Supplementary Information for

An in situ high-throughput screen identifies inhibitors of intracellular Burkholderia pseudomallei with therapeutic efficacy


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Materials and Methods

Ethics statement
All research with laboratory animals was conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida (protocol #: 201609601), in full compliance with the Animal Welfare Act and other federal regulations and statutes pertaining to animals. All in vivo experiments were performed in an ABSL-3 facility at the UF Communicore’s accredited animal research facility, managed by UF Animal Care Services. Humane care and treatment protocols were conducted according to i) 9 CFR Parts 1-4 (U.S.C. 2131-2156), and ii) the "Guide for the Care and Use of Laboratory Animals", NIH Publication No. 86-23.

Select Agent experiments
In vitro experiments with Bp and Bm were performed in a BSL-3 high containment facility at UCLA. Personnel wore Tyvek suits and powered air purifying respirators. The BSL-3 facilities at UCLA and UF are registered with the CDC DSAT and approved for possession, use, and transfer of Bp and Bm (Tier-1 Select Agents) under entity registration numbers C20090508-0836 and A20150312-1681 for UCLA and UF, respectively.

Reagents
BFX was purchased from ChemBridge (San Diego, CA), and flucytosine was purchased from Selleckchem (Houston, TX). Dimethyl sulfoxide was used as solvent for high-throughput screening and follow-up studies. Hcp antibodies were provided by Mary Burtnick at the University of South Alabama (Mobile, AL).

Bacterial strains and mutant construction
Bt E264 (1), Bp 1026b (2), MSHR305 (3), and Bm ATCC 23344 (4) were grown in LB medium without NaCl (LB-NS) or with NaCl (in the case of Bm). In-frame mutations were constructed using allelic exchange as described previously (5, 6). Strains constitutively expressing VirA and VirG were constructed by insertion of a mini-Tn7 transposon containing the virAG genes from Bp340 downstream of the S12 ribosomal subunit promoter, as described previously (6). Plasmid construction was performed using a derivative of the broad-host range plasmid pBBR1-MCS2 containing the nptII kanamycin resistance gene, as previously described (5, 6). Bt E264 transposon mutants were obtained from a near-saturating transposon mutant library, generated using two Tn5 derivatives: \( Tn_7 \) producing translational lacZ when inserted in frame and encoding tetracycline resistance (60 \( \mu g/ml \)), and \( Tn_5 \) producing transcriptional lacZ fusions and encoding trimethoprim resistance (50 \( \mu g/ml \)) (7). Bt transposon mutants were provided by Colin Manoil at the University of Washington (Seattle, WA).

Small molecule library
The small molecule library housed at the UCLA molecular screening shared resource (MSSR, https://cnsi.ucla.edu/facilities/technology-centers/mssr/) contains ~220,000 small molecules. Among these are 1,120 FDA-approved drugs (Prestwick library), 1,280 pharmacologically-active drug-like molecules (LOPAC collection), 8,000 molecules from the Microsource Spectrum Collection, 8,000 molecules which target kinases, proteases, ion channels and GPCRs (druggable compound set), 20,000 compounds from a lead-like compound set, 30,000 compounds from the ChemBridge DiverSet E, 50,000 diverse molecules from Life Chemicals, 5,000 compounds from the UCLA in-house collection, and ~100,000 diverse molecules from libraries proprietary to the UCLA MSSR.

High-throughput screen
25 \( \mu l \) of cell culture media [Dulbecco’s Modified Eagle’s Medium (DMEM) + 10% bovine growth serum (BGS)] was dispensed into black clear-bottom 384-well plates (E&K Scientific, EK-30091) using a multidrop reagent dispenser (Thermo Fischer Scientific). Small molecules were pinned into plates using a Biomek FX robot to achieve a final well concentration of 5 \( \mu M \) (250 nl of 10mM DMSO solution). eGFP expressing HEK293 cells were seeded onto the 384-well plates
for a final well volume of 50 μl and cell count of 35,000/well. Plates were incubated for 24 hours at 37°C and then infected with Bt E264 at a multiplicity of infection (MOI) of 0.01. Validation with Bp 1026b or Bm 23344 was conducted similarly, except that infections and subsequent steps were performed in our CDC-approved Tier 1 Select Agent-certified BSL3 facility. Plates were gently centrifuged (200 x g for 5 min) to allow bacterial attachment to cells, and incubated at 37°C for 1 h. Following incubation, media containing kanamycin was added to the wells for a final well concentration of 125 μg/ml to kill extracellular bacteria. Plates were incubated for 18-22 hours, fixed with 4% paraformaldehyde (final well concentration), and imaged by laser scanning cytometry (Image Express XL plate reader). Burkholderia infection results in lesions (i.e. plaques) of uniform size (~1 mm diameter) on HEK 293 monolayers at 18-22 hours post infection. These represent MNCs that have or are about to lyse, and aberrancies in size and number are readily identified by eye. Well images were analyzed qualitatively in a blinded, independent observer fashion to identify compounds inhibiting lesion formation, which were deemed positive hits. Given that all selected compounds entered a systematic validation process, a low-threshold approach was taken during initial screens such that any compound that had even a slight effect on the number or size of plaques was selected for follow-up. Uninfected wells treated with DMSO and wells treated with DMSO and infected with the wild-type parental strain served as negative and positive controls, respectively. False-positive and false negative hit rates were estimated as the percentage of positive control wells without plaques and the percentage of negative control wells with plaques, respectively.

Cell culture, infection, and intercellular lifecycle assays

HEK293 (ATCC CRL-1573) cells were grown in DMEM + 10% BGS and 5% CO₂. Prior to experiments, plate wells were incubated at room temperature for 30 minutes with a 1:30 dilution of Matrigel liquid (BD) in serum-free DMEM for improved adherence of cells. Cell-based infection experiments were performed as previously described (5, 6). For plaque assays to measure cell fusion, cells were infected as described above but with a MOI of 4 x 10⁻⁴ to prevent overcrowding of plaques, overlaid with 125 μg/ml kanamycin (Km), and imaged 18-22 hours later by fluorescence microscopy (5, 6). When quantitative data was desired, plaques were visually counted by scanning the entire well at low power magnification by fluorescence microscopy. Plaque forming efficiency was calculated as the number of plaques (plaque forming units; PFU) divided by the number of colony forming units initially used for the infection (CFU), which was assessed by plating dilutions of the bacterial suspension. Plaques were defined as defects in the monolayer resulting from MNCs that have enlarged and are about to burst, as well as MNCs that have already lysed. For assessments of invasion and intracellular replication, cells were seeded at 1.8 x 10⁶ cells per well in 6-well plates or 7.2 x 10⁵ cells per well in 12-well plates. HEK293 cells were infected at a MOI of 1.0. Following addition of bacteria to wells, plates were gently centrifuged as described above. One hour after infection, extracellular bacteria were killed by the addition of 1,000 μg/ml Km, which remained present for the remainder of the infection (5, 6). At different timepoints, infected cells were washed with Hank’s Buffered Salt (HBS), harvested with 0.25% trypsin, and lysed with 0.2% Triton X-100 + 20mM MgSO₄ and 50 μg/ml DNase I (to reduce lysate viscosity). Intracellular colony-forming units (CFUs) were enumerated by plating serial dilutions of the lysate at indicated time points.

Toxic dose and effective dose determinations

HEK293 cell viability in the presence of small molecules was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). HEK293 cells (3,300/well) were seeded into 384-well plates treated with widely varying concentrations of small molecules (increasing in two-fold increments from 0.0001 to 50 μM) in duplicate and incubated for 48 hours, then treated with CellTiter-Glo reagent and incubated for 10 minutes, at which point the luminescent signal of compound-treated and DMSO-treated control wells was measured using a plate reader (Perkin Elmer Wallac). The toxic dose (TD) was defined as the dose at which luminescence (indicative of HEK293 ATP levels and viability) was decreased by a statistically significant margin relative to DMSO-treated control wells (Student’s T test, P<0.05). The effective dose (ED), defined as the dose that completely inhibited plaque formation, was determined with dose-response plaque assays in 384-well plates using the same plaque assay as described in the HT screening
methodology, with compounds pinned at the same dose-response range described above (two-fold increments from 0.0001 to 50 μM).

**Minimum Inhibitory Concentration (MIC) determinations**

MICs were determined by broth microdilution. Compounds were diluted in LB or LB-NS broth to concentrations ranging from 4μM to 22μM (in 2μM steps) in 96 well plates (100μl per well). DMSO-treated wells (carrier-only) were used as negative controls. Wells at the edge of the plate were not used to avoid broth evaporation during incubation. Overnight cultures of *B. thailandensis*, *P. aeruginosa*, or *E. coli* were diluted 1:200 in compound-treated 96-well plates, and plates were sealed with a plastic cover and tape. Inoculated plates were gently shaken (150 RPM) at 37°C for 16hrs, at which point the optical density at 600nm (OD600) for each well was determined using an Epoch microplate spectrophotometer (BioTek Instruments). For the attenuated strain Bp82 (SI Appendix, Table S2), MICs were determined using cation-adjusted Mueller-Hinton broth supplemented with 80 μg/ml adenine (8).

**Chemical mutagenesis and 5-FC forward genetic screen**

Wild-type *Bt* were grown to mid-exponential phase (OD600 ~ 1), washed three times with PBS, resuspended in PBS + 1% ethyl methanesulfonate (EMS) and shaken at 37°C for 10-15 minutes. After EMS treatment, bacteria were washed three times with PBS, then resuspended in LB-NS and allowed to recover at 37°C for 1 h. The mutagenesis protocol was designed based on published EMS mutagenesis protocols for *E. coli* (9, 10) and optimized using survival rates with different EMS treatments to obtain roughly 5-10 SNPs per genome. Pooled mutants were then used to infect eGFP-expressing HEK293 cells treated with 25 μM 5-FU in 384-well plate format at an MOI of 0.03. 5-FU was used instead of 5-FC to bypass early steps in the pyrimidine salvage pathway and avoid selecting for inactivating mutations in *codB* and *codA*, which are already known to confer resistance. Plates were centrifuged at 200 x g for 5 min and a 125 μg/ml Km overlay was added after 1 h. After 16 hours of incubation at 37°C, plates were scanned by laser scanning cytometry. Wells with MNCs were trypsinized, cells were lysed with 0.2% Triton X-100 + 20mM MgSO4 and 50 μg/ml DNase I, and dilutions were plated on LB-NS plates. 10 colonies from each MNC were picked, grown in LB-NS, and used to infect 5-FU-treated cell monolayers to validate their resistance. Validated, resistant mutants were screened for mutations in the *upp* gene by PCR amplification of *upp* and upstream sequences (*upp* forward primer: TCGGTAAACTTCGGCGTGC, *upp* reverse primer: AATCGTCCGCGAATCGTTCC, *upp* upstream forward primer: CAGCAGGTGTTGTGATCGTCA, *upp* upstream reverse primer: ATGTGCGTGAGTTTGTGCTG) and amplicons were sequenced to detect mutations. For resistant mutants without SNPs in *upp* (7 total), and the parental wild-type strain, genomic DNA was extracted and analyzed by whole genome sequencing. WGS confirmed an average of 8 SNPs per genome, validating our EMS mutagenesis protocol.

**Mouse model of *B. pseudomallei* infection**

All Select Agent animal work was carried out in a CDC/USDA Tier 1 approved facility at the University of Florida following Tier 1 regulations. This study was approved by the UF Institutional Animal Care and Use Committee (protocol #: 201609601). Female BALB/c mice between 4 and 6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in Allentown microisolator cages under pathogen-free conditions. *Bp* 1026b was grown overnight to an OD600 of ~1 and frozen in 20% glycerol aliquots overnight at -80°C. An aliquot of each was thawed and CFU enumerated by dilution plating on LB medium. The target inoculation of 4,500 CFU in 20 µl was achieved by thawing an aliquot and dilution in PBS immediately prior to challenge. The challenge dose was serially diluted and plated to confirm the desired inoculum. Animals were anesthetized with a KX cocktail containing 87.5 mg/kg of ketamine (Patterson Veterinary) and 12.5 mg/kg xylazine (Alfa Aesar) of body weight. Once fully anesthetized, groups of 8 mice (n=8) were challenged with the 20 μl inoculum by pipetting into the nares of the mouse alternating nostrils until fully inhaled. Starting at five hours post-infection, mice were treated twice daily via intraperitoneal injection of 100 μL of PBS + 20% DMSO (negative control), 65 mg/kg of ceftazidime in 100 μl PBS (positive control), 50 mg/kg of 5-FC in 100 μl PBS, or 5 mg/kg of BFX in 100 μl PBS + 20% DMSO. Two daily 50 mg/kg doses of 5-FC
correspond to 100 mg/kg/day, which was selected to fall within the recommended dosage range for humans (infants < 1mo: 25-100 mg/kg/day; adults: 50-150 mg/kg/day) [https://www.drugs.com/dosage/ceftazidime.html]. Two daily 5 mg/kg doses of BFX correspond to 10 mg/kg/day, which was selected to fall within the recommended dosage range for Cip in humans (pediatric: 6-30 mg/kg/day; adult: 5.7-21.4 mg/kg/day, assuming adult weight of 70 kg) [https://www.drugs.com/dosage/ciprofloxacin.html]. Two daily 65 mg/kg doses of ceftazidime correspond to 130mg/kg/day, which was selected as it approximates the therapeutic dosage used in humans (https://www.drugs.com/dosage/ceftazidime.html). Each treatment group contained 8 mice. Overall survival was followed over a 10-day period. Mice were sacrificed when moribund. Humane euthanasia endpoints included severe weight loss, shivering, labored breathing, uncoordinated movements, and unresponsiveness to touch. Mice surviving to the tenth day were euthanized, and lungs, spleens, and livers were excised and homogenized in 5 ml of 1xPBS using a stomacher (Seward). Undiluted and diluted homogenate were plated on LB agar to determine organ bacterial loads. Colonies were positively identified as Bp by spot-testing with the latex agglutination test as previously described (11, 12). A separate pre-determined endpoint study was undertaken to determine organ bacterial loads at 48 hours post-infection. Four mice per treatment group were infected and treated as described above, and then humanely euthanized and processed for lung, spleen, and liver organ bacterial loads as described above. One additional mouse per treatment group was infected as described and processed for histopathological analysis of the lungs, liver, and spleen. For this, organs were excised, fixed in 10% formalin, and processed for paraffin embedding at the University of Florida Molecular Pathology Core. 5 μM sections were obtained at regular intervals from the middle of each organ and stained with H&E and analyzed by microscopy.

**E. coli DNA gyrase assays**

Supercoiled pUC18 plasmid DNA was treated with Topoisomerase I (New England Biolabs) for 30 min at 37°C to generate relaxed circular pUC18 DNA, and then heated to 65°C to inactivate Topo 1. Relaxed circular pUC18 was then treated with one unit of *E. coli* DNA gyrase (New England Biolabs) in the presence of water, Cip (100 μM or 500 μM), or BFX (100 μM or 500 μM), incubated at 37°C for 30 minutes, and then run on a 1% agarose gel without ethidium bromide (EtBr), as EtBr intercalates DNA. After electrophoresis, the gel was stained with EtBr, destained briefly in water, and then imaged with a UV transilluminator.

**In vitro type VI secretion assays**

*Bt* E264 and *Bp* 1026b strains constitutively expressing VirAG were used for in vitro T6SS-5 secretion experiments as previously described (6). In the case of 5-FC-resistant *Bt* transposon mutants, complementation with a pBBR plasmid expressing virG was used to induce T6SS-5 expression and secretion in vitro. Overnight bacterial cultures were diluted to an optical density at 600nm (OD600) of 0.05 in LB-NS containing antibiotics when necessary. At an OD600 of 0.8-1.2, 0.5ml aliquots were centrifuged, washed, and resuspended in 200 μl of Laemmli buffer (pellet fractions). The remainder of the culture was centrifuged at 4,750 X g for 15 min, and the supernatants were filtered through a 0.2 μM syringe tip filter and precipitated in 10% trichloroacetic acid (TCA) overnight at 4°C. The supernatants were centrifuged at 18,900 X g for 15 min at 4°C and the pellets were washed with 1ml acetone and resuspended in 200 μl Laemmli buffer (supernatant fractions). Pellet and supernatant samples were normalized according to the OD600 of the bacterial culture at the time of harvest. Samples were analyzed by Western Blot as previously described (6) and treatment or mutant bands were quantified relative to control bands using densitometry.

**Microscopy**

For actin tail visualization experiments, a 1:1 mixture of HEK293 GFP- and RFP-expressing cells were grown on glass coverslips treated with Matrigel solution, incubated overnight with compound or DMSO, infected with *Bt* E264 at MOI = 1, and then washed and fixed 9 hours after infection with 4% paraformaldehyde in PBS containing 3 mM MgCl2 and 10 mM EGTA for 15 min. Cells were permeabilized with 0.2% Triton X-100 in PBS, then incubated with Alexa-Fluor 488-labeled phalloidin, rabbit *Bt* antiserum, and secondary antibodies in blocking
buffer. Permanent mounts of specimens were made with Prolong Gold (Invitrogen), analyzed with a Leica SP5-II AOBS confocal microscope setup, and imaged with a Zeiss Axiovert 40CFL inverted fluorescence microscope fitted with a Canon digital camera. Separate experiments were performed in multiplicate. The proportion of bacteria expressing actin tails was calculated as the number of bacteria associated with an actin tail/total bacteria, per high-powered field.

RT-PCR
HEK293 cell monolayers were infected with Bt as described above and lysed 9 hours after infection with 1ml TRIzol. RNA was isolated according to manufacturer instructions, and residual DNA was removed with the Ambion TURBO DNA-free kit (Thermo Fisher). The SuperScript III first strand synthesis kit (Thermo Fisher) was used to synthesize cDNA, and the Bio-Rad iQ SYBR Green supermix and Bio-Rad iQ5 machine were used to perform quantitative PCR. RNA levels were normalized to recA and the $\Delta\Delta$CT method was used to calculate relative expression levels. The following RT-PCR primers were used: RecA Forward: CGTGATGTTCGGCAATCC, Reverse: GGCAGACCCCTGTTCTTGAC; VirG Forward: GATCTCCTTTGAGCACGTCCA, Reverse: ATGAAGCTCAATGGAATGAC; BsaN Forward: ATACCGGACGTCATGAAAAC, Reverse: CCGTTTTCGACAAGAATA; BsaM Forward: TGAAGCTCTGCAACTCCGAATC, Reverse: CAATACTTCCCCTGACAGAATCG; BopE Forward: TGACTTACAACCGAGAATCG, Reverse: CTGAAGATGCGCTTGATCTGT; FliC2 Forward: GCGAACAACCTGACGAACAT, Reverse: TGCTGCAGAATGTTCAGCTT; BimA Forward: GCCGCTTTCGAACCTC, Reverse: GACGTGAGGCGATGCGA; ClpV Forward: GAGGACTTCACCGCGAAAG, Reverse: GCACACCGGATTGTTCTTG; VgrG5 Forward: GTCCGGCAACAAGTTCGATT, Reverse: GACGTAGATGTCGGTGTTG.

Library prep and whole genome sequencing
Libraries were prepared with the Nextera XT kit (Illumina) starting from 1 ng of genomic DNA according to manufacturer's instructions with minor modifications. The initial tagmentation step was extended to 8 min and the post-PCR purification was performed using a 1:1 ratio of PCR product and AMPure XP beads (Beckman Coulter). Normalized libraries were pooled and sequenced as 100 single-end reads on a HiSeq2500 (Illumina) Rapid Run Mode.

WGS analysis
Reads for each sample were aligned to the Burkholderia thailandensis E264; ATCC 700388 reference genome using BWA-MEM v.0.7.12-r1039 (Heng Li 2013 arXiv:1303.3997). An average of 1600 Mb were successfully mapped per sample; minimum was 960 Mb for the wild type sample. Variant discovery was performed with Genome Analysis ToolKit's (GATK) HaplotypeCaller v.3.6-0-g89b7209 (13). Read alignments for variant regions were inspected for quality assurance using Integrative Genomics Viewer (IGV) (14). The functional impact of variants was predicted using SnpEff 4.2 (15).

Data analysis
Figures and graphs were prepared using Graphpad Prism and Keynote. Statistical analyses were performed with Student's t test or ANOVA implemented in Graphpad Prism. Animal survival analyses were performed using the Log-rank (Mantel-Cox) test and corroborated with a Gehan-Breslow-Wilcoxon test to account for the possibility of non-proportional hazards, with concordance of results. Results were considered statistically significant if the $P$ value was less than 0.05. For figures showing mean values of technical replicates, error bars are shown that represent the standard deviation (SD). For figures showing mean values of biological replicates, error bars are shown that represent the standard error of the mean (SEM).
Supplementary Table S1. 5-FC and BFX are non-toxic to HEK293 cells at concentrations far exceeding those which effectively inhibit intercellular spread of *Bp*. Cellular toxicity was assessed using the Celltitre-Glo cell viability assay following 48 hour HEK293 cell incubation in 384-well plates with the compound of interest over a wide range of concentrations, increasing in two-fold increments from 0.0001 to 50 μM. The toxic dose (TD) was defined as the lowest dose of compound at which HEK293 cell viability was decreased by a statistically significant margin relative to DMSO-treated control wells (Student’s T test, \( P < 0.05 \)). The effective dose (ED; IC100) was determined using dose-response plaque assays in 384-well plates with compound concentrations increasing in two-fold increments from 0.0001 to 50 μM. The ED was defined as the dose that completely inhibited plaque formation by *Bp*.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ED (μM)</th>
<th>TD (μM)</th>
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<tbody>
<tr>
<td>BFX</td>
<td>0.1</td>
<td>&gt;50</td>
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<tr>
<td>5-FC</td>
<td>3.1</td>
<td>&gt;50</td>
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Supplementary Table S2. *B. thailandensis* E264, *B. pseudomallei* 82, *P. aeruginosa* 27853, and *E. coli* 25922 are susceptible to BFX. MIC values for ciprofloxacin (Cip) and burkfloxacin (BFX) were determined by broth microdilution and found to be similar for *Bt* E264, *Bp* 82, *Pa* 27853, and *Ec* 25922. *Bp* 82 is an attenuated select agent-excluded ΔpurM derivative of *Bp* 1026b (16).

<table>
<thead>
<tr>
<th>Species</th>
<th>BFX MIC (µg/ml)</th>
<th>Cip MIC (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td><em>Bt</em> E264</td>
<td>2.6</td>
<td>1.3</td>
</tr>
<tr>
<td><em>Bp</em> 82</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>Pa</em> 27853</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><em>Ec</em> 25922</td>
<td>5</td>
<td>3</td>
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Supplementary Fig. S1. BFX is a more potent inhibitor of intercellular spread by *Burkholderia thailandensis* than Cip. Images show multinucleate cell (MNC) formation 18 hours of infection, in the presence of BFX or Cip at concentrations ranging from (0.125 μM to 8 μM). BFX prevented plaque formation at 8-fold lower concentrations than Cip, despite the fact that the two compounds have comparable *in vitro* MICs (1.3 μg/ml for Cip and 2.6 μg/ml for BFX). This suggests that the superior efficacy of BFX in cells is a result of more efficient intracellular accumulation, rather than higher bactericidal/bacteriostatic activity.
Supplementary Fig. S2. Bacterial loads in the lungs, liver, and spleen of mice surviving the entire study duration (10 days) following \textit{B. pseudomallei} infection. For almost all mice surviving until day 10 post-infection (one 5-FC-treated and five BFX-treated), bacterial loads in the liver and spleen were considerably lower than in the lung. This suggests that 5-FC and BFX may reduce mortality by limiting bacterial dissemination from lungs.
References


15. P. Cingolani et al., A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin)* **6**, 80-92 (2012).