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mel_feany@hms.harvard.edu

A neuroprotective role for the DNA damage checkpoint in tauopathy

Vikram Khurana[1*], Paola Merlo[1*], Brian DuBoff[1], Tudor A. Fulga[1], Katherine A. Sharp[1], Shelagh D. Campbell[3], Jürgen Götz[4], and Mel B. Feany[1]

Department of Pathology[1], Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA; [3]Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada; and [4]Brain and Mind Research Institute, University of Sydney, Camperdown, Australia

*These authors contributed equally.

Corresponding author:
Mel B. Feany, M.D., Ph.D.
Department of Pathology
Brigham and Women's Hospital
Harvard Medical School
77 Louis Pasteur Ave., Room 630
Boston, MA 02115
Tel: (617) 525-4405
FAX: (617) 525-4422

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Summary

ATM and p53, effectors of the DNA damage checkpoint, are generally considered pro-apoptotic in neurons. We show that DNA damage and checkpoint activation occurs in postmitotic neurons in animal models of tauopathy, neurodegenerative disorders that include Alzheimer's disease. Surprisingly, checkpoint attenuation potently increases neurodegeneration through aberrant cell cycle re-entry of postmitotic neurons. These data suggest an unexpected neuroprotective role for the DNA damage checkpoint in tauopathies.

A number of studies have demonstrated DNA replication and cell cycle activation within postmitotic neurons in tauopathies, including Alzheimer's disease and related disorders (Andorfer *et al*., 2005; Herrup & Yang 2007). A growing literature also demonstrates that DNA damage accompanies neurodegeneration (Kruman *et al.* 2004; Kim *et al.* 2008). In the major DNA damage checkpoint responding to DNA double-stranded breaks (DSBs), the Ser/Thr PI3 kinase family member ATM activates targets including p53 and Chk2, a transcription factor and Ser/Thr kinase respectively, to mediate either cell cycle arrest or apoptosis (Lee & McKinnon 2000). Reports of upregulation of p53 and its homolog p73 in Alzheimer's disease brains (Kitamura *et al.* 1997; Wilson *et al*., 2004), coupled with studies indicating ATM and p53 inhibition in cultured or developing neurons is neuroprotective, has led to arguements that ATM and p53 may be pro-apoptotic in Alzheimer's disease (Kruman *et al.* 2004; Culmsee &

Mattson 2005; Kim *et al.* 2008). Here we probe the relationship of the DNA damage checkpoint to tau-dependent neurodegeneration in mature postmitotic neurons of the genetically tractable fruit fly.

To show activation of a DSB checkpoint in experimental tauopathy, we first demonstrated that p53 was upregulated in cortical neurons in tau transgenic mice (Fig. 1a, arrow; Fig. S1a; Ittner et al., 2008) compared to age-matched control brains. The phosphorylation at Ser-139 of the histone variant H2AX (pH2AX) is a specific marker for DSBs in mammalian cells. We found expression of pH2AX in neurons from tau transgenic mice (Fig. 1c, arrow; Fig. S1b), but not in control tissue. We then investigated whether these changes were mirrored in a *Drosophila* tauopathy model, in which pan-neuronal expression of human tau recapitulates key features of human tauopathies (Khurana *et al.* 2006). In the fly model, as in tau transgenic mice, there was upregulation of neuronal p53 (Fig. 1b; Fig. S2), along with induction of Chk1 (Fig. S3) and the p53 target gene Gadd45 (Fig. S4). In *Drosophila*, the homologous histone modification to pH2AX is phosphorylation at Ser-137 of H2Av (pH2Av). We found that antibodies to pH2Av labeled neuronal chromatin in tau-expressing adult animals (Fig. 1d, arrow), but not in age-matched controls. To demonstrate physical evidence of DNA damage, brains from tau-expressing *Drosophila* were analyzed with the comet tail assay in which DNA single- or double-stranded breaks are demonstrated using single-cell gel electrophoresis. We observed that nuclei of tau-expressing flies displayed greater than a 2-fold longer comet tails (Fig. 1e).

Having established that a DSB response occurs in tauopathy models, we next determined the effect of genetically manipulating the checkpoint. Neurodegeneration in our model can be assessed by TUNEL staining, caspase cleavage, and standard histology (Khurana *et al.* 2006). We first determined whether the *Drosophila* homolog of *ATM*, *dATM*, is a modifier of tau-induced toxicity in the adult fly brain. We found that removing one copy of *dATM* significantly enhanced tau-induced neuronal apoptosis (Fig. 2a), with two separate *dATM* loss-of-function alleles, indicating a protective role for dATM in tauopathy.

Fly homologs of Chk2 and p53, dChk2 and Dmp53, also play a homologous role in the DNA damage response. Dominant-negative transgenic constructs of these modifiers have been created. A dominant-negative *Chk2* transgene and two separate dominant-negative *p53* transgenes led to a marked increase in tau-induced apoptosis (Fig. 2b; Fig. S5a,b). Conversely, increasing expression of p53 rescued toxicity (Fig. S6). Consistent with *ATM*, *Dmp53* and *Chk2* acting in the same pathway, combining these individual genetic modifiers demonstrated a non-additive modifying effect (Fig. 2c). Importantly, genetic modifiers did not alter tau expression (Fig. S7). These data support a neuroprotective role for an ATM-dependent DSB checkpoint in the fly tauopathy model.

We reasoned that the DSB checkpoint could be neuroprotective by inhibiting the cell cycle. We have previously shown that abnormal markers of cell cycle activation can be detected in adult neurons within the brain of tau-expressing flies, including proliferating cell nuclear antigen (PCNA), a marker also upregulated in Alzheimer's disease and related tauopathies (Fig. 2; Khurana *et al.* 2006; Herrup & Yang 2007).

Furthermore, cell cycle re-activation is causally linked to tau-induced neurodegeneration in fly and mouse Alzheimer's disease models (Andorfer *et al.* 2005; Khurana *et al.* 2006; Kim *et al.* 2008). We found that decreasing function of ATM, Chk2 or p53 strongly upregulated tau-induced PCNA (Fig. 2d, e, arrowheads). These data implicated re-entry of postmitotic neurons into the cell cycle as a mechanism through which checkpoint inhibition exacerbates neurotoxicity.

There is a growing literature on aberrant cell cycle re-entry, DNA damage and checkpoint activation in Alzheimer's disease and related tauopathies (Herrup & Yang 2007; Kim *et al.* 2008; Myung et al., 2008). The relationship among these three processes, however, has remained unclear. In this paper, we provide direct evidence of DNA damage and checkpoint activation in tauopathy models. Our previous work identifying oxidative stress as a critical component of tau-induced neurodegeneration provides one potential mechanism for tau-induced DNA damage (Dias-Santagata et al., 2007; Barlow et al., 1999). The genetic data presented in this study argue for a neuroprotective role of the DNA damage checkpoint and suggest caution in designing p53 inhibitors as therapeutic agents in Alzheimer's disease and related tauopathies. A number of recent studies have demonstrated an early protective role for the DNA damage checkpoint in tumorigenesis (Bartkova *et al.* 2005). Together with these findings and the known concomitant neurodegeneration and tumorigenesis that occurs with *ATM* loss-of-function in the disease ataxia telangiectasia, our data raise the possibility of a generally protective function for the DNA damage checkpoint in diseases of aging.

Andorfer C, Acker CM, Kress Y, Hof PR, et al. (2005). Cell-cycle reentry and cell death in transgenic mice expressing nonmutant human tau isoforms. *J Neurosci*. **25**, 5446-5454.

Barlow C, Dennery PA, Shigenaga MK, Smith MA, Morrow JD, Roberts LJ 2nd, Wynshaw-Boris A, Levine RL (1999). Loss of the ataxia-telangiectasia gene product causes oxidative damage in target organs. *Proc Natl Acad Sci U S A*. **96**, 9915-9919.

Bartkova J, Horejsi Z, Koed K, Kramer A, et al. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*. **434**, 864-870.

Culmsee C, Mattson MP (2005). p53 in neuronal apoptosis. *Biochem Biophys Res Commun*. **331**, 761-777.

Dias-Santagata D, Fulga TA, Duttaroy A , Feany MB (2007). Oxidative stress mediates tau-induced neurodegeneration in Drosophila. *J Clin Invest*. **117**, 236-245.

Herrup K , Yang Y (2007). Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? *Nat Rev Neurosci*. **8**, 368-378.

Ittner LM, Fath T, Ke YD, Bi M, et al. (2008) Parkinsonism and impaired axonal transport in a mouse model of frontotemporal dementia. *Proc Natl Acad Sci U S A*. **105**, 15997-16002.

Khurana V, Lu Y, Steinhilb ML, Oldham S, et al. (2006). TOR-mediated cell-cycle activation causes neurodegeneration in a Drosophila tauopathy model. *Curr Biol*. **16**, 230-241.

Kim D, Frank CL, Dobbin MM, Tsunemoto RK, et al. (2008). Deregulation of HDAC1 by p25/Cdk5 in neurotoxicity. *Neuron*. **60**, 803-817.

Kitamura Y, Shimohama S, Kamoshima W, Matsuoka Y, et al. (1997). Changes of p53 in the brains of patients with Alzheimer's disease. *Biochem Biophys Res Commun*. **232**, 418-421.

Kruman, II, Wersto RP, Cardozo-Pelaez F, Smilenov L, et al. (2004). Cell cycle activation linked to neuronal cell death initiated by DNA damage. *Neuron*. **41**, 549-561.

Lee Y, McKinnon PJ (2000). ATM dependent apoptosis in the nervous system. *Apoptosis*. **5**, 523-529.

Myung NH, Zhu X, Kruman II, Castellani RJ, et al. (2008). Evidence of DNA damage in Alzheimer disease: phosphorylation of histone H2AX in astrocytes. *Age (Dordr).* 30, 209-215.

Wilson C, Henry S, Smith MA, Bowser R (2004). The p53 homologue p73 accumulates in the nucleus and localizes to neurites and neurofibrillary tangles in Alzheimer disease brain. *Neuropathol Appl Neurobiol*. **30**, 19-29.

FIGURE LEGENDS

Figure 1. Upregulation of p53 and DNA damage in tauopathy models. (a,c) Increased p53 (a) or pH2AX (c) in neurons of tau transgenic mice (arrows). Scale bars are 10 µm. (b,d) Elevated p53 (b) or pH2AX (d) in neurons of tau transgenic flies (arrows). Scale bars are 3 µm. (e) Comet assay shows an increase in tail moment in tau transgenic fly brains compared to control. *P<0.01, unpaired t-test. Control genotype: *elav-GAL4/+*. Flies are 10 days old.

Figure 2. Neurodegeneration and cell cycle are promoted by reducing checkpoint function. (a,c,d,e) Reducing checkpoint function increases neurodegeneration (a-c, TUNEL) and cell cycle reentry (d,e, PCNA, arrowheads). (c) No further increases in tau neurotoxicity are observed when two checkpoints are reduced simultaneously, consistent with a nonlinear interaction. *P<0.01, ANOVA with Student-Neuman-Keuls. Control genotype: *elav-GAL4/+*. Flies are 10 days old.

SUPPLEMENTARY INFORMATION

METHODS

Fly Stocks, Genetics, Mice

All fly crosses and experiments were performed at 25°C. The tauopathy model used is based on expression of human 0N,4R tau carrying the R406W mutant version of tau found in patients with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). In this model the bipartite UAS/GAL4 expression system is used to express human tau in a panneural pattern with the *elav-GAL4* driver. The model has been previously described in detail (Wittmann *et al.* 2001; Khurana *et al.* 2006; Dias-Santagata *et al.* 2007). All flies were analyzed at 10 days of age in experiments using the *elav-GAL4* driver. Since developmental expression of wild type p53 causes lethality with the *elav-GAL4* driver, we used the conditional GeneSwitch system and the panneural driver *elav-GeneSwitch* (Osterwalder et al., 2001) to express wild type p53 (Fig. S6). To induce expression of p53 flies were fed the steroid RU486 following eclosion. RU486 was added to instant fly food (Carolina Biological) at a final concentration of 1 mg/ml. Flies were analyzed at 20 days of age in the GeneSwitch experiment.

The *elav-GAL4* driver and *CycE-lacZ* ($CycE^{05206}$) lines were obtained from the Bloomington *Drosophila* Stock Center. The *dAtm* mutants were previously described (Silva *et al.* 2004). *UAS-Chk2*^{DN} was described in Peters et al. (2002). *UAS-p53*$^{DN(1)}$ (*UAS-p53*^{D259H}) and *UAS-p53*$^{DN(2)}$ (*UAS-p53*^{Ct}) were the dominant negative constructs as described and characterized in Brodsky et al. (2000). $p53^{11\text{-}1B\text{-}1}$ was described in Rong et al. (2002) and was kindly provided by Michael Brodsky. The presence of the $p53^{11\text{-}1B\text{-}1}$ mutant allele in relevant stocks was confirmed using PCR on genomic DNA (primers: (1) 5'-GTTCGCCTGGATCTTAATTA-3'; (2) 5'-GTTCGCCTGGATCTGAATGT-3'; and (3) 5'-AATCGCTGCATGCGGTAGTA-3') as described by Kent Golic (Tweedie et al., 2009). Additional stocks were generously provided by the indicated investigators: UAS-

human PARP transgenic flies, Darren Williams (Williams et al., 2006); *hid-lacZ* (*hid*$^{20\text{-}10}$-*lacZ*) reporter, Andreas Bergmann (Fan et al., 2010); *th-GFP,* Jin Jiang (Zhang et al., 2008); *Chk1-GFP*, William Sullivan (Royou et al., 2008).

Transgenic mice express 1N,4R tau carrying the K369I mutant version of tau reported in a patient with Pick's disease tauopathy pathology driven by the mThy1.2 promoter (Ittner et al., 2008). Mice were analyzed at 10 months of age, and compared to age-matched littermate controls.

Sectioning, Immunostaining, and Histology

Adult flies were fixed in formalin at 10 days and embedded in paraffin. Serial frontal 4 µm sections including the entire brain were prepared. Mouse tissue was also paraffin embedded, and was cut at 3 µm. Antigen retrieval was performed by microwaving in sodium citrate buffer. Immunostaining was performed with an avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories) or with secondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 555. Primary antibodies included anti-elav (Developmental Studies Hybridoma Bank), anti-pH2AX (Cell Signaling), anti-pH2Av (Rockland), anti-MAP2 (Chemicon), anti-*Drosophila* p53 (d-200 and E-5, Santa Cruz; H3, Developmental Studies Hybridoma Bank), anti-mouse p53 (FL-393, Santa Cruz), anti-human PARP (E51, Abcam), and anti-PCNA (Biomeda). p53 knockout mice (not shown) or flies (Fig. S2) were used to ensure the specificity of the p53 antibodies.

For PCNA quantification, the number of PCNA-positive foci were counted in the entire brain. For PARP quantification, cleaved PARP immunoreactive neurons were counted throughout the entire brain (Fig. S5a). For vacuole quantification, H&E stained sections were analyzed and vacuoles larger than 5 µm were counted throughout the entire brain (Fig. S5b). For PCNA, PARP and vacuole quantification at least six brains were analyzed per genotype; statistical significance was established one-way ANOVA with post hoc test for multiple comparisons (Student-Neuman-Keuls; $P< 0.01$). For quantitation of p53 or pH2AX in K3 (n=5) and nontransgenic control (n=4) mice, the number of positive nuclei (out of 50 neuronal nuclei) were counted in the lateral frontal cortex, an area of high tau expression in this model. Statistical significance was evaluated using an unpaired t-test ($P<0.001$).

TUNEL Staining

Neuronal apoptosis was detected with the TUNEL assay using a commercially available kit (TdT FragEl, Oncogene). Neurodegeneration was quantified by counting the number of TUNEL-positive cells per hemibrain in consecutive frontal sections between the ellipsoid body anteriorly and the mushroom body posteriorly. At least six hemibrains were examined per time point for each genotype. Statistical significance was established by one-way ANOVA with post hoc test for multiple comparisons (Student-Neuman-Keuls; $P< 0.01$).

Comet Assay

The comet assay was performed using commercially available reagents (CometAssay, Trevigen) under neutral electrophoresis conditions. Images were analyzed using the software Comet Score version 1.5. The assay was repeated three times. At least 6 animals per genotype were analyzed. Statistical significance was evaluated using an unpaired t-test ($P<0.01$).

Western blot

Adult *Drosophila* heads were homogenized in 2X Laemli's buffer (Sigma-Aldrich). Samples were boiled for 10 minutes, briefly centrifuged, and subjected to SDS-PAGE in a 10-20% gradient gel (Cambrex). Proteins were transferred to nitrocellulose membranes (Bio-Rad), blocked in 2% milk in PBS with 0.05% Tween 20, and immunoblotted using a phosphorylation-independent rabbit polyclonal anti-tau antibody (Dako). The blot was reprobed with an anti-actin antibody (JLA20, Developmental Studies Hybridoma Bank) to illustrate equivalent protein loading.

RNA extraction and real-time quantitative PCR

RNA was isolated from fly heads using Trizol solution as suggested by the manufacturer with an additional centrifugation (11,000 x g, 10 minutes) to remove debris prior to the addition of chloroform. RNA quality was evaluated on 1% agarose gel. 1 µgm of total RNA was used in the reverse-transcription reaction (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Primers were designed using Primer Express 3.0 (Applied Biosystems) and were used at a final concentration of 150 nM. PCR reactions were set up in a 16 µl reaction volume using SYBR Green PCR Master Mix (Applied Biosystems). PCR amplifications were performed with an Applied Biosystem Step One machine. The RpL32 ribosomal protein *L32* transcript was used as an endogenous control. Primers were as follows: GADD45 for – TGCTGGAGGCCTTTTGCT, GADD45 rev – GCGTCGTCCACCTTGATCA, RpL32 for – GGCCCAAGATCGTGAAGAAG, RpL32 rev – TTTGTGCGACAGCTTAGCATATC. Experiments were run in triplicate and the experiment was repeated four times.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Induction of p53 and DNA damage in neurons from K3 transgenic mice. (a) Quantification demonstrates a significant increase in the number of p53-immunoreactive nuclei in frontal cortical neurons from mice expressing mutant human tau (n=5) compared to nontransgenic controls (n=4). (b) Quantification demonstrates a significant increase in the number of pH2AX-immunoreactive nuclei in frontal cortical neurons from mice expressing mutant human tau (n=5) compared to nontransgenic controls (n=4). The Y-axis indicates the percent of neurons with positive nuclei. *indicates P<0.01, unpaired t-test. Mice are 10 months old.

Supplementary Figure 2. No p53 immunoreactivity is observed in p53 null neurons. To confirm the specificity of the anti-*Drosophila* p53 antibody used in Fig. 1b (Santa Cruz, d-200), brains from flies expressing tau in a *p53* null background were immunostained in parallel with flies expressing tau in the presence of two normal copies of *p53*. No significant immunofluorescence is observed in the p53 null animals (green, upper panels). An antibody to the neuronal protein elav is used to identify neuronal nuclei (red). Scale bar is 10 µm and pertains to all panels. Driver is *elav-GAL4*. Flies are 10 days old.

Supplementary Figure 3. Cell death and cell cycle pathway markers are specifically expressed in tau transgenic flies. Reporters of *hid* (*hid*$^{20\text{-}10}$*-lacZ*), *th* (also known as *Diap1*), *Chk1* (*grapes*), and *cyclin E* expression are all positive in tau transgenic fly brains (right panels, arrows), but are negative in controls (left panels). Expression of the reporters was assayed by immunohistochemical detection of ß-galactosidase or GFP on sections of adult fly brains. Positive signals are indicated by the brown (DAB) reaction product (arrows). A light hematoxylin (blue) counterstain highlights nuclei in panels showing the *Chk1-GFP* and *CycE-lacZ* reporters. Scale bar is 10 µm and pertains to all panels. Control genotype: *elav-GAL4/+*. Flies are 10 days old.

Supplementary Figure 4. Gadd45 mRNA is upregulated in tau transgenic flies as measured by quantitative real time PCR. Data is presented as fold increase over control (*elav-GAL4/+*) and is normalized to an endogenous control transcript (RpL32 ribosomal protein *L32*). Experiments were run in triplicate and the experiment was repeated four times. The data shown represent the average of four experiments. Flies are 10 days old.

Supplementary Figure 5. Neurodegeneration is promoted by reducing checkpoint function as measured by caspase-cleaved PARP (a) and vacuole formation (b). Removing one copy of *dAtm* using heterozygous loss of function mutants (*dAtm*^{8} and *dAtm*^{3}) significantly increases tau neurotoxicity. Reducing Chk2 or p53 function using dominant negative forms of the proteins significantly significantly increases tau neurotoxicity. (a) Caspase-cleaved PARP was monitored by introducing a transgene including the caspase cleavage site of human PARP and detecting cleaved human PARP on tissue sections comprising the entire brain using a specific antibody. The number of cleaved PARP-positive foci was counted. (b) Vacuoles greater than 5 µm were counted in sections comprising the entire brain. n=6 per genotype. *indicates P<0.01, one-way

ANOVA with Student-Neuman-Keuls post hoc test for multiple comparisons. Control genotype (a): *elav-GAL4/+; UAS-PARP/+*. Flies are 10 days old.

Supplementary Figure 6. Overexpression of wild type p53 rescues tau neurotoxicity. The inducible GeneSwitch system was used to circumvent developmental lethality of p53 overexpression. Wild type p53 was expressed for the first 20 days of adult life. Although reduced toxicity is seen when tau was expressed using GeneSwitch, most likely due to significantly reduced levels of expression mediated by *elav-GeneSwitch* compared with *elav-GAL4* system, rescue of tau neurotoxicity is nonetheless observed. n=7 per genotype. *indicates $P<0.05$, unpaired t-test. Flies are 20 days old.

Supplementary Figure 7. Western blot analysis reveals no change in tau levels in the presence of genetic modifiers (upper panel). A phosphorylation-independent polyclonal antibody (Dako) was used to detect tau. The blot was reprobed with an antibody to actin to illustrate equivalent protein loading (lower panel). Driver is *elav-GAL4*. Flies are 2-3 days old.

SUPPLEMENTARY REFERENCES

Brodsky MH, Nordstrom W, Tsang G, Kwan E, Rubin GM , Abrams JM (2000). Drosophila p53 binds a damage response element at the reaper locus. *Cell*. 101, 103-113.

Dias-Santagata D, Fulga TA, Duttaroy A , Feany MB (2007). Oxidative stress mediates tau-induced neurodegeneration in Drosophila. *J Clin Invest*. 117, 236-245.

Fan Y, Lee TV, Xu D, Chen Z, Lamblin AF, Steller H, Bergmann A (2010). Dual roles of Drosophila p53 in cell death and cell differentiation. *Cell Death Differ*. 17, 912-921.

Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ , De Bakker PIW (2008). SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics*. 24, 2938-2939.

Khurana V, Lu Y, Steinhilb ML, Oldham S, Shulman JM , Feany MB (2006). TOR-mediated cell-cycle activation causes neurodegeneration in a Drosophila tauopathy model. *Curr Biol*. 16, 230-241.

Madigan JP, Chotkowski HL , Glaser RL (2002). DNA double-strand break-induced phosphorylation of Drosophila histone variant H2Av helps prevent radiation-induced apoptosis. *Nucleic Acids Res*. 30, 3698-3705.

Osterwalder T, Yoon KS, White BH, Keshishian H (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proc Natl Acad Sci U S A*. 98, 12596-12601.

Peters M, DeLuca C, Hirao A, Stambolic V, Potter J, Zhou L, Liepa J, Snow B, Arya S, Wong J, Bouchard D, Binari R, Manoukian AS , Mak TW (2002). Chk2 regulates irradiation-induced, p53-mediated apoptosis in Drosophila. *Proc Natl Acad Sci U S A*. 99, 11305-11310.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira M, Bender D, Maller J, Sklar P, de Bakker P, Daly M , Sham P (2007). PLINK: a tool set for whole-genome

association and population-based linkage analyses. *Am J Hum Genet*. 81, 559-575.

Rong YS, Titen SW, Xie HB, Golic MM, Bastiani M, Bandyopadhyay P, Olivera BM, Brodsky M, Rubin GM, Golic KG (2002). Targeted mutagenesis by homologous recombination in D. melanogaster. Genes Dev. 16, 1568-1581.

Royou A, McCusker D, Kellogg DR, Sullivan W (2008). Grapes(Chk1) prevents nuclear CDK1 activation by delaying cyclin B nuclear accumulation. J Cell Biol. 183, 63-75.

Silva E, Tiong S, Pedersen M, Homola E, Royou A, Fasulo B, Siriaco G , Campbell SD (2004). ATM is required for telomere maintenance and chromosome stability during Drosophila development. *Curr Biol*. 14, 1341-1347.

Tweedie S, Ashburner M, Falls K, Leyland P, McQuilton P, Marygold S, Millburn G, Osumi-Sutherland D, Schroeder A, Seal R, Zhang H, and The FlyBase Consortium (2009). FlyBase: enhancing Drosophila Gene Ontology annotations. *Nucleic Acids Research.* 37, D555-D559; doi:10.1093/nar/gkn788.

Williams DW, Kondo S, Krzyzanowska A, Hiromi Y, Truman JW (2006). Local caspase activity directs engulfment of dendrites during pruning. Nat Neurosci. 9, 1234–1236.

Wittmann CW, Wszolek MF, Shulman JM, Salvaterra PM, Lewis J, Hutton M , Feany MB (2001). Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. *Science*. **293**, 711-714.

Zhang L, Ren F, Zhang Q, Chen Y, Wang B, Jiang J (2008). The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev Cell. 14, 377-387.