SAPIENZA
Università di Roma
Facoltà di Scienze Matematiche Fisiche e Naturali

DOTTORATO DI RICERCA
IN GENETICA E BIOLOGIA MOLECOLARE

XXIX Ciclo
(A.A. 2016/2017)

Stress, transposons and genome evolution

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SUMMARY

After Darwin's book on the origin of species by the natural selection, the theory of his precursor Lamarck was never completely abandoned. Over time, the observation of strange natural phenomena has occasionally resurrected the concept of the heredity of acquired characters. To explain, in Darwinian sense, some of the apparent Lamarckian-like phenomena, Waddington elaborated the “canalization and assimilation” concepts (Waddington, 1959). He observed that some phenotypic traits induced in *Drosophila* pupae by heat shock treatment and selected for a number of generations in the presence of the same stress, became heritable, thereby showing that an induced phenotypic trait could be inherited trough the germ line. Waddington hypothesized the existence of a cryptic genetic variation that is maintained hidden due to the robustness of the developmental process that he indicated as “canalization”. If an environmental stress is strong enough to overcome this robustness, the development pathway can change because of the expression of a cryptic genetic variant. Then, this variant can be selected and become heritable by an “assimilation” process. During the last few years, data supporting this view and providing possible molecular explanations were published. Rutheford and Lindquist (1998) showed that, in *Drosophila*, impairment of Hsp90 function induces morphogenetic variants that occasionally became fixed and stably transmitted. The interpretation was that Hsp90 is a capacitor of morphological evolution and buffers a pre-existing genetic variation that is not expressed and accumulates in neutral conditions. The stress sensitive storage and release of genetic variation by Hsp90 would favour adaptive evolution. However, our recent study has suggested a different explanation of these results (Specchia et al., 2010). It has been demonstrated that Hsp90 is involved in repression of transcription and mobilization of transposable elements in germ cells by affecting piRNA biogenesis. The reduction of HSP90 causes stress response-like activation and

transposition of mobile elements along with a wide range of phenotypic variants due to the transposon insertions to the corresponding genes. On the basis of these observations, we have suggested that Hsp90, rather than functioning as a capacitor, acts, when absent, as a mutator, capable of causing activation and transposition of mobile elements through impairment of piRNAi silencing. Consequently, we propose that stress causes the activation of transposons that would induce *de novo* gene mutations, affecting development pathways; mutations can be expressed and fixed across subsequent generations by an assimilation process consisting of a co-selection of a somatic and a germinal event giving the same phenotype. This view implies that transposon activation is a major reaction of genomes to environmental stresses and represents a powerful adaptive response.

INTRODUCTION

"Nothing in biology makes sense except in the light of evolution"(Dobzhansky, 1973)

"Evolution may lay claim to be considered the most central and the most important of the problems of biology."(Huxley, Evolution: The Modern Synthesis)

"E' la teoria biologica più generale e più coerente di cui si disponga: è la sola spiegazione scientifica che possa dar ragione di una grande quantità di fenomeni biologici, quali le successioni delle faune e delle flore nelle grandi ere e periodi geologici, le affinità di struttura più o meno strette fra i vari organismi, cioè l'aspetto attuale dell'insieme dei viventi come risulta dal quadro della classificazione, la distribuzione geografica delle piante e degli animali, l'idoneità all'ambiente in cui vivono, e vari altri ancora. In mancanza di questa teoria, numerosi fenomeni che ora ci sono chiari, almeno in linea di principio, ripiomberebbero nel mistero." (Montalenti, L'Evoluzione)

"MAN HAS ALWAYS been fascinated by the great diversity of organisms which live in the world around him."(Dobzhansky, Genetics and the Origin of Species)

Although evolution has been widely accepted (by the scientific community and not) as historical fact, many of its mechanisms of action are not yet well understood.
For example is not yet clear how natural selection operates on phenotypes, and how, during the history of life, this has led to the remarkable phenotypic variation observed within and among populations. Although the importance of phenotypic variation is well established, in that it provides the raw material for natural selection and enables organisms to adapt to changing or novel

environments (Warner, 2014), the mode of induction of this variability is not yet very clear if not at speculative level.
We know that often in nature the structure of animals or plants is particularly suited to the functions to be performed and to the environment that surrounds them, but for what concerns the processes by which a similar result has been achieved in the course of the evolutionary process is, to date, one of the deepest biological problems.
Two main theories were proposed to explain the mechanisms by which evolution occurs.
According to the hypothesis of the inheritance of acquired characters (Lamarck, 1809), characteristics arisen due to certain physiological performance (as a result of environmental changes) would be somehow transmitted to descendants, making them particularly suited to new environmental conditions (Fig. 1a). Although this theory has been widely discredited after the modern synthesis (Huxley, 1942), it has never been completely abandoned until today (Sano, 2010).
According to the theory of natural selection (Darwin, 1859), instead, changes made by mutations occur completely at random, and adaptation to the environment is nothing more than the result of a process of natural selection (Fig. 1b).

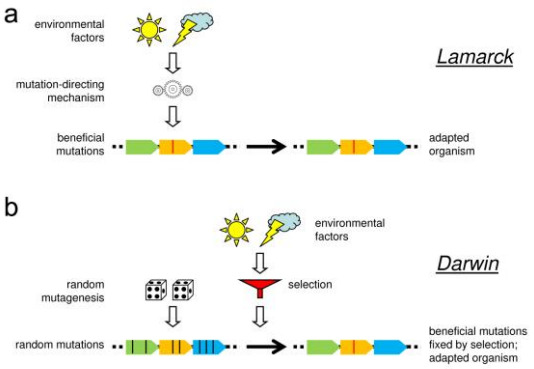

Figure 1. According to Lamarckian theory environment play an active role in adaptation (a); according to Darwinian theory environment plays only a secondary role in adaptation (b) (modified from Koonin, 2009).

The eclipse of Darwinism

This term was used by Julian Huxley (1942) to describe a period between the end of the 19th century and the first decades of the 20th century when Darwinism was under constant attack from evolutionary scientists who did not believe in natural selection as the main driving force of evolution.
Except for Weismann and few others, who believed in selection theory as the only acceptable evolution mechanism, at that time, the majority of biologists were inclined to invoke at least some non-Darwinian processes.
Some alternative theories were derived from the Haeckel's recapitulation theory, also called *"embryological parallelism"*, according to which the course of evolution was modeled on the embryological development of individual organism (*"ontogeny recapitulates phylogeny"*).
The Lamarckian theory of inheritance of acquired characteristics, not denied even by Darwin himself, became to be considered as a real alternative to selectionism from the school of thought known as neo-Lamarckism.
According to the lamarckian-related Orthogenesis theory (Eimer, 1890; 1898), organisms have evolved in non-adaptive mode due to internal forces that predispose to fixed paths.
Biologists were reluctant to recognize that evolution is a random process by "trial and error", and preferred to believe that the development is designed to advance in the improvement and in ordered directions.
On the other hand mutationism theory (De Vries, 1901; 1903) believed that in evolution major changes between forms occur all-at-once by great saltational jumps (through random "not gradual" mutational events) rather than Darwinian gradualism, basically suggesting a form of saltationism. In this case new species arise as a result of a single mutational step causing novel fully functional and adapted phenotypes.

In the early years of the new century, however, Mendelian genetics (in support of Darwin's theory) was able to show that the individual growth was not a suitable model for the evolution, thus threatening the indirect evidence that had been used to sustain Lamarckism and orthogenesis.

Lamarckian (putative) phenomena

Experimental data (all discredited) showed that the heredity of acquired characteristics could be demonstrated.

..the guinea pigs unfortunate hereditary adventure

Figure 2. C.E Brown-Sequard

In 1859 Brown-Sequard (Fig. 2) published a paper in which the inheritance of acquired characteristics was demonstrated. He cut the spinal cord in the dorsal region of guinea pigs, causing abnormal nervous condition resembling epileptic symptoms. Then he allowed animals to breed and observed that a certain number of the offspring was epileptic like the parents (Brown-Sequard, 1859; 1860). This interesting investigation has been promptly accepted by the supporters of the transmission of acquired characters because of its direct evidence of the inheritance of somatic modifications.

In 1890 Romanes unsuccessfully tried to repeat Sequard's results (Romanes, 1895).

..a flounder story

Several years later J.T. Cunningham **(**1891; 1893; 1895**)** tried to demonstrate Lamarckian theory by using fishes.
In his experiments, Cunningham used flounders. In nature these (and others) flat-fishes shows a different coloration pattern before and after metamorphosis. During young stage they are dark-coloured on both sides; at the adult stage, only the upper side (exposed to light) is dark-coloured, while the lower side (usually in contact with the ground and protected from the light) is white.
He put larval flat fishes into aquaria lighted from below in order to obtain fishes equally coloured on both sides. Although he failed to retain the original bilateral pattern, it was found in a fair percentage of cases that after several months of ventral illumination some pigment begins to come back again in the lower side (Fig. 3).

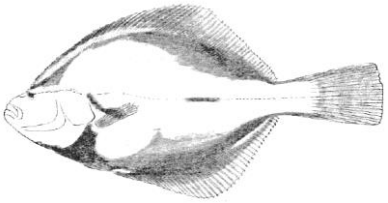

Figure 3. Under side of flounder showing pigmentation (modified from Cunningam, 1895)

The weakness of the data provided by Cunningham was the subject of much criticism by other scientists who did not believe he proved evidence for Lamarckism (Morgan, 1903).

.. the case of the midwife toad

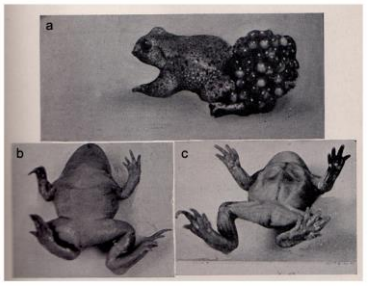

Figure 4. Male, with tightly packed eggs around the things (a); control specimen (b); specimen of the fifth generation bred in water (c) (from Kammerer, 1924)

During the 1920s, Paul Kammerer designed a series of experiments with amphibians trying to demonstrate his Lamarckian theories (Kammerer, 1924). The most famous, although well known in the history of biology as fraudulent, was the case of the midwife toad (*Alytes obstetricans*). Many toads mate in water and have nuptial pads, but midwife toad males (*Alytes*) generally do not. By increasing significantly the temperature, Kammerer induced the *Alytes* toads to move to a tank full of water allowing them to mate there. Midwife toad females deposited their eggs directly in the water, as opposed to on land. As consequence he claimed to have obtained, after some generation, toads with nuptial pads (Fig. 4). Unfortunately the story had a tragic outcome because in 1926 it was published a note in Nature (Noble, 1926) which declared Kammerer's experiments as fraudolent. It was established that the only remaining toad sample examined had no calluses and that the pads black coloration was due to India ink injection. A few weeks later (September 23) P. Kammerer committed suicide, leaving a letter in which he acknowledged that someone had to have manipulated that exemplary, but did not indicate any suspicious person (Przibram, 1926).

..a very debated experiment

Merrifield (1894) and Standfuss (1896), by exposing caterpillars and chrysalises for varying periods to high or low temperatures, produced changes in specific characters of the butterflies which have emerged from them (named "phenocopies" by Goldschmidt in 1935) resembling geographically distant races (Fig. 5). Standfuss (1900) showed also that, after several generations, by continuing the action of cold shock on the caterpillars, the induced variations appeared also in the subsequent generation, even after the cold ceased to act.

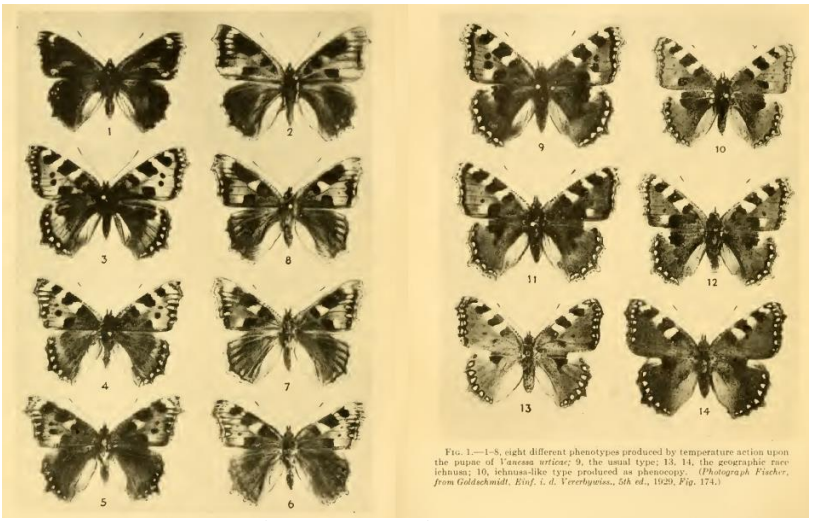

Figure 5. Cold-induced butterflies phenocopies (from Goldschmidt, 1938)

These experiments on stress-induced modifications in butterflies were frequently cited as an example of non-Darwinian heredity. It is a really good example on how an observation could be explained in several ways. In this case, such phenomenon was interpreted in "saltational" sense (Punnett, 1915) as well as in "orthogenetic" sense (Eimer, 1898), but especially in Lamarckian sense as argued by Standfuss himself (1898) and others (Delage and Goldsmith, 1915).

..a way to Darwinism

To explain, in Darwinian sense, some of these apparent Lamarckian phenomena, a series of similar theories were done. According to the organic selection theory (later known as the Baldwin effect), proposed independently in 1896 by the psychologists James Mark Baldwin and C. Lloyd Morgan, individuals subjected to environmental change would choose the most appropriate response, and their bodies would acquire the characteristics suitable for the new environment. These acquired characteristics need not to be inherited, since these species have had sufficient flexibility for short-term adaptations. Eventually, they would produce random variations in a heritable version of the same feature, and the selection would be able to move the species to the path traced by the new character.
Few months later the paleontologist H.F. Osborn proposed a very close theory ("coincident selection") in a more orthogenetic sense. Organic selection, by contrast, denied the inheritance of acquired characteristics and orthogenesis, suggesting that the selection could be stimulated by the positive response of living things to their environment.

The developmental pathways are canalized

Two very closed theories, also similar to Organic Selection, were proposed by Ivan Schmalhausen (1949) and Waddington (1942).

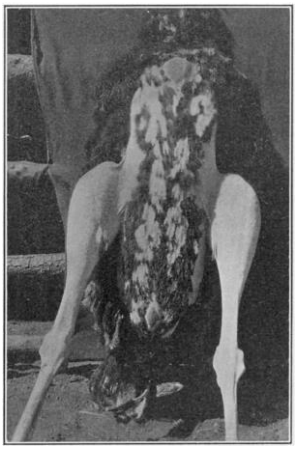

Figure 6. Under surface of ostrich showing the large sternal callosity (from Duerden, 1920).

Schmalhausen and Waddington utilized the example of the callousness of the ostrich (Fig. 6) to emphasize the adaptive potential of genomes. The genome of ostrich has the ability to allow the formation of calluses when the skin is abraded. This "ability to respond" is the essential point. If the presence of calluses is adaptive, then this phenotype may be selected in order to be formed without abrasions already in the embryo. This reaction which led to formation of the callus was transferred from an external stimulus to a genetic one (Gilbert, 2003).

Waddington was able to elaborate the "canalization and assimilation" concepts (Waddington, 1959; Fig. 7) through its experimental evidences made on wild fruit flies.
In his experiments (1942; 1953), Waddington chose an environmental stimulus that produced phenotypes were not usually present in a natural population of *Drosophila melanogaster*.

Figure 7. Waddington epigenetic landscape as a way to represent developmental canalization.

He subjected pupae aged between 21 and 23 hours at a temperature of 40° C for 4 hours, and a certain percentage of these flies displayed abnormal wings in which transverse veins were interrupted or missing (crossveinless phenotype, Fig. 8).

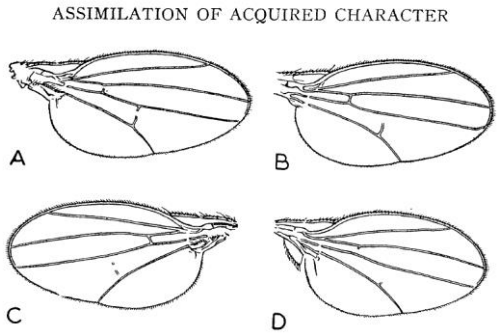

Figure 8. Heat-induced crossveinless phenotypes. (A) grade 4; (B) grade 3; (C) grade 2; (D) grade 1 (adapted from Waddington, 1953).

Waddington established two selective lines, one with flies with interrupted veins and the other with those who had not reacted to the stimulus (Fig. 9).

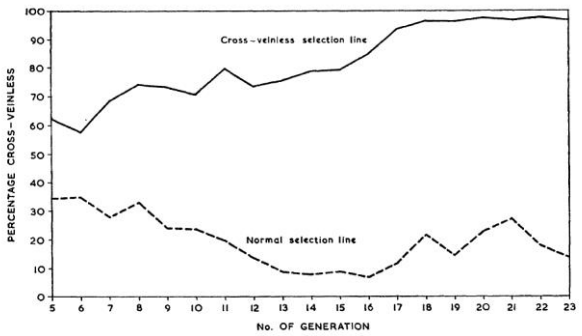

Figure 9. Experiment selection lines. The response to selection, from generation 5 onwards, for crossveinless wings ("upward" selection) and normal wings ("downward" selection) (adapted from Waddington, 1953).

In both these lines the percentage of reactive individuals changed in subsequent generations, increasing in a line and decreasing in the other. After a number of generations he was able to stabilize the crossveinless phenotype, obtaining strains without the horizontal wing vein even when the individuals were bred in normal conditions without being subjected to high temperatures. This alteration, that before appeared only in response to an external agent, was now present in the selected stocks regardless of environmental characteristics.
To explain this really controversial and well known result (Standfuss experiment is only one of the several other examples in that period), Waddington hypothesized the existence of a cryptic genetic variation that is maintained hidden due to the robustness of the developmental process that he indicated as “canalization”. If an environmental stress is strong enough to overcome this robustness, the development pathway can change because of the expression of a cryptic genetic variant. Then, this cryptic variant can be selected and become heritable by an “assimilation” process.

Further experiments using heat stress at different times of the development (Bateman, 1959a; 1959b), or exposure of the embryos to ether vapors (Waddington, 1956) confirmed the concept of "canalization and assimilation" (Fig. 10).

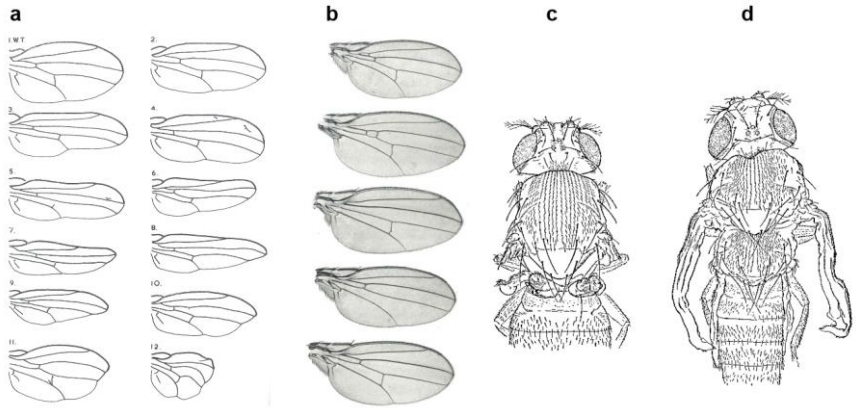

Figure 10. (a) The series grades from wild-type, through narrow-wing to extreme dumpy (modified from Bateman, 1959a); (b) four extravenation phenocopies (adapted from Bateman, 1959b); ether induced bithorax-like phenocopies (c) medium grade; (d) extreme grade (modified from Waddington, 1956).

Waddington suggested that in organisms the developmental processes are canalized in that they have acquired a robustness in respect of environmental perturbations and potential injuries such as mutations through constant selection mechanisms thus maintaining the same phenotype. Given that cells take discrete decisions about their fate, there must be only a finite number of distinct potential trajectories of development, and every path should be stable against small perturbations (Waddington, 1940; 1957; 1962).

A metaphorical representation of the canalization, the epigenetic landscape of Waddington, represents the development system as a landscape of hills and valleys in which a ball rolls down (Fig. 11).

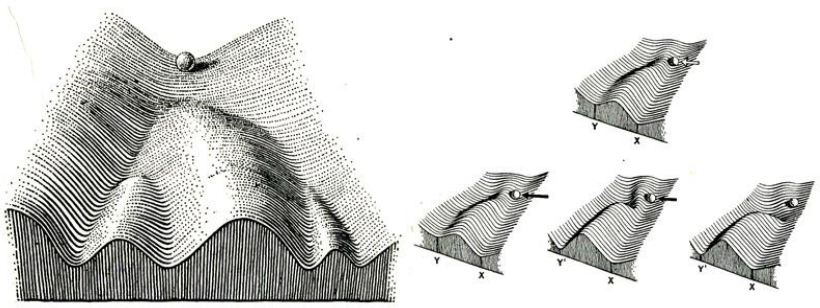

Figure 11. The epigenetic landscape.

The ball represents a cell and the selection of the developmental pathway occurs at each branch point, thanks to the action of embryonic induction factors or genes. Each valley corresponds to a collection of similar developmental trajectories. A small change could lead to different trajectories even within the same valley. This analogy allows us to understand the meaning of the canalization: up to a certain threshold, any genetic variation or environmental noise will be buffered and has no effect on development, but beyond this threshold, the cell could divert to another pathway of development.

The molecular basis of canalization

HSP90 as capacitor for morphological evolution

The molecular mechanism in support of Waddington's theory has remained a mystery until 1998, when Rutherford and Lindquist have proposed a molecular explanation.
They stressed that HSP90 meets requirements to be a key component of developmental canalization and assimilation phenomena (Rutherford and Lindquist, 1998).
HSP90 is a highly abundant protein in eukaryotes, representing approximately 1% of the soluble proteins even in the absence of stress.
Unlike other heat shock proteins, HSP90 possesses a range of more selective substrates, the majority of which are key regulators of growth and development.
This feature allows to speculate that HSP90 could be a good candidate for morphological evolution.
Lindquist and co-workers believed in this hypothesis and showed that in *Drosophila* (1998), as well as in *Arabidopsis* (Queitsch et al., 2002), the genetic or pharmacological inactivation of HSP90 induces the expression of cryptic variability, resulting with the appearance of previously hidden phenotypic variants.
Furthermore, this phenotypic variability can be selected and eventually assimilated into the population (Rutherford and Lindquist, 1998).
In *Drosophila melanogaster* HSP90 is a protein of about 83 kDa (HSP83) coded by a single gene located on the third chromosome called *hsp83*.

The authors have selected, in adult *hsp83* mutant flies, several morphological abnormalities, as an altered abdominal pattern, duplication of bristles, deformed eyes or legs and alterations in the form or in the venation of the wings (Fig. 12).

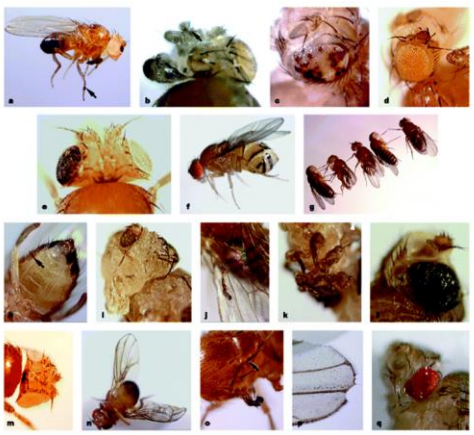

Table 1 Developmental defects associated with Hsp90 deficit

Body part	Code	Description	No. of observations	Temperature (°C)	F_1?	F_2?
Abdomen	A1	Disorganized tergites	14	25	Yes	–
	A2	External trachea?	7	25	No	n.d.
Bristles	B1	Duplications	36	30, 18	Yes	Yes
	B2	Extra scutellar bristles	48	18	Yes	n.d.
	B3	Split scutellars	8	18	Yes	n.d.
	B4	Forked	5	25	No	n.d.
Eyes	E1	Deformed	22	30	Yes	Yes
	E2	Transformed	7	18	No	–
	E3	Smooth	18	18	Yes	Yes
	E4	Rough	16	25	Yes	Yes
	E5	Black facets	24	18	Yes	Yes
	E6	Eyes absent	3	18	Yes	n.d.
Halteres	H1	Ubx transformations	9	25	Yes	Yes
Legs	L1	Deformed	28	18	Yes	Yes
	L2	Transformed	3	18	No	–
Thorax	T1	Disc eversion	12	25	No	–
	T2	Humeral 'balls'	5	26	Yes	n.d.
	T3	Duplication	6	25	No	n.d.
Wings	W1	Small round	26	18	Yes	Yes
	W2	Notched	6	18	Yes	–
	W3	Wing veins	7	18	No	Yes
	W4	Wing border	5	25	No	–
	W5	Transformed	9	18	Yes	n.d.

Developmental abnormalities produced in *Hsp83* mutants, coded according to the part of the fly affected. The approximate number of observations of, and the temperature most frequently producing, each trait are indicated. The observation of at least one cross producing multiple F_1 flies with a given trait is indicated by 'Yes', as is any instance of transmission of the trait to the F_2 generation. A dash indicates not observed; n.d., not done. Many fewer flies were tested at 30°C, so this situation is under-represented.

Figure 12. Developmental abnormalities associated with Hsp90 functional inactivation. (left side) Morphological variants observed among *hsp83* mutant flies; (right side) Table showing detailed description of abnormalities (modified from Rutherford and Lindquist, 1998).

Retracing the experience of Waddington with crossveinless phenotype, the authors observed that the selection for 10 or more generations of a particular adult structural abnormality has led to the assimilation of the new phenotype in the population, even after recovery of HSP83 levels.

According to the authors, HSP90 can be an essential component in "buffering" phenotypic variability that, through the storage and release of genetic variability in dependence from stress conditions, promotes the adaptive evolution.

Figure 13 shows a schematic representation of the hypothesis proposed by Lindquist. Normal levels of HSP90 maintain pre-existing morphological variants in a "hidden" state in the individual genotype (Fig. 13a).
When HSP90 levels decrease beyond a certain threshold, the cryptic variants, which were previously hidden, come out (Fig. 13b).

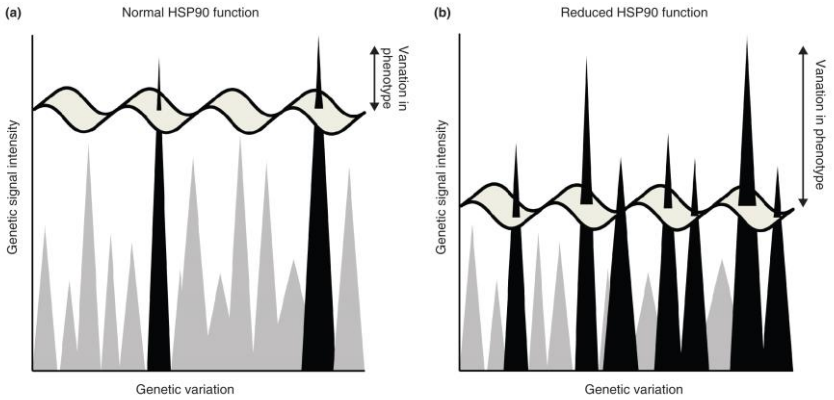

Figure 13. A model for the buffering role of HSP90 in canalization (adapted from Sato and Siomi, 2010).

HSP90 as a mutator

A recent study (Specchia et al., 2010) suggested an additional, perhaps alternative, explanation for a concrete mechanism underlying canalization.
The authors demonstrated that in *Drosophila*, functional inactivation of HSP83, resulted with the appearance of the typical stellate-made crystals in spermatocytes (Tritto et al., 2003), usually absent in wild-type testes (Fig. 14), because of the post-transcriptional silencing mediated by Piwi-interacting RNAs (piRNAs), a class of germ line-specific small RNAs known to maintain repetitive sequences and transposons in a repressed state.

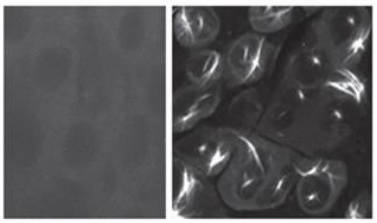

Figure 14. *hsp83* mutations activate Stellate sequences. Testes of Ore-R (on the left) and *hsp83*$^{-}$*/hsp83*$^{-}$ (on the right) males immunostained with anti-Stellate (modified from Specchia et al., 2010).

The impairment of the piRNA biogenesis pathway results in transposable elements de-repression and mobilization.
The authors showed that, in *hsp83* mutant germ line, piRNAs diminution (Fig. 15a) corresponded to a very significantly increase of transposon transcript levels respect to the control (Fig. 15b).
They used Southern blot analysis to look at the effect of *hsp83* mutations on transposon mobility, and observed new bands in the genomic DNA of the F_1 progeny compared to the parental DNA, thus suggesting a mutation-induced mobilization of these elements (Fig. 15c).

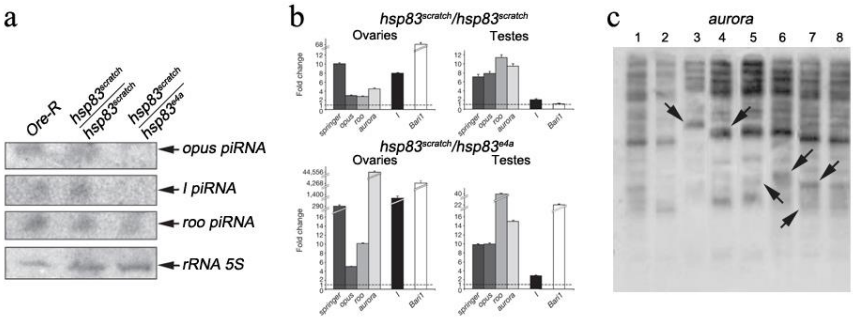

Figure 15. (a) Northern blot analysis shows that for all transposons, the piRNAs are strongly reduced in homozygotes and trans-heterozygotes; (b) qRT PCR shows that *hsp83* mutations activate transposon transcription. (c) Southern blot analysis shows significant differences between parents and F1 progeny (modified from Specchia et al., 2010).

Moreover, a molecular analysis of a phenotypic variant isolated in a *hsp83* mutant strain and resembling the dominant *Scutoid* mutation, showed a I-element transposon insertion in the corresponding *noc* gene (Fig. 16).

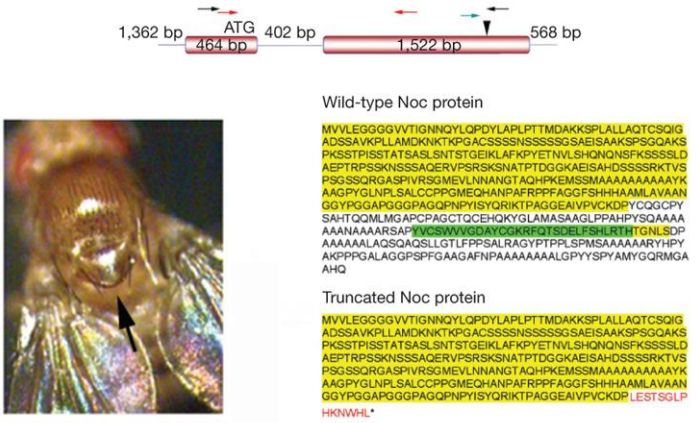

Figure 16. Analysis of the morphological mutant identified among *hsp83*^{scratch} homozygous flies (modified from Specchia et al., 2010).

On the basis of these observations, the authors have suggested that HSP90, rather than functioning as a capacitor, could act, when absent, as a mutator capable of causing activation and transposition of mobile elements through impairment of RNAi silencing (Fig. 17).

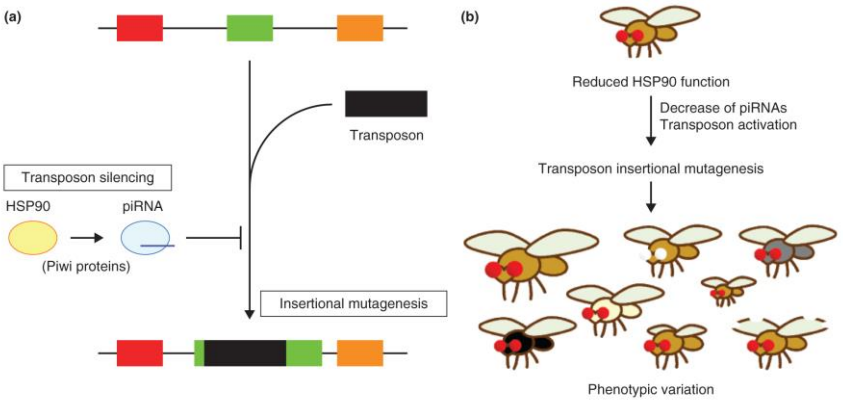

Figure 17. HSP90 functions in the suppression of transposon-mediated 'canonical' mutagenesis via the piRNA pathway (adapted from Sato and Siomi, 2010).

Transposable elements and the impact on genome evolution

Evolution cannot proceed without variation.
After their identification (McClintock, 1950), the idea that transposable elements could have played a major role in genome shaping and evolution took hold only after several dark years during which they had been seen as "junk" and "selfish" DNA pieces (McClintock, 1984; Kazazian, 2004).

"The history of these genomic elements provides one of the best examples of how scientific concepts in biology emerge and then evolve into new concepts." (Biemont, 2010)

With very few exceptions, transposons are present in almost all genomes and persist through independent replication of their sequences.
TEs are distinguished by their mode of propagation in two major classes.
Class I elements (retrotransposons; Fig. 18a) include non-autonomous short interspersed repeat elements (SINEs), autonomous long interspersed repeat elements (LINEs) and retrovirus-like elements with long-terminal repeats (LTRs). These transposable elements are propagated by a "copy and paste" mechanism mediated by reverse transcription of an RNA intermediate (non-autonomous SINE elements require reverse-transcriptase encoded by LINEs to transpose).
By contrast, Class II elements (DNA transposons; Fig. 18b) move through a direct "cut-and-paste" mechanism. The majority have terminal inverted repeats (TIR) and encode a transposase. There are also small non-autonomous DNA transposons, such as MITEs (miniature inverted-repeat transposable elements), which lack the coding potential and need of autonomous DNA to transpose.

Most of the transposon families leave duplications of a characteristic length on target sites at the time of integration in the genome (see Huang et al., 2012 and Lisch, 2013 for reviews).
Another class of transposable elements, Helitrons (Fig. 18c), transpose via a rolling circle mechanism (Kapitonov and Jurka, 2007).

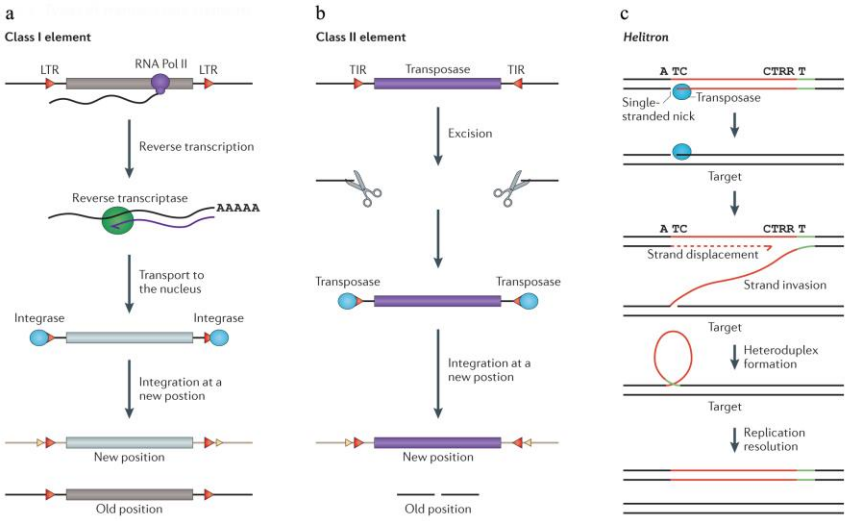

Figure 18. Types of transposable elements (adapted from Lisch, 2013).

TEs themselves are subject to complex regulation and require proper cis-regulatory sequences for that function to mimic host promoters this regulation (Fig. 19).
These regulatory sites as well as RNA polymerase II promoters are present in each transposon class.
These sequences are present in both class I and class II elements: within each of the two LTR domains of LTRs elements, in 5´ untranslated region (UTR) of LINE elements and in within the terminal inversion repeats of class I elements (Mager et al., 2015).
Intact full length SINE elements contains internal sequence motifs that are capable of recruiting Pol III (Richardson et al., 2015).

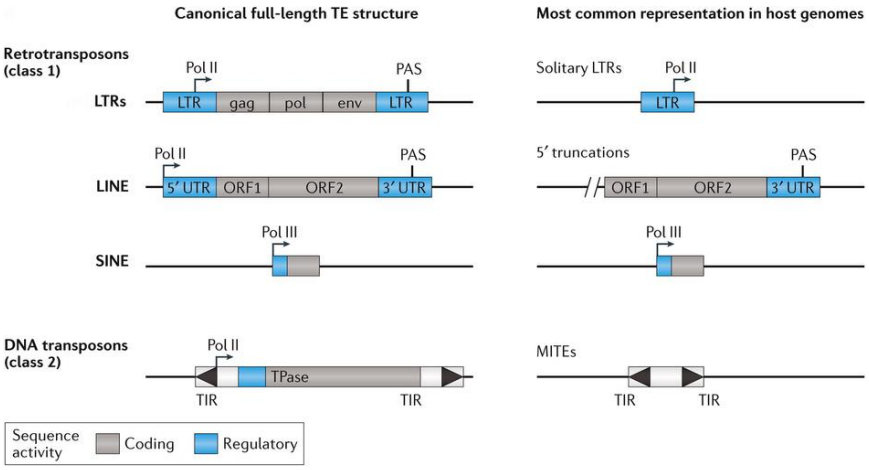

Figure 19. Schematic of major transposable element (TE) classes and their typical genetic organization. The left panel depicts the general full-length version of each TE type. Most TEs harbour regulatory sequences that function to promote their own transcription and regulation, such as promoters for RNA polymerase II (Pol II) or Pol III, and polyadenylation signals (PASs). The right panel shows the structures of each type of TE as they most commonly occur in the genome, which can differ substantially depending on the TE (modified from Chuong et al., 2016).

These sites allow the TEs to maintain a proper regulation in response to stress or developmental cues (Hirsch and Springer, 2016). However, it is possible that these cis-acting regulatory sites would also be able to influence expression of nearby genic promoters as well.

A Transposon insertion can disrupt existing regulatory information (Fig. 20a). When TEs insert within these cis-acting regulatory sequences they can disrupt the transcription factor binding and result in aberrant gene expression.

A TE insertion upstream of the gene could result in increased or ectopic expression in novel tissues or cell types thanks to the novel cis-regulatory site provided (Fig. 20b) that is bound by another transcription factor. Transposons can also influence expression of genes by changing the local chromatin states (Fig. 20c). TEs are generally marked with high levels of heterochromatic marks that may interfere with transcriptional regulation. If these marks are not

confined to the transposon, but can spread to flanking sequences, they may influence epigenetic regulation of adjacent genes.

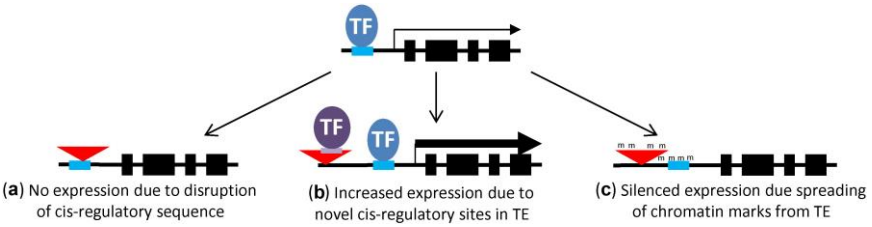

Figure 20. Mechanisms by which TEs can influence expression of plant genes. A typical plant gene is diagramed with a cis-acting regulatory site that provides a binding site (blue square) for a transcription factor (blue circle) and moderate expression (indicated by black arrow). A TE (red triangle) insertion can have different potential influences on the expression of this gene. In (a) the TE inserts within the regulatory sequence and prevents binding of the TF which reduces expression of the gene. In (b) the TE inserts upstream of the gene and contains a novel cis-regulatory site (purple rectangle) that is bound by another transcription factorwhich results in increased expression or expression in novel tissues or cell types. In (c) the TE is subject to chromatin modifications (m) that can spread to cover nearby regulatory sequences or the promoter resulting in reduced expression of the gene (modified from Hirsch and Springer, 2016).

The concept that TEs may act to influence expression of nearby genes fits with McClintock's suggestion that TEs can play important roles in the response of genomes to environmental stress and controlling gene expression (McClintock, 1956; 1963; 1984). This suggestion has been widely demonstrated and, to date, we have several examples regarding the influence of transposable elements on gene variation and genome adaptive responses both in plants and in animals.
For example, among the natural populations, transposable elements can play a very important role in the generation of the flower color polymorphisms (Clegg and Durbin, 2003; Fig. 21a) as well as in maize or in grapevine or oranges (Lisch, 2013; Fig. 21b,c and d).

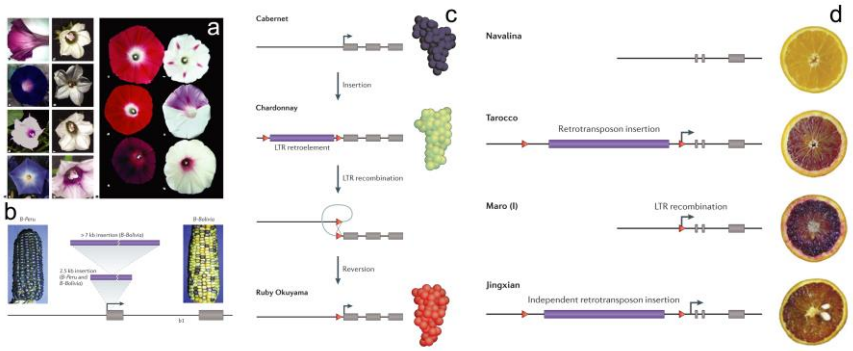

Figure 21. The role of transposons in creating variety (modifed from Clegg and Durbin, 2003; Lisch, 2013).

Many observations were provided also in the animal kingdom. It has been demonstrated the role of transposable elements in regulating gene expression both in invertebrates and vertebrates (Sela et al. 2010; Feschotte 2008; Elbarbary et al., 2016; Shapiro 2016). For example it has been demonstrated the contribution to the evolution of cephalopod neural complexity (Albertin et al., 2015) or in species diversification (Hoffmann et al., 2015). Moreover, in mammals, they have contributed to the evolution of the pregnancy. More precisely, it has been demonstrated that a transposon (MER20), has contributed to the origin of a new regulatory network in the recruitment of signaling pathways of adenosine monophosphate (cAMP) in endometrial stromal cells (Lynch, 2011). Beyond the benefits on the variability of genomes, the random insertion of the TEs may cause (more frequently) deleterious effects on the viability of the cell, going to be inserted into essential genes (Callinan and Batzer, 2006; Hancks and Kazazian, 2012). For this reason transposons are maintained compartmentalized and silenced in specific heterochromatic regions within the genome (Levin and Moran, 2011; Slotkin and Martienssen, 2007) through epigenetic mechanisms mediated by small non-coding RNAs (Fedoroff, 2012).

The extended evolutionary synthesis

Waddington's theory on genetic assimilation, has resulted in the formulation of an extended theory of evolution (***"extended evolutionary synthesis-EES"***).
According to the supporters of this theory (Pigliucci, 2006;2008; West-Eberhard, 2003; 2005), it must also consider other aspects not detailed in the modern synthesis.
More precisely, the modern synthesis is not able to fully clarify the evolution of new phenotypic traits, if not thanks to the addition of the field of evolutionary developmental biology (*evo-devo*) as a driver for it.
According to this theory, the centrality of genes that mediates phenotype variants through mutational events is replaced by the "non-random" phenotypic plasticity's faster response to the environment. In this way, selection will act only on phenotypes without necessarily the implication of a genetic component.
Phenotype developmental alterations, induced by genomic or environmental inputs, are therefore a source of immediate selectable variation. If an alternative phenotype has a fitness effect (reproductive success), selection occurs. If this alteration has a genetic component, selection leads to "genetic accommodation" event.
It has been proposed also an active role of phenotypes in the construction of niches.
For example, many ant and termite species regulate temperature by plugging nest entrances at night or in the cold, by adjusting the height or shape of their mounds to optimize the intake of the sun's rays, or by carrying their brood around their nest to the place with the optimal temperature and humidity for the brood's development (Laland et al., 1999). In this case organisms do not passively adapt to conditions in their environment, but actively construct and modify environmental conditions that may influence other environmental sources of selection (Laland et al., 2016).

Hence, there can be a continuous, reciprocal interplay between phenotype and environment. Altered environments induce phenotypic changes in individuals, and altered individuals may alter their environment in a continuous phenotypic plasticity-environment feedback loop (Whitman and Ananthakrishnan, 2009).
So in this case selection is not the primary force for adaptation but plays a secondary role.
Unlike standard evolutionary biology living things do not evolve to fit into pre-existing environments, but co-construct and co-evolve with their environments, in the process changing the structure of ecosystems.

Aims

Inspired from these really attractive and exciting observations and theories, we decided to repeat Waddington's experiment to clarify which hypothesis best fits with genome environmental adaptations. We think it's important to define a correlation between environment and genome evolution because the scientific thought on canalization is, to date, not uniform.
Waddington's assimilation concept could be explained by the assumption of a possible mutagenic effect of the environment by acting on the stability of transposon's silencing pathway.
The phenomenon of canalization could correlate perfectly with the natural selection theory through the induction of *de novo* mutations rather than the pre-existing cryptic variation.
In our opinion it's important to make clear this point because the debate on this topic is still very heated (Laland et al., 2014) and especially because, although there are many indications regarding the canalization phenomenon (Peuß et al., 2015; Fares, 2015; Horne et al., 2014; Sato et al., 2015; Pievani, 2015; Rohner et al., 2013; Burgess, 2014), experimental evidence on the mechanisms have not yet been provided, nor in favor of cryptic variation, not even for induction of *de novo* mutations.

According to this point of view, Waddington's assimilation concept could be explained as follows (Piacentini et al., 2014; Fig. 22):

stress alters developmental processes and produces phenotypic variants. The induction of transposon activity may generate germ line mutations producing the same phenotypic variants. Selection, under stress, in following generations of a somatically induced phenotypic variant may also allow the co-selection of a corresponding germ line mutation thus making it apparently heritable such as phenotypic variant.

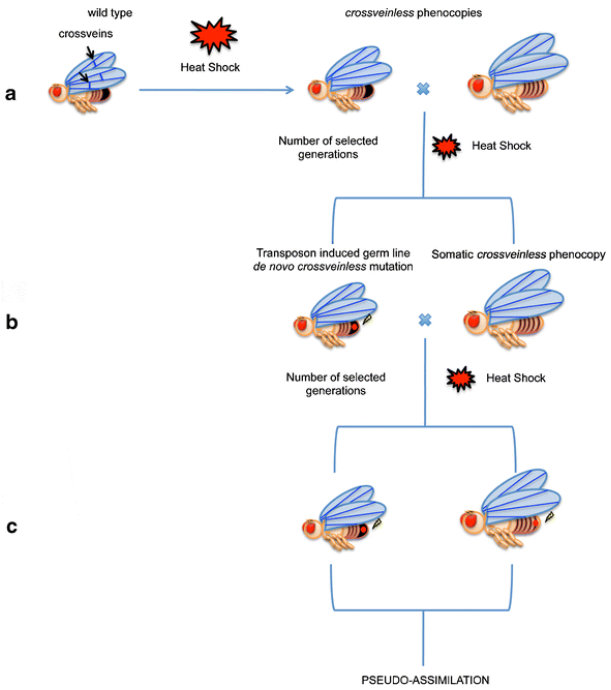

Figure 22. Schematic reinterpretation of Waddington results. (a) Heat-shock induces phenotypic variants; (b) heat-shock could induce transposons activation and mobilization through the impairment of silencing pathway; (c) co-selection of the somatically-induced morphological change with a corresponding germ line mutation eventually make the phenocopy apparently heritable (assimilation) (from Piacentini et al., 2014).

Results

Stressors and transposable elements derepression

To demonstrate our hypothesis, we firstly investigated if environmental stressors were able to derepress transposons through the perturbation of piRNA pathway. We subjected wild type adult fruit flies to the following types of environmental stress in order to evaluate the impact of different stressors on the derepression of several families of transposable elements in *Drosophila* wild type strain. Figure 23 shows qRT-PCR analysis in which the amount of transposon transcript levels in both germ line and somatic tissues is higher respect to the control.

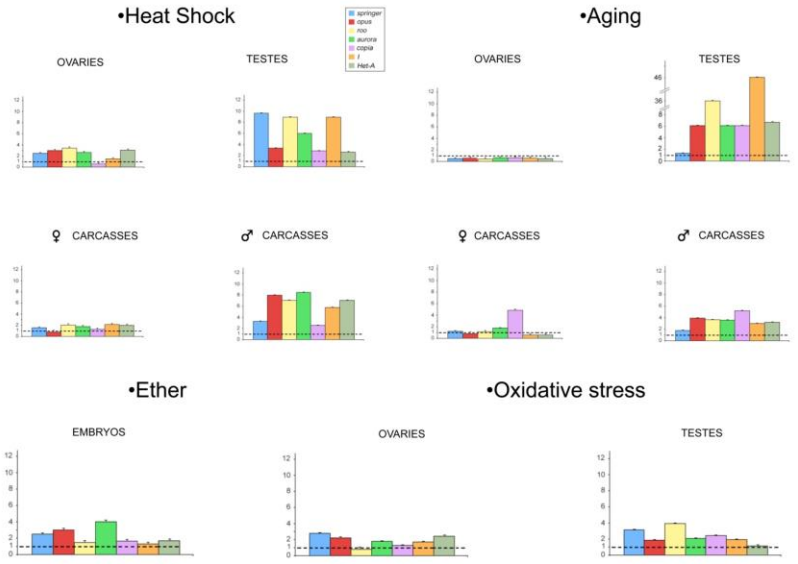

Figure 23. Stressors effect on TEs transcription. Chronic and heavy heat shock; oxidative stress (treatment with 5 mM paraquat); aging; embryos exposure to ether saturated atmosphere. Somatic and germ tissues of treated flies were analyzed by qRT-PCR and the results confirmed a significant increase of various transposable element families.

Also a biotic stress, as Wolbachia infection (Chrostek et al., 2013; 2015) resulted in transposable elements activation in the female germ line and in the appearance morphological variants (Fig. 24), similarly to Waddington experience with heat shock experiment.

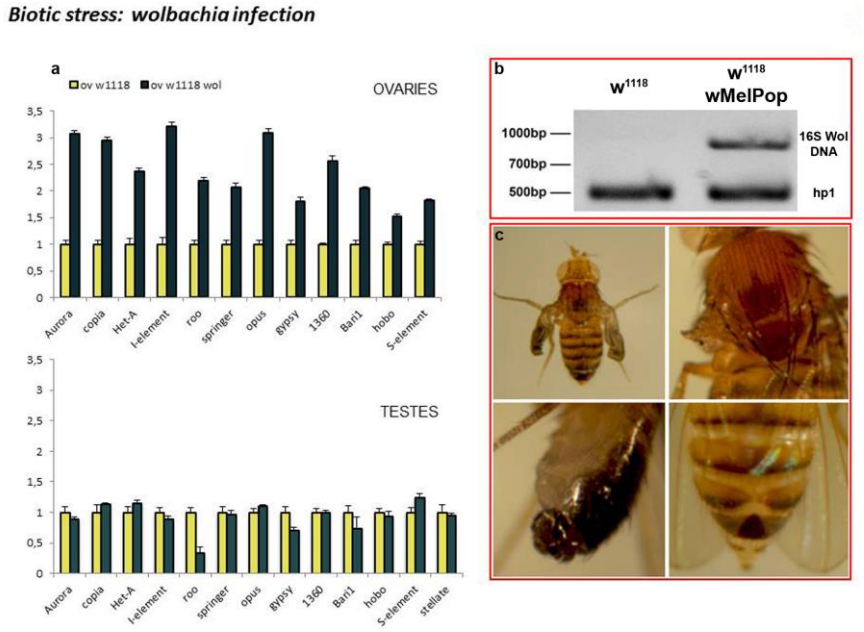

Figure 24. Biotic stress and transposable elements. (a) qRT PCR showing the effect of Wolbachia infection in female germ line; (b) PCR analysis with 16S Wolbachia primers confirms the presence of infection only in the w^{1118} wMelPop line and not in the control line (w^{1118}); (c) morphological abnormalities observed in w^{1118} wMelPop strain.

These results clearly show that stress-induced activity of transposons could provide an important tool by which environmental factors can finely modulate the genome functions. According to our working hypothesis, stress-induced mobilization of TE could generate de novo somatic mutations (that produce non-heritable phenotypic variants) and germline mutation.

Heat-shock transcriptional control on transposons

To test a possible effect of stress on the transcriptional control of transposons, we made ChIP-seq experiments in ovaries extracts from heat treated and untreated females using antibodies against histone H3K9me3 and H3K4me3 which are two specific epigenetic markers for transcriptionally inactive and active chromatin respectively. Bioinformatics analysis demonstrated that the patterns of enrichment of H3K9me3 and H3K4me3 on chromatin of TE genomic sequences of all the families of transposable elements, were unchanged before and after the treatments (Fig. 25).

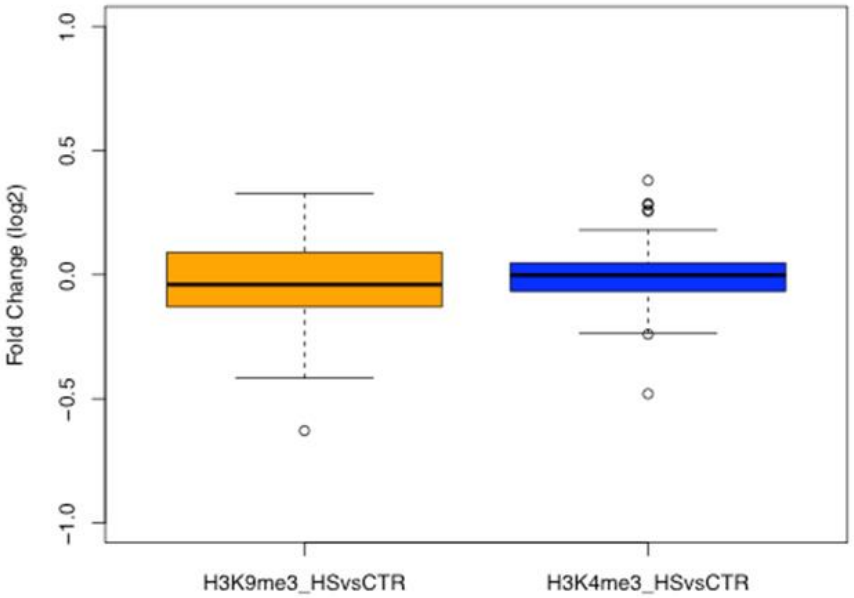

Figure 25. ChIP-seq analysis shows no abundance difference of H3K9me3 and H3K4me3 on transposon sequences before and after stress.

These data strongly suggest that the derepression of transposons after stress should take place mainly at post transcriptional level.

Heat-shock effect on TEs mobilization

In order to investigate if transposons activation results also in their mobilization we performed FISH analysis on salivary glands of F1 3rd stage instar larvae, born from heat shocked wild type males and not shocked wild type females showing new heterozygous insertions in polytene chromosomes as indicated by arrows (Fig. 26).

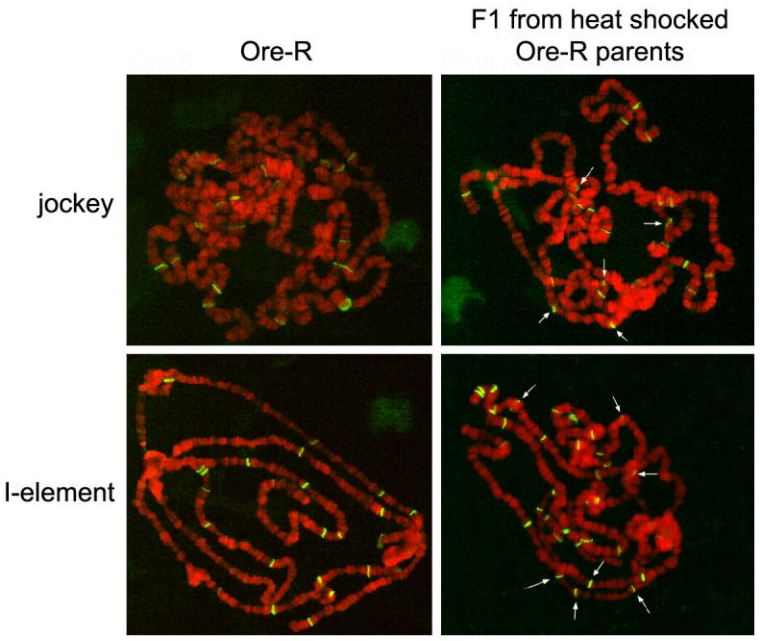

Figure 26. FISH analysis reveal new heterozygous insertions in the offspring polytene chromosomes born from heat-shocked male flies.

The mobilization after heat treatment has been confirmed also by Southern Blot analysis. Figure 27 shows a different distribution pattern of these transposable elements in Ore-R strain and two natural populations (collected by us in different areas of central Italy around the towns of Paliano and Carpineto Romano) shocked at pupal stage for some generations.

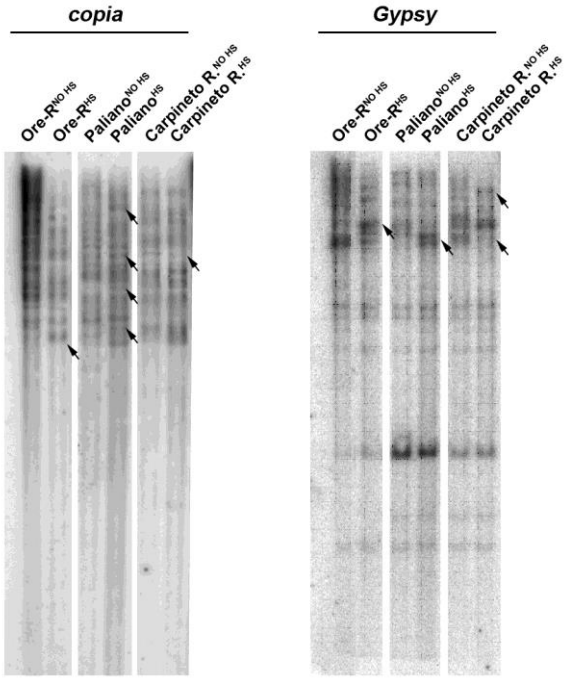

Figure 27. Southern blot analysis shows different hybridization pattern of *copia* and *Gypsy* retroelements between not-shocked and heat-shocked wild type strains as indicated by arrows.

Taken together, these data allowed us to repeat Waddington's experience to possibly demonstrate that assimilated phenocopies were the result of *de novo* induced germ line mutations by transposon insertions.

Waddington re-examinated

For our experiment we used two natural populations (Paliano and Carpineto Romano) to have a wider spectrum of genetic variability compared to lab strains. For each population, we selected 20 non-virgin females that were allowed lay eggs in vials to establish F1 stocks. Then, we selected and crossed 50 virgin females to 50 males from the Paliano F1 offspring, and 47 virgin females to 51 males from Carpineto Romano offspring; after egg deposition, we purified the genomic DNA from each of F1 flies that represented the parental generation in our experiments.
We started Waddington experiment by shocking the F2 progeny from each population to 40°C for 4 hours at the pupal stage. After eclosion, we intercrossed treated flies and heat-shocked their progeny again at the pupal stage.
For both stressed strains, we found different types of not-fixable phenotypic abnormalities (Fig. 28a-e) with a significant frequency (about 25% and 12% per generation in the Paliano and Carpineto R. populations, respectively; Fig. 28f).

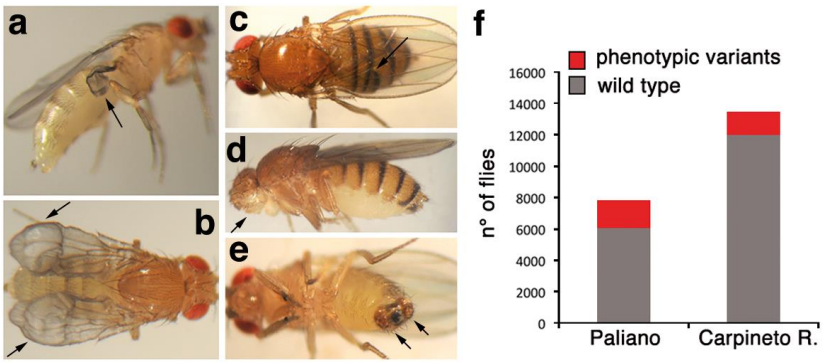

Figure 28. Phenotypic variants induced by heat-shock stress. (a) One haltere transformed to a wing; (b) blistered wings; (c) abnormal tergites; (d) abnormal eye morphology and white color; (e) male with two genital apparatus; (f) diagram showing the total number of examined flies in both the populations and the number of phenotypic variants (red).

The same protocol was repeated for several generations until the emergence of a fair number of flies showing morphological abnormalities resembling different classical mutants. These flies were then selected and crossed to each other in separate vials and their progeny were again treated with heat-shock at the pupal stage until the stabilization of the phenotypic variant even in the absence of stress. Fixed phenotypic variants corresponding to mutations at different loci were determined by complementation tests.
In the Paliano strain we fixed the *sepia* (Fig. 29a) and *forked* (Fig. 29b) phenotypic variants by isolating a corresponding autosomal *sepia* mutation and the X-linked *forked* mutation, which respectively affect eye color and bristles; in the Carpineto Romano strain we isolated the X-linked bristle mutation *singed* (Fig. 29c) and we were able to fix phenotypic variants showing tumors in both larvae and adults by isolating the tumor-inducer *cactus* mutation (Fig. 29d). For all these mutations we made stable stocks.

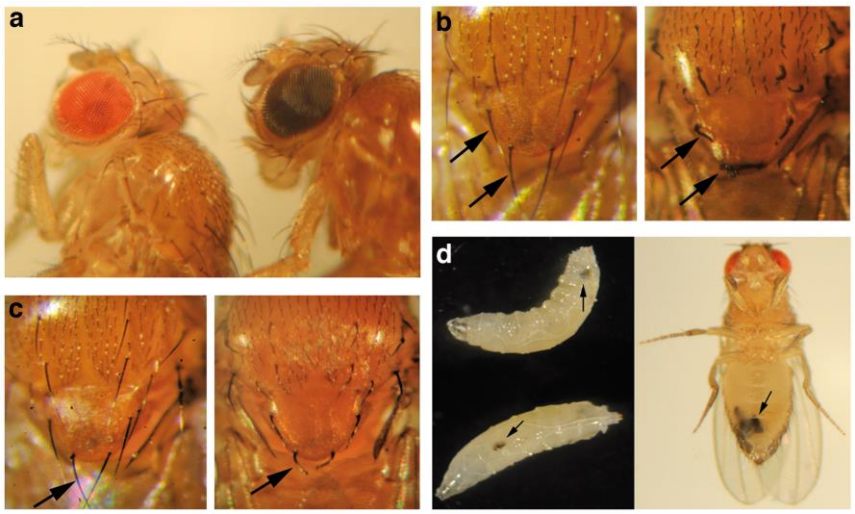

Figure 29. Fixed phenocopies from Paliano (a,b) and Carpineto R. (c,d) natural populations. (a) Eye colour phenotypes of wild-type (left) and *sepia* mutant (right); (b) fly displaying the *forked* phenotype (right) has dorsal bristles shortened, gnarled and bent compared to the wild-type (left) (arrows); (c) abnormal shape of dorsal bristles (right) compared to the wild type (left) in the *singed* mutant (arrows); (d) phenotype of hypomorphic *cactus* fixed mutation in third instar larvae (left) and adult flies (right). Melanotic nodules in the hemocoel are seen as black spots, as indicated by arrows.

During our experiment, we screened also not shocked generations from F1 established stock without observe any morphological variant.
Among the heat-shocked pupae from the Paliano strain, some flies with the *sepia* phenotype and others with a clear *forked* phenotype appeared with variable numbers during the first few generations. We collected males from these variants and crossed them firstly to female flies from classical *sepia* or *forked* mutant strains in separate vials for 5 days, and then with their *sepia* or *forked* female variant sisters to continue with the heat-shock treatments of their pupae progeny. Complementation test resulted with the appearance of all wild type progeny from *sepia* cross and only wild type females from *forked* cross, showing that we were observing true phenocopies.
In addition, in the second cross we also examined the progeny of females showing the *forked* phenotype who were first kept in a vial for 5 days and then, as above described, transferred with *forked* male variants to another vial for heat-shock procedure. If these forked females had been true mutants, we would expect to see several forked males in F1 progeny whether or not these females were virgins, but we observed only wild type males.
We repeated the same test at each generation to verify the fixation of such phenotypes.
At the fourth generation, 12 flies showing the *sepia* phenotype were collected and the pupae of progeny were subjected to heat-shock. After eclosion we found 11 wild type flies and 59 *sepia* flies. We collected the *sepia* flies and again their progeny were shocked at the pupal stage showing an increase in the number of flies showing the *sepia* phenotype (from 84% to 92%) until its fixation at the ninth generation. For what concern the other stress-induced phenocopy, instead, at the sixth generation we observed 6 males showing the *forked* phenotype. Since the *forked* mutation is located on the X chromosome, we hypothesized that such males were derived from a cluster of *forked* mutations which arose in a grandfather's germ line. We directly tested these males by crossing

them with females carrying an attached-X compound chromosome. This cross produces male progeny who receive the X chromosome directly from their fathers. We observed that all the male progeny were in fact *forked*, indicating that the phenotypic variants actually carried a mutation at the corresponding *forked* gene.
Similarly, at the twelfth generation of heat-shock stress in the Carpineto Romano strain, we observed the appearance of 5 males showing the X-linked *singed* phenotype. We again directly crossed these males with females carrying an attached-X compound and observed that all the progeny males were *singed*, thus indicating that the mutant males were generated by a mutation at the *singed* locus. Since we did not observed *singed* phenocopies in previous generations, this case corresponds to a classical mutagenic event rapidly fixed. In addition, in the Carpineto R. strain, we observed from the first generations a variable number of flies showing melanotic tumors in the abdomen. Since melanotic tumors could be caused by mutations at several different genes we could not probe such variants by complementation tests. However, we collected and inter-crossed the variants in a vial for 5 days before transferring them to another vial for the heat-shock procedure. We did not observe any mutant phenotypes in either F1 or F2 non-heat-treated offspring. These results strongly suggested that the variants were just phenocopies induced by heat-shock. At the seventh generation, the frequency of flies with tumors began to increase. We collected these flies and subjected their progeny to heat-shock at the pupal stage. We repeated this procedure for a number of generations and for each generation we observed a gradual and significant increase in the frequency of the tumor phenotype until its fixation at the twelfth generation. Chromosome mapping and complementation tests told us that the phenotype was caused by a hypomorphic mutation at the *cactus* locus, already known to induce such tumors (Sparrow, 1978).

Molecular analysis of assimilated phenocopies

For all these mutations we did a molecular analysis of the corresponding loci by PCR amplification and Sanger sequencing using appropriate overlapping primers. We found that the *sepia* mutation was caused by an extended deletion that includes the first and almost the entire second exon of the *sepia* gene and the entire second exon of the *Gst03* adjacent gene (Fig. 30a,b). Since these two genes share a strong homology and the same orientation along the chromosome 3, this suggests that the deletion was probably generated by a pairing of the *sepia* gene with the *Gst03* gene followed by a heat-shock-induced recombination.

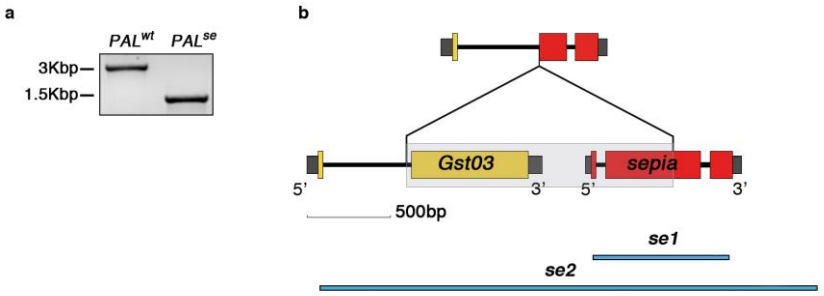

Figure 30. Molecular characterization of heat-induced *sepia* mutation.
(a) PCR analysis of the genomic region spanning *sepia* reveals that the *sepia* allele is caused by a large deletion. (b) The deletion includes the first and almost the entire second exon of the *sepia* gene and the entire second exon of the *Gst03* adjacent gene. Below the schematic representation of *Gst03* and *sepia* genes are indicated the positions of overlapping primers used to completely amplify such genes and to map the deletion. PAL^{wt} = Paliano wild type strain; PAL^{se} = Paliano *sepia* mutant.

This unexpected result is supported in literature by a paper which discusses heat shock as responsible for double-strand brakes (Velichko et al., 2012)
Also *forked* assimilated phenotype was caused by a 4 base pair deletion (in the antepenultimate exon of the gene) which affects the coding of all the 9 different transcripts by the "frameshift mutation" as a consequence (Fig. 31a,b).

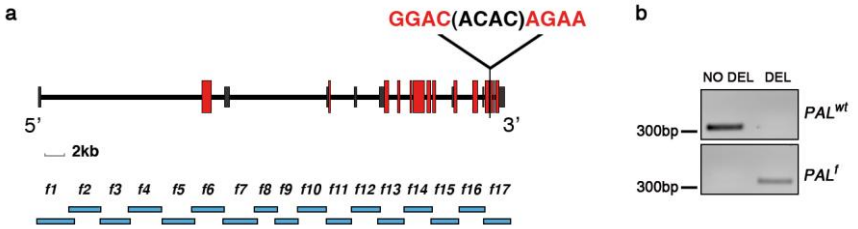

Figure 31. Molecular characterization of heat-induced *forked* mutation.
(a) The *forked* mutation is associated with a four-nucleotide frameshift deletion in the antepenultimate exon of the gene as determined by Sanger sequencing. Below the schematic representation the *forked* gene are indicated the positions of overlapping primers used to completely amplify such gene. (b) The four-nucleotide deletion was validated by PCR analysis using primers specific for the normal *forked* gene that amplify only the wild type DNA and primers spanning the deleted *forked* region that amplify only the *forked* DNA. PAL^{wt} = Paliano wild type strain; PAL^{f} = Paliano *forked* mutant.

The *singed* mutation is correlated with an insertion of the KP transposon (Lee et al., 1996) in correspondence to the 5' UTR of four of the five transcripts (Fig. 32a).
By semiquantitative RT-PCR we demonstrated the lower amount of singed transcripts (affected by the insertion) respect to the wild type control, and the equal aboundance of *sn-RG* transcript that is not affected by the insertion (Fig. 32b,c). Figure 32d shows the lower amount of SINGED protein respect to the control.

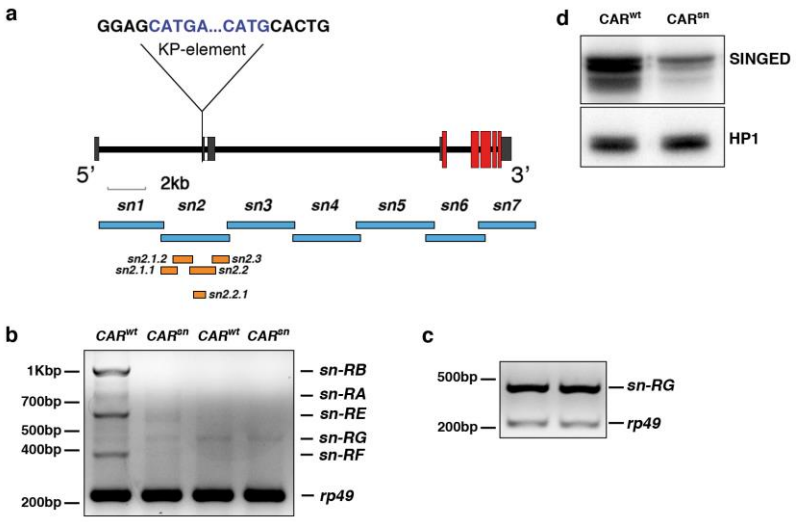

Figure 32. Molecular characterization of heat-induced *singed* mutation.
(a) The *singed* mutation (*CAR*^{sn}) is correlated to a single copy KP-element insertion in the first intron of *sn-RG* transcript; the same genomic position corresponds to the 5'-UTR region of other four singed transcripts, *sn-RA, sn-RB, sn-RE and sn-RF*. Below the schematic representation of *singed* gene are indicated the positions of overlapping primers used to completely amplify such gene. In orange are indicated the primers that have allowed to sequence and identify the KP transposon along with its insertion point. (b) Semiquantitative RT-PCR analysis shows that such four transcripts are significantly affected by the KP-insertion in *singed* mutant larvae respect to the control wild type larvae. The KP-insertion does not affect the *sn-RG* transcript neither in larvae where is poorly expressed, nor in adult heads **(c)** where is highly expressed (*rp49* was used as an internal control). (d) Western blot assay shows an incomplete but significant reduction of Singed protein levels in *CAR*^{sn} larvae compared to the control strain (HP1 was used as internal control). (e) PCR analysis of genomic DNA from a *singed* revertant strain (REV) demonstrates the precise excisions of KP-element. *CAR* = Carpineto wild type strain; *CAR*^{sn} = Carpineto *singed* mutant.

We performed also FISH analysis on polytene chromosomes with specific probes for KP-element and *singed* gene. The results clearly show the insertion of the KP-element in the singed locus (Fig. 33).

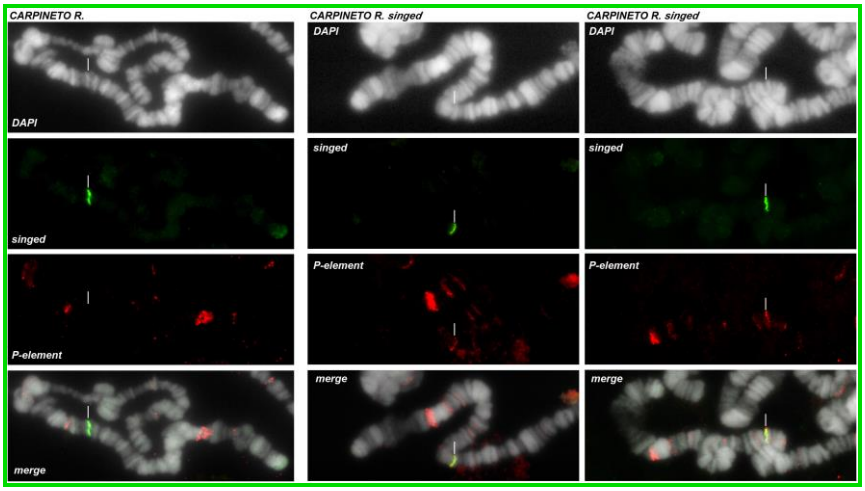

Figure 33. FISH analysis on polytene chromosomes shows the KP-element insertion in correspondence of *singed* locus.

We were also able to obtain revertants by the remobilization of the KP-element using an active transposase demonstrating that the insertion is responsible for the phenotype (Fig. 34).

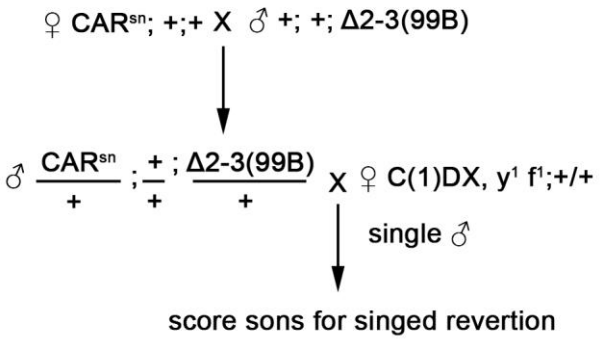

Figure 34. Genetic crosses to obtain *singed* revertants.

The molecular analysis of the genetic revertants clearly shows the precise KP-element excision (Fig. 35).

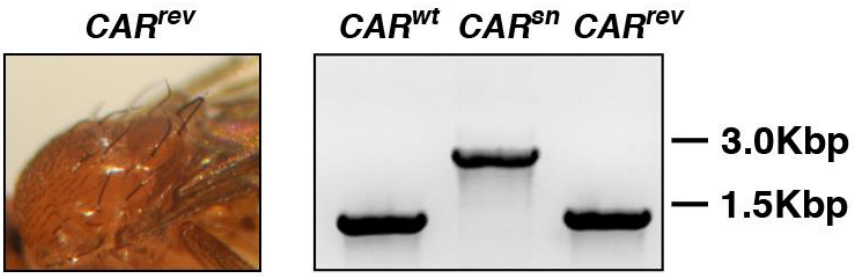

Figure 35. PCR analysis of genomic DNA from a *singed* revertant strain (on the right) demonstrates the precise excisions of KP-element that induces a complete recovery of wild-type phenotype (on the left).

Regarding the *cactus* hypomorphic mutation, we found an insertion of the micropia retrotransposon (Lankenau et al., 1988) into the 3' UTR of the gene (Fig. 36a,b).

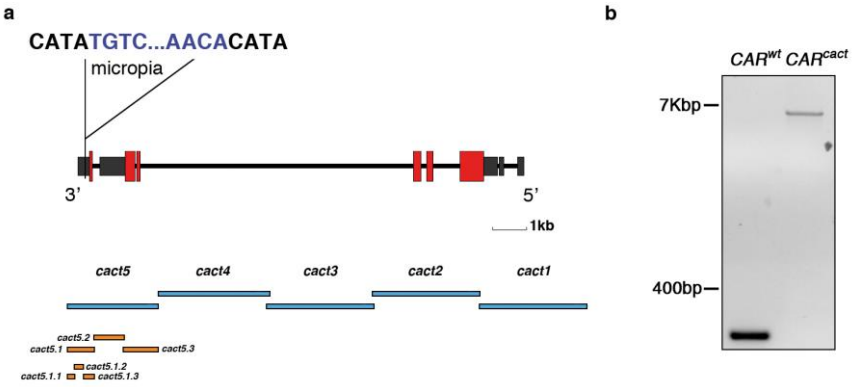

Figure 36. Molecular characterization of heat-induced *cactus* mutation.
(a) The genetic lesion of the *cactus* allele is a full length insertion of micropia retrotransposon into the 3'-UTR of the gene as demonstrated (b) by PCR analysis. Below the schematic representation of *cactus* gene are indicated the positions of overlapping primers used to completely amplify such gene. In orange are indicated the primers that have allowed to sequence and identify the micropia retrotransposon along with its insertion point.

To test whether these mutations were *de novo* induced by the heat-shock stress or were already present in the original population, we realized PCR experiments to analyze all the flies collected from the parental generation. We did not find any genomic lesions at the corresponding genes (Fig. 37), thus excluding the existence of any cryptic variants underlying the fixed mutations.

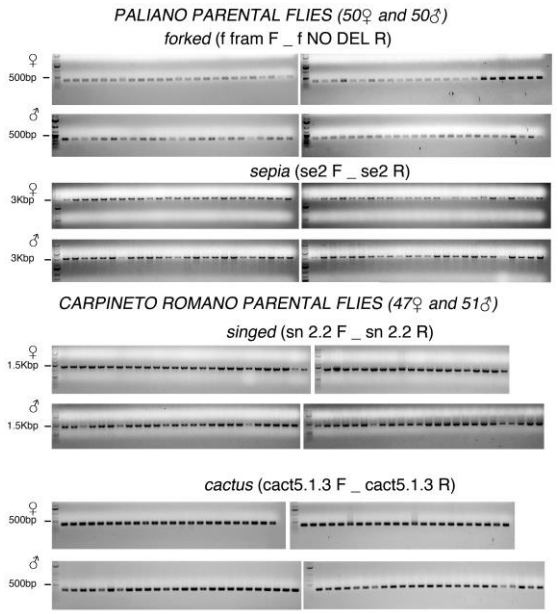

Figure 37. PCR analysis of the genes corresponding to the fixed phenocopies in the wild type Paliano and Carpineto R. parental flies. PCR analysis was performed with genomic DNA purified from each single fly of the Paliano parental generation using specific primers flanking the gene regions altered in *forked* and *sepia* induced mutations. For *forked* gene we used a pair of primers (f fram F_f NO DEL R) that amplify only in absence of the 4 base pairs deletion. The presence of the 400-bp amplicon in all samples confirms the absence of the molecular lesion responsible for the stress-induced *forked* phenotype. For *sepia* we used the same primers that allowed us to identify the *Gst03-sepia* deficiency (se2 F_se2 R). We obtained a 3Kbp amplified product in all parental flies as expected for a wild type genomic sequence. PCR analysis of *singed* and *cactus* genes in each single fly of the Carpineto Romano parental strain was performed using specific primers flanking the KP-element (sn2.2 F_sn2.2 R) and micropia (cact5.1.3 F_cact5.1.3 R) insertion sites, respectively. In both cases, the expected wild type molecular weight of the PCR products (1.5 Kbp for *singed* gene and 400bp for *cactus* gene) allows us to exclude the presence of a pre-existing transposon insertion in the parental generation.

DISCUSSION

These results clearly show that heat-shock stress is capable of inducing DNA deletions and transpositions and strongly suggest that the main mechanism in heat-shock is the production of *de novo* variants through its mutagenic properties. Consequently, the prevalent effect of a temperature stress applied at the pupal stage for a number of generations is to induce morphological alterations that can be fixed by the selection of *de novo* induced corresponding mutations. The number of generations required for the complete fixation of a specific phenocopy depends on the number of genetic factors involved in its determination, their autosomal or sex chromosomal localization, their degree of penetrance and the time of their appearance. In addition, our selection procedures do not exclude the presence of non-virgin females, which could extend the time necessary for fixation. Thus, it is reasonable to expect the spectrum of results that we observed in our experiments.
Finally, in cases such as that described by Waddington, who observed that the assimilation of crossveinless phenocopies was related to two mutations localized on two different autosomes, a higher number of generations before the complete assimilation is clearly expected.

We think then, that these data strongly support our previous hypothesis of pseudo-assimilation by the co-selection over generation of somatically and genetically stress-induced phenotypes (Fig. 38b) rather than Waddington's one (Fig. 38a).

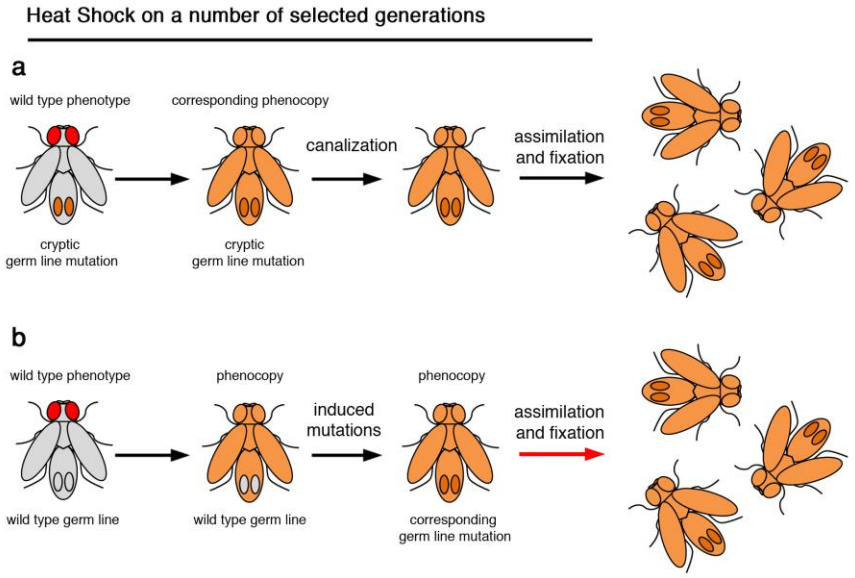

Figure 38. Models for the assimilation of a stress-induced phenocopy.
(a) According to Waddington the phenocopy induced by stress could be assimilated by the selection of preexisting cryptic mutations at one (monogenic determination) or more genes (polygenic determination). (b) According to our model the phenocopy could be fixed by a co-selection of a corresponding germ line mutation *de novo* induced by stress. Note that this view is also adaptable to mutant phenotypes with a polygenic determination. As discussed in the text, the assimilation process could take several generations also after the mutation induction and before the complete fixation of the mutant phenotype (red).

Intriguingly, this mechanism was already considered and rejected at the same time by Waddington (1953) himself with the following sentence:

"*One could therefore at best suppose that the shock treatment increased the production of many different types of mutations, tending to mimic all the relevant phenocopies. But since there are no considerations which force us to postulate the occurrence of any new mutations at all, it does not seem necessary to pursue the argument any further*".

Curiously, this sentence does not seem to take into account previous work that had already suggested a mutagenic effect of repeated heat-shock treatment on *Drosophila* larvae, and more strikingly, with the formulation of a similar hypothesis.
That hypothesis, called *"parallelism between somatic variants and their fixation by induction of corresponding mutations"* by Jollos (1934) is summarized by the following sentence:
"Heat treatment causes non-inherited variations within the treated generation - Sooner or later, with repeated treatment, the same variations are produced as true mutations".
It's impressive to realize how this forgotten opinion correlates perfectly with "Organic selection" theory!
It is also hard to believe how it is difficult, to date, to have a clear overview on this phenomenon, despite the advanced technologies we have available.
However, our data reflect the evidence that evolutionary phenomena similar to organic selection could play a major role in biological evolution and should be introduced forcefully into current debates on evolutionary theory.

MATERIALS AND METHODS

Drosophila strains

The natural population of *Drosophila melanogaster* were collected in two different areas of Central Italy in the year 2012.
Ore-R strain, the balanced and multi- or single marked stocks used for genetic mapping and complementation tests have been kept in our laboratory for many years.
w; ry^{506} Sb^{1} P{Δ2-3}99B/TM2, ry^{SC} red^{1}* (#1798) and *FM7i/C(1)DX, y^{1} f^{1}* (#5263) stocks were obtained from Bloomington Stock Center. *D. melanogaster w^{1118}* control stock and *w^{1118}* stock with Wolbachia wMelPop were kindly provided by professor William Sullivan (University of California Santa Cruz) (Chrostek et al., 2013; 2015). Cultures were maintained at 24°C on standard cornmeal-sucrose-yeast-agar medium.

Total RNA extraction and qRT-PCR

RNA samples from whole larvae or ovaries and testes were isolated by Qiazol reagent (Qiagen) according to the manufacturer's instructions. The concentrations were determined using a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific). 5μg of total RNA were used as a template for oligonucleotide dT primed reverse transcription using SuperScript III RNaseH-reverse transcriptase (Invitrogen), according to manufacturer's instructions.
Real-time RT–PCR was performed in 7300 Real-Time PCR System (Applied biosystems). Relative abundance of the different TE transcripts was determined using QuantiFast SYBR Green PCR Kit (Qiagen) according to manufacturer's protocol. For quantification of the transcripts we used the 2-ΔΔCt method (Livak KJ, 2001).

The primers used were:

RP49 for	GCGCACCAAGCACTTCATC
RP49 rev	TTGGGCTTGCGCCATT
Het-A F	ACTGCTGAAGCTCGGATTCC
Het-A R	TGTAGCCGGATTCGTCATATTTC
roo F	CGTCTGCAATGTACTGGCTCT
roo R	CGGCACTCCACTAACTTCTCC
aurora F	GAAGGAACTGAGCGTGTTCCA
aurora R	CGTCTACCGCAACTAATGCAAA
copia F	TGGAGGTTGTGCCTCCACTT
copia R	CAATACCACGCTTAGTGGCATAAA
springer F	CCATAACACCAGGGGCA
springer R	CGAGTGCTGGTCTGTCA
I element F	CAATCACAACAACAAAATCC
I element R	GGTGTTGGTGTGGTTGGTTG
opus F	CGAGGAGTGGGGAGAGATTG
opus R	TGCGAAAATCTGCCTGAACC
Idefix F	AACAAAATCGTGGCAGGAAG
Idefix R	TCCATTTTTCGCGTTTACTG
Gypsy F	CTTCACGTTCTGCGAGCGGTCT
Gypsy R	CGCTCGAAGGTTACCAGGTAGGTTC
1360 F	TCGTGCAAGACAATGAGAGG
1360 R	GCAACTGGATCCCTTAGCAA
hobo F	AAACTGTTCTGGACGGATGG
hobo R	TTATGGCGGGATAAATTGGA
S-element F	TTTAAATCGTGCGGGAAAAG'
S-element R	TTCGCATAATAAGGCGGTCT
Bari1 F	TTGAAAACGTTTGGGCTTTT
Bari1 R	TAACACCACCTTTGGCATCA
Stellate F	GGCGATGAAAAGAAGTGG
Stellate R	CAGCGAGAAGAAGATGTC

ChIP-seq

Approximately 300 ovaries were dissected from 7 to 10 days-old heat-shocked and not-shocked females in 1X PBS (8.06mM sodium phosphate, 1.94mM potassium phosphate, 137mM NaCl, 2.7mM KCl and pH adjusted to 7.4), and were cross-linked in buffer A1 (60 mM KCl, 15 mM NaCl, 15 mM Hepes (pH 7.6), 4 mM MgCl2, 0.5% Triton X-100, 0.5 mM DTT, and complete EDTA-free protease inhibitor cocktail [ROCHE]), in the presence of 1.8% formaldehyde, by homogenization in a Potter and then in a Dounce homogenizer with type A pestle (three strokes each), followed by incubation for 15 min at room temperature. Cross-linking was stopped by adding glycin to 225 mM for 5 min. The homogenate was centrifuged for 5 min, 4000g at 4°C. The supernatant was discarded and the crude nuclei pellet was washed three times in 3 ml A1 buffer and once in 3 ml A2 buffer (140 mM NaCl, 15 mM Hepes (pH 7.6), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, and protease inhibitors) at 4°C. After the washes, nuclei were resuspended in A2 buffer in the presence of 0.1% SDS and 0.5% N-lauroylsarcosine, and incubated for 10 min in a rotating wheel at 4°C. After sonication (four pulses of 30 s with 1-min intervals, using a Hielscher Ultrasonic processor UP100H and 10 min high speed centrifugation, fragmented chromatin (DNA fragment size ranging from 200 bp to 500bp) was recovered in the supernatant. For each immunoprecipitation, 50 µg of chromatin was incubated in the presence of 10 µg of H3K4me3 (Active Motif) or H3K9me3 (Active Motif) antibodies (3 hours at 4°C in a rotating wheel). Then, 50 µl of dynabeads protein G (invitrogen) was added and incubation was continued over night at 4°C. The supernatants were discarded and samples were washed four times in A2 buffer + 0.05% SDS and twice in 1 mM EDTA, 10 mM Tris (pH 8) (TE) buffer (each wash 5 min at 4°C). Chromatin was eluted from beads in two steps; first in 100 µl of 10 mM EDTA, 1% SDS, 50 mM Tris (pH 8) at 65°C for 10 min, followed by centrifugation and

recovery of the supernatant. beads material was re-extracted in 150 µl of TE, 0.67% SDS. The combined eluate (250 µl) was incubated 6 h at 65°C to reverse cross-links and treated by 50 µg /ml RNaseA for 15 min at 65°C and by 500 µg /ml Proteinase K (Invitrogen) for 3 h at 65°C. Samples were phenol–chloroform extracted and ethanol precipitated. DNA was resuspended in 25 µl of water and sequenced by high-throughput Illumina technology.

Fluorescent in situ hybridization.

DNA probes for copia, jockey or P-element ORFs was labelled with digoxigenin-11-dUTP (DIG-Nick Translation Mix, Roche) while *singed* PCR fragment were labelled with biotin-11-dUTP according to the manufacturer's instructions. DNA probes were ethanol precipitated and resuspended in 50% formamide, 10% dextran sulfate and 2X SSC.
For polytene chromosomes preparations, third larval stage salivary glands were dissected in physiological solution (0.7% NaCl), fixed with 3.7% paraformaldehyde in PBS1X for 10 min and after fixation, frozen in liquid nitrogen.
Chromosome preparations were dehydrated by sequential immersion in 70%, 90% and 100% ethanol, and then denaturated for 2 min at 70°C in 70% formamide and 2XSSC. After sequential immersion in cold 70%, 90% and 100% ethanol, slides were incubated with 10 ml of probe at 37°C in wet chamber.
After overnight incubation, slides were whashed three times at 42°C with 50% formamide and 2X SSC, followed by three whashes at 60°C in 0.1X SSC.
Chromosomes preparations were blocked 30 min at 37°C in 3% BSA, 0.1% Tween-20 and 4X SSC, and then incubated with 1:100 diluition of anti-digoxigenin FITC (Roche) or 1:200 diluition of Avidin Rhodamine coniugated in 0.1% BSA, 0.1% Tween-20 and 4XSSC for 30 min at 37°C.

After three washes at 42 C in 4XSSC and 0.1% Tween-20, slides were air dried, stained with DAPI and mounted in antifading medium.

Southern Blot

About 10 µg of genomic DNA were digested with Hind III restriction enzyme (New England BioLabs) at 37°C overnight and separated on 1% agarose gel. The DNA was then denaturated (in NaOH 0.5M, NaCl 1.5M) for 1h, neutralized (in NaCl 1.5 M, Tris-HCl 1M pH 8) and transferred to positively charged nylon membrane (Hybond N filters Biorad) in SSC 10X (300mM sodium citrate, pH 7, 1M sodium chloride) for 16 hours. After washing in 2X SSC, UV cross-linking was applied to the membrane. As Probes for Southern analysis we used clones of copia and gypsy. Hybridizations were performed in solutions of 5X SSC, 5X Denhardt's, 0.5% SDS, 20 µg/ml salmon sperm DNA. After a pre-hybridization performed at 65°C for 2 hours , filters were probed with denaturated DNA probes nick translated using [alpha-32P] CTP at 42°C for 12 hours. Membranes were washed under the following conditions: 10 min at room temperature with 2X SSC, 0.1% SDS; 15 min at 65°C with 1X SSC, 0.1%SDS and 10 min at 65°C with 0.1X SSC, 0.1%SDS.
Typhoon scan was used to acquire radioactive signals.

Isolation of genomic DNA.

Genomic DNA was prepared either from 20-30 flies or from single fly. Single fly was homogenized in 100µl of extraction buffer (120mM Tris-HCl, pH 8.0, 60mM EDTA, pH 8.0, 80mM NaCl, 160mM sucrose, 0.5% v/v SDS, 200µg/mL RNase DNase free) and incubated at 65°C for 60min. After cooling at room temperature for a few minutes, 14µl 8M K-acetate was added. After 30 min on ice, the samples were spun at 10000 rpm for 15 min at 4°C; DNA was precipitated by adding to the to the

supernatant 0.5 volume of isopropanol and after 10 min at room temperature the samples were spun again for 10 min. The pellet was washed with 70% ethanol, dried and redissolved in 20µl H2O. For DNA extraction from pools of flies the volume of the extraction buffer was adjusted to 1mL and the volumes of the subsequent components were changed accordingly.

Primers design and PCR amplifications.

All PCR specific Primers (18–25mers with a minimum GC content of 50% and average Tm of 60°C) were designed using the Invitrogen OligoPerfect™ designer web tool and oligonucleotide sequences were screened using a BLAST search to confirm the specificity.
PCR amplifications were performed in 1X PCR Buffer with 50-100ng of genomic DNA, primers (0.4µM), $MgCl_2$ (1.5mM), dNTPs (0.2mM each), and 2U/rxn of PlatinumTM Taq DNA Polymerase (2U)(Invitrogen). The thermal profile for PCR amplifications was as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 58-62°C for 1 min, 72°C for 2 min, and ending with a final extension at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis, purified to remove primers and excess nucleotides and sequenced in two separate reactions, each using one of the PCR primers as a sequencing primer (Bio-Fab Research, Rome, Italy). Gene diagrams was obtained using GSDS (Gene structure display server: GSDS: http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015).

List of primers used in PCR amplification. In red are indicated specific oligonucleotides used for mapping and identification of heat-induced mutations.

	Primer sequences	Annealing temperatures
Wolbachia detection		
16S Wol F	TTGTAGCCTGCTATGGTATAACT	55°C
16S Wol R	GAATAGGTATGATTTTCATGT	55°C
HP1 F	ATCCCGAAACTGAGAACACG	55°C
HP1 R	CCATTCTTGCGAAGGACAAA	55°C
singed		
sn1 F	TCCTGACCGTCTTCAATGATAGT	58°C
sn1 R	AATAGTTTCGTGGGAGAAAGGAC	58°C
sn2 F	CCAGTTTTCCAATGACTCTGCTAC	58°C
sn2 R	GAAACGAGTCAGAAAAGAGAGGAG	58°C
sn3 F	CTCCTCTCTTTTCTGACTCGTTTC	58°C
sn3 R	CTAGTAAGCCAGCCTTCCAGTTT	58°C
sn4 F	GAAACTGGAAGGCTGGCTTACTA	58°C
sn4 R	GAAAGACTAAAGACCCCTGGACAT	58°C
sn5 F	CCAGGGGTCTTTAGTCTTTCAGT	58°C
sn5 R	CTGTCTTCACTGTCTGGACTGTCT	58°C
sn 6 F	gtcgaagtcgaagtcagagttgt	58°C
sn 6 R	TTGAGTCCGGCTATAAAGGAAG	58°C
sn 7 F	TACGTGCAACAACAAgtgagtg	58°C
sn 7 R	ggacgaagccaaagatagagaa	58°C
sn 2.1.1 F	CCAGTTTTCCAATGACTCTGCTAC	60°C
sn 2.1.1 R	TCGACTTTGTATGGTCACTCTAGC	60°C
sn 2.1.2 F	GTATCGATTCGCTTTGATGGTG	60°C
sn 2.1.2 R	GAGACTCTGGTGGAGATGCTTTAT	60°C
sn 2.2 F	**CAAGTGCAGCTTGGGTTATTAGAG**	60°C
sn 2.2 R	**CTCCTTCTTTTTGGCTCTATGCTC**	60°C
sn 2.3 F	CAACAGTTACAACTTCGGCAAC	60°C

sn 2.3 R	GAAACGAGTCAGAAAAGAGAGGAG	60°C
sn 2.2.1 F	ggatcgcacatatagctactcctt	60°C
sn 2.2.1 R	GCAGGTTTTTCTAAGACGAGACG	60°C
forked		
f1 F	CTACATTGGGGAATACTCATCCTC	60°C
f1 R	gataactgtttcccacctgatctc	60°C
f2 F	gaatcgcttagtcttggtcttgtc	60°C
f2 R	gtctttccaacggagtagtaaacc	60°C
f3 F	cggagtttgagatagagtggtgtt	60°C
f3 R	AAACTCTACGAGGAGTTCACCAAC	60°C
f4 F	CTAAAGCGGAAGATACGGTACAC	60°C
f4 R	CCTCTGCCTATATGCCAGTAGAAT	60°C
f5 F	GAGAAAAGCCAGACTACCGACTAA	57°C
f5 R	GTACAAGAAGTACAAGCCGCTGAC	57°C
f6 F	CTTCTCTTGCTTCTTACCCTTCC	60°C
f6 R	AGAGCAGATACCACGCAGAGTT	60°C
f7 F	ctccacaaagcgtacataactcag	60°C
f7 R	gttctctgtgtacctctcttgct	60°C
f8 F	ctgtcacaacttgggtgagtctt	60°C
f8 R	tatagagtttgtgcccctagaacg	60°C
f9 F	cagagtttgagttcgaggttgag	60°C
f9 R	GATTGTAATCCTCCACTGCTGTC	60°C
f10 F	ATTTGACAGCAGTGGAGGATTAC	60°C
f10 R	TAGTTTGGTGTTGTGGCTTAGGT	60°C
f11 F	tccttacgaaccattctgctctac	60°C
f11 R	cggtagctggtaaacagtaacaac	60°C
f12 F	ggtctcgtgcagcataatacaac	60°C
f12 R	CACACTTATATGCACACACACAGG	60°C
f13 F	gatgtagatgtgaggcaagaagc	60°C
f13 R	gtcacttgtccacttcgctagtt	60°C
f14 F	taccaagtagccaactagcgaag	60°C
f14 R	GGAGGTGGTCCATTCActaaag	60°C
f15 F	TCAGAGTAGCAGGAATCTCAAGC	60°C

f15 R	tctttaagagctgtccactctcg	60°C
f16 F	tcgagagtggacagctcttaaa	58°C
f16 R	agagatctcggctactttcactg	58°C
f17 F	ctgccacttttcttaccacagA	58°C
f17 R	GCCAATAGACAGACTTCCTGGAT	58°C
***forked* frameshift sequencing**		
f fram seq F	CCATGTCTGGCAAATCACAC	
f fram seq R	ACCAGTTGGCCAGAACTCAC	
***forked* frameshift detection**		
f fram F	**GCCTCTACCCCACGGAATG**	62°C ($MgCl_2$ 1.28mM
f NO DEL R	**CTGTGTGTCCGTGCGACG**	62°C ($MgCl_2$ 1.28mM
f DEL R	TTCTGTCCGTGCGACGC	62°C ($MgCl_2$ 1.28mM
cactus		
cact1 F	CTCCCAGGCGTTCATGATATT	58°C
cact1 R	TCTAGCGCTTCTTTCACTACGG	58°C
cact2 F	aaacccagaggtttcgaatagg	58°C
cact2 R	CCTGGGAGCAGTTCTATCAACA	58°C
cact3 F	ccgcccgatctactaccactat	58°C
cact3 R	caagaaacgagacgttcagacg	58°C
cact4 F	aagacgagagtgacgaggtacg	58°C
cact4 R	agaaagtcaacaaggcgagtcc	58°C
cact5 F	CATCCGTTGATATGCTCAGACC	58°C
cact5 R	cgtacctcgtcactctcgtctt	58°C
cact5.1 F	ACACGTCACGAGTTCTCCAGAT	60°C
cact5.1 R	cctcttgttcttgcagATGTACG	60°C
cact5.2 F	CGTACATctgcaagaacaagagg	60°C
cact5.2 R	GTACCCAGCTGAACTGGTATTCA	60°C
cact5.3 F	ACCAGTTCAGCTGGGTACTTCTC	60°C
cact5.3 R	gtacctcgtcactctcgtctttg	60°C

cact5.1.1 F	TACCCAACAATGGTGAAGCAAG	58°C
cact5.1.1 R	AGGTCTGAGCATATCAACGGATG	58°C
cact5.1.2 F	AGCATCCGTTGATATGCTCAGA	58°C
cact5.1.2 R	ACAAGGATGCAGAATGTCAAGC	58°C
cact5.1.3 F	**GCTTGACATTCTGCATCCTTGT**	58°C
cact5.1.3 R	**GGTGATCCTCGCTATTTTGTCAG**	58°C
micropia sequencing		
micropia1 F	CTGTCATGAAGTGGAGCGAGTT	
micropia2 F	TGTCTGAGCCAACAGGAGTCAT	
micropia3 F	TATCCGCCCAAGTAGTTCTCCA	
micropia4 F	GTTACACACTGACGCCAGTTCC	
micropia5 F	CATCGTTCAGATTGATGGCTTC	
sepia		
se1 F	CGCACATCATGAGTAACGGCAG	60°C
se1 R	ATCAGGCATCCTTGACCAGCAG	60°C
se2 F	**AAGgtaagagagcaagcaaaacc**	60°C
se2 R	**CCATTTATCCTGTGCTGACTTGT**	60°C

Western blot analysis.

To perform Western blots, larvae were homogenized in SDS gel-loading buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 10 mM dithiothreitol, and 0.1% bromophenol blue) in the presence of protease inhibitors (10 µM benzamidine HCl, 1 mM PMSF, 1 µg/ml phenanthroline, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A) and heated at 95°C for 4 min. Insolubles were pelleted by centrifugation before electrophoresis. Proteins fractionated by 10% SDS-PAGE were electroblotted onto Immobilion-P polyvinyldifluoride membranes (Bio-Rad Laboratories) in a buffer containing 10mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) (Sigma-Aldrich), pH 11, and 20%

methanol, in a semi-dry transfer apparatus (Amersham Biosciences). The filter was blocked with 5% nonfat dry milk in Tris-buffered saline with T ween 20 (TBST) buffer (20mM Tris pH 7.5, 150mM NaCl, 0.1% Tween 20). After blocking, proteins were probed with a monoclonal antibody against singed (DSHB Hybridoma Product sn 7C) (1:20) and α-tubulin (Sigma) (1:3000), and detected with a 1:10000 dilution of goat anti–mouse conjugated to protein A Horseradish Peroxidase (HRP) linked. The Enhanced Chemiluminescence kit was purchased from GE Healthcare. Images were acquired with the ChemiDoc imaging system (Bio-Rad Laboratories).

semiquantitative RT-PCR analysis.

For semiquantitative PCR amplification, 6µl of the cDNA synthesized were used as template with Platinum Taq (Invitrogen), according to the manufacturer's recommendations. The cycling conditions were as follow: 94 °C for 3 min; 27 cycles of 30 s denaturation at 94 °C, 30 s at 58 °C and 60 s at 72 °C. PCR was performed using the following primer pairs designed using NCBI/Primer-BLAST:

sn-variants R(A,B,E,F) For	ACTGGAGTGCAGTTCGTGAG
sn-variants R(A,B,E,F) Rev	TGCCAAACTGATCGACCGAA
sn-variant RG For	CAAAGCGGTGAACAGTACGC
sn-variant RG Rev	TGCCAAACTGATCGACCGAA
rp49 For	CCCAAGGGTATCGACAACAG
rp49 Rev	GACAATCTCCTTGCGCTTCT

The final PCR products were electrophoresed on 1.5% agarose gels.

KP-element excision by Δ2-3 transposase.

CAR^{sn} (*CAR^{sn}/CAR^{sn};+/+)* virgin females were crossed with males carrying the transposase Δ2-3 (*w*/Y; ry^{506}Sb^{1}P{Δ2-3}99B/TM2, ry$^{[SC]}$red^{1}*). F1 males carrying both the singed mutation and the transposase (stubble bristles, Sb^{1}) (*CAR^{sn}/Y; ry^{506}Sb^{1}P{Δ2-3}99B/+*) were mated with virgin females carrying an attached X (*C(1)DX,y^{1}f^{1}/Y;+/+)* and the male progeny phenotype was screened to isolate singed revertants. Finally, each male revertant was backcrossed with *C(1)DX* virgin females to establish a stock.

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LIST OF PUBLICATIONS

“Transposon's jumping into the canalization and assimilation concepts”
Laura Fanti, Lucia Piacentini, Ugo Cappucci, Assunta M. Casale and Sergio Pimpinelli. ***Submitted to Science Advances***

“Drosophila CG3303 is an essential endoribonuclease linked to TDP-43-mediated neurodegeneration”
Pietro Laneve, Lucia Piacentini, Assunta Maria Casale, Davide Capauto, Ubaldo Gioia, Ugo Cappucci, Valerio Di Carlo, Irene Bozzoni, Patrizio Di Micco, Veronica Morea, Carmela Antonia Di Franco, Elisa Caffarelli. ***Scientific Report (in press)***

“Drosophilidae monitoring in Apulia (Italy) reveals Drosophila suzukii as one of the four most abundant species”
Rachele Antonacci, Patrizia Tritto, Ugo Cappucci, Laura Fanti, Lucia Piacentini, Maria Berloco. ***Under revision to Bulletin of Insectology***