Vapors of benzene, a parquet varnish and a synthetic thinner induce chromosome loss in cells of the Drosophila wing primordia

Ph.D. Thesis

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2013

Publications related to the Ph.D. Thesis

Scientific publications

I.

István Soós and János Szabad, Assaying benzene, a parquet varnish, and a synthetic thinner for inducing *in vivo* chromosome loss in Drosophila wing primordia cells. Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 2014 Feb 11. pii: S1383-5718(14)00033-3. doi: 10.1016/j.mrgentox.2013.11.007. (Impact factor: 2.220)

II.

Szabad J, **Soós I**, Polgár G, Héjja G, Testing the mutagenicity of malondialdehyde and formaldehyde by the Drosophila mosaic and the sex-linked recessive lethal tests. Mutation Research **113**, 117-133, 1983. (Impact factor: 2.220)

Making science popular paper article

III.

Soós István és Szabad János, Kromoszómáink stabilitása. Természet Világa **143**, 386-389, 2012. (www.termeszetvilaga.hu/szamok/tv2012/tv1209/szabad.html)

Poster

Szabad, J. Venken, K., Bellen, H. and **Soós, I.**, Detection and quantitative evaluation of chromosome loss induced in Drosophila wing primordial cells. 12th International Conference on Preimplantation Genetic Diagnosis. Istanbul, Turkey, May 8-11, 2013. Poster P1.

Oral presentation

Soós István, Kromoszóma vesztés kimutatása foltos szárnyú muslicákkal. Az élhető város és vidéke szakmai szimpózium a magyar tudomány ünnepe keretében. Békéscsaba, 2012. november 22; p. 46.

LIST OF CONTENTS

SUMMARY

The consequences of environmental pollution and occupational hazards called much attention all over the world, from especially the 1970-es. Agents that cause adverse effects through changing the meaning of the genetic information and/or its amount in the cells are of special importance due to their usual delayed action in time and the induction of cancer. Of the so-called genetic hazards those are of special interests that bring about aneuploidy, one of the preconditions of cancer formation. No wonder why test procedures have been elaborated to identify and characterize environmental and genetic agents that can generate aneuploidy through the induction of gain and/or loss of chromosomes in the course of cell proliferation. One of the test procedures, the so-called CLADS technique (Chromosome Loss Assay in the Drosophila Soma) was published recently. It is based on the *in vivo* loss of $mwh^{+}Y$, a genetically engineered *Y* chromosome in cells of the developing wing primordia in Drosophila. The mwh^{+} transgene in the $mwh^{+}Y$ chromosome prevents the manifestation of the mutant phenotype of *mwh* (*m*ultiple *w*ing *h*airs), an excellent recessive cell marker mutation. Following the loss of the $mwh^{+}Y$ chromosome in any cell of the wing primordia, the *mwh* mutant phenotype becomes expressed, naturally only in *mwh* homozygous genetic background. Descendants of the by now genetically marked cell remain together during the subsequent rounds of mitoses and form an *mwh* clone (or mosaic spot) in midst of the normal cells (with only one hair per cell) in the wing blade. Significant elevation in the *mwh* clone frequency - as compared to the control - shows the ability of the analyzed agent to induce chromosome loss. The intensity of chromosome loss can be determined based on the frequency and size of the *mwh* clones. During my Ph.D. work I analyzed the chromosome loss-inducing ability of benzene, a reference chemical, a common environmental pollutant that has been known to induce chromosome loss. In fact its ability to induce leukemia appears to be related to its aneugenic capacity. In addition to benzene, I studied the effects of vapors of a commonly used parquet varnish and a synthetic thinner. While making use of the novel CLADS technique I confirmed the chromosome loss-inducing ability of benzene and showed that vapors of both the parquet varnish and the thinner bring about chromosome loss, albeit less efficiently as benzene. It is also my conclusion that the CLADS technique is an appropriate tool for high-throughput analysis of environmental and/or genetic agents to analyze their abilities to induce chromosome loss. The technique is sensitive, simple, very inexpensive and quite fast.

INTRODUCTION

In the civilized world cancer takes about 13% of the human lifes. Most of the cancers originate due to mutations in the cells, i.e. sudden and heritable changes in the DNA sequence and/or in the amount of the genetic material. The mutation-causing effects, the so-called mutagens, are frequently environmental, often man-made pollutants. It is generally believed that over 90% of the cancers are caused by environmental or occupational pollutions. Since every mutagen is also carcinogen, the identification of mutagens - through the so-called mutagenicity test procedures - provides helpful knowledge to avoid cancer formation.

Changes in the cell's heritable material can be classified into three major types: (i) point mutations, (ii) chromosomal breaks that may alter the amount of DNA and/or bring about rearrangements in the genetic material and (iii) changes in chromosome number. Aneuploidy is one consequence of the latter type of events and means departure from diploidy by usually one chromosome. The aneuploid cells may become the source of mental retardation, miscarriage or cancer (Pellman 2007; Li et al., 2010; Holland and Cleveland 2012; Pfau and Amon 2012). In fact, aneuploidy has been regarded as a hallmark of cancer in humans (Parry et al. 2002; Gordon et al., 2012).

There have been a number of assays developed to detect the first and the second types of mutations and several of those have been used on a large scale (Zeiger 2004; Claxton et al. 2010; Deepa et al. 2011). To detect gain and/or loss of the chromosomes, a number of the so-called aneuploidy test procedures were elaborated. They have been proficiently overviewed in panel reports like the FDA Redbook (http://www.fda.gov/ Food/GuidanceRegulation/ GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078321.htm) or in the OECD Test Guidelines for Genotoxicity and Mutagenicity Testing (http://alttox.org/ttrc/toxicity-tests/genotoxicity/).

The aneuploidy test procedures make use of yeasts, Drosophila, mice or cultured mammalian cells. There are three main reasons why they are not routinely used and included in the batteries of mutagenicity test procedures. (i) The aneuploidy detecting assays are not sensitive enough to observe rare events in the generally limited number of target cells. (ii) The high background noise, especially in the karyotyping-based procedures, sets a strong limit on the use of several of the proposed procedures. (iii) Most of the aneuploidy test procedures are quite sophisticated and are usually rather expensive.

To overcome the above difficulties Szabad, Bellen and Koen (2012) developed the so-called CLADS technique (Chromosome Loss Assay in the Drosophila Soma) and reported its use for four known mutagens (X-rays, colchicine, ethyl-methanesulfonate and formaldehyde) as well as for loss- and gain-of function *lodestar* mutant alleles. In my Ph.D. research I made use of the

CLADS technique, analyzed and confirmed the aneugenic activity of benzene and studied the effects of vapors of a regularly used parquet varnish and a synthetic thinner for their abilities to induce chromosome loss.

The CLADS technique

Principles of the CLADS technique are summarized in Figure 1. It is based on the use of *mwh*$^{+}$*Y*, a genetically engineered *Y* chromosome (Szabad et al. 2012). The *mwh*$^{+}$*Y* chromosome carries an *mwh*$^{+}$ transgene that prevents the expression of the phenotype of *mwh*, an excellent wing blade recessive marker mutation of *Drosophila melanogaster*. (For an explanation of the genetic symbols visit the FlyBase homepage at http://flybase.org/ and see below.) Following the loss of the *mwh*$^{+}$*Y* chromosome the cells express the *mwh* mutant phenotype - in *mwh* homozygous background - and can be identified on the forming wing blades. In practice *X*/*mwh*$^{+}$*Y*; *mwh*/*mwh* larvae, with the developing wing discs inside, are treated for the induction of chromosome loss. Loss of the *mwh*$^{+}$*Y* chromosome in any cell of the developing wing primordia leads to the formation of an *X0*; *mwh*/*mwh* cell. (*X* stands for an *X* chromosome labeled with the *w* - *white eyes* - recessive marker mutation and *0* for the lack of the *mwh*$^{+}$*Y* chromosome.) The *X0*; *mwh*/*mwh* cell as well as its descendants - that stay together and form an *mwh* clone (mosaic spot) - that can be recognized on the forming wing blade. Significant elevation in the *mwh* clone frequency shows the ability of the studied treatment to induce chromosome loss.

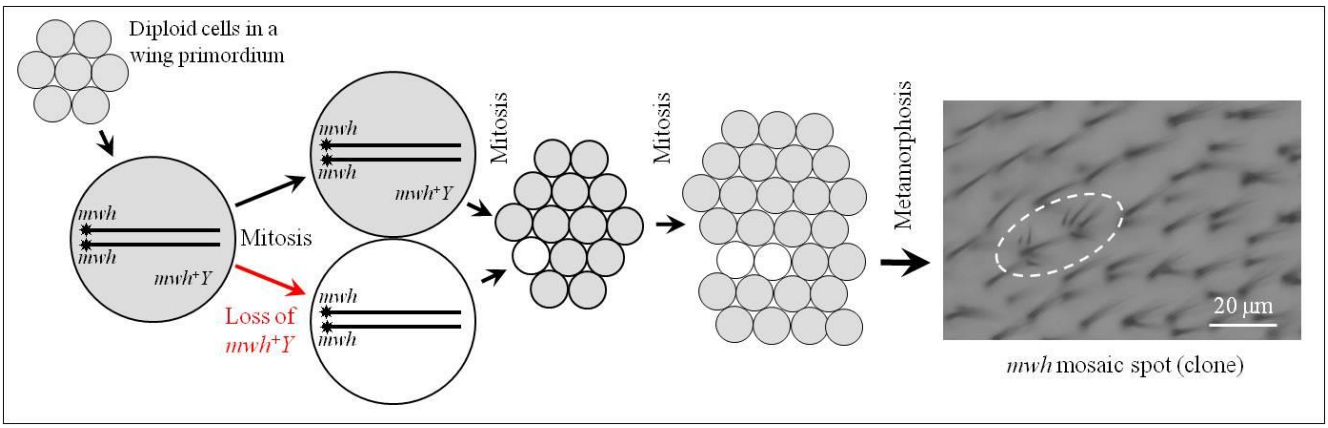

Figure 1. Principles of the CLADS technique. The *mwh*$^{+}$ transgene - inserted into a *Y* chromosome prevents the manifestation of the *mwh* mutant phenotype. Following the loss of the *mwh*$^{+}$*Y* chromosome one of the daughter cells becomes *mwh*-labeled. Its descending cells form a *mwh* clone in the wing blade. Each of the *mwh* homozygous cells produces 2-7 hairs instead of the usual one hair per cells.

Efficiency of the treatment to induce chromosome loss can be measured based on the frequency and size of the *mwh* clones and through the following considerations (Szabad et al. 2012). Assuming equal contribution of the wing disc cells to the wing blade, the number of cells in a wing disc primordium at the time of *mwh* clone induction is $C/2m$, where C is the number of the screened cells in a wing blade (C=30,000; [Garcia-Bellido and Merriam 1971]) and m is the average clone size. Since generally only one of the daughter cells becomes *mwh*-labeled following the loss of the mwh^+Y chromosome during mitosis, m needs to be multiplied by two. Screening N wings implies the analysis of $N\ C/2m$ target cells exposed to the treatment and - as described below - the number of these target cells can easily reach 10^5. Considering that a single *mwh*-labeled cell will give rise to one *mwh* clone, n - the number of *mwh* clones in N wings - equals the number of the target cells that gave rise to daughter cells without the mwh^+Y chromosome. Therefore f - the frequency of mwh^+Y chromosome loss - is $f = n\ 2m/N\ C$ (Szabad et al. 2012).

The CLADS technique makes use of the following achievements of the highly elaborated Drosophila developmental genetics.

1. In *Drosophila melanogaster* loss or gain of the *Y* chromosome do not alter cell viability. (The *Y* chromosome carries only few genes most of which are involved in sperm motility; Carvalho et al., 2001). This fact is elegantly illustrated by the facts that (i) although the Drosophila *X*0 males are sterile their viability is not different from those of the *XY* ones. (0 denotes the lack of the *Y* chromosome.) The *XYY* Drosophila males are also normal and fertile although they produce - through secondary nondisjunction - rather high frequencies of aneuploid germ cells (Bridges 1916).
2. There is a huge collection of marker mutations in Drosophila of which *mwh* (*m*ultiple *w*ing *h*airs) is one of the best and has been used for several decades by now. Wing cells homozygous for the *mwh* recessive marker mutation produce 2-5 trichomes (hairs) per cell and are usually short and possess abnormal polarity (Yan et al., 2008). The normal and the mwh/mwh^+ heterozygous wing blade cells each develop a single trichome (Figures 1 and 2). The *mwh* mutant phenotype is cell autonomous and single *mwh* homozygous cells can easily be detected in the midst of the wild type cells (Szabad et al., 1983).
3. The diploid cells of the imaginal discs - primordia of the future imaginal epidermis - proliferate throughout the larval life. A wing primordium (a wing disc) forms from about 30-50 cells of the embryo seven hours following the commencement of embryogenesis (that happens during egg deposition). The founder cells start to proliferate toward the end of the 1st larval instar, 37 hours after egg deposition. Cells in the wing discs proliferate through 10-11 rounds of mitoses - with a cell cycle length of about ten hours - throughout the oncoming

larval life. Mitoses cease within one day after pupariation, when wing blades form. In summary, during the roughly 100 hours of the exponential proliferative period the number of cells increase by a thousand fold and reaches about 50,000 (Fig. 3; Bryant and Levinson 1995). Of the 50,000 cells about 30,000 compose the wing blade (García-Bellido and Merriam 1971; Bryant 1975). Each wing blade cell produces a single hair within 1.5 day after pupariation (Ren et al., 2005). After eclosion of the adult flies from the pupal case the wings appear as chitinous organs in which every cell develops a hair (trichome; Fig. 2). In summary, the wing discs with their mitotically active cells are ideal organs to induce and detect genetic changes in its cells.

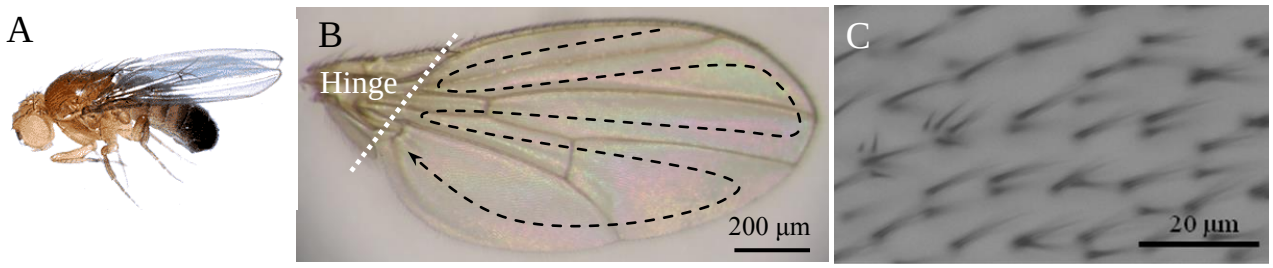

Figure 2. A *white*-eyed Drosophila male, a wing blade and wing hairs. (B) The path (dashed arrow) of screening the wings for *mwh* mosaic spots was drawn onto the photograph. The hinge region (left of the white dotted line) was omitted from the clone screen. (C) Micrograph of part of a wing blade with an *mwh* clone composed from two *mwh* homozygous cells. Note a normal cell produces one hair and that both wing surfaces are hairy.

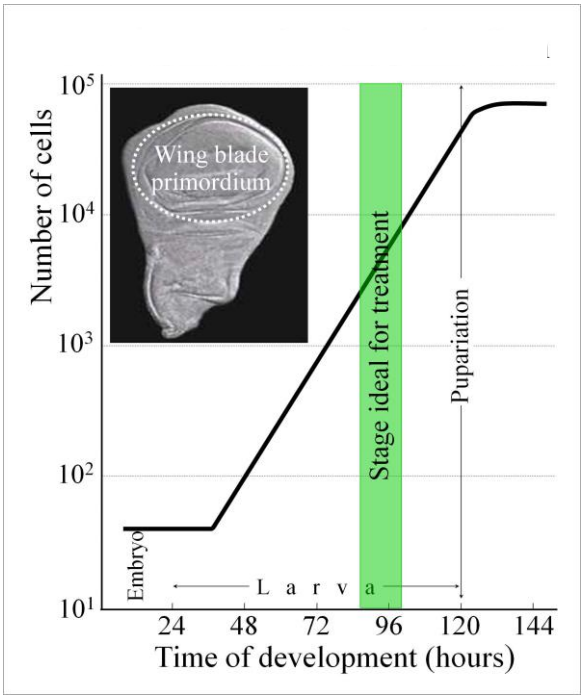

Figure 3. Features of wing disc development. (After Bryant and Levinson 1985.) The insert is a micrograph of a wing disc dissected from a late 3rd instar larva. The green bar corresponds to 84-92 hours in development when the larvae are still feeding (Rodriguez et al. 2009) and the wing disc cells will go through about three more rounds of mitoses.

Benzene, parquet varnish and synthetic thinner

Benzene, one of the aromatic hydrocarbons, is a common pollutant present in e.g. gasoline, cigarette smoke, automobile exhaust, and emissions from fires. Benzene has been used in the synthesis of - among others - dyes, detergents, plastics, pesticides, and synthetic fibers. A number of epidemiological studies have shown that benzene is a human carcinogen and tends to induce leukemia through the formation of aneuploid cells (Lynge et al. 1997; Chilcott, 2007). It is generally accepted that benzene exerts its adverse effects through its metabolites, among others benzene oxide, a rather stable molecule (Whysner et al. 2004; Khan 2007; Chilcott 2007; Smith 2010; Zhang et al. 2011). Benzene was used as a reference chemical in the present study as it has been known to induce aneuploidy (Zhang et al. 1999; Zhang et al. 2011).

In addition to benzene, vapor of a parquet varnish and a synthetic thinner were also included in the present study. The synthetic thinner contains aromatic hydrocarbons that are also present in the yet liquid parquet varnish. Aromatic hydrocarbons, or rather their metabolites, have been known to induce the risk of cancer in humans (Boffetta et al. 1997; Boström et al. 2002). Environmental and occupational exposures to aromatic hydrocarbons have been the suspected sources of cancer among people who work as painters, employees in filling stations, repair cars and in general work with organic solvents (Lynge 1997; Lindbohm et al. 2009).

MATERIALS AND METHODS

Treatments, preparation and screening of the wings

Eggs were collected in the *w*/*mwh*$^{+}$*Y*; *mwh* strain for eight hours in small glass Petri dishes filled with a layer of 5-6 mm standard *Drosophila* corn meal medium with drops of live yeast on the food surface. The hatching larvae developed in this medium at 25°C. For treatment, a glass Petri dish with 84-92-hour-old 3rd instar larvae in the medium was transferred into the center of the slightly arched bottom of a 0.5 liter IKEA SLOM glass jar (Fig. 4; article number 400.658.67). During the experiments the jar was sealed air-tight with a silicon rubber band and a wire clamp.

Figure 4. The glass jar with a Petri-dish filled with standard Drosophila food.

There are about 5000-6000 cells in a wing blade primordium in the 84-92-hour-old larvae. The remaining 28-36 hours till pupariation (at 120 hours) allow for about three rounds of mitoses (Bryant and Levinson 1985). Concerning the treatment of 84-92-hour-old larvae, the largest *mwh* clones are thus expected to be composed from four *mwh* homozygous cells.

As control, 84-92-hour-old larvae on standard *Drosophila* medium in Petri dishes were placed into a jar and kept under air-tight conditions for 2, 6, 12 and 24 hours. (Since similar frequencies of *mwh* clones were observed for the different time periods in the control wings, the data were pooled and appear as "Control" in Table 1 on page 11). To perform a treatment, a Petri dish was placed onto the bottom of a jar. Benzene (3, 7, 27, 80 or 100 µl) was pipetted into the bottom of the 500 ml jar and evaporated within minutes. (Note that partial pressure of 100 µl evaporating benzene in 500 ml is 41 mmHg, about 2.5 times less than the saturation level.) To expose the larvae to the vapors of the liquid parquet varnish or the thinner, a Petri dish with 84-92-hour-old larvae on the medium was placed into a jar. Ten ml liquid parquet varnish from a freshly opened tin can or ten ml synthetic thinner was pipetted onto the bottom of the jar, which was then sealed instantaneously. The larvae remained in the air-tight closed jar for well-defined periods of time, as listed in Table 1. Following the treatments, the Petri dishes - with the medium and the larvae inside - were removed from the jar and transferred into a plastic cup as shown in Fig. 5. The surviving larvae completed their development, wings of the eclosing *w*/*mwh*$^{+}$*Y*; *mwh*/*mwh* adult males were removed, mounted and screened for the presence of *mwh* clones in a compound microscope at 400x (Figs 2 and 6). The number and the size of the *mwh* clones were recorded.

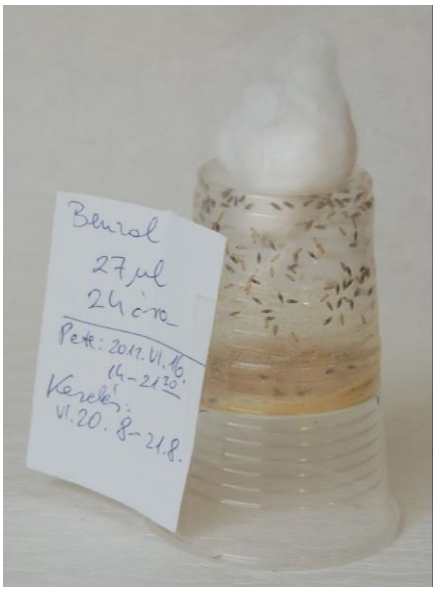

Figure 5. The Petri-dishes with the food and the treated larvae inside were fixed in an upside-down standing plastic cup where they completed development. A hole was drilled on the bottom of the cup and closed with cotton stopper.

Figure 6. The wings were mounted in Faure's water-based medium. In the permanent preparations wings of the same male were arranged in pairs and take identical positions in e.g. rows one and two.

Toxicity of the benzene, vapors of the liquid parquet varnish and the thinner were assessed by exposing samples of hundred 84-92-hour-old larvae (kept in 5-cm diameter glass Petri dishes filled with standard *Drosophila* corn meal medium) to vapors of the above agents for hours as listed in Table I. Percentage of the larvae that developed to adulthood was determined.

The alkyd-resin-based, polyurethane-reinforced single component parquet varnish was a product of Akzo Nobel Coatings Co. Tiszaujvaros, Hungary (Fig. 7). (Product number: MONOLAKK 163091.) Its main solvent is white spirit (25-50%). The H-100 synthetic thinner, another product of the Akzo Nobel Coatings Co., is recommended for setting the consistency of e.g. the above alkyd-resin-based parquet varnish (Fig. 7). It is a mixture of white spirit (50-94%), *n*-butyl alcohol (6-10%) and contains slight amounts of aromatic hydrocarbons. (Product number: 166126.)

Figure 7. The parquet varnish and the The H-100 synthetic thinner were products of the Akzo Nobel Coatings Company.

RESULTS

Toxicity

As reported earlier (e.g. by Singh et al., 2010) and is confirmed here, even short exposures of the *Drosophila* larvae to low concentrations of benzene vapor is rather toxic (Table 1). Exposure to vapors of the liquid parquet varnish for one day significantly reduced the survival of the *Drosophila* larvae to adulthood. However, larvae tolerated the shorter than twelve-hour exposures remarkably well. Apparently, a six-hour exposure of the *Drosophila* larvae to vapors of the synthetic thinner did not reduce the viability of the larvae (Table 1).

Table 1. Features of *mwh* mosaicism

Treatment			Survival to adulthood[1]	Wing	*mwh* clone	*mwh* clone frequency	Size class[2] and the number of *mwh* cells per clone					Frequency of clones with ≥ 2 *mwh* cells	Average clone size[3] (*mwh* cell per clone)	Frequency of clone induction
							I. 1	II. 2	III. 3-4	IV. 5-8	V. 9-16			
				N	*n*	*n/N*							*m*	*f = n 2m/NC*
Control in Szabad et al., 2012			-	108	164	1.5	96 (0.9)	39	22	5	2	0.6	1.70±0.97	$1.7x10^{-4}$
Control			78.2±1.8 (5)	64	259	4.0	216 (3.4)	33	7	2	1	0.67	1.17±0.78	$3.2x10^{-4}$
Benzene (µg/ml)	0.005	24 hours	77.0±1.2 (3)	40	188	4.7	163 (4.1)	18	6	1	0	0.63	1.14±0.74	$3.6x10^{-4}$
	0.016		68.1±1.6** (3)	30	173	5.8	147 (4.9)	21	4	1	0	0.83	1.14±0.71	$4.4x10^{-4}$
	0.047		59.0±2.6** (3)	30	218	7.3*	179 (5.9*)	31	5	2	1	1.30*	1.18±0.79	$5.7x10^{-4}$
	0.140		26.0±2.0** (3)	12	107	8.9*	84 (7.0*)	15	5	3	0	1.92*	1.24±0.85	$7.4x10^{-4}$
	0.175		9.4±3.6** (5)	18	227	12.6**	180 (10.0**)	33	12	2	0	2.61**	1.22±0.81	$10.3x10^{-4}$
	0.175	1 hour	66.5±2.8** (3)	38	176	4.6	145 (3.8)	23	7	1	0	0.82	1.18±0.76	$3.6x10^{-4}$
		2 hours	46.7±4.7** (3)	12	80	6.7	65 (5.4)	10	4	1	0	1.25	1.20±0.79	$5.3x10^{-4}$
		4 hours	19.2±2.8** (3)	20	236	11.8**	195 (9.8**)	31	9	1	0	2.05**	1.17±0.76	$9.2x10^{-4}$
Parquet varnish (Supralux)		2 hours	-	36	189	5.3	166 (4.6)	17	5	0	1	0.64	1.13±0.74	$4.0x10^{-4}$
		6 hours	78.5±2.0 (3)	40	188	4.7	160 (4.0)	24	4	0	0	0.70	1.14±0.72	$3.9x10^{-4}$
		12 hours	76.3±2.1 (3)	38	226	5.9	193 (5.1)	25	6	2	0	0.87	1.15±0.75	$4.6x10^{-4}$
		24 hours	68.0±5.3** (4)	28	216	7.7*	176 (6.3*)	28	9	2	1	1.43*	1.22±0.81	$6.3x10^{-4}$
Thinner (H-100)		1 hour	-	38	180	4.7	157 (4.1)	16	3	3	1	0.61	1.15±0.80	$3.6x10^{-4}$
		2 hours	-	40	202	5.1	177 (4.4)	17	4	3	1	0.63	1.15±0.79	$3.9x10^{-4}$
		4 hours	-	44	310	7.0*	248 (5.6*)	51	10	1	0	1.41*	1.19±0.76	$5.9x10^{-4}$
		8 hours	79.0±3.9 (4)	12	107	8.9*	87 (7.3*)	15	4	1	0	1.67*	1.19±0.78	$7.1x10^{-4}$

Notes
- Wings of *w*/*mwh*$^{+}$*Y*; *mwh*/*mwh* males were screened for *mwh* mosaic spots (clones).
- * and ** represent values significantly different from the control at P<0.05 and P<0.01, respectively; χ^2 test for the clone frequencies and t-probe for the survival rates.
- The saturation concentration for benzene is 0.44 µg/ml as calculated on the basis of its 95.1 mm Hg partial pressure at 25^{o}C.

[1] The number of adults (average ± standard deviation) that developed from 100 larvae in the *w*/*mwh*$^{+}$*Y*; *mwh* strain. (In brackets: the number of repeats.)

[2] The minimum number of cell divisions (I-V) required - following the loss of the *mwh*$^{+}$*Y* chromosome - for the formation of *mwh* clones composed from 1, 2, 3-4, 5-8 or 9-16 *mwh* cells. It is assumed that only one of the daughter cells becomes *mwh*-labeled following the loss of the *mwh*$^{+}$*Y* chromosome during mitosis. (In brackets in size class I: frequency of the single *mwh* cell clones.)

[3] The average clone size was calculated from the average size class by making use of the linear relationship between the number of size classes (I-V) and the log average clone size within the size classes: y = 0.27x - 0.26 where x and y stand for the size class and the log average clone size, respectively.

The air-tight condition raises the frequency of single cell *mwh* clones

Unexpectedly, single *mwh* cell clones appeared with a much higher frequency in the control than published earlier (Szabad et al. 2012): while 216 such clones emerged in 64 wings (3.4) in the present control only 96 in 108 wings (0.9) were reported earlier ($P<0.01$, χ^2 test; Table 1). The excess of the single *mwh* clones is most likely related to the air-tight condition and not to changes in the *w*/*mwh*$^+$*Y*; *mwh* strain since a recent analysis revealed 20 such clones in 20 wings of the *w*/*mwh*$^+$*Y*; *mwh*/*mwh* males that developed under normal condition (Soós I., unpublished).

Apparently, a time period as short as one or two hours under air-tight condition was sufficient to elevate the frequency of the single cell *mwh* clones: following the exposure of larvae for one or two hours to vapors of any of the three tested agents, such clones emerged with similar frequencies as the 216/64 control value ($P>0.05$; Table 1). It is very unlikely that the single *mwh* cell clones formed due to phenocopy of the *mwh* mutation and/or abnormal trichome formation since no such clones appeared on 40 wings of the *w*/Y; *mwh*$^+$/*mwh*$^+$ and 42 wings of the *w*/Y; *mwh*/*TM2* males. (*TM2* is the symbol of a balancer chromosome that carries the *mwh*$^+$ gene and prevents mitotic recombination; Surján et al. 1985. Such events can happen in the normal *mwh*/*mwh*$^+$ cells and lead to the formation of *mwh* mosaic spots.) Whether some of the air-tight condition-related single *mwh* cell clones developed as the consequence of a reduced expression of the *mwh*$^+$ transgene in the *mwh*$^+$*Y* chromosome or loss of the *mwh*$^+$*Y* chromosome due to reduced oxygen and/or increased CO_2 concentrations awaits further analyses. It is also possible that approaching metamorphosis, cells in the matured wing primordia are not as alert to maintaining chromosomes as during their proliferative period. Although chromosome(s) can be lost, perdurance of the gene products may well support the life of the already non-dividing aneuploid cells (Garcia-Bellido and Merriam, 1971). The fact that cells in the mature wing disc may indeed be less sensitive to chromosome loss as compared to those that proliferate intensively in the growing primordia is indicated by the following observation: frequencies of the clones with ≥2 *mwh* cells were not different in (i) the formerly published and the present control (68/108 versus 43/64; $P<0.05$; Table 1) and (ii) the controls and those treatments that did not increase significantly the frequencies of the *mwh* clones. It is remarkable that whichever treatment brought about a significant elevation in the *mwh* clone frequency it also significantly elevated the frequencies of both the single and the ≥2 *mwh* cells per clones (Table 1). Apparently, in such cases the single *mwh* cell clones originated as a result of both the air-tight condition and the vapors of the benzene, the parquet varnish, or the thinner.

Benzene effectively induces loss of the *mwh*$^{+}$*Y* chromosome

Although the frequency of *mwh* clones slightly exceeded the control level following exposure to 0.005 or 0.016 μg/ml benzene for one day, the frequencies - as compared to the control - were not significantly different (P>0.05; Table I). However, a one day exposure to 0.047 or to 0.140 μg/ml benzene vapor significantly elevated the frequency of *mwh* clones confirming the ability of benzene to induce chromosome loss (Table 1). As expected, the exposure of the larvae to 0.175 μg/ml benzene for one day not only strongly reduced their survival to adulthood but also induced a rather high frequency of *mwh* clones (P<0.01; Table 1). Under such conditions, the frequency of clone induction, i.e. loss of the *mwh*$^{+}$*Y* chromosome, exceeded the $3.6x10^{-4}$ control level almost three times (Table 1). Exposure of the larvae to 0.175 μg/ml benzene for one or two hours significantly reduced their survival rate to adulthood and the frequencies of the *mwh* clones increased slightly, though the differences were not significant when compared to the control (Table 1). However, a four-hour exposure of the larvae to 0.175 μg/ml benzene vapor significantly increased the frequency of *mwh* clones (P<0.01; Table 1).

There appears to be a linear relationship between the toxicity of benzene and its ability to induce loss of the *mwh*$^{+}$*Y* chromosome (Fig. 8). This observation suggests that the toxic effect of benzene may well be related to its ability to induce chromosome loss. (It has been reported earlier that benzene does not induce chromosome breaks and point mutations [Nylander et al., 1978; Kale and Baum, 1983].) This assumption is supported by the observation that most of the larvae exposed to benzene die following pupariation (Soós I. unpublished) suggesting that benzene disturbs the function of the mitotically active imaginal discs and neuroblast cells and has little if any effect on the endoreplicating larval cells (Yan and Li, 2011).

Results related to benzene clearly confirm the former observations: benzene does induce chromosome loss and the formation of aneuploid cells. To determine whether benzene induces chromosome loss through disturbing the spindle assembly checkpoint and/or the mitotic machinery remains to be elucidated.

The present study made use of *Drosophila* to detect chromosome loss and lead to the same conclusion as the former benzene-related studies suggesting that the CLADS technique may be an appropriate tool to study other environmental and genetic agents for their abilities to induce chromosome loss.

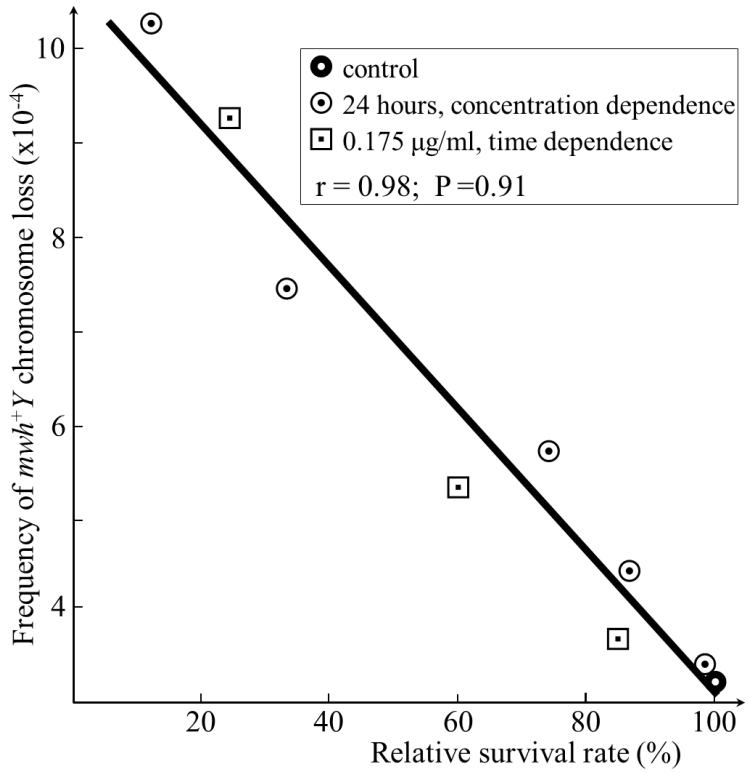

Figure 8. The correlation between toxicity of benzene - measured as the relative survival rate of the larvae to adulthood - and its ability to induce loss of the *mwh*$^{+}$*Y* chromosome *in vivo* in Drosophila wing primordial cells.

Liquid parquet varnish and synthetic thinner can induce chromosome loss

The parquet varnish has been used extensively to build resistant coating layers on wooden floors. It is dissolved in a thinner and stored in tin cans before use. Following spreading on the floor it solidifies through oxidization while the solvent evaporates. It's thinner contains - in addition to white spirit and *n*-butyl alcohol - aromatic hydrocarbons that have been known to increase the risk of cancer (Lynge et al. 1997; Boström et al. 2002). We aimed to determine whether vapors of the parquet varnish and the thinner can induce the loss of the *mwh*$^{+}$*Y* chromosome in cells of the Drosophila wing primordia. Although short (2-12 hour) exposures to vapor of the yet liquid parquet varnish elevated the frequencies of the *mwh* mosaic spots, the differences - as compared to the control - were not significant ($P>0.05$; Table 1.) However, following the exposure for one day the frequency of the *mwh* mosaic spots significantly exceeded the control level showing that vapor of the liquid parquet varnish induce chromosome loss ($P<0.01$; Table 1). The frequency of chromosome loss is 6.3×10^{-4}, about twice of the 3.2×10^{-4} control value (Table 1).

Upon exposure for four or eight hours to saturated vapor of the H-100 synthetic thinner the frequency of the *mwh* mosaic spots significantly exceeded the control level and the frequencies

of *mwh*$^{+}$*Y* chromosome loss were 5.9x10^{-4} and 7.1x10^{-4}, respectively (Table I.). Although the effects of the vapors of the parquet varnish and the thinner are rather weak - when it is concerned that (i) the larvae spent several hours in the saturated vapors and that (ii) 1 μg/ml colchicine induced chromosome loss with the frequency of 29.4x10^{-4} (Szabad et al. 2012) - it clearly shows the ability of the vapors to induce chromosome loss. It may be assumed that the vapors can induce chromosome loss not only in cells of the Drosophila wing primordia but also in other cell types and can thus have adverse effects through the induction of aneuploidy. Surprisingly, it is not recommended for either product to use them under open air condition also with appropriate protective mask.

DISCUSSION

Mitotic nondisjunction, chromosome loss, triplo- and haplo-mosaics

During sexual reproduction life of most of the orgianisms begins through the fusion of two haploid (n) germ cells. The forming zygote is diploid (2n) and propagates this ideal condition onto the descending cells throughout the oncoming rounds of mitoses. Such diploid cells perform their normal functions and ensure normal life of the organisms. Abnormal mitoses may lead to gain (during mitotic nondisjunction) and/or loss of usually one chromosome. The forming trisomic (2n+1) or the monosomic (2n-1) conditions disrupt the normal (2n) gene dosage relationship in the aneuploid cells. As mitotic nondisjuntion and/or chromsome loss happens in one of the cells during mitosis, genetic mosaics form, a conditon in which cells with different genetic composition make up the body of an organism (Fig. 9). The aneuploid cells may survive, divide and transmit their unusual genetic composition to the descending cells. Such cells - as mentioned in the Intruduction - can cause a number of different types of complications and are of potential danger for the life of the organisms. The reasons behind the tri- and the monosomy-associated complications are the so-called triplo-abnormal and the haplo-insufficient conditions, respectively.

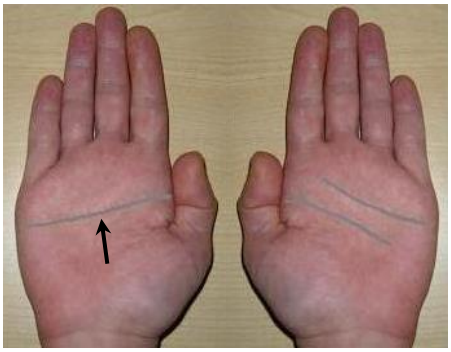

Figure 9. Palms of a Down mosaic. Note the single and straight Simian line (↑) on the palm on the left side. The Simian line pattern is normal on the palm on the right side. The functionally normal gene that resides on the 21st chromosome and brings about a single Simian line has not been identified yet.

Expression of most of the genes is regulated in our cells and concentrations of the encoded gene products are set according to the needs of the cells. However, the expression of some of the genes are not regulated. An extra copy of such genes leads to 50% elevation of the gene product in the cells and this phenomenon is known as the triplo-abnormal condition. For example an extra copy of the *DYRK1A* gene (a member of the dual-specificity tyrosine phosphorylation-regulated kinase family, linked to the 21st chromosome in humans) leads to increased risk of developing acute megakaryoblastic leukemia and acute lymphoblastic leukemia (Birger and Izraeli 2012). Naturally, only if the 21st trisomic cells function in the blood-producing cell populations.

Similarly, cells monosomic for the 22nd chromosome may survive and if they happen to form a mosaic spot in the central nervous system the haplo-22 cells can be the sources of meningiomas (Ruttledge et al. 1994). The corresponding haplo-insufficient gene that resides in the 22nd chromosome is NF2 (neurofibromin 2) that encodes merlin, a protein that was proposed to control cell shape, cell movement and communication and functions as a tumor suppressor protein preventing cells from dividing in an uncontrolled way (McClatchey and Giovannini 2005).

Chromosome/genome stability

Understandably, mechanisms evolved along with the emergence of the eukaryotes that ensure stability of the chromosomes/genomes and avoid the formation of aneuploid cells that pose threats for the organisms. Perhaps surprisingly, the mechansims that ensure chromosome/genome stability accomplish this "goal" not only by taking care of the chromosomes but also be eliminating the aneuploid cells. There are two such mechanisms kown: the spindle assemby checkpoint (SAC) and the mitotic catastrophe (Mussachio 2011; Vitale at al. 2011; Lara-Gonzalez et al. 2012; Foley and Kapoor 2013).

SAC makes sure that chromosome segregation happens correctly during both mitosis and meiosis. It prevents the onset of anaphase until all the metaphase chromsomes are properly attached to the spindle apparatus, i.e. the kinetochores on their sister chromatids are attached to opposite spindle poles (Fig. 10). Such an arrangement ensures that each daughter cell will receive one set of the chromosomes and stay diploid. Failures in the SAC mechanism lead to frequent formation of aneuploid cells. For example, in absence of *BubR1* gene function (a component of the SAC machinery) the spindle assembly checkpoint is eliminated such that metaphase can proceed without control. Failure of the *BubR1* gene function leads thus to frequent formation of aneuploid cells as was reported for a man who developed colon and stomach cancers by the age of 34 (Frio et al. 2010).

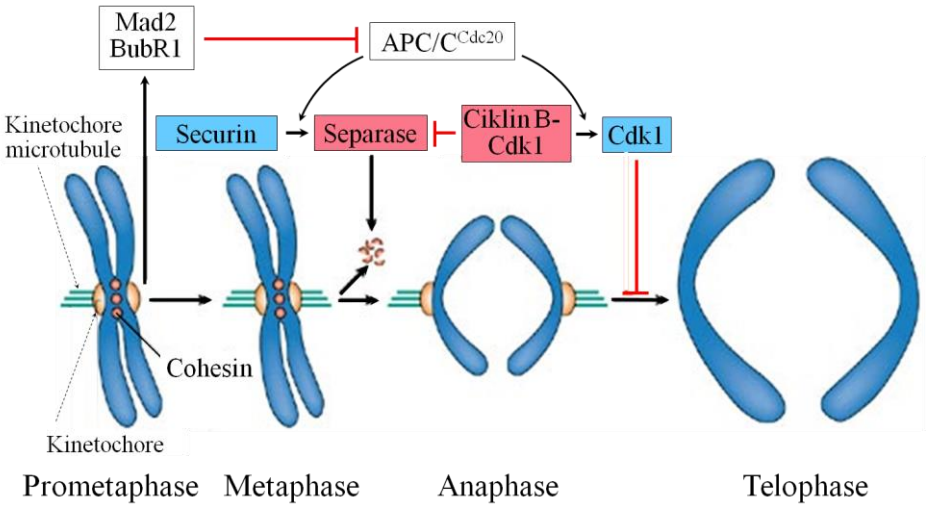

Figure 10. Components and functions of the Spindle Assembly Checkpoint. Prior to metaphase proteins like Mad2 and BubR1 join the kinetochores. These proteins are active in those kinetochores that do not or improperly bind the microtubules. The active Mad2 (mitotic arrest deficient) and BubR1 (budding uninhibited by benzymidazole) proteins inhibit the function of the APC/C^{Cdc20} (anaphase-promoting complex/cyclosome complex) and remain active until all the kinetochore microtubules are attached to one of the centrosomes and all the others to the other centrosome. The Mad2/BubR1 inhibition cease at this stage, the APC/C^{Cdc20} complex becomes active and decompose the Securin and the Cyclin B molecules. Securin is an inhibitor of Separase. Following the decomposition of Securin, the Separase molecules become active and decompose the Cohesion complex. The Cohesin complexes keep the sister chromatids together. Following the decomposition of the Cohesin complexes the sister chromatids are set free and are transported toward the centrosomes as chromosomes. (Source http://mol-biol4masters.masters.grkraj.org/html/Cell_Cycle_And_Its_Regulation.htm6.)

In case the SAC mechanism fails and aneuploid cells form, a second line of defense steps into action, the so-called mitotic catastrophe, a set of events through which the cells with unusual numbers of chromosomes are destroyed (Peters 2002; Takada et al. 2003; Vakifahmetoglu et al. 2008; Vitale et al. 2011). The mitotic catastrophe mechanism also eliminates cells with abnormal DNA and/or chromosome composition. Chk2 (checkpoint kinase 2) is a key component of the mitotic catastrophe mechanism (Fig. 11).

People homozygous for *Chk2* loss-of-function mutant alleles possess symptoms of the Li-Fraumeni (also known as the sarcoma, breast, leukemia and adrenal gland) syndrome (Lee et al. 2001). They are highly susceptible to cancer. Although the Drosophila flies homozygous for loss-of-function *Chk2* mutant alleles are viable, they show defects in maintaining genome stability and are also highly sensitive to ionizing radiation (Xu et al. 2001). Mutations in the Drosophila *lodestar* gene, that appears to be engaged in the same pathway as *Chk2* (Szalontai et al. 2009) have been shown to bring about chromosome instability through the induction of both gain and loss of chromosomes (Szabad et al. 1995; Szabad et al. 2012).

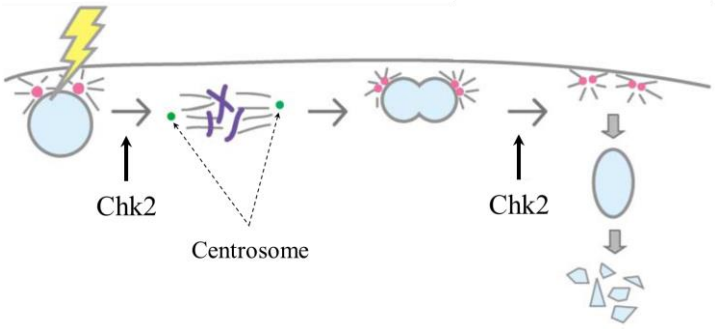

Figure 11. The mechanism of the mitotic catastrophe. Chk2 (checkpoint kinase 2) molecules are released from the nuclei that have unusual DNA/chromosome composition. In the first step, the Chk2 molecules inactivate the centrosomes such that they can not organize a sindle apparatus. In the second step action of the Chk2 molecules leads to the fragmentation of the nuclei and the eventual death of the cell (Peters et al. 2002). (After Takada et al. 2003.)

Components of the SAC and the mitotic catastrophe mechanisms are proteins encoded by genes. Mutations in these genes lead to reduced chromosome/genome stability, to the formation of aneuploid cells which, if survive, can be the sources of different types of troubles. Naturally, both the SAC and the mitotic catastrophe mechanisms have capacities which, when reduced or exhausted allow the formation of aneuploid cells with unusually high frequencies. Disturbed or depleted functions of the above mechanisms as well as that of the spindle apparatus by environmental agents may lead to the formation of excess frequencies of aneuploid cells. This concept motivated the generation of the so-called aneuploidy test procedures including those that make use of the model species *Drosophila melanogaster*.

Aneuploidy screening procedures

Making use of genetically labeled X and Y chromosomes Szabad (1986) developed an assay to detect meiotic nondisjunction and chromosome loss in both the female and male germ line cells of *Drosophila melanogaster*. In the procedure *y* w^{co}*/fs(1)K10 w* females were mated with *y* $w^{co}/B^{s}Y$ males and the offspring was screened for exceptional flies (see footnote 1). It could be determined, based on the phenotype of the exceptional flies, whether they originated as the consequence of nondisjunction and/or chromosome loss. Although the system worked as expected and clearly revealed the effect of the spindle poison colchicine, it was never used routinely. Most likely due to reasons: (i) The number of target cells are limited in the Drosophila female and male germ lines and (ii) the two generation test takes much time and labor.

Footnote 1. Meaning of the genetic symbols are as follows: *y*, yellow body; *w*, white eyes; w^{co}, an allele of *w*, coral-red eyes; *fs(1)K10* dorsalized eggs; B^{s} barr-shaped eyes.

While analyzing *Horka*^{D}, a dominant female-sterile mutation of *Drosophila melanogaster*, it has turned out that *Horka*^{D} induces nondisjunction during spermatogenesis and renders chromosomes unstable such that they may be lost during embryogenesis in the descending zygotes (Erdélyi and Szabad 1989; Szabad et al. 1995; Szalontai et al. 2009). The nondisjunction and/or the chromosome loss inducing ability of *Horka*^{D} was characterized by the following system: *C(2)EN, bw sp* females were mated with *cn bw*/*ed dp cl*; *Horka*^{D}/*TM3, Sb Ser* males (Szabad et al. 1995). Genotype of the different types of germ cells and the possible combinations in the zygotes and their fates and phenotypes are summarized in Table 2. The scheme not only allows the detection of nondisjunction and/or chromosome loss but also to determine the type and stage of abnormal chromosome segregation. The frequencies of the different types of events can be determined based on the number of deposited eggs (Szabad et al. 1995). The proposed procedure is slightly tedious, time consuming and the number of target cells is rather low in the male germ line.

Table 2. Features of the *C(2)EN* system.

Female germ cell genotypes	Male germ cell genotypes							
	Normally		Following reductional (1st meiotic division) nondisjunction		Following equational nondisjunction			Following chromosome loss
	cn bw	*ed dp cl*	*cn bw* *ed dp cl*	0	*cn bw* *cn bw*	*ed dp cl* *ed dp cl*	0	0
C(2)EN, bw sp	†	†	†	*bw sp*	†	†	*bw sp*	*bw sp*
0	†	†	Wild type	†	White eyes	Rough, brown eses, oblique wings	†	†

Meaning of the genetic symbols are as follows: *C(2)EN*, two entire 2nd chromosomes are fused into one; *bw*, brown eyes; *sp*, axils of wings have black specks; *cn*, bright red eyes; eyes of the *cn bw*, homozygotes are white; *ed*, eyes large and rough; *dp*, oblique wings; *cl*, dark dark maroon eyes.

To overcome the problem of the limited number of target cells in the germ line and to include the soma to detect both nondisjunction and chromosome loss, Szabad and Würgler (1987) developed a system that made use of the so-called *zeste-wite* interaction (Lifschytz and Green 1984). While eyes of the *z* w^{+}/*z* w^{+} females are yellow that of the *z w*/w^{+}*Y* males are dark red. Szabad and Würgler (1987) constructed *z w*/w^{+}*Y* males in which the *X* chromosome carried the *z* and the *w* marker mutations. The *Y* chromosome carried the w^{+} segment of an *X*

chromosome. Eyes of the *z w*/w^+Y males are dark red. However, if nondisjunction happens during the course of mitotic proliferation of the eye primordia cells, a *z w*/w^+Y w^+Y and a *z w*/0 cell forms (Fig. 12). Descendants of these cells remain together and form a yellow (*z w*/w^+Y w^+Y) and a white (*z w*/0, colorless) so-called twin spot in the eye in the otherwise red (*z w*/w^+Y) background. Evidently, loss of the w^+Y chromosome results in the formation of single *white* mosaic spot. Although the *z w*/w^+Y system works well, it was not used for screening genetic or environmental agents for their abilities to induce nondisjunction and/or chromosome loss. This is largely due to reasons: (i) Screening for eye mosaic spots is tedious and needs much experience. (ii) Borders of the ommatidia and the eye clones do not coincide and the small eye clones go undetected (Becker 1957). This feature of the eye mosaics imply insensitivity of the *zeste-wite* system: the clones must be induced during early development when the the clones can grow large enough to be noticed. The number of the target cells is rather low at such early stages of development.

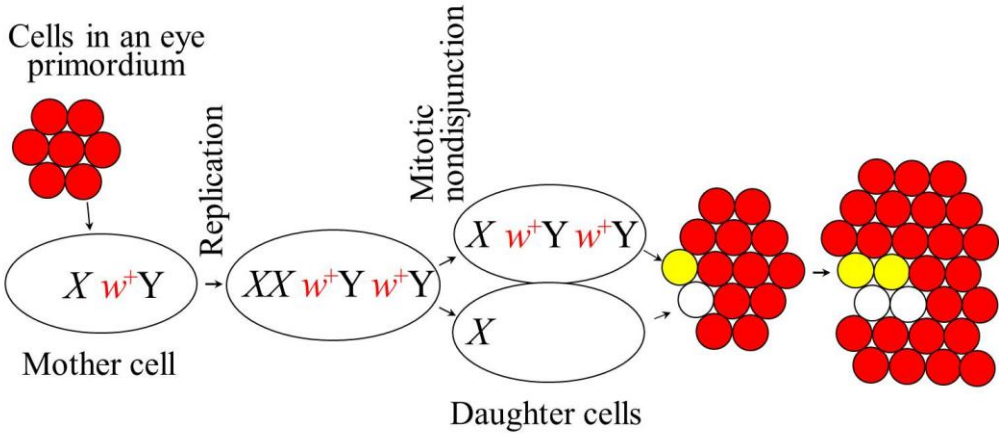

Figure 12. The *zeste-wite* system. The *X*/w^+Y cells are red. (*X* symbolizes an X chromosome labeled with the *z* and the *w* recessive marker mutations.) Following mitotic nondisjunction an *X*/w^+Y w^+Y and an *X0* cell form. (Where 0 stands for the lack of the w^+Y chromosome.) The descending *X*/w^+Y w^+Y and the *X0* cells form a *yellow*/*white* twin spot among the otherwise red (*X*/w^+Y) cells. Loss of the *X*/w^+Y chromosome leads to the formation of a single *white* clone.

The CLADS technique overcomes several of the drawbacks of the other methods that detect the gain- and/or the loss of chromosomes. Values of the CLADS technique can be summarized as follows.

1. The high number of target cells. If 84-92 hours old mid 3rd instar larvae are exposed to the analyzed environmental agent, the number of the target cells are about 5000-6000 in a single developing wing blade primordium. Such larvae forage for 4-12 more hours and thus the

tested agent may have acces - through e.g. the digestive system and the hemolymph - to the wing disc cells. These cells go through about three rounds of mitoses before cessation of cell proliferation and thus cells without the *mwh*$^{+}$*Y* chromosome can proliferate and form *mwh* clones in the developing wing blades.

2. The *mwh* marker mutation allows the detection of single genetically-labeled cells among the about 30,000 wing blade cells.
3. The method detects *in vivo* chromosome loss.
4. Exposing the larvae to the tested environmental agent is convenient.
5. Mounting and screening the wings is relatively simple and the permanent preparations are available for re-examinations. Screening one wing - if contains none or only few clones - takes about two minutes.
6. The CLADS technique offers quantitative evaluation of the strengths of the chromosome loss-inducing abilities of the studied agents.
7. Cells of the Drosophila wing primordia have been used to detect chromosome breaks and/or point mutations (Szabad et al. 1983; Surján et al. 1985; for a review see Deepa et al. 2011). (The method has been known as SMART, somatic mutation and recombination technique.) When e.g. *mwh*/*mwh* females are mated with *flr*/*TM2* males and the descending larvae are treated, some of the chromosome breaks in cells of the *mwh*/*flr* larvae lead to mitotic recombination and the formation of *mwh*/*mwh* and *flr*/*flr* homozygous cells (Fig. 13). Descendants of these cells form an *mwh*//*flr* twin spot in the developing wing and thus the mosaic spots reflect chromosome breaks (Szabad et al. 1983; Fig. 13). (Cells homozygous for the *flr* marker mutation produce multiple short outgrowths which appear as swellings on the wing blade; Fig. 13; Garcia-Bellido and Dapena 1974.) Based on the frequency and size of the mosaic spots strength of the treatment of the tested agent can be determined, similarly as described for the CLADS technique (Szabad et al. 1983).

   Mitotic recombination can not occur in cells of the sibling *mwh*/*TM2* larvae as the *TM2* so-called balancer chromosome prevents it through its four inversions. (For details visit the http://flybase.org/reports/FBab0005473.html website. The *TM2* chromosome carries the *mwh*$^{+}$ normal allele, a site for newly induced *mwh'* mutant alleles. Thus point mutations in the *mwh*$^{+}$ gene in the *mwh*/*TM2* cells will result in the formation of an *mwh*/*mwh'* cell and its descendants will form a single *mwh* clone in the wing blade (Surján et al. 1985). The *mwh* mosaic spots in wings of the *mwh*/*TM2* flies allow thus the determination of the studied treatments to induce point mutations. The combination of the SMART and the CLADS techniques offer thus comparative analysis of genetic and/or environmental agents to induce chromosome breaks, point mutations and abnormal chromosome segregtion.

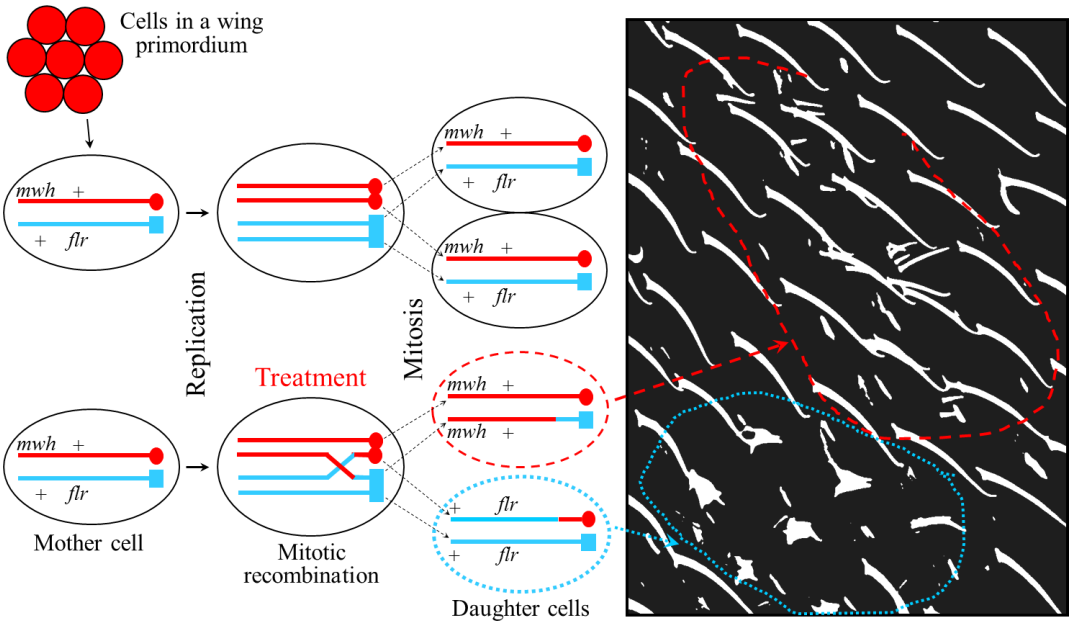

Figure 13. Mitotic recombination as a tool to detect chromosome breaks. Typically, mitoses in the cells trans-hetrozygous for the recessive marker mutations *mwh* and *flr* result in cells that develop a single hair following metamorphosis (Ren et al. 2005). Breakage and unusual reunion of the chromatids may lead to the formation - through mitotic recombination - of two daughter cells, one of which is now *mwh*- the other one is *flr*-labeled. The genetically labeled cells proliferate and their descendants form an *mwh*//*flr* twin spot in the developping wing. Chromosome breaks between the *mwh* and the *flr* marker mutations result in the formation of *mwh* single clones. Double chromosome breaks between *mwh* and *flr* plus the centromere and *flr* lead to the formation of single *flr* clones (Szabad et al. 1983).

Besides the many attractive features of the CLADS technique one apparent weakness is obvious: it detects only chromosome loss and does not sense gain of chromosomes. Perhaps the modern genetic technologies will create a system that will enable the simultaneous detection of the gain- and the loss of chromosomes. The Drosophila *Y* chromosome, the wing primordia, the large number of marker mutations and the advanced transgene technologies may solve the challenging task some day.

ACKNOWLEDGEMENTS

I am thankful to Professor Janos Szabad for his generous and helpful guidance throughout my Ph.D. work. Let me express special thanks to Kissne Ani for her excellent skills in aligning and preparing wings to screen. The project was supported by OTKA NI69180 from the Hungarian

Scientific Research Fund to JSz. I also thank the support of my family and workplace during the Ph.D. work.

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