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Review

The cellular substrate of evolutionary novelty

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SUMMARY

A major challenge in biology is comprehending how complex multicellular novelties evolve. Central to this problem is explaining how qualitatively new phenotypic traits — typically the focus of comparative developmental and macroevolutionary studies above the species level — can become established through population genetic processes. Here, we suggest that a resolution may be found by acknowledging the fundamental entities from which functional organismal phenotypes are constructed. We argue that these are not genes, proteins or cell types, but rather gene expression programs (GEPs): sets of co-expressed transcripts that collectively encode cellular subfunctions. We advance that, because GEPs are the smallest, elemental functional units underlying phenotypes, it follows that they represent the substrate upon which population genetic processes must act to explain the origin of evolutionary novelty at the cellular level and above. Novelty arises through the evolution of novel GEPs, through novel synergisms between GEPs that become co-expressed within the same cell or through interactions between different GEPs juxtaposed in cooperating cells within organs. The revolution in single cell biology offers the chance to trace evolution at the resolution of GEPs in populations and across clades, potentially unifying our view of multicellular phenotypic evolution.

Introduction

Many features of multicellular organisms are exclusive to the lineages that possess them. Evolutionary biologists term such characteristics 'novelties' — functional traits such as insect wings, turtle shells, mammalian placentas and vertebrate camera eyes, which occur in specific taxa but are absent in outgroups or serially homologous body parts[1,2]. Novelties play a leading role in evolutionary diversification: without qualitatively 'new' traits, the biological world would be phenotypically impoverished. Yet, one of evolutionary biology's enduring problems is explaining the origin of these pronounced discontinuities in phenotypic variation[3–8]. First, novelties are often complex traits composed of multiple cell types that together generate emergent, organ-level functions. How structures evolve in which each cell type's contribution is effective only in the context of other, interdependent cell types is difficult to understand[9]. Second, novelties are traditionally the purview of macroevolution: characters that differ between rather than within species (many novelties in fact separate higher taxonomic groups, such as families or phyla). Novelties are thus perplexing given that evolutionary change takes place at the population level through mutation, selection, drift, and recombination operating on genomic loci[10]. How these four forces build complex, multicellular structures, which in the eyes of natural selection are a functionally indivisible unit, is likewise hard to explain.

The field of evolutionary developmental biology (evo-devo)[11] has provided many examples of how modified developmental programs or specific genomic changes have contributed to novelties in particular clades[12]. Yet, if macroevolutionary change is the accumulation of changes happening within populations[13–15], then we must reconcile the establishment of these qualitative, macroevolutionary differences between taxa with the microevolutionary scale at which variation is generated. What is lacking is a rigorous theory of novelty — one that both empiricists and evolutionary theorists can work together to synthesize. Developing such a theory depends on our ability to recognize novelties as they are emerging, so that we may measure them in individual organisms and model their evolution at the population level. A crucial step towards this goal is identifying the entities that should be measured and modeled: the biological 'substrate' from which novelties are built.

Here, we argue that advances in single cell biology have uncovered a key piece of this puzzle. The development of single cell transcriptomics (scRNA-seq) has enabled biologists to profile the transcriptomes of thousands of individual cells simultaneously[16]. scRNA-seq technology has yielded a deluge of cell 'atlases' from different species, revealing the diversity and transcriptomic composition of cell types comprising an organ or whole organism[17–22]. The parallel development of computational methods to analyze scRNA-seq data[23] has led to a key outcome, which is that scRNA-seq data decompose fundamentally not into cell types, but into features termed gene expression programs (GEPs)[24]. A GEP is a set of transcripts that are co-expressed within a cell, and which collectively encode a specific process or activity, referred to as a 'subfunction'. This work is transforming our understanding of how the phenotypic properties of cells are encoded molecularly. We propose that GEPs are precisely the substrate for which evolutionary biologists studying novelty have been searching. Because GEPs encode the basic functions from which cell types, tissues, organs and whole organisms are built, it follows that they are the entities on which population genetic processes must act to build

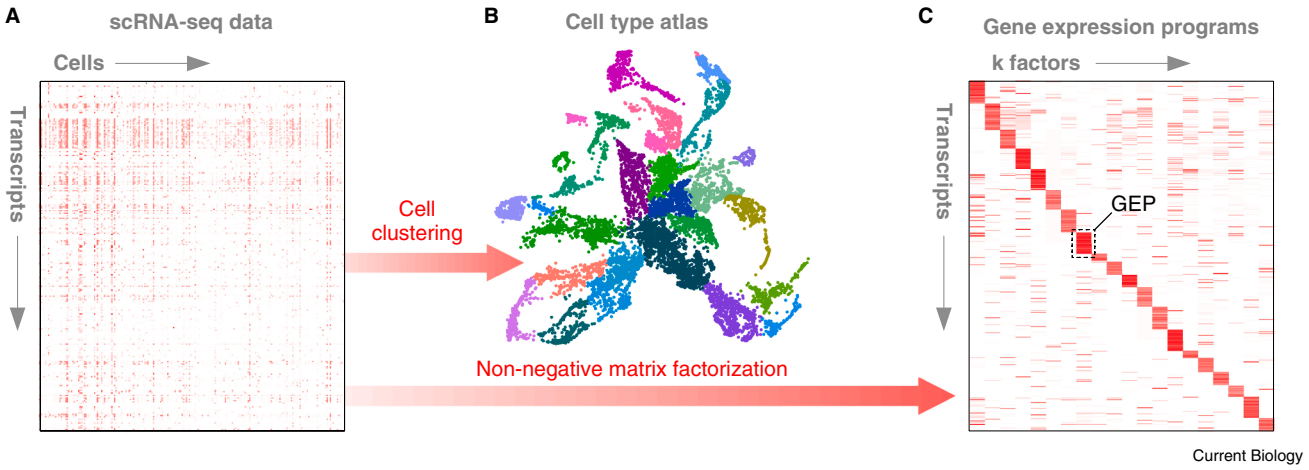

Figure 1. Identifying GEPs in scRNA-seq data.
(A) scRNA-seq of a cell population generates a transcripts × cells matrix. (B) Clustering cells based on similarity of their profiled transcriptomes creates an atlas of cell types, depicted by a uniform manifold approximation and projection (UMAP) plot, with colors corresponding to cell types. (C) Non-negative matrix factorization of scRNA-seq data decomposes gene expression into groups of co-expressed transcripts (k-factors), which correspond to GEPs. See Box 1 for further explanation.

novelties. A GEP-focused view may yield a theory of novelty that unifies our understanding of evolution across temporal scales and levels of organismal complexity.

The GEP framework for studying cell function

The development and application of methods to identify and study GEPs has happened outside of evolutionary biology. It is in the context of biomedical efforts to interpret large-scale scRNA-seq data sets that the fundamental importance of GEPs to understanding cell behavior has been recognized. To understand what a GEP is, and why they are important, consider the following example: patients with severe bacterial or SARS-CoV2 infections can exhibit dysregulated immune responses. By performing scRNA-seq on blood samples from sick and healthy patients, the transcriptomes of thousands of circulating blood and innate immune cells can be sequenced (Figure 1A)[25]. Individual cells can then be grouped based on transcriptomic similarity and classified, yielding an 'atlas' comprising clusters of cells of different type or expression state (Figure 1B). Analyzing these data in a different way, one can factorize the entire transcriptome — encompassing all cell types — into a small number of sets of transcripts that tend to be co-expressed (Figure 1C). This is to say that, across all sequenced cells from the blood of all sick and healthy patients, if a given gene is expressed in some of them, then so too is a relatively constant set of tens to hundreds of other genes (Box 1). Remarkably, factorizing the data in this way reveals that a certain type of monocyte within the immune cell repertoire of infected patients consistently upregulates a specific module of ∼65 genes. The collective action of these genes renders these monocytes immunosuppressive[26].

This module of co-expressed transcripts represents a GEP. The power of using GEPs to study processes within single cells is attested to by the explosion in the number of studies that are examining them. Variation at the level of GEPs has been found to capture the functional heterogeneity of human immune cells[27], as well as their responses to antigens[28] and immunomodulatory drugs[29]. Disease-associated alleles have been shown to exert damaging impacts on cellular function via disruption of specific GEPs[30]. GEPs appear to be both natural units into which scRNA-seq data have been found to decompose, as well as the basic elements that govern how cells function. They are quasi-discrete in nature, meaning that although their core composition is consistent, some transcripts may contribute (pleiotropically) to multiple GEPs, while certain transcripts may be present in one cell type but absent when the same GEP is used in another. GEPs are also multivariate features of the transcriptome: across cells, relative transcript levels in a GEP can vary, as can the level of the entire GEP relative to others comprising the transcriptome. We argue that the GEP framework is useful beyond fundamental cell biology and biomedicine. These quasi-discrete, multivariate components of gene expression also provide a powerful conceptual lens for the study of evolution.

Constructing a novelty using GEPs

To explain how GEPs can help us understand the origin of novelty, we draw on a study system developed in one of our labs (Parker). Rove beetles (Staphylinidae) are small insects, many of which have evolved chemical defense glands[31]. Species within one subfamily, Aleocharinae, possess a tergal gland — a structure within the dorsal abdomen that is composed of two unique secretory cell types (Figure 2A)[32–34]: the 'BQ cells' produce benzoquinones — toxic compounds that activate TRPA1 pain channels[35], but which by themselves are inert solids (Figure 2B). The second cell type, the 'solvent cells', however, produces a mixture of alkanes and esters into which the benzoquinones dissolve, yielding a potent secretion. The solvent cells are continuous with the beetle's exoskeleton, forming a chitin-lined invagination that acts as a common reservoir into which both cell types secrete their products[33] (Figure 2B). The tergal gland is a novelty unique to aleocharines, and how it originated can be explained by investigating evolution at the level of GEPs.

Box 1. Finding GEPs in scRNA-seq data.

Single-cell RNA sequencing (scRNA-seq) provides an unparalleled resolution of the patterns of gene (co)expression. For studying variation at the cellular level, this resolution is essential; earlier technologies for measuring gene co-expression, such as bulk tissue RNA-seq and microarrays, can confound changes in patterns of co-expression between samples with changes in cell-type composition[110]. This technological advance has spurred the development of new methods and adaptations of existing approaches to address unique challenges, including data sparsity, dropout events, and increased technical noise[111]. There are two closely related, yet distinct, types of co-expression patterns extractable from transcriptome data. First, networks of co-expressed genes can be identified from transcript abundance correlations across samples, with methods like weighted gene co-expression network analysis (WGCNA)[112]. Commonly used for bulk RNA sequencing experiments involving different tissues, single-cell specific extensions have recently emerged[113,114].

Second, and more relevant to the study of GEPs, one can infer *modules* of co-expression. A popular example of such a technique is consensus non-negative matrix factorization (cNMF)[24]. cNMF decomposes the gene expression matrix X (transcripts × cells) (Figure 1A) into two non-negative matrices. The first matrix, W, is a transcripts × k factors matrix where each k factor represents a GEP — a set of genes that tend to be co-expressed with each other across the cell population. In the W matrix, genes with high loadings are key components of that GEP (Figure 1C). The 'consensus' approach involves running NMF multiple times with different initializations and combining results to identify consistent patterns. cNMF addresses challenges of scRNA-seq data by incorporating steps such as overdispersion-based gene filtering and stability analysis across runs. The method determines the optimal number of GEPs by assessing factor stability and interpretability across different k values. The second matrix, H, is a k factors × cells matrix (e.g. Figure 2C). This matrix indicates GEP activity in each individual cell. If cells have been independently clustered into cell types based on overall transcriptome similarity (Figure 1B), each cell type's average 'GEP usage' can then be calculated (the relative proportions of its transcriptome made up of different GEPs)[24].

We emphasize that these are still early days in the study of GEPs and that current methods for identifying GEPs in scRNA-seq data offer only a working approximation of the biologically 'true' GEPs that are the products of evolution. And for the study of novelty, there are some major technological hurdles to be overcome. For example, we do not yet know how best to homologize GEPs across samples structured by phylogeny; one source of inspiration may be phylogenetic principal component analyses[115] and related techniques, in which the phylogenetic structure is used to determine the major axes of variation. While scRNA-seq factorization may suffice to recover extant GEPs, other methods must be employed to infer how a GEP assembled ancestrally, or how it may have broken apart in other species. Furthermore, current scRNA-seq methods, though scalable to hundreds of thousands of cells, tend to capture a fraction of each cell's transcriptome (usually a few thousand transcripts per cell)[116,117]. This bias towards highly expressed genes may limit inferences about GEP evolution; indeed, we hypothesize that much of novelty originates from weak, stochastic expression of GEPs in new cellular contexts, at expression levels that may be missed using low read-depth methods. Given the rapid pace of technological innovation, however, we are optimistic that these problems will be solved. Moreover, we speculate that evolutionary information will itself play an increasing role in the bioinformatic identification of GEPs.

First, by generating scRNA-seq data for entire rove beetle abdominal segments, the thousands of genes the beetle expresses within its abdomen can be assigned to different cell types, including neurons, muscle cells, cuticle cells, adipocyte-like fat body cells and cells comprising the tergal gland (Figure 2C). One can also factorize the entire transcriptome into GEPs, as described above (Box 1). In this case, twenty GEPs contribute to the cell types within the rove beetle abdominal segment. Taking these data a step further, one can ask to what extent each cell type's transcriptome is composed, proportionally, from each of these GEPs (Figure 2C). Some cell types use a single, main GEP, which likely confers that cell type's functional identity. Conversely, some GEPs are expressed by most cell types, indicating shared or constitutive functions. Still other cell types, however, are formed from different proportions of GEPs, which is the case for the solvent cells. This cell type is a combination of two GEPs: one — the 'cuticle GEP' — is the principal GEP used by cells comprising the beetle's chitinous exoskeleton (Figure 2C). This is not surprising given that solvent cells form part of the beetle's exoskeleton and secrete chitin themselves (Figure 2B). The second GEP, however, is the main GEP used by two ancient metabolic cell types — the pheromone-producing oenocytes and cells within the beetle's fat body — both of which play conserved roles in fatty acid metabolism in all insects (Figure 2B). This 'fatty acid GEP' includes several dozen enzymes and supporting proteins that carry out the biosynthesis of fatty acids and their modification into alkanes and esters.

The novel solvent cells, then, arose when an ancient GEP that evolved in the oenocytes and fat body became co-opted into cells comprising a region of cuticle, which had previously expressed only the cuticular GEP (Figure 1D). This conferred to cuticular cells the novel ability to synthesize fatty acids, convert them into alkanes or esters and secrete them from the abdomen (Figure 1E).

A 'periodic table' of cellular functions

Solvent cells exemplify how the GEP is the category of feature within a cell's transcriptome that has the property of conferring a specific subcellular process, or subfunction (analogous to an 'apomere' in the terminology of Arendt and colleagues[36,37]). Moving that function into a new cellular context can create a novel feature — in this case, cuticular cells that are also glandular. A cell type can thus be considered the outcome of the different activities encoded by its constituent GEPs. That scRNA-seq data decompose not into cell types,

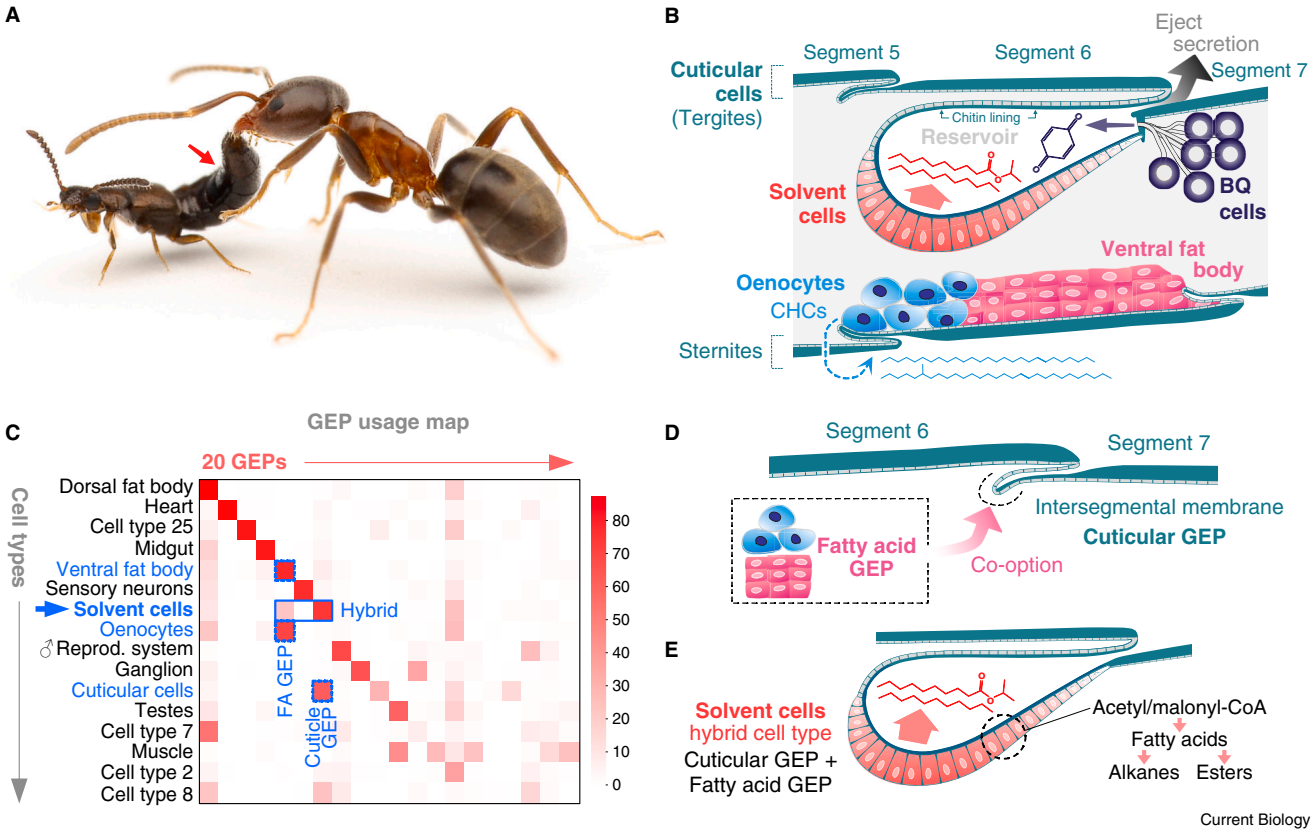

Figure 2. Evolving novelty with GEPs.
(A) Aleocharine rove beetles possess a defensive tergal gland located in the dorsal abdomen (arrow) (photo: T. Shimada). (B) Cartoon of rove beetle abdominal segments showing novel solvent and BQ cells comprising the tergal gland, both secreting their products into a common reservoir between segments 6 and 7. Also shown are more ancient cell types: oenocytes that secrete fatty acid-derived cuticular hydrocarbons, ventral fat body cells that function in fatty acid metabolism, and cuticular cells that secrete the chitinous exoskeleton (with which the solvent cells are continuous). (C) GEP usage map, showing proportional use of 20 GEPs across abdominal cell types. Solvent cells are a hybrid cell type (blue box, solid line) encompassing the cuticle GEP and fatty acid (FA) GEP (blue boxes, dashed lines). (D) Co-option of the fatty acid GEP into a region of intersegmental membrane that previously expressed the cuticular GEP alone. (E) Co-option created a hybrid cell identity — the solvent cells — encoded by both the fatty acid and cuticular GEPs. Chitin-secreting intersegmental membrane cells gained the capacity to synthesize high levels of fatty acids and convert them into alkanes and esters.

but into GEPs, reveals that there is a level of biological organization above genes and transcripts, but below cells and cell types, at which functions become realized. It is at this level — through the activities encoded by GEPs — that the phenotype emerges. One can think of GEPs as modular elements underlying an organism's phenotype, each element requiring co-expression of its constituent loci for a specific function to become realized within a cell. An organism's GEPs are thus analogous to a 'periodic table' of cellular subfunctions, deployed during ontogeny, out of which the organism's cell types are built.

Crucially, if GEPs confer functionality to cells, and thus tissues and organs, then GEPs also render these structures visible to natural selection. It follows that GEPs are the substrate on which selection necessarily acts to build, preserve or modify the function of a cell type, tissue or organ. This insight is critical, because it helps us understand how novel structures originate at the population level. Historically, it has been hard to comprehend how a new multicellular structure that confers a new function might emerge. After all, evolution proceeds as population genetic forces act on allelic variants — a gene-centered view that is both empirically and theoretically well established[38]. GEPs, though, enable us to reconcile the gene-centered view of evolution with the emergence of complex novelties. Understanding how novelties originate and become fixed attributes of species hinges on our capacity to trace the origins, spread and eventual co-regulation of the genes that encode new, function-generating GEPs within evolving populations. It also depends on our being able to follow population-level variation in patterns of GEP usage across the cells of multicellular taxa. And to more realistically understand adaptive evolution in multicellular organisms, we need to comprehend how single alleles can alter the phenotype — not unilaterally, but by modifying the properties of the GEP(s) to which that locus contributes. Taking the periodic table metaphor further, one can envisage different modes by which evolution might act on an organism's repertoire of GEPs, by adding to it, or using existing elements in new ways. Accordingly, we describe three principal modes by which novelty may originate. We outline below examples of each type of novelty, and how each mode may have contributed to formation of the rove beetle tergal gland.

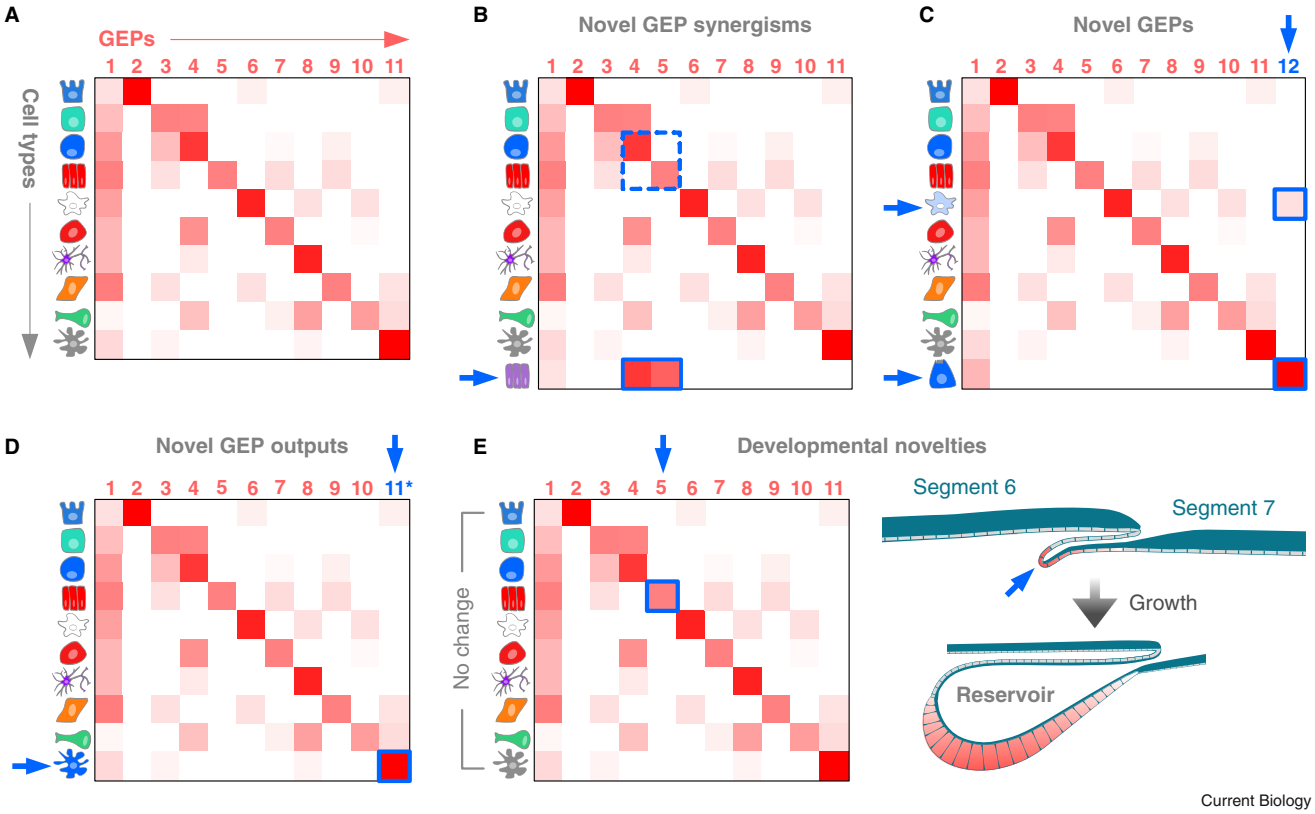

Figure 3. Modes of evolutionary novelty.
(A) GEP usage map with proportional usage of 11 GEPs across 10 cell types. (B) Novelty by new GEP synergism, where GEPs 4 and 5 are combined to create a novel cell type (blue arrow). (C) Novelty by new GEP, used within a novel cell type but also partially by an earlier-evolved cell type (blue arrows). (D) Novelty via modification of an ancestral GEP (11) that qualitatively changes its phenotypic output in cells expressing it (11*). (E) Developmental novelty, where a novel trait arises without a change in the usage map, but developmental processes modify body growth or pattern to create a qualitatively new function. An example is the tergal gland reservoir, formed from a large invagination of solvent cells (depicted as expressing 'GEP 5' for the purpose of explanation).

Mode 1: New GEP synergisms

Combining different GEPs can create synergistic functions and is one mode by which the periodic table of GEPs can fuel novelty (Figure 3A,B). Synergisms between GEPs may emerge from their co-expression within the same cell, as in the case of rove beetle solvent cells, as well as several analogous 'hybrid' cell types that have recently been discovered from studies in other systems[39–44]. Similarly, juxtaposing different GEPs in neighboring cells can also lead to new, local functions emerging from their combined activities. An example is the production of an active defensive cocktail via the fatty acid GEP in solvent cells and the benzoquinone biosynthesis program in BQ cells[33]. Such cooperative systems may in fact themselves derive evolutionarily from ancestral multifunctional or hybrid cell types. This may occur when the constituent GEPs operating within the same cell become split into adjacent, now-functionally interdependent cell types[9,45–48]. This process is termed 'subfunctionalization' and has been proposed as a mechanism by which different cell types evolve division of labor — the defining hallmark of multicellular organs[9,45].

Mode 2: New GEP activities

Cellular novelty can arise with the addition of a new GEP to the 'periodic table' (Figure 3A,C), or the functional divergence of a pre-existing GEP (Figure 3A,D). Factorizing scRNA-seq data from differentiating butterfly wings has identified putative novel GEPs for sculpting the structural morphology of scales, and another for pheromone biosynthesis and secretion from specialized glandular cells that adorn the wings of males[49]. The complex tissue and organ environment of a multicellular organism presents many niches for the evolution of such novel programs. In the rove beetle tergal gland, the solvent cells are thought to have evolved first, potentiating the later evolution of benzoquinones that could be solubilized in the solvent cells' alkane/ester secretion[9,33]. The benzoquinone biosynthesis program is a mosaic of co-opted or duplicated enzymes from different primary metabolic pathways[33,34], hinting at how new GEPs originate within functional niches created by earlier-evolved cell types. Novel functions may also arise as GEPs diverge, either between species or between different cell types from the same species. For example, the fatty acid GEP used by solvent cells has diverged in critical ways from its ancestral usage in the oenocytes. In oenocytes, elongase enzymes are encoded by the GEP, ensuring very-long-chain hydrocarbons are produced that function as waxy contact pheromones. Conversely, in the solvent cells, the GEP encodes no elongases, resulting in a volatile, medium-chain hydrocarbon liquid that is near-optimal for dissolving benzoquinones (Figure 2B)[33].

Mode 3: Developmental novelties

The two modes of novelty described above stem from changes in cellular behavior caused by underlying GEP evolution. Novelty can also result from phenotypic changes above the cellular level. Developmental novelties are traits where an organism's GEPs remain unaltered, but changes in developmental patterning or tissue growth remodel the distribution of cell types or patterns of GEP usage in ways that achieve new functions. For example, the fighting 'horns' of male dung beetles[50–52] and the 'helmet' of treehoppers[53] are considered novelties — the former a sexually selected trait that mediates male–male combat, the latter an adaptation for camouflage on host plants. Both protrusions are outgrowths of the body wall comprising cells of cuticular identity that are not known to be markedly different to those forming other parts of the exoskeleton. These 'novelties' are thus effectively quantitative traits, yet each one confers a new qualitative function at the organismal level. During tergal gland evolution, we speculate that an equivalent process created the reservoir, from an initial patch of solvent cells to a large invagination into which the cells could secrete and store alkanes and esters (Figure 3E). A similar scenario can arise when a previously evolved GEP is developmentally re-deployed in a new location and simply replicates its previous role in the new location. This is the case with the co-option of the pteridine eye pigmentation program in embryonic appendages of water striders, putatively providing aposematic coloration to the embryo[54]. No new function originated at the cellular level, and no novel, cooperative synergism between GEPs emerged, but the organism still acquired a qualitatively new character simply by depositing pteridines in a different place.

A substrate-based view of the phenotype

The three modes of novelty above underscore how GEPs give granularity to phenotypes: they permit organismal differences to be defined concretely in terms of an underlying substrate. We suggest that the GEP framework provides the basis for a theory of novelty across temporal scales. GEPs offer a level of resolution with which we can draw comparisons either within or between species, and in each case objectively characterize those differences in terms of divergence in the composition or usage of GEPs. At the micro-scale, we can pinpoint when a novelty arises in a population as a segregating variant belonging to one of the three modes described above — origination of a novel GEP, for example, or formation of a new hybrid cell type via GEP co-option. We may even infer earlier events that potentiated the novelty — both the prior molecular evolutionary steps and the antecedent population genetic conditions. At the other (macro-) end of the timescale, complex multicellular novelties that distinguish species or higher taxa can be deconstructed as products of the accrual of more such variants (potentially encompassing all three modes of novelty, as illustrated by the tergal gland).

GEPs as 'go-betweens' of gene regulation and cell function

GEPs sit at a unique position in the hierarchy of biological organization. Through the cellular subfunctions they encode, GEPs are direct surrogates of phenotypes defined at the lowest level. Simultaneously, GEPs are the final outputs of developmental gene regulatory networks (GRNs) that operate during ontogeny. For a given organism, then, both the complexity of its phenotype and the dimensionality of its total gene expression space reduce down to a set of GEPs. One can thus conceive of GEPs as operational intermediaries — 'go-betweens' — that connect gene expression to realized functions within cells. Herein lies the powerful utility of GEPs: they enable us to integrate structuralist and functionalist accounts of novelty[55]. The structuralist paradigm traces how novelty arises from evolutionary changes in mechanisms of trait construction and has been the typical purview of GRN-focused studies of character evolution. Conversely, functionalist explanations view phenotypic evolution through the lens of environmental pressures, trait performance and adaptation — why a trait exists, in the form that it does, in service to the organism. Recognition of GEPs as go-betweens, linking these two perspectives, has significance for studying novelty for three reasons.

The GEP gambit

First, as the elemental substrate comprising the phenotype, GEPs permit us to correctly individuate the foci of functionalist explanations of cell-, organ- and organismal-level novelties. They allow us to delineate what selection truly 'sees'. An important corollary is that, to study novelty, GEPs enable us to invoke the phenotypic gambit — or the transcriptomic equivalent thereof. The phenotypic gambit is a powerful heuristic device in evolutionary theory. It asserts that to understand why traits originate and change in frequency within populations, we do not necessarily need detailed information about their entire underlying genetic architecture[56]. The trait itself is the catch-all manifestation of the upstream genetic mechanisms that resulted in its expression. Analogously, if GEPs encode phenotypes at the lowest, most elemental level, we can identify when novelties originate and track how they spread in populations by following GEPs at the resolution of cells within individuals within populations. In other words, we can quantify and model the evolution of novelty without necessarily knowing the precise changes in upstream GRNs that, for example, caused a new GEP to assemble, or led to a GEP becoming co-opted into a new cellular context. GEPs are a proxy both of novel phenotypes and their causal regulatory variants. By invoking the 'GEP gambit', we have a valuable heuristic tool to identify and track novelties at the microevolutionary scale.

Bottom-up GRN inference

Second, if we do want to understand the molecular evolutionary changes within GRNs that bring about novelty, GEPs provide an entry point. Finding causal changes in GRNs can be challenging, because the focus is on upstream signaling pathways, transcription factors and regulatory mutations that may be several steps removed from the phenotype. GEPs, however, demarcate molecular features of novelties that are actually new, offering a tangible manifestation of GRN variation. This permits an actionable, bottom-up approach, exposing terminal loci from which one can extrapolate backwards through regulatory cascades, disentangling changes in GRNs and pinpointing phenotypically relevant mutations[57,58]. Yet, perhaps more radically, by anchoring GRN variation to its phenotypic consequences, GEPs potentially offer functionalist explanations for (traditionally structuralist) accounts of GRN evolution: they enable us to ask why a given novelty is constructed in the fashion we observe. How such a GEP-directed approach can disambiguate GRN

complexity and tie it to cellular phenotype is underscored by a recent study in which the transcriptomes of several human cell types were profiled by scRNA-seq following a broad range of signaling pathway perturbations[29]. By modeling how these perturbations push a cell to use different combinations of GEPs, an underlying GRN architecture could be inferred. This GRN could then be harnessed to predict how GEP usage would respond to novel perturbations — validating its biological reality. One can envision extending this approach, measuring GEP usage across individuals or species, yielding models of cellular evolution that reify GRN variation and the perturbative impact of mutation.

GEPs as the natural outputs of GRNs

Finally, much focus in the study of novelty has been on the relationship between GRNs and cell types[36,59,60], but we suggest it is more precise to think of GRNs as controlling the spatiotemporal deployment of GEPs rather than the specification of cell types *per se*. As discussed above, single cell data reveal the promiscuity of many GEPs across cell types within multicellular organisms; cases of hybrid cell types expressing multiple GEPs are being discovered, as well as the converse situation of GEPs becoming evolutionarily segregated into now-subfunctionalized cell types[9]. GEPs are thus co-optable entities that can be combined with each other in cells but also separated during evolution. GEPs thus comprise the final target loci of GRNs, with cell types, by contrast, emerging as the second order product of differential GEP activation across cells. GRNs culminate in the activities of 'terminal selector' transcription factors[61], or multiprotein complexes thereof (co-regulatory complexes)[36,62]. Accordingly, we argue that terminal selectors activate GEPs as opposed to straightforwardly conferring cell type identities. One may offer counterevidence: several of the best-studied terminal selectors can unilaterally induce particular cell identities when expressed[61], often homeotically suppressing alternative fates[63,64]. Rather than implying a one-to-one correspondence between terminal selectors and cell types, however, we suggest these represent cases where terminal selectors have gained an ability to suppress expression or activity of other GEPs. Consistent with such a scenario, certain terminal selectors that repress each other in mammalian neurons appear to nevertheless function in parallel, within the same cell, in reptiles[47].

Tracing GEP evolution

If GEPs can be studied as quasi-discrete entities, an important question is how they become assembled, both into function-generating mechanisms and into individuated modules that can be co-opted or segregated during evolution. A useful concept for considering this problem is the 'character identity mechanism', which describes an assemblage of biological components that collectively possesses a recognizable activity profile[65]. Individual components within a character identity mechanism may change during evolution, but the assemblage as a whole is nevertheless traceable as a discrete entity across the branches of a phylogenetic tree. GEPs are archetypal character identity mechanisms. Their constituent transcripts may be gained and lost, and both the upstream regulatory inputs and downstream phenotypic outputs may even change. The key point is that, throughout all of this, the GEP's phenotypic output remains unitary — its activity can be studied as a single mechanism. This framework can help us think about the molecular evolution of a GEP 'from cradle to grave'. For example, the genes comprising a GEP may start out with different functions, even in different cell types. Sequentially, the genes become co-expressed within the same cell where their new, collective function as a GEP becomes realized; if the function happens to be adaptive, their visibility to natural selection may sustain their co-expression long enough that they fall under the eventual control of a common transcriptional mechanism. At this point, the GEP may become transformed into a co-optable unit. Conversely, under relaxed selection, the GEP may break apart.

Walking through this hypothetical scenario, a key implication is that a GEP's unitary function may precede its unitary genetic regulation. Gene expression within a cell is influenced by multiple, parallel inputs, including secreted patterning morphogens[66–68], hormones and metabolic cues[69,70], stress responses and immune activity[39,71], transcriptional noise[72–74], intercellular contacts[75,76], as well as mechanical forces[77]. Evolutionary (or environmentally induced) changes in any one of these can bring about variation in gene expression, enabling new functional programs to emerge from the multitude of transcripts that are co-expressed in the cell. Some of these new programs may be adaptive, and their visibility to natural selection may afford sufficient time for mechanisms to evolve that consolidate their transcriptional regulation, creating new, genetically individuated GEPs. For example, stabilizing selection on these parallel inputs may permit their respective transcriptional effectors to eventually evolve protein–protein binding interactions with each other, forming a co-regulatory complex[36]. Similarly, stabilizing selection on a GEP's nascent function may allow DNA binding sites for a pre-existing transcription factor to evolve in the GEP's constituent loci, leading to terminal-selector control. While this may seem like a reversal of logic, the visibility of a collective function to selection may be the sustaining force enabling co-regulatory mechanisms to originate — analogous to the evolution of bacterial operons from previously unclustered loci[78].

This view of GEPs as character identity mechanisms helps relate the GEP concept to earlier ideas in the gene regulation field. Gene collectives that encode cell functions have been referred to as 'regulons'[79,80] — a set of genes co-regulated by the same transcription factor — and 'differentiation gene batteries'[81] — downstream target genes within a GRN, encoding the terminally differentiated state of a cell type. While both regulons and differentiation gene batteries may correspond to GEPs, from an evolutionary standpoint, the functional profile of a GEP may predate its regulation by a single transcriptional mechanism. Furthermore, a GEP may originate and persist within a transcriptome silently, without exerting influence on differentiation. By contrast, the term 'gene expression program' — emanating from the study of single-cell transcriptomics — removes the constraint of tying a GEP to a specific mode of regulation or role in cell differentiation. The term instead emphasizes the modular nature of the GEP itself and the unitary activity it encodes. How the GEP is regulated, and how it functions within cells, can of course be studied: a GEP may evolve into a regulon or differentiation gene battery, and yet will remain a GEP.

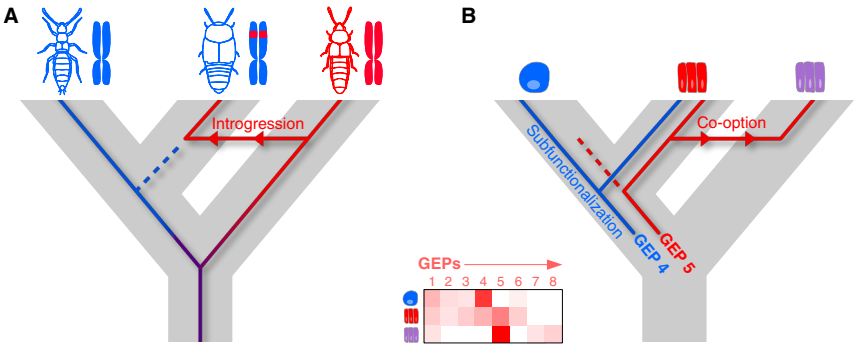

Figure 4. GEP trees in cell type trees.
(A) A three-species tree produced by two sequential speciation events is largely congruent across the species' genomes, but an introgressed chromosomal region from the red species into one of the blue species' genomes supports an alternative topology. (B) A three-cell type tree recovered from whole transcriptome data is incongruent with how the cell types originated via changes in GEP usage. The red and blue cell types have more similar transcriptomes overall compared with the purple cell type (represented by GEP 4 in the tree and additional uniquely shared GEPs 2, 3, and 6 in the heatmap). They became distinct cell types following a subfunctionalization event in which some cells lost GEP 5, yielding the blue cell type. The red cell type retained GEP 5, which was subsequently co-opted into some otherwise transcriptomically distant cells, yielding the purple cell type.

GEP trees in cell-type trees

How do GEPs reframe our view of cell-type evolution? Single-cell methods have recently inspired evolutionary biologists to chart tree-like evolutionary relationships between cell types, both within and between species[19,36,46,82–86]. We agree that tree-thinking is a powerful framework for understanding the origin of cellular novelty, and that a GEP-based view is not in conflict with this endeavor. We think it helpful, however, to draw an analogy between this practice and the discordance that often exists between gene trees and species trees[87]. The possibility that gene trees can have different evolutionary histories (i.e., different topologies) than the species tree, owing to incomplete lineage sorting and other evolutionary processes, has long been recognized by evolutionists. However, in the last decade, as phylogenomics has become routine, it has become apparent that such discordance is the rule rather than the exception[88]. Analogously, one can think of cell types as being like species, and GEPs as genes, so that a cell type's transcriptome is composed of multiple, semi-independently evolving entities. Cell-type trees constructed from whole transcriptome data are thus 'GEP consensus trees'.

Refocusing the study of novelty to the level of GEPs, one may expect frequent incongruence between GEP and cell-type histories. Even if cell types are homologizeable across species based on cell lineage information[89], whole transcriptomic similarity[18,19,86,90], GRNs or molecular markers such as identity-specifying transcription factors[91], they may still embody novelties due to divergence in GEP usage [92]. One source of discordance is GEP co-option — analogous to introgression between species (Figure 4A) — where a GEP that was predominantly expressed in one cell type became co-opted into another (Figures 3B and 4B). Further incongruence will arise from instances in which new GEPs originate within earlier-evolved cell types, or from cases where GEPs are lost or segregate through subfunctionalization (Figures 3C and 4B). Factorization of scRNA-seq has revealed that many GEPs function promiscuously across several cell types, implying that these phenomena are likely to be pervasive. This observation may explain why many non-tree-like topologies are recovered even when whole transcriptome data are used to infer cell-type relationships[90]. In many systems, there is also evidence of 'developmental systems drift'[93] — a possible further source of discord, in which the cell lineages or GRNs of closely related species diverge and yet still generate homologizeable traits, presumably as a consequence of stabilizing selection on the functions generated by GEPs, while the upstream developmental mechanisms have some freedom to evolve under neutrality.

A roadmap for evolutionary single-cell biology

If phenotypes can be meaningfully decomposed into GEP-level granularity, as emerging evidence suggests they can, a number of exciting research avenues open up. We can start thinking about a truly comparative biology of GEPs and envision how we might study their evolution. Collecting the scRNA-seq data necessary to describe the pattern of GEPs within an organism (or even at the level of organs) can be laborious, involving meticulous dissection of organisms, and expensive. Given the massive innovations in single-cell biology, it is clear that at least the latter will quickly change and that research groups will be able to collect scRNA-seq from multiple organs and tissues from multiple individuals and multiple species, as is becoming more common in bulk RNAseq studies[94–97]. With these data in hand, we can ask new empirical questions or address old ones in new ways. Most basically, we could ask descriptive questions (what has been referred to as 'phylogenetic natural history'[98]). For example, how much variation is there in the 'periodic table' of GEPs within versus between species? Can we infer where across phylogeny a GEP became assembled and retrace its stepwise evolution? Within a population, can we detect standing variation in GEPs, identify when GEP-level novelties emerge, and track their frequency over generations?

With these descriptive insights, one could ask fundamental questions about the relative importance of different evolutionary and molecular processes in shaping GEP evolution. This can be done by deriving the phylogenetic expectations for GEP evolution given some set of microevolutionary processes. Several studies[95,99–104] have used the distribution of mRNA transcripts across lineages to draw inferences about the roles of drift and selection in driving divergence[105]. However, with few exceptions[106], these analyses have looked at expression evolution on a gene-by-gene basis. As we have argued throughout, the evolutionary dynamics of individual genes should be highly dependent on the GEP context under which they are used. We anticipate a future set of phylogenetic models for gene

expression evolution that would allow rich inferences into both how the GEP context shapes how selection acts on single genes, as well as how new GEPs originate and how existing GEPs become repurposed in new cellular contexts (Box 1).

Crucial to this roadmap is the simultaneous development of methods to functionally study GEPs. As a concept, GEPs may be tangible entities. Yet, factorizing scRNA-seq data has revealed many GEPs that elude straightforward functional prediction based solely on the transcripts they comprise. It is becoming clear that our search image of subcellular functionality is rudimentary, and we anticipate that the functions of many GEPs will not be intuitive in the way those of the rove beetle solvent cells proved to be (Figure 2D). Computational methods that incorporate prior biological knowledge of gene function may be useful starting points[107,108], but are no substitute for the hard work of genetically or biochemically revealing the cell biological process that a GEP encodes. For the study of novelty, the goal lies beyond this still, in demonstrating how GEPs function as go-betweens of gene regulatory and phenotypic variation. This means on the one hand rigorously connecting GEPs to cellular activities that manifest in (potentially selectable) variation at the organismal level; and on the other, retracing causal GRN variation and phenotypically relevant mutations underlying those changes in GEP activity[57]. Illuminating how molecular evolutionary processes and population genetic forces together build and functionalize GEPs, and alter patterns of GEP usage, will no doubt prove challenging, but such an endeavor is central to establishing a theory of novelty. Encouragingly, GEPs discretize the vastness of an organism's transcriptome into units that can be amenable to this work[33].

Conclusion

The supposed failure of established theoretical models to explain the origin of new organismal features has led some researchers to call for a fundamental rethink of evolutionary theory. Others have dismissed such calls, arguing that the core processes of evolution, which have been recognized for decades, are sufficient to explain all kinds of phenotypic change[109]. While we agree that there is no reason to think that additional processes need to be invoked to explain novelty, we also think the core problem has tended to be explained away. Exceptional work in evo-devo over recent decades has revealed how major differences in organismal form are underlain by changes in the architecture of GRNs. It has, nevertheless, been challenging to study how variations in GRNs spread through populations to establish novelty, or even what, at a functional level, is 'seen' by natural selection. Our thesis is that single-cell technology has led to the new discovery of the existence of quasi-discrete GEPs. This finding provides a clearer path towards addressing the problem of novelty, both empirically by offering us the units to measure, and theoretically by giving us the entities to model. We believe that there is important conceptual overlap between the questions currently being asked by evolutionary biologists, single-cell genomicists and cell biologists. A GEP-centered view of how phenotypes are constructed, function and evolve will be transformational for our understanding of multicellular living systems and can be fully realized by creative interaction between these fields.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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