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Protein phosphatase 2A (PP2A) is required for the maintenance of *Drosophila* chromosome integrity

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Cover image: Examples of chromosome aberrations (CABs) observed in *Drosophila tws* mutant larval brains (C. Merigliano).

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GLOSSARY

Break induced replication (BIR)
a homologous recombination (HR) pathway variant employed to repair DNA double-strand breaks (DSBs) when homology is restricted to one end.

Breakage-fusion-bridge (BFB) cycle
a cyclic process in which broken chromosomes fuse and then break during anaphase when the two centromeres are pulled in opposite direction.

Chromatid deletion
break of a single chromatid generated during S or G2.

Chromosome bridge
chromosome that bridges two separating groups of anaphase chromosomes, formed when the two centromeres of a dicentric chromosome are being drawn to opposite poles.

Exchange
aberration generated in G1, S or G2 in which the broken ends of different chromosomes fuse, giving rise to either dicentric chromosomes or translocations.

Deficiency
a chromosomal aberration that is characterized by the deletion of a part of the genome.

Deletion mapping
genetic strategy (based on pseudodominance phenomenon) that employs overlapping deficiencies to localize a gene of interest in a defined chromosome interval.

Dicentric chromosome

aberrant chromosome with two centromeres, often accompanied by two acentric fragments and generated by a chromosome exchange event.

DNA damage checkpoint

control mechanism that prevents or delays progression through the cell cycle in case of DNA damaged.

DNA damage response (DDR)

cellular response to DNA damage consisting in DNA repair promotion, cell cyce arrest and, in case of severe DNA lesions, apoptosis induction.

DNA-repair foci

cytologically visible nuclear sites, in correspondence of DSBs, where DDR factors accumulate.

Genomic instability

an abnormal increase in the rate at which genes and chromosomes are mutated, rearranged or lost.

Homology directed repair (HR)

a major double-strand break repair pathway that is template-mediated and therefore considered to be highly accurate. Particularly important for sister chromatid regulation in S/G2 phase.

Hypomorphic mutation

a type of mutation that results in a reduced level of activity of the product encoded by the mutated gene.

Isochromatid deletion

aberration generated in G1, S or G2 in which both sister chromatids are broken at same level.

Lagging chromosome

a chromosome that is unattached to the mitotic spindle during anaphase.

Loss of heterozygosity (LOH)

a genetic event at a particular locus heterozygous for a mutant allele and a wild-type allele in which the wild-type allele is either deleted (rendering the cell hemizygous for the mutant allele) or mutated (rendering the cell homozygous for the mutant allele).

Mitosis

division of the nucleus of an eukaryotic cell, involving condensation of the DNA into visible chromosomes, and separation of the duplicated chromosomes to form two identical genetic sets.

Non-homologous end joining (NHEJ)

a major double-strand break repair pathway that involves the ligation of free ends, sometimes after processing that leads to the loss or gain of sequence. This pathway is particularly well studied in the context of V(D)J recombination.

Polytene chromosomes

giant chromosomes, found primarily in the salivary glands of certain insects, formed by repeated rounds of DNA replication without any cell division (endoreduplication). Replicated DNA molecules tightly align side-by-side in parallel register, creating a characteristic banding pattern.

Ortholog

a gene or a protein that belongs to a different species and that has a similar nucleic-acid or amino acid sequence, respectively.

Recombination mapping

method based on recombination frequency to establish the relative distance (measured in map unit, m.u.) between genes.

RNA interference (RNAi)

a form of gene silencing in which dsRNA induces the degradation of the homologous endogenous mRNA transcripts, thereby mimicking the effect of the reduction, or loss, of gene activity.

Single-strand annealing (SSA)

a subpathway of double strand breaks (DSB)-repair by homologous recombination, requiring only limited sequence homology of the ends joined and without the formation of recombination junctions.

Tumour suppressor genes

genes that normally interfere with tumorigenesis. Deletion or loss-of-function mutations in these genes are permissive for the development of cancer.

V(D)J recombination

a recombination reaction that assembles genes encoding diverse T-cell receptor and immunoglobulin molecules from variable (V), diversity (D) and joining (J) gene segments. V(D)J recombination is necessary for the recognition of diverse foreign antigens.

SUMMARY

Cellular responses to DNA damage are based on signal-transduction pathways involving phosphorylation-dephosphorylation events. Recent literature has demonstrated that protein serine/threonine phosphatases have important functions in DNA damage response (DDR). In particular growing evidence indicate that the protein phosphatase 2A (PP2A) plays a crucial role in genome stability maintenance, acts as tumor suppressor and is mutated in some cancer types. However current knowledge on the mechanisms and the pathways linking PP2A to DDR is still rudimentary.

Although most of the roles of PP2A are evolutionarily conserved, there are at present very few data suggesting an involvement of *Drosophila* PP2A in DNA repair. In the course of a screening aimed at identifying new *Drosophila* genes involved in the maintenance of genome stability we found an allele of *twins (tws)* gene, encoding the regulatory PP2A B subunit, that caused frequent chromosome aberrations (CABs), suggesting that also in *Drosophila* this phosphatase is involved in DNA repair. We observed that all previously identified alleles at the *tws* locus also caused CABs and high frequency of spontaneous γ-H2Av foci. Moreover *tws* mutations determined γ-H2Av foci persistence in irradiated brain cells, indicating that Tws promotes foci regression by dephosphorylating γ-H2Av. We also demonstrated that mutants in the *Pp4-19C* gene, that encodes the PP4 catalytic subunit, affected γ-H2Av foci dissolution but not exhibited CABs suggesting that impaired foci regression is not sufficient to cause CABs.

PP2A and PP4 are also involved in the G2/M checkpoint. In irradiated *tws* mutant brains the mitotic index (MI) did not drop at 15 minutes (min) as in control cells, but remained similar to that of non-irradiated controls without significant variations over time. In contrast in *Pp4-19C* mutant cells MI dropped at 15 min after irradiation but the recovery was significantly delayed. These data

indicate that PP2A and PP4 are both implicated in the G2/M checkpoint although with different roles.

To better understand the origin of CABs in *tws* mutants we tried to individuate Tws substrates by cytological examination of double mutants carrying *tws* mutation and mutations in genes involved in DDR pathway. This analysis revealed that mutations in the ATM-coding gene *tefu* and mutations in *ku70* gene, encoding a component of NHEJ system, are both perfectly epistatic to *tws* mutations. From these data we deduced that Tws controls genome integrity through a pathway in which Ku70 is first phosphorylated by ATM and then dephosphorylated by Tws (that perhaps dephosphorylates also ATM itself) to allow DNA repair. Therefore, in *tws* mutants CABs are induced by the hyperphosphorylation status of Ku70.

INTRODUCTION

The ultimate goal of cell division is to accurately duplicate the genome, ensuring that the resultant daughter cells will receive exact copies of the genetic material from the parent cell. Failure to accomplish this objective allows genomic instability, a prerequisite for oncogenic transformation.

The diploid state of mammalian somatic cells is guarded by control mechanisms that operate throughout the cell cycle to prevent both the occurrence of genetic aberrations and the proliferation of cells with deviant DNA content. The cyclin dependent kinases (Cdks) and their cyclin subunits regulate key protein phosphorylation events such that DNA replication and mitosis, driving the cell cycle forward. The initiation of DNA replication is controlled by a system that prevents re-replication; moreover two types of checkpoints (the DNA damage checkpoints and the spindle assembly checkpoint (SAC)) ensure the integrity of the genome and the proper separation of newly replicated sister chromatids. The DNA damage response pathways monitor the integrity of the diploid chromosome complement, integrating DNA repair and cell cycle progression. Chromosomal stability is maintained by protecting the linear chromosomes end with nucleoprotein structures called telomeres, which prevent fusion events that lead to genomic rearrangements.

DNA Double strand breaks (DSBs)

Genomes are incessantly exposed to environmental and endogenous agents that create thousands of DNA lesions per cell each day (Lindahl, 1993). Damage to DNA can arise from external sources, such as exposure to ionizing radiation (IR), ultraviolet radiation (UV) or environmental toxins, or from endogenous sources such as reactive oxygen species or errors during DNA replication. These events can generate a wide range of DNA

lesions, including modified bases or sugar residues, formation of DNA adducts, cross-linking of the DNA strands and production of single and double strand breaks. Consequently, cells have evolved at least six different DNA repair pathways to deal with these distinct types of DNA damage (Kennedy and D'Andrea, 2006). Of the various types of DNA lesions that arise within the cell, DNA double-strand breaks (DSBs) are particularly dangerous as their improper repair can cause chromosomal rearrangements that promote oncogenic transformation (Löbrich and Jeggo, 2007; Kastan and Bartek, 2004).

Exogenous factors such as ionizing radiation, crosslinking agents, and topoisomerase poisons, contribute to DSBs formation. Moreover DSBs are associated with oxidative metabolism, form during the normal S phase, when replication forks collapse and are generated during physiological processes such as V(D)J recombination, which occur to initiate rearrangements during maturation of immunoglobulin genes (O'Driscoll and Jeggo, 2002), yeast mating type switching (Paques and Haber 1999), and meiosis (Neale and Keeney, 2006). Last, but not least, naturally occurring DSBs at chromosome ends are associated with human cell aging, as they are exposed when telomeres become critically short during replicative senescence (d'Adda di Fagagna *et al*., 2003).

Chromosome aberrations (CABs)

Abundant evidence indicates that unrepaired or improperly repaired DSBs cause chromosome aberrations (CABs). CABs include stable and unstable rearrangements. Stable chromosome rearrangements can be transmitted to the progeny and are: inversions (that occur when a chromosome breaks in two places and the region between the break rotates 180° before re-joining with the two end fragments), translocations (that take place when a chromosome break occurs in each of two non homologous chromosomes and the two breaks re-join in a new arrangement),

deletions (the complete loss of a portion of one chromosome) and duplications (an extra copy of a segment of a chromosome). Unstable chromosome rearrangements fail to undergo regular cell division and include: rings (circular chromosomes), acentric fragments and dicentric chromosomes (chromosomes with two centromeres, derived from an exchange event).

Inversions and translocations are further classified as balanced rearrangements since they do not present visible gain or loss of chromosome material. Deletions and duplications are instead classified as unbalanced rearrangements, since they exhibit gain or loss of whole chromosome or portions of them.

Since the beginning of the twentieth century, the observations of Theodor Boveri suggested that acquired chromosome abnormalities could have a causative role in the origin of cancer (Boveri, 1914). Half a century later the discovery of the *Philadelphia* chromosome in chronic myeloid leukaemia (CML) was the first chromosome change observed in a human tumour and supported the view that chromosome abnormalities may have an important role in the initiation of carcinogenesis (Rowley, 1973). With the introduction of banding techniques many additional chromosome aberrations were soon detected in various tumour types. Moreover, sophisticated mouse modelling approaches confirmed that chromosome instability (CIN) plays a causative role in a substantial proportion of malignancies (Ricke *et al*., 2008).

It is now well known that cancer is produced or by deletion of tumour suppressor genes or by deregulation of proto-oncogenes. Thus, deletions, duplications and chromosome exchanges such as dicentrics and translocations can ultimately result in the loss of genetic material (loss of heterozygosity), leading to failure of tumour suppressor functions. Inversely, DNA amplification and formation of aberrant gene fusions can allow to increased expression of proto-oncogenes, also leading the cell to carcinogenesis (Mitelman *et al*., 2007; Aguilera and Gòmez-Gonzàlez, 2008).

Mechanisms of DNA DSB repair

DSBs are repaired through two distinct but interconnected mechanisms -non-homologous end joining (NHEJ) and homologous recombination (HR)- both of which are mediated by evolutionarily conserved proteins. NHEJ can operate throughout the cell cycle without the need for template DNA. In contrast, HR requires the presence of a homologous template, usually a sister chromatid, which allows accurate repair of postreplicative DSBs in S and G2 phases of the cell cycle.

Although the term "non-homologous" is used to describe this repair pathway, a tiny 1-6 bp region of sequence homology (micro homology) near the DNA end often facilitates re-joining mediated by NHJE. In contrast the HR repair is guided by much longer stretches of homology, generally encompassing 100bp or more. Thus, a major difference between NHEJ end HR repair is the span of homologous sequences associated with repair processing.

It is now widely accepted that non-homologous end joining (NHEJ) and homologous recombination (HR) repair pathways can both promote chromosomal rearrangements that contribute to oncogenic transformation. While NHEJ repair of perfectly compatible overhangs is generally error-free, repair of less compatible ends is error-prone. Moreover the oncogenic capacity of NHEJ lies in its ability to inappropriately join non-contiguous sequences if there is more than one DSB, resulting in deletions, insertions or translocations. This can lead to generation of dicentric chromosomes and subsequent chromosomal rearrangements through the breakage-fusion-bridge (BFB) cycle, as was first described for telomeric fusions. Furthermore, translocations can generate novel gene fusions with potential to initiate neoplastic cell growth (Figure 1). Although HR is considered an error free system, its utilization in G1 promotes use of a homologous chromosome as a template, resulting in localized loss of heterozygosity (LOH) and potentially reciprocal translocations if associated with crossovers. Moreover HR variants, such as single strand annealing (SSA) and

break induced replication (BIR), can generate deletions of varying size (Kasparek and Humphrey, 2011) (Figure 1).

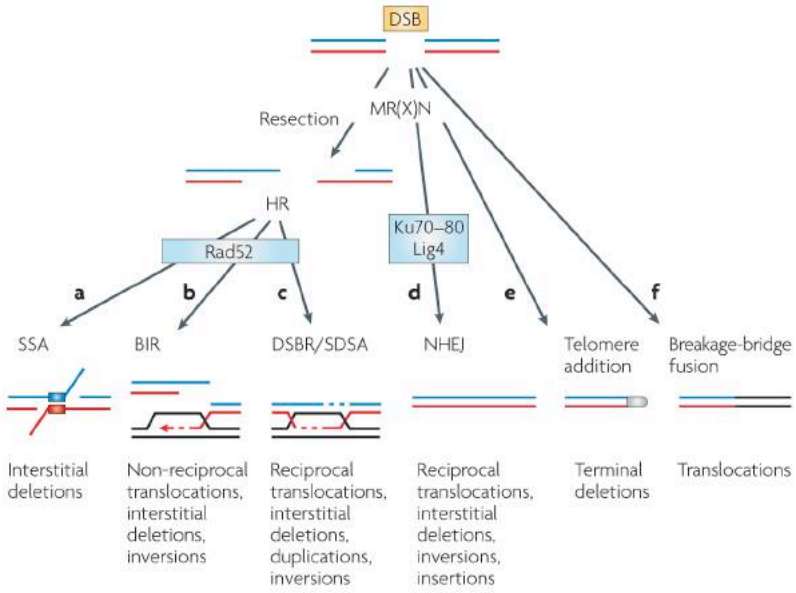


Figure 1. Mechanisms of double-stranded break repair leading to different chromosome rearrangements. (a) A double-stranded break (DSB) flanked by two homologous DNA repeats can be repaired by single-strand annealing (SSA). **(b)** One-ended DSB can be repaired by break-induced replication (BIR). **(c)** Standard homologous recombination (HR) events that occur by either the standard DSB-repair (DSBR) model, requiring resolution of two Holliday junction structures, or the synthesis-dependent strand-annealing (SDSA) pathway. **(d)** In the absence of sequence homology, the ends of two different DSB can be joined together by non-homologous end joining (NHEJ). **(e)** A broken chromosome can be stabilized by telomere addition. **(f)** Breakage–bridge fusion can generate translocations and other types of rearrangements (Adapted from Aguilera and Gòmez-Gonzaléz, 2008).

NHEJ repair system

In the NHEJ process, DNA ends are first bound by the Ku70/Ku80 heterodimer, which recruits and activates the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK holoenzyme. Broken DNA ends juxtaposed by DNA-PK are then acted on by factors such as the nuclease Artemis, polynucleotide kinase (PNK), Aprataxin, and APLF (Aprataxin and PNK-like factor) before being ligated by the XLF–XRCC4 (X-ray crosscomplementing-4) LigaseIV complex (Lieber, 2010) (Figure 2).

Not all DNA ends are however readily joinable. DNA ends can contain aberrant 3′ phosphate groups, 5′ hydroxyl groups, damaged backbone sugar residues and damaged DNA bases. Such DNA ends require processing before proper joining can proceed. Several other genes have been found to be required for efficient repair of a subset of ill-defined 'difficult' breaks (also referred to as 'dirty' or 'complex' breaks). Although the mechanistic details of such non-homologous end joining sub-pathways are not yet fully elucidated, they probably involve the ATM, 53BP1 and Mre11/Rad50/Nbs1 proteins and require phosphorylation of histone H2AX (Riballo *et al*., 2004).

HR repair system

The first step in HR is resection of the DSB that involves Mre11-Rad50-Nbs1 (MRN) complex. Resulting ssDNA overhangs are then coated by the ssDNA-binding complex RPA (replication protein A) before being substituted by RAD51 proteins with the help of factors such as RAD51 paralogs, RAD52, and other proteins that comprise the FA (Fanconi anemia) pathway such as FANCD1 (FA-associated nuclease CD1)/BRCA2 (breast cancer-2, early onset) and FANCN/PALB2 (partner and localizer of BRCA2). These proteins play key roles in detecting and repairing interstrand cross-links, particularly at sites of stalled DNA

replication. The RAD51 nucleofilament, together with various other HR factors, then mediates homology search along the sister chromatid, followed by strand invasion into the homologous template. After the actions of DNA polymerases and DNA end ligation by Ligase I, DNA helicase and resolvase enzymes then mediate the cleavage and resolution of HR intermediates to yield intact, repaired DNA molecules (San Filippo *et al*., 2008; Moynahan and Jasin 2010) (Figure 2).

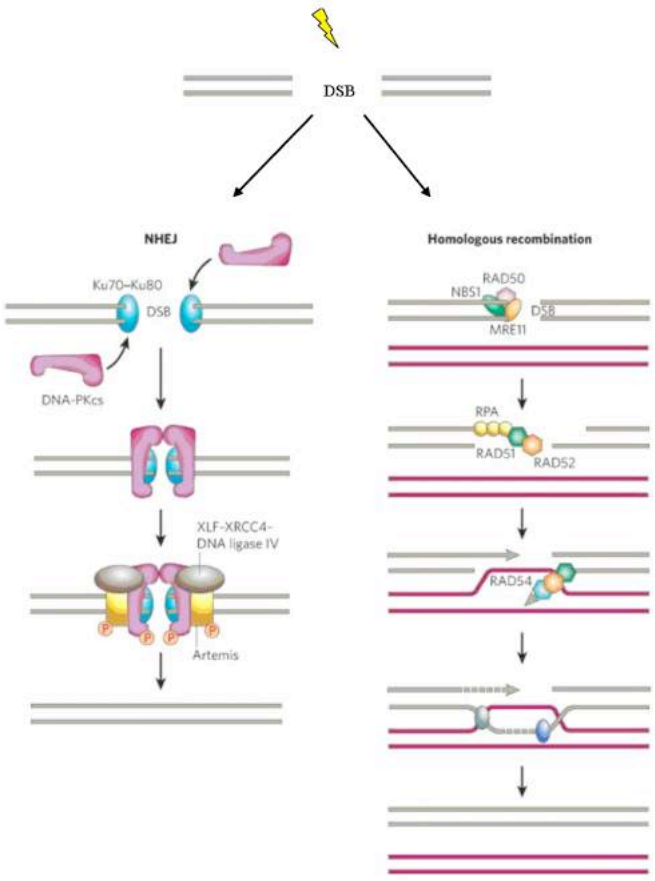


Figure 2. Schematic representation of non-homologous end-joining (NHEJ) and homologous recombination (HR) repair systems (Adapted from Downs *et al*., 2007).

SSA repair system

SSA can occur following end resection if sequence repeats exist on both sides of the DSB. In this case the two 3′ overhangs are simply aligned and annealed (Helladay *et al*., 2007) (Figure 3). SSA is restricted to repair of DNA breaks that are flanked by direct repeats that can be as short as 30 nt (Sugawara *et al*., 2000; Villarreal *et al*., 2012). Resection exposes the complementary strands of homologous sequences, which recombine resulting in a deletion containing a single copy of the repeated sequence. Such deletions have been observed between homologous segments of ALU elements in germ-line mutations of several tumor suppressor genes (Kolomietz *et al*., 2002). This repair system is therefore considered to be highly mutagenic (Mehta and Haber, 2014). SSA requires the strand-annealing activity of Rad52 and is aided by the Rad52 homolog Rad59; however, SSA does not involve DNA-strand invasion and thus is independent of the Rad51 recombinase (Sugawara *et al*., 2000).

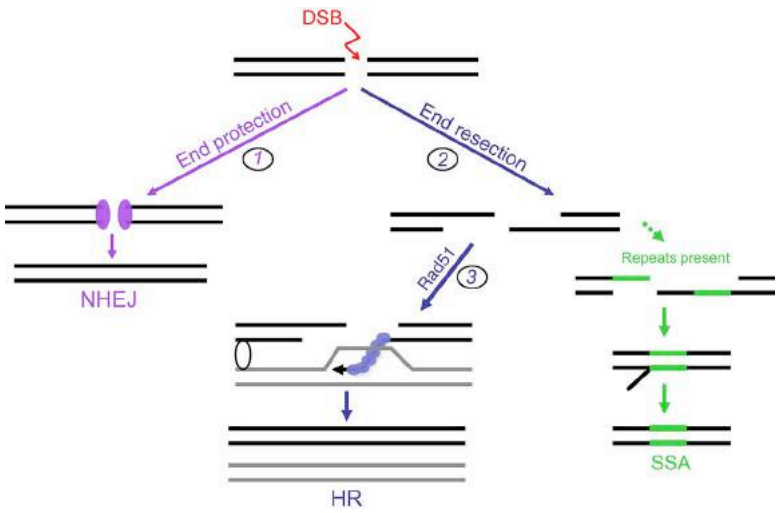


Figure 3. Schematic representation of DNA double strand breaks-repair system pathways (From Kass and Jasin, 2010).

DNA damage response pathways (DDR)

Cellular genomic integrity is closely monitored by processes, collectively known as the DNA damage response (DDR), those detect and repair DSBs and that also arrest cell cycle progression until the repair is complete. Sensing the lesion is the main step in the cellular response to DSBs. Several studies implicate the Mre11/Rad50/Nbs1 (MRN) complex as having an early role in the detection of the DSB, recruiting the damage response kinases, where they initiate downstream signaling to the DNA repair machinery and the cell cycle checkpoints (O'Driscoll and Jeggo, 2006). The masters regulators of DDR are the proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family—ATM, ATR, and DNA-PK—and the members of the poly(ADP-ribose) polymerase (PARP) family (Figure 4). ATM and DNA-PK are activated by DNA-damaging agents that create DSBs (Harper and Elledge, 2007; Meek *et al*., 2008). In contrast to ATM, which has hundreds of substrates, DNA-PK primarily regulates a smaller group of proteins involved in DSB end joining. ATR, in complex with its partner protein ATRIP (ATR-interacting prontein), is activated following recruitment to RPA-coated ssDNA regions generated at stalled replication forks and DSBs (Cimprich and Cortez, 2008). Within the PARP family only PARP1 and PARP2 have been implicated in the DDR. PARP1 and PARP2 are activated either by SSBs and DSBs and catalyze the addition of poly (ADP-ribose) chains on proteins to recruit DDR factors to chromatin at breaks (Schreiber *et al*., 2006).

Significant contributions to the field of DDR have been provided by studies on ATM and ATR kinases. Following the recognition of DNA lesions by sensor proteins, ATM and ATR initially phosphorylate mediator proteins, which then led to the amplification of the DDR by acting as recruiters of ATM/ATR substrates (Zhou and Elledge, 2000). Effector proteins of the DDR are directly phosphorylated by ATM/ATR or by the CHK1 and CHK2 kinases (Harper and Elledge, 2007) (Figure 4). One of the

essential aspects of these DNA-damage-signalling events is the activation of the so-called "checkpoint" mechanisms that slow down or stop cell-cycle progression while the damage persists, helping in this way to prevent the replication of damaged DNA or segregation of damaged chromosomes during mitosis. Additional aspects of the DDR-signalling mechanisms include alterations in chromatin structure and, particularly when the damage persists, the triggering of programmed cell death or long-term cell-cycle arrest known as senescence (Jackson and Bartek, 2009). Defective regulation of any of these activities results in genomic instability after DNA damage.

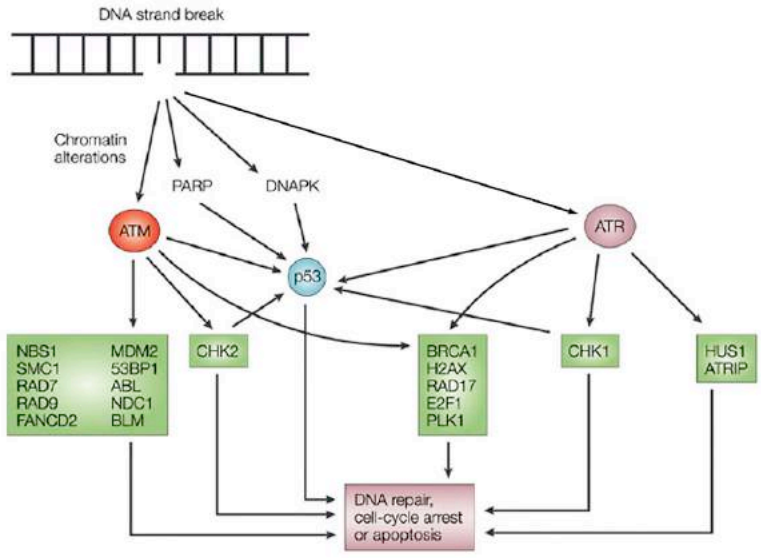


Figure 4. Schematic representation of the DNA damage response pathway (Adapted from Ljungman and Lane, 2004).

Mutations in ATM, MRE11, NBS1, BRCA1-2, ATR and Ligase 4 genes cause human syndromes characterized by both CABs and cancer predisposition, highlighting the connection between CABs and cancer (O'Driscoll and Jeggo, 2006). Inactivation of ATM leads to Ataxia Telangectasia (AT) an autosomal-recessive neurodegenerative disease clinically characterized by cerebellar ataxia, immunological dysfunction, cancer predisposition, sensitivity to ionizing radiation and premature ageing (Lavin, 2008). Phenotypic overlap between AT and diseases caused by MRE11 or NBS1 mutation support the physiologic relevance of interactions between ATM and the MRN complex. Hypomorphic mutations in Mre11 have been discovered in individuals exhibiting milder characteristics of AT, resulting in AT-like disease (ATLD; Stewart *et al*., 1999). MRE11 also has been shown to be frequently mutated in nonfamilial cancers (Wang *et al*., 2004). Likewise, hypomorphic mutations in NBS1 have been identified as the cause of the Nijmegen Breakage Syndrome (Varon *et al*., 1998). In contrast to AT, NBS is characterized by microcephaly and is not associated with neurodegeneration or ataxia (Digweed and Sperling, 2004). Defects in ATM and rad3-related (ATR) protein are also involved in aspects of the DNA damage response, and hypomorphic mutations can lead to Seckel syndrome (O'Driscoll and Jeggo, 2006).

DNA-repair foci

The DNA-repair process manifests itself cytologically in the form of DNA-repair foci. These foci are structures formed by the recruitment and accumulation of DNA-repair factors at damage sites (Figure 5a). As said before, ATM and ATR promote DSB repair in part through phosphorylation-dependent recruitment of DDR factors to sites of DNA damage. One of the earliest targets of ATM and ATR is the histone protein variant H2AX. Immediately upon DSB formation, H2AX becomes rapidly phosphorylated on four serine residues to form γ-H2AX at rising DSB sites (Bonner *et al*., 2008). Within 30 minutes after DSB formation, large numbers of γ-H2AX molecules form in the chromatin surrounding the break site, creating a focus where proteins involved in DNA repair, chromatin remodelling and DNA damage checkpoint accumulate. This striking cytological manifestation of DSB repair activity makes it possible to detect individual DSBs with antibodies directed against γ-H2AX or other proteins that accumulates at break foci, such as MDC1, Rad51 or the components of the MRN complex (Bonner *et al*., 2008). Foci can be experimentally induced by γ-irradiation, radiometric drugs, laser micro irradiation or sequence-specific endonucleases (Lukas *et al*., 2005). They are ubiquitous and form in organisms ranging from yeast to mammalian cells.

The structural organization of repair foci has not been elucidated; however, it appears that they are not merely random aggregates of repair components, as different factors exhibit distinct patterns of association with the DNA lesion (Bekker-Jensen *et al*., 2006) (Figure 5b-d). The recombination factors RAD51 and RAD52, the ATR kinase and its interacting partners ATRIP and RPA, and the DNA-clamp proteins RAD17 and RAD9 are restricted to a small area around the DSB, which corresponds mainly to stretches of ssDNA to which these proteins bind (Figure 5c). By contrast, several factors, including the sensor complex MRN, the DDR mediator MDC1, the ATM kinase and the

downstream factors 53BP1 and BRCA1, spread up to a megabase away from the break (Figure 5e). Interestingly, the downstream effector kinases CHK1 and CHK2 and other effectors of DDR such as p53 and CDC25A seems to not accumulate at damage sites, suggesting that these proteins only interact transiently with the sites of damage, where they presumably become activated, and then rapidly spread to the entire nucleus. Members of the NHEJ pathway, such as the Ku80–Ku70 heterodimer and the DNA protein kinase DNA-PK (Bekker-Jensen *et al*., 2006), do not accumulate in foci either, it has been speculated because they are only required at low copy numbers at sites of damage. Repair foci can form at all stages of the cell cycle, but their appearance is linked to the involved repair process.

Whether the accumulation of repair factors in foci is essential for efficient repair is unclear. The accumulation of factors could play a structural role by keeping the broken ends in spatial proximity, enhancing the efficiency of repair. Another likely, and potentially essential function of foci is the amplification of the DNA-damage signal by the recruitment of multiple copies of signalling kinases to sites of damage. Consistent with this idea, activation of cellular DNA damage response can be triggered by stable association of single repair factors with chromatin also in absence of DNA lesions, in both yeast and mammalian cells (Bonilla *et al*., 2008; Soutoglou and Misteli, 2008). Although repair foci are stable cytological structures once they are formed, they are highly dynamic structures subjected to precise spatiotemporal regulation (Essers *et al*., 2002; *Lukas et al*., 2004). Most of the factors involved are in constant dynamic exchange between the chromatin-bound pool and the freely diffusing nucleoplasmic fraction, and their residence time in the foci depends on the nature of their interaction with damaged chromatin as well as on their affinity for other components of the repair machinery (Misteli and Soutoglou, 2009).

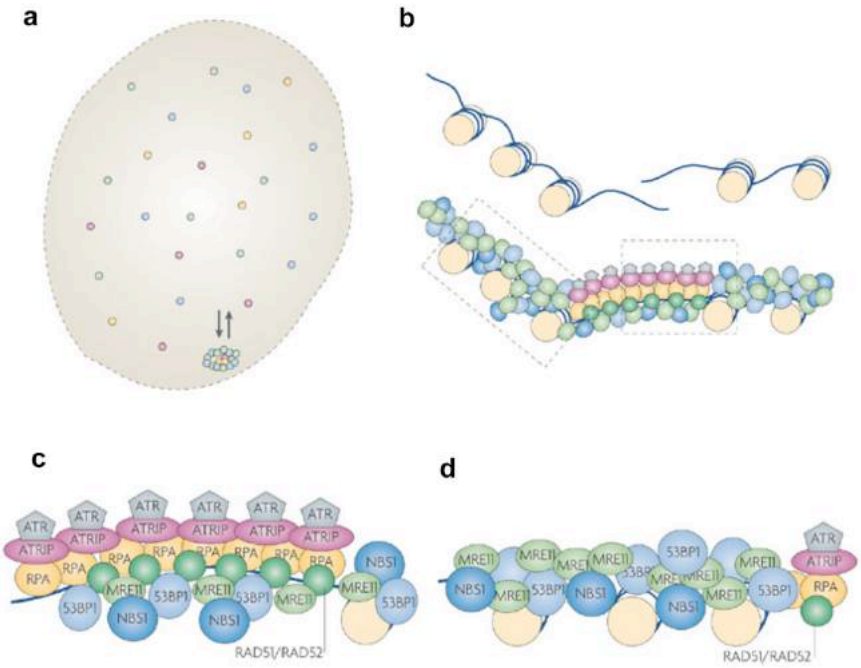


Figure 5. DNA-repair foci and their dynamics. (a) Repair foci are stable, yet highly dynamic structures. Although they persist for long time, their resident proteins are recruited from a freely diffusible nucleoplasmic pool that is in continuous dynamic exchange with repair foci. Species of repair factors are indicated in different colours. **(b)** Schematic illustration of the 'microstructure' of repair foci. The centre of the repair focus contains resected single-stranded DNA (ssDNA). This region is occupied by a specific set of factors that generate and have affinity for ssDNA regions (grey, purple, yellow and dark green). The regions flanking the actual break are occupied by a distinct set of proteins (blue and light green) that is involved in spreading and amplifying the DNA-damage response (DDR) signal. **(c)** Hypothetical organization of a ssDNA microcompartmen. **(d)** Hypothetical organization of a region that flanks a DSB (Adapted from Misteli and Soutoglou, 2009).

Although the formation of DNA repair foci and their roles in promoting protein recruitment to the sites of DNA damage are becoming increasingly understood, much less is known about how and why they are removed from chromatin once DNA repair has been effected. The removal of γ-H2AX from sites of DNA damage is apparently tightly regulated, as it is closely correlated with DNA repair. In many cases, foci dissolution occurs mainly by reversing the post-translational modifications that led to focal DDR protein assembly. Several γ-H2AX phosphatases have been identified, including Pph3 and PP1 in budding yeast (Keogh *et al*., 2006; Bazzi *et al*., 2010) and PP1, PP2A, PP4, PP6, and WIP1 in mammals (Nazarov *et al*., 2003; Chowdhury *et al*., 2005, 2008; Nakada *et al*., 2008; Cha *et al*., 2010; Douglas *et al*., 2010). Interestingly, H2AX dephosphorylation can take place on chromatin, as reported in human cells (Chowdhury *et al*., 2005, 2008; Nakada *et al*., 2008), or after histone eviction from chromatin in yeast (Keogh *et al*., 2006). Furthermore, the reversal of H2AX phosphorylation also involves chromatin remodelling complexes as Tip60 (Kusch *et al*., 2004; Jha *et al*., 2008) although until now it was not clear when they exactly works.

Ser/thr protein phosphatase 2A (PP2A)

Complex biological processes require cellular activities to quickly switch from one state to another. One of the most important mechanisms that allow activation and silencing of these activities is reversible phosphorylation. Multiple kinases and phosphatases have been indeed 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). PP2A is highly 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 (Hunter and Cooper, 1985).

In mammals, PP2A is a heterotrimeric enzyme consisting 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 more than 90 different complexes, which are 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 harbours two genes, *PP2A-29B* and *microtubule star (mts),* that 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* (*vrd*; B'/B56 type 2) and *CG4733* (B''/PR72) (Uemura *et al*., 1993;

Snaith *et al*., 1996; 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 (Perrimon *et al*., 2007; 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 (SV40). Other studies exploited mutations or RNAi to deplete specific PP2A subunits. Work on the B55 subunit showed that the PP2A-B55 enzyme is responsible for dephosphorylation of many mitotic proteins, so as to allow mitotic exit and progression through interphase. It has been demonstrated that the activity of this enzyme is high during interphase but is inhibited when cells enter mitosis to avoid premature reversion of CDK1-driven phosphorylation of mitotic proteins. The PP2A-B55 regulation in this pathway depends on the activity of the Greatwall (Gwl) kinase and its endosulphyne substrate proteins (the Xenopus Ensa and Arpp 19, and their single *Drosophila* orthologue Endos) (reviewed by Glover, 2012; 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; Tang *et al*., 2006; Chen *et al*., 2007). In addition to its mitotic functions, PP2A play important roles in nervous system functioning and maintenance and DNA repair (reviewed by 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) (Chowdhuri *et al*., 2005; Wang *et al*., 2009; Kalev *et al*., 2012).

Several non-mutually exclusive hypothesis have been proposed to explain the role of PP2A in the repair DSBs in mammalian cells. 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 than in control cells, suggesting that foci persistence leads to incomplete DSB repair (Chowdhuri *et al*., 2005). Another study suggested that PP2A dephosphorylates and activates both Ku and the DNA PK catalytic subunit (DNA-PKcs) that mediate the nonhomologous end joining pathway (NHEJ; reviewed by Lieber, 2010), and that PP2A-deficient cells accumulate DSBs due to defects in this pathway (Wang *et al*., 2009). In contrast with this interpretation, a third study 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; reviewed in Holthausen *et al*., 2010) pathway of DSB repair (Kalev *et al*., 2012).

AIMS

It has been demonstrated that the serine/threonine phosphatase PP2A is involved in DNA damage response (DDR) (Chowdhury *et al*., 2005), but to date there are contrasting data about the pathway in which PP2A operates to control chromosome integrity. In the laboratory where I have carried out my PhD thesis, a screen to identify *Drosophila* genes required for chromosome stability yielded the mutant tws^{430}, which exhibited a high frequency of chromosome aberrations (CABs).
Molecular analysis showed that the gene specified by tws^{430} encodes the B regulative subunit of PP2A demonstrating, for the first time, the involvement of *Drosophila* PP2A in DNA damage response. Moreover this finding has provided us the opportunity to take advantage of the sophisticated genetic analysis of *Drosophila* to obtain insight into the role of PP2A in maintenance of chromosome stability.

My primary aim was to precisely define the mitotic phenotype caused by the loss of Twins function performing a cytological analysis of tws^{430} and previously isolated *tws* alleles. Moreover I investigated the role of *tws* in the formation and persistence of γ-H2Av repair foci employing immunofluorescence and biochemical techniques.

The results obtained during the first year of research set the **aims for the second year**: understanding the mechanisms leading to CAB formation in *tws* mutants. In particular I tried to determinate if CABs were induced by the persistence of γ-H2Av repair foci or if they were caused by a dephosphorylation failure of some component of repair systems. These studies showed that, in *Drosophila*, impaired foci regression is not sufficient to cause CABs.

My third aim was to identify PP2A substrates and define the pathway in which *tws*^{430} works using a genetic approach. Thus I constructed and examined the phenotypes of double mutants carrying the *tws* mutant allele and a mutation in another gene involved in the DDR. From this analysis I obtained substantial insight into the mechanisms that cause CABs in *tws* mutants.

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 (Figure 6) 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. We therefore named our mutation tws^{430} (430 is the number of the *tws*-bearing stock in the collection we screened).

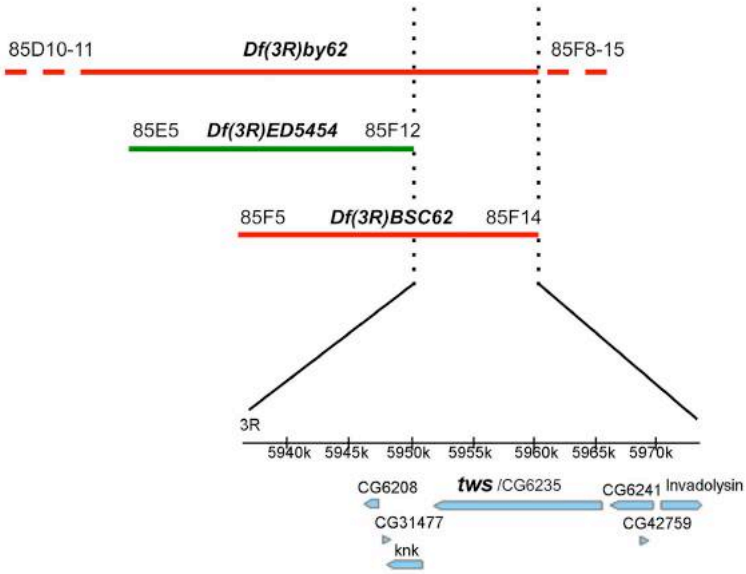


Figure 6. Deficiency mapping of tws^{430} mutation. The deficiencies that uncover the mutation are depicted in red.

tws encodes 8 transcripts that differ at the 5' UTR; all these transcripts give rise to two polypeptides of 499 (isoforms A) and 433 aa (isoforms B) that differ only in 56 aa at the N terminus. DNA sequencing showed that tws^{430} carries a G->A transition in a splicing acceptor site located in an intron shared by all transcripts (Figure 7A). Consequently (as demonstrated by RT-PCR and sequence analysis) the first AG in the downstream exonic sequence is used as a splicing site, leading to transcripts lacking 19 nucleotides that would result in truncated proteins of 142 and 86 amino acids (Figure 7B).

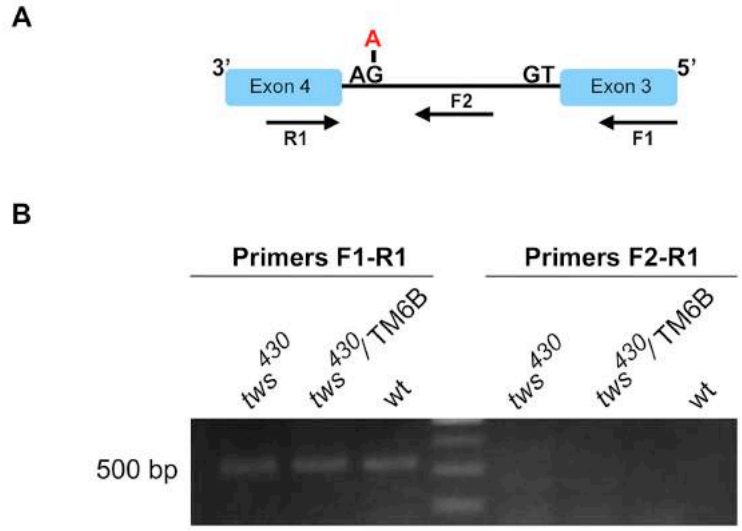


Figure 7. ***tws*** **mutation is a single nucleotide substitution falling within a splice acceptor site (A)** Zoomed region of the *tws* gene showing the intron between exons 3 and 4 and the splicing acceptor site mutated in the tws^{430} allele. Arrows indicate the localization of primers used in RT-PCR experiments. **(B)** RT PCR in tws^{430} homozygotes, tws^{430}*/TM6B* heterozygotes and wild type. The amplification with the couple of primers F1-R1 gives rise to fragments of similar sizes in both mutants and controls. The amplification with the couple F2-R1 gives rise to any amplification, indicating that also in the mutants the intron between exon 3 and exon 4 is removed.

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 as *Df*, is a deficiency that removes tws^{+}), tws^{430}/tws^{P} , tws^{430}/tws^{196}, , tws^{P}/tws^{196}, tws^{P}/Df and tws^{196}/Df (Figure 8). The chromosomes carrying the tws^{196} and tws^{P} also carry a second site lethal mutation, which prevented the analysis of tws^{P} and tws^{196} homozygotes.

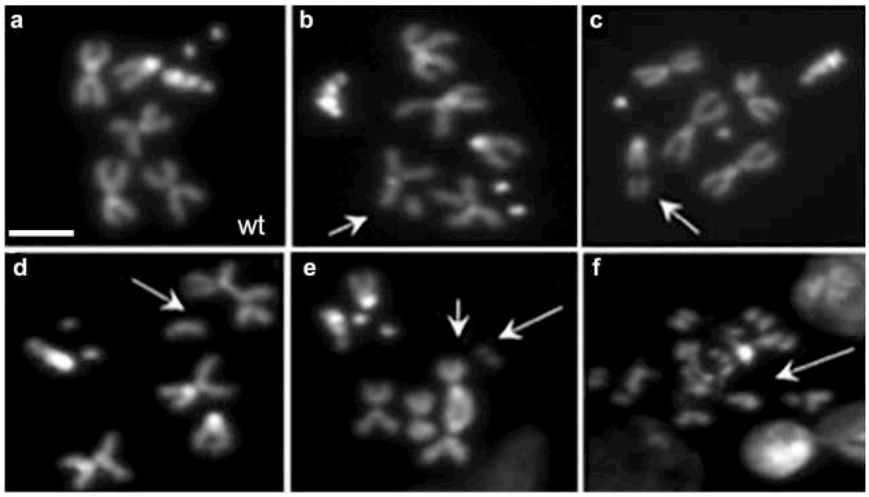


Figure 8. CABs in *tws* mutants. Examples of CABs (a) wild type metaphase; (b) chromatid deletion; (c) isochromatid deletion; (d) chromatid exchange; (e) dicentric chromosome with acentric fragment; (f) metaphase with extensive chromosome fragmentation. Scale bar, 5 μm.

The brains of all mutant genotypes examined displayed very high frequencies of cells with CABs (ranging from 37 to 57%) compared to wild type controls (0.8%) (Table1). In all mutants, we observed metaphases displaying from one to five breaks and metaphases with more than 5 CABs often showing an extensive chromosome fragmentation (Figure 8). Since the latter cells do not permit a reliable evaluation of the type and number of CABs, in Table 1 we reported only the frequencies of cells showing CABs instead of the CAB frequency per cell.

Genotype	# of cells scored	# of brains	% of cells with CABs (§)	% of cells with 5 or > 5 CABs
wt	751	6	0.8	0.0
tws^{430}	1245	15	40.5	10.6
tws^{430}/ *Df*	489	10	46.5	15.4
tws^{430}/ tws^{P}	703	9	37.3	9.5
tws^{430}/ tws^{196}	658	7	50.4	17.0
tws^{P}/ tws^{196}	1091	8	41.1	15.0
tws^{P} / *Df*	1123	13	56.9	25.2
tws^{196}/ *Df*	1002	13	40.5	10.5

Table 1. Frequencies of CABs in *tws* mutants. CAB frequencies observed in wild type and in different *tws* alleles. §, includes cells with 5 or > 5 CABs.

The analysis of the CAB frequencies indicates that *tws*^{P} is the strongest mutant allele (Table 1). Consistent with this result, Western blotting showed that the Tws protein is undetectable in *tws*^{P}*/Df* mutant brains and strongly reduced respect to control in brains from *tws*^{430}*/Df* and *tws*^{196}*/Df* mutants (Figure 9).

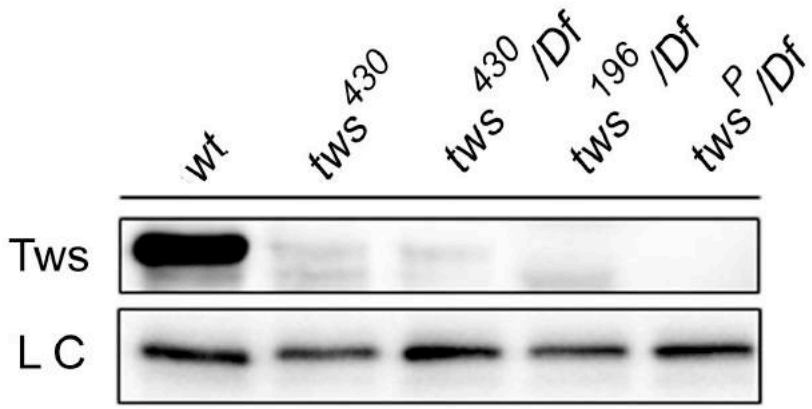


Figure 9. Tws protein levels in different *tws* alleles. Western blotting showing a strong reduction of Tws protein in different *tws* mutant alleles. The loading control (LC) is Giotto, a *Drososphila* Phosphatidylinositol transfer protein (see Methods).

The frequencies of the different types of CABs observed in metaphases with 1 to 5 CABs are reported in Table 2. As shown in Figure 8 and in Table 2, *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 (Gatti *et al*., 1974). Thus PP2A-B55 dowregulation 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

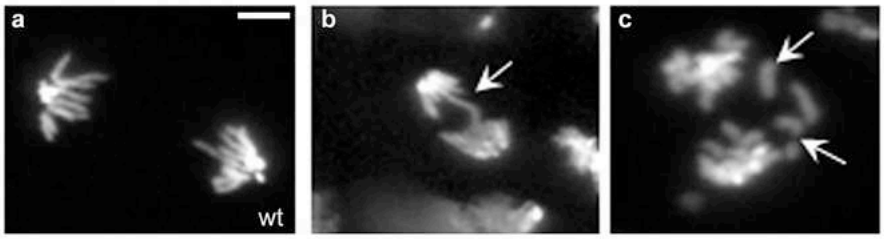
all the elements that gave rise to the CAB. For example, the large majority of isochromatid deletions consisted of both the centric and the acentric fragment. “Incomplete” isochromatid breaks consisting of either a centric fragment without the corresponding acentric element or of an acentric fragment 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 that exhibit frequent 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.

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

Table 2. Type and frequency of CABs in *tws* mutants. Cd=chromatid deletion; Iso=isochromatid deletion; C-tid exch=chromatid exchange; C-some exch= chromosome exchange.

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 a higher mitotic index (MI) than control (1.7 *vs* 0.6) but a lower anaphase frequency (3.5% *vs* 14% of control) (Figure 10A-B); 44% of anaphases showed chromatin bridges and lagging acentric chromosome fragments probably originated by dicentric chromosomes (or chromatids; see Figure 8) and sister union isochromatid deletions (in which the proximal end of the broken chromosome are fused) (Figure 8). These results are consistent with our observations on colchicine-treated metaphases (Figure 8), with previous work on *aar*/*tws* mutants (Gomes *et al*., 1993) and with the known role of *tws* in mitotic progression (Mochida *et al*., 2009; Kim *et al*., 2012).

A



B

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 10. Mitotic parameters in *tws* mutants. (A) Examples of irregular anaphases (a) wild type anaphase; (b) mutant anaphase with a chromatin bridge; (c) mutant anaphase with lagging acentric fragments. **(B)** Mitotic parameters in wild type and *tws* mutant brains. The mitotic index (MI) is the average number of mitotic figures per optic field (see Materials and Methods). Scale bar, 5 μm.

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 (Chowdhuri *et al.,* 2005), we asked if *tws* mutant cells exhibit γ-H2Av DNA repair foci. In brain cell nuclei from *tws* mutants the average frequency of cells with γ-H2Av foci ranged from 30% to 45%, while only 5% of wild type cells displayed γ-H2Av accumulations (Figure 11). Because γ-H2AX foci form around DSBs (Polo and Jackson, 2011), these results indicated that Tws downregulation results in DSBs.

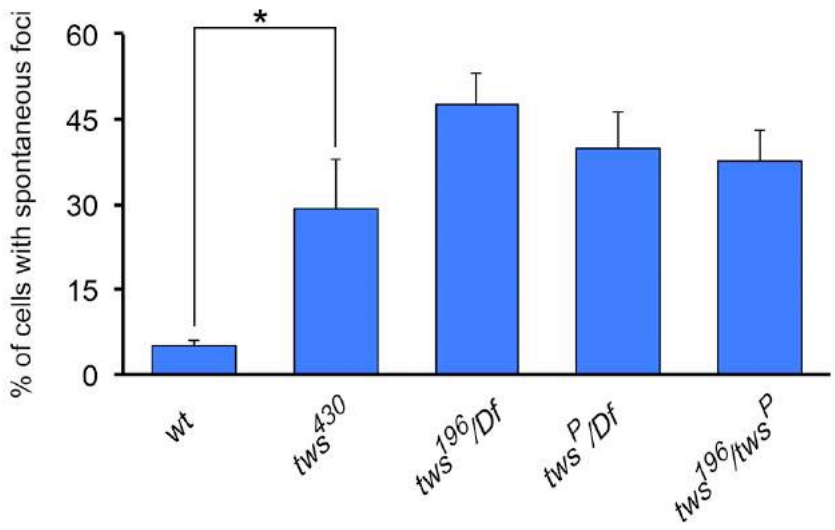
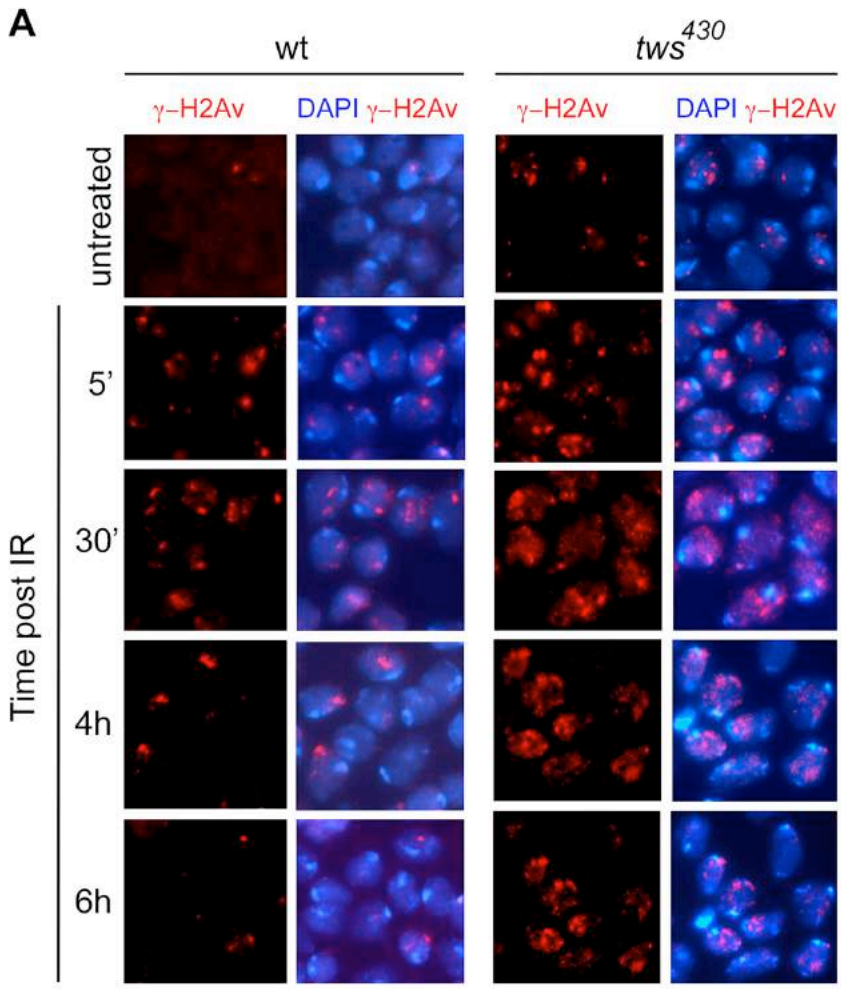


Figure 11. *tws* mutants exhibit high frequencies of spontaneous γ-H2Av foci in neuroblast cells. Frequencies of γ-H2Av positive cells in wild type e *tws* mutant alleles. Bars represent the mean frequencies of γ-H2Av positive cells (± SE) obtained by examining at least 300 cells/brain in 4 brains; * significantly different in Student t test with P <0.001

We next asked whether *tws* mutation affects γ-H2Av dephosphorylation and increases the persistence of irradiation (IR)-induced γ-H2Av foci. We thus performed time course experiments to analyze the kinetics of X-rays-induced γ-H2Av foci in *tws* and wild type brains. 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 both control and mutant brains the frequency of γ-H2Av foci peaked at 5 minutes (min) post-irradiation (PIR) and remained high at 30 min PIR to progressively decrease at 1 and 2 hours (h) PIR (Figure 12A, B). However, at 4 and 6 h PIR, the frequency of nuclei with foci remained high in *tws* mutants but dropped in wild type controls; at 6 h PIR, only 25% of wild type nuclei were still displaying foci, whereas approximately 65% of nuclei from *tws* mutants showed γ-H2Av foci (Figure 12A, B).


A
wt
tws430
γ–H2Av
DAPI γ–H2Av
γ–H2Av
DAPI γ–H2Av
untreated
5'
30'
4h
6h
Time post IR

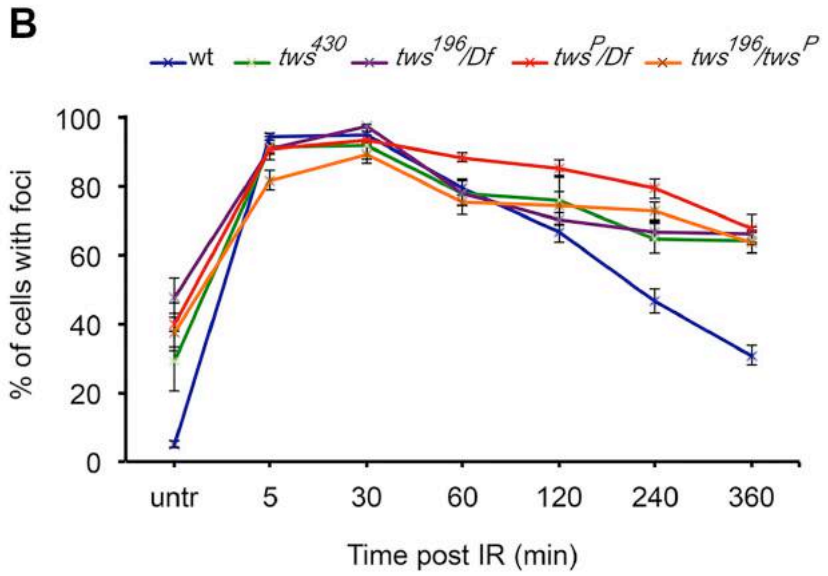


Figure 12. *tws* mutants exhibit delayed dissolution of γ-H2Av foci. **(A)** γ-H2Av foci in wild type and *tws* nuclei at different times PIR **(B)** Kinetics of γ-H2Av foci dissolution in wild type and in different *tws* mutant alleles. Bars represent the mean frequencies of γ-H2Av positive cells (± SE) obtained by examining at least 200 cells/brain in 4 brains.

These findings are consistent with the Western blotting analysis shown in Figure 13. In blots from mock-treated brain

extracts the intensities of the γ-H2Av bands were high from 15 min to 2 h PIR, but then decreased at 4 and 6 h 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 h PIR to decrease at 6 h PIR. 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.

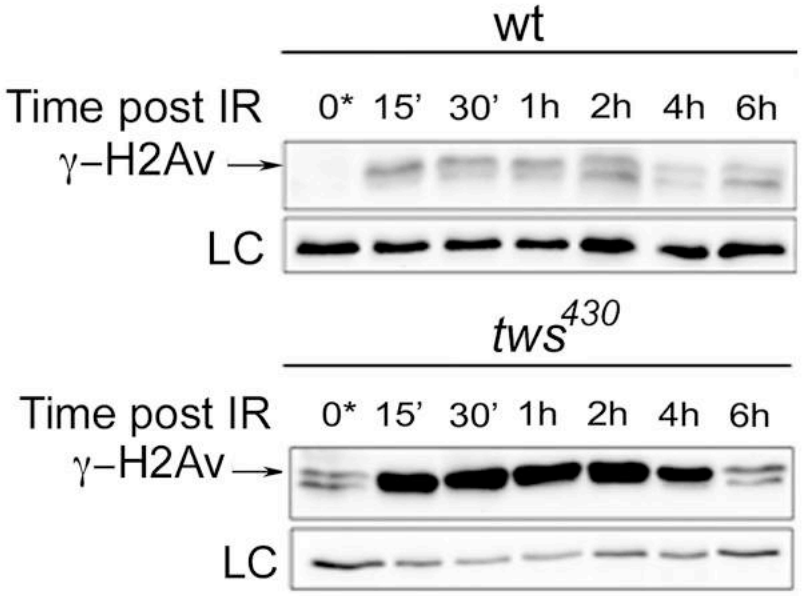


Figure 13. γ-H2Av levels remain high 6 h PIR in *tws*^{430} mutants. Western blotting showing γ-H2Av levels in wild type and *tws*^{430} brain extracts at indicated PIR times. The loading control (LC) is Giotto, a *Drosophila* Phosphatidylinositol transfer protein (see Methods).

We also asked whether PP2A associates with γ-H2AX foci as occurs in mammalian cells (Chowdhuri *et al*., 2005). We thus immunostained with an anti-Tws antibody wild type cells before and after and X-ray treatment. In unirradiated cells, the Tws protein is mainly localized in the cytoplasm. However, 2 h PIR, it became almost exclusively nuclear where it self-aggregated forming ciytologically detectable foci. At 6 h PIR, Tws returned to the cytoplam and the nuclear foci disappeared (Figure 14A). We next analyzed brain cells imunostained 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 14B). These results provide further evidence for a role of Tws in γ-H2Av foci regulation.

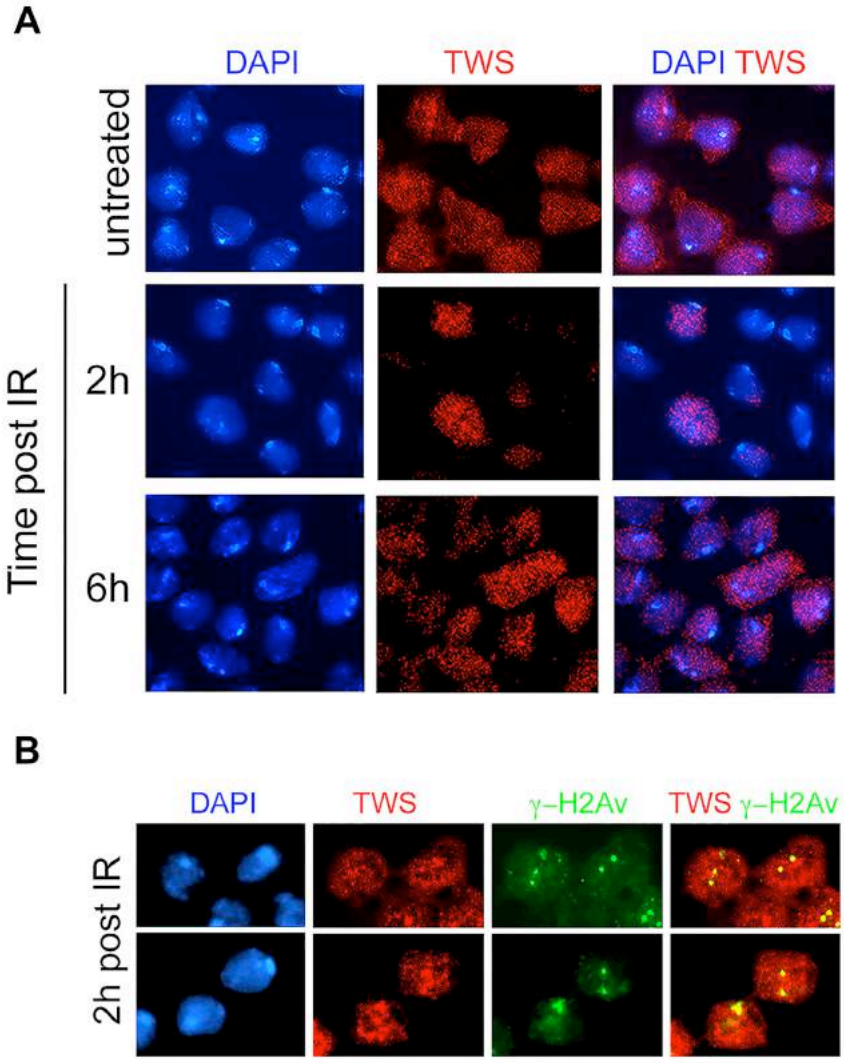


Figure 14. Subcellular localization of Tws protein. (A) Tws protein localizes in both nuclei and cytoplasm in untreated wild type brain cells and accumulates within the nuclei 2 h PIR. It returns to the cytoplasm 6 h PIR **(B)** Colocalization of Twins protein and γ-H2Av 2 h PIR.

We finally asked whether the ATM and ATR kinases (encoded respectively by *tefu*^{atm} and *mei-41 Drosophila* genes) are required for γ-H2Av foci formation. Previous work on mammalian cells has shown that H2AX can be phosphorylated by both ATM and ATR (Burma *et al*., 2001; Ward and Chen, 2001; Park *et al*., 2003). Wild type and mutant brains were treated with X-ray and fixed 5 min PIR. Immunostaining with an anti phospho-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*^{29D} mutants displayed the same frequency of cells with foci as non-mutant controls. These results indicate that formation of *Drosophila* γ-H2Av foci is mediated by ATM and not by ATR (Figure 15).

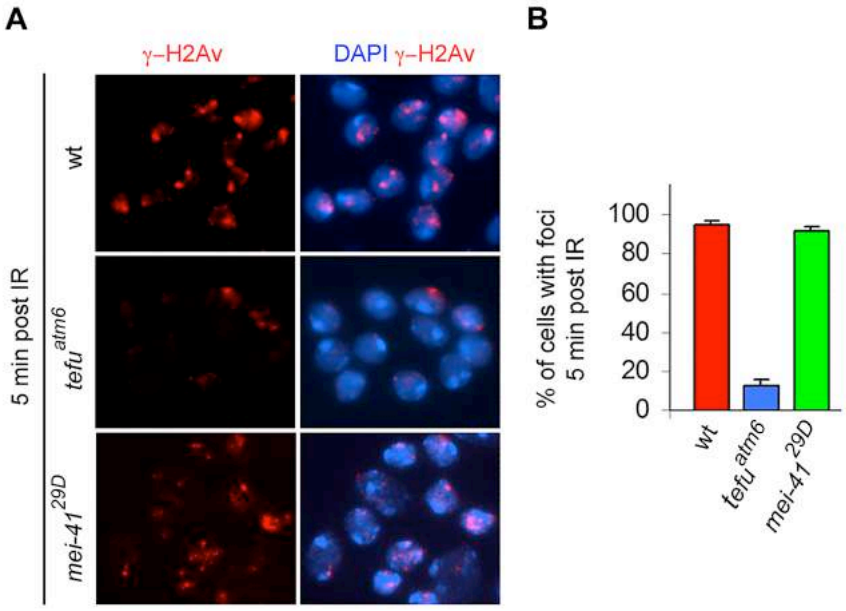


Figure 15. *tefu*^{atm6} mutants exhibit decreased γ-H2Av foci. (A) γ-H2Av foci in wild type, *tefu*^{atm6} and *mei-41*^{29D} irradiated brain cells. **(B)** Quantification of results in A.

Drosophila PP4 controls foci dissolution

To obtain insight into the mechanisms through which PP2A depletion leads to CABs, we asked whether other *Drosophila* phosphatases control γ-H2Av foci dissolution. Because previous studied showed that mammalian PP4 and PP6 phosphatases affect γ-H2AX foci regression (Chowdhury *et al*., 2008; Douglas *et al*., 2010), we tested whether the *Drosophila* homologues of these enzymes are also required for proper γ-H2Av foci behavior. Thus, we irradiated *Pp4-19C* larvae carrying a mutation in the catalytic subunit of PP4 and transgenic *PpV* RNAi larvae (*PpV* encodes the PP6 ortholog) and then followed the dynamic of γ-H2Av foci in a time course experiment. At 4 and 6 h PIR approximately 65% of brain nuclei of *Pp4-19C* mutants displayed foci (Figure 16). In contrast, at the same PIR times both *PpV* ^{RNAi} and wild type controls showed only 30-40% of the nuclei with foci (*PpV* interfered cells showed 70% reduction of protein as determined by RT PCR analysis). These findings indicate that *Drosophila* PP4 is required for γ-H2Av foci regression just like Tws (compare Figures 12B and 16), while PP6 is not involved in this process. In contrast, both PP4 and PP6 are required for γ-H2AX foci dissolution in mammalian cells (Chowdhury *et al*., 2008; Douglas *et al*., 2010).

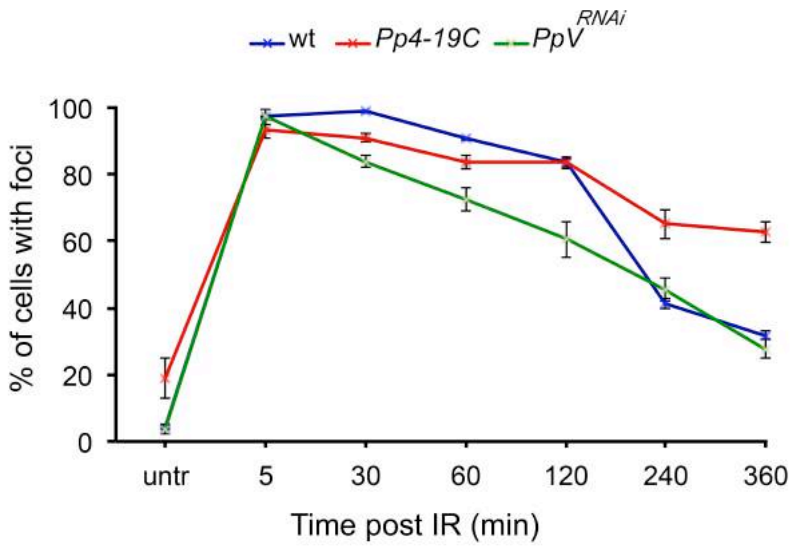


Figure 16. *Pp4-19C* mutants affect γ-H2Av foci dissolution. Kinetics of γ-H2Av foci dissolution in *Pp4-19C* and PpV^{RNAi} mutant brain cells. Bars represent the mean frequencies of γ-H2Av positive cells (± SE) obtained by examining at least 200 cells/brain in 4 brains

We then examined *Pp4-19C* mutant brains for the presence of CABs and found a CAB frequency comparable to that of wild type controls (0.5% CABs, in 500 cells examined from 4 mutant brains). Collectively, these results indicate that the persistence of γ-H2Av foci is not sufficient to induce CABs in *Drosophila* brain cells.

tws and *Pp4-19C* genes control the G2/M checkpoint

To further characterize the roles of *Drosophila* PP2A and PP4 in the DNA damage response (DDR) pathway, we asked whether they are involved in the regulation of the G2/M checkpoint like their mammalian counterparts (Yan *et al*., 2010). The G2/M checkpoint arrests cell cycle progression to prevent cells from initiating mitosis with damaged DNA (Yasutis and Kozminski, 2013). Thus, we performed a checkpoint assay, evaluating the mitotic index (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*^{29D} and *tefu*^{atm6} mutant flies as positive controls, since they are known to have a defective G2/M checkpoint (Laurençon *et al*., 2003, Brodsky *et al*., 2000, Bi *et al*., 2005). As shown in Figure 17A, the MI of the Oregon R control dropped at 15 min PIR and came back to a normal value only at 2 h PIR. In contrast, irradiated *mei-41*^{29D}, *tefu*^{atm6} and *tws*^{430} 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* mutants the MI dropped at 30 min PIR as occurs in control cells. However, *Pp4-19C* mutant cells began re-entering mitosis 2 h later than controls (Figure 17B). These results indicate that PP2A and PP4 play different roles in the *Drosophila* G2/M checkpoint; PP2A appears to be required for checkpoint signaling and PP4 for checkpoint recovery.

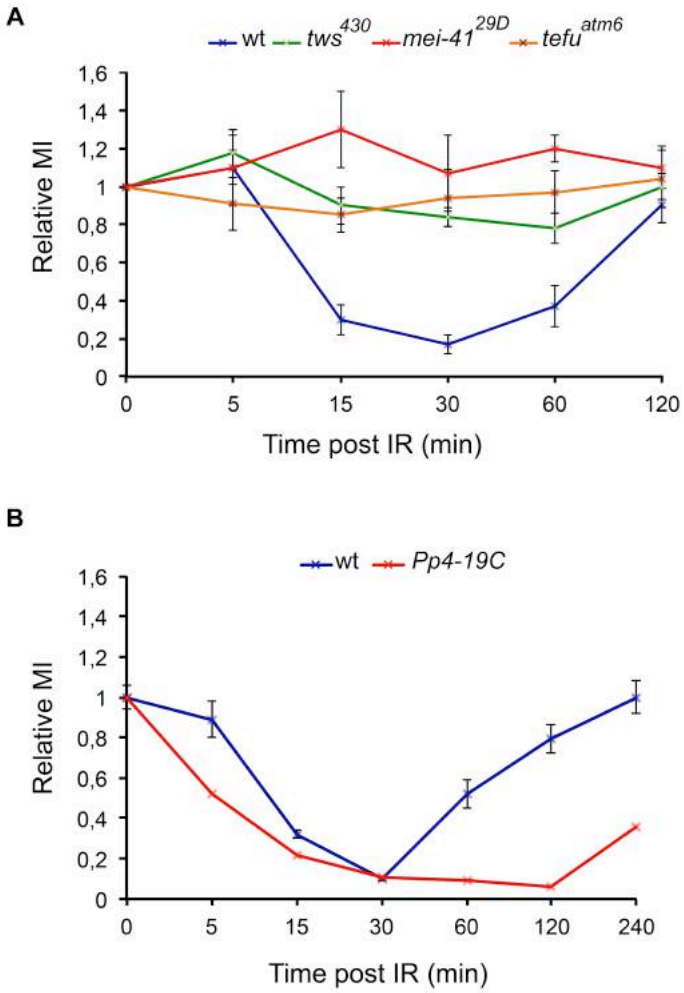


Figure 17. *tws* and *Pp4-19C* control the G2/M checkpoint. (A) Mitotic index (MI) variation at different PIR times in wild type, tws^{430}, $tefu^{atm6}$ and $mei\text{-}41^{29D}$ brains. MIs are normalized with respect to the MIs of unirradiated brains. In wild type control strain, MI drops 15 min PIR and comes back to normal value only 2 h PIR. In contrast, in tws^{430}, $tefu^{atm6}$ and $mei\text{-}41^{29D}$ irradiated brains, MI remains similar to that of unirradiated controls. **(B)** In *Pp4-19C* mutants MI drops 15 min PIR but cells restart to divide 2 h later than control. Bars represent the mean of MI (± SE) obtained by examining at least 500 cells.

Interactions between *tws* and other genes involved in the DDR pathway

To investigate molecular mechanisms underlying CAB formation in *tws* mutants we used a genetic approach. We constructed and examined citologically double mutants carrying the tws^{430} mutant allele and a mutation in another gene involved in the DDR. We first examined tws^{430} $tefu^{atm6}$double mutants because studies on mammalian cells have shown that PP2A dephosphorylates and physically interacts with ATM (Goodarzi *et al*., 2004; Kalev *et al*., 2012). The ATM kinase is at the apex of the DSB repair pathways; it is recruited at the DSB by the NBS subunit of the MRN complex and phosphorylates a series of repair factors including the H2AX histone variant (Shiloh and Ziv, 2013). Consistent with a previous work (Ciapponi *et al.,* 2004; Ciapponi *et al*., 2006), single *tefu* null mutants ($tefu^{atm6}$) showed 9% of cells with CABs and 50% telomeric fusions (TFs) (Table 3). Strikingly, $tefu^{atm6}$ tws^{430} double mutants displayed CABs and TF frequencies very similar to those seen in the *tefu* single mutants, while the CAB frequency observed in tws^{430} single mutants was about 4-fold higher than that seen in the double mutants (Table 3). Thus, mutations in *tefu* are perfectly epistatic to mutation in *tws*.

We then examined the interactions between mutations in *tws* and mutations in *nbs* (Table 3). In tws^{430} nbs^{1} double mutants, the frequency of cells with CABs (33%) was slightly (but not significantly) lower than that seen in *tws* single mutants (38%). However, the TF frequency in the double mutants was significantly lower than that seen in *nbs* single mutant. A possible explanation of this result is that since tws^{430} nbs^{1} double mutants die as second instar larvae they still probably retain residual maternal products (Gatti and Baker, 1989).

We finally analyzed the interactions between mutations in *tws* and some mutations in genes involved in the three major DSB repair pathways: non-homologous end joining (NHEJ), single-strand annealing (SSA) and homologous recombination (HR).

These pathways are evolutionarily conserved, and several *Drosophila* genes involved in these pathways have been characterized (Johnson-Schlitz *et al*., 2007; Klovstad *et al*., 2008). We examined mutations in *ligase4* (*lig4*) and *ku70* genes that encode components of non-homologous end joining system (NHEJ); mutations in genes involved in the SSA repair that encode Mei-9 endonuclease (orthologous to human XPF and yeast Rad1p) and the Mei-41/ATR kinase and mutations in the genes encoding respectively the *Drosophila* homolog of the human tumor suppressor BRCA2 and Spindle A (SpnA, homologous to Rad51) both involved in HR system.

lig4^{5}, *brca*^{56E} and *brca*^{KO} single mutants did not exhibit CABs (Table 3). However *lig4*^{5} *tws*^{430}, *brca*^{56E} *tws*^{430} and *brca*^{KO} *tws*^{430} double mutants died during embryogenesis or in the very early larval stages preventing cytological analysis.

mei-41^{29D} *tws*^{430} double mutants exhibited a CAB frequency (48%) close to the sum of the frequencies observed in the single mutants (44%) (Table 3) suggesting that Tws and ATR work in different pathways. *mei-9*^{A1} and *rad-51*^{1} single mutants showed CAB frequencies similar to control. Although mei-*9*^{A1} *tws*^{430} and *rad-51*^{1} *tws*^{430} double mutants exhibited CAB frequencies (respectively 24.3% and 24.2%) slightly lower (but not significantly, as determined by Student t test) than those showed by single *t*ws mutants, we considered additive these frequencies. Since these double mutants did not reach the third instar larval we suppose that they retained some *tws* residual maternal product that reduced CAB frequency. These data indicated that CABs induced by Tws depletion are not processed by HR nor SSA repair systems.

When we examined *ku70*^{Ex8} single mutants we found CAB frequencies comparable to those observed in wild type controls (0.2%). Remarkably also *tws*^{430} *ku70*^{Ex8} double mutants showed wild type CAB frequencies (0.3%) indicating that mutations in *ku70* are epistatic to mutations in *tws*. These results suggested that Ku70 is a Tws substrate and must be dephosphorylate by Tws to prevent CAB formation.

Genotype	# of brains	# of cells scored	% of cells with CABs (§)	% of cells with 5 or > 5 CABs	TF per cell
wt	5	460	0.4	0.0	0.0
tws^{430}	5	406	38.2	14.0	0.0
tefu^{atm6}	8	531	8.7	0.0	0.5
tws^{430} *tefu*^{atm6}	11	581	12.0	0.0	0.5
nbs^{1}	16	796	8.3	0.0	0.4
tws^{430} *nbs*^{1}	15	769	33.0	7.5	0.2
ku70^{Ex8}	7	850	0.2	0.0	0.0
tws^{430} *ku70*^{Ex8}	15	1392	0.3	0.0	0.0
mei-9^{A1}	10	950	0.2	0.0	0.0
mei-9^{A1} *tws*^{430}	12	423	24.3	1.7	0.0
mei-41^{29D}	5	415	5.8	0.0	0.0
mei-41^{29D} *tws*^{430}	8	632	47.6	10.4	0.0
rad51^{1}	7	840	0.4	0.0	0.0
tws^{430} *rad51*^{1}	16	1088	24.2	3.4	0.0

Table 3. *tws* mutants genetically interact with *tefu*^{atm6} and *Ku70*^{Ex8} mutants. CAB and Telomeric fusion (TF) frequencies in different double mutants. Note that *tws*^{430} *tefu*^{atm6} double mutants exhibit the same CAB frequencies of *tefu*^{atm6} single mutant and *tws*^{430} *ku70*^{Ex8} double mutants show the same CAB frequency of *ku70*^{Ex8} single mutant. §, includes cells with 5 or > 5 CABs.

To confirm these results we performed an epistasis analysis by RNAi experiments on S2 *Drosophila* cultured cells. Namely, we determined the frequency of cells with CABs after single depletion of Tws, Ku70, Rad51 and Brca2, or after double depletion of Tws and each of the Ku70, Rad51 and Brca2 proteins. Then we asked whether the doubly depleted cultures displayed a frequency of cells with CABs significantly different from the additive one expected for proteins functioning in different pathways. Because 10.7% of untreated cells exhibited spontaneous CABs we used a correction factor to calculate the expected additive frequency of cells with CABs. We calculated this frequency by summing the frequencies of cells with CABs observed in Tws -and Ku70 (or Brca2, or Rad51)- depleted cultures and subtracting from this value the expected frequency (10.7%) of cells with spontaneous aberrations. With this correction we avoided to consider twice spontaneous CABs, whose frequency is expected to be the same in singly and doubly depleted cells.

As showed in Table 4, cultures treated only with *tws* dsRNA showed a frequency of cells with CABs significantly higher (41.9%) than untreated cultures (10.7%). In cells lacking only Ku70, CAB frequency (8.2%) was similar to that found in untreated cells (10.7%). Interestingly cultures lacking both Tws and Ku70 exhibited a frequency of cells with CABs (24.8%) significantly lower than the additive one (39.4%). Thus, also in S2 cells, Ku70 depletion turned out to be epistatic over Tws depletion for effects on CABs confirming that Tws and Ku70 act in the same pathway.

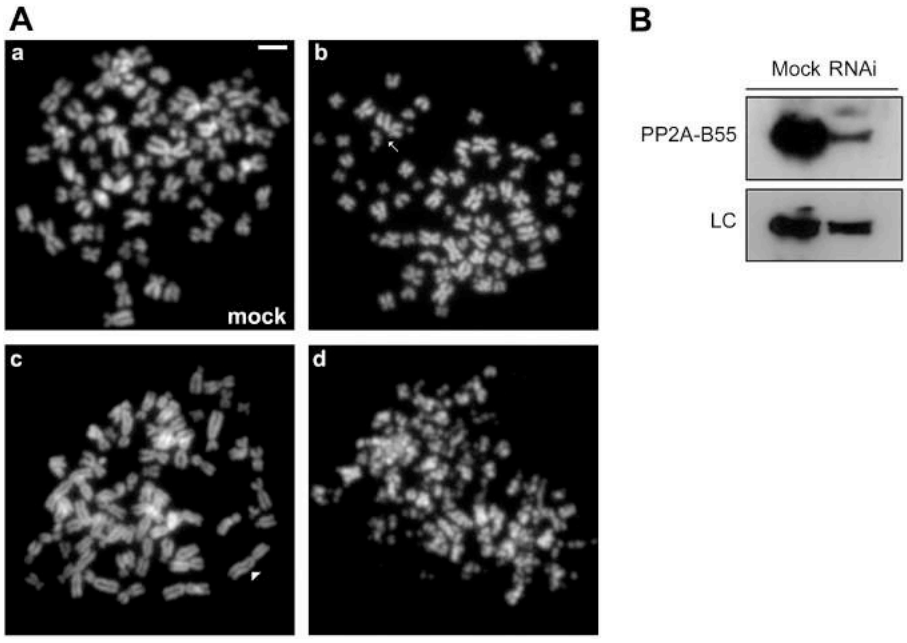
In contrast, cultures doubly depleted of Tws and Rad51 or Tws and Brca2 displayed CAB frequencies (respectively 51.6% and 50%) not significantly different from the expected additive values (respectively 41.4%. and 44.4%) These data indicated that Tws acts in a pathway different from that of Rad51 and Brca2. Therefore lesions generated by Tws depletion are not processed by HR repair system.

RNAi	# of cells scored	% of cells with CABs
control	131	10.7
tws	86	41.9
ku70	146	8.2
tws ku70	113	24.8
rad51	90	10.2
tws rad51	64	51.6
brca2	121	13.2
tws brca2	92	50.0

Table 4. Frequencies of CABs in RNAi cells. The simultaneous depletion of Tws and Ku70 resulted in significantly ($P<0.04$ in the Chi-square test) decreased CAB frequency respect to the expected additive one.

Loss of Human Twins causes chromosome aberrations in HeLa cells

We finally asked whether a specific reduction of the PP2A regulatory B55 subunits causes CABs in human cells. HeLa cells treated for 4 days with *B55 PP2A* siRNAs showed a reduction of the B55 protein to the 40% of the control level and displayed a significant increase in the CAB frequency (23%; n = 109) compared to untreated controls (2.45%; n= 122) (Figure 18). These data suggest that the B/B55 subunit of PP2A has an evolutionary conserved role in the maintenance of chromosome integrity.



C

Genotype	# of cells scored	% of cells with CABs
Mock	122	2.45
RNAi	109	22.9

Figure 18. Depletion of PP2A-B55 subunit causes CABs in HeLa cells. (A) Examples of CABs (a) mock-metaphase (b) metaphase with a chromosome fragmented (c) metaphase with a chromatid deletion (d) metaphase with extensive chromosome breakage. Scale bar, 10 μm. **(B)** Western blot showing that RNAi against B55 in HeLa cells reduces the level of the B55 protein to the 40% of the control level. **(C)** CAB frequencies in mock-treated and *B55* RNAi cells.

DISCUSSION

tws mutations induce CABs

PP2A is a major serine/threonine phosphatase, highly conserved, that plays a series of roles in cellular signalling and cell cycle regulation (Hunter and Cooper, 1985). In mammals and in *Drosophila* PP2A is a heterotrimeric enzyme formed by a catalytic (C) and a structural (A) subunit (the core enzyme) and one of various regulatory B subunits (B, B', B'', B'''), which governs its subcellular localization and substrate specificity.

In recent years growing evidence demonstrated that PP2A is involved in DNA damage response (DDR), although mechanisms by which it regulates the repair are not yet clarified.

In a previous screen, performed in our laboratory, we isolated the mutation tws^{430} that causes high frequencies of CABs in mutant larval brains and maps in the B regulative subunit of PP2A. This finding demonstrated, for the first time, that also in *Drosophila*, PP2A has a role in DNA damage. Thus, we decided to exploit the *Drosophila* model to obtain novel insights into the mechanisms by which PP2A controls genome stability. We first conducted a cytological analysis of previously identified *tws* alleles confirming the involvement of PP2A in DNA repair. Moreover, results obtained from cytological examination of mutant strains or in vivo RNA interference of the other regulative PP2A subunits (Wdb and PP2A-B'') (data not shown) demonstrated that CABs are caused by the specific depletion of Tws subunit.

In a previous work Gomes and coworkers (Gomes *et al*., 1993) showed that aar^{1} mutant allele (the first *tws* allele isolated) exhibited an elevated mitotic index and high frequencies of irregular anaphases with chromosome bridges and lagging chromatids. Based on these phenotypes the authors proposed that *aar (tws)* gene is required to promote a correct anaphase progression. Although we also found in *tws* mutants irregular anaphases and an elevated mitotic index, our data are not in agreement with this interpretation. We showed that in *tws* mutants most of aberrations are "complete" containing both centric and acentric fragments. Conversely if was impaired the mechanism to decatenate sister chromatids we would expect to find high frequency of cells with incomplete aberrations resulting by the

breakage of anaphase bridges. Thus, we deduce that most of CABs observed in *tws* mutants are not generated during anaphase but by DNA lesions produced during the interphase that precedes the mitotic divisions examined.

tws controls γ-H2Av foci dissolution

We observed that *tws* mutants exhibited, in neuroblast nuclei, an increased basal level of γ-H2Av as compared to wild type control (approximately 40% versus 5%). Since γ-H2Av is a marker of DSBs (Polo and Jackson, 2011) these results indicated that Tws depletion causes DSBs. Moreover, by time course experiments, we demonstrated that PP2A affects regression of γ-H2Av X-rays induced foci. In *tws* mutant alleles about 60% of cells remained positive to foci 6 hours (h) post irradiation (PIR), differently from control where the γ-H2Av persisted only in the 20% of cells. We confirmed these results by western blotting analysis, demonstrating that even 6 h PIR phospho-H2Av levels remained high in tws^{430} brains, whereas they resulted undetectable in wild type control brains.

In agreement with the hypothesis that *Drosophila* PP2A dephosphorylates γ-H2Av, immunolocalization experiments with an antibody directed against Tws showed that it moves from cytoplasm to nucleus 2 h PIR and co-localize with γ-H2Av foci. Our results are in perfect agreement with data reported in literature showing that specific PP2A inhibition by okadaic acid (OA) or by RNA interference, determine persistence of γ-H2AX foci and affects DNA repair in HeLA cells treated with Camptotecin. Moreover the catalytic subunit of PP2A (PP2A[C]) is recruited to DNA damage foci in wild-type cells and coimmunoprecipitate with γ-H2AX in cell extracts 2.5 h after CPT-induced DNA damage (Chowdhury *et al*., 2005).

Although the formation of γ-H2AX and its role in promoting DNA repair begin to be understood, much less it is known about its removal from chromatin. It is accepted that foci dissolution is regulated by both phosphatases and chromatin remodelling complexes. In yeast *S. Cerevisiae* γ-H2A is first displaced from chromatin (by chromatin remodelling complexes

not yet identified) and subsequently dephosphoryilated by the trimetric complex HTP-C, containing the Pph3 phosphatase (the hortolog of PP4) (Keogh *et al*., 2006). In mammals many phosphatases as PP2A, PP4, PP6, and Wild-type p53-induced phosphatase (WIP1) regulate foci dissolution probably working on γ-H2AX molecules chromatin bound (Nazarov *et al*., 2003; Chowdhury *et al*., 2005; Nakada *et al*., 2008; Cha *et al*., 2010; Douglas *et al*., 2010), but it is not clear why cells need multiple phosphatases to eliminate γ-H2AX. One hypothesis is that distinct phosphatases differentially regulate γ-H2AX that originates from different stressors and/or from different degrees of DNA damage. In support of this hypothesis PP4 phosphatase complex in mammalian cells specifically dephosphorylates ATR-mediated γ-H2AX generated during DNA replication (Chowdhury *et al*., 2008). Also in mammals chromatin remodelling complex as TIP60 and FACT (Spt16/SSRP1) have been implicated in γ-H2AX elimination but their exact role has not yet fully elucidated (Jha *et al*., 2008, Heo *et al*., 2008). The most likely scenario is that the chromatin should be first remodelled to allow the phosphatases to operate (Jha *et al*., 2008).

In *Drosophila* there are not so far data indicating an involvement of phosphatases in phospho-H2Av removal. Previous studies (Kusch *et al*., 2004) indicated that foci regression is only based on TIP60 complex that acetylates nucleosomal phospho-H2Av and exchanges it with an unmodified H2Av. In particular the histone-exchange reaction is catalyzed by the ATPase Domino and enhanced by dTip60 mediated acetylation of nucleosomal phospho-H2Av. Accordingly to this, S2 *Drosophila* cells depleted of dTip60 and dMgr15 (two TIP60 subunits) by RNAi and also *mgr15* mutant embryos exhibited phospho-H2Av persistence 3 h PIR when in control cells is not more detectable (Kusch *et al*., 2004).

Our data represent the first demonstration that also in *Drosophila* PP2A and PP4 phosphatase are involved in γ-H2Av repair foci dissolution. At present we don't know if PP2A and PP4 act on γ-H2Av molecules induced by different stress or located at different distances from DSB sites. Moreover, how phosphatase activity integrates with that of TIP60 complex remains also to be determined.

Given that *tws* affects foci kinetics it might be expected that in *tws* mutants CABs are caused by impaired γ-H2Av foci resolution that would prevent recruitment or activation of some critical repair factor(s). Surprisingly we found that mutations in the PP4 catalitic subunit (*Pp4-19C)*, although impaired foci regression, didn't cause CABs. This demonstrated that persistence of γ-H2Av foci is not sufficient to induce CABs in *Drosophila* brain cells.

tws plays a role in the G2/M DNA damage checkpoint

We also showed that *tws* affects cell cycle progression after X-rays exposure. In *tws* mutants Mitotic index (MI), evaluated through a checkpoint assay, revealed in fact that after irradiation cell cycle doesn't slow, as occurs in wild type control strain, to allow repair. A similar effect was caused by mutations in $tefu^{atm}$ and *mei-41* genes that are known to play a role in the G2/M checkpoint. This suggested that also PP2A controls cell cycle arrest maybe dephosphorylating factors involved in cell cycle regulation. Our data are consistent with those recently obtained by da Yan and coworkers (Yan *et al*., 2010) that demonstrated that PP2A inhibition by OA, or RNA interference of catalytic subunit attenuates the cell cycle arrest in various types of irradiated cells. It is known that G2/M transition is controlled by the complex Cdc2/CyclinB and that DNA damage induces phosphorylation of the Tyr-15 residue of Cdc2 resulting in G2/M arrest. Yan and coworkers (Yan *et al*., 2010) showed that depletion of PP2A resulted in decreased IR induced Cdc2-Tyr15 dephosphorylation and also in impaired dephosphorylation of ATR kinase and its downstream target CHK1. If Tws acts through the same pathway has to be investigated.

In addition we demonstrated that also *Drosophila* PP4 plays a role in the G2/M checkpoint. In *Pp4-19C* mutant cells MI dropped at 15 min after irradiation but failed to return to normal values 2 h PIR. The same results have been obtained in yeast and human cells (Keogh *et al*., 2006; Nakada *et al*., 2008). PP4C depleted cells began to re-enter mitosis more than 9 h after the initial arrest, differently from control cells that began to divide 7 h post-irradiation (Nakada *et al*., 2008).

Collectively our results suggest that also in *Drosophila* PP2A and PP4 are both involved in the G2/M damage checkpoint

but with different functions: PP2A is required for checkpoint signaling and PP4 for checkpoint recovery.

Tws works in the same pathway of ATM and Ku70 to maintain chromosome integrity

Using a classical genetic approach consisting in cytological examination of double mutants carrying *tws*^{430} mutation and a mutation in genes involved in DNA damage control, we were able to isolate two genes that interact with *tws*: *tefu*^{atm} and *ku70*. This analysis allowed us to individuate the pathway for maintaining chromosome integrity in which *tws* is involved; moreover it enabled us to understand the origin of CABs induced by Tws depletion. In particular we found that *tws*^{430} *tefu*^{atm6} double mutants exhibited the same CAB frequency showed by *tefu*^{atm6} single mutants, demonstrating a clear epistatic relationship of *tefu*^{atm6} mutation on *tws*^{430} mutation. This result clearly indicates that Tws works in the same pathway of ATM, dephosphorylating ATM itself and/or some ATM substrates. Interestingly also in human cells there are evidences indicating an interaction between ATM and PP2A. It has been demonstrated in fact that PP2A counteracts ATM autophosphorylation on 1981 serine (Goodarzi *et al*., 2004). Moreover it has been recently reported that ATM phosphorylation at S1981 is dispensable for the ability of ATM to localize to DSBs but it is necessary for its retention at damages sites (So *et al*., 2009). Accordingly with these results Kalev and coworkers (Kalev *et al*., 2012) showed that cells depleted for PPP2R2A (the counterpart of Tws subunit) displayed increased ATM foci and impaired repair in human cells suggesting that the ATM hyperphosphorylation causes genome instability.

Our most interesting result obtained from the analysis of double mutants was that mutations in *ku70* gene, one of the key players of the NHEJ repair pathway, resulted epistatic to mutations in *tws* gene. *tws*^{430} *ku70* double mutants showed in fact the phenotype of *ku70* single mutants (absence of CABs) suggesting that Ku70 is a PP2A substrate. This epistatic relationship was confirmed by RNAi experiments in *Drosophila* S2 cells. Cultures doubly depleted of both Tws and Ku70 exhibited a frequency of cells with CABs (24.8%) significantly lower than the additive one (39.4%) expected for Tws and Ku70 functioning in different pathways.

These results suggested that 1) Ku70 is a Tws substrate that must be dephosphorylated to fulfil a correct DNA repair, 2) in *tws* mutants CABs are generated by dephosphorylation failure of Ku70, 3) Ku70 is phosphorylated by ATM, 4) the hyperphosphorylation of Ku70 is more toxic to cells respect to its depletion.

Ku70 is part of the Ku heterodimer (Ku70/Ku80) involved in DNA repair, chromosome maintenance, transcription regulation, and V(D)J recombination. In particular Ku heterodimer is the DNA-binding component of the NHEJ repair machinery. Upon recognition and binding to the broken DNA ends, Ku recruits DNA-PKcs to form the active complex DNA-PK. It has been demonstrated that DNA-PK undergoes phosphorylation of all its components in vitro, and phosphorylation of the DNA-PK complex correlates with loss of protein kinase activity and disruption of DNA-PKcs from the Ku-DNA complex (Chan and Lees-Miller, 1996). Previous studies demonstrated that PP2A directly dephosphorylates Ku as well as the DNA-PK catalytic subunit (DNA-PKcs) in vitro and in vivo, enhancing the formation of a functional Ku/DNA-PKcs complex. Moreover disruption of PP2A by either expression of SV40 small tumor antigen or depletion of endogenous PP2A/C by RNA interference inhibits Ku DNA binding and DNA-PK activities and causes the suppression of the NHEJ pathway with consequent increase of genetic instability (Douglas *et al*., 2001; Wang *et al*., 2009).

In *Drosophila* no obvious homolog of DNA-PKcs has been identified. The analysis of double mutants, however, suggested us that Ku70 is phophorylated by ATM. If Ku70 was the substrate of another kinase, in $tws^{430}tefu^{atm6}$ double mutants it would remain hyperphosphorylated causing CAB frequency comparable with that of *tws* single mutant. In contrast $tws^{430}tefu^{atm6}$ double mutants exhibited CAB frequencies lower than that showed by *tws* single mutants and very similar to that exhibited by $tefu^{atm}$ single mutants. This indicated that in absence of ATM, Ku70 is not phosphorylated and, as a consequence, Tws activity is not required.

Although an interaction between PP2A and Ku70 has already been suggested on the basis of previous biochemical evidences (Douglas *et al*., 2001; Wang *et al*., 2009) our data provided the first demonstration of the functional relationship between Ku70 and PP2A.

In addition, with respect to other studies (Wang *et al*., 2009), we showed that Twins is the specific regulative B subunit that links PP2A to DNA repair accomplished by NHEJ system. Moreover our findings suggested that hyperphosphorylation of Ku70 is more dangerous to cells respect to its depletion since null mutations *ku70* show CAB frequencies comparable to wild type control. Thus, a possible explanation of CAB origin in *tws* mutant is that when Ku70 fails to be deposphorylated, the NHEJ system is not fully active resulting in DSBs accumulation. Alternatively the hyperphosphorylation could modify the overall structure of Ku70 rendering this enzyme in some way genotoxic.

Recent studies carried out in human cells showed that depletion of PP2A results in an increased level of ATM autophosphorylation accompanied by downregulation of two component of HR repair system: RAD51 and BRCA1 suggesting that PP2A controls genome stability through HR system (Kalev *et al*., 2012). However when we analyzed double mutants carrying *tws* mutation and mutations in genes involved in HR system we got different data. *rad-51*^{1} *tws*^{430} double mutants showed an additive effect suggesting that Tws and Rad51 work in parallel pathways. In contrast *brca tws*^{430} double mutants died precociously preventing their cytological analysis. To overcome this problem we carried out RNAi against *tws* and *brca2* genes, on *Drosophila* S2 cultured cells, and found CAB frequencies close to the sum of frequencies observed in cells treated with single dsRNA. We obtained the same result performing double RNAi experiments against *tws* and *rad51*. Thus we concluded that PP2A in *Drosophila* doesn't work in HR pathway.

Finally we demonstrated that PP2A B55 subunit (Tws homolog) depletion causes chromosome aberrations also in human cells suggesting a conserved role of PP2A in chromosome integrity maintenance.

Concluding remarks

We demonstrated that in *Drosophila* the serine/threonine phosphatase PP2A also plays a crucial role in DNA damage response. Mutations in the B regulative PP2A subunit, Tws, cause CABs in neuroblast cells. Moreover they affect γ-H2Av repair foci dissolution and influence cell cycle progression after damage. We showed that *tws* interacts with ATM, the master protein kinase of DDR, and with Ku70, a component of NHEJ DNA DSB repair system. Our findings strongly suggested a model in which Ku70 need to be phosphorylated by ATM and dephosphorylated by Tws to promote repair.

In the absence of the *tws* function, Ku70 is abnormally and/or untimely phosphorylated, and as such interferes with the normal repair processes leading to DSBs and CABs.

Collectively, our results point to a mutagenic effect of an improperly phosphorylated Ku protein. Identification of the putative mutagenic phosphorylated forms of Ku70 and definition of the mechanisms underlying their effects on DNA metabolism will be the goal for future studies.

MATERIALS AND METHODS

Drosophila strains and crosses

tws 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. tws^{P} *and* tws^{196} were obtained from M.L. Goldberg laboratory (Cornell University, Ithaca NY). $brca^{56E}$ and $brca^{K0}$ were obtained from Schüpbach laboratory (Princeton University, New Jersey); $tefu^{atm6}$, $mei\text{-}41^{29D}$ and nbs^{1} mutations have been described previously (Silva *et al*., 2004; Laurençon *et al*., 2003; Ciapponi *et al*., 2006). *Df(3L)by62, Df(3L)ED54541* and *Df(3L)BSC621*, *Pp4-19C, brca2*^{KG03961}, $lig4^{5}$, $ku70^{Ex8}$, $mei\text{-}9^{A1}$, *rad51*/SpnA^{1} were all obtained from the Bloomington Stock Center. PpV^{RNAi} strain was obtained from the VDRC Stock Center.

Mutations on the third chromosome were kept in stock over the third chromosome balancer *TM6C* carrying the *Stubble (Sb)* and *Tb* dominant markers. Mutations on second chromosome were kept in stocks over the second chromosome balancer *CyOTbA*, bearing the *Tb* dominant marker. Homozygous and hemi-zygous mutant larvae were recognized for their non-Tubby phenotype.
Mutations on X chromosome were kept in stocks over the *FM7-GFP* balancer. Homozygous and hemi-zygous mutant larvae were recognized for their non-GFP phenotype.

For in vivo RNAi-experiments, females carrying the RNAi construct were crossed to males carrying Actin-Gal4 driver. The Oregon R laboratory 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/)

Chromosomes bearing both *tws*^{430} and *tefu*^{atm6} (or *nbs*^{1}, *rad51*^{1}, *ku70*^{Ex8}) were generated by recombination and balanced over *TM6B*.
Double mutants *tws*^{430} and *brca2*^{KG03961} were constructed crossing *tws*^{430}*/TM6B; Cy*^{GFP}*/Sco* females to *MKRS/TM6B; brca2*^{KG03961}*/Cy*^{GFP} males. Progeny *tws*^{430}/TM6B; *brca2*^{KG03961}/Cy ^{GFP} resulting from this cross, were mated inter se to obtain a stable stock.
To generate larvae *brca*^{56E}*/ brca*^{K0} *;tws*^{430}*/ tws*^{430} we crossed *tws*^{430}*/ TM6B*; *brca*^{56E}*/Cy* ^{GFP} females to *tws*^{430}*/ TM6B*; *brca*^{K0}*/ Cy* ^{GFP} males.
mei41^{29D} *tws*^{430} *(or lig4*^{5}*, mei-9*^{A1}*)* double mutants were generated by crossing *mei41*^{29D}*/FM7-GFP; l/TM6B* (*l* is a lethal mutation) females to w/Y; *tws*^{430}/TM6C. The *w/FM7-GFP ; tws*^{430}*/TM6B* females and *mei41*^{29D}*/Y ; tws*^{430}*/TM6B* males resulting from this cross were mated each other. The *mei41*^{29D}*/ FM7-GFP; tws*^{430}*/TM6B* females and *FM7-GFP/Y tws*^{430}*/TM6B* males were then crossed to obtain a stable stock. Double mutant larvae from these crosses were unambiguously identified on the basis of their non-GFP and/or non-*Tubby* phenotypes.

Chromosome cytology

Drosophila metaphase chromosome preparations were obtained according to published methods (Gatti and Goldberg, 1991). Preparations for the analysis of mitotic index (MI) and anaphase frequency were obtained in the same way, excluding both colchicine and hypotonic treatments. To estimate the mitotic index (MI) we calculated the average number of mitotic figures per optic field as described previously (Gatti and Goldberg, 1991). The percentage of anaphases was calculating from the ratio of the total number of anaphases and the total number of mitotic figures observed.
The checkpoint assay was performed exposing wild type and mutant larvae to 10 Gy of X-rays. Brains from irradiated larvae

were then dissected at the indicated times and fixed to determine the mitotic index. The relative MIs were calculated normalizing the MIs values of irradiated brains respect to those of untreated brains. All preparations are mounted in Vectashield H-1200 with DAPI (Vector Laboratories) to stain the chromosomes.

Immunostaining

For immunostaining experiments, brains from third instar larvae were dissected and fixed as described in Bonaccorsi *et al*., 2000. To induce γ-H2Av foci, wild type and mutant larvae were irradiated with 5 Gy of X-rays. At indicated times brains were dissected and fixed. To induce Twins foci wild type larvae were exposed to 5 Gy of X-rays. Brains were dissected from irradiated larvae after 2 and 6 h and then fixed. After several rinses in phosphate buffered saline 0.1% Triton (PBSt) brain preparations were incubated overnight at 4°C with primary antibodies. After two rinses in PBSt detection was performed by 1h incubation at room temperature with secondary antibodies. For γ-H2Av immunostainig brain preparations were incubated with anti-Histone H2AvD pS137 (Rockland code #600-401-914) diluted 1:100 in PBSt and detected with Alexa-Fluor-555-conjugate anti-rabbit IgG (Molecular Probes), diluted 1:300 in PBSt. For Twins immunustaining brain preparations were incubated with rat anti-Twins antibody (gift from T. Uemura, Kyoto University, Japan) diluited 1:50 in PBSt and detected with FITC-conjugated antirat IgG + IgM (Jackson Laboratories) diluited 1:20 in PBS. In all cases, immunostained preparations were mounted in Vectashield medium H-1200 (Vector Laboratories) containing the DNA dye DAPI (4,6 diamidino-2-phenylindole). To quantify the % of cells positive to foci at least 400 cells were analyzed for each fixing time post IR and the standard error was calculated on the media values.

Microscopy

All cytological preparations were examined with a Zeiss Axioplan fluorescence microscope, equipped with an HBO100W mercury lamp and a cooled charged-coupled device (CCD camera; Photometrics CoolSnap HQ). Grayscale images were collected separately, converted to Photoshop (Adobe Systems), pseudocolored and merged.

Western Blot

Extracts for Western blotting of *Drosophila* proteins 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 Antibody (#600-401-914 Rockland) diluited 1: 500 in TBSt and rat anti-Tws (a gift of T. Uemura) diluited 1: 500 in TBSt.
To determine the kinetics of H2Av phosphorylation we collected sample of 20 brains from wild type and *tws* larvae at different time after X-rays exposure (10 Gy). In each experiment we determined the intensities of H2AvD pS137 bands respect to the loading control [Giotto (Gio) a Drosophila Phosphatidylinositol transfer protein; (Giansanti *et al*., 2006)]. Measurements were performed on unsaturated bands using Image J software (http://rsb.info.nih.gov/ij/) for band quantification and normalization.

RNA interference in *Drosophila* S2 cultured cells

RNAi treatments in S2 cells were performed according to published methods (Somma *et al*., 2008).

Primers used for dsRNA:

rad51-5' AATACGACTCACTATAGGGAGGCAGTAACCTGGT
rad51-3' AATACGACTCACTATAGGGAGGCCAGTCTGCACA
brca2-5' AATACGACTCACTATAGGGAGGAACCGCATCAAC
brca2-3' AATACGACTCACTATAGGGAGGAAGGCTTGGGAG
ku70-5' AATACGACTCACTATAGGGAGGTGCCAGATGCCC
ku70-3' AATACGACTCACTATAGGGAGGACTTGGCTGCTC
tws-5' AATACGACTCACTATAGGGAGGTGGTCAACCAGA
tws-3' AATACGACTCACTATAGGGAGGAGAACATGGATG

Human cell procedures

HeLa cells were grown in DMEM (Gibco BRL) with 10% fetal bovine serum (FBS, Gibco BRL) in a humidified 5% CO_2 atmosphere. PP2A-B55-α/β/γ siRNA (44034 Santa Cruz Biotechnology*)* were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Mock-transfected and siRNA-transfected cells where grown for 72 hours in normal medium, after that colcemid (0.05 μg/ml, Gibco BRL) was added to the cultures for 3 hours before fixation according to Revenkova's group (Revenkova *et al*., 2009). Chromosome preparations were mounted in Vectashield H-1200 with DAPI. Human cell extracts were prepared according to Cherubini *et al*., (Cherubini *et al*., 2011), and Western blotting was performed as described in Somma *et al*., 2002; PP2A B Subunit was detected using a rabbit anti- PP2A B Subunit antibody (#2290 Cell Signaling) diluited 1:1000 in TBSt, BSA 5%.

Nucleic acid extraction, PCR, RT-PCR

Preparations of fly genomic DNA, PCR, RT-PCR, agarose gel electrophoresis, DNA sequencing, and sequence analysis were performed with standard procedures.
For the RNA extraction was used the RNeasy Mini Kit (Qiagen, Germany).
From 20 ng of the total RNA collected was next synthetized the corresponding cDNA using the Superscript kit (Invitrogen).

Primers used for RT-PCR:

tws F1 GTAATGGAGAGGCGTCTGGT

tws R1 TCTCCTGATCCGAATTAACGC

tws F2 AATATATCATGAAACTATTGC

PP6 for. GTCCTTGGTCACATTGTGGCC

PP6 rev. CTATGCGAGATGGTCTGCGAT

PCR products were sequence by Bio-Fab Research Genomics Company of Rome.

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