From linear genes to epigenetic inheritance of three-dimensional epigenomes

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Abstract

Fifty years ago Jacob and Monod reported their findings on the regulation of gene activity. Working on lambda bacteriophage lysogeny and the regulation of the production of an enzyme that cleaves lactose, they observed that its production was induced by the presence of lactose in the medium and came to general conclusions about gene expression that still hold true today. Thanks to decades of intense multidisciplinary research, these conclusions have been extended by several fundamental discoveries. The first is that gene regulatory circuits include the ability to propagate the memory of a specific gene regulatory state long after it being established, and even when the original inducer is no longer present. The second is that, in addition to being regulated by binding of regulators, such as RNAs or proteins, in the vicinity of the site of transcription initiation, genes can be regulated by factor binding at incredible distances from their transcriptional start sites. Prominent among the regulatory components involved in these processes are Polycomb and Trithorax group proteins, pleiotropic gene regulators of critical importance in development, physiology and disease.

Introduction

The concept of the operon itself, a functional unit of genomic material containing a cluster of genes under the control of a single regulatory signal, is not universal in biology since it is used mainly in a set of bacterial genes. On the other hand, the research leading to the identification of the operon led to the discovery of the universal basis of gene regulation. Indeed, Jacob and Monod's work identified two classes of genes, those coding for structural and metabolic components of cells and those coding for gene regulators, which act by binding to their regulatory regions in a combinatorial manner. The broader significance of this research is that it defines the basis for most gene regulatory processes in biology. In addition to this giant accomplishment, François Jacob contributed the concept of evolutionary tinkering, which opposes the idea that evolution may be like an engineer producing sophisticated works out of exquisite blueprints. Instead, he proposed that most novelty in evolution emerges from the casual and mostly erratic process of

assembling already available spare parts (i.e. new proteins may be generated by mutation-driven fusion of existing protein domains). Again, this idea has had profound impact on current thinking regarding the processes underlying genome evolution and continues to be highly influential in the field of evolutionary biology.

With these monumental achievements in his pocket, François Jacob undertook research in the 70's in a new area, focusing on the study of mouse embryogenesis. Although the concepts derived from his research in prokaryotes have had a profound impact on biology in general, he embraced this new challenge because he had the intuition that eukaryotic organisms have many wonders with which to amaze the mind of all scientists.

One of these wonders is that eukaryotic genomes are not only much larger in size and contain more genes than prokaryotes, but also that they are compartmentalized in the cell nucleus, where they are packaged in a highly organized manner into chromatin. Chromatin contains the genomic material, DNA, and highly basic histone proteins that form octamers. Approximately 147 bp of DNA folds around these histone octamers to form a left-handed superhelical structure called the nucleosome, which is the fundamental packaging unit of DNA in all eukaryotes [1; 2]. In vitro, a string of nucleosomes is essentially a fiber 11 nm in diameter and this string can be compacted to form higher-order chromatin fibers of 30 nm in diameter. These fibers represent only one layer of chromatin organization and may, at least in some circumstances, be present in vivo [3]. Beyond this level of organization, little is known about chromatin folding since the tools to observe chromatin at high-resolution in vitro as well as in vivo are lacking, but we know that further levels of folding exist, that they involve the formation of chromatin loops, and that they contribute to the regulation of eukaryotic gene expression [4]. Obviously, the identification and functional dissection of cellular components that regulate gene expression via chromatin folding is a major issue in biology. Part of this regulation involves the post-translation modification of histones [5] as well as ATP-dependent chromatin remodeling [6]. Another set of components that modulate nucleosome and higher-order chromatin structure are the Polycomb and Trithorax group (PcG and TrxG, respectively) proteins, which are known to play critical roles in gene regulation during development and disease. As such, they have been studied in considerable detail both in terms of their molecular function as well as in their roles in regulation of gene

expression [7; 8; 9; 10; 11; 12]. Here, I briefly recapitulate the function of PcG proteins and discuss some intriguing molecular features that may allow them to regulate the memory of cellular states.

PcG and TrxG proteins and nucleosome regulation

Originally discovered during genetic analysis of the regulation of the antero-posterior body plan of *Drosophila*, *Polycomb* was identified as a repressor of the expression of Hox genes, thus explaining the homeotic phenotypes observed in mutants [13]. Cloning and characterization of the protein showed that it is chromatin associated and binds to many genomic loci [14]. Moreover, many other PcG genes were identified in screens for modifiers of Hox phenotypes, and a set of genes, named the trxG, having the opposing function of transcriptional activation, have been identified and characterized genetically and molecularly [15]. In particular, biochemical analysis identified two PcG protein complexes named PRC1 and PRC2 (Figure 1A). These two complexes are recruited by a set of sequence-specific DNA binding proteins, and possibly by noncoding RNAs, to specific target sequences called Polycomb response elements or PREs [16; 17; 18; 19; 20; 21; 22; 23; 24; 25; 26; 27]. Once recruited, the PRC2 complex trimethylates histone H3 at lysine 27 (Figure 1B) in the region surrounding its binding sites [28; 29]. This activity is catalyzed by the protein E(z) in *Drosophila* and by its homolog, EZH2, in vertebrates. A second vertebrate homolog of E(z), EZH1, is much less active [30; 31]. The H3K27me3 mark is bound by the PRC1 complex via its PC subunit [32; 33] in vitro and assists its tethering to chromatin being targeted [34], although PRC1 can also be recruited to chromatin in a PRC2 and H3K27me3-independent manner, both in vitro and in vivo [35; 36]. PRC1 itself contains a protein (dRing/Sce in *Drosophila*, Ring1B in mammals) that can mono-ubiquitylate histone H2A at K119 (Figure 1B). The dRing protein is also found within another complex, named dRAF, and this may actually be the most important source of H2A K119 ubiquitylation, at least in *Drosophila* [37]. Mono ubiquitylated H2AK119 (H2AK119ub) can inhibit transcription by inhibiting RNA polymerase II elongation [38; 39]. Much however, remains to be learned regarding the role of this histone modification. In particular, another PcG complex, PR-DUB, deubiquitylates H2AK119ub [40] and it remains to be understood how two complexes with opposing functions both lead to gene silencing. Furthermore, a reader of the H2AK119ub mark has been recently identified as the ZRF1 protein. However, this protein has the surprising role of counteracting PRC1

chromatin binding and its gene silencing function [35]. Whether other cellular activities read the same mark to repress transcription remains to be seen.

In addition to repressing transcription via H2A ubiquitylation, PRC1 can prevent ATP-dependent chromatin remodeling in vitro [41] and antagonizes SWI/SNF complexes in vivo [15]. It is unclear whether the H3K27me3 mark generated by PRC2 can repress transcription on its own. However, in addition to playing roles at the nucleosomal level and by directly inhibiting RNA polymerase II function, both PRC2 and PRC1 seem to silence gene expression by affecting higher order chromatin folding.

Regulation of higher-order chromatin structure by Polycomb proteins and chromatin insulators

It has been proposed that the PcG-mediated repression of transcription may depend on chromatin compaction. It is difficult to measure chromatin condensation in vivo because of the lack of reliable assays. However, in vivo evidence for PcG-dependent chromatin condensation in *Drosophila* came from the inability of the transcriptional activator GAL4 to drive transcription of a gene in the embryonic regions where it was silenced in a PcG-dependent manner [42]. By assaying the accessibility of PcG target chromatin to DNA methylation carried out by the bacterial Dam methylase, Boivin and coworkers came to similar conclusions, although the difference in accessibility in the presence versus absence of PcG proteins was small [43]. Another study involving chromatin digestion by restriction enzymes did not reveal increased condensation [44], suggesting that the effect of PcG proteins on chromatin condensation may be modest and/or context dependent.

Later studies, however, have shown that the PRC1 complex can drive chromatin compaction in vitro [45]. This activity was dependent on the protein Psc, the fly homolog of Bmi1. More recently, a variant of the PRC2 complex containing EZH1 was also shown to condense chromatin in vitro [30]. These studies have revived attention on this subject and prompted new analysis using fluorescent in situ DNA hybridization (FISH). By using two probes well spaced along the chromosome within an imprinted locus that is bound by PcG proteins in mouse, it was shown that the locus is contracted as the distance between probes is small when the locus is silent. In both PRC1 and PRC2 mutants, the probe interdistance increased, suggesting that

both complexes contribute to locus contraction [46]. In a similar study performed at the murine Hoxb and Hoxd PcG target loci, contraction was shown to be dependent on Ring1B [47]. Intriguingly however, locus contraction and gene silencing were both shown to be independent of the histone ubiquitylation activity of Ring1B, suggesting that this activity is not essential for PcG-mediated silencing.

While a PcG-dependent decrease in the distance between two FISH probes is compatible with chromatin condensation, there are other possible interpretations of the data. In *Drosophila*, a similar analysis was performed at the Hox gene cluster called BX-C and, again, the silent locus was shown to be contracted by FISH [48]. Furthermore, by combining FISH with Polycomb immunostaining, the authors showed that the contraction corresponded to the colocalization of distant FISH probes within nuclear bodies enriched in Polycomb, called Polycomb bodies. However, it was shown that the decreased interprobe distance did not simply reflect global chromatin condensation. Using chromatin conformation capture (3C), they instead showed that silent BX-C adopts a multi-looped structure where PREs contact each other and their target gene promoters [48]. Thus, interprobe distances evaluated in FISH studies may not reflect chromatin condensation but rather formation of more comlex topological structures. Whether this also applies to mammalian Hox or imprinted loci is not clear, but the ability of PcG components to participate in long-distance chromatin loops has been documented at least in certain chromosomal regions in mammalian cells [49; 50].

Perhaps even more striking is the ability of Polycomb proteins to engage in very long range chromatin contacts. These contacts may involve endogenous genes or transgenes containing PREs very distant from each other, either within the same chromosome or even in different chromosomes [16; 51; 52; 53; 54]. Long range contacts have been shown to reinforce PcG-mediated gene silencing [51; 52] and are severely reduced when PcG proteins are mutated, suggesting that PcG components play important roles in forming or maintaining these distant contacts. Indeed, studies using immunostaining or GFP fusion protein show that PcG proteins are located in a number of discrete Polycomb bodies within the nucleus [16; 55; 56]. PcG proteins, however, are not the sole driver of gene contacts. Indeed, chromatin elements that are able to induce long-range 3D chromatin contacts are also regulated by other factors. Two regions from the bithorax complex, (BX-C) named *Fab-7* and *Mcp*,

contain so-called chromatin insulators adjacent to PREs and a careful analysis comparing the effects of insulators and PREs showed that the major components driving long range contacts between multiple copies of these two elements are the insulators [53]. Multiple proteins bind to these regions, among these CTCF, GAGA factor and CP190 seem to be good candidates that could drive long-range contacts. CTCF is also able to induce looping in mammalian cells [57; 58; 59]. Other cellular components may also be able to perform similar activities. One such example is *Drosophila* insulator binding protein Su(Hw) [60; 61; 62; 63; 64; 65]. Finally, both PcG components and CTCF are also involved in the modulation of regulatory processes such as genomic imprinting and X chromosome inactivation that involve long-distance pairing [66; 67; 68], suggesting that long-range chromatin contacts may regulate a variety of genomic processes in diverse species.

Chromatin and epigenetic memory of gene expression

While the molecular details of Polycomb protein function are still full of secrets, the most intriguing and fascinating property of Polycomb-mediated silencing is undoubtedly its ability to convey memory of chromatin states through cell division. This so-called cellular memory was already uncovered during the analysis of Polycomb in the early years. In a PcG mutant, repression of Hox genes was correctly specified outside their normal expression domains along the antero-posterior axis of early embryos, but later in development Hox genes became derepressed throughout the body [69]. This observation showed that PcG proteins are not required for the initiation of gene silencing, but rather for their maintenance. Later work illustrated this memory function molecularly, by analysis of a transgene carrying a PRE flanking a GAL4 activatable reporter gene (Figure 2). A short pulse of transcriptional activation during embryogenesis was able to revert PcG-dependent silencing into a TrxG-dependent activation mode that could be inherited throughout development all the way to adulthood. Most strikingly, inheritance of the active state could also be revealed in subsequent generations and it was shown to involve induction of histone acetylation at the derepressed transgene [70; 71]. This transgenerational inheritance was later seen in other model systems in both flies and mammals [72; 73]. The mechanisms by which chromatin states can survive DNA replication, during both mitosis and meiosis, remain to be elucidated, however, they might involve retention of modified histones on the chromatin template during these critical phases of cell

life. Indeed, it has recently been shown that histone modifications are retained on developmental genes even in sperm, the most condensed chromatin state known and a condition where most histones are replaced by protamines [74]. Currently it is not known whether this retention of histone modifications occurs in every species and whether the histone marks retained in sperm contribute to epigenetic memory [75]. However, the recent demonstration of epigenetic transmission of chromatin states through the male mouse germline seems to suggest that this might be the case [76].

A particularly surprising case of epigenetic inheritance involves the transgenerational transmission of features of nuclear architecture of PcG-targeted chromatin [51]. Transgenic lines carrying the *Fab-7* DNA element were shown to induce interchromosomal contact of the locus of insertion with the endogenous *Fab-7* locus found at BX-C. These contacts induced strong silencing of a reporter gene that was contained in the transgene and of the flanking locus of insertion. By deleting the endogenous *Fab-7* found at BX-C the contacts were removed and a loss of silencing was observed in the transgene insertion locus. Surprisingly, however, the derepressed state was propagated even upon re-insertion of the endogenous sequence in a fraction of the flies (Figure 3). This transgenerational inheritance was transmitted through the female germline in both transgenic lines, but the male germline could also carry the memory in at least one of them. Therefore, not only histone marks but also different three-dimensional chromosomal architectures can be inherited through cell division and meiosis.

Recently, two reports demonstrate that an environmental input, such as the type of paternal diet, can affect the expression of hundreds of genes in the offspring [76; 77]. This is in agreement with the possibility that histone marks transmitted via the sperm may contribute to the traits of the progeny. In one of the papers, the pattern of PcG-dependent H3K27me3 mark was shown to be altered in the progeny of low-protein diet male mice. This observation suggests that the modification of Polycomb function can be an important driver of transgenerational inheritance, although it is perfectly possible that many other factors convey chromatin inheritance as well. The biological implications of such inheritance processes are far reaching. Moreover, recent work on PcG and TrxG proteins shows that they are not only involved in the maintenance of cellular memory, they also modulate gene expression in a dynamic way and participate in reprogramming of cell fate upon various developmental and

environmental stimuli [9; 10]. Thus, one exciting research avenue will be to analyze the molecular mechanisms for the transmission of chromatin information to the progeny and under which circumstances this information can be transmitted, as opposed to other cases in which it is reprogrammed. Despite the giant achievements of the operon times and of the subsequent decades, many formidable challenges rise ahead of us and fascinating discoveries are to come. Jacob and Monod would just love to be at the bench today!

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Figure legends

Fig. 1. Polycomb complexes and mechanisms of gene silencing. A) The two main PcG complexes PRC2 and PRC1 are shown. *Drosophila* subunits are indicated. The two enzymatic subunits, E(z) for PRC2 and dRing for PRC1 are indicated in lighter color. **B)** Action of PcG proteins on chromatin. PcG complexes are recruited at PREs via DNA-binding components and, possibly, noncoding RNA molecules that are not indicated here for simplicity. The core PRE region is depleted of nucleosomes (or alternatively contains partially unfolded or disassembled nucleosomes), while flanking regions contain nucleosomes that are modified by PRC2 and PRC1. PRC2 makes the H3K27me3 mark (Me octagon in the panel), while PRC1 binds to the H3K27me3 mark and makes the H2AK119ub mark (Ub circle). This ultimately inhibits RNA pol II recruitment to promoters and interferes with transcriptional elongation of engaged RNA polymerases.

Fig. 2. Polycomb and trithorax proteins and the maintenance of cellular memory.

In *Drosophila* transgenes, when the *Fab-7* regulatory region of the BX-C flanks a reporter gene responsible for red pigmentation of the eye it silences its expression such that eyes have a light color. This silencing is PcG-dependent since it is lost in

PcG mutants. The transgene also contains binding sites for the transcriptional activator GAL4. When a short pulse of GAL4 is supplied during embryogenesis, GAL4 is able to revert PcG-dependent silencing into a trxG-dependent activation mode that is inherited throughout development all the way to adulthood. This is reflected in strong red pigmentation of the eye (top in the figure). In contrast, when a similar experiment is done in a line with a transgene that does not contain *Fab-7* GAL4 induces transcription in embryos but the active state is then lost when the GAL4 levels decay in late embryogenesis. Therefore, adult flies emerge with light eye pigmentation. These chromatin elements that are able to convey memory of either PcG-dependent silencing or of trxG-dependent activation have been defined as cellular memory modules or CMMs [70; 78].

Fig.3 Cellular memory dependence on nuclear organization of CMM elements.

In fly lines containing the *Fab-7* CMM, the locus of insertion (locus 1) of the transgene is frequently colocalized with the endogenous *Fab-7* locus, BX-C. This increases silencing, as is seen from the light eye color and the variegated eye phenotype. Deletion of the endogenous *Fab-7* element induces loss of silencing. Surprisingly, re-introduction of the endogenous *Fab-7* by crossing does not restore silencing in all flies. A significant proportion of them maintain the loss of pairing and silencing, suggesting that nuclear architecture is a heritable feature and, once perturbed, cellular memory of the original architecture is lost and can not faithfully be established in all cells.

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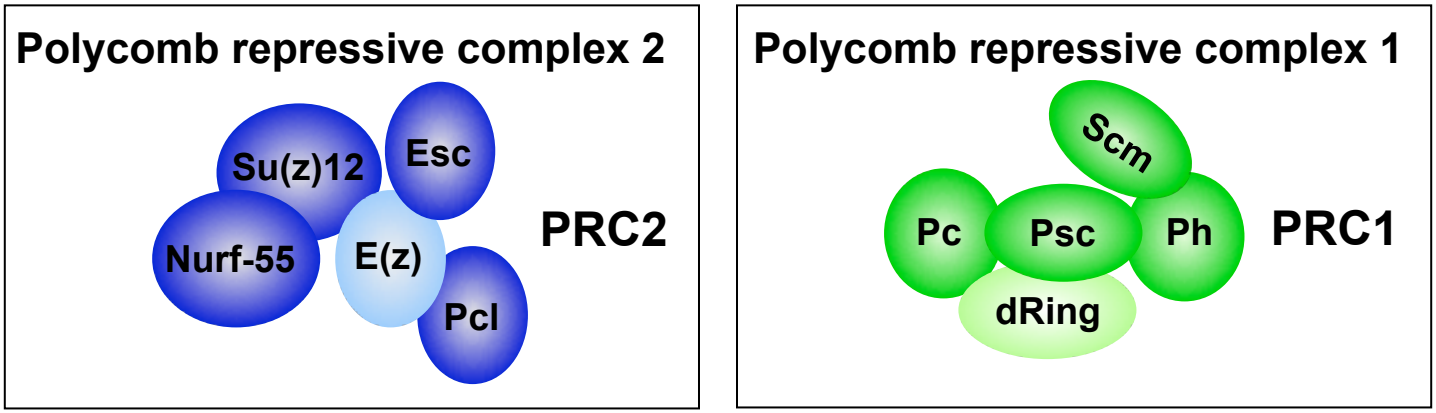
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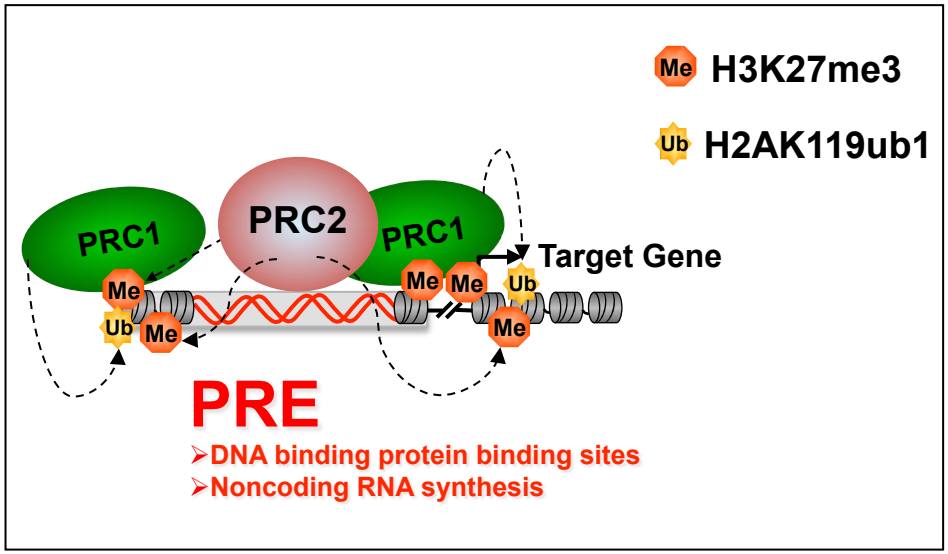
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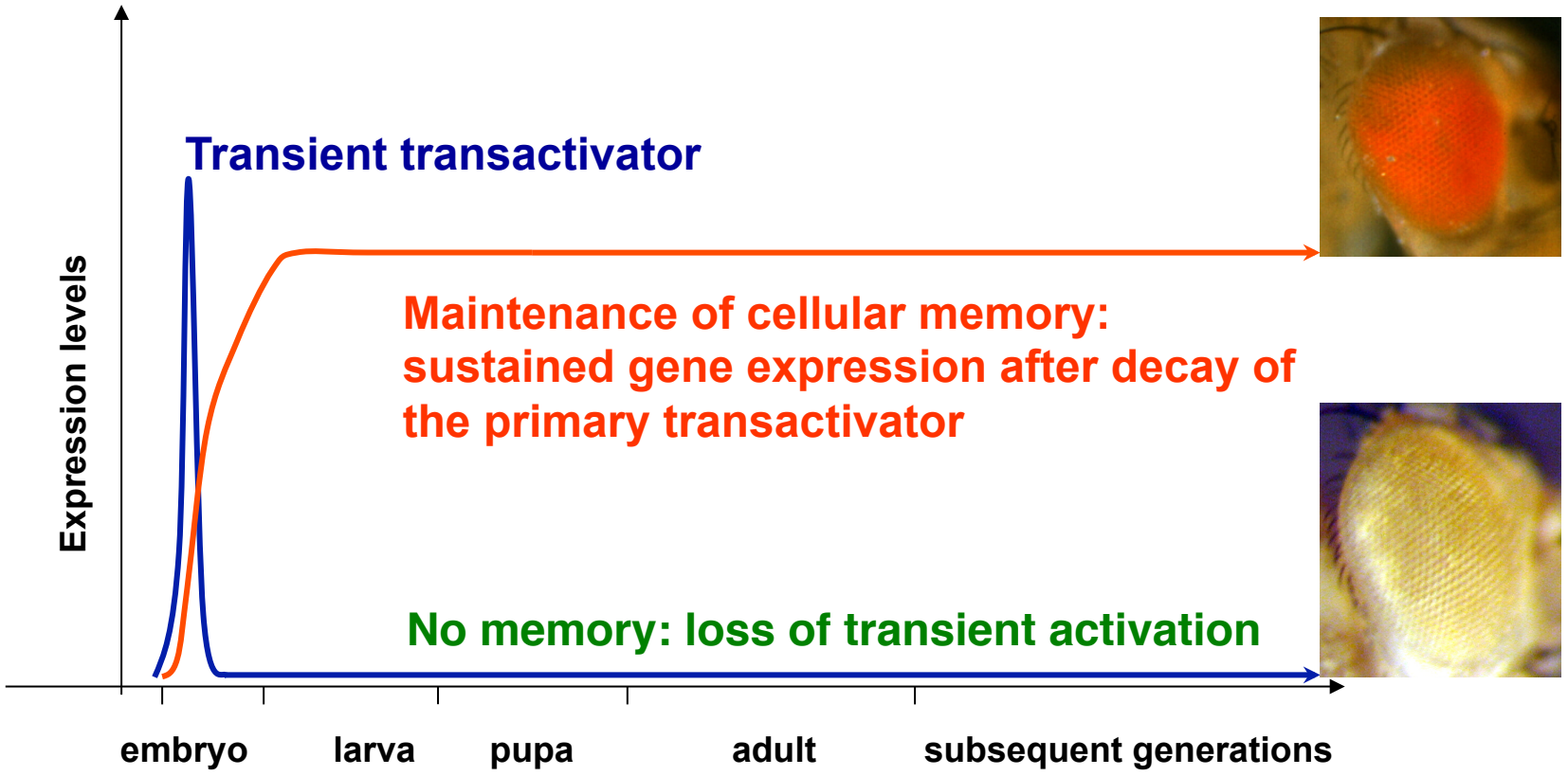
Transient transactivator
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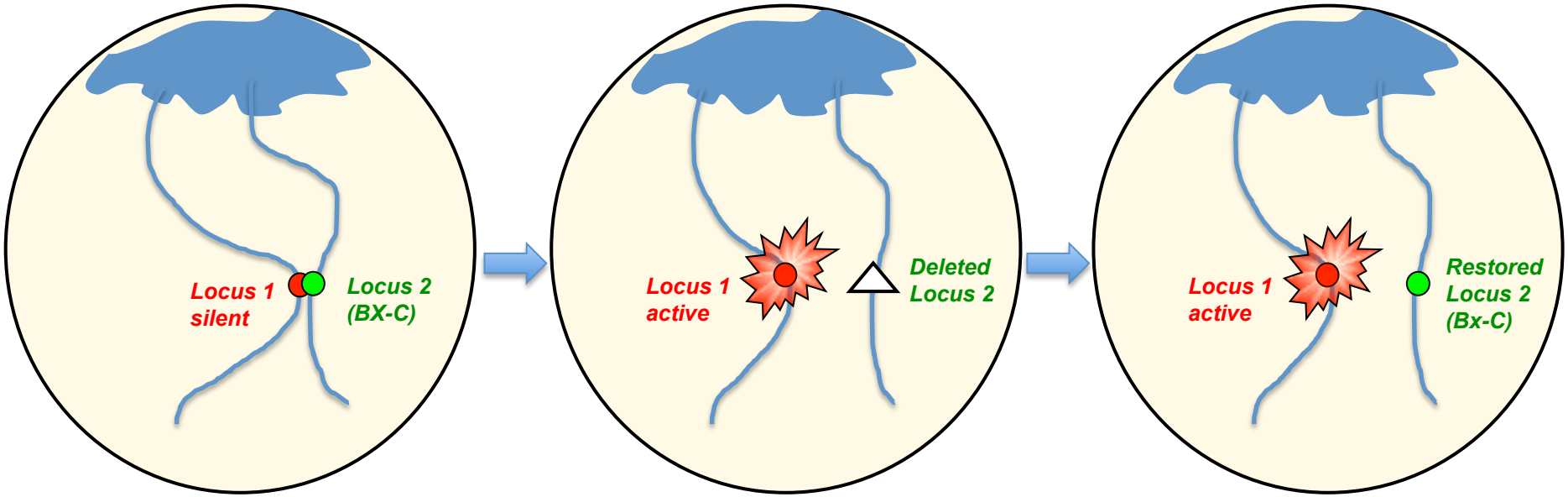
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