A Role for the Twins Protein Phosphatase (PP2A-B55) in the Maintenance of *Drosophila* Genome Integrity

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ABSTRACT The protein phosphatase 2A (PP2A) is a conserved heterotrimeric enzyme that regulates several cellular processes including the DNA damage response and mitosis. Consistent with these functions, PP2A is mutated in many types of cancer and acts as a tumor suppressor. In mammalian cells, PP2A inhibition results in DNA double strand breaks (DSBs) and chromosome aberrations (CABs). However, the mechanisms through which PP2A prevents DNA damage are still unclear. Here, we focus on the role of the *Drosophila twins* (*tws*) gene in the maintenance of chromosome integrity; *tws* encodes the B regulatory subunit (B/B55) of PP2A. Mutations in *tws* cause high frequencies of CABs (0.5 CABs/cell) in *Drosophila* larval brain cells and lead to an abnormal persistence of γ-H2Av repair foci. However, mutations that disrupt the PP4 phosphatase activity impair foci dissolution but do not cause CABs, suggesting that a delayed foci regression is not clastogenic. We also show that Tws is required for activation of the G2/M DNA damage checkpoint while PP4 is required for checkpoint recovery, a result that points to a conserved function of these phosphatases from flies to humans. Mutations in the ATM-coding gene *tefu* are strictly epistatic to *tws* mutations for the CAB phenotype, suggesting that failure to dephosphorylate an ATM substrate(s) impairs DNA DSBs repair. In addition, mutations in the *Ku70* gene, which do not cause CABs, completely suppress CAB formation in *tws Ku70* double mutants. These results suggest the hypothesis that an improperly phosphorylated Ku70 protein can lead to DNA damage and CABs.

KEYWORDS Tws; PP2A-B55; chromosome aberrations; ATM; Ku70; *Drosophila*

COMPLEX biological processes require cellular activities to quickly switch from one state to another. One of the most important mechanisms that allows activation and silencing of these activities is reversible phosphorylation. Multiple kinases and phosphatases have been identified that work in concert to regulate fundamental processes such as the cell cycle and the DNA damage response (DDR) pathways (Freeman and Monteiro 2010). The protein phosphatase 2A (PP2A) is one of the major serine/threonine phosphatases; it regulates several cellular processes including DDR and mitosis. Consistent with these functions, accumulating evidence indicates that PP2A is mutated in many types of cancer and acts as a tumor suppressor (reviewed in Eichhorn *et al.* 2009; Khanna *et al.* 2013; Perrotti and Neviani 2013). PP2A is conserved from yeast to humans and is one of the most abundant enzymes, accounting for up to 1% of total cellular proteins in some tissues (Eichhorn *et al.* 2009; Shi 2009).

In mammals, PP2A is a heterotrimeric enzyme consisting of a core dimer, formed by a catalytic (C) and a structural (A) subunit, associated with a third regulatory B subunit, which governs subcellular localization and substrate specificity. PP2A A and PP2A C are encoded by two distinct genes, each of which produces two protein isoforms; the B subunits (B/B55, B′/B56, B″/PR72, and B‴/STRN) are encoded by four genes that also produce several isoforms. It has been estimated that the combinatorial association of the PP2A subunits can give rise to >90 different complexes, which are

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likely to mediate different physiological processes (Janssens and Goris 2001; Eichhorn *et al.* 2009). PP2A is a heterotrimeric complex also in *Drosophila*. The fly genome harbors two genes, *PP2A-29B* and *microtubule star* (*mts*), which encode the PP2A A and PP2A C subunits, respectively, and four genes that specify the B regulatory subunits: *twins* (*tws*; B/B55), *widerborst* (*wdb*; B′/B56 type 1), *well rounded* (*wrd*; B′/B56 type 2), and *CG4733* (B″/PR72) (Mayer-Jaekel *et al.* 1992; Uemura *et al.* 1993; Snaith *et al.* 1996; Hannus *et al.* 2002; Viquez *et al.* 2006; see also FlyBase). The fly genome also contains the *connector to kinase to AP-1* (*cka*) gene that encodes a protein partially homologous to B‴/STRN (Ribeiro *et al.* 2010; see also FlyBase).

Studies carried out in several metazoan organisms, including *Drosophila* and humans, have shown that PP2A plays an important role in the regulation of cell division. In some studies, the role of PP2A was addressed by inhibiting the enzyme activity with either okadaic acid or the small tumor antigen (ST) of Simian virus 40. Other studies exploited mutations or RNA interference (RNAi) to deplete specific PP2A subunits (see, for example, Tournebize *et al.* 1997; Chen *et al.* 2007; Kotadia *et al.* 2008; Foley *et al.* 2011). Work on the B55 subunit showed that the PP2A-B55 enzyme is responsible for dephosphorylation of many mitotic proteins, allowing mitotic exit and progression through interphase. The activity of this enzyme is high during interphase but is inhibited when cells enter mitosis to avoid premature reversion of cyclin-dependent kinase 1 (CDK1)-driven phosphorylation of mitotic proteins. PP2A-B55 is regulated by a conserved pathway involving the Greatwall (Gwl) kinase; in preparation of the M phase, CDK1 phosphorylates and activates Gwl, which in turn phosphorylates Endosulfine that binds and inactivates PP2A-B55 (Glover 2012; Hunt 2013; Lorca and Castro 2013; Williams *et al.* 2014).

Besides exerting a general control on mitotic progression and exit, PP2A has been implicated in specific aspects of mitosis. For example, PP2A inhibition with okadaic acid or ST, or disruption of its core subunits (A or C) affects centrosome behavior, spindle formation, and chromosome segregation in both *Drosophila* and vertebrates (Snaith *et al.* 1996; Tournebize *et al.* 1997; Kitajima *et al.* 2006; Tang *et al.* 2006; Chen *et al.* 2007; Kotadia *et al.* 2008). Defects in microtubule-kinetochore interaction and chromosome segregation have been also observed in HeLa and *Drosophila* cells depleted of the B56 regulatory subunits (Chen *et al.* 2007; Foley *et al.* 2011; Porter *et al.* 2013), while B55 depletion in *Drosophila* S2 cells counteracted Plk4 autophosphorylation leading to centrosome amplification (Brownlee *et al.* 2011).

In addition to its mitotic functions, PP2A plays important roles in nervous system maintenance and functioning, and in DNA repair (reviewed in Lambrecht *et al.* 2013). Studies in mammalian cells have shown that PP2A inhibitors or RNAi against the core subunits of the complex cause DNA double strand breaks (DSBs) and chromosome aberrations (CABs) (Chowdhury *et al.* 2005; Lankoff *et al.* 2006; Wang *et al.* 2009; Kalev *et al.* 2012). Several nonmutually exclusive hypotheses have been proposed to explain the role of PP2A in the repair of DSBs in mammalian cells. It has been suggested that loss of PP2A affects the dephosphorylation kinetics of γ-H2AX DNA repair foci associated with DSBs (Chowdhury *et al.* 2005). Immediately after their occurrence, DSBs recruit a series of DNA repair factors, starting with the Mre11-Rad50-Nbs (MRN) complex and the ATM kinase. ATM phosphorylates the histone variant H2AX (H2Av in *Drosophila*) at Ser139 to form γ-H2AX, which spreads in both directions from the DSB and helps recruiting additional DNA repair factors; leading to the formation of cytologically detectable foci, which disassemble after DNA repair, following γ-H2AX dephosphorylation (reviewed in Bekker-Jensen and Mailand 2010 and Polo and Jackson 2011). It has been shown that PP2A directly binds and dephosphorylates γ-H2AX at DNA repair foci and that in PP2A-deficient cells, γ-H2AX foci persist longer that in control cells, suggesting that foci persistence leads to incomplete DSB repair (Chowdhury *et al.* 2005). It has been also reported that PP2A-deficient cells display an increase in the level of ATM autophosphorylation/activation accompanied by upregulation of the ATM downstream kinase CHK2 and downregulation of the RAD51 and BRCA1 factors, which mediate the homologous recombination (HR) pathway of DSB repair (Kalev *et al.* 2012). Other studies suggested that PP2A dephosphorylates the Ku complex that mediates the nonhomologous end joining pathway (NHEJ), and that PP2A-deficient cells accumulate DSBs due to defects in this pathway (Wang *et al.* 2009). Finally, consistent with a general role of PP2A in DNA repair, several studies have shown that PP2A dephosphorylates not only ATM and CHK2 but also the ATR kinase and its downstream target CHK1, and it is required for the G2/M cell cycle arrest induced by DNA damage (Leung-Pineda *et al.* 2006; Yan *et al.* 2010).

Although most of the roles of PP2A are evolutionarily conserved, there are currently very few data suggesting an involvement of *Drosophila* PP2A in DNA repair. The only indication of a possible role of PP2A in the maintenance of chromosome integrity is provided by the observation that mutants in the B55-coding gene *tws* (also called *aar*) exhibit chromatin bridges at anaphase (Gomes *et al.* 1993; Mayer-Jaekel *et al.* 1993). Here we show that mutations in *tws* cause high frequencies of CABs and abnormal persistence of γ-H2Av DNA repair foci. We also show that mutations in the ATM-coding gene *tefu* and the *ku70* gene are strictly epistatic to *tws* mutations for the CAB phenotype. Collectively, our genetic analyses suggest that failure of Tws-mediated dephosphorylation generates abnormally and/or untimely phosphorylated Ku70, which interferes with normal DNA repair leading to DNA damage and CABs.

Materials and Methods

Drosophila strains and crosses

tws^{430} was isolated by a cytological screen of larval brain squashes from a collection of 1680 EMS-induced late lethals generated in Charles Zuker's laboratory (University of California,

San Diego, CA). *tws^{P} and tws^{196}* were obtained from M.L. Goldberg (Cornell University, Ithaca, NY), *brca^{56E}* and *brca^{K0}* from T. Schüpbach (Princeton University, Princeton, NJ), and *ku70^{Ex8}* from W. R. Engels (University of Wisconsin, Madison, WI); *tefu^{atm6}*, *mei-41^{29D}*, *nbs^{1}*, *His2av ^{810}*, and *grapes^{1}* mutations have been described previously (van Daal and Elgin 1992; Sullivan *et al.* 1993; Laurençon *et al.* 2003; Silva *et al.* 2004; Ciapponi *et al.* 2006). *Df(3L)by62*, *Df(3L)ED54541*, and *Df(3L)BSC621*, *Pp4-19C*, *brca2^{KG03961}*, *lig4^{5}*, *okr$^{17-11}$*, *okr^{A19-10}*, *mus301^{D4}/SpnC^{D4}*, *mei-9^{A1}*, *rad51^{1}/SpnA^{1}* were all obtained from the Bloomington Stock Center. Mutations and deficiencies on the third and second chromosomes were kept in stock over the *TM6C Sb Tb* and *CyO-TbA* (Lattao *et al.* 2011) balancers, respectively; homozygous and hemizygous mutant larvae were recognized for their non-*Tubby* phenotype. Mutations on the *X* chromosome were balanced over *FM7-GFP* and mutant larvae were recognized for their non-GFP phenotype. *tws^{430}* and *brca2^{KG03961}* double mutants were constructed by crossing *tws^{430}/TM6B*; *CyO-GFP/Sco* females to *MKRS/TM6B*; *brca2^{KG03961}/CyO-GFP* males; *tws^{430}*/TM6B; *brca2^{KG03961}/CyO-GFP* progeny were mated *inter se* to obtain a stable stock. To generate *brca^{56E}/brca^{K0}*; *tws^{430}/tws^{430}* and *okr$^{17-11}$/okr^{A19-10}*; *tws^{430}/tws^{430}* double mutants, *tws^{430}/TM6B*; *brca^{56E}/CyO-GFP* and *tws^{430}/TM6B*; *okr$^{17-11}$/CyO-GFP* females were crossed to *tws^{430}/TM6B*; *brca^{K0}/CyO-GFP* and *tws^{430}/TM6B*; *okr^{A19-10}/CyO-GFP* males, respectively. *mei41^{29D} tws^{430}*, *lig4^{5} tws^{430}*, and *mei-9^{A1} tws^{430}* double mutants were generated by crossing *mei41^{29D}/FM7-GFP*; *+/TM6B*, *lig4^{5}/FM7-GFP*; *+/TM6B* and *mei-9^{A1}/FM7-GFP*; *+/TM6B* females to *w*; *tws^{430}/* TM6C males, respectively. The *w/FM7-GFP*; *tws^{430}/TM6B* females resulting from this cross were then mated to *mei41^{29D}*; *tws^{430}/TM6B*, *lig4^{5}*; *tws^{430}/TM6B* and *mei-9^{A1}*; *tws^{430}/TM6B* males to generate *mei41^{29D}/FM7-GFP*; *tws^{430}/TM6B*, *lig4^{5}/FM7-GFP*; *tws^{430}/TM6B* and *mei-9^{A1}/FM7-GFP*; *tws^{430}/TM6B* females that were crossed to *FM7-GFP*; *tws^{430}/TM6B* males to generate stable stocks. Double mutant larvae from these crosses were unambiguously identified on the basis of their non-GFP and/or non-*Tubby* phenotypes. *tws^{430} tefu^{atm6}*, *nbs^{1} tws^{430}*, *tws^{430} H2Av^{810}*, *tws^{430} rad51^{1}*, *tws^{430} ku70^{Ex8}*, and *mus301^{D4} tws^{430}* double mutants were generated by recombination and balanced over *TM6B*. To check for the presence of both mutations on the recombinant third chromosomes we performed complementation tests; the presence of both *tws^{430} ku70^{Ex8}* on the recombinant chromosome was also confirmed by PCR. Also, the *ku70MT tws^{430} ku70^{Ex8}* chromosome was constructed by recombination and the presence of *ku70MT*, *tws^{430}*, and *ku70^{Ex8}* in this chromosome was confirmed by complementation and PCR analyses. We designate as *ku70MT* a *ku70/irbp* gene carrying the 2xTY1-sGFP-V5-Pre-TEV-BLRP-3xFLAG multi-tag at its C terminus. *ku70MT* is a component of the fosmid clone FlyFos 015211, which contains the *nina*, *CG6723*, *Ranbp9*, *ku70/irbp*, *mgr*, and *mRpL40* genes and part of the *pros* gene. *ku70/irbp* was tagged *in vitro*, and the entire fosmid construct was then inserted into a fly stock carrying an attP landing site at 65B using a *nanos*-ΦC31 integrase source [Sarov *et al.* 2016; the *ku70MT* bearing line was obtained from the Vienna *Drosophila* Resource Center (VDRC) stock center]. *Tip60* and *Mrg15* RNAi flies were generated by crossing females bearing the RNAi construct (Tip60, 22233 from VDRC; *Mrg15*, 35241 from Bloomington Stock Center) to males carrying the *69B-Gal4* driver. To determine the effect of *ku70* RNAi in a *tws^{430}* mutant background, we used the 110,409/kk VDRC construct (*ku70*-RNAi) mapping to the second chromosome; *CyO-GFP/actin-GAL4*, *tws^{430}/TM6B* females were crossed to *CyO-GFP/ku70*-RNAi, *tws^{430}/TM6B* males to obtain *ku70*-RNAi*/actin-GAL4*; *tws^{430}/tws^{430}* larvae that were recognized for their non-GFP and non-*Tubby* phenotype. The Oregon R strain was used as wild-type control. All stocks were maintained, and crosses were made at 25° on standard *Drosophila* medium. The balancers and the genetic markers used in these crosses are described in detail in FlyBase (http://flybase.bio.indiana.edu/)

Chromosome cytology

Colchicine-treated larval brain metaphases for CAB scoring and noncolchicine-treated preparations for the analysis of anaphases and the mitotic index (MI) were obtained as described (Gatti and Goldberg 1991). The MI is the average number of mitotic figures per optic field; the frequency of anaphases is the ratio between the number of anaphases and the total number of mitotic figures observed (Gatti and Goldberg 1991). To perform the checkpoint assay, wild-type and mutant larvae were treated with 10 Gy of X rays. Brains from irradiated larvae were then dissected and fixed at the indicated times to determine the MI. The relative MIs were calculated normalizing the MIs of irradiated brains with respect to that of untreated brains. All fixed preparations were mounted in Vectashield H-1200 with DAPI (Vector Laboratories, Burlingame, CA) to stain the chromosomes.

Tests for X-ray and hydroxyurea sensitivity

To determine the X-ray sensitivity of *tws* mutants, homozygous *tws^{430}* larvae and wild-type larvae were irradiated with 2.5 Gy. Then, 3 hr after irradiation (IR), larval brains were dissected in saline (NaCl 0.7%), incubated for 1 hr with colchicine (10^{-5} M), and then fixed. To determine the hydroxyurea (HU) sensitivity, brains from homozygous *tws^{430}* mutant larvae and wild-type larvae were incubated for 20 min in 1 mM HU dissolved in saline. They were then rinsed, incubated in saline for 3.5 hr, and fixed. Finally, 1 hr before fixation, colchicine was added to the saline to collect metaphases.

Immunostaining and γ-H2Av foci detection

For immunostaining, brains from third instar larvae were dissected and fixed as described in Bonaccorsi *et al.* (2000). To induce γ-H2Av and Tws foci, larvae were irradiated with 5 Gy of X rays; larval brains were then dissected and fixed at various postirradiation (PIR) times. Brain preparations were then rinsed several times in PBS 0.1% Triton (PBST), incubated overnight at 4° with primary antibodies diluted in PBST, rinsed in PBST, and then incubated for 1 hr at room

temperature with the pertinent secondary antibodies. The primary antibodies were: rabbit anti-Histone H2AvD pS137 (1:100; Rockland code #600-401-914) and rat anti-Twins (1:50; a gift from T. Uemura, Kyoto University, Japan). Antibodies were detected with Alexa-Fluor-555-conjugated anti-rabbit (1:300 in PBSt; Molecular Probes, Eugene, OR) and FITC-conjugated anti-rat (1:20 in PBSt; Jackson Laboratories). Immunostained preparations were mounted in Vectashield H-1200 (Vector Laboratories) containing DAPI (4,6 diamidino-2-phenylindole). To quantify the foci, at least 400 cells were analyzed for each PIR fixation time. All cytological preparations were examined with a Carl Zeiss (Thornwood, NY) Axioplan fluorescence microscope, equipped with an HBO100W mercury lamp and a cooled charged-coupled device (CCD camera; Photometrics CoolSnap HQ).

Western blotting

Extracts for Western blotting were prepared by lysing samples of 20 brains in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 30 mM NaF, 25 mM b-glycerophosphate, 0.2 mM Na_3VO_4, Triton X-100 1%, and cOmplete Protease Inhibitor Cocktail (Roche). Extracts were immunoblotted according to Somma *et al.* (2002); blotted proteins were detected using rabbit anti-Histone H2AvD pS137 (1: 500 in TBST; Rockland code #600-401-914) and rat anti-Tws (a gift form T. Uemura; diluted 1:500 in TBST). To determine the kinetics of H2Av phosphorylation, larvae were irradiated with X rays (10 Gy) and 20 brain samples were collected at different PIR times. The loading control was always Giotto (Gio), a *Drosophila* Phosphatidylinositol transfer protein (Giansanti *et al.* 2006).

RNAi in Drosophila S2 cultured cells

RNAi treatments in S2 cells were performed as described in Somma *et al.* (2008). To produce double-stranded RNAs we used the following primers:

lig4 F: ATGTGACCACCA; *lig4* R: ATGCCTTCGCGA.
Blm F: CAACCGCCATGT; *Blm* R: ATGCTCGTAAGG.
mus-301 F: CAGTTGGACTGC; *mus-301* R: CGATTCAGCTGC.
okr F: CTGTAGTACTCA; *okr* R: ATTACGGTGGTA.
tws F: TGGTCAACCAGA; *tws* R: AGAACATGGATG.
brca2 F: AACCGCATCAAC; *brca2* R: AAGGCTTGGGAG.
His2Av F: AGTTGGCAGGCA; *His2Av* R: ATGCGTGCGGGT.

Each of these primers contained the T7 polymerase binding sequence:

5′-TAATACGACTCACTATAGGGAGG-3′.

Nucleic acid extraction, PCR, and RT- PCR

Preparation of fly genomic DNA and RNA from S2 cells, PCR, RT-PCR, agarose gel electrophoresis, DNA sequencing, and sequence analysis were performed with standard procedures. RNA extraction was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany). For RT-PCR we used 20 ng of RNA to synthesize complementary DNAs (cDNAs) using the Superscript kit (Invitrogen, Carlsbad, CA). For cDNA amplification were used the following primers:

rp49 F: TACAGGCCCAAGATCGTGAA; *rp49* R: ACGTTGTGCACCAGGAACTT.
tws F: GTAATGGAGAGGCGTCTGGT; *tws* R: TCTCCTGATCCGAATTAACGC.
brca2 F: CTGGACGACAAGGAGCAACC; *brca2* R: TCAAGTCCAACAGACGTCGG.
lig4 F: ACGATCACGGCACCTTAACG; *lig4* R: CTTGTCCTTGTACCACCCAC.
His2Av F: GCTGGCGGTAAAGCAGGCAA; *His2Av* R: AATGACGTTGCCCTTCCGCT.
mus-301 F: AAATGGTGGGACGAGCAGGT; *mus-301* R: ACAGAATGCTGGCATCTGCC.

To test for the presence of *kuMT* and $ku70^{Ex8}$, we used the following *GFP* and *ku70* primers:

GFP F: AAGGGCGAGGAGCTGTTCA; *GFP* R: TTGTACAGCTCATCCATGCCCA.
Ku70 F: GACTCATCTTCGCCAACACCA; *ku70* R: ATGATGCTGCTGGGCTTCAAG.

Data availability

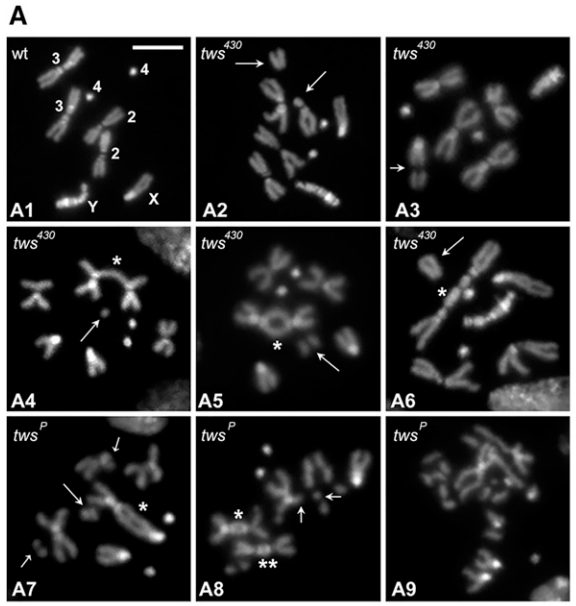
All data needed to confirm the results presented in this study are included in the main article and in the supplemental material. Fly strains are available upon request.

Results

Mutations in tws induce CABs

In the course of a screen aimed at the isolation of new *Drosophila* mitotic mutants (see *Materials and Methods*), we identified a lethal mutation that causes frequent CABs in larval brain cells. Recombination and deletion mapping showed that this mutation is included in the 85F12–85F14 polytene chromosome interval and fails to complement mutations in the *tws* gene that maps to the same region (Supplemental Material, Figure S1A). We therefore named our mutation tws^{430} (430 is the number of the *tws*-bearing stock in the collection we screened). *tws* encodes eight transcripts that differ at the 5′ UTR; all these transcripts give rise to two polypeptides of 499 (a isoform) and 433 aa (b isoform) that differ only in 56 aa at the N terminus (Figure S1B). DNA sequencing showed that tws^{430} carries a G→A transition in a splice site at the 5′ of an intron shared by all transcripts (Figure S1B). Consequently, the first AG in the downstream exonic sequence is used as a splice site, leading to a stop codon that would result in truncated proteins of 142 and 86 amino acids (Figure S1C).

To define the frequency and pattern of CABs induced by mutations in *tws* we examined DAPI-stained brain preparations from third instar larvae of various genotypes: tws^{430}/tws^{430}, $tws^{430}/Df(3R)by62$ (*Df(3R)by62*, henceforth designated

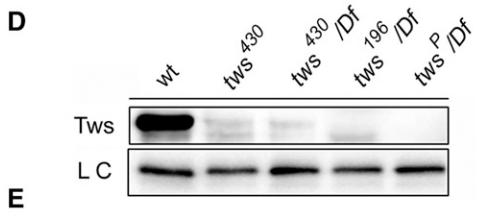


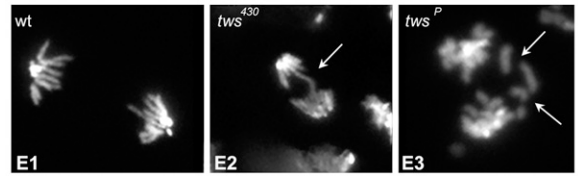
B

Genotype	# of cells scored	# of brains	Cells with CABs (%) (§)	Cells with 5 or >5 CABs (%)	CABs/cell
wt	751	6	0.8	0.0	0.008
tws^{430}	1245	15	40.5	10.6	0.92
tws^{430}/ *Df*	707	10	46.5	15.4	1.16
tws^{430}/ tws^{P}	703	9	37.3	9.5	0.82
tws^{430}/ tws^{196}	658	7	50.4	17.0	1.29
tws^{P}/ tws^{196}	1091	8	47.1	15.0	1.15
tws^{P} / *Df*	1123	13	56.9	25.2	1.67
tws^{196}/ *Df*	1002	13	40.5	10.5	0.92

C

Genotype	Cd (%)	Iso (%)	C-tid exch. (%)	C-some exch. (%)
tws^{430}	26.6	58.7	10.6	4.1
tws^{430}/ *Df*	15.4	58.6	15.0	11.0
tws^{430}/ tws^{P}	20.2	62.1	10.7	7.0
tws^{430}/ tws^{196}	16.1	61.6	12.3	9.9
tws^{P}/ tws^{196}	23.2	51.0	15.5	10.2
tws^{P} / *Df*	10.6	56.5	22.7	10.2
tws^{196}/ *Df*	24.6	58.4	8.7	8.3





F

Genotype	# of optic fields	Mitotic index (MI)	Total anaphases (%)	Irregular anaphases (%)
wt	400	0.6	13.7	0.0
tws^{430}	1059	1.7	3.5	44.4

Figure 1 *tws* mutants exhibit frequent CABs. (A) Examples of CABs: (A1) Wild-type male metaphase. (A2) Isochromatid deletion (iso) of a major autosome (Au), arrows. (A3) Iso of the *X* chromosome, arrow. (A4) Asymmetric Au-Au chromatid exchange (*) with acentric fragment (AF; arrow). (A5) Au-Au dicentric chromosome (*) with the AF (arrow). (A6) Au-Au dicentric chromosome (*) with the AF (arrow). (A7) Au-XL dicentric chromosome (*) with AF (big arrow) and autosomal isochromatid deletion (small arrows). (A8) XR-Au (*) and Au-Au (**) dicentric chromosomes with the AFs; and chromatid deletion (Cd) of a major autosome (arrows). (A9) Metaphase with extensive chromosome fragmentation. Bar, 5 μm. (B) Frequencies and (C) types of CABs observed in *tws* mutants. (D) Western blotting showing strong reductions of the Tws protein in different *tws* mutants. (E) Examples of irregular anaphases: (E1) wild type, (E2) mutant anaphase with a chromatin bridge, (E3) mutant anaphase with lagging acentric fragments. (F) Mitotic parameters in wild-type and *tws* mutant brains. The MI is the average number of mitotic figures per optic field (see *Materials and Methods*). #, number; C-tid exch., chromatid exchange; C-some exch., chromosome exchange; LC, loading control; wt, wild type.

as *Df*, is a deficiency that removes tws^{+}), tws^{430}/tws^{196}, tws^{430}/tws^{P}, tws^{P}/tws^{196}, tws^{196}/Df, and tws^{P}/Df. The chromosomes carrying the tws^{196} and tws^{P} also carry second site lethal mutations, which prevented the analysis of $tws^{P}$and tws^{196} homozygotes. The brains of all mutant genotypes examined displayed very high frequencies of cells with CABs (ranging from 28 to 57%) compared to wild-type controls (0.8%). In all mutants, we observed metaphases displaying from one to five CABs and metaphases with more than five CABs often showing an extensive chromosome fragmentation (Figure 1, A and B). Since the latter cells did not permit a reliable evaluation of the type and number of CABs, in calculating the frequencies of CABs per cell we arbitrarily assumed that each of them contained only five CABs. Thus the CABs/cell frequencies reported in Figure 1B are an underestimation of the actual frequencies. An analysis of the CAB frequencies indicates that tws^{P} is the strongest mutant allele (Figure 1B). Consistent with this result, Western blotting showed that the Tws protein is undetectable in tws^{P}/Df mutant brains and strongly reduced with respect to control in brains from tws^{430}/Df and tws^{196}/Df mutants (Figure 1D).

The types and frequencies of CABs observed in metaphases with one to five CABs are reported in Figure 1C. As shown in Figure 1, A and C, *tws* mutant cells displayed chromatid and isochromatid deletions and exchanges of chromatid and chromosome type; chromosome exchanges included mostly dicentric chromosomes with only a few translocations, as in *Drosophila* most exchanges involve the homologous chromosomes due to the somatic pairing, and chromosome type symmetrical exchanges between homologs are usually not

detectable (Gatti *et al.* 1974). All dicentric chromosomes observed in *tws* mutants were accompanied by acentric fragments, indicating that they are not telomeric fusions (TFs) (Figure 1A). Thus PP2A-B55 downregulation results in chromosome-type CABs generated during G1 and chromatid-type CABs formed during S/G2. Notably, most aberrations and exchanges were "complete," namely they contained all the elements that give rise to the CAB. For example, the large majority of isochromatid deletions consisted of both the centric and the acentric fragment. "Incomplete" isochromatid breaks consisting of either a centric fragment without the corresponding acentric element or of an acentric fragment associated with a normal chromosome complement were very rare. Incomplete isochromatid breaks are the expected outcome of the rupture of chromosome bridges during anaphase and are very frequent in *Topoisomerase2* (*Top2*) mutants which exhibit anaphase bridges generated by failure to decatenate sister chromatids (see Mengoli *et al.* 2014 for a detailed analysis of the types of CABs generated by the rupture of anaphase bridges). Thus, we conclude that most CABs observed in *tws* mutants are not generated by breakage of anaphase bridges but by DNA lesions produced during the interphase that precedes the mitotic division examined.

We also asked whether *tws* mutations affect the cell cycle progression. Preparations from tws^{430} mutant brains not treated with colchicine and hypotonic solution showed an MI higher than controls (1.7 *vs.* 0.6) but a lower anaphase frequency (3.5 *vs.* 13.7% of control) (Figure 1, E and F). Consistent with previous work on *aar/tws* mutants (Gomes *et al.* 1993), 44% of the anaphases observed in *tws* mutant brains displayed chromatin bridges and lagging acentric chromosome fragments. These aberrant anaphases are probably originated by the dicentric chromosomes present in metaphase (Figure 1, A and C) and not by events directly occurring during anaphase, which would generate "incomplete aberrations" that are not found in brain preparations from *tws* mutants.

To obtain some insight into the mechanisms underlying CAB formation in *tws* mutants, we asked whether these mutants are sensitive to mutagenic agents such as X rays and HU. X rays directly induce DNA breaks; while HU inhibits the production of deoxyribonucleotides, causing replication fork collapse which ultimately results in DSBs. As shown in Table S1, *tws* mutants are sensitive to both agents. Treatments that produce ~0.05 CABs/cell in wild-type controls resulted in ~2.5-fold increases of the CAB frequency in *tws* mutants.

Collectively, these results indicate that the Tws/B55 PP2A subunit is required to prevent chromosome damage in *Drosophila*. Previous work showed that the human PP2A C catalytic subunit is also required to prevent CABs (Wang *et al.* 2009; Bouley *et al.* 2015). Altogether, these findings indicate that PP2A plays an evolutionarily conserved role in the maintenance of genome integrity.

PP2A is required for γ-H2Av dephosphorylation

Because *tws* mutations exhibit CABs and previous work has shown that PP2A dephosphorylates γ-H2AX in mammalian cells (Chowdhury *et al.* 2005), we asked whether *tws* mutant cells exhibit γ-H2Av DNA repair foci. Brains were fixed and immunostained with the pS137 anti-phospho-histone antibody that specifically recognizes γ-H2Av (Madigan *et al.* 2002), and the frequency of cells with foci was quantified by microscope analysis. In brain cells from *tws* mutants, the average frequency of nuclei with γ-H2Av foci ranged from 30 to 45%, while only 5% of wild-type cells displayed γ-H2Av accumulations (Figure 2, A and B). Because γ-H2AX foci form around DSBs (Polo and Jackson 2011), these results indicate that *tws* downregulation results in DSBs.

We next asked whether *tws* mutations affect γ-H2Av dephosphorylation and increase the persistence of IR-induced γ-H2Av foci. We performed time course experiments to analyze the kinetics of X-ray-induced γ-H2Av foci in *tws* and wild-type brains. In both control and mutant brains, the frequency of nuclei with γ-H2Av-positive foci peaked at 5 min PIR and remained high at 30 min PIR to progressively decrease at 1 and 2 hr PIR. However, at 4 and 6 hr PIR, the frequencies of nuclei with foci remained high in *tws* mutants but dropped in wild-type controls; at 4 hr PIR, <50% wild-type nuclei were still displaying foci, whereas ~80% of nuclei from *tws* mutants showed γ-H2Av foci; and at 6 hr PIR the frequencies of nuclei with foci were ~30% in wild type and ~70% in *tws* mutants (Figure 2, A and C). These findings are consistent with a Western blot analysis of H2Av phosphorylation. In blots from mock-treated brain extracts, the intensities of the γ-H2Av bands were high from 15 min to 2 hr PIR, but then decreased at 4 and 6 hr PIR. In blots from *tws* mutant brain extracts, the intensities of the γ-H2Av bands were much higher than those of controls and remained high until 4 hr PIR to decrease at 6 hr PIR (Figure 2D). Collectively, these results indicate that *Drosophila* PP2A has a role in γ-H2Av dephosphorylation and is required for the timely dissolution of γ-H2Av DNA repair foci. These findings are consistent with previous work showing that inhibition of PP2A in HeLa cells results in the persistence of Camptotecin-induced γ-H2AX foci (Chowdhury *et al.* 2005).

We also asked whether PP2A associates with γ-H2AX foci as occurs in mammalian cells (Chowdhury *et al.* 2005). We immunostained with an anti-Tws antibody wild-type cells before and after X-ray treatment. In unirradiated cells, the Tws protein was present in both the nucleus and the cytoplasm. However, at 2 hr PIR, Tws accumulated almost exclusively in the nucleus, where it self-aggregated forming cytologically detectable foci; at 6 hr PIR, Tws returned to the cytoplasm (Figure 2, E and F). We next analyzed brain cells immunostained with both anti-Tws and anti γ-H2Av antibodies. Although the number of γ-H2Av foci was lower than that of Tws foci, nearly all γ-H2Av foci colocalized with Tws aggregates (Figure 2F). These results provide further evidence for a role of Tws in γ-H2Av foci regulation.

We finally asked whether the ATM and ATR kinases are required for γ-H2Av foci formation. Previous work on mammalian cells has shown that H2AX can be phosphorylated by

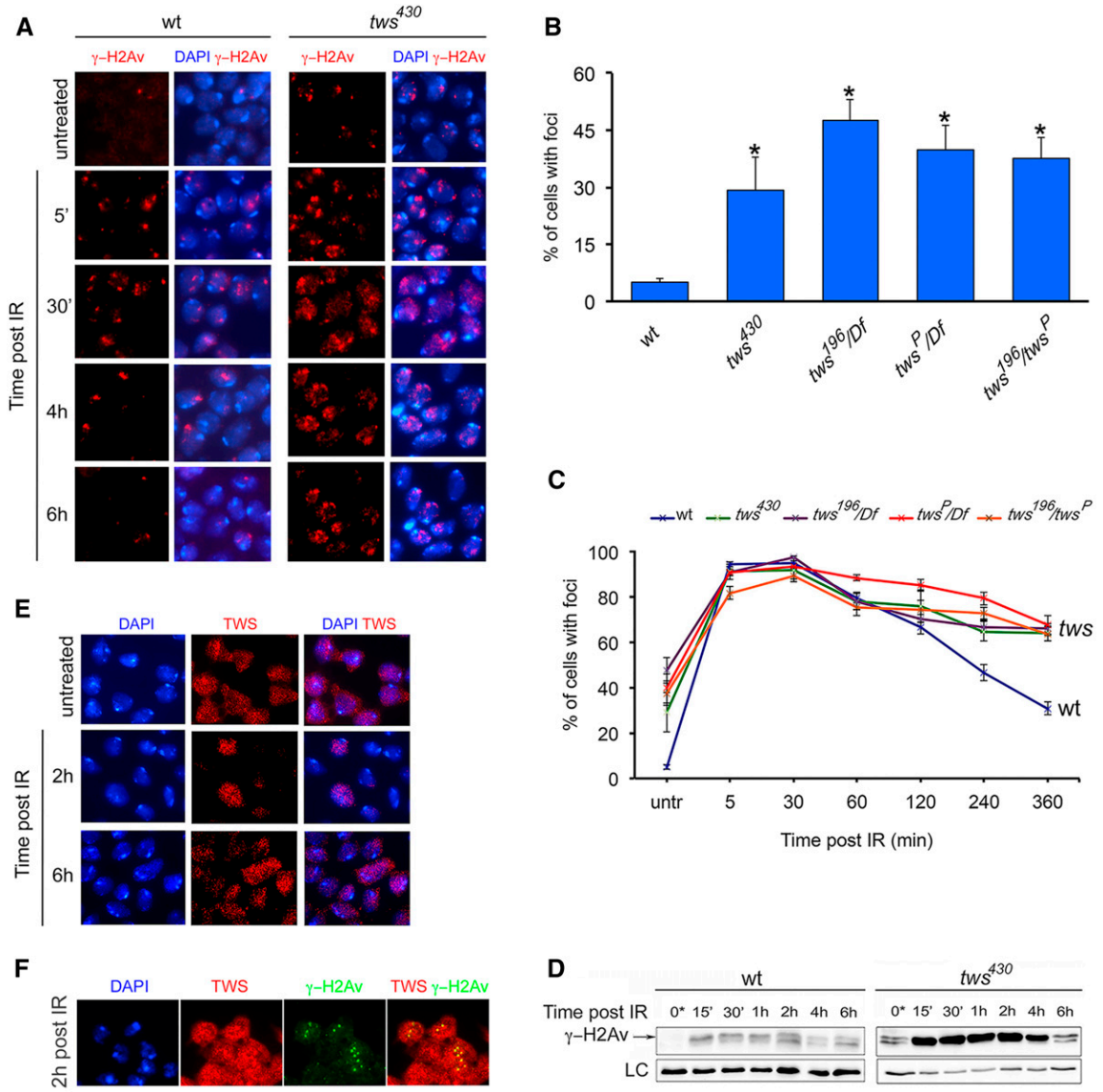


Figure 2 *tws* mutants exhibit delayed dissolution of γ-H2Av foci. (A) Examples and (B) frequencies of γ-H2Av foci observed in nuclei of wild-type (wt) and unirradiated *tws* mutant brains. Bars show the mean values of three independent experiments ± SEM; * significant in the Student's *t*-test with $P < 0.001$. (A) Examples and (C) dissolution kinetics of X-ray-induced (5 Gy) γ-H2Av foci in wild-type and *tws* mutant brains; each PIR time represents the mean value from three independent experiments ± SEM; at least three brains per PIR time examined in each experiment. (D) Western blotting showing the persistence of γ-H2Av at 6 hr PIR in *tws* mutants; Giotto was used as a loading control (LC). (E) The Tws protein localizes in both the nucleus and the cytoplasm of untreated wild-type brain cells, it is enriched within the nuclei at 2 hr PIR, and diffuses back in the cytoplasm at 6 hr PIR. (F) Colocalization of Tws protein and γ-H2Av at 2 hr PIR.

these two kinases and the DNA-PK kinase (Burma *et al.* 2001; Ward and Chen 2001; Park *et al.* 2003; Chowdhury *et al.* 2008); the DNA-PK kinase does not have a *Drosophila* homolog (FlyBase). Wild-type and mutant brains were treated with X ray (5 Gy) and fixed 5 min PIR. Immunostaining with an anti γ-H2Av antibody revealed that $tefu^{atm6}$ mutant cells display a significantly lower frequency of γ-H2Av foci compared to wild-type controls. In contrast, irradiated *mei-41* mutants displayed the same frequency of cells with foci as nonmutant controls (Figure 3). These results indicate that formation of X-ray-induced γ-H2Av foci is mediated by the ATM kinase. They also suggest that ATR might not be involved in foci formation. However, we cannot completely exclude a minor role of ATR in γ-H2Av phosphorylation. *mei-41* (ATR) and *tefu* (ATM) mutants are 10-fold and 3-fold more sensitive than wild type to X-ray-induced CABs, respectively (for *mei-41* sensitivity see Gatti *et al.* 1980; to determine the *tefu* sensitivity we examined 300 metaphases from both wild-type and $tefu^{atm6}/tefu^{atm6}$ brains irradiated with 1 Gy of X rays). Thus, it is possible that the observed frequency of foci in irradiated *mei-41* mutants is the combined outcome of an increased DNA damage and a low/moderate level of ATR-mediated H2Av phosphorylation.

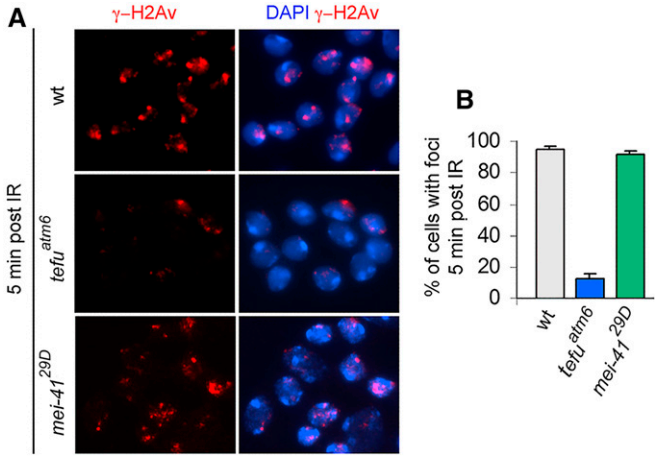


Figure 3 Formation of *Drosophila* γ-H2Av foci is mediated by ATM. (A) Representative examples of nuclei from irradiated (5 Gy) wild-type, *tefu* (ATM), and *mei-41* (ATR) mutant brains stained for γ-H2Av. (B) Frequencies of nuclei with γ-H2Av foci in irradiated wild-type, *tefu*, and *mei-41* mutant brains. Bars show the mean values of three independent experiments ± SEM.

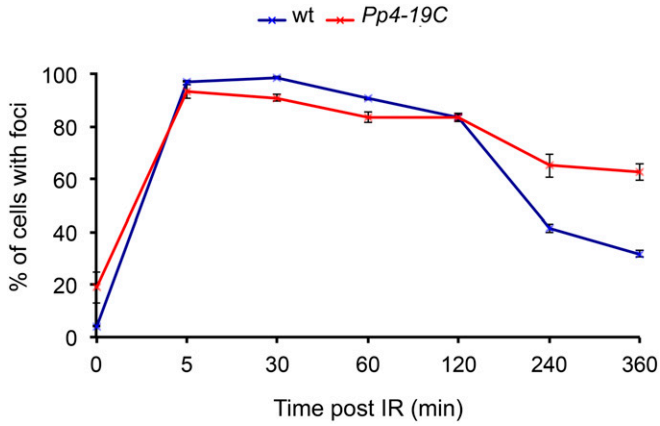


Figure 4 PP4 is required for γ-H2Av foci regression in *Drosophila*. Each PIR time represents the mean value from three independent experiments ± SEM. Larvae were irradiated with X ray (5 Gy) and dissected brains were fixed at various PIR times; at least three brains per time examined in each experiment.

Relationships between foci dissolution and CAB formation in Drosophila

Previous work on mammalian cells has shown that both PP2A and PP4 can dephosphorylate γ-H2AX and promote foci dissolution; it has also been proposed that abnormal γ-H2AX foci persistence could result in DNA damage (Chowdhury *et al.* 2005, 2008). We thus asked whether *Drosophila* PP4 is also required for proper γ-H2Av foci behavior. We irradiated *Pp4-19C* larvae carrying a mutation in the catalytic subunit of PP4, and then followed the dynamics of γ-H2Av foci in a time course experiment. Both control and mutant brains displayed similar frequencies of nuclei with γ-H2Av foci until 2 hr PIR. However, at 4 and 6 hr PIR, mutant brains showed significantly higher frequencies of nuclei with foci than controls (∼65 *vs.* 40% at 4 hr, and ∼65 *vs.* 30% at 6 hr) (Figure 4). These findings indicate that *Drosophila* PP4 is required for γ-H2Av foci regression just like Tws (compare Figure 2C and Figure 4).

We then examined unirradiated *Pp4-19C* mutant brains for the presence of CABs and found a CAB frequency comparable to that of wild-type controls (0.006 CABs/cell, in 500 cells examined from 4 mutant brains). This finding suggests that a delay in γ-H2Av foci dissolution is not sufficient to induce CABs in *Drosophila* brain cells. An additional support for this conclusion comes from the analysis of mutants that disrupt the Tip60 pathway. In both *Drosophila* and humans, depletion of the TIP60 complex leads to foci persistence (Kusch *et al.* 2004; Jha *et al.* 2008). This complex mediates acetylation of γ-H2AX/H2Av, a post-translation modification that is thought to facilitate the access of phosphatases to the phospho-histone variant (Ikura *et al.* 2007). Specifically, an abnormal persistence of foci was observed in *Drosophila* cells depleted of the Tip60 and dMgr15 subunits of the complex (Kusch *et al.* 2004). We performed *in vivo* RNAi against *Tip60* and *Mrg15* using specific RNAi constructs and a 69B-GAL4 driver. RNAi flies died at the larval/pupal transition but their brains did not show an increase in CABs compared to wild-type controls (in *Tip60* and *Mrg15* RNAi brains, the frequencies of CABs were 0.002 and 0.003 per cell, respectively; 600 cells scored from at least three RNAi brains).

The tws and Pp4-29C genes control the G2/M checkpoint

To further characterize the roles of *Drosophila* PP2A and PP4 in the DDR pathway, we asked whether they are involved in the regulation of the G2/M checkpoint like their mammalian counterparts (Nakada *et al.* 2008; Yan *et al.* 2010). The G2/M checkpoint is a complex signaling machinery that includes CDKs and opposing phosphatases; when this checkpoint detects lesions in the nuclear DNA, it arrests cell cycle progression to prevent cells from initiating mitosis with damaged DNA (Yasutis and Kozminski 2013). We performed a checkpoint assay, evaluating the MI in larval brains fixed at different times after X-ray exposure (10 Gy). In these experiments, we used the Oregon R wild-type strain as a negative control and the *mei-41* (ATR) and *tefu* (ATM) mutant flies as positive controls, since they are known to have a defective G2/M checkpoint (Hari *et al.* 1995; Oikemus *et al.* 2004). As shown in Figure 5A, the MI of the Oregon R control brains dropped at 5–15 min PIR, remained low for ∼1 hr and came back to a normal value only at 2 hr PIR. In contrast, irradiated *mei-41*, *tefu*, and *tws* mutant brains did not show significant variations in the MI over time, indicating a defect in the G2/M checkpoint.

Differently from *tws* mutants, in *Pp4-19C* mutant brains, the MI dropped at 5–15 min PIR as occurs in controls, but then remained low for >3 hr, in contrast to wild-type brains where the cells began reentering mitosis at 30–60 min PIR (Figure 5B). These results indicate that PP2A and PP4 play different roles in the *Drosophila* G2/M checkpoint. PP2A appears to be required for initial checkpoint signaling like ATM

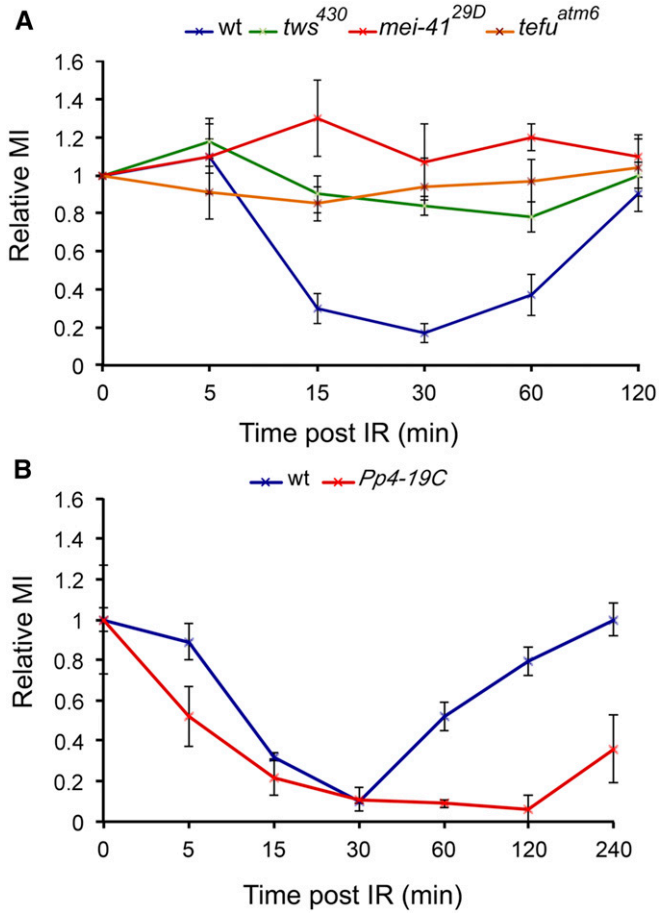


Figure 5 PP2A-Tws and PP4 play different roles in the *Drosophila* G2/M DNA damage checkpoint. Each PIR time represents the mean value from three independent experiments ± SEM. Larvae were irradiated with X ray (10 Gy) and dissected brains were fixed at various PIR times; at least three brains per time examined in each experiment. The MI values reported in the graph are normalized with respect to the MI of untreated brains. Note that (A) *tws* is required for checkpoint signaling like *tefu* (ATM) and *mei-41* (ATR), while (B) PP4 appears to be dispensable for signaling but necessary for checkpoint recovery. wt, wild type.

and ATR. In contrast, PP4 is not involved in checkpoint initiation but it is required for termination of signaling and checkpoint recovery. A requirement of PP4 for checkpoint recovery has been previously demonstrated in budding yeast and human cells (Keogh *et al.* 2006; Nakada *et al.* 2008), pointing to a highly conserved function of this phosphatase in the regulation of the G2/M checkpoint.

Interactions between tws and the ATM kinase

We have shown that ATM mediates H2Av phosphorylation and that PP2A dephosphorylates this histone. In addition, previous work has shown that PP2A and ATM physically interact and that PP2A dephosphorylates ATM (Goodarzi *et al.* 2004; Kalev *et al.* 2012). We thus decided to use a genetic approach to investigate the relationships between PP2A and ATM in CAB formation. We performed an epistasis analysis by comparing the types and frequencies of chromosome abnormalities found in *tefu* (ATM) and *tws* single mutants with those observed in *tws tefu* double mutants. Consistent with our previous work (Ciapponi *et al.* 2006), single *tefu* null mutants ($tefu^{atm6}$) showed 0.09 CABs/cell and an average of 0.5 TFs/cell (Table 1). Strikingly, tws^{430} $tefu^{atm6}$ double mutants displayed CAB and TF frequencies very similar to those seen in the *tefu* single mutants, while the CAB frequency observed in tws^{430} single mutants was ~10-fold higher than that seen in the double mutants (Table 1). Thus, mutations in *tefu* are perfectly epistatic to mutation in *tws*. These results indicate that ATM and PP2A function in the same pathway that leads to CABs in *Drosophila tws* mutants, and strongly suggest that CAB formation in these mutants depends on ATM activity. However, the role of ATM in CAB generation is unclear. In mammalian cells, quiescent ATM exists as a homodimer and is activated by autophosphorylation at serine 1981 (S1987 in mouse) and at least three additional sites. Abrogation of these modifications hampers the ATM activity in the DDR in human cells but not in mouse cells (reviewed in Shiloh and Ziv 2013). In addition, studies in human cells have shown that ATM phosphorylation at S1981 is dispensable for ATM recruitment at the DSBs but is required for ATM retention at DSBs (So *et al.* 2009). Based on these findings, we can envisage that dysregulation of ATM phosphorylation can interfere with the DNA repair processes, ultimately leading to DSBs and the ensuing CABs. Alternatively, it is possible that loss of B55/PP2A activity leads to an excessive and/or untimely phosphorylation of a specific ATM substrate(s), which would interfere with proper DNA repair, resulting in DNA damage. Should this hypothesis be correct, and should these "phospho-mutagens" exist, one would expect that loss-of-function mutations in the genes they specify would act as suppressors of the CAB phenotype elicited by *tws* mutations.

Search for suppressors of the CAB phenotype of tws mutants

To identify the potential suppressors/modifiers of the *tws*-dependent CAB phenotype, we constructed a series of double mutants carrying both tws^{430} and a mutation in another gene involved in DNA repair. For this analysis, we selected genes whose protein products have two characteristics: they are involved in DNA repair and have phosphorylated mammalian orthologs. To assess the phosphorylation state of these gene products we exploited the extant literature and the PhosphoSitePlus database (Hornbeck *et al.* 2015). The list of the genes selected for double mutant analysis and their main features are reported in Table 2.

We examined double mutants for *tws* and *His2Av*, *nbs*, or *grapes* (*grp*), whose protein products have mammalian orthologs that are phosphorylated by ATM (Shiloh and Ziv 2013). Nbs is a component of the MRN complex which binds to DSBs and participates in ATM activation, thus acting upstream of the major DNA repair pathways (Williams *et al.* 2010); *grp* is the *Drosophila* homolog of the Chk1 kinase which plays a major role in the DNA damage checkpoint (Fogarty *et al.*

Table 1 Frequencies of CABs and TFs observed in larval brains of *tws*^{430} mutants and *tws*^{430}-bearing double mutants

Genotype	No. of brains	No. of cells scored	Cells with CABs[a] (%)	Cells with ≥5 CABs (%)	CABs/cell[b]	TFs/cell
wt[c]	5	460	0.4	0.0	0.004	0.0
tws^{430c}	5	406	38.2	14.0	1.017	0.0
tefu^{atm6c}	8	531	8.7[d]	0.0	0.087	0.5
tws^{430} *tefu*^{atm6c}	11	581	12.0[d]	0.0	0.120	0.5
nbs^{1c}	16	796	8.3	0.0	0.083	0.4
nbs^{1} *tws*^{430e}	15	769	33.0	7.5	0.631	0.2
ku70 ^{Ex8c}	7	850	0.2	0.0	0.002	0.0
tws^{430} *ku70*^{Ex8c}	15	1392	0.3	0.0	0.003	0.0
ku70MT tws^{430} *ku70*^{Ex8c,f}	5	451	13.1	2.2	0.155	0.0
ku70 RNAi *tws*^{430c,g}	5	513	2.7	0.0	0.027	0.0
mei-9^{A1c}	10	950	4.0	0.0	0.040	0.0
mei-9^{A1} *tws*^{430e}	12	423	24.3	1.7	0.391	0.0
mei-41^{29Dc}	5	415	5.8	0.0	0.058	0.0
mei-41^{29D} *tws*^{430c}	8	632	47.6	10.4	0.950	0.0
rad51^{1c}	7	840	0.4	0.0	0.004	0.0
tws^{430} *rad51*^{1e}	16	1088	24.2	3.4	0.413	0.0
grp^{1c}	10	650	0.0	0.0	0.000	0.0
grp^{1} *tws*^{430e}	29	1137	32.9	7.4	0.664	0.0

[a] Includes cells with more than five CABs.
[b] The CABs/cell frequencies have been calculated assuming that the cells with more than five CABs contained only five CABs, and are therefore an underestimation of the actual frequencies.
[c] Normal wandering third larvae with comparable body and brain sizes.
[d] Not significantly different in the Chi square test.
[e] Small larvae dying at the late second/early third instar.
[f] *tws*^{430} *ku70* ^{Ex8}/*ku70MT tws*^{430} *ku70*^{Ex8} heterozygous larvae.
[g] *ku70*-RNAi construct/*actin-GAL4*; *tws*^{430}/*tws*^{430}.

1997; Patil *et al.* 2013). We also characterized the *mei-41 tws* double mutant; the *Drosophila* Mei-41/ATR kinase is not only involved in DNA damage signaling but has also a direct role in DSB repair (Oikemus *et al.* 2006; LaRocque *et al.* 2007; Benna *et al.* 2010).

Moreover, we analyzed the interactions between mutations in *tws* and mutations in genes known to be involved in either the NHEJ or the HR DSB repair pathway. These pathways are evolutionarily conserved from flies to humans, and several *Drosophila* genes functioning in these pathways have been characterized (Johnson-Schlitz *et al.* 2007; Wei and Rong 2007). In NHEJ, the DNA ends flanking the break are directly ligated, but this repair process often results in the insertion or loss of nucleotides at the site of ligation (Khanna and Jackson 2001; Mehta and Haber 2014). In *Drosophila*, NHEJ exploits the activities of at least two conserved genes: *ligase4* (*lig4*) and *ku70*, which encodes a subunit of the Ku70/Ku80 heterodimer (Johnson-Schlitz *et al.* 2007; Wei and Rong 2007). HR is based on recombination with homologous genomic sequences and is not mutagenic, leading to an accurate repair of the DSB (Khanna and Jackson 2001; Mehta and Haber 2014). The factors that mediate HR in flies include Blm/Mus309 (a recQ-like protein homologous to the human BLM helicase responsible for the Bloom syndrome), the *Drosophila* homolog of the human tumor suppressor BRCA2, the conserved recombinases Okra (Okr, homologous to Rad54), and Spindle A (SpnA, homologous to Rad51) (Johnson-Schlitz *et al.* 2007; Wei and Rong 2007; Klovstad *et al.* 2008).

Finally, we examined double mutants in *tws* and either *mei-9* or *mus301/spnC*, which encode an endonuclease orthologous to human XPF and a helicase homologous to human HELQ, respectively (Sekelsky *et al.* 1995; McCaffrey *et al.* 2006). *mei-9* and *mus301/spnC* have been implicated in the SSA repair of DSBs, but there are conflicting results on their involvement in this process (Johnson-Schlitz *et al.* 2007; Wei and Rong 2007).

The *tws*^{430} *His2av*^{810}, *okr*$^{17\text{-}1}$/*okr*$^{A19\text{-}10}$ *tws*^{430}, *lig4*^{5} *tws*^{430}, *brca2* ^{KG03961} *tws*^{430}, *brca2*^{ko}/*brca2*^{56E} *tws*^{430}, and *mus301*^{D4} *tws*^{430} double mutants displayed an early lethality (before the second instar), preventing cytological analysis of larval brain chromosomes. All other double mutants were lethal but reached a larval stage that allowed preparation of brain squashes for CAB scoring. Specifically, *nbs*^{1} *tws*^{430}, *mei-9*^{A1} *tws*^{430}, *tws*^{430} *rad51*^{1}, and *grp*^{1} *tws*^{430} died at the late second instar/early third instar larval stage, while, *mei-41*^{29D} *tws*^{430}, and *tws*^{430} *ku70* ^{Ex8} reached the mature third instar larval stage (wandering stage). We emphasize that larvae of *mei-41*^{29D} *tws*^{430} and *tws*^{430} *ku70*^{Ex8} double mutants used for CAB analysis were comparable in body and brain size to larvae of *mei-41*^{29D}, *tws*^{430}, or *ku70*^{Ex8} single mutants. The frequency and types of CABs observed in single and double mutants are shown in Table 1 and Table S2. All mutants showed similar types and relative frequencies of chromatid- and chromosome-type CAB patterns (Table S2), suggesting that the CABs were generated throughout all phases of the cell cycle. However, the double mutants displayed different frequencies of CABs in relation to the *tws* single mutants. *nbs*^{1} *tws*^{430}, *mei-9*^{A1} *tws*^{430}, *tws*^{430} *rad51*^{1}, and *grp*^{1} *tws*^{430} double mutants displayed CAB frequencies significantly lower than the single *tws* mutants. *mei-41*^{29D} *tws*^{430} double mutants showed a CAB frequency

Table 2 Loss-of-function phenotypes of *Drosophila* and orthologous mouse and human genes required for DDR and DSBs repair

Drosophila gene name	Human gene name	Protein function[a]	Loss-of-function phenotype in flies	Loss-of-function phenotype in mice//human diseases	References[b]
HisH2Av	*H2AFX*	Nucleosome component; repair of DSBs	Lethal; CABs	Viable; growth retardation; male infertility; CABs//None	van Daal and Elgin 1992; Celeste *et al.* 2002; Vernì and Cenci 2015
tefu	*ATM*	Serine/threonine kinase, DDR, DNA damage checkpoint	Lethal; CABs and TFs	Viable; CABs// ataxia telangiectasia; CABs	Oikemus *et al.* 2004; Silva *et al.* 2004; Song *et al.* 2004; Ciapponi *et al.* 2006; Shiloh and Ziv 2013
mei-41	*ATR*	Serine/threonine kinase, DDR, DNA replication; DNA damage checkpoint	Viable, female sterile; recombination defective; mutagen sensitive; CABs	Lethal; CABs// Seckel syndrome	Baker *et al.* 1976; Gatti 1979; Hari *et al.* 1995; Laurençon *et al.* 2003; Johnson-Schlitz *et al.* 2007; Wei and Rong 2007; Maréchal and Zou 2013
grp	*CHEK1*	Serine/threonine kinase, DDR, DNA damage checkpoint	Viable and fertile	Lethal//none	Fogarty *et al.* 1997; Liu *et al.* 2000; Takai *et al.* 2000
nbs	*NBN (NBS1)*	Component of the rad50-Mre11-Nbs complex; DNA repair	Lethal; CABs and TFs	Lethal; CABs// Nijmegen breakage syndrome; CABs	Zhu *et al.* 2001; Bi *et al.* 2005; Ciapponi *et al.* 2006; Oikemus *et al.* 2006; Stracker and Petrini 2011
ku70/irpb	*XRCC6 (Ku70)*	Forms a complex with Ku80; binds DNA ends; DNA helicase activity; NHEJ repair	Viable; telomere maintenance	Viable; telomere maintenance// none	Melnikova *et al.* 2005; Johnson-Schlitz *et al.* 2007; Fell and Schild-Poulter 2015
lig4	*LIG4*	DNA ligase; NHEJ repair	Viable and fertile	Lethal//LIG4 syndrome	Gorski *et al.* 2003; Johnson-Schlitz *et al.* 2007; Wei and Rong 2007; Woodbine *et al.* 2014
mei-9	*ERCC4 (XFP)*	Forms a complex with ERCC1; endonuclease activity; DNA repair	Viable and fertile; mutagen sensitive; recombination defective; CABs	Lethal//Fanconi anemia, xeroderma pigmentosum, Cockayne syndrome, XFE progeroid syndrome	Baker *et al.* 1976; Gatti 1979; Sekelsky *et al.* 1995; Johnson-Schlitz *et al.* 2007; Wei and Rong 2007; Manandhar *et al.* 2015
mus301/spnC	*HELQ*	DNA helicase; DNA repair	Viable, female sterile; mutagen sensitive; CABs (this report)	Viable, subfertile// none	Boyd *et al.* 1981; Johnson-Schlitz *et al.* 2007; Wei and Rong 2007; Adelman *et al.* 2013
Blm/mus309	*BLM*	RecQ-like DNA helicase; DNA replication; HR repair	Viable, male and female sterile; mutagen sensitive; CABs	Lethal//Bloom syndrome; CABs; high SCE rate	Boyd *et al.* 1981; Kusano *et al.* 2001; Johnson-Schlitz *et al.* 2007; Croteau *et al.* 2014; Cenci *et al.* 2015
brca2	*BRCA2*	Binds single-stranded DNA; binds RAD51; HR repair	Viable, female sterile; HR repair; CABs (this report)	Lethal//breast-ovarian cancer susceptibility; Fanconi anemia D1	Klovstad *et al.* 2008; Prakash *et al.* 2015
spnA/rad51	*RAD51*	Binds DNA; binds BRCA2; HR repair	Viable, female sterile; HR repair	Lethal//breast cancer susceptibility	Tsuzuki *et al.* 1996; Staeva-Vieira *et al.* 2003; Johnson-Schlitz *et al.* 2007; Wei and Rong 2007
okr/rad54	*RAD54L*	Binds DNA; helicase activity; HR repair	Viable, female sterile; HR repair; CABs (this report)	Viable//tumor susceptibility	Kooistra *et al.* 1997; Ghabrial *et al.* 1998; Johnson-Schlitz *et al.* 2007; Wei and Rong 2007; Mazin *et al.* 2010

SCE, sister chromatid exchange.
[a] All protein products of the human genes are phosphorylated (see text).
[b] For *Drosophila* genes we reported the main references; for mammalian genes, when possible, we cited reviews.

comparable to that seen in *tws* single mutants. Most strikingly, in tws^{430} $ku70^{Ex8}$ double mutants CAB formation was completely suppressed and doubly mutant brains exhibited the same CAB frequency as wild-type controls. We note that the $ku70^{Ex8}$ mutation is most likely a null allele, as it carries a very large deletion of the gene coding sequence (Johnson-Schlitz

et al. 2007). However, $ku70^{Ex8}$ homozygotes are viable, indicating that *ku70* is not an essential gene in *Drosophila*.

To confirm the epistatic relationship between mutations in *ku70*, which do not cause CABs, and mutations in *tws*, we first performed a rescue experiment. We generated a recombinant chromosome carrying $ku70^{Ex8}$, tws^{430}, and $ku70^{+}$ insertion in region 65B; this insertion, designated as *ku70MT*, consists of a fosmid clone containing the *ku70* gene fused with the 2xTY1-sGFP-V5-Pre-TEV-BLRP-3xFLAG multi-tag, as well as the *nina*, *CG6723*, *Ranbp9*, and *mRpL40* genes (Sarov *et al.* 2016; see *Materials and Methods*). We then used this recombinant chromosome to generate $ku70^{Ex8}$ tws^{430}/ *ku70MT* $ku70^{Ex8}$ tws^{430} third instar larvae; these larvae displayed normal body and brain sizes and showed relatively high frequencies of CABs (0.16 per cell; Table 1) compared tws^{430} $ku70^{Ex8}$ double mutants (0.003 per cell; Table 1). We next analyzed brain cells from tws^{430}/tws^{430} larvae bearing a *ku70* RNAi construct and an *actin-GAL4* driver. These brains displayed a much lower frequency of CABs (0.027 per cell; Table1) than those of tws^{430} homozygotes (1.017 per cell; Table1). Collectively, these results confirm that mutations in *ku70* are *strictly* epistatic to mutations in *tws*, indicating that the expression of a normal ku70 protein is essential for CAB formation in a *tws* mutant background.

Establishing epistasis relationships between the other couples of mutations ($mei\text{-}41^{29D}$ tws^{430}, nbs^{1} tws^{430}, $mei\text{-}9^{A1}$ tws^{430}, tws^{430} $rad51^{1}$, and grp^{1} tws^{430}) is more difficult. Given that *mei-41* has only a few CABs, it is not possible to determine whether the CAB frequency found in the $mei\text{-}41^{29D}$ tws^{430} double mutant is due to epistasis of *tws* over *mei-41* or instead reflects an additive pattern of CABs. The relatively low CAB frequency observed in the double mutants bearing tws^{430} and either nbs^{1}, grp^{1}, $mei\text{-}9^{A1}$, or $rad51^{1}$ would suggest epistasis of these mutations over tws^{430}. However, it is likely that these doubly mutant larvae exhibit a relatively low CAB frequency because they die earlier (at the late second/early third instar stage) than tws^{430} homozygous larvae. It has been shown that mothers heterozygous for a mutation in a gene specifying an essential cell cycle function accumulate into the egg a substantial amount of wild-type product, which is progressively depleted during development but is usually sufficient to allow larvae to reach the third instar. As a result, young larvae contain a higher amount of wild-type product than old larvae and thus exhibit a milder phenotype (Gatti and Baker 1989; Gatti and Goldberg 1991). We verified this notion by Western blot analysis, showing that in third instar larvae the level of the Tws protein is substantially lower than in first or second instar larvae (Figure S2). Thus, the apparent epistasis of *nbs*, *grp*, *mei*-9 and *rad51* over *tws* might simply reflect the fact that double mutants have a higher level of wild-type Tws protein than *tws* single mutants and are therefore less subject to DNA damage.

The reason why several double mutants die early during development is unclear. $His2av^{810}$ and tws^{430} homozygotes both die as third instar larvae, and it is thus understandable that the double mutant dies earlier than either single mutant. However, $okr^{17\text{-}1}/okr^{A19\text{-}10}$ and $brca2^{ko}/brca2^{56E}$ heterozygous flies, and flies homozygous for $lig4^{5}$, $brca2^{KG03961}$, or $mus301^{D4}$ are viable, suggesting that these mutations interact synergistically with the tws^{430} mutant allele leading to early death. To ascertain whether the early lethal phase of these double mutants is due to an intolerable level of chromosome breakage, we exploited RNAi-mediated gene silencing in S2 cells. For each couple of mutations resulting in early lethality in flies, we performed single RNAi against each of the genes they identify and double RNAi against both genes. We included *Blm* in this analysis because the close map position of *Blm* and *tws* prevented the construction of a double mutant by recombination. In all cases, we checked the efficiency of RNAi and found that target messenger RNAs were not detectable by RT-PCR (Figure S3). We anticipate that S2 cells are not an ideal system for the analysis of CABs because they exhibit a relatively high frequency of cells (~12%) with spontaneous CABs (Figure 6 and Figure S4). The main purpose of our RNAi experiments was to discover possible synergistic interactions between genes in CAB formation. S2 cells allow detection of such interactions, as they manage to undergo mitosis even in the presence of an extremely high frequency of CABs (Somma *et al.* 2008).

A comparison of the CAB frequencies induced by RNAi against *His2Av*, *okr*, *lig4*, *brca2*, *mus301*, or *Blm* with those elicited by double RNAi against *tws* and each of these genes did not reveal synergistic effects leading to extensive chromosome damage (Figure 6), suggesting that the embryonic lethality observed in the corresponding double mutants is not the consequence of an intolerable CAB level. RNAi against *lig4*, *brca2*, or *mus301* did not cause a CAB increase compared to control, and double RNAi against *tws* and each of these genes resulted in a CAB frequency similar to that seen in *tws* RNAi cells (Figure 6); indicating that *lig4*, *brca2*, and *mus301* are not involved in the pathway leading to CABs in Tws-depleted cells. RNAi against *His2av* or *okr* resulted in a substantial increase of CABs compared to control; *His2av tws* and *okr tws* double RNAi cells displayed CAB frequencies similar to those observed after RNAi against single genes, suggesting that *His2av*, *okr*, and *tws* function in the same CAB formation pathway. Finally, *Blm tws* double RNAi cells showed an additive CAB pattern with respect to *Blm* and *tws* RNAi cells (Figure 6), suggesting that *Blm* and *tws* function in parallel pathways leading to CABs.

Discussion

It is generally accepted that DNA DSBs are the lesions that lead to CAB formation and that the CAB frequency is a reliable measure of DNA damage (Obe *et al.* 2002; Durante *et al.* 2013). DSBs can be generated by the exposure to a variety of exogenous physical and chemical mutagens or by the action of endogenous agents such as viruses, reactive oxygen species, and mutations in genes required for proper DNA metabolism (Khanna and Jackson 2001; Mehta and Haber 2014). Many studies have established that CABs can promote

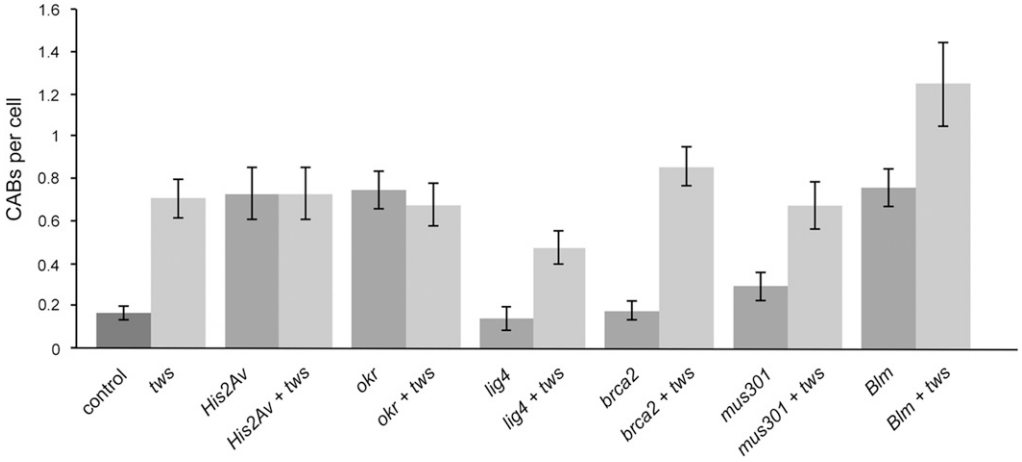


Figure 6 Frequencies of CABs observed in S2 cells after RNAi against the indicated genes. At least 100 cells examined for each RNAi or double RNAi experiment; error bars indicate SEM. The CAB frequency observed in double *Blm tws* RNAi cells is significantly higher ($P < 0.01$) than that seen in *tws* RNAi cells. In the other double RNAi cells, the CAB frequencies are not significantly different from that detected after RNAi against *tws* only.

carcinogenesis, and CABs are therefore considered as one of the main hallmarks of cancer (Kasparek and Humphrey 2011). However, despite their involvement in tumorigenesis, the mechanisms underlying CAB formation are still ill-defined. We believe that our results on *Drosophila* PP2A/B55 provide substantial insight into the role of phosphatases in the maintenance of genome integrity, suggesting possible mechanisms through which loss of phosphatase activity can lead to CABs.

Previous studies on *Drosophila* Tws focused on the role of this PP2A subunit in mitotic division (Gomes *et al.* 1993; Mayer-Jaekel *et al.* 1993; Chen *et al.* 2007; Brownlee *et al.* 2011; Wang *et al.* 2011; Kim *et al.* 2012). Here, we have shown that Tws is also required for the maintenance of chromosome integrity, and that *tws* mutants are threefold more sensitive than wild type to CAB induction by either X ray or HU. In addition, we have shown that Tws/PP2A is required for γ-H2Av dephosphorylation and DNA repair foci regression, and that it is involved in the DNA damage G2/M checkpoint. All these roles of PP2A have been previously described in mammalian cells (Chowdhury *et al.* 2005, 2008; Wang *et al.* 2009; Yan *et al.* 2010; Kalev *et al.* 2012) but have never been demonstrated in *Drosophila*. Thus, our results strongly suggest that Tws/PP2A serves a highly conserved function in the maintenance of genome integrity, and that *Drosophila* is an excellent model system for the analysis of this function.

Previous work in mammalian cells has shown that loss of PP2A leads to DSBs and CABs. However, there are at least three different hypotheses on the mechanisms leading to DNA damage in PP2A deficient cells. It has been proposed that PP2A depletion leads to an abnormal persistence of γ-H2AX foci impairing normal DSB repair (Chowdhury *et al.* 2005, 2008). It has been also suggested that PP2A downregulation inhibits the HR pathway by decreasing the expression of both BRCA1 and RAD51 (Kalev *et al.* 2012). Other studies have shown that the PP2A C catalytic subunit associates with Ku and promotes its dephosphorylation, and that PP2A C overexpression accelerates DSB repair in wild-type cells but not in Ku-depleted cells. These results have led to the hypothesis that PP2A-mediated Ku dephosphorylation activates Ku, promoting DSB repair and preventing CAB formation (Wang *et al.* 2009). However, it has been also reported that a specific phosphorylated form of Ku70 (pS27-S33 Ku70), which is overexpressed in aggressive forms of chronic lymphocytic leukemia, contributes to a faster, error-prone DNA repair process that results in a high CAB level. In addition, this study suggested that both DNA PK and ATM redundantly mediate S27 Ku70 phosphorylation (Bouley *et al.* 2015).

Our analysis of γ-H2Av foci regression in *tws* and *Pp4-29C* mutants argues against the possibility that a simple delay in foci dissolution can lead to CAB formation. We have also shown that *rad51* null mutations do not cause CABs, ruling out the possibility that *tws* mutations lead to DNA damage through disruption of the *rad51* function. Finally, our observation that null mutations in *ku70* do not exhibit CABs exclude the possibility that *tws* mutations lead to CABs via inhibition of Ku activity. We found that mutations in both *tefu* (ATM) and *ku70* are perfectly epistatic over *tws* mutations for the CAB phenotype. Specifically, we have shown that *tws tefu* double mutants exhibit the same chromosomal phenotype as *tefu* single mutants, namely a low CAB frequency (0.12 *vs.* 1.0 CABs/cell in *tws* mutants) and frequent TFs (0.5 per cell), which are absent in *tws* mutants. Most strikingly, we have shown that *tws ku70* double mutants exhibit the same CAB frequency as *ku70* single mutants, which is comparable to the wild-type frequency (∼0.005 per cell). One of the most straightforward explanations for these epistatic relationships is that Ku70 is a substrate of ATM and that, in the absence of the *tws* function, Ku70 is not properly dephosphorylated and as such interferes with the normal repair processes leading to DSBs and CABs.

A mutagenic activity of phosphorylated *Drosophila* Ku70 is consistent with the finding that a phosphorylated Ku70 form (pS27-S33 Ku70) leads to CAB formation in human cells by affecting the NHEJ DNA repair process (Bouley *et al.* 2015). However, we would like to point out that *Drosophila* Ku70 has only a limited homology with its human counterpart (only 21% identity) and that the "mutagenic" phosphorylation sites found in human Ku70 (S27 and S33) are not conserved in the fly protein. Thus, the mechanisms underlying the possible mutagenic activity of *Drosophila* phospho-Ku70 remain to

be defined. At the moment, we can only speculate on these mechanisms, which could also involve Ku80, as in mammalian cells Ku70 and Ku80 form a binary complex and are mutually dependent for their stability (reviewed in Fell and Schild-Poulter 2015). The Ku complex binds the double-stranded DNA ends, forming a ring that encircles DNA and bridges the broken ends making them compatible. After end ligation, Ku keeps encircling repaired linear DNA and it is eventually removed. It is currently unclear whether Ku is removed through ubiquitin-mediated degradation or through direct DNA nicking which allows Ku to escape from the topological trap (Fell and Schild-Poulter 2015). In this scenario, it is not difficult to conceive that an abnormally persisting post-translational modification of Ku can affect its behavior and leads to DNA damage.

To the best of our knowledge, there are no examples in the literature of an improperly phosphorylated protein that acts as a mutagen. However, previous studies on DNA repair have provided examples in which the presence of an abnormal protein is more harmful than its absence. For example, the expression of catalytically inactive ATM in mice causes a stronger genomic instability and is more detrimental than ATM loss (Daniel *et al.* 2012; Yamamoto *et al.* 2012). Similarly, while loss of Topoisomerase II does not activate a G2/M checkpoint, catalytically inactive forms of this enzyme interrupt the DNA decatenation process and trigger a G2 arrest (Luo *et al.* 2009; Furniss *et al.* 2013).

In summary, our genetic analyses have shown that loss-of-function mutations in the *tws* gene lead to CABs in a Ku-dependent manner, raising the possibility that failure to dephosphorylate Ku leads to DNA damage. Identification of the putative mutagenic phospho-forms of Ku70, and possibly of Ku80, and definition of the mechanisms underlying their effects on DNA metabolism will be the goal for future studies.

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