reactions. Intermediary metabolism, for example, is run by enzymatic machines that are revved up and down by allosteric responses to the binding of small molecules. And in bacteria we know of one set of genes that is not regulated by recruitment: the inactive promoters bear a special form of tightly-bound RNA polymerase, and the activator uses energy in the form of ATP to turn on transcription. In this system the basal level of transcription is vanishingly low, and so no repressor is required (or found). But as we encounter ever more complex organisms (and leave intermediary metabolism essentially unchanged) we find increasing roles played by the kinds of binding reactions discussed here. According to the following line of argument, this should not be surprising.

In *The Origin of Species* Darwin was, paradoxical as it might at first seem, looking at the simplest task evolution undertook — the elaboration of plants and animals. Unlike the evolution of bacteria that grow in disparate environments, the 'recent' evolution of these complex organisms required few new enzymatic activities — we have essentially the same set of such activities as do flies and other animals and plants. It's as though once evolution had produced the enzymes found in eukaryotic cells, including those that make/break binding sites, it was easy to quickly deploy these enzymes, using recruiting reactions — specificity determinants — to different ends.

Development of higher organisms is made possible by elaborate programs of intercellular signaling, and the signals are usually in the form of proteins or other macromolecules. The reiterated use of binding reactions to give meaning to these signals, as we have seen, comes with unavoidable dangers. Things can go awry in many ways, and, unfortunately, it can be hard to decipher what has gone wrong in any given case, and even harder to fix it. It would be easier if we had been intelligently designed and were made of neat machines. Like Ferrari engines.

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Primer

Transcriptional autoregulation in development

Stephen T. Crews and Joseph C. Pearson

A sober moderation stands sure, no violent extremities endure.

Charles Aleyn, 'The Battaile of Crescey' (1633)

One of the key features of animal development is the precise control of gene expression. This can range from regulating rapid changes of transcript levels in both space and time to maintaining concentrations at a constant level for extended developmental periods. This latter case includes stabilizing or locking-in the transcriptional pattern of differentiated cell types. Much of the complexity of transcriptional control involves the interplay of transcriptional activators and repressors on *cis*-regulatory modules that reside close to target genes: these consist of transcription factor binding sites with specific arrangements and affinities that integrate the contributions from combinations of transcription factors, leading to transcriptional output. Many developmentally important transcription factors control sizeable gene batteries. Consequently, it is important that their levels are tightly controlled so that target genes can dependably perform their specific developmental functions. Numerous examples exist in which developmental defects are observed when levels of important transcription factors are only mildly altered by genetic lesions. These include the thoracic phenotype caused by a *Drosophila Ultrabithorax* (*Ubx*) homeotic mutation in just a single copy (haploinsufficiency), and human developmental disorders of the skeleton (cleidocranial dysplasia) and heart (Holt-Oram syndrome) caused by haploinsufficient reductions in the RUNX2 and TBX5 transcription factors, respectively.

There are a number of biochemical rationales for why levels of transcription factors are maintained within defined limits during

Box 1. Phage lambda — genetic switches and autoregulation.

Autoregulatory loops underlie one of the classic genetic switches, the decision by bacteriophage lambda to enter either a lytic or lysogenic phase. The transcriptional mechanisms that govern this decision are elegantly described in Mark Ptashne's book '*A Genetic Switch: Phage Lambda Revisited*', and provide useful paradigms for studying the roles of autoregulation during development. The key regulatory molecules are the CI and Cro DNA-binding proteins. The CI protein (the lambda repressor) is required for lysogeny, in which lambda remains dormant. Expression of *cro*, repressed by CI, is required for bacterial lysis and viral production. The CI protein positively regulates its own expression by cooperatively binding to adjacent operator binding sites (O_R1 and O_R2) and recruiting RNA polymerase. It also negatively regulates its own expression when CI levels increase by binding to a low affinity site (O_R3) that blocks RNA polymerase binding. These feedback loops stabilize CI protein levels. This is important, because CI levels must be kept low enough so that inactivation of CI will efficiently occur when environmental conditions dictate lytic growth. As CI levels decline during induction of the lytic phase, it can no longer repress the adjacent *cro* gene. Cro, which is a repressor, binds O_R3 to block *cI* transcription, and then later negatively regulates its own expression. Thus, the balance between lysogeny and lysis is dictated by a bistable system of double-negative autoregulation in which CI and Cro can repress each other's expression (Figure 2A). This system is well-suited for decisively responding when bacterial conditions change. These regulatory events are an excellent example of how the arrangement and affinities of transcription factor binding sites, and cooperative interactions between transcription factors mediate regulatory phenomena.

development, and there are also multiple mechanisms that control those levels. Cooperative interactions between transcription factors are a key feature of gene regulation and can contribute to the rapid changes in gene expression that are often dictated by cell signaling events. Cooperativity can only occur when the levels of transcription factors are kept below concentrations in which a single transcription factor could activate transcription by itself. In other cases, the levels of transcription factors can directly influence their activity. For example, low concentrations of the *Drosophila* Krüppel zinc finger protein activate transcription, whereas high levels lead to homodimerization and repression of transcription.

One mechanism that evolved to refine and maintain transcription factor levels is autoregulation, a transcriptional strategy employed from bacteriophage (Box 1) to humans to control genetic pathways. It has been argued that the occurrence of autoregulation positively correlates with the developmental or physiological importance of the transcription factor. Thus, master developmental regulators that control large numbers of genes will be autoregulated, because their levels of expression must be tightly controlled. Similarly, autoregulation of important developmental regulatory proteins may be important in maintaining cell fate and differentiation — its self-maintaining property means that it can mediate the epigenetic memory of a differentiation state, as classically shown in the case of the lambda (epi)genetic switch (Box 1). Autoregulatory mechanisms come in a diverse variety of forms: they can be positive or negative; they may be direct or indirect; they can mediate maintenance or refinement; and they can involve simple or complex *cis*-regulatory modules. And they have been studied using a variety of molecular and genetic methods (Box 2). This primer summarizes the multiplicity of autoregulatory modes used during animal development, and compares autoregulation with the kind of transcriptional regulation mediated by Polycomb (Pc) and Trithorax (Trx) proteins, which have also been implicated in the transcriptional 'memory' that maintains cell states over time and through cell division.

Positive autoregulation

Positive autoregulation, in which a transcription factor either directly or indirectly activates its own expression, results in maintenance of transcription in the absence of the factors that initiated expression (Figure 1A–C). One common role involves sustaining the expression of target genes throughout a developmental period — for example, controlling expression of genes that mediate cell adhesion of a developing tissue. Another function is developmental patterning, in which expression is reinforced in some cells and not others, leading to differences in cell fates. Positive autoregulation can also be used in a quantitative role to boost expression levels — this appears to be a function of the *Drosophila fushi tarazu* autoregulatory upstream element, which increases *fushi tarazu* stripe levels many-fold. While autoregulation can maintain transcription levels, it is not irreversible and can be altered or extinguished by regulatory inputs.

Positive autoregulation thus provides flexibility in addition to stability. One consequence is that expression of some autoregulated genes is maintained for long time intervals, while in other cases it is maintained for relatively short time periods. Positive autoregulation is a common feature of developmental transcription factors with dozens of examples. There are, however, also instances of developmentally-expressed transcription factors that do not obviously autoregulate. And in some cases transcription factors autoregulate in some cell types in

Box 2. Methodology.

Genetic and molecular approaches are commonly used to study autoregulation. Analysis of loss-of-function mutants of transcription factor genes for alterations in their own expression is frequently employed, and the direction of those changes (down or up) reveal whether the autoregulation is positive or negative. Experiments using transgenic organisms in which the transcription factor is overexpressed are similarly useful. Direct autoregulation (in which the transcription factor binds to *cis*-regulatory modules within its own regulatory DNA; Figure 1A) is demonstrated by mutating transcription factor binding sites within the *cis*-regulatory module and assaying expression of a reporter transgene. The use of whole-genome *in vivo* chromatin immunoprecipitation or DNA adenine methyltransferase identification approaches to identify direct target genes of a transcription factor can reveal binding within its own gene (as well as that of other genes). Carrying out these experiments throughout development can assess the timing and duration of autoregulation.

which they are expressed, but not in others. Autoregulation is often direct, but, in other cases, it is achieved indirectly, involving intercellular signaling pathways. One alternative pathway to positive autoregulation is the use of a double-negative feedback loop. The next several examples demonstrate the general features of positive and double-negative autoregulation.

Autoregulatory and cross-regulatory circuits

The *Myocyte enhancing factor 2* (*Mef2*) and *twist* (*twi*) genes are important regulators of *Drosophila* embryonic muscle development. Both maintain expression throughout embryonic development and control hundreds of muscle-expressed genes. Two genome-wide studies employed chromatin immunoprecipitation to identify Mef2 and Twist (Twi) binding sites at multiple stages of embryonic development. Both Mef2 and Twi proteins are expressed during early phases of mesodermal development. The *twi* gene is expressed first and may act as a competence factor to allow the mesodermal developmental program to advance; its expression is extinguished before muscle cell differentiation. The *Mef2* gene is activated by Twi, and then remains on throughout mesodermal development, including the period of muscle differentiation.

Thus, both genes are expressed over extended developmental periods, necessitating a mechanism to maintain their expression. Each protein binds to its own gene, suggesting direct autoactivation, something previously suggested for both proteins from the results of experiments using other genetic and molecular approaches. Interestingly, both Mef2 and Twi proteins also bind to each other's gene: this illustrates a regulatory paradigm that can apply to autoregulation: the feed-forward loop (Figure 1B). In this case, Twi activates expression of *Mef2*, and Mef2 and Twi act together to regulate expression of muscle-expressed genes, including themselves. Why use two transcription factors to maintain expression, instead of just one? It is argued that a dual-component autoregulatory/cross-regulatory loop is more resistant to noise and system fluctuations than a single-component autoregulatory loop, because of the requirement that the second component (*Mef2*) must be activated by the initial component (*twi*).

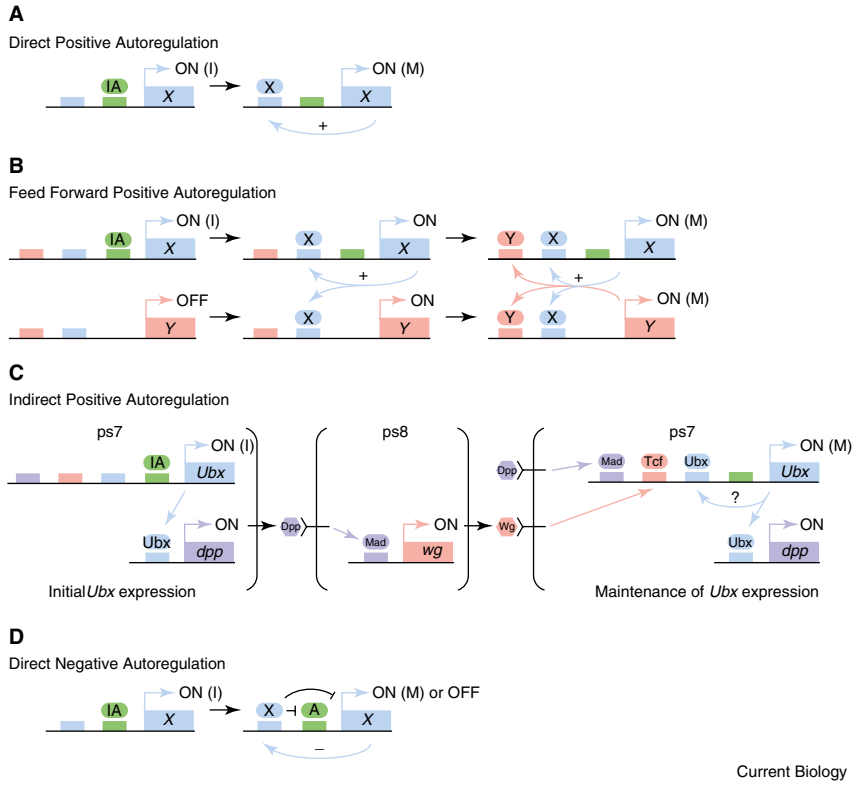

Figure 1. Paradigms of developmental autoregulation.

(A) An initial transcriptional activator (IA) initiates (I) expression of Gene *X* (box with arrow), which maintains (M) its expression by direct binding. (B) Feed-forward positive autoregulation involves Gene *X* activating expression of Gene *Y*, followed by autoregulatory and cross-regulatory binding to maintain expression of both genes. (C) Autoregulation of *Ubx* expression in mesodermal parasegment 7 (ps7) requires an intersegmental signaling loop with: Ubx activation of *dpp* expression in ps7; Dpp activation of *wg* expression in ps8; and Wg and Dpp activation of *Ubx* expression in ps7. The Mad and Tcf proteins mediate the transcriptional response of Dpp and Wg signaling, respectively. Ubx may also directly autoregulate. (D) Expression of the negative autoregulatory protein is initiated by a transcriptional activator (IA). The autorepressor reduces expression of an activator (A), and, depending on the strength of repression, this results in either the maintenance of expression (M) or the termination of expression (OFF).

In another well-studied example, six important regulators of human hepatocyte transcription were examined by chromatin immunoprecipitation. Five of the six transcription factors — HNF1α, HNF4α, HNF6, FOXA2 and CREB1 — were found to be bound to their own promoters, and most of these promoters also bound at least one of the other factors. This study reinforced the view that autoregulation is both common and multi-component.

Structure of autoregulatory *cis*-regulatory modules

Just as nearly all transcription factors that act during development are present in multiple cell types at various developmental times, autoregulation itself is also often spatially and temporally restricted — that is, operates in some cell types and not others. Even the same transcription factor gene can be positively or negatively autoregulated depending on the cell type. Thus, most autoregulatory *cis*-regulatory modules are unlikely to simply be multiple binding sites for the autoregulating transcription factor, but rather are complex, as in the case of Mef2 and Twi, with additional binding sites for co-regulatory proteins that may be present or absent in particular cell types. As with any *cis*-regulatory module, positive autoregulatory *cis*-regulatory modules control gene expression according to the nature of the module, in a way that is determined by parameters such as the strength of DNA binding, the number of binding sites, and interactions with additional transcription factors.

Genes can have multiple autoregulatory *cis*-regulatory modules. The *Drosophila Distal-less* (*Dll*) gene, for example, has a promoter-distal autoregulatory fragment, LT, which maintains *Dll* expression in head and thoracic primordia during embryogenesis. During larval development, however, another promoter-proximal fragment, M, is required for autoregulation throughout the leg disc. The M fragment contains Dll binding sites, but also requires sequences within LT to maintain expression, even though LT itself is unable by itself to maintain expression throughout the disc. The *Drosophila Deformed* (*Dfd*) homeobox gene has at least five autoregulatory *cis*-regulatory modules; each controls subdomains of *Dfd* ectodermal expression, and their sum comprises much of the *Dfd*-dependent expression pattern.

Refining morphogenetic signals

Early developmental patterning often involves an initial transcriptional response to a morphogenetic gradient followed by refinement of the expression domain. One mechanism that can facilitate refinement is autoregulation. If maintenance of expression requires a certain threshold for activation, then areas of weak initial expression will not be maintained, whereas regions of higher expression will be strengthened, which leads to refinement.

As an example, during mouse embryogenesis, an initial gradient of retinoic acid emanating from the mesoderm induces gene expression of three *Hox* genes — *Hoxb3*, *Hoxb4* and *Hoxd4* — in the hindbrain. Interestingly, another key component, the *Retinoic acid receptor β* (*Rarb*) nuclear receptor gene, is also activated in a similar pattern by retinoic acid signaling (via different retinoic acid receptors). The diffuse anterior expression of these genes is subsequently refined to a sharp border at the rhombomere 6/7 boundary by direct *Hox* and RARβ autoregulatory and cross-regulatory interactions. This feedback circuit is able to refine the initial graded pattern into a well-defined, robust domain of expression.

Indirect positive autoregulation

An alternative regulatory mode, indirect autoregulation, involves a transcription factor which activates target genes which, in turn, maintain expression of the transcription factor via cell signaling (Figure 1C). The *Drosophila Ubx* gene, for example, is expressed in a segmental pattern in the mesoderm: it is absent in parasegment 8 and present in parasegment 7, where it plays a key role in midgut development. Expression of *Ubx* in parasegment 7 is controlled by cell-signaling pathways that are influenced by positive, indirect autoregulation. *Ubx* activates expression of *decapentaplegic* (*dpp*), and Dpp signaling activates expression of *wingless* (*wg*) in the adjacent parasegment 8. The Wg protein signals back to parasegment 7 and activates expression of *Ubx*. Dpp also signals within parasegment 7, activating *Ubx* expression, and *Ubx* may also directly activate its own expression. Thus, there exist multiple autoregulatory inputs controlling *Ubx* expression levels, and *Ubx* levels are coordinated with the signaling pathways that create sharp segmental boundaries in the mesoderm.

Double-negative feedback autoregulation

Just as positive autoregulation can maintain levels of gene expression, a double-negative feedback loop containing two repressors can achieve the same goal. The phage lambda CI and Cro repressors use this mode of regulation to control the switch between lytic and lysogenic pathways (see Box 1; Figure 2A). In the nematode *Caenorhabditis elegans*, the fates of two functionally different chemosensory neurons, ASEL and ASER, are stabilized by a double-negative microRNA (miRNA) and transcription factor feedback loop (Figure 2B). The DIE-1 transcription factor controls expression of ASEL/ASER-specific gene expression: when *die-1* expression is ON, ASEL-specific expression occurs; when *die-1* is OFF, ASER-specific expression appears. The COG-1 and DIE-1 transcription factors and *lsy-6* and *mir-273* miRNA genes constitute a double-negative feedback loop that maintains expression of *lsy-6* and *die-1* expression in ASEL and *cog-1* and *mir-273* expression in ASER. It will be interesting to see how often double-negative feedback loops and miRNAs are employed as constituents of autoregulatory feedback loops in other developmental decisions.

Comparison with Trx-mediated transcriptional maintenance

As initially described for *Drosophila Hox* gene expression, *Trx* (*trx*) and associated *trx* group genes are required for the maintenance, but not the initiation, of expression of many genes that are important in development. This is, of course, a function also carried out by positive autoregulatory loops. In the case of *Ubx* expression, gap and pair-rule genes initiate segmental *Ubx* expression in the embryonic ectoderm, and that expression is subsequently refined by cross-repressive *Hox* gene interactions. While the gap and pair-rule proteins quickly disappear, maintenance of *Ubx* expression is sustained in part by action of the Trx group proteins. They form multi-protein complexes capable of modifying nucleosomal histones, remodeling chromatin, and contributing to the general transcription machinery.

There are a number of issues regarding Trx regulation relevant to autoregulation. Trx group proteins can maintain expression of target genes for extended periods of time, including cycles of cell division. How is this achieved? Do the Trx group proteins require the continued presence of target-specific transcriptional activators, or do they act by themselves (see Ptashne, 2007)? Thus, in the case of transcription factor gene regulation, do Trx group proteins work in conjunction with positive autoregulation, or do individual genes use autoregulation in some cell types and Trx regulation in others? If so, are the two mechanisms functionally different in their control of gene expression; for example, is one more amenable to transcriptional plasticity than the other? What are the relative numbers of genes regulated by each mechanism?

Comprehensive answers to these questions remain unknown, and will require systematic genetic and molecular studies, but there are relevant data available. Genome-wide molecular and bioinformatic data suggest that dozens, possibly hundreds, of genes are regulated by Trx. But there is also genetic evidence that many genes do not require Trx for transcriptional maintenance, and

that Trx regulation can be cell-type specific. The *Ubx* gene maintains expression via a Trx-independent positive autoregulatory loop in the visceral mesoderm, yet requires Trx for stable expression in the ectoderm. Both *Dfd* and *Dll* also autoactivate independently of Trx. These results indicate that at least some genes employing autoregulatory loops do not require *trx* for transcriptional maintenance, although other types of chromatin maintenance complexes devoid of Trx could be involved — for example, the Trx-related (Trr) protein may control the expression of different sets of genes than Trx.

With regard to functional differences between the operations of autoregulatory loops and Trx, one possibility is that maintenance by Trx is more sustained and less dynamic than autoregulatory loops. One potential example involves the *Drosophila engrailed* (*en*) gene, which is expressed in three phases. It is first activated early in embryogenesis by pair-rule genes, and then modulated by Wg signaling and En autoregulation. However, this expression is transient: *en* expression persists only in a subset of cells, and this long-lived expression requires Trx. Thus, an earlier autoregulatory phase is short-lived, whereas a Trx-mediated phase is prolonged. Additional comparative data or direct experimental tests will ultimately indicate the relative merits of each mechanism.

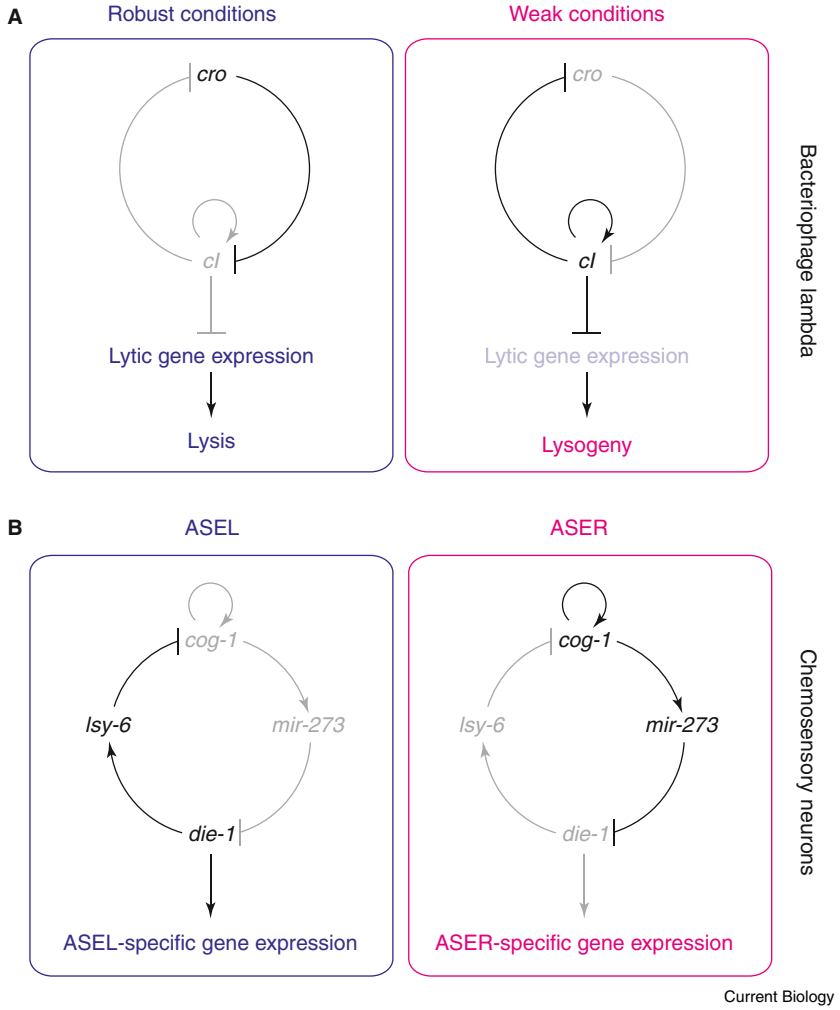

Figure 2. Double-negative autoregulatory loops.

(A) Double-negative feedback loop underlies the phage lambda lysis/lysogenic switch. Active circuits are indicated by dark lines and genes, and inactive circuits and genes are gray. (B) Double-negative feedback loops consisting of transcription factors (COG-1 and DIE-1) and miRNAs (*lsy-6* and *mir-273*) control differences in *Caenorhabditis elegans* ASEL and ASER chemosensory cell fates and gene expression. The schematic is adapted from Johnston *et al.* (2005), which can be consulted for details along with Ptashne (2004).

Negative autoregulation

Negative autoregulation is a common mechanism of developmental control in which transcription factors repress their own expression (Figure 1D). Theoretical and experimental observations have revealed that negative autoregulation is an effective mechanism for homeostatically controlling expression levels, something classically established for the lambda repressor (Box 1). Autorepression can also lead to termination of expression: this may occur when repressor binding is sufficiently strong to extinguish expression. In principle, the strength of autorepression could also influence the timing of gene expression, with the stronger the repression, the shorter the time interval that transcription persists. Negative autoregulation will generally require the presence of transcriptional activators, whose expression is then repressed, since eukaryotic basal transcription is usually low.

Transcription levels and embryonic patterning

Negative autoregulatory loops can influence embryonic patterning. The *Drosophila Ubx* gene is negatively autoregulated in multiple embryonic cell types and imaginal tissues, and the degree of autorepression varies depending on cell type. *Ubx* expression is absent in segment T2p of the imaginal leg disc, and genetic experiments have shown that T2p repression is dependent on *Ubx*. The T2p repression is functionally relevant, because ectopic *Ubx* expression was found to result in transformation of T2p into the more posterior segment type T3p. Thus, *Ubx* abolishes its own expression, and this repression may be further maintained by non-Ubx factors, such as Pc (see below).

In a less extreme mode, *Ubx* employs negative autoregulation to stabilize expression in other cell types. This autorepression is also likely to be developmentally relevant, as increases in *Ubx* gene dosage lead to developmental abnormalities, such as changes in haltere size. Not surprising for a gene as developmentally complex and important as *Ubx*, it shows both positive and negative autoregulation. Similarly, other

Drosophila homeotic genes and vertebrate *Hox* genes — as well as the lambda repressor (Box 1) — show both positive and negative autoregulation.

Indirect negative autoregulation and developmental patterning

Indirect negative feedback loops are used in developmental patterning. During *Drosophila* oogenesis, the *broad* (*br*) gene, which encodes a zinc-finger transcription factor, is initially expressed throughout the dorsal follicle cells (roof cells) that surround the developing oocyte. Its pattern is refined during oogenesis, and ultimately extinguished. One component of the refined pattern is an indirect negative autoregulatory loop, in which Br first activates expression of the Thick-vein (Tkv) Dpp receptor in the roof cells. Dpp signaling from adjacent anterior follicle cells activates Tkv, which via phosphorylated Mad — an intracellular component of the signaling pathway downstream of Tkv — represses *br* expression. Thus, both negative and positive autoregulation can be mediated via indirect signaling mechanisms to influence developmental patterning.

Comparison with Pc-mediated repression

The study of Pc and related Pc group of repressor proteins began with the discovery that *Drosophila Pc* mutants are unable to maintain *Hox* gene repression. Recent work has shown that Pc group proteins form two complexes: PRC1 and PRC2. These complexes work together to modify histones into a repressive state. They act at specific sites in the genome referred to as *Pc* response elements. Do Pc group proteins work with negative autoregulation? In some cases, they may. The *Drosophila myc* (*dmyc*) gene negatively autoregulates its own levels of expression (as does vertebrate c-*myc*), and this autorepression requires Pc. In addition, many *dmyc* target genes are also regulated by Pc.

In a more extreme case in which negative autoregulation terminates expression, Pc group proteins are good candidates for being the components that maintain repression, as the autorepressor is no longer present. For example, both *Ubx* and *Pc* mutants result in ectopic *Ubx* expression in imaginal discs. One interpretation is that, in some cells, Ubx initially autorepresses, and this is followed by repression by Pc group proteins. Consistent with this model, when high levels of Ubx are achieved by transient overexpression, this can result in a *Pc*-dependent silencing of *Ubx* expression. Mechanistically, it was proposed that *Ubx* transcription extends through a nearby *Pc* response element, thus inhibiting the formation of repressive Pc complexes. When Ubx levels are sufficiently high to abolish *Ubx* transcription, the *Pc* response element is now able to recruit an active Pc complex that leads to permanent repression.

Another intriguing case of negative autoregulation concerns the Pc group and Trx group genes themselves. One component of PRC1, the *Posterior sex combs* (*Psc*) gene product (a RING-finger protein required for histone ubiquitination), negatively regulates its own expression. Similarly, the *Trx-like* gene, which encodes the GAGA factor transcription factor, is a key element of the Trx group proteins, and negatively autoregulates its expression. Since some (but not all) Pc group and Trx group mutants are dosage-sensitive, the negative autoregulation may help maintain expression levels below potentially deleterious concentrations.

Final thoughts

As the regulatory circuitry is revealed for an increasing number of developmental pathways, the mechanisms and functions of autoregulation will be further illuminated by both genome-scale projects and smaller, focused approaches. Large-scale transgenic identification of *cis*-regulatory modules is in progress, and will likely reveal many autoregulatory *cis*-regulatory modules. Whole-genome chromatin immunoprecipitation experiments performed with numerous transcription factors will reveal sites of direct autoregulation. Genetic studies of transcription factor or reporter gene expression in their respective mutant backgrounds will provide evidence for autoregulation, and indicate the relative frequencies of autoactivation and autorepression during development.

Mechanistically, it will be possible to compare autoregulatory *cis*-regulatory modules of different transcription factors for similar binding site configurations, and to understand how the autoregulatory *cis*-regulatory modules compare to each other and to non-autoregulatory *cis*-regulatory modules residing on target genes for each transcription factor. Phylogenetic studies will provide insight into how autoregulatory circuits change during evolution, both functionally and mechanistically. Rapid progress is being achieved in understanding Pc group/Trx group proteins, and this will allow a more complete assessment of the relative contributions (or convergence) of autoregulatory loops and chromatin-based maintenance/repression mechanisms. Finally, it is widely assumed that autoregulatory loops lock-in developmental states. While reasonable, critical tests have generally been lacking: this needs to be assessed *in vivo* by specifically abolishing autoregulation and assaying developmental stability.

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Further reading

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