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Chromatin dynamics in regeneration epithelia: Lessons from *Drosophila* imaginal discs

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ABSTRACT

During the process of regeneration, a switch in the transcription program occurs in cells that contribute to the reconstruction of the missing tissue. Early signals released upon damage are integrated into the chromatin of responding cells to change its activity and function. Changes in chromatin dynamics result in transcriptional reprogramming, this is the coordinated regulation of expression of a specific subset of genes required for the regeneration process. Here we summarize changes in gene expression and chromatin dynamics that occurs during the process of regeneration of *Drosophila* imaginal discs.

1. Introduction

Regeneration is the process by which organisms reconstruct the original shape, size and function of body parts that have been physically or functionally lost or damaged. Regeneration can denote both the continuous cellular self-renewal of particular structures or tissues (physiological regeneration or tissue homeostasis) and the restoration of damaged tissue or lost structures (reparative regeneration) (reviewed in [1]). In 1901 Thomas H. Morgan attempted to refine the concept of regeneration by coining the terms *epimorphosis* to refer to regenerative phenomena in which the formation of the new part involves cellular proliferation and *morphallaxis* to refer to those cases in which regeneration results from the remodeling of existing material without cellular proliferation [2]. *Epimorphosis* can be found, for instance, in zebrafish heart and fin regeneration [3] and *Hydra* is one example where *morphallaxis* takes place [4].

Regeneration can occur at multiple levels of biological organization throughout metazoans and, accordingly, a classification has been proposed to describe regeneration, ranging from single-cell to tissue, organ, structural and whole-body regeneration [5,6]. Moreover, as already suggested by Morgan [2], regenerative capacity seems to be regulated by a number of fundamental traits, such as age, body size, life stage or wound-healing response (reviewed in [7–9]).

The capacity to regenerate is not universal and varies greatly, not only from one species to another, but also between tissues and organs or between developmental stages of the same species (reviewed in [5,10–12]). Planarians, for instance, can reconstruct their whole body from a tiny piece of almost any of their body parts; other Platyhelminthes, however, are unable to regenerate their heads and die after head amputation [13,14]. Such regenerative differences between closely related species do not only occur at high levels of biological organization, where patterning, development, and production of many different cell types occur; regeneration following less complex levels of biological organization, such as tissue regeneration, can also be similarly divergent. This, for example, is the case of differing skin regeneration between the mouse lab model (*Mus musculus*) and the African spiny mouse (*Acomys*). While the African spiny mouse can regenerate skin perfectly, the mouse laboratory model is unable to regenerate and instead forms fibrotic scars [15]. In addition, regeneration also depends on the developmental stage or maturation of the individual. In mammals, fetuses and newborn individuals have a relatively high degree of regenerative capacity, which is lost in the adult: newborn mice can heal their heart or skin better than adults [16,17]. In humans, distal phalanx regeneration after amputation has been observed in young children but not in adults (reviewed in [18]). To a certain extent, the same occurs in some insects: the capacity to regenerate specific organs that is observed at larval stages seems to be lost in the *Drosophila* adult (reviewed in [19,20]).

Injury is unavoidable for animals in the wild, where they can lose body parts to predators or due to other natural distresses. Thus, reparative regeneration involves the well-coordinated restoration of cells, tissues, and organs that have been physically or functionally lost. This

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process must therefore recognize missing structures and recapitulate them, while simultaneously achieving functional integration between recently formed and preexisting tissues. Cells that contribute to the restoration process are guided to regions where they are needed; and once regeneration is complete, specific cues are required for signal termination. However, regeneration is not constrained to recovery of lost parts, which are rare and often caused by accidents, but also occurs in the renewal of cells that become damaged due to altered homeostatic conditions. On the other hand, in all instances, regeneration, whether reparative or physiological, stands as a widespread and advantageous trait of survival [21].

Different models for understanding regeneration, from invertebrates to mammals, are currently used and have provided considerable insight into the molecular mechanisms underlying regeneration, which seem to be remarkably conserved. A key question nowadays is how cells trigger nuclear reprogramming and transcriptional programs specific for regeneration. In this review, we explore the current understanding of changes in chromatin dynamics that contribute to the regenerative response of *Drosophila* imaginal discs. Imaginal discs are epithelial sacs that are the primordia of adult appendages and other cuticular structures. Imaginal discs can also serve as an example of reparative regeneration, since they are capable to regenerate after damage (reviewed in [19,20,22,23]). Damage can be induced physically, by microsurgery, or genetically, by genetic induction of cell death (reviewed in [20,24]). Whether discs undergo regeneration in the wild after cell death induced by cold or heat shocks, or other natural distresses, is currently unknown.

2. Signaling to chromatin

Damage, one way or another, activates regeneration programs in the living cells that proliferate to recover the injured area. These regeneration programs include several signaling pathways that are essential for normal development. Soon after an injury occurs, damaged cells release signals as reactive oxygen species (ROS), calcium waves or bioelectrical stimuli that are sensed as pro-regenerative signals by the adjacent living cells [25–31]. At the same time, injury causes inflammation, which results in the recruitment of immune cells to the wounded area. These immune cells release cytokines that are also sensed as pro-regenerative signals [32–37]. Altogether, these signals ultimately regulate the activation of signaling pathways such as, JNK and p38, WNT, Jak-STAT, EGFR/Ras/MAPK or Hippo [29,31,38–45]. Upon receiving such input, cells undergo extensive changes in chromatin activity and switch their transcriptional programs to rebuild the tissue that has been lost or injured (Fig. 1).

Chromatin plays a key role in determining the cellular fate and identity. Changes in chromatin dynamics underlie cell plasticity (the capacity of cells to take on characteristics of other cells) following injury [46,47]. The number and type of cells to be restored, as well as the source of new cells, may be different, however, in different species. Planarians, for example, use a population of stem cells called neoblasts that self-renew and also generate new cell types (reviewed in [48–50]). Among vertebrates, lens regeneration is basically restricted to some amphibians; whereas in frogs, the lens is regenerated by transdifferentiation of the cornea. Meanwhile, pigment epithelial cells of the newt dorsal iris can regenerate a new lens via transdifferentiation [51]. In other systems, such as *Hydra*, a combination of the two, stem cells and transdifferentiation processes, is required [52,53]. In the regenerating zebrafish heart, existing differentiated cardiomyocytes undergo dedifferentiation, reduce their contractile state and start cell division to generate new cardiomyocytes that replace lost heart mass [54,55]. The mammalian liver is also able to regenerate after injury in order to maintain proper homeostasis. This regeneration process consists of hepatocytes undergoing compensatory hyperplasia (an increase in cell number) as well as an increase in cell size (reviewed in [56]). Recent studies provide evidence that adult fly tissues such as flight muscles and the gut may also harbor quiescent stem cells, which can regenerate the tissue upon injury [57]. In the case of the wing disc, in addition to an increase in proliferation near the damaged tissue, there is reorientation of cell division and local respecification of vein and intervein fates [40,58,59].

Determining how signaling pathways integrate with chromatin dynamics during regeneration would provide a basis to understand the cell plasticity that is required to allow reconstitution of the missing tissue while disc identity is maintained [60]. During the course of regeneration, imaginal disc cells may also undergo transdetermination: a process whereby determined cells change their fate to that of a different disc identity [60–62]. In addition, transgression of compartment borders has been reported after massive damage in one compartment of the wing imaginal disc; the transgressing cells are genetically reprogrammed and acquire a new identity [63].

3. Signaling integration

The outcome of signaling is the transcriptional regulation of target genes that will elicit the final response. After entering the nucleus, the transmitted signals may modulate the activity of transcription factors that together with chromatin-remodelers and modifying enzymes and other epigenetic pathways, including non-coding RNAs (ncRNAs), will influence chromatin structure and activity in the cells contributing to the regeneration process (Fig. 1).

The fact that adult fruit flies cannot reset the transcriptional programs needed for regeneration could be explained by differences in the spatial and temporal regulation of gene expression, and may not be a consequence of the genes encoded in their genome. If regenerative signals are not properly sensed or integrated into the genome, then the

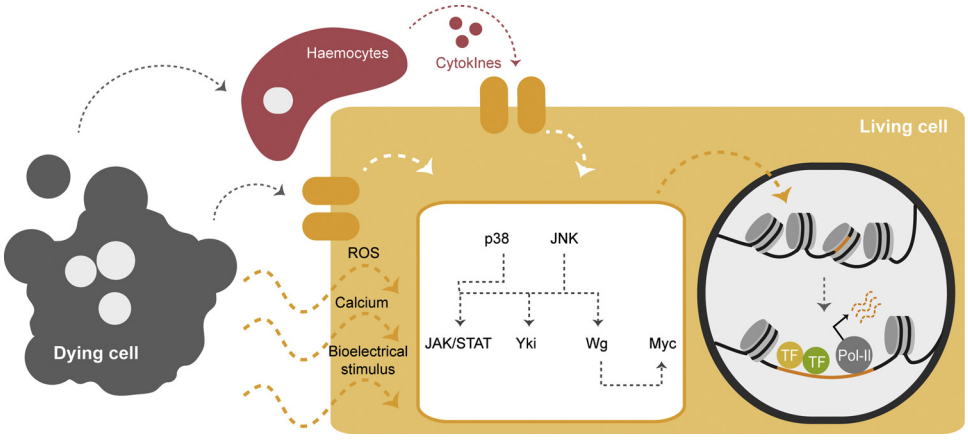

Fig. 1. Early regeneration signals. Depiction of how a living cell can sense different signals (ROS, calcium, bioelectrical stimuli, inflammatory signals and the release of different ligands). These signals are released from the damaged tissue (dying cell) to promote regeneration. As a consequence, several signaling pathways are activated. These are integrated into the nucleus to promote the transcription of regeneration genes. TF: transcription factor; Pol-II: RNA polymerase II.

whole process fails. Indeed, it has previously been hypothesized that the chromatin landscape could determine the capacity for regeneration. Species that have acquired fully repressed states upon maturation would not allow the transcription of genes involved in regeneration. In contrast, animals that allow switches in chromatin states could more easily reprogram gene expression to cover the emerging needs of regeneration (reviewed in [64]).

3.1. Polycomb and Trithorax groups in regeneration

Historically, one of the most studied regulatory systems of chromatin-modifying factors in charge of the chromatin states involves the Polycomb group (PcG) and the Trithorax group (TrxG) of proteins, which act antagonistically to orchestrate the expression of key developmental genes. Originally discovered in *Drosophila,* PcG and TrxG are evolutionarily conserved proteins that play a role in cellular memory systems that maintain specific patterns of gene expression: repressed or active respectively (reviewed in [65]). Repressed states are best described by the presence of the repressive mark H3K27me3, meanwhile active states are depleted of it and enriched in other as H3K4me3 and H3K27ac. Proteins belonging to TrxG and PcG have been proven to be crucial to the process of regeneration by integrating signaling cues into the chromatin.

Studies in mouse skin epithelium have demonstrated that the depletion of epigenetic silencing mediated by PcG proteins helps to mediate upregulation of repair genes, after physical injury. Moreover, upregulation of H3K27 demethylases of the TrxG (Utx histone demethylase (UTX) and JmjC domain-containing protein 3 (JMJD3) is required in the blastema area to promote gene expression [66]. Similarly, studies in the fly indicate that transdifferentiation events in regeneration require an enhanced transcription state in which silencing is weakened by the coordinated action of the JNK pathway and PcG/TrxG members [67]. More recently it has been found that animals that are heterozygous for *trithorax* (*trx*) are unable to maintain activation of a developmental checkpoint that allows regeneration to occur. This defect is likely to be caused by abnormally high expression of *puckered (puc)*, a negative regulator of Jun N-terminal kinase (JNK) signaling, at the wound site [68]. Additionally, it was discovered that the chromatin regulator Taranis (Tara), which belongs to the TrxG, stabilizes compartmental identities during the same transdifferentiation events [69]. In zebrafish fin regeneration, bivalent promoters containing H3K4me3 and H3K27me3 histone modifications are converted to an active state by the action of an H3K27me3 demethylase [70]. Finally, it has been shown that separate modules of the WNT enhancer mediate damage response and age-dependent silencing in wing imaginal disc regeneration. PcG-mediated silencing of this enhancer limits the damage-responsive *wg* expression in mature discs [71]. Altogether, signaling cues are sensed by the PcG and TrxG to allow the relaxed and active chromatin state in charge of the transcriptome of regeneration.

3.2. The transcriptome of regeneration

The regeneration transcriptome seems to be best described as the modulation of transcript levels of genes already expressed, rather than initiation of gene transcription *de novo*. For instance, in newt lens regeneration, RNA-seq results show that dorsal and ventral irides, which present different regeneration capacities, mostly differ in the number of transcripts, rather than their uniqueness [72]. In adult skeletal muscle, severe trauma induces transcriptional and post-transcriptional regulation of both coding and non-coding transcripts [73]. Moreover, transcriptomic studies in zebrafish and *Drosophila* have reported that such gene modulation is a burst of transcription that mainly occurs during the early regeneration stage and reverts over time [36,74,75]. In addition, by studying the transcriptome of regenerating wing imaginal discs, we found that there is global co-regulation of genes involved in regeneration; genes induced early on tend to be located close to one another in the linear genome. These clusters of upregulated genes are enriched in signaling pathway genes [75]. This observation suggests that co-regulation of gene expression could represent an efficient mechanism, as several clustered genes could be turned on at once by the same regulatory event. The fact that gene transcription is mostly explained by gene modulation indicates that regulatory elements, such as regeneration enhancers, could play a crucial role in the regulation of gene expression during the restoration process (reviewed in [76]).

4. Enhancers as key regulatory elements in regeneration

Enhancers are *cis*-regulatory DNA-regions that control gene expression. Some examples of specific damage-responsive enhancers have been reported in *Drosophila* and zebrafish in the context of regeneration. After damage, ectopic expression of *WNT* genes is found in early wing imaginal discs. This injury-induced expression is regulated by a bipartite enhancer at the *WNT* locus that is required for proper regeneration [58,71,77]. Transcriptomic analysis and epigenetic profiling have also revealed that the *leptin b* gene (*lepb*) is strongly induced in regenerating hearts and fins of zebrafish; and a short DNA sequence element upstream and distal to *lepb* has been identified as a tissue regeneration enhancer element (TREE) [78]. By combining chromatin accessibility analysis with functional studies, the regulatory regions involved in whole-body regeneration have been recently characterized in Acoels [79].

Several properties of enhancers underlie their regulatory function. When bound to specific proteins, enhancers increase the level of transcription of an associated gene independently of the orientation and distance to its core promoter. Despite their position, enhancers can target their promoters through chromatin loops, which bring them in close spatial proximity [80–83]. It has been suggested that chromatin loops assemble an active chromatin hub, providing a more supportive environment for transcription than that created by transcription factors bound directly to their promoter alone. Indeed, many enhancer–promoter combinations usually share binding sites for common transcription factors, potentially leading to eRNA transcription (reviewed in [84,85]). The regulatory information provided by enhancers is encoded in short sequences that are recognized by transcription factors which bind to them and recruit cofactors, thus forming a complex that ultimately mediates activation of gene transcription [86–88] (Fig. 2A).

Many features that determine enhancer activity have been characterized; yet none of them seems to be a universal trait. Although active enhancers are located at accessible positions within the chromatin, enhancers *per se* are found in a default *off* state, determined by nucleosome positioning; they only become accessible under specific environmental conditions [89,90]. Chromatin accessibility is thus a key requirement for gene regulation and is one of the features that best predict enhancer activation [91].

When active, both, enhancers or promoters, are nucleosome depleted; however, the histones in the flanking nucleosomes often carry post-translational modifications, which provide a useful readout of enhancer activity. In active chromatin states, promoters are usually marked with H3K4me3, enhancers with H3K4me1, and both of them with H3K27ac [87,92–95]. Changes in H3K56ac seem an indicator of enhancer activation on the response to Notch signaling [96]. Furthermore, in silent chromatin states, promoters and enhancers are labeled with H3K27me3 (reviewed in [65,97]); while H3K9me3 is found in silent heterochromatin regions [98]. Thanks to the combinatorial action of histone marks, other chromatin states have been predicted. For instance, poised bivalent enhancers are those containing both H3K4me1 and H3K27me3 [99]; while latent enhancers are those that are not labeled with any type of mark, which means they can only be activated upon stimulation through signaling pathways [100].

Although histone modifications are one of the best predictors of enhancer activity, they present two major weak points. Usually, there is

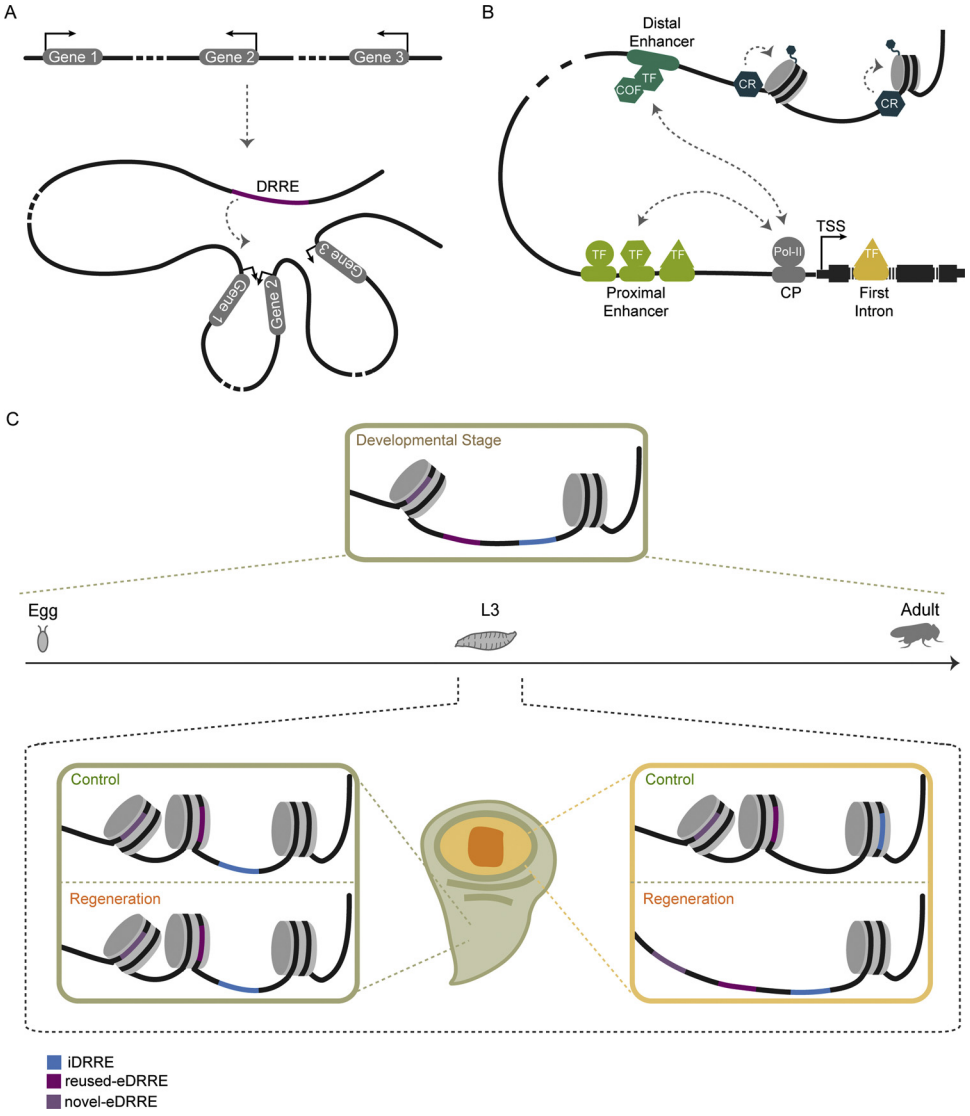

Fig. 2. Chromatin regeneration hubs. **a)** Illustration showing a putative chromatin regeneration hub. Spatial proximity generated by chromatin loops brings three genes, located at different genomic locations, into close contact, so they can be co-regulated by a single eDRRE; **b)** Illustration showing the three DRRE types and their accessibility patterns in control, regeneration and other tissues and stages. The model does not reflect a real situation, in that enhancers are not necessarily located in the genomic distribution depicted. TF: transcription factor; COF: co-factor; Pol-II: RNA polymerase II; CR: chromatin remodelers; DRREs: damage-responsive regulatory elements; L3: third instar larva.

a correlation between histone modifications and states; however, there is no mark or combination that perfectly matches any one state. For example, around 40% of *Drosophila* embryonic enhancers lack H3K27ac, yet they are active [101]. Moreover, there is no evidence that such marks are either sufficient or necessary for transcription. One recent study in *Drosophila* has demonstrated that correlation does not imply causation; and that it is not in fact the mark (H3K4me1) that is required for transcription, but the histone methyltransferase governing that mark [102,103]. Additionally, it has been demonstrated that transcription can occur in the absence of histone marks in promoters of regulated genes in *Drosophila* [104].

Transcription factors bound to enhancer sequences also provide information on enhancer activity. Often, each enhancer is bound to a specific transcription factor or to a particular combination of them [105]. Together with the search for universal rules for enhancer activation, other properties have been studied. Enhancers possess some inherent promoter capacity and can recruit Pol-II and transcription factors [106] leading to the transcription of enhancer-RNA (eRNA) [107–109]. In a recent study, it was demonstrated that the degree of enhancer or promoter activity is reflected in the level and directionality of eRNA transcription in the fruit fly [110].

Finally, one of the most fascinating properties of enhancers is their functional autonomy: their capacity to retain transcription-activating functions outside their endogenous loci ([111]; reviewed in [88,94]. Taking advantage of this feature and by using reporter lines, enhancer activation has been described in cells involved in regeneration in both zebrafish and *Drosophila* [71,75,78].

4.1. Enhancer accessibility during regeneration

Different types of regulatory elements have recently been identified based on their accessibility in zebrafish heart (TREEs) and *Drosophila* wing disc regeneration, using high- throughput genome-wide analyses such as chromatin profiling and ATAC-seq [75,112]. In the case of fruit flies, three main types of damage-responsive regulatory elements (DRREs) have been defined [75] (Fig. 2B). The first, named *increasing* DRREs (iDRREs), are found in chromatin regions that are already accessible in the wild-type tissue under study, but become more accessible during regeneration, indicating fine-tuning of gene expression. The damage enhancer found at the WNT locus in regeneration of the wing disc [71] is included in this class. Although it may seem that these enhancers are already active before regeneration occurs, analysis using reporter lines has shown that compared to their basal activation pattern, some iDRREs are indeed ectopically activated in the wound [75].

The second type of DRREs have been named *emerging* DRREs (eDRREs) because they correspond to open regions only detected after damage [75]. Some eDRREs can be further classified into *reused* eDRREs, which are co-opted from other developmental stages or tissues

and reused in regeneration and *novel* eDRREs, which act exclusively in the damaged tissue. *Reused* eDRREs account for around 50% of the enhancers thought to be specific to wing disc regeneration [75]. As mentioned, during regeneration, cells have to undergo respecification and proliferate to replace lost tissue; in this manner, the tissue recapitulates some necessary developmental traits. Reactivation of two embryonic enhancers in the epicardial cell layer of zebrafish and mouse hearts explains how some epicardial genes that are transcriptionally activated during embryonic development can be re-induced after injury [113]. Similarly, an enhancer that triggers *Bone morphogenetic protein 5* (*Bmp5*) expression during mouse skeletal development is also used in bone repair. Interestingly, the same enhancer is sufficient to trigger gene expression in mesenchymal and epithelial cells in multiple tissues, suggesting that it might contain an injury-responsive enhancer element [114].

Novel eDRREs account for almost 50%, approximately, of the enhancers thought to be specific of wing disc regeneration [75]. This last category could, in theory, represent unique regeneration enhancers. The *leptin B* enhancer found in zebrafish regeneration seems to be this type of enhancer: it has been proved that it plays a crucial role in regeneration, but it does not seem to be required at all, either during development or in basal heart homeostasis [78]. However, further comparative analysis with more tissues and stages is needed to allow us to refine our knowledge of the occurrence of the different enhancer types.

4.2. Post-translational chromatin modifications at regeneration enhancers

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) has been used to map globally the chromatin state of various cis-regulatory elements at different times after acute muscle trauma and the total number of enhancer elements has been determined based on the presence of H3K4me1 and H3K27ac [73]. Similarly, ChIP-seq experiments on such histone marks have demonstrated that 90% and 50% of DRREs in zebrafish heart and wing disc respectively, are marked during regeneration [75,112]. Intriguingly, despite some DRREs not being marked by any of the common modifications, they are still activated upon damage [75]. On the other hand, the need to lose repressive modifications seems to be crucial for activation [66,67,69,70].

4.3. Transcription factors: the link between signaling and chromatin

Accumulating evidence from many model systems indicates that the combinatorial interplay of multiple transcription factors, each with its own partially overlapping temporal window of expression, is a prominent regulator of context-specific binding. A recent study has identified four transcription factors involved in the de novo epithelialization of skin ulcers in mice [115]. Moreover, some studies indicate that there could be global regulatory rules, represented as codes for motif composition that ultimately determine enhancer activity (reviewed in [116]. Such codes are simultaneously regulated by the transcription factors found at each time in the cell. Hence, variations in this spectrum will generate different target gene expression profiles in different cells. Thus, transcription factors can induce alterations and enable patterning through development in the same cell type (reviewed in [105,116]). The same kind of logic is postulated for regeneration, where a plethora of these proteins are specifically activated upon damage, leading to a variation of the preexistent spectrum. As mentioned above, regeneration does not depend only on the genome sequence but on genome activity and cell physiology. Cell physiology, represented as pro-regeneration signals, governs the activation of the set of specific transcription factors that help to modulate genome activity through the recognition of their binding sites, written as codes of motif composition in enhancers.

Motif analysis has enabled to identify the binding sites required in zebrafish heart and fly wing disc regeneration. RUNX2B, STAT1, RFX2, SPI1 and NFBKB are the transcription factors whose binding sites are the most enriched within the enhancers required in zebrafish heart regeneration; whereas STAT92E, Trl, Fd68A and Grh motifs are those most enriched within *Drosophila*'s wing disc regeneration enhancers [75,112]. Moreover, the recent comparison of transcriptomic data obtained from three different organs in three different species (mouse liver, zebrafish heart and *Drosophila* wing disc) has resulted in identifying a set of 21 transcription factors conserved and upregulated in all three of them [75,112,117]. Interestingly, some of these transcription factors (STAT92E, Sd, and Myb*)* present a motif enriched in DRREs.

5. Conclusions and future challenges

Drosophila is currently shedding some light into the basic and conserved mechanisms behind the regenerative process, although it is still not clear if imaginal discs can regenerate in the wild under adverse conditions. Comparative studies between model organisms should provide valuable information to help us understand how transcription associated with regeneration is regulated.

An increase in transcription that correlates with several changes associated to chromatin dynamics has been observed after damage in the wing imaginal disc. These change include: increased chromatin accessibility, increase in the expression levels of specific transcription factors and formation of new chromatin loops, and loss of repressive chromatin modifications (Fig. 3).

New approaches should provide more insight into the how changes in chromatin dynamics contribute to changes in cell plasticity during the regeneration process: 1) Using high-throughput techniques, such as 4C and High-C conformation capture, that allow us to interrogate the 3D structure of the genome. The information this could provide would be important to map the contacts between different chromosomal regions, such as those between enhancers and promoters; 2) Genome

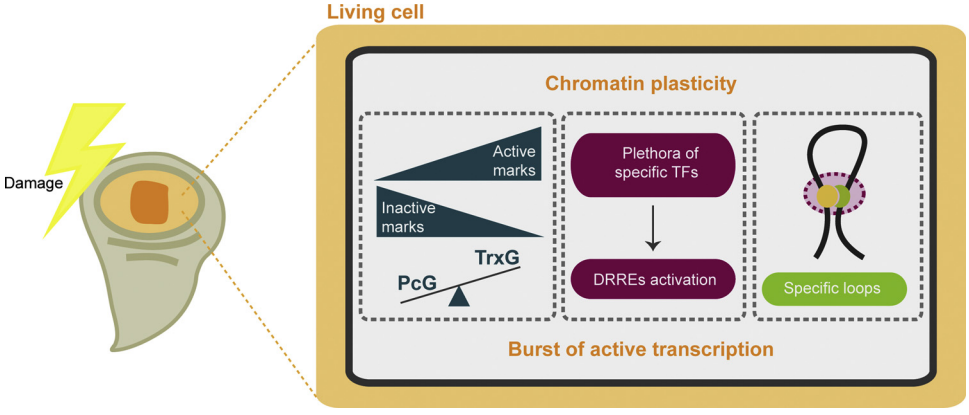

Fig. 3. Changes in transcription and chromatin plasticity after damage. An increase in transcription in cells responding to damage correlates with several changes associated to chromatin dynamics and increase of cell plasticity. These changes include: loss of repressive chromatin modifications, increase in the expression levels of specific transcription factors and formation of new chromatin loops.

editing, using the CRISPR/Cas9 system or similar ones, is needed to validate the requirement for regeneration-associated enhancers as well as to demonstrate that their removal affects transcription; the same CRISPR technology may be a useful tool to modify enhancer architecture or to study the role of specific histone modifications at enhancers. 3) Determining the contribution of the non-coding genome to regeneration. Information on such transcripts is already available for the systems on which RNA-seq has been performed. Focusing on transcripts that are induced during regeneration and producing the appropriate mutations should provide instrumental information regarding their potential contribution to the process.

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