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Organ Evolution: Emergence of Multicellular Function

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Abstract

Instances of multicellularity across the tree of life have fostered the evolution of complex organs composed of distinct cell types that cooperate, producing emergent biological functions. How organs originate is a fundamental evolutionary problem that has eluded deep mechanistic and conceptual understanding. Here I propose a cell- to organ-level transitions framework, whereby cooperative division of labor originates and becomes entrenched between cell types through a process of functional niche creation, cell-type subfunctionalization, and irreversible ratcheting of cell interdependencies. Comprehending this transition hinges on explaining how these processes unfold molecularly in evolving populations. Recent single-cell transcriptomic studies and analyses of terminal fate specification indicate that cellular functions are conferred by modular gene expression programs. These discrete components of functional variation may be deployed or combined within cells to introduce new properties into multicellular niches, or partitioned across cells to establish division of labor. Tracing gene expression program evolution at the level of single cells in populations may reveal transitions toward organ complexity.

Contents

INTRODUCTION

Across the unicellular backbone of the tree of life, a small handful of clades have undergone the transition to obligate, complex multicellularity (Buss 1987, Fisher et al. 2013, Knoll 2011). These groups include animals (Brunet & King 2017), embryophyte (land) plants (Bowman 2022), brown algae (Cock et al. 2010, 2014), several lineages of fungi (Nagy et al. 2018), and two clades of red algae (Borg et al. 2023). The fundamental trait shared by these taxa is the mutual dependence among the cells comprising the organism—a property that confers individuality to the assemblage of cells as a whole. It is in these lineages where the multicellular condition has become irreversibly entrenched, marking a shift to multilevel selection that embodies a major evolutionary transition (MET) (Okasha 2005, Smith & Szathmáry 1997). Yet perhaps none of these groups would be especially prominent were it not for the second feature that they have in common. This is division of labor—the partitioning of tasks across differentiated cell types (West & Cooper 2016, West et al. 2015). Following the origins of multicellularity in stem groups of these clades, higher-order biological functions became allocated to specialized cells arranged into organ-level structures. It is via this phenomenon—the evolution of organs—that manifest functional and morphological diversity has arisen, enriching the biosphere with macroscopic complexity.

Organs are typically discrete, multicellular structures. Often subjectively delineated, they are nevertheless built from different cell types that operate collectively, generating emergent properties of the organism that no single cell type possesses (Adler et al. 2023, Kishi & Parker 2021). In multicellular taxa, the pattern of organ evolution is one of profound innovation in early branching lineages—a phase that established Bauplans diagnostic of phyla or classes. Within each clade, qualitatively new organs have also continued to originate, even at the lowest phylogenetic divergences separating genera and species. How did ancient organs that typify Bauplans evolve? How have novel ones emerged? To ask these questions is both to enquire how new cell types evolve and to wonder how different cell types engage in cooperative interactions, creating new multicellular functions. Here, I synthesize knowledge of organ evolution and propose a framework that connects molecular variation within populations to the evolution of new cellular functions, cooperation between cell types, and the emergence of organ-level complexity within multicellular clades.

ORGAN EVOLUTION: DEFINING THE PROBLEM

What is an organ? The term lacks an unequivocal definition: Historically, organs have been variously defined by their functions, morphologies, or positions in the body (Minelli 2021, Schmidt-Rhaesa 2007). They are structures that sit at a particular level in the hierarchical organization of multicellular organisms: Organs are composed of cell types that are commonly organized into tissues; organs can themselves be components within larger, multi-organ systems. From an evolutionary standpoint, however, organs possess a defining hallmark: They embody the emergence of cooperative division of labor between cells—a conceptual challenge to explain at whatever scale it arises. Consequently, the term organ is operationally defined herein as the lowest indivisible level of organization at which an organismal function is accomplished via cooperation between cell types. Following this definition, an organ need not be composed of tissues. Insect sensilla, for example, accomplish mechanosensory or chemosensory functions via cooperation between a single sensory neuron, a trichogen (cuticle-secreting) cell, a tormogen (socket-forming) cell, a thecogen (dendritic-cap-forming) cell, and a glial cell (Shields 2004). Each cell type is indispensable for the performance of the sensillum as a functional unit. Similarly, the simplest eyes of insects—ocelli and stemmata—confer light sensitivity through cooperation between four cone cells, two pigment cells, and a small number of photoreceptors (Gilbert 1994). Such minimally complex structures embody the central feature that we wish to explain: functional synergism between cell types that generates an emergent, multicellular activity—one that bestows a distinct phenotypic property onto the organism.

Relatively early in the evolution of each multicellular taxon, stem lineages settled upon organ-level solutions for executing core, organismal functions. These have been mostly conserved over deep time by long-term stabilizing selection. Early in the evolutionary history of land plants, for example, multiple convergent origins of leaves are posited to have occurred in stem group liverworts, lycophytes, ferns, mosses, and angiosperms (flowering plants)—each with differing characteristic morphologies (Harrison 2017, Szövényi et al. 2018). In a similar fashion, roots arose independently in lycophytes and the remaining vascular plants (Euphyllophytes) (Yang et al. 2023). In the Metazoa, major discontinuities in organ composition exist between the 37 phyla, yet each is defined by characteristic sets of organs that have been largely conserved despite the great antiquity and often massive species richness of these clades (Schmidt-Rhaesa 2007). Analogously, across the 19 orders of brown algae (Phaeophyceae), the largest and most structurally complex taxa each possess a stereotyped anatomy comprising a holdfast, a stem-like stipe, and leaf-life blades (Bringloe et al. 2020, Charrier et al. 2012). Interwoven into these ancient Bauplans are evolutionary novel organs, specific to certain lineages. Such structures often facilitate ecological or life history specialization. In animals, profound multicellular innovation exists in their sensory structures (Jacobs et al. 2007, Oteiza & Baldwin 2021), secretory glands (Brückner & Parker 2020, Kishi & Parker 2021), symbiotic organs (Douglas 2020), and diverse types of defensive apparatus (Mackie 1999, Surm & Moran 2021, Zancolli & Casewell 2020). Novel animal organs are not restricted to the organism's periphery, however: In vertebrates, placentas have evolved convergently, potentially 137 times across viviparous lineages (Griffith & Wagner 2017). In plants, organ-level novelties abound in angiosperm floral organs (Specht & Bartlett 2009), floral and extrafloral nectaries (Roy et al. 2017), trichome glands (Wagner 1991), salt glands (Dassanayake & Larkin 2017), and remarkable, prey-trapping pitchers (Fukushima et al. 2017). Brown algae have similarly evolved novel air bladders (pneumatocysts) and specialized reproductive organs (receptacles and conceptacles) (Bringloe et al. 2020).

Attempting to explain the extraordinary diversity of organs across multicellular clades may appear to pose a macroevolutionary problem—a task of accounting for organismal features relevant

to the species level and above. Yet a theory of organ evolution must root such diversity in evolutionary processes operating at the level at which variation arises—that is, within populations. A challenge thus exists in connecting the emergence of new multicellular functions to the fundamental population genetic forces of mutation, selection, and drift. How these processes act on genomic loci to build cooperative structures, where a cell type's role is meaningful only in the context of other, contributing cell types, remains profoundly mysterious. Retracing the origins of cellular cooperation and organ function contrasts with asking how organs evolve structurally through changes in patterning, morphogenesis, and growth—the traditional scope of evolutionary development biology (Arthur 2002). The emphasis is instead on explaining the origins of function-producing interactions between terminally differentiated cell types comprising the individual. What phenomena—mechanistic and population genetic—shaped these multicellular configurations?

THE CELL- TO ORGAN-LEVEL TRANSITION

The change from the cellular to the organ level of biological organization marks a qualitative leap in complexity in which functions are now carried out by groups. A useful framework for comprehending radical shifts in complexity is major evolutionary transitions (METs) theory. In METs, entities capable of independent replication evolve to cooperate, forming a composite individual that can itself replicate (Cooper & West 2018, Michod 1999, Smith & Szathmáry 1997, West et al. 2015) (**Figure 1*a***). Examples include the transition from unicellularity to complex, obligate multicellularity; the origin of essential organelles in eukaryotic cells via endosymbiosis; and the evolution of eusocial insect colonies with reproductive and sterile worker castes (Szathmáry 2015). Common to all METs are three features. The first is mutual dependence. Here, ancestral lower-level entities (e.g., individual cells or solitary insects) sacrifice their propensity for selfish replication in favor of promoting the replication of the higher-order, composite individual (i.e., the multicellular organism, the eukaryotic cell, or the superorganismal colony). The second is division of labor, which, as mentioned above, is the separation of functions across lower-level

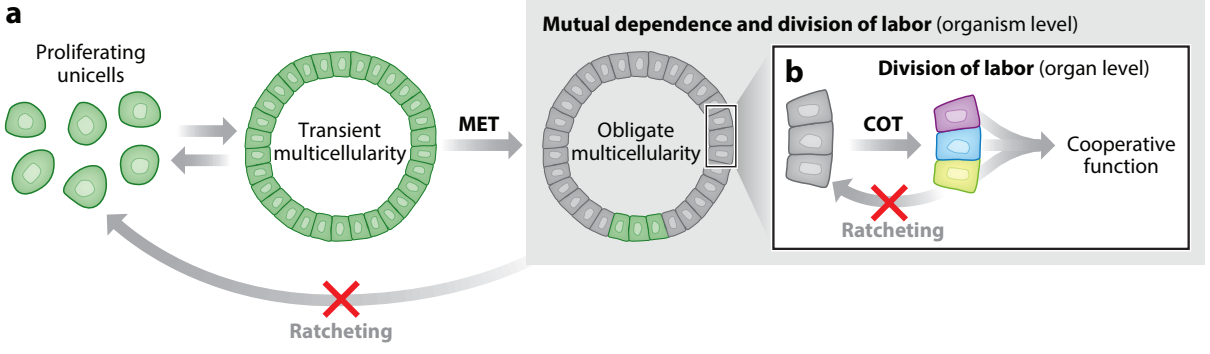

Figure 1

METs and COTs. (*a*) MET from unicellularity to obligate multicellularity occurs as ancestral unicellular organisms associate to form a multicellular assemblage. Cells evolve to facilitate the viability and replication of a unitary, multicellular individual by dividing labor organism-wide—a process that eventually locks them into the multicellular condition (in this example, reproductive division of labor has evolved, apparent in the *green* germ line and *gray* nonreplicative soma). (*b*) COTs occur within the multicellular individual as functional differences arise between cells, enabling them to divide labor and execute cooperative, organ-level functions. Specialization of cell types leads to organ-level ratcheting, whereby a cell type's adaptive value becomes contingent on the functions of other cell types within the organ. Organ-level division of labor and ratcheting contribute to the irreversibility of the MET at the whole-organism level. Abbreviations: COT, cell-level to organ-level transition; MET, major evolutionary transition.

entities. Examples include the distinct functions of cell types within a multicellular organism; the complementary roles of the nuclear and mitochondrial genomes within eukaryotic cells; and the tasks executed by different worker castes, queens, and males within social insect colonies (Cooper & West 2018). The third essential feature of all METs is contingent irreversibility (Smith & Szathmáry 1997), otherwise termed ratcheting (Libby & Ratcliff 2014, Libby et al. 2016). As selection acts to cement cohesion of the new, composite individual, its constituent lower-level entities may eventually lose the capacity for independent replication. Turning of the evolutionary ratchet may stem from mutations that enhance fitness in the cooperative group condition being disadvantageous in the solitary condition. Alternatively, mutations that are adaptive or neutral in the group condition may inhibit reversion by making it increasingly mechanistically inaccessible (Libby et al. 2016). At this point, switching back to a viable ancestral condition is no longer feasible, the higher-level phenotype having become evolutionarily entrenched (**Figure 1*a***).

The METs framework is a valuable intellectual device. The evolution of organs can be seen as a perpetual manifestation of the organism-wide division of labor that is fundamental to all METs from unicellularity to obligate multicellularity. Moreover, the assembling of cells into organs may have been a profound cause of ratcheting. The molecular evolution of specialized cell types—virtuoso for some functions, incapable of others—likely spawned many of the contingencies that established the irreversibility of these METs, locking clades into the multicellular state. Yet not only has the process of organ evolution contributed to METs via division of labor and ratcheting but these same key features of METs are themselves also intrinsically embedded, fractal-like, within the process of organ evolution itself. Organ-level complexity arises whenever cells evolve differentiated roles that contribute to a collective function. Organ evolution can thus also be interpreted not as a MET itself but as an evolutionary transition of a precise kind—one in which a new layer of functional organization is introduced between the cell and whole-organism levels. By way of its impact on the organism's phenotype, selection may act on this new layer to stabilize or directionally modify the collective function. The emergence of cooperative interactions between cell types within individuals is herein termed a cell-level to organ-level transition (COT) (**Figure 1*b***). Division of labor in the context of a COT refers to a local allocation of functions across two or more cell types comprising a multicellular structure. Ratcheting in COTs is pervasive in the seemingly irreversible evolution of obligate interdependencies between different cell types, such that only as a collective, multicellular unit can each cell type confer an adaptive function to the organism (with loss of one cell type diminishing or abolishing this adaptive function).

That a cell type's contribution to fitness is maximized—or realized exclusively—through its role within a group of other cell types is perhaps the key criterion by which the organ level of biological organization may be defined. A COT can thus be said to have occurred whenever such nonadditive cooperativity arises between cell types. In essence, in obligately multicellular clades—those that have evolved mutual dependence—iterations of division of labor and ratcheting have occurred at scales within the organism, entrenching the collective functions we recognize as organs. Hence, nested within each of the METs toward obligate multicellularity are myriad COTs (**Figure 1**).

MODELS OF ORGAN EVOLUTION

Concrete examples revealing the evolutionary steps comprising a COT are lacking, but two models, described below, have been proposed for how this type of transition may occur.

The Subfunctionalization Model

Drawing from models of gene duplication (Ohno 1970), subfunctionalization posits that ancestral cell types within obligately multicellular clades were multifunctional, executing several tasks

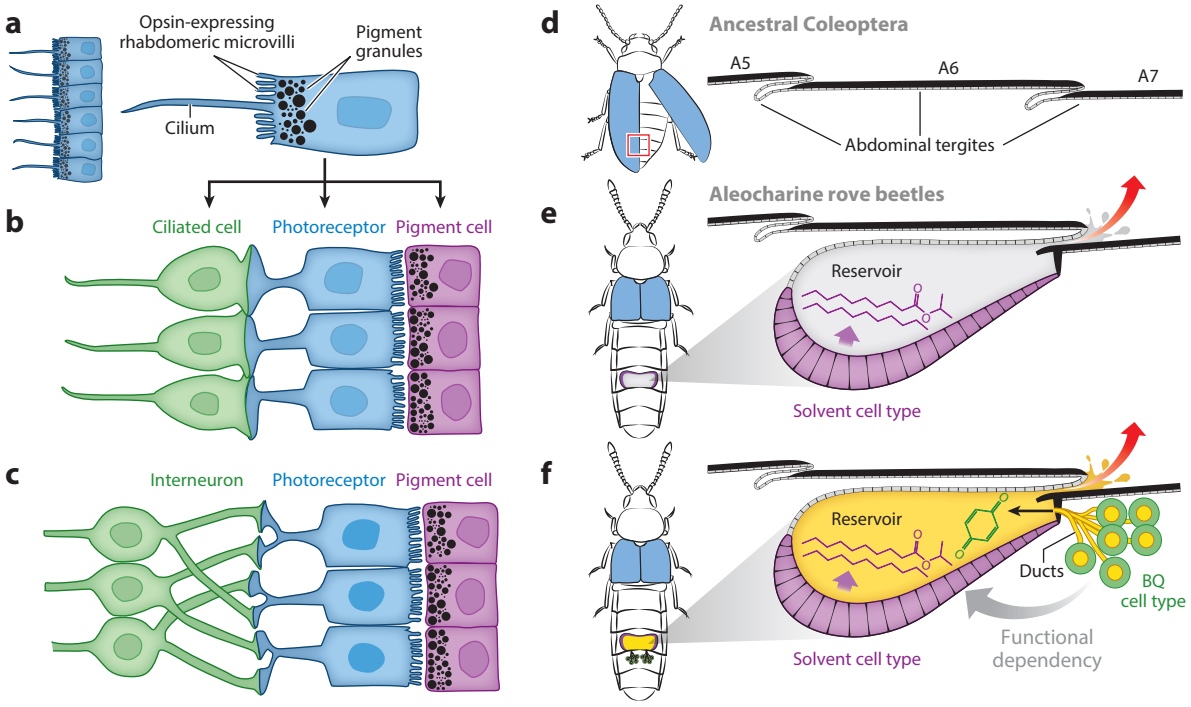

Figure 2

Models of organ evolution. (*a–c*) Subfunctionalization. (*a*) Shown is an epithelium composed of identical, multifunctional cells with photoreceptive, pigment, and locomotor subfunctions. (*b*) Cells undergo subfunctionalization, leading to division of labor between separate cell types. (*c*) Further divergence of ciliated cells leads to interneurons with axonal connections to photoreceptors. (*d–f*) Niche creation. (*d*) Shown is a cross section through dorsal abdominal segments (tergites) A5 to A7 of an ancestral beetle. (*e*) Elytron reduction in aleocharine rove beetles enabled a gland to evolve through invagination of the A6 to A7 intersegmental membrane, forming a chemical reservoir, with differentiation of cuticle cells into alkane/ester-producing solvent cells. (*f*) Solvent cells created a niche for the subsequent evolution of BQ cells, producing toxic BQs that require the alkane/esters to dissolve. Abbreviation: BQ, benzoquinone.

autonomously by way of molecularly separable subfunctions. COTs occur when these subfunctions become individually reallocated across multiple, now-specialized cell types, the ancestral function becoming executed by the collective (functional segregation) (Arendt 2008). Animal eyes have been widely studied as models of complex phenotypic evolution (Darwin 1859, Gregory 2008, Nilsson 2009, Oakley & Speiser 2012). Accordingly, Arendt and coauthors (2009) depicted an explicit, hypothetical scenario by which a multicellular eye may have arisen through the subfunctionalization of an ancestral photoreceptor cell. In this model, the progenitor of complex eyes comprised perhaps a single cell type with a photoreceptive function, granules of shading pigment, and a cilium—the latter two subfunctions conferring directional phototaxis onto the cell (Randel & Jékely 2016) (**Figure 2*a***). Such multifunctional cells exist within the pigment rings of motile sponge larvae (Leys & Degnan 2001), as well as in cnidarian planulae (Nordstrom et al. 2003). During subsequent evolution, the three subfunctions are posited to have become segregated across separate but physically interconnected photoreceptor cells, shading pigment cells, and locomotor ciliary cells (**Figure 2*b***). Complementary losses of subfunctions in each cell type established their division of labor in phototransduction and locomotion. Further subfunctionalization may have led to visual eyes, with projections to the central nervous system mediated by interneurons, arising via the loss of all three ancestral subfunctions in cells that retained axonal connectivity to the

photoreceptors (Arendt et al. 2009, Murphy et al. 2019) (**Figure 2*c***). Additional losses, such as the microvilli from pigment cells, may have further ratcheted the cell types into functional interdependence.

Subfunctionalization has been invoked to explain division of labor in other organ contexts, particularly the nervous system. Ancestral mechanoreceptors that possess axons, for example, may have subfunctionalized within the vertebrate inner ear, yielding axon-less hair cells, each innervated by a sensory neuron (Fritzsch et al. 2000). Certain neurons comprising regions of the mammalian hippocampus are thought to have arisen via the segregation of subfunctions present in primordial cortical neurons, represented today in reptilian (sauropsid) cortex where the subfunctions remain unsegregated (Tosches et al. 2018). More generally, dividing labor across increasing numbers of neurons that remain synaptically connected has been proposed as a mechanism by which neural circuits and brain regions may typically originate (Arendt 2008, Arendt et al. 2016b, Jékely 2011, Tosches 2017). Cell types that diverged from the same ancestral cell type have been considered sister cell types (Arendt 2008, Arendt et al. 2009, Mah & Dunn 2023). In the case of subfunctionalizing ancestors, however, molecular features that confer distinct identities onto descendants may be largely nonhomologous. Central to the subfunctionalization model is the idea that the tasks performed by cell types within an organ predate their segregation. A corollary is that subfunctionalization erodes ancestral unicellular sophistication—an unsustainable complexity drain with a finite potential to generate different cell types (McShea 2002). Ancestral unicellular photoreceptors, for example, housed sufficient complexity to fuel the essential cell types comprising simple phototactic eyes but not the complete repertoire of functions found in complex, image-forming eyes of bilaterians (with corneas, ciliary muscles, lenses, and multiple photoreceptor classes) (Randel & Jékely 2016). What is missing from models of organ evolution that invoke subfunctionalization alone is thus novelty—the evolution of new cellular functions.

The Niche Creation Model

Organs are multicellular environments, presenting opportunities for the integration of new cellular behaviors that adaptively modify organ performance. Niche creation posits that organs both originate and undergo further complexification via the sequential addition of novel cell types into functional niches created by earlier-evolved cell types. A COT commences on the first iteration, whereby a structure composed of a single cell type creates a niche for a second cell type that is functionally dependent on the first. A paradigmatic example of niche creation is the chemical defense gland of rove beetles (Staphylinidae). Within this species-rich family, the coleopteran body plan has become modified such that the elytra (wing cases) are reduced in size, exposing an elongate, flexible abdomen (**Figure 2*d*,*f***) (Parker 2017, Thayer 2005). Countering this loss of physical protection, various staphylinid lineages have evolved abdominal defensive glands (Dettner 1993, Kishi & Parker 2021). Members of the largest subfamily, Aleocharinae, possess a tergal gland in the dorsal abdomen, composed of two secretory cell types that are unique to aleocharines (Brückner et al. 2021, Jordan 1913, Steidle & Dettner 1993). The benzoquinone (BQ) cells produce cyclic BQs—potent irritants that activate nociceptive TRPA1 channels (Ibarra & Blair 2013). However, by themselves, BQs are inert solids; hence, the second cell type—the so-called solvent cells—produces fatty acid derivatives that solubilize the BQs, activating the total secretion (**Figure 2*f***).

A sequential process of niche creation, novelty, and ratcheting has been proposed for the evolution of this cooperative system (**Figure 2*d–f***) (Brückner et al. 2021). The precondition for this scenario was exposure of the abdomen through elytron reduction—a step that both created the vulnerability that triggered selection for chemical defense and exposed a body surface on which glandular innovations could develop. Invagination of the intersegmental membrane between

abdominal tergites 6 and 7 (A6 and A7) formed a reservoir, with epidermal cells surrounding it evolving into solvent cells with biosynthetic capacity. Given the ineffectiveness of solidified BQs as defensive compounds, the evolution of the solvent cells is hypothesized to have been the first step in gland evolution (**Figure 2*e***). Their fatty acid–derived secretion may have provided a weak chemical defense or functioned as a pheromone or abdominal lubricant. Prior emergence of the solvent cell type is proposed to have created the niche for the subsequent evolution of the BQ cells (**Figure 2*f***). The potency of the BQs, dissolved by the fatty acid derivatives, was likely strongly adaptive, locking the BQ cells and solvent cells into a functional, modular unit, with selection now acting on the emergent phenotype of the two cell types working together. The tergal gland has been conserved over ∼150 million years as Aleocharinae radiated into tens of thousands of species (Kitchen et al. 2024). Clade-wide, a small number of specific BQs have been highly conserved, but the compounds produced by solvent cells vary considerably, including mixtures of alkanes, alkenes, esters, and aldehydes, spanning a range of different chain lengths and combinations (Kitchen et al. 2024, Steidle & Dettner 1993). Ratcheting appears to have occurred in many such taxa where the solvent cells' secretion is unlikely to have any stand-alone adaptive value without being mixed with BQs.

Biosynthetic synergism between the BQ cells and solvent cells embodies the simplest case of organ evolution, where just two cell types enter a cooperative division of labor. Intuitively, iterations of this scenario could generate more complex structures. The sequential incorporation of novel cell types into organs is a feature of phylogenetically broad depictions of organ evolution, including eyes (Gregory 2008, Randel & Jékely 2016) and the heart [from a myoepithelial tube in stem bilaterians to three- or four-chambered amniote hearts (Olson 2006) composed of numerous cardiac cell types (Litviňuková et al. 2020)]. The evolution of neural circuits, via the duplication of neurons and their subsequent divergence into distinct sister cell types, can be seen as a category within the more general process of niche creation (Arendt 2008, Tosches 2017). Iterations of this process may have led to the formation of ganglia and, in bilaterians, still larger neuronal populations comprising the central nervous system (Arendt et al. 2016b).

CYCLES OF NICHE CREATION, NOVELTY, AND SUBFUNCTIONALIZATION DRIVE ORGAN EVOLUTION

The subfunctionalization and niche creation models are not mutually exclusive. By reconciling them, a more general scenario of organ evolution can be synthesized. Because novel cellular processes must originate in a preexisting cellular environment, one can speculate that, across obligately multicellular clades, new cellular subfunctions must have repeatedly evolved within—or been co-opted into—earlier-evolved cell types or epithelia. Filling vacant niches, new subfunctions established adaptive, cooperative interactions with earlier-evolved subfunctions (either present within the same cell type or elsewhere within the same organ) before becoming segregated into novel, functionally discrete cell types themselves. As selection acted on the emergent functions stemming from their cooperation, reciprocal coevolution between novel and more ancient cell types ratcheted them into obligate interdependencies. One can envisage cycles of this process driving the complexification of organs into structures composed of multiple cooperating cell types.

How might subfunctions evolve to fill vacant niches? One mechanism is via the acquisition of additional, novel subfunctions. Echoing the multifunctional cell types believed to have existed in stem groups and early branching animals that underwent subfunctionalization (e.g., **Figure 2*a–c***) (Arendt 2008), derived cell types must have frequently originated that expressed both the ancestral function(s) and one or more subfunctions that were qualitatively new within the cell type's evolutionary history. Such new subfunctions may have undergone evolutionary assembly de novo (in situ) and were hence cell-type-specific, or were co-opted from elsewhere within the

organism (effectively creating hybrid cell types). Examples of novel, multifunctional cell types are gradually being discovered: astrocytes in mammalian hippocampus capable of glutamate transmission (de Ceglia et al. 2023); ephemeral Cajal-Retzius neurons in developing amniote cortex expressing a multiciliation apparatus shared by ependymal cells of the choroid plexus (Moreau et al. 2023); outer radial glia of human cortex with putative cholesterol biosynthesis capacity (Moriano et al. 2023); the *Drosophila* R8 photoreceptor that separates image-forming and irradiance signals by cotransmitting both histamine and acetylcholine (Xiao et al. 2023); structural epithelial, endothelial, and fibroblast cells in mice that express inducible immune gene repertoires typical of hematopoietic cell types (Krausgruber et al. 2020); intestinal enterocytes and lung epithelial cells from bats that express the complement system (Levinger et al. 2023); and solvent cells of rove beetles that form part of the exoskeleton but have acquired glandular functionality (Brückner et al. 2021) (discussed above).

In these examples, the coexpression of different subfunctions within the same cell may create an adaptive synergism. Many other composite cell types may have historically originated in multicellular taxa, but ultimately became subfunctionalized if coexpression was not itself more advantageous than segregating functions into separate cells. Discovering such post-subfunctionalization cell types is more difficult, and depends on comparisons with outgroups where the pre-subfunctionalized state persists. Examples are starting to emerge, however. One is the hippocampal neurons mentioned above that are subfunctionalized in mammals but remain unsegregated in reptiles (Tosches et al. 2018). Another is the class I Kenyon cells (KCs) of the insect mushroom body—a brain region with important roles in learning and memory. In the earliest-branching lineage of Hymenoptera—sawflies (Symphyta)—KCs comprise one principal cell type; in honeybees, several subfunctions conserved with sawfly KCs have become segregated across three spatially discrete KC populations (Kuwabara et al. 2023).

A second mechanism of niche filling is the molecular divergence of a preexisting subfunction. Many closely homologous sister cell types originated via divergence in formerly identical subfunctions—for example, through differential use of paralogous loci (Arendt 2008). The divergence of sensory cell types through receptor duplication is one example (Baldwin & Ko 2020), producing photoreceptor subtypes (van der Kooi et al. 2020, Lamb 2013, Ogawa & Corbo 2021) and olfactory sensory neurons (Bontonou et al. 2024, Nei et al. 2008). Further examples are the recent discovery of paralogous effector loci underlying the divergence of fast and slow twitch muscle cells in cnidarians (Cole et al. 2023) and hypothalamic cell types in teleosts (Shafer et al. 2022). Diverging sister cell types automatically engage in division of labor if the cells topologically depend on each other, as in the case of duplicated interneurons that remain synaptically connected along a sensorimotor pathway. This contrasts with diverging sensory neurons: Here, duplication and divergence yield parallel, afferent inputs, the functions of which are not necessarily contingent on each other. In the vertebrate inner ear, for example, ancestral, gravity-sensing hair cells are believed to have diverged into additional sensors for audition and motion (Fritzsch & Straka 2014, Fritzsch et al. 2002). The loss of one such sensory cell type may not diminish contributions of its sisters to inner ear performance.

In a third scenario, a niche may become filled via the wholesale co-option of a preexisting cell identity through homeotic respecification. Evolutionary switching between cell identities has been hypothesized to occur in the context of neural circuit evolution, where neuron subtypes are specified by terminal selectors—transcription factors, or complexes thereof, that regulate batteries of loci involved in the biosynthesis, secretion, and reception of specific neurotransmitters, along with ion channels and cytoskeletal and extracellular matrix proteins (Hobert 2016, 2021). The control of cell identity via terminal selectors has been posited to apply to cell types in general (Arendt et al. 2016a, Brückner & Parker 2020). In cases of apparent evolutionary homeosis, however, it is

unknown whether a binary change occurred, or whether an intermediate condition of ancestral and novel fates in fact existed (with the ensuing loss of the ancestral fate). Terminal selectors often simultaneously promote one fate while repressing alternative fates (Arlotta & Hobert 2015), but repression may have arisen secondarily, with apparent cases of homeosis in reality stemming from co-option and subfunctionalization. Support for this interpretation comes from the hippocampus where, in mammals, neuronal subtypes are segregated by the expression of mutually repressive terminal selectors, which in outgroup reptiles are coexpressed and function in parallel within the same cells (Tosches et al. 2018). Co-option of new cell identities into organs may also occur through patterning or morphogenetic changes earlier in development. In vertebrate eyes, altered tissue-level morphogenetic movements and progenitor cell migration have led to novel juxtapositions of fates, enabling the integration of cell types derived from the neural ectoderm, surface ectoderm, and periocular mesenchyme (Heavner & Pevny 2012).

GENE EXPRESSION PROGRAMS: FUNDAMENTAL UNITS OF CELL-TYPE AND ORGAN FUNCTION

By whichever mechanism it occurs, organ evolution decomposes into changes in cooperative, function-generating interactions between different cell types (both the gain and loss of such interactions). There are two facets to comprehending how such interactions might originate and spread in a population. The first is understanding the nature of molecular evolutionary variation relevant to functional evolution at the cellular level. The second is knowledge of the population genetic forces that act upon this variation, guiding the steps of organ evolution at the population level. A prerequisite for comprehending both aspects is identifying the fundamental parts from which organ functions are constructed. It is these components of function that molecular evolutionary processes must build for higher-level organ functions to emerge. Correspondingly, it is upon these units that population genetic forces must act to drive COTs and organ complexification. A key insight from considering the scenarios of organ evolution described above is that cell types per se are not these fundamental units. Rather, it is the subfunctions that cell types express. Often, a cell type will perform multiple subfunctions, evolution having brought them together in the same intracellular environment. Conversely, in many cases evolution has split subfunctions apart into different cell types. Some subfunctions may operate alone within cells, giving the impression of being synonymous with cell types; this is especially pronounced when subfunctions appear seemingly incompatible—one precluding others from being coexpressed or suppressing their phenotypic effects. It nevertheless follows that cellular subfunctions are the elemental units from which cell-type and organ functions are built.

Encoding every subfunction is a gene expression program (GEP): a set of transcripts that are coexpressed within the same cell under terminal selector control. In the eyes of evolution, GEPs comprise the heritable substrate with which evolution builds functionality at the cell-type level and above. It is GEPs—not cell types—that have undergone genetic individuation (Arendt et al. 2016a), despite instances of direct correspondence between single GEPs and specific cell types. This distinction is critical: A cell type can be viewed as the outcome of operationalizing one or more GEPs within a cell. GEPs are thus the key constituents of molecular variation on which we need to focus if we are to have hope of retracing how organ complexity evolves at the population level. Understanding this process reduces down to our ability to track how GEPs originate in populations, becoming consolidated entities that are visible to natural selection; how they diverge, both between species and—in cases of GEP co-option—between the different cell types that employ them; and how and why they become combined within or segregated across cells within organisms.

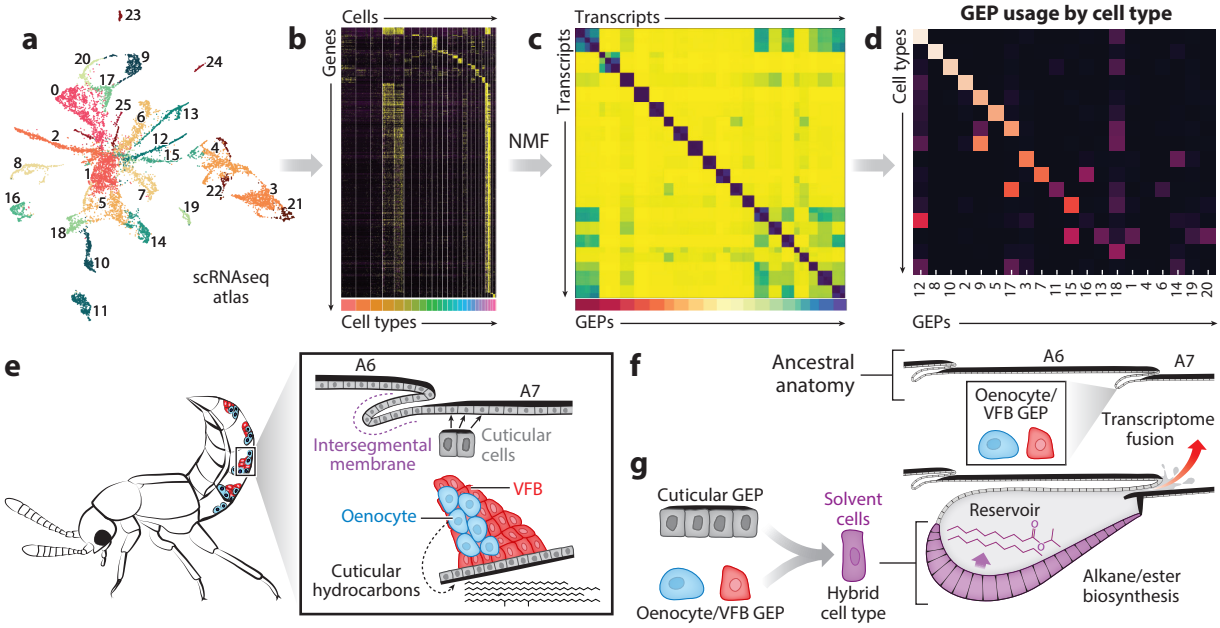

Figure 3

GEPs are the elemental units of cell-type and organ function. (*a*) A single-cell atlas of cell types from animal tissue. (*b*) scRNAseq revealing cell-type diversity and gene expression of individual cells. (*c*) Factorizing the transcripts × transcripts matrix revealing GEPs. (*d*) GEP usage across cell types. Transcriptomes of some cell types are composed of one principal GEP, others of GEP combinations. (*e–g*) Evolution of the solvent cell type. (*e*) Ancestral abdominal segments show ancient cuticle, oenocyte, and VFB cell types. (*f*) Co-option of oenocyte/VFB-GEP into intersegmental membrane cells. (*g*) A hybrid cell type, the solvent cells, which evolved through fusion of the cuticle and oenocyte/VFB GEPs, bestowing alkane/ester production onto intersegmental membrane cells. Abbreviations: GEP, gene expression program; NMF, non-negative matrix factorization; scRNAseq, single-cell RNA sequencing; VFB, ventral fat body. Figure adapted with permission from Brückner et al. (2021).

The development of methods for single-cell RNA sequencing (scRNAseq) has brought GEPs into view, providing an unprecedented opportunity to start retracing the evolutionary steps involved in cell-type and organ evolution. scRNAseq has empowered the analysis of transcriptional variation in large populations of cells within organisms, leading to a recent flood of cell atlases from a variety of multicellular taxa (Aldridge & Teichmann 2020). Atlases simultaneously reveal the diversity of cell types comprising an organ or body region (**Figure 3*a***) as well as the loci each cell type expresses (**Figure 3*b***). To recover GEPs within scRNAseq data, a key advance has been the development of unsupervised methods that factorize the matrix of gene expression into clusters of transcripts that show significant coexpression across cells (e.g., Jiang et al. 2023, Kotliar et al. 2019) (**Figure 3*c***). A cell type's transcriptome is typically built from some combination of these fundamental, quasi-discrete building blocks of gene expression. By plotting each cell type's proportional usage of each GEP, the correspondence between GEPs and cell types is revealed (**Figure 3*d***). Many cell types are amalgams of multiple GEPs, while others have a single, principal GEP; certain GEPs also appear to be used by the majority of cell types—an indication that they confer a constitutive activity rather than a feature of cell identity (**Figure 3*d***) (Kotliar et al. 2019).

The rove beetle tergal gland provides a case study for how cell-type and organ novelties evolve at the level of GEPs. Here, solvent cells were found to be a hybrid cell type formed from the fusion of two GEPs: one conferring cuticular identity, consistent with the solvent cells comprising part of the intersegmental membrane between tergites and secreting chitin that lines the

reservoir, and another GEP that defines two metabolic cell types—the oenocytes and ventral fat body (VFB) (Brückner et al. 2021). Both of these latter cell types are ancient in insects and perform roles in fatty acid and lipid metabolism (**Figure 3*e***). Oenocytes are specialized secretory cells found within the abdomen that convert fatty acids into cuticular hydrocarbons—very-long-chain alkanes and alkenes that are secreted onto the cuticle where they function as pheromones and form a waxy barrier to prevent water loss (Makki et al. 2014). The fat body is an organ composed of adipocyte-like cells that, among other metabolic and immune functions, synthesize fatty acids and store them as lipid droplets (Arrese & Soulages 2010). The inferred initial step in tergal gland evolution was the co-option of the oenocyte/VFB GEP into a patch of dorsal abdominal exoskeleton (**Figure 3*f***). This event equipped cuticle cells with the capacity for cell-autonomous production of fatty acids and their modification into alkanes and esters that could be secreted. Further invagination of the intersegmental membrane created a large chemical reservoir into which the solvent cell product could be stored and deployed on abdominal flexion (**Figure 3*g***).

The solvent cells provide a clear example of how a new cell type arises by combining GEPs, creating a bona fide evolutionary novelty—a qualitatively new organismal function. This example naturally raises another question of where GEPs come from. In this case, the beetle's primary metabolism provided a major biosynthetic program comprising several dozen loci that could act collectively to manufacture a chemical solvent. Wholesale co-option of this GEP may stem from the recruitment of a terminal selector that originally coordinated the expression of these loci in the oenocytes and fat body—the ancient cell types in which the GEP evolved. GEP co-option appears to have drawn certain loci into a pleiotropic conflict arising from their roles in both ancestral and novel cell types. The terminal enzyme in alkane synthesis is a decarbonylase, CYP4G (Brückner et al. 2021), which in the earliest-branching aleocharine lineage that possesses the gland exists as a single copy that functions in both oenocytes and solvent cells (Kitchen et al. 2024). In the stem lineage of the sister clade (the remaining Aleocharinae), CYP4G duplicated into separate oenocyte and solvent cell copies (Kitchen et al. 2024). Following this event, both the solvent cell and oenocyte copies underwent periodic positive selection, consistent with each paralog having escaped from adaptive conflict (Innan & Kondrashov 2010, Marais & Rausher 2008).

Co-option of a GEP from the primary metabolism implies that perhaps many GEPs are ancient, modular programs, some of which can be co-opted and modified in new contexts. Love & Wagner (2022) describe how ancient cellular stress and immune response mechanisms represent a similarly ancient source of GEPs that provided substrates for the evolution of new cell types. An attractive model is that immune-related GEPs became activated in cells under stress, and their plastic expression sustained long enough to allow for the genetic assimilation of advantageous molecular features. Yet co-option only gets us so far in explaining GEP origins: How might qualitatively new GEPs become evolutionarily stitched together? Their stepwise assembly seems the only feasible route, and some insight may be gained from the tergal gland—specifically the BQ cell type. Here, precursors of the BQs—hydroquinones—are produced by a modified version of a constitutively expressed mitochondrial pathway that produces ubiquinol (coenzyme Q_{10})—a redox-active compound involved in cellular respiration. Hydroquinone synthesis appears to have evolved via the partial co-option of this pathway, but with unique paralogs of certain enzymes (such as Methoxyless, a rove beetle–specific duplicate of COQ3) (Brückner et al. 2021). The hydroquinones are then loaded onto a sugar molecule for secretion into the BQ cell lumen; there, they are cleaved by a co-opted β-glucosidase enzyme, which originated earlier in insect evolution. Finally, to oxidize the hydroquinones into toxic BQs, the BQ cells express a secreted, aleocharine-specific laccase, named Decommissioned. Laccases comprise a family of enzymes that are involved in cuticle hardening and pigmentation (tanning) (Asano et al. 2019). Foreshadowing the evolution of Decommissioned was a major expansion of laccases in the genomes of aleocharine rove

beetles—many of which have gained localized expression patterns in different beetle organs (Kitchen et al. 2024). Hence, the BQ pathway is a chimera, stitched together from multiple independent biosynthetic pathways. Presumably, the entire GEP that confers BQ cell identity arose this way.

GEPs that confer functions onto conserved cell types likely underwent an analogous process of stepwise assembly in stem- and early branching lineages of obligately multicellular clades. A GEP for core neural identity is inferred to have assembled in a stepwise manner during early animal cladogenesis. Placozoans, members of a nonbilaterian phylum that lack neurons, possess a seemingly transitional form of this GEP (Najle et al. 2023). Complex multicellularity itself may have hinged on the prior evolution of GEPs that could be partitioned across cells within nascent multicellular organisms. The first animal GEPs likely had a pre-crown-group metazoan origin, perhaps existing as temporally or environmentally regulated transcriptional programs in unicellular opisthokont protists or animal stem groups (Brunet & King 2017, Mikhailov et al. 2009, Ros-Rocher et al. 2021). It follows that it is not the presence of differentiated cell types per se that distinguishes animals but the partitioning of GEPs in a way that permits regionalization.

Indeed, for understanding COTs, the essential counterpart to explaining how GEPs originate and recombine within cell types is the problem of how they become segregated across cell types. Complementary losses of terminal selectors in different cells provide a molecular evolutionary explanation for how subfunctionalization may occur (Kishi & Parker 2021, Tosches et al. 2018). Yet, under what population genetic conditions is this process feasible, or even likely?

WHY SUBFUNCTIONALIZE?

Execution of functions by different cell types is a defining hallmark of organs. Segregation of functions is pervasive in exocrine glands (Kishi & Parker 2021), exemplified by small molecule defensive glands where spatially separated cell types produce toxins and solvents (Brückner et al. 2021, Dettner 1993, Eisner & Meinwald 1966) (**Figure 2*f***) or toxin precursors and activating enzymes (Bourguignon et al. 2016). Similarly, distinct venom proteins and peptides have been traced to parcellated cell populations in venom glands of animals (Surm & Moran 2021), including snakes (Hamilton et al. 2020, Kazandjian et al. 2022, Post et al. 2020), centipedes (Undheim et al. 2015), cone snails (Dutertre et al. 2014), and assassin bugs (Walker et al. 2018). In male insect reproductive tracts, seminal fluid is a mixture of proteins, small molecules, and vesicles produced by multiple secretory cell types within the accessory gland (Bayram et al. 2018, Hopkins et al. 2019). Regionalized secretion of digestive enzymes occurs in insect salivary glands (Ribeiro 1995, Swart et al. 2006), while fibroin and sericin derive from neighboring cell types in silk moth glands (Suzuki et al. 1990). Joining these examples are many cases of functional segregation in the nervous system—between parallel afferent inputs, and between sequential neurons in sensorimotor pathways. Graded changes in cell function also occur across many organs, a phenomenon termed zonation. In the liver, hepatocytes exhibit pronounced zonation, reflected in a continuum of GEPs that are active along lobule axes (Ben-Moshe & Itzkovitz 2019, Halpern et al. 2017). Enterocytes are functionally and transcriptomically zonated along small intestinal villi (Moor et al. 2018), as are vascular endothelial cells along the arteriovenous axes of blood vessels in the mouse brain (Vanlandewijck et al. 2018).

Pervasive segregation demands a population genetic explanation, as does the converse problem of why subfunctions have not become recombined in cells more commonly than we observe. These problems become still more pertinent when confronted by the dramatic potential for unicellular multifunctionality exhibited by protists, the paraphyletic, microbial backbone from which all obligately multicellular clades evolved (Marshall 2020). The question of why cells

subfunctionalize warrants a closer consideration of microbes: It is in the context of METs from microbial unicellularity to multicellularity that the evolution of division of labor between cells has been most scrutinized. Focusing primarily on soma–germ line separation, two selective drivers of microbial division of labor have been identified. The first is the potential for accelerating fitness returns on a cell's investment in a task. If, through specialization, a cell becomes more efficient at converting energy into a fitness payoff, then dividing labor between cells within a group can be adaptive (Cooper & West 2018, Michod 2007, West & Cooper 2016). Second, the geometry of interactions between cells inside the group can favor division of labor. If the cooperative benefits of dividing labor are shared exclusively between close neighbors, reproductive division of labor can again be favored—even if the individual specialization of cells partially diminishes their efficiency (Cooper et al. 2021, Yanni et al. 2020).

Lessons can be learned from this body of theory about the possible drivers of subfunctionalization during COTs, with both accelerating fitness returns and topological constraints playing plausible roles. An important consideration is how a multifunctional cell type's performance might be encumbered by trade-offs between different GEPs. Executing multiple tasks may be constrained by cytoskeletal or membrane architecture, for example, or a limited ribosome pool that restricts protein synthesis supporting different subfunctions. Such inter-GEP conflicts have been demonstrated in some multifunctional cell types. In *Drosophila*, scRNAseq of fat body cells uncovered how this organ comprises a heterogeneous population of six cell types with probable specialized metabolic functions (Gupta et al. 2022). The fat body also performs an immune function against bacterial infection, however—an effect that diverts transcription from lipogenic and glycogenic enzyme loci to antimicrobial peptides (Clark et al. 2013). Mounting an immune response compromises the fat body's metabolic activities, with all six cell types exhibiting reductions in ribosome biogenesis and protein translation (Gupta et al. 2022). Aspects of cells that are drawn into pleiotropic conflict between competing GEPs render multifunctional cell types Pareto efficient: Their evolution is constrained along a surface corresponding to a trade-off between the different subfunctions (Shoval et al. 2012). Subfunctionalization, then, provides a means to escape intracellular Pareto trade-offs by freeing up cell types to specialize (**Figure 4*a*,*b***). In principle, subfunctionalization thus permits GEPs that were formerly coexpressed to escape adaptive conflict (**Figure 4*b***). A challenge remains, however, in explaining how subfunctionalized phenotypes persist in a population before adaptive mutations have had the opportunity to arise.

Here and in the following section, adaptive and nonadaptive explanations for how subfunctionalized phenotypes might spread in populations are hypothesized. Selection-based hypotheses emerge from considering the theoretical work of Rueffler and coauthors (2012), who presented a general mathematical framework for why division of labor might evolve through the functional specialization of repeated modules. Here, modules—which could be genes, cells, or appendages—are equivalent, each performing two activities that positively impact fitness but are constrained by a Pareto trade-off. An example would be an epithelium composed of a single cell type that expresses two, functionally distinct GEPs, where specialization toward one GEP activity detracts from investment in the other. Applying the model to this specific context, two explanations surface for why subfunctionalization could be a selective driver, not a mere consequence, of a COT.

The first invokes accelerating fitness returns as an automatic selective advantage of subfunctionalization. If a cell's increasing metabolic investment in a GEP has a disproportionally large fitness return, such that twice the investment more than doubles the positive impact on organismal fitness, subfunctionalization may be favored. In this case, greater fitness is achieved by way of enhanced cell- or organ-level performance (**Figure 4*b***). The second expectation is that the geometry of cell types, post-subfunctionalization, may be adaptive. If the descendent (sister) cell types organize spatially such that whole organ performance is enhanced, selection may

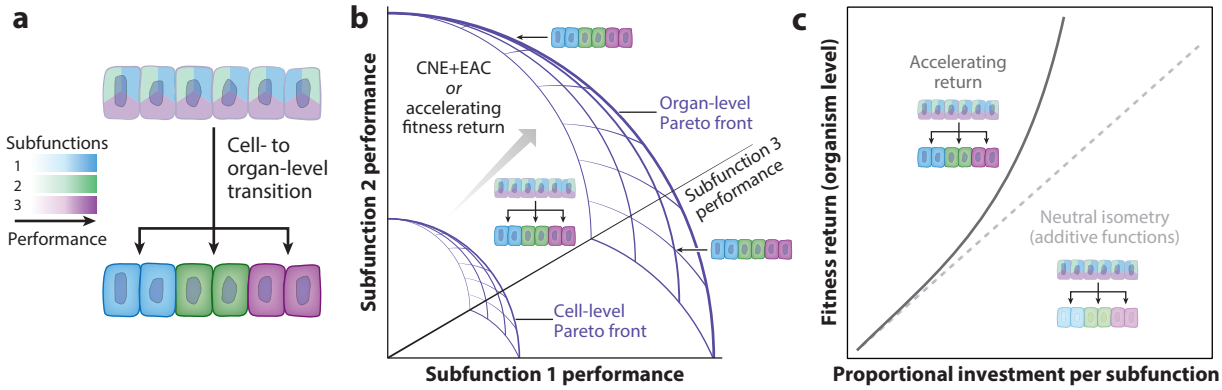

Figure 4

Subfunctionalization and adaptation. (*a*) Shown is an ancestral epithelium composed of a single multifunctional cell type in which three subfunctions trade off with each other. Subfunctionalization can free up cells to undergo adaptive specialization, increasing individual cell-type (and now organ) performance. (*b*) The performance of a multifunctional cell type is constrained across a Pareto front defined by relative investment in the three subfunctions. Subfunctionalization enables cell-type specialization and adaptive increases in whole-organ performance; a new, organ-level Pareto front is defined by the relative proportions of the three cell types. How a shift from the cell-level Pareto front to the organ-level Pareto front occurs in an evolving population may be explained by CNE followed by a period of drift, until adaptive changes arise in each cell type (EAC). Alternatively, subfunctionalization itself may confer an advantage if increasing relative investment in a subfunction has an accelerating relationship with fitness. (*c*) The relationship between subfunction investment and fitness return per cell dictates whether subfunctionalization will be neutral or adaptive. Abbreviations: CNE, constructive neutral evolution; EAC, escape from adaptive conflict.

favor subfunctionalization—even if it partially reduces each cell type's efficiency at performing its respective task. Hence, chance configuration of the topology of sister cell types might be automatically adaptive. One can imagine how, in the model of early eye evolution (Arendt et al. 2009) (**Figure 2*a–c***), the subfunctionalization of the ancestral photoreceptor-pigment cell might have been instantly adaptive if it restricted the localization of pigment granules to the desirable side of the photoreceptive sister cell, enabling more precise phototaxis. Positional effects might also explain subfunctionalization in chemical defense glands. Here, precursor compounds could be secreted into a reservoir, only undergoing activation from enzymes secreted at the site of ejection, as in the case of hydroquinone to BQ oxidation in bombardier beetle defense glands (Aneshansley et al. 1969). Extending this idea, it has been shown how, when spatial gradients of metabolites exist along an organ axis—as is the case in intestinal villi and vascularized structures such as liver lobules—the zonation of GEPs across cells in the epithelium provides an optimal solution (Adler et al. 2019).

CONSTRUCTIVE NEUTRAL EVOLUTION OF ORGAN COMPLEXITY

If no automatic, positive effect on fitness derives from subfunctionalization, consequent division of labor is nonadaptive. There is a possibility that certain steps in COTs are routinely driven not by selection, however, but instead by random genetic drift—a pervasive force in many multicellular taxa with small effective population sizes (Lynch 2007). Subfunctionalization that establishes cooperative division of labor between cell types is itself such a step—one that, in principle, may happen unavoidably via a phenomenon termed constructive neutral evolution (CNE). CNE is a mechanism by which biological complexity increases via the entrenchment of nonadaptive interdependencies between component parts. CNE draws on the observation that some biological activities are produced in excess of the needs of the organism or as nonadaptive by-products of

other functions (Gray et al. 2010, Muñoz-Gómez et al. 2021, Stoltzfus 1999). The presence of such excess capacities can epistatically mask (presuppress) a subsequent loss of functionality elsewhere in the organism that would otherwise prove deleterious. Once lost, purifying selection ensures both components—the suppressor and the suppressed—remain obligately associated.

The CNE framework was developed to explain certain molecular-level phenomena that are hard to interpret as simple products of natural selection (Stoltzfus 1999). One example is the profusion of introns in eukaryotic genomes. Here, the evolution of a catalytically efficient spliceosome is conjectured to have presuppressed the neutral proliferation of introns in loci genome-wide (Muñoz-Gómez et al. 2021). Another example is the retention of sister genes following duplication. In this case, the duplicate copies mask complementary loss-of-function regulatory mutations that restrict each copy to subdivisions of the ancestral, single-copy gene's expression pattern (Force et al. 1999). Experimentally, CNE has been demonstrated as a probable pervasive force in the evolution of multiprotein complexes, where neutral binding between subunits shields the accumulation of hydrophobic residues at the binding interface that would otherwise be detrimental to function (Finnigan et al. 2012, Hochberg et al. 2020). In all cases of CNE, reversion to independence of the interacting parts requires mutational paths of vanishingly low probability; hence CNE ratchets formerly separate components into obligate interdependencies.

CNE need not be confined to the subcellular level (Brunet 2022). **Figure 5** depicts how a COT can be driven by a combination of CNE and selection (Kishi & Parker 2021). Here, an epithelium is composed of one cell type executing a certain subfunction (encoded by GEP1) (**Figure 5*a***). GEP1 creates a niche for the adaptive evolution of a novel subfunction (encoded by GEP2) that is functionally dependent on GEP1. The emergent phenotype produced by GEP1+2 is beneficial and experiences positive selection. Importantly, the amount of GEP1 activity produced by the epithelium evolved prior to GEP2; in the new context, GEP1 activity is produced in excess of that needed for cooperation with GEP2. Excess GEP1 capacity can thus mask (presuppress) the loss of GEP1 in GEP2-expressing cells (**Figure 5*b***). Any inactivating mutations in regulatory regions that control GEP1 expression in GEP2-expressing cells become neutral. These mutations arise, subfunctionalizing the cell type and ratcheting the now-distinct GEP1- and GEP2-expressing cells into a cooperative division of labor (**Figure 5*c***). This neutral, subfunctionalized phenotype randomly drifts to fixation, or for long enough that an adaptive mutation arises with which it can hitchhike to fixation (depicted by the CNE + EAC scenario in **Figure 4*b***). In a subsequent step, selection on the cooperative GEP1+2 function permits GEP1 to acquire adaptive or neutral changes that abolish its former, stand-alone utility—reciprocal coevolution between cell types thus further ratcheting their interdependency (**Figure 5*d***).

One can envisage such a sequence in the evolution of the aleocharine tergal gland. Here, alkane/ester-producing solvent cells opened a functional niche for the production of BQs by these same cells (**Figure 5*e***). The potency of the newly produced cocktail was strongly adaptive, presuppressing the neutral loss of alkane/ester production in some cells, creating the conditions for subfunctionalization leading to the BQ cell type (**Figure 5*f***). Subsequently, in some lineages, the effectiveness of esters purely as a BQ solvent permitted the neutral loss of alkane production by solvent cells, the fitness contribution of which became exclusively realized through cooperation with the BQ cells (**Figure 5*g***). Further subfunctionalization of the BQ cell type may have occurred, yielding distinct but physically connected glandular (acinar) and duct cell types (**Figure 5*g***). Evidence supporting one aspect of this model is the presence of low, seemingly inconsequential transcription of solvent pathway enzymes in BQ cells of some aleocharine taxa—a possible vestige of their former hybrid function, pre-subfunctionalization (Kitchen et al. 2024).

Previous explanations of organ complexity have implicated neutral phenomena. Without explicitly invoking CNE, Arendt and coauthors (2009) acknowledged the possibility of neutral

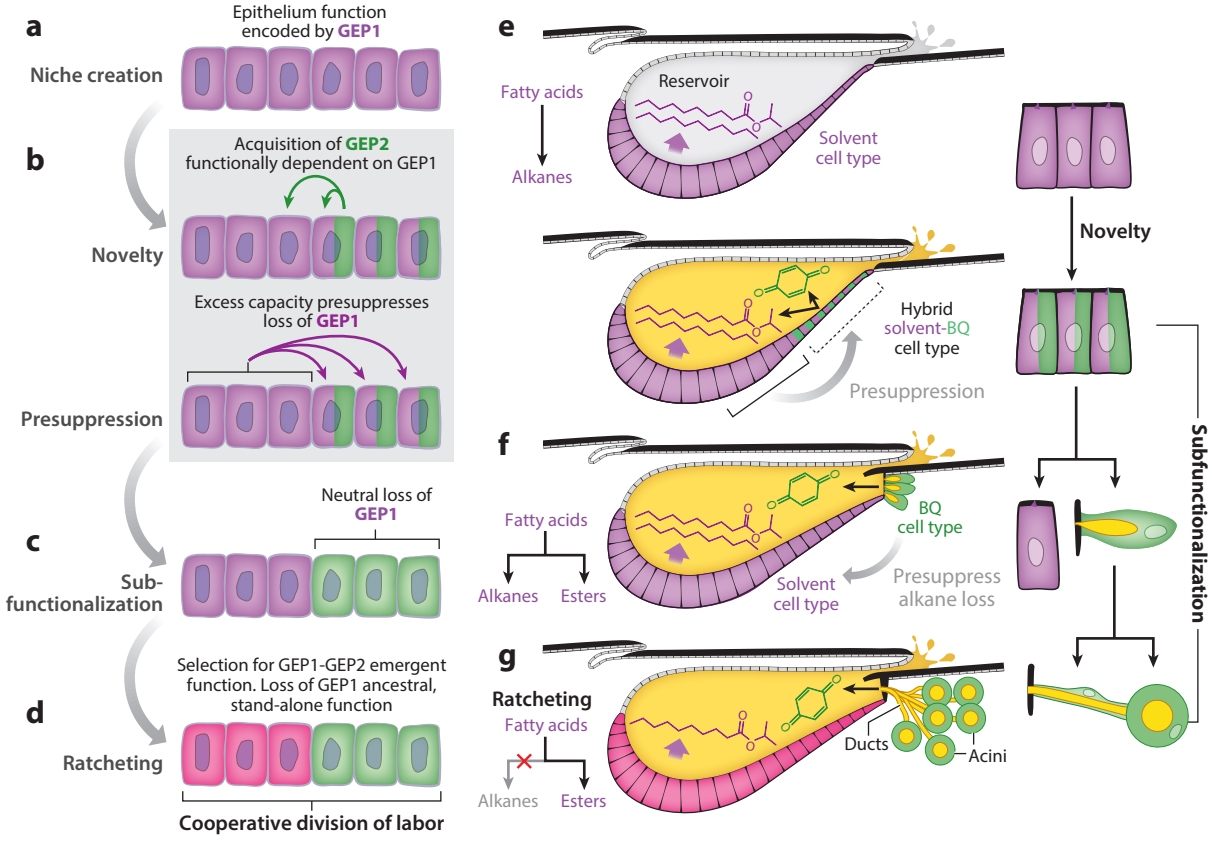

Figure 5

Constructive neutral evolution of organ complexity. (*a–d*) A COT driven by CNE and selection. Ancestral cells express GEP1 (*a*), forming a niche for dependent GEP2 to evolve. Positive selection acts on the GEP1+2 cooperative function. GEP1 excess capacity presuppresses the loss of GEP1 in the GEP1-GEP2 hybrid cells (*b*). Subfunctionalization occurs in GEP1-GEP2 hybrid cells via neutral loss of GEP1. Selection on the GEP1+2 cooperative function entrenches the two cell types (*c*). Divergence of GEP1 causes the loss of ancestral functionality depicted in panel *a* (*d*). (*e–g*) Hypothetical COT of the rove beetle tergal gland involving CNE and selection. Solvent cells create a niche for BQs to evolve, raising the adaptive value of the gland. An excess of solvent presuppresses the loss of solvent production in hybrid solvent-BQ cells (*e*). Subfunctionalization segregates the BQ cell type, and the potency of the solubilized BQs in esters alone presuppresses the loss of alkanes from solvent cells (*f*). Solvent cells undergo the neutral loss of alkanes. BQ cells further subfunctionalize into acinar cells and connective duct cells that transport BQs to the reservoir (*g*). Abbreviations: BQ, benzoquinone; CNE, constructive neutral evolution; COT, cell-level to organ-level transition; GEP, gene expression program.

subfunctionalization in their scenario for early eye evolution. Adapting the CNE framework to their model, excess capacities inherent to the ancestral, multifunctional cell type (**Figure 2*a***) presuppressed complementary losses of subfunctions, yielding separate photoreceptors, pigment cells, and ciliated cells (**Figure 2*b***). Here again, each sister cell type exhibits seemingly residual expression of the other subfunctions—remnants of their multifunctional ancestor (Arendt et al. 2009). Brunet (2022) proposed a hypothesis for how CNE might explain the origins of the liver. The range of tasks performed by the liver is impressive, including bile and albumin production, glycogenesis and glycogenolysis, lipid metabolism, blood detoxification, mineral and vitamin storage, regulation of blood amino acid levels, and plasma protein biosynthesis (Kmieć 2001). The

liver is a vertebrate novelty, however; hence, many of these functions may have ancestrally been distributed throughout the body. Plausibly, cells performing these functions that were connected directly to the circulatory system could exert effects body-wide, presuppressing losses of these functions elsewhere in the body, and consolidating them within a nascent liver (Brunet 2022).

CONCLUSION

Alluring macroevolutionary patterns of organ gain, loss, and divergence have their origins in population-level evolutionary events that altered the group behaviors of cell types. The COT framework advanced herein codifies a set of mechanistic and evolutionary phenomena that are hypothesized to drive organ evolution at the population level. I have proposed that the defining feature of an organ is cooperative division of labor between constituent cell types. I have argued that the key to comprehending the origins of division of labor between cell types is acknowledging GEPs as the fundamental substrate underlying cell-type function. Understanding how GEPs originate, tracing how they combine within or partition across cell types, and illuminating why these processes occur in evolving populations are central to explaining organ evolution and multicellular complexity.

With this goal in mind, I advance that an important future aim of single-cell biology should be to better enable evolutionary biologists to retrace evolution at the level of GEPs within a species. By examining quantitative patterns of GEP variation in populations, we may pinpoint examples of new GEPs forming, GEPs undergoing co-option, GEPs segregating in cells undergoing subfunctionalization, and GEPs combining with other GEPs with which they may functionally synergize. Connecting patterns of GEP variation to population genetic processes, and to phenotypic effects on cell and organ function, will no doubt prove challenging. Demonstrating the contribution of a GEP to the function of the cell type and organ is difficult enough; proving cooperative division of labor between GEPs or cell types is harder still. Studies in which cell types have been selectively inhibited, and organ function and organismal fitness tested, are scarce, but they point the way forward. In the aleocharine tergal gland, for example, impeding biosynthesis of either the solvent or BQ cells compromises the beetle's chemical defense; further, only when the full cocktail of compounds produced by these cell types is combined can a potent secretion be produced, with desirable physicochemical properties (Brückner et al. 2021). In contrast, in male *Drosophila* accessory glands, experimental inhibition of so-called secondary cells revealed their unique contribution to the seminal fluid without compromising total gland function (Hopkins et al. 2019). These studies provide a glimpse of the approaches needed to delineate cooperative groups of cell types that are products of COTs and that, in the eyes of natural selection, comprise indivisible, functional entities. Difficult problems remain as we unravel the population genetic drivers of cell-type evolutionary processes—some especially so, such as distinguishing between adaptive and neutral accounts of features of organ complexity. Yet confronting hard problems is where evolutionary cell biology must go if we wish to explain the origins of multicellular form and function.

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