Adult eyes representative of the most frequent phenotypic class in the analysis of animals carrying the indicated genotypes and concerning the data included in Figure 4.

Supplemental Bibliography

1. Freeman M. Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. Cell. 1996;87(4):651-660.

2. Basler K, Yen D, Tomlinson A, Hafen E. Reprogramming cell fate in the developing *Drosophila* retina: transformation of R7 cells by ectopic expression of rough. Genes Dev. 1990;4(5):728-739.

3. Bourbon HM, Gonzy-Treboul G, Peronnet F, et al. A P-insertion screen identifying novel X-linked essential genes in *Drosophila*. Mech Dev. 2002;110(1-2):71-83.

4. Henriksen MA, Betz A, Fuccillo MV, Darnell JE, Jr. Negative regulation of STAT92E by an N-terminally truncated STAT protein derived from an alternative promoter site. Genes Dev. 2002;16(18):2379-2389.

5. McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL. Spatiotemporal rescue of memory dysfunction in *Drosophila*. Science. 2003;302(5651):1765-1768.

6. McGuire SE, Mao Z, Davis RL. Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in *Drosophila*. Sci STKE. 2004;2004(220):pl6.

7. McGuire SE, Roman G, Davis RL. Gene expression systems in *Drosophila*: a synthesis of time and space. Trends Genet. 2004;20(8):384-391.