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Ultraconservation of enhancers is not ultranecessary.

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increased placental size. In summary, vPRC1 has a key role in establishing repressive modifications transmitted from oocytes to embryos.

Genetic deletion of *Eed* (which is essential for PRC2 activity) revealed that while H3K27me3 was globally lost, H2AK119ub1 was largely unaffected in oocytes and morula-stage embryos (Fig. 1c). Exceptionally, H2AK119ub1 was lost over non-canonically imprinted regions and *Xist*^{14,15}. Hence, a notably strong link appears to exist between PRC1 and PRC2 in regulating this class of genes.

Chen et al. interrogated the role of H2AK119ub1 by directly injecting mRNA encoding PR-DUB into fertilized zygotes (Fig. 1d). Interestingly, whereas H2AK119ub1 was lost, H3K27me3 was unaffected, and RNA-sequencing analysis showed no change in imprinted-gene expression in four-cell embryos. Together, the results suggest that PRC1 regulation of imprints probably occurs in oocytes, possibly through PRC2 recruitment, but is dispensable in embryos. Crucially, however, depletion of H2AK119ub1 resulted in premature activation of canonical target

Polycomb-regulated genes and cell arrest at the four-cell stage.

The PR-DUB overexpression experiment strongly indicates that H2AK119ub1 is immensely important in the earliest stages of embryo progression. Both groups made the striking finding that PRC1 regulation at this class of genes occurred seemingly independently of PRC2, thus perhaps explaining the milder PRC2 mutant phenotype. Although formal confirmation remains to be reported, these findings suggest that upstream vPRC1 activity represses Polycomb-regulated genes. However, this finding prompts the question of why PRC2 does not nucleate at these regions soon after fertilization. Are key factors that recruit PRC2 to vPRC1-marked regions absent, or are antagonistic factors perhaps present? Moreover, what is the biological rationale for limiting PRC2 activity at these stages? Finally, what is the mechanistic explanation for the sufficiency of vPRC1 for repression in this window? Future work will ideally address these exciting questions, thus further elucidating Polycomb-mediated gene regulation

in the context of in vivo mammalian development.

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FUNCTIONAL GENOMICS

Ultraconservation of enhancers is not ultranecessary

Stretches of non-coding DNA that have remained identical across millions of years of evolution are typically assumed to have functional regulatory roles that would be compromised by any amount of nucleotide substitution. A new study finds that these ultraconserved regions are more robust to mutagenesis than their level of conservation would suggest.

Maureen Pittman and Katherine S. Pollard

As organisms reproduce, mistakes in DNA replication create novel mutations. Most of these changes are neutral, meaning they are tolerated, and a small percentage of them persist just by chance. In contrast, deleterious mutations that decrease an organism's likelihood of surviving and reproducing are much less likely to persist in the gene pool, owing to a process known as purifying selection^{1,2}. Regions of the genome that are necessary for embryonic development exemplify this phenomenon. They are often conserved, meaning that they are devoid of DNA substitutions even across huge evolutionary

timescales, thus suggesting that every nucleotide is necessary, and any mutations would disrupt their function. A new study from Snetkova et al.³ challenges this idea.

In the non-coding genome, conserved sequences often function as enhancers, which recruit transcription factors present in specific spatiotemporal patterns; the transcription factors in turn recruit the machinery to 'switch on' gene expression in the right place at the right time⁴. Despite this key role, enhancers and their transcription-factor-binding sites are typically redundant and degenerate, thus making them tolerant to small

changes or even complete loss^{5,6}. At odds with this observation is the existence of ultraconserved elements—segments of hundreds of consecutive non-coding DNA base pairs that retain 100% sequence identity between humans and rodents⁷. This apparent contradiction is the subject of the study by Snetkova et al., which shows that several ultraconserved developmental enhancers are robust to mutagenesis.

Enhancer activity is robust to mutagenesis

Snetkova et al. first mutagenized 23 ultraconserved enhancers at variable

Fig. 1 | Diagram of a transgenic mouse embryo at embryonic day 11.5 (akin to those examined by Snetkova et al.). Blue staining indicates enhancer activity in the developing brain.

saturations: mutants had 2%, 5% or 20% of base pairs altered. The authors measured the effects on enhancer function in mouse transgenic reporter assays (Fig. 1) at a time point in embryonic development when the major organs have appeared. Ten were nonfunctional after 20% of the sequence had been mutated—a somewhat unexpected finding given that the sequences are identical in the genomes of humans and mice, which are separated by 65 million years.

Having shown that subsets of base pairs can be mutated without an apparent loss of enhancer activity, the authors investigated the possible role of gain of activity, which could be detrimental in a developmental context in which finely tuned localized expression of genes is key to cellular differentiation. Only two mutants showed activity in new tissues, thereby indicating that gain-of-function mutations are uncommon. However, these experiments tested for new enhancer activity at only a single time point in mouse development.

Snetkova et al. note that these ultraconserved enhancers show evidence of activity at multiple developmental stages. Therefore, the authors repeated a subset of these experiments in mouse embryos at a later time point. For nine enhancer sequences mutagenized at 5% and showing

no decrease in activity at the original time point, five retained normal activity at the later stage, but four showed diminished or complete loss of activity. These four indicate that pleiotropy could contribute to ultraconservation.

How can we know whether ultraconserved enhancers function at time points not observed in these assays? The authors' solution was to create knock-in mouse lines for a subset of mutant enhancers to observe postnatal phenotypes. This approach has the additional advantage of mutating ultraconserved enhancer sequences at their endogenous loci. These experiments showed that 5% of base pairs mutated in an enhancer of *Arx* decreased the dentate gyrus length—the same phenotype observed when the entire sequence was deleted. For another enhancer, three versions of sequence were tested: full enhancer deletion; 5% mutagenesis that caused loss of activity in the transgenic assay; and 5% mutagenesis that did not cause loss of activity in the transgenic assay. Both complete enhancer deletion and replacement by the loss mutant led to increased density of a specific type of interneuron, whereas the no-loss mutant did not. Together, these findings suggest that the transgenic assays translate to organismal phenotypes, although the small number of tested enhancers does not clarify whether this concordance is generalizable.

The mystery of ultraconservation deepens

Given that most tested ultraconserved enhancers can tolerate sequence changes while maintaining necessary regulatory activities, the question arises as to why they defy the expected rates of neutral substitution. One hypothesis is that mutations at these sites may cause phenotypes that compromise fitness in ways not measured in this study, such as the ability to grow to adulthood, find food, escape prey or reproduce. The authors' observation of discordant results at two embryonic time points for approximately half the enhancers hints that we must look across lifespan to understand ultraconservation. In agreement with this idea, many enhancers can function as silencers—sequences that repress rather than activate genes—at different developmental stages. Perhaps it is the repressor activity of these sequences that is intolerant to mutation.

Although this study represents a heroic amount of work, it only scratches the surface of potential functions of

ultraconserved sequences across cellular contexts. Quantifying changes in gene expression as a consequence of many more ultraconserved enhancer mutations in the endogenous loci would be useful. Investigating these questions in more species, cell types and developmental stages would also be important. Such an enormous undertaking is currently limited by the scalability of enhancer assays in whole organisms, although massively parallel versions of enhancer assays and genome editing are possible in cell lines⁸. We expect that decoding ultraconserved elements will also require high-throughput assays for repression, insulation and other functions.

Nevertheless, some avenues for immediate further investigation exist. For example, some species of mammals, such as canines, have evolved slightly different sequences in ultraconserved regions. Perhaps associating the mutated nucleotides with differences in dog physiology could improve understanding of the importance of perfect conservation between rodents and humans.

Snetkova et al. have probed an old puzzle: is perfect sequence conservation necessary for the endogenous activity of ultraconserved enhancers? They find that the answer is often no, at least for measurable phenotypes between fertilization and birth in mice. The most exciting implication that follows from these findings is the possibility that some yet-to-be-identified mechanism might be responsible for the ultraconservation of these enhancers. □

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Competing interests

The authors declare no competing interests.