Trends in Genetics

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Spotlight

Silencers, Enhancers, and the Multifunctional Regulatory Genome

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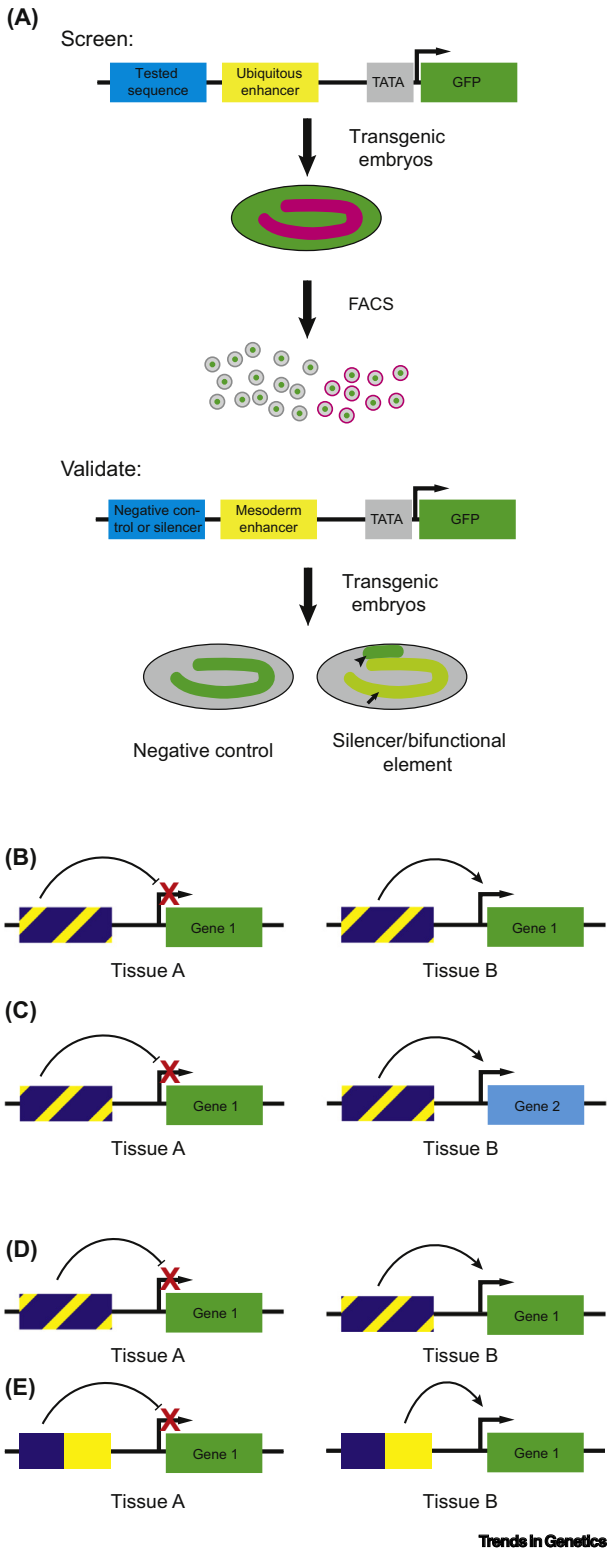
Negative regulation of gene expression by transcriptional silencers has been difficult to study due to limited defined examples. A new study by Gisselbrecht *et al.* has dramatically increased the number of identified silencers and reveals that they are bifunctional regulatory sequences that also act as gene expression-promoting enhancers.

Exquisite spatial and temporal control of gene expression is a hallmark of metazoan development. Much of this regulation is carried out by distal positive-acting 'enhancers', a well-studied class of *cis*-regulatory elements with a substantial literature exploring their function. No less important, but less well-studied, is negative gene regulation. The action of short-range repressors bound at enhancers to keep them in an 'off' state is one common mechanism [1], but at least two other forms of negative regulation have been described: Polycomb-mediated silencing and transcriptional 'silencer' elements. Proteins of the Polycomb group (PcG) mediate chromatin-based gene silencing marked by histone 3 lysine 27 trimethylation (H3K27me3), which often spreads broadly throughout the silenced locus [2]. In *Drosophila*, PcG proteins are recruited via a specific class of negative *cis*-regulatory element, the Polycomb response element (PRE), whereas the method of targeted PcG recruitment remains unclear in mammalian cells. By contrast, transcriptional silencers are the negative-regulatory counterpart of enhancers: defined *cis*-regulatory sequences that repress transcription from otherwise active promoters [3]. Although initially described over 30 years ago, the difficulty of assaying for silencers has resulted in a paucity of clearly defined examples, and little understanding of their characteristics and mechanism of action. However, a recent first-of-its-kind large-scale screen has now led to a significant increase in the number of known silencers [4]. Rather than being the negative counterparts to enhancers, the data suggest that most, if not all, silencers are in fact enhancers, and that activation and silencing are merely two sides of the same *cis*-regulatory coin.

Gisselbrecht *et al.* [4] screened for silencers by placing putative silencer sequences upstream of a reporter cassette comprising a ubiquitous enhancer driving GFP expression. By looking for tissue-specific (in the *Drosophila* mesoderm) reductions in GFP activity (Figure 1A), their assay was able to detect sequences with an affirmative silencing ability while ruling out nonsilencer mechanisms, such as short-range repression, failure to activate transcription, and insulator activity (problems that have confounded previous studies). Close to 10% of >300 assayed sequences (29) were revealed to be silencers. No particular histone modification signature was associated with these sequences, consistent with the idea that commonly cited sets of histone marks may not in fact be sufficient to reliably identify specific regulatory sequences [5]. There was little overlap with Polycomb-bound PREs, and there were no universally present transcription factor-binding sites or bound co-repressors (based on extensive existing data sets). However, 40% of the sequences were co-enriched for a set of known mesodermal repressors, including *snail* (*sna*), and mutation of *sna* binding sites abrogated silencer function. Interestingly, the presence or absence of *sna* binding appeared to divide the identified silencers into two functional classes: Hi-C studies demonstrated that non-*sna*-binding silencers made contact with their target-gene promoters, whereas *sna*-binding silencers failed to do so. Remarkably, all but one of the identified silencers were known, or shown, to function as enhancers, based on reporter gene analysis, in a different tissue.

Although such 'bifunctional' elements have been observed previously, they had appeared to be exceptions rather than the rule. However, current results imply that all silencers may also be enhancers. By contrast, most known enhancers tested in [4] did not show silencer activity. It remains unknown whether most enhancers therefore lack this ability or whether these enhancers have silencing activity in a tissue or time point not observable in the study.

Could the silencing results merely be an artifact of the reporter gene assay used? This was addressed for one bifunctional silencer via CRISPR-mediated deletion of the genomic sequence. Transcription of the target gene was upregulated, demonstrating that the element functions as a silencer in its native genomic context. However, transcript levels from the tissue where the element functions as an enhancer were not assessed. This is unfortunate, because it leaves open the possibility that the enhancer activity is an artifact of the reporter gene assay. Reporter gene assays are considered a 'gold standard' for enhancer function, but it is not in fact known how often they lead to either false-positive (ectopic activity) or false-negative results. Therefore, a combination of both reporter gene and genomic deletion studies might be needed to provide final definitive proof of bifunctionality [5,6]. More comprehensive studies of deleted putative bifunctional elements would also be able to ascertain whether silencer and enhancer functions are directed to the same target gene, which cannot be determined from the reporter gene assay (Figure 1B,C). However, recent studies in *Heliconius* butterflies confirm that this is at least sometimes the case [7]. While not seeking to define



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silencers, CRISPR-based knockout of a putative regulatory sequence revealed instances of both gained and lost expression from what appears to be a single gene, suggesting the presence of a bifunctional regulatory element. These results highlight the power and promise of CRISPR-mediated genome editing for interrogating putative regulatory sequences and, in particular, its ability to uncover silencers and other negative regulatory sequences. As these methods become increasingly mainstream, we should expect that a richer diversity of regulatory elements will be revealed.

An important unsettled question is whether enhancer/silencer elements are truly bifunctional or whether they instead comprise adjacent silencer and enhancer sequences (Figure 1D,E). Given that the boundaries of regulatory elements, such as enhancers and silencers, are not clearly demarcated based on sequence, only detailed functional dissections can address this question. Even then, the limits of where one regulatory feature ends and another begins can be a matter of interpretation, as revealed recently in conflicting views over pleiotropic versus functionally distinct enhancers [5,8].

The emerging and unprecedented in-depth view of the regulatory genome reveals both that there is a greater complexity and abundance of regulatory elements than previously had been appreciated, and that these regulatory elements may exist more on a continuum of functional types than as a series of discrete features with unique properties. The study by Gisselbrecht *et al.* [4] raises the possibility of both bifunctional and monofunctional enhancers, and their identified silencers appear to fall into at least two mechanistically distinct classes (*sna*-bound, promoter not contacted vs *sna*-unbound, promoter contacting). It is also tempting to speculate that observed differences from many studies at known enhancer regions in,

among others, histone modifications, transcription of enhancer RNAs (eRNA), and binding of mediator or other cofactors (e.g., at 'superenhancers'), may distinguish enhancer subclasses, perhaps with distinct regulatory roles in RNA polymerase II recruitment, release of paused RNA polymerase II, or targeting of genes to nuclear subregions [5,6]. Not only may there be distinct subclasses of enhancers and silencers, but it has also been suggested that there is significant functional overlap between enhancers and promoters [9], promoters and insulators [10], enhancers and PREs [2], and enhancers and silencers [4].

Our understanding of the regulatory genome is still in its infancy, and we should expect to see many 'settled' ideas upended in the coming years as our ability to delve deeply into regulatory features and mechanisms increases.

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Figure 1. Transcriptional Silencers as Bifunctional Regulatory Elements. (A) The screen for silencers used by Gisselbrecht *et al.* [4]. Transgenic flies are constructed bearing the sequences to be tested for silencer activity placed upstream of a ubiquitously active enhancer driving GFP expression ('Screen'). The embryos are dissociated and mesodermal cells assayed by fluorescence activated cell sorting (FACS) based on a red-fluorescent mesodermal cell marker (magenta outline). Cells with reduced GFP expression are indicative of silencing and are sequenced to recover the identity of the silencer sequence. The identified putative silencers are then validated by placing them upstream of a mesoderm-specific enhancer driving GFP in stabile, individual transgenic fly lines ('Validate'). Silencer activity leads to a reduction in mesodermal GFP expression relative to negative controls (light green, arrow). Enhancer function of the same sequences manifests as GFP expression in non-mesodermal tissues (dark green, arrowhead). (B,C) Two possibilities for silencer/enhancer bifunctionality. In (B), the bifunctional regulatory element regulates a single gene, silencing 'gene 1' in tissue A but activating 'gene 1' in tissue B. In (C), the bifunctional element is a silencer with respect to 'gene 1' in tissue A, but an enhancer with respect to 'gene 2' in tissue B. The current data are insufficient to clearly support either model, although the evidence slightly favors the case shown in (B). (D,E) Integrated bifunctional elements versus adjacent silencers and enhancers. In (D), silencer functions (blue) and enhancer functions (yellow) are mixed within a single bifunctional sequence. An alternative is shown in (E), where silencer and enhancer functions reside in separable but adjacent sequence elements.