

Trends in symbiont-induced host cellular differentiation

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Abstract:

Bacteria participate in a wide diversity of symbiotic associations with eukaryotic hosts that require precise interactions for bacterial recognition and persistence. Most commonly, host-associated bacteria interfere with host gene expression to modulate the immune response to the infection. However, many of these bacteria also interfere with host cellular differentiation pathways to create a hospitable niche, resulting in the formation of novel cell types, tissues, and organs. In both of these situations, bacterial symbionts must interact with eukaryotic regulatory pathways. Here, we detail what is known about how bacterial symbionts, from pathogens to mutualists, control host cellular differentiation across the central dogma, from epigenetic chromatin modifications, to transcription and mRNA processing, to translation and protein modifications. We identify four main trends from this survey. First, mechanisms for controlling host gene expression appear to evolve from symbionts co-opting cross-talk between host signalling pathways. Second, symbiont regulatory capacity is constrained by the processes that drive reductive genome evolution in host-associated bacteria. Third, the regulatory mechanisms symbionts exhibit correlate with the cost/benefit nature of the association. And, fourth symbiont mechanisms for interacting with host genetic regulatory elements are not bound by native bacterial capabilities. Using this knowledge, we explore how the ubiquitous intracellular *Wolbachia* symbiont of arthropods and nematodes may modulate host cellular differentiation to manipulate host reproduction. Our survey of the literature on how infection alters gene expression in *Wolbachia* and its hosts revealed that, despite their intermediate-sized genomes, different strains appear capable of a wide diversity of regulatory manipulations. Given this and *Wolbachia*'s diversity of phenotypes and eukaryotic-like proteins, we expect that many symbiont-induced host differentiation mechanisms will be discovered in this system.

Keywords: *Wolbachia*, *Drosophila*, Symbiosis, Cellular microbiology, Cellular differentiation, Epigenetics, Transcription, Translation, Proteolysis,

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1. The symbiotic lifestyle requires cellular remodelling

Bacterial symbionts of eukaryotic hosts form stable associations by colonizing host tissues or cells. This lifestyle requires an added layer of cellular regulation relative to non-symbiotic lifestyles because symbionts need to integrate with and control the host environment to create a hospitable niche (La et al. 2008; Schwartzman and Ruby 2016; Borges 2017). Without this ability, the bacteria are quickly eliminated by the host's immune system (Medzhitov 2007). Symbionts are benefitted by their ability to control the host environment, as their free-living relatives cannot do much to influence their abiotic environments. However, influencing host cells and tissues is not a trivial task. To do so, symbionts must decode another organism's regulatory pathways and interfere with them without causing too much damage. This is true for costly parasitisms and beneficial mutualisms, as well as extracellular and intracellular lifestyles: in all types of associations, bacteria must subvert host defenses to create a replicative niche (Medzhitov 2007; Mergaert 2018). Furthermore, owing to the deep, two billion year divergence between host and symbiont taxonomic domains, the eukaryotic regulatory pathways that need to be subverted are often completely unique from what the bacterial symbiont uses for its own genetic regulation (Cashin et al. 2006).

Nevertheless, bacterial symbionts have repeatedly found ways of controlling host gene expression for their own purposes. In many instances, this means finding ways of integrating with the biology of their multicellular hosts to be recognized as part of the "self" and colonize particular cell types. Naturally, many of the well-known examples of symbiont control of host gene expression involve mechanisms for limiting and modulating immune responses (Grabiec and Potempa 2018), solving the self/non-self issue. While these abilities are fascinating and essential for host-associated bacteria, they have been explored in depth elsewhere (see (Hamon and Cossart 2008; Zhong et al. 2013; Silmon de Monerri and Kim 2014; Cheeseman and Weitzman 2015; Pereira et al. 2016; Vilcinskas 2017; Cornejo et al. 2017)). Instead, here, we explore the evidence for bacterial symbiont control of host cellular differentiation, which can be used to control the identity of infected host cells, the size of the infection niche, and host reproduction.

In this review, we summarize what is known about how and why symbionts ranging from pathogens to mutualists control host cellular differentiation to create novel cell, tissue, or organ types for their habitation (Figure 1). We focus on cellular, tissue, and organ-levels of differentiation, as different symbiont taxa can target regulatory mechanisms at any of these levels of organization. In particular, we are interested in the processes of immortality maintenance and dedifferentiation/redifferentiation, as these strategies enable the stable manipulation of host gene expression and cell identity for symbiont purposes. In parasitisms, these are often viewed as neoplastic structures, *i.e.*, abnormal growths. Whereas, in mutualisms, these structures are generally a part of normal host morphology. After presenting on the diversity of symbiont-induced tissue differentiation mechanisms reported from nature, we focus specifically on the ubiquitous intracellular alphaproteobacterial symbiont of arthropods and nematodes, *Wolbachia*. We focus on *Wolbachia* in particular because of the myriad of remarkable phenotypes it is able to induce in its hosts (discussed below and reviewed in

(Werren et al. 2008)) and the tantalizing data that has been accumulating, which suggests that these bacteria have significant capabilities to control host cell differentiation pathways. Given the recent growth and progress in the field of *Wolbachia* research, the aim of this review is to inform on the experimental avenues to explore in the future.

2. Shared and unique mechanisms of gene regulation in eukaryotic-bacterial symbioses

The central dogma - DNA encodes RNA, which encodes proteins - holds across the diversity of life (Piras et al. 2012). Meaning, regulation points exist for bacteria and eukaryotes at 1) pre-transcription (e.g., epigenetic DNA/histone modifications), 2) transcription, 3) post-transcription (e.g., mRNA processing or regulation), 4) translation, 5) post-translation (e.g., protein modifications), and 6) proteolysis. However, as depicted in Figure 2, how these regulatory mechanisms work in real-time can differ greatly between domains (Kozak 1992; Blumenthal et al. 2002; Belasco 2010; Gur et al. 2011). For example, while both domains of organisms can regulate DNA access for transcription through DNA methylation (Sánchez-Romero et al. 2015), eukaryotes also have histones, which can be modified to be more or less permissive to the entry of transcriptional machinery (Verdone et al. 2005). Following transcription, eukaryotes have additional ways to modify their mRNA relative to bacteria, including RNA splicing, poly-A-tailing, and 5'-capping (Belasco 2010), to alter its identity, stability, or accessibility for protein translation, respectively. Although, bacteria do have a range of post-transcriptional regulatory strategies (Dar and Sorek 2018).

In addition to phylogenetic constraints, the different body plans and life histories among hosts and symbionts also underlie their different genetic regulatory capabilities. Multicellular hosts with complex tissue types and body plans require precise mechanisms for controlling gene expression across both space and time to properly control tissue differentiation and maintain stem cell pluripotency. Many plants and animals epigenetically alter their DNA by packaging it into chromatin, which helps maintain differential gene expression in different cell types over the lifespan of the host (Meissner 2010; Li et al. 2011). Interestingly, epigenetic alterations also underlie the transitions between parasite life stages that are evoked by different hosts, both in multicellular (Roquis et al. 2018) and single-cellular (Duraisingh and Horn 2016) eukaryotic parasites. By binding to the DNA promoters and regulatory regions made accessible by epigenetic modifications, transcription factors are also very important to cellular differentiation. This is true for both eukaryotes as well as bacteria, which use transcription factors to differentiate into different metabolic or motility states in response to environmental signals (Laub et al. 2007; Cole and Young 2008; Losick and Desplan 2008; Wolański et al. 2014).

Using these similarities and differences in genetic regulation, many host-associated bacteria have evolved ways to interact with host regulatory pathways. The simplest model for how a bacterium evolves control over its host's gene expression is through the co-option of one of its own pathways. In this situation, the majority of required machinery for the pathway would already be in place, and only modifier components would need to be added for controlling host gene expression. In contrast, it is also possible for bacteria to evolve strategies for interfering with eukaryotic-specific mechanisms of gene expression, such as histone modifications or

splicing. In fact, this strategy appears to be quite common among pathogenic bacteria, which can possess proteases, acetyltransferases, kinases, phosphatases, ubiquitin ligases, and deubiquitinases for altering host gene expression (Guvén-Maiorov et al. 2017). It is unlikely that genes lacking functions specific to the bacterial cell evolved in concert with the endogenous bacterial gene expression regulatory networks. Thus, their presence implies either introduction via horizontal gene transfer (e.g., (Patrick and Blakely 2012)) or functional convergence (e.g., (Alvarez-Venegas 2014)), often resulting in structural mimicry of the host protein (Frank 2019).

The nature of bacterial regulation of host gene expression likely depends on the host cell type and the desired outcome of the interaction. In terms of host cell differentiation, bacterial influence can either cause a host cell to become less differentiated, *i.e.*, more stem-cell-like with pluripotent capabilities, or it can cause a host cell to become more differentiated towards some particular fate. Less differentiated fates could facilitate bacterial transmission, especially if they are proliferative because bacteria can be inherited by both daughter cells during cell division. For example, the intracellular symbiont *Wolbachia* has been shown to segregate evenly between dividing embryonic cells in *Drosophila melanogaster* (Albertson et al. 2009). More differentiated fates could have a variety of impacts depending on whether the interaction is mutualistic or pathogenic, respectively. For example, the differentiation of host cells into bacteriocytes in mutualistic associations (see Figure 1B) provides an environment for bacterial symbionts to live at high densities and perform metabolic functions necessary to the host (Braendle et al. 2003; Hongo et al. 2013; Matsuura et al. 2015). In pathogenic interactions, bacteria often induce host cell differentiation to reach a metabolic state where more resources are provided to the bacteria for replication, increasing bacterial virulence and infectivity (Cornejo et al. 2017).

3. Making a house a home: bacterial symbionts influence host cellular differentiation during infection and establishment

In the sections below, we describe examples from the literature of different ways in which pathogenic and mutualistic symbionts have been found to control host cellular differentiation. These examples are generally organized by their place in the molecular biology hierarchy, from DNA to RNA to protein. Bacterial influence may occur at early points in the hierarchy and have cascading effects on the subsequent stages of gene expression, which are discussed when possible. As regulation becomes circular at the ends of the hierarchy, e.g., post-translational modifications of histone proteins affect DNA accessibility for transcription, this framework serves to organize the discussion.

3a. Epigenetic control of host gene expression

Multicellular organisms control the differentiation of their cells and tissues through epigenetic modifications put in place during development (Meissner 2010), and bacterial symbionts often use this mechanism to influence host cellular differentiation too (Hamon and Cossart 2008). Indeed, abundant evidence exists that a variety of host-associated bacteria, including

Legionella, *Listeria*, *Clostridium*, *Streptococcus*, *Helicobacter*, and *Salmonella*, are able to influence host DNA methylation or histone post-translational modifications to alter chromatin transcriptional accessibility and attenuate the immunological responses their infections solicit (Bierne et al. 2012). Immune responses include the upregulation of inflammatory cytokines, chemokines, toll-like receptors, and antimicrobial peptides, including cationic antimicrobial peptides (CAMPs). Bacterial symbionts can inhibit gene expression underlying these responses by directly altering chromatin packaging with their own enzymes (Alvarez-Venegas 2014). They can also indirectly alter the activities of host proteins such as DNA methyltransferases (DMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases (HMTs) through protein-protein inhibition or signalling, e.g., through short chain fatty acids (Grabiec and Potempa 2018). Depending on the molecular specificity of the interactions, epigenetic alterations can be highly targeted to a particular host gene or can be global across the host genome. For example *Shigella flexneri* produces and secretes an effector protein, OspF, that ultimately prevents histone phosphorylation and NF- κ B access to transcription binding sites, thus inhibiting an immune response (Arbibe et al. 2007). Importantly, these anti-inflammatory mechanisms are also used by commensal bacteria to a more beneficial effect because chronic inflammation is harmful to hosts (Grabiec and Potempa 2018).

Interestingly, in some instances, pathogen control of the host immune response can also induce developmental effects. For example, in the greater wax moth, *Galleria mellonella*, infection with *Listeria monocytogenes* increases the expression of both HATs and HDACs, resulting in a developmental delay that extends the time until metamorphosis (Mukherjee et al. 2012). Developmental effects such as these could have initially arisen as a side effect of cross-talk between epigenetic mechanisms mediating development and immunity (Vilcinskas 2017) or immune activation being required for nuclear reprogramming (Lee et al. 2012), and been maintained by the pathogen for its benefit. Developmental delays could be beneficial for reallocating resources from the host to the pathogen (Vilcinskas 2017). Thus, influence of host cellular differentiation can be a byproduct of the mechanisms used for infection and virulence (Vilcinskas 2017), and might facilitate the evolution of more intrinsic manipulations that change the identity of the host cell for symbiont purposes.

The known cases of bacterial epigenetic reprogramming of host cellular differentiation are from pathogenic bacteria, potentially due to pathway cross-talk. In an exquisite display of cellular manipulation, the intracellular pathogen that causes leprosy, *Mycobacterium leprae*, has been shown to reprogram the Schwann cells it inhabits to reach a stem cell-like state (Figure 1A). It does this via changes in methylation patterns and gene expression profiles that turn off Schwann cell differentiation genes/transcription factors and turn on developmental and embryonic genes/transcription factors. From this reprogrammed state, these infected stem cell-like cells can then differentiate into different tissue types and migrate from the peripheral nervous system to the surrounding connective tissues and muscles, helping to disseminate the bacteria throughout the host. Interestingly, they also use this reprogrammed state to attract and infect macrophages, further spreading the infection (Masaki et al. 2013).

A more brute-force approach to epigenetic reprogramming of host cells has been reported for the male-killing spirochete parasite of *D. melanogaster*, *Spiroplasma*. While epigenetic regulation via DNA methylation does not occur in *D. melanogaster* because it lacks functional DNA methyltransferase enzymes (Goll and Bestor 2005), it does regulate its gene expression with histone acetylation. In males, acetylation is used to double the expression of X chromosome-linked genes. *Spiroplasma* symbionts are able to interfere with this process to induce male killing, which eliminates these “dead-end” infections from the population so that more resources are available for the females, through which these bacteria will be maternally transmitted (Veneti et al. 2005). Recent work by (Cheng et al. 2016) found that these bacteria accomplish this by interfering with the male specific lethal 2 (MSL-2) protein of the dosage compensation complex, which is only active in male embryos, causing it to be randomly mislocalized across the genome. Mislocalization causes randomly elevated transcription across the genome through elevated acetylation, resulting in male lethality (Cheng et al. 2016).

While we are still in the early days of characterizing how bacterial symbionts can epigenetically modify host cellular differentiation through chromatin modifications, a number of preliminary data points suggest that this will be a productive area of research in future years. For example, host-pathogen associations have been reported to have long-lasting or transgenerational effects, likely mediated through epigenetic mechanisms, although they have not yet been identified (Fridmann-Sirkis et al. 2014; Mukherjee et al. 2017; Yang et al. 2018; Gegner et al. 2019). Epigenetic-based gene regulation is also implicated in eukaryote-eukaryote mutualisms such as coral-algal symbioses (Li et al. 2018). Furthermore, bioinformatic evidence suggests that many host-associated bacteria contain SET-domain proteins in their genomes (named for their discovery in *Drosophila* proteins **Su**(var)3-9, **E**nhancer-of-zeste and **T**riThorax), which are known to encode lysine histone methyltransferases (Alvarez-Venegas 2014). Given that bacteria do not contain histones, it is highly likely that many of these proteins are used to alter eukaryotic cellular functions.

3b. Symbiont co-option of host signalling pathways and transcription machinery mimicry

Studying the intertwined and intimate interactions between host and symbiont is often a difficult task, however, the advent of microarrays and next generation sequencing opened up one avenue of investigation significantly: host and symbiont transcriptomics. While these methods enabled the high throughput collection of gene expression data from hosts and symbionts, challenges continue to this day regarding the amount of mRNA that can be obtained from host-associated bacteria *in situ*. One of the main issues involves the drastic differences in relative abundance of bacterial versus eukaryotic mRNA. Furthermore, bacterial mRNA only comprises ~4% of total cellular bacterial RNA, with rRNAs and tRNAs making up the bulk of the transcripts. In addition, the half-life of bacterial mRNAs is far shorter than that of eukaryotic mRNAs, making it difficult to accurately capture bacterial gene expression in real-time. On top of all of this, bacteria do not A-tail their transcripts unless they are being marked for degradation. So, while eukaryotic mRNAs can be selected for by poly-dT priming, bacterial mRNAs cannot be directly selected, and instead must be depleted of rRNA (La et al. 2008). Nonetheless, many

methodological tricks have been developed over the years to deplete rRNAs and host transcripts or enrich for microbial mRNAs (Güell et al. 2011), and so this is the step of gene expression that we have the most data for presently.

These studies have revealed a few trends in host-symbiont transcriptomics. Importantly, it appears that some, but not all bacterial symbionts are capable of modulating their own or their host's transcription in response to the association. Those that cannot typically exhibit severe degrees of genome erosion, and are discussed later in this section. However, it is worth noting that even the symbionts with extreme levels of genome degradation are able to induce the differentiation of the specific host cells and/or organs they reside in, termed bacteriocytes and bacteriome, respectively (see Figure 1B). While the mechanism(s) of induction have not been identified, and may involve other elements of host signalling pathways (Smith and Moran 2020), upregulation of the host homeobox transcription factor *Ultrabiothorax* has been shown to be necessary for bacteriocyte differentiation in seed bug insects (Matsuura et al. 2015) and aphids (Braendle et al. 2003). In general, the symbionts that can influence host transcription do so by either modulating host signalling pathways upstream of transcriptional responses (Rogan et al. 2019) or by mimicking host transcription factors, activators, and suppressors (Saijo and Schulze-Lefert 2008). Examples of these two strategies have been reported for diverse symbiotic systems and are detailed below.

Interaction with host signalling pathways to induce transcriptional changes is the most commonly reported strategy for symbiont-induced modulation of host transcription. Symbionts may be predisposed to evolving this strategy because components of the host signalling cascades that induce immune responses are also used during development (Rogan et al. 2019). This is likely another manifestation of pathway cross-talk discussed above. Of the known signalling pathways, pathogens have been shown to frequently interact with the Notch, Wnt, and STAT3 signalling pathways (Hannemann et al. 2013; Rogan et al. 2019). The Wnt pathway is especially fruitful to exploit because its induction through canonical and non-canonical pathways can alter gene expression to manipulate immune responses and increase cell proliferation (Rogan et al. 2019). Additionally, Wnt signalling-induced transcription factor β -catenin is important for the activation of many genes including ones for adherens junction integrity, which is essential for epithelial integrity. Given that many pathogens are benefited by breaking down epithelial barriers for further dissemination, being able to target Wnt signalling may be strongly selected for. Thus, by increasing the translocation of transcription factors such as β -catenin to the nucleus, symbionts can simultaneously make a more hospitable and a larger niche for themselves in the host.

Transcription-level bacterial control of host cellular differentiation via the Wnt pathway is also displayed by *Helicobacter pylori*, the leading cause for chronic gastric inflammation and cancer worldwide. This epsilonproteobacterium colonizes the mammalian stomach epithelium through controlling cell differentiation, proliferation, and apoptosis (see Figure 1C). It accomplishes this via direct interactions with cell adhesion and polarity factors (Amieva 2003; Bagnoli et al. 2005; Wessler and Backert 2008) and indirect interactions with host transcription factors, including β -catenin and (Hatakeyama 2006; Wessler and Backert 2008) and Nuclear factor of activated T-

cells (NFAT) (Yokoyama et al. 2005). *H. pylori*-induced transformation of host gastric epithelial cells resembles the process of epithelial-to-mesenchymal cellular transition during embryogenesis, and produces an invasive migratory phenotype through altering the localization and expression of genes involved in controlling cell shape, polarity, and division. While there are several identified mechanisms underlying these abilities, most involve the effector protein cytotoxin-associated gene A (CagA) (e.g., (Yokoyama et al. 2005; Bagnoli et al. 2005; Suzuki et al. 2009; Bertaux-Skeirik et al. 2015)), which *H. pylori* injects into host epithelial cells with its type IV secretion system. The large, 1200 amino acid CagA protein causes a range of effects due to interactions between host factors and its N- and C-terminal domains, which have different activities in different phosphorylation states (Bagnoli et al. 2005; Hatakeyama 2006; Wessler and Backert 2008). Interestingly, CagA exhibits structural and functional similarity to eukaryotic Grb-2 associated binder (Gab) adapter proteins, although it does not exhibit any sequence similarity, and likely evolved to mimic Gab interactions with host cellular machinery (Botham et al. 2008).

The nitrogen-fixing mutualistic rhizobia bacteria of leguminous plants (consisting of alpha- and betaproteobacterial lineages) co-opt host signalling cascades to alter host root tissue differentiation in order to create the nodule structure where the symbionts are housed (see Figure 1D). This structure is essential to the bacteria, as they need an oxygen-free environment to fix atmospheric dinitrogen into biologically-available compounds such as ammonium. Nodule development is induced by bacterial colonization from the surrounding soil, and follows an intricate signalling cascade between rhizobia and the root (Oldroyd 2013). The interaction begins when rhizobia encounter legume flavonoids in the soil, which induce the bacteria to produce and secrete nodulation (nod) factors, which bind to host membrane receptors, inducing oscillations in nuclear calcium concentration. The nuclear calcium concentration-dependent transcriptional response is thought to activate the nuclear calcium- and calmodulin-dependent kinase (CCaMK). CCaMK phosphorylates the transcriptional activator CYCLOPS, inducing the expression of genes essential for symbiosis establishment, including infection thread formation and mitotic reactivation at the root cortex. Underscoring the importance of these host genes, CCaMK or CYCLOPS activation alone, without the presence of symbionts, is sufficient to induce nodule formation (Singh et al. 2014). Interestingly, many of the host genes in these pathways have homologs in non-legumes and are also involved in mycorrhizae establishment, suggesting that they may have evolved for that association first, and were co-opted for the later-evolving rhizobial associations (Singh et al. 2014).

While the full details are not yet available, preliminary evidence suggests that *Vibrio fischeri*-induced development of the squid light organ is also mediated through host transcription factor signalling pathways (Peyer et al. 2017). In this association, bioluminescent gammaproteobacterial *V. fischeri* are lured from the complex community in the surrounding seawater by host production of chitin-like compounds (Mandel et al. 2012). Upon localizing to the juvenile squid's nascent light organ epithelium, general microbe-associated molecular patterns, such as peptidoglycan, induce changes in host gene expression and mucus production. The bacteria then migrate through this mucus to colonize the light organ crypts (Kremer et al. 2013). This process is specific because the bacteria must endure acidic and free

radical bombardment by nitric oxide (Nyholm and McFall-Ngai 2004). Once within the crypts, *V. fischeri* induce apoptosis and loss of external appendage structures (see Figure 1E) through interactions with Crumb, the protein regulator of apical-basal polarity and adherens junctions (Peyer et al. 2017). Interestingly, *V. fischeri*-induced tissue differentiation does not end there. In the adult squid, bacterial interactions with genes involved in squid retinal regeneration mediate daily changes in light organ epithelial microvilli density (Heath-Heckman et al. 2016; Kremer et al. 2018).

A second mechanism for influencing host transcription has been reported for a range of pathogens and operates through direct interaction with the process itself via mimicking or influencing host transcription factors, activators, or suppressors. For example, plant pathogenic bacteria, such as *Xanthomonas*, the etiological agent of bacterial blight in rice, synthesize and secrete transcription activator-like effector (TALE) proteins through their type III secretion systems. These proteins cross into the host nucleus and mimic host transcription activators. In susceptible plants, the binding of TALEs to host transcription factors alters transcription start sites and induces the expression of host genes that increase cell size, which facilitates dissemination of the bacteria from the intercellular spaces (Saijo and Schulze-Lefert 2008; Yuan et al. 2016).

Epigenetic and transcriptional control of host differentiation are obviously effective strategies, however, genome erosion in host-associated bacteria has repeatedly limited the capacity for these types of mechanisms in many taxa. Pathogens with no or limited degrees of genome degradation are capable of modulating their gene expression at the transcriptional level (La et al. 2008). Even obligate intracellular pathogens with moderate levels of genome degradation such as the Chlamydiae exhibit evidence of using transcription factors to modulate their own gene expression (de Barsey et al. 2016). In contrast, obligate intracellular pathogens, e.g., *Treponema pallidum* (La et al. 2008), or mutualists, e.g., *Buchnera* (Hansen and Degnan 2014), with extreme levels of genome erosion (genome sizes ≤ 1 Mb) generally have relatively stable transcriptional states, although exceptions do exist (see the *Baumannia* symbiont of the glassy winged sharpshooter (Bennett and Chong 2017)). However, it is clear that some form of post-transcriptional or translational regulation has replaced these mechanisms because, in many associations, differentially expressed mRNA abundances do not correlate with translational abundances (i.e., proteins or “translatomes”, which are the ribosome-associated population of mRNAs) (Traubenik et al. 2019).

The loss of reliance on transcriptional regulation for endogenous or host genetic regulation is likely a direct consequence of genome erosion, as many of these bacteria have lost the majority of their transcription factor genes and other proteins required for transcriptional regulation (Galán-Vásquez et al. 2016). Indeed, the intracellular pathogen *Mycoplasma pneumoniae* encodes only eight predicted transcription factors in its 0.82 Mb genome (Güell et al. 2009) (compared to *E. coli*'s 314 transcription factors (Güell et al. 2011)) and expresses an abundance of antisense RNA and polycistronic operons relative to free-living bacteria (Güell et al. 2009). Interestingly, the substitution of transcriptional regulation with post-transcriptional mechanisms has not resulted in higher transcription errors (Traverse and Ochman 2016). Next, we explore

how these restricted regulatory capacities have impacted symbionts' abilities to interact with host biology.

3c. The pervasiveness of post-transcriptional mechanisms for control of host cell state

The control of host gene expression through small RNA (sRNA) pathways is a common feature among symbiotic bacteria, likely because both bacteria and eukaryotes use various types of sRNAs to regulate the turnover of their transcripts. While eukaryotes make a diversity of specific sRNA classes, such as microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA) (Palazzo and Lee 2015), bacteria make more general types of sRNA that are short to long (~50-1000s nt) and highly structured (Bobrovskyy and Vanderpool 2013). Bacterial sRNAs are either cis- or trans-acting, depending on whether they regulate the gene they were transcribed from (in the case of antisense RNAs), or whether they regulate a gene far away, respectively. Trans-acting sRNAs often have multiple targets, making them akin to post-translational transcription factors (Güell et al. 2011). Although eukaryotes and bacteria have very different endogenous mechanisms for sRNA-mediated genetic regulation, the sRNAs themselves have enough similarities to make cross-domain transfer and their function possible (reviewed in (Simonov et al. 2016; Zeng et al. 2019)). In some cases, host RNA-processing proteins are even involved in converting bacterial sRNAs into miRNA molecules (Gu et al. 2017).

Bacterial symbionts with extreme levels of genome degradation appear to have reverted to an RNA-based strategy of genetic regulation, similar to mitochondrial and plastid organelles (Cognat et al. 2017; Thairu and Hansen 2019). This is an efficient strategy for bacteria with highly degraded or streamlined genomes for three reasons. First, cis-encoded sRNA-based mechanisms of gene regulation are self-contained within the genetic element, making this regulatory approach independent of additional coding sequence, which may not be maintained during genome erosion. As trans-acting sRNAs often require an RNA chaperone protein., e.g., Hfq, for localization, the smallest endosymbiont genomes tend to not have these elements. Second, hosts use sRNA-based gene regulation, making this regulatory mechanism effective for both endogenous and host genetic regulation (Kim et al. 2016). Third, sRNAs have been shown to be critical to bacterial metabolic regulation (Bobrovskyy and Vanderpool 2013). As metabolic functions are often what intracellular mutualists are responsible for in their associations, the retention of their primary regulatory mechanism likely helps to maintain function in the face of coding sequence loss. Consistent with this, the aphid symbiont *Buchnera* has been shown to use its sRNAs to regulate its own arginine biosynthesis (Thairu et al. 2018). Of course, not all expressed sRNAs may be functional, as the often AT-rich sequence content of these genomes may produce spurious promoters (Lloréns-Rico et al. 2016). However, as pointed out by (Thairu and Hansen 2019), this “noise” may produce regulatory raw material for symbionts to select upon.

Pathogens employ sRNAs to regulate their own virulence gene expression as well as host miRNA-mediated immune responses (Sharma and Heidrich 2012; Sesto et al. 2014; Knip et al.

2014; Ortega et al. 2014; Vilcinskas 2017). Bacterial sRNA-based influence of host gene expression is exemplified by the food-borne pathogen *Salmonella*. This intracellular bacterium uses the host Argonaute RNA processing protein to modify double stranded bacterial non-coding RNA derived from the 5'-UTR of its ribosomal transcripts into ~22 bp miRNA, which it uses to promote intracellular survival (Gu et al. 2017) via mechanisms such as inhibiting nitric oxide production (Zhao et al. 2017). Despite these clear functions in host genetic regulation, facultatively host-associated enteric bacteria such as *Escherichia coli* and *Salmonella enterica* exhibit low conservation of antisense RNA expression (Raghavan et al. 2012). Given that pathogens such as *Listeria monocytogenes* do not share sRNAs with their non-pathogenic relatives, these data suggest that sRNAs may be involved in the evolution of virulence (Sesto et al. 2014). Consistent with this notion, similar mechanisms of controlling host gene expression have been reported for eukaryotic pathogens (Knip et al. 2014). For example, the fungal pathogen *Botrytis* secretes its own effector sRNAs into host cells that bind to host Argonaute proteins to inhibit immune gene expression via RNA interference (RNAi) (Weiberg et al. 2013). Bacterial pathogen-produced sRNAs may even mediate an epigenetic memory of the infection, as *Pseudomonas aeruginosa*'s sRNAs have been recently shown to induce pathogen avoidance up to four generations after infection (Kaletsky et al. 2019).

Although the majority of reported examples of symbiont-induced host post-transcriptional gene regulation involve modulating immunity or uncharacterized phenotypic effects, one example does exist of a symbiont that uses sRNA to regulate host tissue differentiation. Plant pathogenic bacteria in the genus *Agrobacterium* inhabit soils and, depending on the species, cause neoplastic tumors (galls; see Figure 1F) or excess adventitious roots (hairy roots) when they infect wounded plants (Nilsson and Olsson 1997). Within these new tissue structures, *Agrobacterium* induces host cells to synthesize metabolites (termed opines) that only the bacteria can use, effectively forming a symbiont-specific niche in the host plant (Escobar and Dandekar 2003). All pathogenic *Agrobacterium* species examined to date establish infections via transferring a plasmid-encoded section of their genome called T-DNA. Once within the host cytoplasm, T-DNA-encoded genes are expressed by the host because they contain the required eukaryotic regulatory elements (*i.e.*, TATA box, CAAT box, and polyadenylation signals) (Escobar and Dandekar 2003). Oncogenes encoded by T-DNA are responsible for inducing changes in host cell differentiation by synthesizing auxin and cytokinin plant hormones. Depending on the species' T-DNA content, either undifferentiated tumors or proliferation of differentiated tissues results from these alterations. Also encoded on T-DNA are the opine-producing genes, which synthesize these metabolites for bacterial nutrition (Nilsson and Olsson 1997; Escobar and Dandekar 2003). While it is clear that increased hormone signalling induces host plant tissue differentiation, the precise mechanisms of tumor differentiation are still being elucidated. However, recent high throughput sequencing has made it clear that bacterial factors interact with host RNA silencing pathways to induce tumor formation. Specifically, tumor formation by *Agrobacterium tumefaciens* requires host miRNA pathways, but is inhibited by host siRNA pathways. Over the course of tumorigenesis, dedifferentiation induces an anti-silencing state that inhibits siRNA-based immunity against bacterial T-DNA (Peláez et al. 2017).

In addition to sRNA-mediated mechanisms, pathogens also modify host immunogenic gene expression post-transcriptionally through RNA-binding proteins and alternate splicing (Svensson and Sharma 2016). The RNA-binding proteins carbon storage regulator (Csr) and regulator of secondary metabolism (Rsm) are produced by a range of pathogenic bacteria, including *Yersinia pseudotuberculosis* and *Legionella pneumophila*, and bind to the translation initiation region of a large diversity of mRNAs, many of which underlie host immune responses, to inhibit their translation (Svensson and Sharma 2016; Kusmierek and Dersch 2018). As splicing is involved in the activation of normal immune responses to infection, (e.g., via release of membrane-bound pre-mRNAs), pathogen-modified splicing has been proposed to be an understudied mechanism for pathogen manipulation of host gene regulation (Chauhan et al. 2019; Rigo et al. 2019). Indeed, coimmunoprecipitation experiments have shown that *Mycobacterium tuberculosis* produces effector proteins that bind to host splicing factors (Chauhan et al. 2019). In *L. pneumophila* infections, the bacteria inhibit the splicing and activation of response regulator mRNAs, which would otherwise activate the host's immunogenic unfolded protein response as a consequence of the bacteria's co-option of endoplasmic reticulum membrane (Treacy-Abarca and Mukherjee 2015). Bacterially-induced alternative host gene splicing also appears to have been co-opted by mutualistic root symbionts, as many plant transcripts are alternatively spliced during rhizobia-induced root nodule formation, although the responsible bacterial mechanisms have yet to be identified (Rigo et al. 2019).

Although only a single example of symbiont-induced host cellular differentiation via post-transcriptional gene regulation has been reported (*Agrobacterium*-induced tumors), this mode of host manipulation likely occurs more frequently in nature for a couple of reasons. First, bacterial and fungal pathogens have been shown to use their sRNA to manipulate host RNAi-based gene silencing (Weiberg et al. 2013; Gu et al. 2017). Second, this mechanism is not unique to pathogens. Organellar sRNAs have been found to interact with the nuclear-encoded Argonaute protein, suggesting that bacterially-derived organelles have retained the ability to regulate host gene expression through host RNA interference pathways (Cognat et al. 2017).

3d. Symbiont influence on host protein translation

Eukaryotic translation involves a complex suite of interactions with various protein complexes to bind the 5' cap and 3' poly-A tail of mRNA molecules, initiate translation, and elongate the growing peptide. The timing and location of this process influences protein localization and cellular patterning. Initiation is the rate limiting step of translation because it requires the recruitment of multiple initiation factor proteins to the 5' cap, recruitment of the poly-A binding protein (PABP) to both the 5' cap and 3' poly-A tail, followed by the assembly of elongation factor proteins. Thus, initiation and elongation are the steps most pathogens target to inhibit translation (Mohr and Sonenberg 2012; Jan et al. 2016). In some cases, hosts can overcome translational blocks by overexpressing mRNAs for immune responses, effectively overwhelming the components mediating the block, in a process termed mRNA superinduction (Barry et al. 2017).

Viruses excel at hijacking host translation because all must commandeer it for their own protein synthesis, and many can even induce the host machinery to preferentially translate viral mRNAs (Toribio and Ventoso 2010; Jan et al. 2016; Jaafar and Kieft 2019). One common mechanism for co-opting host ribosomes is through interacting with the cap-initiation complex during translation initiation to inhibit and/or co-opt host factors. For example, picornaviruses such as Poliovirus produce a protease that cleaves the cap-binding domain of host initiation factor protein eIF4G. That protein fragment then binds to viral mRNAs and enables cap-independent translation (Schneider and Mohr 2003). Similarly, RNA viruses like Hepatitis C are able to directly bind host ribosomes with their genome's 5'-untranslated end and a subset of host initiation factor proteins (*i.e.*, eIF3 and eIF2), enabling translation of the full viral genome (Au and Jan 2014). DsDNA adenoviruses phosphorylate host initiation factor protein eIF4E, which inhibits mRNA cap binding and enabling the virus to co-opt the translation machinery for its own mechanism, termed ribosome shunting (Schneider and Mohr 2003). In the previous two examples, viral protein synthesis is accomplished through rendering required host translational components unusable. However, examples also exist in which viral translation is accomplished while host translation is ongoing, such as the human cytomegalovirus (HCMV). Within host cells HCMV increases the expression of host PABPs, which positively regulate the expression of initiation complexes, resulting in an overall increase in the abundance of translation machinery (Au and Jan 2014). Impressively, these strategies are often robust to host interference, as viruses have evolved counter mechanisms that are enacted in response (Jaafar and Kieft 2019).

A range of bacterially produced toxins and effector proteins target host translation in order to inhibit immune responses and scavenge resources (Mohr and Sonenberg 2012). In intestinal infections, *Pseudomonas aeruginosa*-secreted Exotoxin A is endocytosed by adjacent host cells where it inhibits mRNA translation by ribosylating and inactivating host elongation factor EF2 (Dunbar et al. 2012; McEwan et al. 2012). Interestingly, the exotoxins of *Vibrio cholera* and *Corynebacterium diphtheriae* have been shown to inhibit host translation by EF2 ribosylation as well, suggesting this is a common mechanism (McEwan et al. 2012). The intracellular pathogen *Legionella pneumophila* blocks host translation through modifying host translation machinery using five of its effector proteins (Fontana et al. 2011) that act through at least two distinct mechanisms. Host translation elongation factor eEF1A is inhibited via glycosylation by the secreted *L. pneumophila* glucosyltransferases (Lgts), Lgt1, Lgt2, and Lgt3 (Michard and Doublet 2015). Additionally, phosphorylation of host chaperone protein Hsp70 by the *Legionella* eukaryotic-like gene K4 (LegK4), an effector kinase, causes Hsp70 to stall and further lowers the translation rate (Moss et al. 2019). These mechanisms appear to primarily target the host immune response, but may also potentiate the cell for metabolic rewiring (Michard and Doublet 2015). The rewiring process and *L. pneumophila*'s wide diversity of post-translational mechanisms for influencing host gene expression are discussed in the next section. Translation inhibition is essential for the establishment of *L. pneumophila* long-term, as the S-phase of the host's cell cycle is lethal to the bacterium, and blocking translation triggers cell cycle arrest (Sol et al. 2019). Fascinatingly, this attribute may be a side effect of *Legionella*'s history of association with free-living amoebae that live in oligotrophic bodies of water, which likely enter S-phase infrequently due to nutrient limitation (de Jesús-Díaz et al. 2017).

From the existing literature, it appears that mutualistic bacteria are unlikely to target host translation for two reasons: first, inhibiting translation induces strong antimicrobial responses and second, the genomes of these bacteria likely do not encode the necessary machinery. Given that all viruses hijack protein translation and many pathogens secrete effector proteins to inhibit translation, hosts have evolved signalling mechanisms to detect this perturbation and induce apoptosis (McEwan et al. 2012; Mohr and Sonenberg 2012; Cornejo et al. 2017). Thus, it is likely in the best interest of a symbiont whose strategy is to live in harmony with its host to not interfere with protein translation. Sensitivity to translational inhibition may also underlie why we were unable to find examples of symbiont-induced host cellular differentiation. Furthermore, the limited genomic coding capacity of these bacteria suggests that they do not encode the proteins necessary to do so. For example, many of these bacteria have lost a subset of their tRNA genes, and instead rely on codon wobble to pair all 61 codons. Furthermore, the 3'-CCA sequence has been lost from many of the tRNAs that remain in the genome and must be added on post-transcriptionally (Hansen and Moran 2012). Thus, these bacteria are ill-equipped to manipulate host translation.

3e. Post-translational modification of host genetic regulatory components

In both eukaryotes and bacteria, protein activity, stability, and physical location is easily altered through post-translational modifications such as phosphorylation, acetylation, methylation, and glycosylation (Macek et al. 2019). Eukaryotes have many more modifications, some of which can be applied to bacterial proteins in host cells, such as prenyl groups for lipidation and membrane attachment (Al-Quadani et al. 2011). While the mechanism of protein modification is simple - a functional group is covalently bound to a protein - the downstream impacts of protein modifications can be quite diverse. For example, ubiquitination can either lead to proteasomal protein degradation or the induction of signalling cascades, depending on the lysine residue ubiquitinated and how many ubiquitins are added (Haglund and Dikic 2005). Amazingly, despite their differences in endogenous post-translational modification capacities, many bacterial symbionts have evolved their own proteins for adding and removing eukaryotic protein modifications such as ubiquitin (Ribet and Cossart 2010; Rolando and Buchrieser 2014; Zhou and Zhu 2015).

One of the most common reasons for symbionts to manipulate host protein modifications is to alter the metabolic balance of the cell to create a nutritive niche. A straight-forward strategy to accomplish this is to increase protein proteolysis via the host's ubiquitination pathway. Short peptides and amino acids alone can go a long way towards meeting a symbiont's complete nutritional needs because many bacteria can use amino acids as both nitrogen and carbon sources (Zhang and Rubin 2013). Using eukaryotic cellular machinery, three enzymes are needed to ubiquitinate a protein, targeting it for degradation by the proteasome: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligating enzyme (E3). These three protein functional classes are not equally represented in eukaryotic genomes, with there being only a few E1 enzymes, several dozen E2 enzymes, and hundreds of E3 enzymes (Zhou and Zhu 2015). Mechanisms of bacterial interference in host ubiquitination have

evolved to mirror the host's pattern of protein diversity: the vast majority of mechanisms involve bacterial protein mimics or new versions of E3 enzymes, whereas E1 and E2 inhibitory mechanisms are less common (reviewed in (Zhou and Zhu 2015)). Some pathogens, such as *Legionella*, have even evolved novel mechanisms of ubiquitination that do not involve the E1 or E2 enzymes or ATP (Qiu et al. 2016).

As with many pathways, the ubiquitination pathway overlaps with immune and general signal transduction, making it a large target for bacterial interference. During the infection process, intracellular bacteria first have to deal with host ubiquitination to evade the innate immune system. Direct ubiquitination of intracellular pathogen membranes with host Parkin E3 ligase marks them for xenophagy (Manzanillo et al. 2013). In the event that this mechanism is insufficient, the host perceives symbiont-induced manipulations that interrupt protein synthesis or increase proteolysis, resulting in an excess of ubiquitinated proteins and amino acids in the cytoplasm. General autophagy is induced in this event, if the bacteria do not interrupt the process by reducing the number of ubiquitinated proteins with bacterially-encoded deubiquitinating enzymes (Zhou and Zhu 2015). Once the threat of ubiquitin-mediated xenophagy has been ameliorated, symbionts can alter patterns of ubiquitination to trigger changes in host gene expression which further alter immune responses and shape the cellular niche. This process is illustrated by the obligate intracellular pathogen *Chlamydia*. This bacterium uses its ChlADub1 effector protein to deubiquitinate β -catenin, preventing its degradation and enabling its transport to the nucleus where it serves as a transcription factor to activate genes invoking cell proliferation, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling, and apoptosis inhibition (Rogan et al. 2019). As these examples illustrate, and given the importance of ubiquitination in normal host biology, it is not surprising that intracellular bacteria have evolved to interact with ubiquitin and its mechanisms for addition and removal.

In addition to ubiquitination, other post-translational modifications are often used by bacterial symbionts to control host gene expression and cellular differentiation (in this case, to create a nutritive niche). The pathogen of amoebas, lung macrophages, and neutrophils, *Legionella pneumophila*, is an excellent example of a bacterium that has become proficient at altering host post-translational protein modifications to metabolically rewire the host cell (see Figure 1G). Within hours of entering a new host cell, *L. pneumophila* induces changes in the cell that cause the *Legionella*-containing vacuole (LCV) to become coated in smooth membrane derived from the endoplasmic reticulum that is lined with mitochondria and ribosomes. It accomplishes these tasks through a diverse array of nearly 300 effector proteins that are able to phosphorylate, alkylate, ubiquitinate, glycosylate, AMPylate, and phosphocholinate host proteins (Michard and Doublet 2015). Furthermore, it is able to co-opt host proteins to perform additional modifications on its own proteins, such as prenylation (e.g., farnesylation) (Al-Quadani et al. 2011). Interestingly, interaction with the endoplasmic reticulum to form a replicative niche is common among pathogens, such as the alphaproteobacterium *Brucella abortus* and the Chlamydiales bacterium *Simkania negevensis* (Cornejo et al. 2017) and is also altered in host-derived bacteriocytes that house mutualistic bacteria (Simonet et al. 2018).

To induce the formation of the LCV, *L. pneumophila* secretes a range of effector proteins into the host cytoplasm to either post-translationally modify host proteins or be post-translationally modified by them. The host-derived membrane surrounding *L. pneumophila* is first altered by the addition of endoplasmic reticulum-derived smooth vesicles, which are directed towards the forming LCV by inactivation of host GTPase Rab1 via adenylation by the effector SidM. Interestingly, *L. pneumophila* secretes two other effectors, SidB and LepD, that antagonize SidM adenylation, as well as one effector, AnkX, that can independently maintain Rab1 in the active state (Michard and Doublet 2015). This genetic redundancy suggests that this step is essential to LCV formation. As this is occurring, the AnkB effector co-opts host machinery to phosphorylate AnkB, enabling it to attach to the LCV membrane. Once attached, the F-box E3-ligase interacting domain of AnkB recruits host ubiquitin ligase complexes to the membrane where together they attach ubiquitins to the membrane underlying the bacteria. The dense polyubiquitinated clusters attract the host proteasome, which proceeds to degrade ubiquitinated proteins and provide amino acids for bacterial nutrition (Bruckert et al. 2014). Simultaneously, epigenetic changes are also induced to increase the availability of ribosomes to embed in the LCV membrane. The LegAS4 effector confers increased transcription of host rDNA via functioning as a lysine histone methyltransferase through its SET domain (Rolando et al. 2013). Thus, with *L. pneumophila*, we come full circle in our classification of symbiont-induced host differentiation because through post-translational modification of host histones, these bacteria are able to influence host gene expression at the epigenetic DNA level.

While obligately intracellular mutualists have not yet been reported to influence host post-translational protein modifications, data from symbiotically-derived organelles suggest some will have this capability, but may have different functions for it within host cells relative to pathogens. Two pieces of evidence support the idea that obligate mutualists may be able to post-translationally modify host proteins. First, the mitochondrial genome has retained genes capable of making post-translational modifications (Gabaldón and Huynen 2007). Second, both mitochondrial proteins encoded by the mitochondrial genome as well as those transferred to the nuclear genome have been shown to be post-translationally modified via phosphorylation, acetylation, and succinylation, indicating that these processes can occur within and by the organellar genome (Hofer and Wenz 2014). However, there may be striking differences between patterns of symbiont-induced host post-transcriptional modifications between mutualists and pathogens. For example, in regards to ubiquitination, amino acid economies are vastly different between pathogenic infections that usurp them from the host (Zhang and Rubin 2013) and mutualistic infections that synthesize them for the host (Feng et al. 2019). Thus, if mutualists are capable of altering host ubiquitination, they may be more likely to use it to control host signalling cascades than to obtain amino acids.

3f. Trends in symbiont-mediated host cellular differentiation mechanisms

From the examples of symbiont-mediated host cellular differentiation described above, it is clear that bacteria are capable of manipulating host gene expression at every step in the process. Some symbionts can induce host epigenetic alterations that impact the access of transcriptional

machinery to chromatin. Many taxa can interfere in transcriptional signalling cascades or transcription factor binding. An abundance of symbionts, including obligate intracellular mutualists, can modify mRNA retention by utilizing the similarities between bacterial sRNA and eukaryotic miRNA pathways. A limited range of pathogens can inhibit translation through the use of toxins and effector proteins. And, lastly, a number of pathogens use effector molecules to post-translationally modify host proteins. Impressively, these host-associated bacteria as a whole are not only able to use their own endogenous regulatory elements to control host gene expression, but they have also repeatedly evolved mechanisms for interacting with elements they do not have in their own genomes, such as histones and ubiquitination machinery.

Looking across this wide diversity of associations, both functionally and taxonomically, a few trends stand out that may reflect shared evolutionary constraints and pressures. First, bacterial symbionts tend to interact with differentiation proteins and pathways that are also involved in innate immune signalling. This may reflect the history of their interactions with their hosts. Symbionts must first evolve strategies to work with the host immune system before they evolve more complex phenotypes. Given that there is a high degree of overlap between immunological pathways and developmental pathways (Cheng et al. 2010), evasion of the immune system may have exapted, or prepared, symbionts to interact with host cellular differentiation pathways. Thus, symbionts have likely evolved the ability to manipulate new host gene regulatory pathways through cross-talk between pathways (Figure 3A).

The second trend that stands out in these examples is that symbiont genome evolution heavily influences the mechanisms available to the symbiont to control host gene expression (Figure 3B). Some symbionts have evolved mechanisms to interfere with host gene expression at every step, from DNA to mRNA to protein (e.g., *Listeria* (Sesto et al. 2014)). Whereas, other symbionts, especially those with degraded genomes, use only one or a few mechanisms. Genome degradation has proceeded far enough in some bacteria, such as the *Nasuia* and *Sulcia* symbionts of leafhoppers with 0.11 and 0.19 Mb genomes, respectively, that control of essential symbiont cellular processes has been ceded to the host (Mao et al. 2018). In these instances, it seems unlikely that the symbionts retain much capacity to manipulate their hosts. However, as many of the host nuclear genes used to maintain symbiont cellular functions were acquired through ancient horizontal gene transfer events from other bacteria (Husnik et al. 2013; Husnik and McCutcheon 2017), it is clearly not straightforward to say who is in control of who in some of these associations.

The temporal and spatial extent of genetic influence may be a factor in constraining what symbiont-mediated host regulatory mechanisms can evolve - mutualists need to live in their organs/tissues/cells for a long time and form large population sizes (discussed in (Russell and Cavanaugh 2017)), whereas pathogens only need to be there to replicate. Due to the intervening steps, the time to reach a protein-coding effect is much longer for an epigenetic alteration than it is for a post-translational modification, which is nearly instant (Hausser et al. 2013; Sasai et al. 2013; Shamir et al. 2016). Thus, the third trend from the data is that symbiont mechanisms for controlling host gene expression correspond to the organismal scale they are trying to influence (cells, tissues, or organs) and the expected duration of the association (days,

weeks, years, or lifetimes) (Figure 3C). Pathogens with highly virulent and acute infection profiles (e.g., *Legionella*, *Salmonella*, *Vibrio*, and *Chlamydia*) implement a diversity of strategies, and are far more dependent on fast-acting, targeted mechanisms such as blocking protein translation or altering post-translational protein modifications within each infected cell. Whereas more chronic types of infection (e.g., *Mycobacterium leprae* and *Helicobacter pylori*) use mechanisms higher up in the gene expression hierarchy, evoking epigenetic and transcriptional of host gene expression to permanently alter the cell fate across tissues. These mechanisms also enable many mutualistic associations (e.g., aphids with *Buchnera*), and the occasional pathogenic association (e.g., *Agrobacterium*) to develop novel symbiont-housing cells, tissues, and organs.

The fourth and final trend from these data is that selection to control host cellular differentiation has driven the evolution of entirely novel proteins and molecular mechanisms. These novel elements conceptually fall in four categories depending on whether bacteria are mimicking host proteins and/or mechanisms to manipulate host gene expression: 1) both host proteins and mechanisms are mimicked, 2) host mechanisms are mimicked using unique proteins, 3) host protein mimics are used in unique mechanisms, or 4) both the protein and mechanism are novel (Zhou and Zhu 2015). For example, SET domains fall in the first category, as these mimic eukaryotic lysine histone methyltransferase in form and function, but evolved in bacteria (Alvarez-Venegas 2014). The AnkX effector of *Legionella* is an excellent example of the second category, as it contains a conserved FIC protein domain that enacts a novel post-translational modification, phosphocholination, to modulate host Rab protein activity (Mukherjee et al. 2011). The OspF protein produced and secreted by *Shigella flexneri*, exemplifies the fourth category, as it is a novel protein that irreversibly dephosphorylates mitogen-activated protein kinase (MAPK) via a unique mechanism, which permanently prevents MAPK from phosphorylating histones for immune gene activation (Cornejo et al. 2017).

Interestingly, some hosts are able to induce some symbiotic bacteria to undergo a differentiation-like process that changes their gene expression globally and often permanently. Examples exist from both mutualists and pathogens. In mutualistic rhizobia root infections, some plants induce their symbionts to terminally differentiate, turning them into highly polyploid, often branching cells that cannot divide again. Host plants appear to accomplish this by delivering a diversity of nodule-specific symbiotic peptides, which are similar to antimicrobial peptides, to intracellular rhizobia (Maróti and Kondorosi 2014). In pathogenic *Chlamydia* infections, host cells starve the intracellular bacteria of amino acids while the bacteria replicate in their active form, termed reticulate bodies. Once amino acids become unavailable, reticulate bodies convert into aberrant bodies with low metabolic rates, which cannot always be reactivated (Zhang and Rubin 2013). These two examples suggest that symbiont metabolic activities and cell division rates can be manipulated by host actions. As more data are collected for symbiotic associations, especially from single cell transcriptomes and proteomes, it will be interesting to see if other symbionts enter these or additional types of differentiated states.

4. A natural aptitude for host manipulation: the intracellular symbiont *Wolbachia*

4a. Known *Wolbachia*-induced host reproductive phenotypes and mechanisms

The obligately intracellular alphaproteobacterium *Wolbachia* is a ubiquitous infection among arthropod and filarial nematode species. Interest in this group has increased in the past couple of decades due to discoveries that have made it suitable as a biological control agent for mosquito populations (Zheng et al. 2019) and their transmissible viruses (Hedges et al. 2008). This maternally-inherited bacterium has achieved high frequencies within and among species through a combination of reproductive manipulation (Werren et al. 2008) and/or mutualism (Gill et al. 2014; Newton and Rice 2019). *Wolbachia*'s reproductive phenotypes include cytoplasmic incompatibility (described below), male-killing, feminization, and parthenogenesis, all which manipulate embryogenesis to increase the frequency of infected females in the population (Werren et al. 2008). However, *Wolbachia*'s capacity for host manipulation does not end there. Even the cases of apparent "mutualism" in *Wolbachia* may have evolved through the manipulative complementation of host cellular and molecular pathways. In contrast to many mutualistic symbionts that imparted novel functions to the host upon their association, many of *Wolbachia*'s mutualistic functions, from apoptosis inhibition (Pannebakker et al. 2007) to oogenesis (Dedeine et al. 2005), involve processes native to the host cell, which the host's ancestors were capable of accomplishing. Thus, *Wolbachia* mutualisms may be more accurately described as "addictive mutualisms" (Sullivan 2017). Clearly, *Wolbachia* is capable of a broad spectrum of host manipulations, which suggests that it encodes a rich diversity of genes and pathways to interact with host gene expression.

Many *Wolbachia*-induced phenotypes occur during host development, and often take place in the germline stem cell, suggesting that this bacterium is able to influence host cellular differentiation. Animal development consists of a series of programmed cell division, migration, and differentiation cascades that create and pattern the adult organism (De Smet and Beeckman 2011). The ability to interact with these processes early-on obviates the need to first dedifferentiate adult host cells, as has been more frequently reported for bacterial pathogens and mutualists acquired from the environment (Wessler and Backert 2008; Masaki et al. 2013; Oldroyd 2013). This is likely due to the differences in transmission mode between these taxa, with vertically inherited *Wolbachia* being present throughout development, opposed to horizontally transmitted pathogens that get taken up by a fully differentiated adult host. Being present in the zygote (Callaini et al. 1994; Albertson et al. 2009; Fast et al. 2011), *Wolbachia* only needs to maintain stem cell status or guide the differentiation process to produce the intended cell type or molecular outcome. And, this is a skill *Wolbachia* has become adept at, as the following examples illustrate.

Often present in host germline stem cells (Russell et al. 2019), *Wolbachia* has been shown to be capable of rescuing or maintaining this cell lineage in different host taxa. In *D. melanogaster*, the wMel strain of *Wolbachia* can rescue mutations in the germline stem cell maintenance genes *sex lethal (sxl)* (Starr and Cline 2002; Sun and Cline 2009) and *bag of marbles (bam)* (Flores et al. 2015). In uninfected flies, both of these genes cause sterility in homozygous females due to the loss of germline stem cell maintenance, resulting in tumorous, over-

proliferated ovaries. Infection with wMel restores the normal ovary phenotype. While it has not yet been shown whether the rescue of these genes involves one or two bacterially-encoded processes, one wMel protein, toxic manipulator of oogenesis (TomO), has been identified that is capable of rescuing part of the phenotype resulting from the loss of *sxl*. TomO is able to maintain host germ cells, preventing their differentiation and loss, by increasing the expression of the germ cell maintenance protein Nanos via binding to *nanos* mRNAs localized within host ribonucleoprotein (RNP) complexes (Ote et al. 2016). Consistent with this mechanism, *Wolbachia* has been reported to interact with other components of host RNPs, such as the protein Gurken (Serbus et al. 2011).

While these germline stem cell maintenance genes are functional in wild-type flies, a scenario could exist in which a *Wolbachia*-infected population goes through a bottleneck and fixes a loss of function allele in the population, converting *Wolbachia* into an “obligate” infection. Wasp species in the genus *Asobara* are potentially an example of this situation. *Asobara tabida* hosts an obligate *Wolbachia* infection that is required for oogenesis, as wasps are unable to reproduce when treated with antibiotics against *Wolbachia*. This appears to have been a very recent occurrence, as all the closely related hymenopteran species do not require *Wolbachia* for reproduction (Dedeine et al. 2005). A similar situation has also been reported for the date stone beetle, *Coccotrypes dactyliperda* (Zchori-Fein et al. 2006). Over time, if a *Wolbachia*-dependent host diversifies and speciates, this process will produce a taxon entirely dependent on these seemingly mutualistic bacteria. This may be what occurred in the filarial nematode lineage. Nearly all of these parasitic worms harbor *Wolbachia* infections that are required for reproduction, development, and survival (Landmann et al. 2011). The requirement for reproduction appears to stem from *Wolbachia*'s ability to maintain quiescence in the female germline stem cell, preventing the expression of differentiation-inducing genes, and preserving its totipotency (Foray et al. 2018).

Many *Wolbachia* strains, especially those found in lepidopterans and isopods, are adept at manipulating the sex-determination systems of their hosts, turning genetic males into females (Werren et al. 2008). The induction of sex-specific gene expression across animal cells during development requires two versions of each differentiation pathway that lead to cell types with male or female-specific characteristics. Animals use cell autonomous and hormonal, non-autonomous, mechanisms to control the sex-specific gene expression profiles of their cells. Thus, both mechanisms are targets for *Wolbachia*-control of host sex-specific gene expression (Negri and Pellicchi 2012). Given *Wolbachia*'s ability to influence host hormone signalling and the overlap between hormone and epigenetic pathways, it has been suggested that *Wolbachia* may have epigenetic mechanisms for controlling host gene expression (discussed in (Negri 2012)). Consistent with this, *Wolbachia* inhibits the expression of the masculinizing gene *masc* in the the adzuki bean borer moth *Ostrinia scapularis*. As *Masc* controls both male-specific splicing and activation of dosage compensation in males, inhibition of this gene results in both female features and mortality, respectively (Sugimoto et al. 2010; Fukui et al. 2015). Similarly, in the leafhopper *Zyginidia pullula*, feminized males exhibit female DNA methylation patterns, whereas males with low *Wolbachia* titer exhibit incomplete feminization and male patterns (Negri et al. 2009). While the full mechanisms underlying these phenotypes are not known, it is

interesting to note that the *Wolbachia* genome contains a DNA adenine methyltransferase encoded on a prophage (Saridaki et al. 2011). Furthermore, a bacterially-induced epigenetic mechanism is reasonable given that many sex-specific differentiation pathways are epigenetically controlled, regardless of the sex-determining mechanism (Piferrer 2013).

The reproductive manipulation termed cytoplasmic incompatibility (CI) involves bacterial modifications of host gamete chromatin packaging, suggesting that this is another example of *Wolbachia* using an epigenetic-like mechanism to control the outcome of host reproduction. CI is a bacterially-induced mating incompatibility between infected males and uninfected females, or females with an incompatible strain of *Wolbachia*. Reproduction between these hosts fails during embryogenesis because modifications made to the sperm by *Wolbachia* fail to be compensated for in the eggs. It has been known for some time that the modifications made by *Wolbachia* result in the male pronucleus exhibiting delayed protamine removal and histone deposition in the zygote, which results in mortality at the first mitosis (Landmann et al. 2009). Recent work has revealed the bacterially-encoded genes underlying these chromatin modifications. In infected males, *Wolbachia* uses the prophage-encoded deubiquitylase CidB and its binding partner CidA (Beckmann et al. 2017; LePage et al. 2017) to alter sperm chromatin. CidB appears to confer these effects through binding to host nuclear import factor karyopherin- α and P32 protamine-histone exchange factor, which may either prevent histone assembly components from reaching the paternal chromosomes or reduce the efficacy of histone assembly (Beckmann et al. 2017). Expression of CidA in the female germline is necessary and sufficient to compensate for the CidA-CidB induced chromatin alterations made to the male sperm by *Wolbachia* (Shropshire and Bordenstein 2019). Thus, CI induction and rescue functions like a toxin-antidote system.

4b. Other known strategies of *Wolbachia*-mediated control of host gene expression

In addition to these bacterial mechanisms of controlling host gene expression that are tied to reproductive manipulations in the host, other mechanisms have been proposed for *Wolbachia*'s more general processes of survival and persistence. Compared to the above examples that were primarily focused on epigenetic or post-translational mechanisms of host genetic regulation, the following examples highlight a wider diversity of mechanisms.

To date, two studies suggest that *Wolbachia* can interfere with host translation through using its own as well as the host's transcription factors. The strain of *Wolbachia* found in *Culex molestus* mosquitoes encodes the transcriptional regulator gene *wtrM* that appears to act as a host transcription factor, upregulating the meiotic gene *grauzone*. While *grauzone* expression correlates with CI strength in the *Wolbachia* variants tested, it is not clear how increased *grauzone* expression impacts this phenotype or others (Pinto et al. 2013). In *Aedes aegypti* mosquitoes, *Wolbachia* induces expression of the host transcription factor GATA4, which suppresses expression of the host ovary-specific genes *blastoderm-specific protein 25D* (*bsg25D*) and *imaginal disc growth factor* (*disc*) (Osei-Amo et al. 2018). Given *Wolbachia*'s propensity to associate with the germline (Fast et al. 2011), high rates of vertical transmission

through oocytes (Narita et al. 2007), and various rescue capabilities in germ stem cells (discussed above), the annotations of these genes suggest that they may be involved in creating or maintaining *Wolbachia*'s niche in the female germline.

Abundant evidence exists that *Wolbachia* is able to interact with host post-transcriptional regulation through the host miRNA pathway. In *Aedes aegypti*, *Wolbachia* expresses its own sRNAs that are exported into the host cell and regulate host mRNAs. For example, *Wolbachia*'s WsnRNA-46 sRNA has been shown to increase the expression of the host motor protein dynein (Mayoral et al. 2014). Additionally, *Wolbachia* has been shown to alter host miRNA expression in *Aedes aegypti*, which impacts the expression of host protein-coding genes. For example, *Wolbachia* increases the expression of host miRNA aae-miR-2940, causing the upregulation of a host metalloprotease needed for normal infection (Hussain et al. 2011). This miRNA also downregulates host DNA cytosine methyltransferase, AaDnmt2, causing methylation to be reduced genome-wide. Interestingly, while inhibition of this miRNA is necessary for *Wolbachia* infection, its inhibition also confers inhibition of *Flavivirus* replication within infected cells (Zhang et al. 2013). In contrast, and potentially suggesting different mechanisms in different hosts or with different viruses, *Wolbachia*-induced upregulation of *D. melanogaster* DNA/RNA methyltransferase was shown to inhibit replication and infectivity of the alphavirus, Sindbis virus (Bhattacharya et al. 2017). *Wolbachia* has also been shown to upregulate aae-miR-981, which downregulates the expression of importin β -4, prohibiting AGO1 from entering the nucleus to regulate transcription (Hussain et al. 2013).

To obtain a reliable source of host amino acids, *Wolbachia* appears to have evolved mechanisms to interfere with their sink and their source, *i.e.*, translation and proteolysis, similar to the pathogens discussed above. A recent cell-based genome-wide RNAi screen in *D. melanogaster* cells infected with the wMel strain of *Wolbachia* found that bacterial density, or titer, increases when host ribosomal and translation initiation proteins are knocked down. This suggests that *Wolbachia* interacts with some of these factors in wild-type cells to alter host translation (Grobler et al. 2018). This is fascinating given the trends we reported in the previous section, which found that generally only highly virulent pathogens interfere with host translation. Supporting a role for translation interference in *Wolbachia* nutrition, this (Grobler et al. 2018) and another cell screen (White et al. 2017), found that *Wolbachia* titer decreased when host ubiquitination was inhibited. Furthermore, White et al. (2017) found that *Wolbachia* infection significantly increases ubiquitination levels in the host cell. Thus, *Wolbachia* may alter host protein synthesis as well as ubiquitination-mediated proteolysis to obtain amino acids as their primary source of nutrition. Consistent with using host protein synthesis and degradation pathways for its own nutrition, *Wolbachia* induces the reorganization of host cell endoplasmic reticulum (ER) and surrounds itself with ER-derived membrane (Fattouh et al. 2019), creating a niche near translation and proteolysis machinery. Given that ubiquitination and protein turnover is involved in host cellular differentiation (Kimata 2019), *Wolbachia* may have co-opted its nutrition-provisioning genes for host manipulation. To take the idea of molecular cross-talk in *Wolbachia* associations a step further, it is possible that *Wolbachia*'s ability to modify host protein ubiquitination was first co-opted from strategies originally evolved for evading xenophagy (*e.g.*, (Manzanillo et al. 2013; Zhou and Zhu 2015)).

4c. Exploring overlooked mechanisms: future prospects in *Wolbachia* research

We surveyed the literature for studies that assayed the impact of infection on gene expression in *Wolbachia* and/or its host and found 68 papers published between 2000 to 2019 (Table S1 and Figure 4). These studies characterized gene expression at all stages, from DNA to protein, and suggest that *Wolbachia* has mechanisms to interfere with host gene expression at many points in the process. Transcription-based studies were over-represented relative to the other gene expression stages, which is likely due to how easy generating transcriptomic data has become since the advent of microarrays and RNAseq. Future work should focus on identifying other *Wolbachia*-mediated post-translational modifications, as these have been studied the least. Furthermore, given the abundance of *Wolbachia*-induced miRNA regulation of host genes in mosquitoes discussed above, evidence for similar mechanisms should be investigated in other *Wolbachia* infections.

Although the *Wolbachia* field is still in its early days, with complete mechanisms underlying host-symbiont interactions just now being elucidated, the abundance of eukaryotic-like elements in the various *Wolbachia* strain genomes suggest a diversity of mechanisms are waiting to be discovered. These elements include deubiquitinating enzymes (Beckmann et al. 2017), ankyrin repeat proteins (Siozios et al. 2013), and proteins with dynamin domains (Rice et al. 2017). Given *Wolbachia*'s known interactions with the host cytoskeleton, including microtubule-dependent motor proteins (Ferree et al. 2005; Serbus and Sullivan 2007; Russell et al. 2018), some of these proteins could mediate these interactions. Indeed, a *Wolbachia* protein containing a synuclein domain that may mediate interactions with host actin has been characterized (Sheehan et al. 2016).

Wolbachia belongs to the Rickettsiales, a taxon with a long history of host-association, suggesting that it possesses ancient mechanisms for host manipulation. Indeed, the ancestor of the mitochondrion was likely a member of this taxon (Andersson et al. 2003) and today, Rickettsiales contains a wide diversity of pathogens, including species in *Rickettsia*, *Orientia*, *Anaplasma*, and *Ehrlichia*. These pathogens have been shown to be capable of modulating host immune responses via epigenetic (Garcia-Garcia et al. 2009) and post-translational (Sahni et al. 2018) modifications, and they themselves encode a diverse set of active sRNAs (Narra et al. 2016). Thus, future investigations of *Wolbachia* associations will likely reveal a wealth of information about the cellular and molecular mechanisms bacterial symbionts use to control host cellular differentiation, as well as how these mechanisms are maintained over evolutionary time.

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Figure 1. Examples of bacterial symbiont-induced cell, tissue, and organ differentiation across the cost-benefit spectrum of bacterial-eukaryotic symbiotic associations, organized by the point in host genetic regulation that they influence. Interestingly, no bacterial examples of translation-mediated host cellular differentiation were found, making viruses and toxin-secreting lytic bacteria the primary representatives for this strategy. A) *M. leprae*-induces dedifferentiation of Schwann cells via altering host epigenetic marks. This produces infected progenitor/stem-like cells (pSLC) that migrate and become new cell types, such as smooth muscle, spreading the infection throughout the host's body (Masaki et al. 2013). B) The fate of host-derived symbiont-housing cells, bacteriocytes, and organs, bacteriomes, is specified through changes in the abundance of host transcription factors (TFs) involved in embryogenesis (Braendle et al. 2003; Matsuura et al. 2015). In the primary aphid endosymbiont, *B. aphidicola*, bacteriocyte formation involves reorganization of the endoplasmic reticulum (orange) to surround dense symbiont (green) aggregates (Simonet et al. 2018). C) Pathogenic *H. pylori* induces host gastric epithelia to dedifferentiate and take on a mesenchymal cell fate via effector-mediated influence of host transcription factor retention and binding. Over the course of a chronic infection, this process produces over-proliferative neoplasms that can develop into gastric cancer (Bessède et al. 2014). D) Soil-dwelling rhizobia bacteria localize to legume plant roots, and induce their uptake into root cells and the formation of the root nodule through interacting with host transcription factor signalling (Oldroyd 2013). E) In juvenile bobtail squid, bioluminescent *V. fischeri* colonize a ciliated epithelium on the outside of the nascent light organ, and induce the degradation of the colonization surface's ciliated appendages through interfering with host transcription factor signalling (Nyholm and McFall-Ngai 2004). F) Plant pathogens in genus *Agrobacterium* transfer a mobile element to the host cell, which manipulates host miRNA-based genetic regulation to induce dedifferentiation and tumor formation (Escobar and Dandekar 2003). G) The intracellular pathogen *L. pneumophila* induces the formation of the *Legionella*-containing vacuole (LCV) through co-opting and mimicking host post-translational modifications to inhibit host translation and increase proteolysis of host proteins and peptides (Xu and Luo 2013).

Figure 2. Coordination between host and symbiont gene expression enables host-symbiont interactions. A) Overview of endogenous general mechanisms of eukaryotic and bacterial gene expression from mRNA transcription from DNA, to protein translation from mRNA, to protein turnover (solid lines). Methodological advancements over the past couple of decades have revealed that eukaryotes and bacteria have more mechanisms in common (pink italicized text) than previously estimated (Güell et al. 2011). Interestingly, bacteria can also regulate their mRNA via poly A-tailing, however, in contrast to eukaryotes, this signals for mRNA degradation and represents a small fraction (<<1%) of transcripts (Güell et al. 2011), which is why it is not listed above. Additionally, it should be noted that post-transcriptional regulatory components contained within mRNAs, such as 5'-untranslated regions, influence the access of proteins and other signalling molecules to transcript translation start sites and riboswitches, but are not

explicitly listed. Reciprocal control over host/symbiont processes works through endogenous and mimicked mechanisms (dashed lines). B) An example of how host-symbiont interactions (straight arrows) function with endogenous mechanisms (curved arrows) to cause phenotypic changes in cell state, such as symbiont-induced formation of an intracellular replicative niche derived from the endoplasmic reticulum membrane, as has been reported for *Wolbachia* (Fattouh et al. 2019) and a variety of other symbionts (see text).

Figure 3. Trends in the distribution of mechanisms for symbiont manipulation of host cellular differentiation. A) Cross-talk between immunological and developmental pathways due to shared components (Cheng et al. 2010) may enable bacterial symbionts (blue star) to develop novel mechanisms of host regulation, such as symbiont-induced cellular differentiation. B) Genetic regulatory capabilities are related to the state of genome erosion in bacterial symbionts. The theory of bacterial endosymbiont genome evolution posits that upon host restriction, bacterial chromosomes begin degrading due to the accumulation of deleterious mutations and the subsequent deletion of pseudogenized regions. This occurs because selection is ineffective in small, host-associated populations. The transmission bottleneck that occurs when a subset of symbionts are transmitted to offspring in vertically-transmitted associations further contributes to genetic drift driving the evolution of these genomes (Toft and Andersson 2010). Based upon the reported coding capacities and mechanisms discussed here, we propose this approximate model for the retention/loss of regulatory capacity at each regulatory level during genome erosion. C) Mechanisms of symbiont-induced host differentiation correlate with the cost/benefit trade-off of the association (depicted in red/green above, respectively) potentially due to temporal constraints. For example, virulent pathogens require fast acting mechanisms to circumvent clearance by the host immune system. Protein regulation generates a quicker response than altering host epigenetics or transcription does (Hausser et al. 2013; Sasai et al. 2013; Shamir et al. 2016). Thus, many pathogens likely first evolved to work with these mechanisms. Although, many have subsequently picked up additional mechanisms.

Figure 4. Distribution of existing literature addressing gene expression in *Wolbachia* and/or its hosts. See Table S1 for the full list of papers included here. The excess of papers studying transcription relative to the other stages of regulation reflects the ease with which transcriptomic data can be acquired since the advent of microarrays and Illumina sequencing. Effect = study found *Wolbachia* infection to have an effect on host gene expression; no effect = study found no effect of *Wolbachia* infection on host gene expression; more data needed = results were ambiguous regarding *Wolbachia*'s influence on host gene expression; and not assessed = *Wolbachia*'s impact on host gene expression was not assessed by the paper (indicated by "NA" in Table S1).

See attached excel file

Table S1. Papers investigating *Wolbachia*'s role in influencing host gene expression.

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