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Immunofluorescence of third instar larval brains

TUNEL labeling of larval brains was performed using the Apotag kit (Appligene Oncor), with the following modifications: a mouse antidigoxigenin antibody (Boehringer) followed by an anti–mouse Alexa 488 secondary was used to detect nick-end–labeled DNA rather than the supplied sheep antidigoxigenin antibody-FITC conjugate, as this allowed enhanced sensitivity.

dsRNA-mediated interference

Template DNA was prepared by PCR, with the following primers: 5′-TTA ATA CGA CTC ACT ATA GGG AGA GGC GGA AAA GCA GCA AAC A-3′ and 5′-TTA ATA CGA CTC ACT ATA GGG AGA AGA GCC GCT TCC TGT TGA C-3′. RNA was synthesized from the PCR template at 37°C for 4 h using the MEGAshortscript T7 kit (Ambion) with the following alterations (per reaction): 2 μl of 10× Reaction buffer, 2 μl each of ATP, CTP, GTP, and UTP mixes, 2 μl of enzyme mix, and 1 μg of total of template DNA, volume made up to 20 μl with Nuclease-free dH_2O. RNA was precipitated by addition of 0.3 M sodium acetate and 2.5 volumes 100% ethanol and incubation on ice for 30 min, followed by centrifugation at 13,000 *g* for 30 min. The pellets were washed in 70% ethanol, air dried, and resuspended in 40 μl of Nuclease-free dH_2O. To prepare dsRNA, the resuspended pellets were heated to 65°C for 30 min, and then placed in a 65°C water bath, which was then allowed to cool slowly to RT. Cells were harvested every 24 h until 144 h, and processed as described.

Cell culture transfections

***Drosophila* S2 cells.** 10^7 cells/ml were transfected with 10 μg of the constructs pMT/EGFP and pMT-DmIX-14~EGFP by electroporation using the following conditions: 400 V, 1000 μF, 200 Ohms, in 0.8 ml of Schneiders media (Sigma-Aldrich). After pulsing, the cells were allowed to recover for 10 min in the cuvettes. Cells were transferred to fresh Schneiders media + 10% FBS (GIBCO BRL) in 6-well plates and grown for the desired time points. Expression from the metallothionine promoter of pMT was induced with 500 μM $CuSO_4$.

Hela cells. 2×10^5 HeLa cells were seeded on 6-well plates and transfected the following day with 1μg of the constructs pEGFP(N1) and pEGFP(N1)-HsIX-14 using the Effectene reagent (QIAGEN). Cells were grown in RPMI media (GIBCO BRL) supplemented with 10% FBS (Sigma-Aldrich).