Wherever I may roam: organellar protein targeting and evolvability

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Many functions of eukaryotic cells are compartmentalized within membrane-bound organelles. One or more cis-encoded signals within a polypeptide sequence typically govern protein targeting to and within destination organelles. Perhaps unexpectedly, organelle targeting does not occur with high specificity, but instead is characterized by considerable degeneracy and inefficiency. Indeed, the same peptide signals can target proteins to more than one location, randomized sequences can easily direct proteins to organelles, and many enzymes appear to traverse different subcellular settings across eukaryotic phylogeny. We discuss the potential benefits provided by flexibility in organelle targeting, with a special emphasis on horizontally transferred and *de novo* proteins. Moreover, we consider how these new organelle residents can be protected and maintained before they contribute to the needs of the cell and promote fitness.

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Introduction

Every organism has emerged from a long evolutionary process of change and selection. However, attention is typically focused upon the specific alterations that appear to increase or decrease fitness, rather than the overarching capacity of a population to find its way across a phenotypic landscape. This evolutionary potential, or evolvability, would itself be heritable and subject to selection within the lineage to which it may ascribe future benefits [1,2]. Evolvability often refers to increased proficiency in accepting, maintaining, distributing, and exploiting genetic variation that drives a specific phenotypic outcome with conventional mechanistic explanations. However, evolvability can also be linked to near-term phenotypic plasticity emerging at the interface of genetic, environmental, and stochastic processes.

Vertically inherited protein sequences can move across fitness landscapes by a series of stepwise substitutions within amino acid sequence space [3]. However, many mechanisms are available that allow eukaryotes to take larger leaps across these landscapes by acquisition of new genetic material. For example, gene duplication has been widely recognized as a contributor of raw genetic material for construction of new cellular components [4]. Moreover, horizontal gene transfer (HGT) from endosymbionts and other prokaryotes appears to have been relatively abundant during early eukaryotic evolution [5], and some speculate that genes may have been shared laterally between the earliest eukaryotic ancestors [6,7]. Accumulating evidence suggests that fixation of HGT events, although potentially reduced in scope at present day [8], continues to contribute to eukaryotic evolution [9]. Finally, we are only beginning to understand, by accumulation of genomic, transcriptomic, and proteomic datasets, the *de novo* appearance of protein coding genes [10•].

A marvelous array of conserved translocation mechanisms and machineries can direct proteins across membranes to selected destinations within the highly compartmentalized eukaryotic cell. Typically, sequential or structural information harbored within a polypeptide allows its direction to a final destination, as initially proposed by Günter Blobel within his well-established 'signal hypothesis' (Box 1). Decades of subsequent research have provided a clearer, yet still incomplete, picture of what properties characterize the organelle targeting signals (OTSs) that allow nucleus-encoded proteins to traverse at least five different membranes *en route* to a final destination [11,12]. Here, we consider how recognition and trafficking of organelle-directed proteins may be a factor in promoting or restricting the evolvability of eukaryotes.

How degenerate are organelle targeting sequences?

Since a primary purpose of organelles is to partition and organize metabolism [13], one might presume that entrance to these compartments would require a distinct and highly specific signal. Instead, the process of organelle targeting appears to be surprisingly permissive. Early experiments applying a genetic approach to OTS identification found that ∼20% of random human genome segments can serve

as an amino-terminal OTS allowing endoplasmic reticulum (ER) translocation and subsequent secretion of an enzyme from the yeast *Saccharomyces cerevisiae* [14]. In conceptually similar experiments, up to 25% of random nonamer peptides could act as targeting sequences driving translocation to the mitochondrial matrix [15]. Experiments focused upon protein transport to the plastid-derived malarial apicoplast demonstrated that nearly 30% of peptides randomly gener-ated on the basis of a biased amino acid composition were apicoplast-targeted [16]. While highly sensitive genetic experiments or *in vitro* assays with purified organelles may not accurately reflect the specificity and import kinetics characteristic of an *in vivo* setting [17], and while similar experimental surveys of random or pseudo-random sequences await completion for other organelles and organisms, it is certainly astounding how much flexibility is inherent to OTSs. Supporting the liberal nature of OTS recognition and delivery, the evolution rate of many signal peptides is several-fold higher than their respective mature protein domains [18], and experimental evidence suggests that signal peptide function can be robust to mutation [19,20].

Given the clear degeneracy in OTS recognition, it is conceivable that every protein family may have had the opportunity to explore every organellar location over billions of years of eukaryotic evolution [21]. Indeed, more than 30% of eukaryotic protein families show evidence of targeting to multiple subcellular compart-ments across eukaryotic phylogeny [22]. Dual-targeting and mis-targeting of polypeptides may be a mechanism by which different vertically inherited proteins find a way to function together within a new location [23], and instances in which the same polypeptide can be targeted to multiple subcellular locations abound [11,24]. More-over, the propensity of otherwise specific OTSs to be mistargeted can be easily revealed by cellular perturba-tion [25•,26]. Beyond nonspecific targeting of the same protein to multiple subcellular compartments, mutation, as well as errors during transcription or translation, can allow sampling of new organelles. Sampling may lead to an initially neutral foothold within an organelle that could later serve toward adaptation. Alternatively, sampling could introduce a new biochemical capacity that leads to an immediate leap across a fitness land-scape. Promiscuous compartmentalization of cellular metabolism is perhaps best exemplified by localization of the ancient pathway of glycolysis, as localization of glycolysis reactions to specific organelles may prevent cross-interference with other pathways that share the same intermediates [27]. While glycolysis occurs exclu-sively in the cytoplasm of human cells, enzymes of glycolysis can be compartmentalized within a peroxi-some-derived organelle in trypanosomes [27], within the mitochondria of stramenopiles [28,29], or within chloroplasts of plants and other photosynthetic organ-isms [30–32]. Other metabolic activities are scattered across different organelles in diverse eukaryotic clades, including isoprene biosynthesis [33] and fatty acid beta-oxidation [34]. Taken together, these findings suggest that the ease with which proteins can be targeted to new locations may be a feature, either taxon-restricted or pan-eukaryotic, that promotes evolvability.

Organelle targeting of novel and foreign proteins

Beyond the acquisition of new targeting information by vertically inherited proteins (Box 2), recent high-throughput sequencing of eukaryotic genes and tran-scripts has revealed a surprising amount of HGT that extends beyond early eukaryogenesis. Some of these proteins become (in the case of HGT from other organ-isms) or commonly remain [in the case of endosymbiotic gene transfer (EGT)] localized to specific organelles after nuclear acquisition (Figure 1a). For example, gut parasites from the genus *Blastocystis* have received a mitochondria-targeted glutamine synthetase from bacteria, potentially leading to enhanced proficiency in nitrogen capture [35]. Several eukaryotic lineages have acquired nucleotide transporters from bacterial donors, and these polypeptides are likely to be initially targeted to the ER [36,37]. Currently, the amoebae *Paulinella chromatophora* is in the process of converting its photosynthetic cyanobacterial endosymbiont to an organelle, providing superb examples of EGT to the nucleus. Even so, an even greater number of bacterial genes transplanted to the nuclear genome have been obtained by HGT from bacteria other than the

Box 2 Acquisition of organelle targeting sequences by vertically inherited proteins

Several molecular mechanisms may lead to novel organellar localization of an ancestral protein. First, gene duplication may generate raw genetic material allowing novel protein compartmentalization while minimally perturbing the existing cellular phenotype, yet sub-functionalization or neofunctionalization would be expected to occur before redundancy leads to loss of a functional gene copy [4]. Next, given the apparent ease with which randomly generated sequences can serve as OTSs, simple extension of an open reading frame at either protein terminus may generate a sequence suitable for directing proteins into one or more organelles. This may occur, for example, by changes to transcription or mRNA splicing coincident with, or subsequent to, the gene duplication event. ORF extension may also be linked to removal, addition, or error-prone recognition of stop and start codons within a transcript [11,76]. Moreover, exon shuffling may have played a historical role in linking OTSs to cargo proteins [77,78]. A recent study in which multiple plastid-directed proteins have acquired identical transit peptides suggests additional, yet poorly understood mechanisms by which a polypeptide might acquire an OTS [79].

P. chromatophora endosymbiont [38]. It is difficult to comprehend how recipient lineages could conceivably benefit from organellar integration of newly acquired prokaryotic proteins if cryptic OTSs found within those polypeptides, or otherwise generated during or subsequent to gene transfer, were not recognizable by eukaryotic protein translocation machineries. Distantly related eukaryotes also pass genes to one another, including membrane-inserted transporters that allow an organism to acquire new nutrients [39**,40], and liberal recognition of OTSs from distantly related eukaryotes may similarly allow benefits to accrue within a given lineage.

Beyond the acquisition of novel genetic information by HGT, *de novo* polypeptides originating from previously non-coding DNA are also potentially subject to organellar recruitment. The formation of *de novo* genes, together with their procurement of OTSs, is poorly understood, but *de novo* gene birth continues to present day, including within primate genomes [41]. Recently, an exciting study demonstrated the likely mechanism by which a newborn piscine antifreeze protein acquired its signal sequence [42**], and as *de novo* genes continue to be identified by computational and experimental means, further studies are required to reveal preferred mechanisms by which *de novo* proteins acquire an OTS.

We note here that while permissive recognition of OTSs may expand the possibilities for adaptation within a lineage, OTS degeneracy may also serve as an Achilles' heel to be exploited. Although much remains to be learned about subcellular targeting of rapidly evolving effectors that are often synthesized by bacterial and eukaryotic pathogens, these organisms may abuse cellular flexibility in OTS recognition while directing their proteins to host organelles [43–45].

## Preservation and integration of nascent organelle residents

While we highlight above the potential benefits of new proteins that may contribute to organellar functions, a novel polypeptide found within the cell can be initially deleterious [46], and if the cell cannot properly buffer this variation, the newly minted organelle resident will be counter-selected. A robust eukaryotic chaperone system may promote the initial maintenance of otherwise deleterious proteins within a population (Figure 1b). Supporting this idea, each common eukaryotic translocation pathway, be it mitochondrial, peroxisomal, nuclear, chloroplastic, or secretory, takes abundant advantage of chaperones that include Hsp70, Hsp90, and their functional binding partners [47–50]. Chaperones can even bind directly to OTSs [51]. Beyond their notable function in protein translocation and their likely role in promoting subcellular location sampling, chaperones are thought to act more generally as evolutionary capacitors that promote evolvability [52,53].

In addition, organelle targeting may provide an initial safe harbor for recently introduced protein sequences that would otherwise disrupt cellular activities, suppressing the effects of gene alteration or addition until further genomic changes, or new environments, reveal the potential for increased fitness [54]. Unwanted proteotoxicity that might arise from accumulation of unselected nascent polypeptides can be countered by vigilant proteostasis pathways, such as organelle-localized AAA domain-containing proteases or the ER-associated ubiquitin-proteasomal degradation pathway. Intriguingly, recent evidence indicates that cells may actively employ organellar targeting to remove pathogenic polypeptides from the cytosol [55**], supporting the idea that a cell may use organelles as a 'dumping ground' for novel proteins.

The concept of constructive neutral evolution [56,57] may also help to explain the maintenance of novel visitors to an organelle before they can make a steadfast contribution to metabolic and other processes occurring at their new location. Specifically, by means of promiscuous physical interactions that might transpire with an existing organelle resident, the new arrival may buffer a mutation that occurs within its interaction partner (Figure 2a). The novel organelle resident would initially provide no benefit from the perspective of survival or reproduction, except to suppress the effect of this otherwise deleterious mutation. Later, stepwise mutations within this new subunit of a macromolecular complex could result in functionalization and a role in conventional adaptation. Support for constructive neutral evolution toward complexity within organelles, and therefore a potential for increased evolvability, can be found in a recent analysis of the mitochondrial ribosome [58**].

After establishment of new recruits within an organelle, some OTSs may be subject to further changes that allow

Figure 1
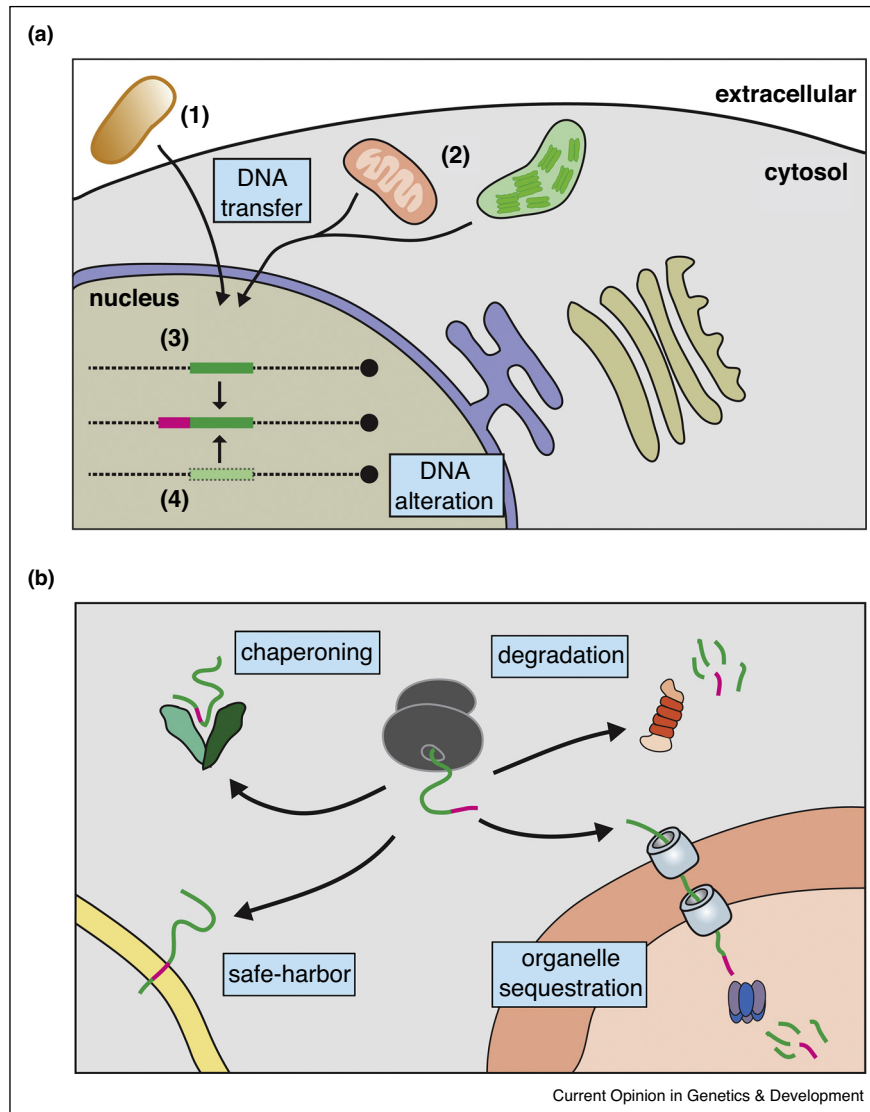
Birth or acquisition of new OTS-containing polypeptides.
(a) Possible mechanisms for cellular acquisition of new OTS-containing genes include (1) HGT from external sources, such as free-living microorganisms (2) EGT from recently acquired endosymbionts or from established organelles (3) acquisition of OTSs by already existing genes after gene duplication or by, for example, exon shuffling, and (4) *de novo* generation of open reading frames that harbor OTSs from sequences that were once non-coding. **(b)** Mechanisms for preventing toxicity prompted by novel proteins may include cytosolic degradation, shielding of protein segments by chaperones, movement of otherwise toxic protein species to 'safe-harbor' locations within the cell, or sequestration of proteins in organelles for potential destruction.

more specific and efficient direction of cargo to its final destination (Figure 2b). Moreover, for proteins initially targeted to more than one organelle, dual localization may be subsequently co-opted by the cell to allow developmentally regulated or environmentally induced adjustment of organellar proteomes [59,60]. OTSs can also act beyond their initial duty in trafficking [61], including during post-translocational folding of mature polypeptides, suggesting that once a protein becomes firmly entrenched in a new location, the OTS may be further selected to

encode for additional functions. So far, there has been scant examination of how OTSs might be fine-tuned over evolutionary time, yet some evidence exists for signal peptide adaptation [62].

Future analysis of organelle targeting sequence generation and evolution

Recent technological advances allow an expanded understanding of OTS generation and evolution. For example, deep mutational scanning of existing polypeptides,

Figure 2

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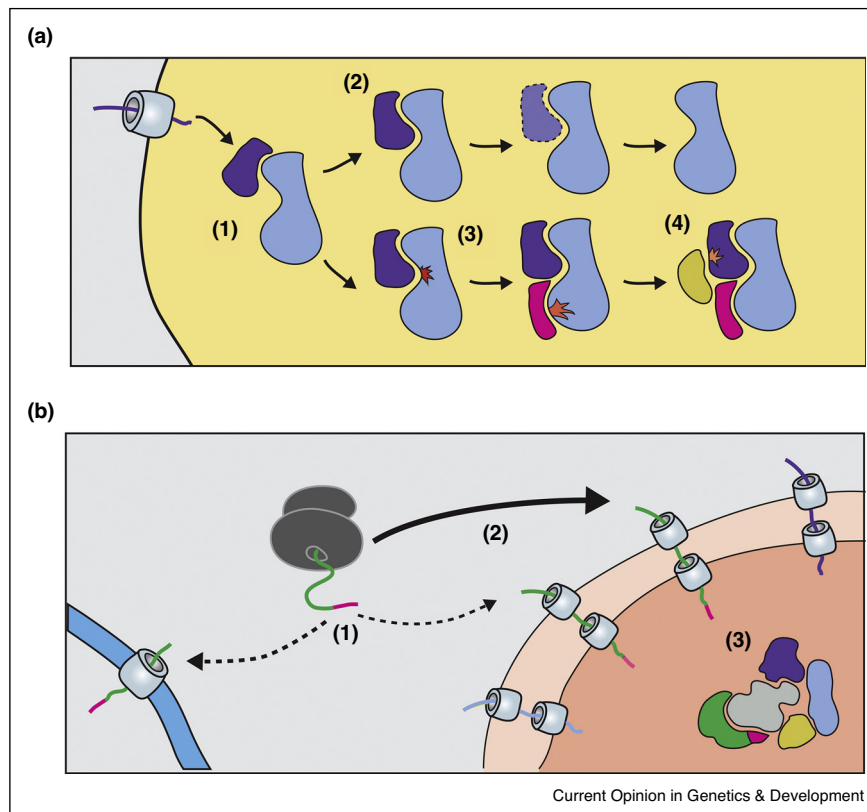
Establishment of new proteins within an organelle.

(a) Constructive neutral evolution may describe how novel organelle-targeted proteins are maintained before integration into organellar activities. (1) A new initiate to an organelle (purple) weakly interacts with a resident protein or RNA. (2) Without gene-level selective pressure to maintain the organelle visitor and its biochemical interactions with existing components, the organelle-initiate is lost. (3) However, if a mutation occurs in the binding partner of the novel organelle resident, the binding event may buffer negative consequences of the mutation, fitness is maintained, and the protein-protein interaction persists. (4) Stepwise addition by the same mechanism leads to increased subunit composition and potential for adaptive benefits. **(b)** OTSs can be under selection following introduction of a protein to a subcellular compartment. (1) Initially, new organelle residents may be targeted to multiple organelles or transported with low efficiency. (2) After further OTS mutation, protein targeting may, under appropriate selection, be improved in efficiency and specificity. (3) Selected OTSs may play additional roles in cargo folding, assembly, or function.

high-throughput surveys of known and predicted OTSs, as well as subsequent machine learning approaches can reveal features that promote OTS specificity or degeneracy [63•,64•,65]. Carefully designed experimental evolution experiments should reveal how a protein may acquire or lose its localization in the cell by subtle or overt changes to OTS structure. Moreover, an ever-increasing set of next-generation sequencing data obtained within and across various eukaryotic clades will enable high-resolution analysis of OTS divergence and selection at different phylogenetic scales. Close study of HGT events leading to nascent organelle targeting, perhaps enriched during the slow conversion of endosymbionts to organelles containing mosaic proteomes [38,66], will further inform our view of OTS acquisition and evolution. Finally, a combination of computational and experimental methods will allow the identification and subsequent study of *de novo* proteins that may have acquired organelle targeting

information [67,68•]. All of these future studies will be profoundly informative regarding whether promiscuous organelle targeting of vertically inherited, horizontally transferred, and *de novo* emerged proteins is a feature of eukaryotes that allows increased mobility across phenotypic space.

Conflict of interest statement

Nothing declared.

Acknowledgements

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• of special interest
•• of outstanding interest

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