METAL BIOLOGY TAKES FLIGHT: THE STUDY OF METAL HOMEOSTASIS AND DETOXIFICATION IN INSECTS

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METAL BIOLOGY TAKES FLIGHT: THE STUDY OF METAL HOMEOSTASIS AND DETOXIFICATION IN INSECTS

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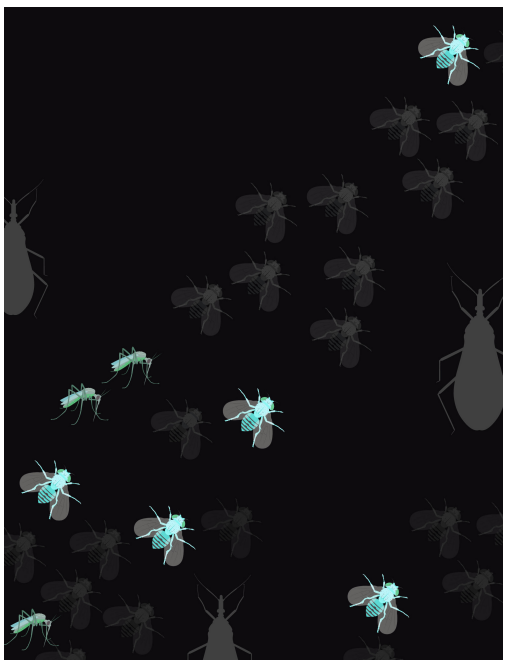

Insects have common and distinct interactions with metals. The study of insect responses to metals, and of metal transport proteins, can shed light on a number of biological and biomedical topics. Illustration designed by Stephanie E. Mohr using the online scientific illustration platform provided by BioRender (https://biorender.io/).

Metals such as copper, iron, manganese, and zinc are clearly required for proper metabolism and development, while imbalances can lead to systemic dysfunction and disease. As a result, organisms have evolved complex genetic systems for the regulation of metal levels, including import, export, and sequestration of metals within cells and sub-cellular compartments.

The study of metal biology in insects has the potential to greatly expand our understanding of metal biology. The results of such studies might point to new possible therapeutic interventions for neurological and other human diseases, as well as new strategies for insect disease vector control.

The articles collected in this Research Topic comprise review and original research on metal biology in insects.

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EDITORIAL
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Editorial: Metal Biology Takes Flight: The Study of Metal Homeostasis and Detoxification in Insects

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Editorial on the Research Topic

Metal Biology Takes Flight: The Study of Metal Homeostasis and Detoxification in Insects

Like all animals, insects must acquire nutrient metals such as copper, iron, manganese, and zinc from their environment or their hosts for essential growth and metabolic functions. When availability of these metals is low, high-affinity metal importers and binding proteins are required to maintain adequate metal intake. Alternatively, when exposure to specific metals is very high, such as iron levels for blood-feeding insects such as mosquitoes, efficient metal exporters and intracellular chelators are needed to prevent toxicity. Furthermore, nutrient metal balance must be achieved while simultaneously minimizing the intake of toxic metals such cadmium, lead, and mercury, necessitating use of selective transporters and stress response systems. All of these processes must be coordinated to maintain the proper balance of metals within insects in the face of variable metal availability.

Humans must also acquire sufficient nutrient metals from their diet while avoiding excess or toxic metals for optimal health. Imbalances in nutrient metals and exposure to toxic metals are both public health concerns since they are linked to numerous pathological conditions, including neurodegenerative disease, cardiovascular dysfunction, and human metabolic disorders (Ayton et al., 2013; Rines and Ardehali, 2013; Dusek et al., 2015; Ferreira and Gahl, 2017). Understanding the pathways and mechanisms of metal imbalances and subsequent toxicity are highly active areas of current biomedical research that have inspired new classes of therapeutics targeting metal metabolism (Barnham and Bush, 2014; Weekley and He, 2017).

Studying metal imbalances in insects provides a number of opportunities since they are diverse, often can be easily maintained in large populations in the lab, and, in the case of *Drosophila*, offer a well-studied model with detailed genetics, a defined life cycle, and a relatively short lifespan. Furthermore, sophisticated genetic tools are available for the study in *Drosophila* of human diseases such as neurodegenerative diseases and genetic disorders that are relevant to metal biology (Calap-Quintana et al., 2017). Moreover, *Drosophila* studies can be complemented by direct study of other insects, including disease vectors such as mosquitoes and kissing bugs in which metal-related cellular activities related to blood feeding can be addressed. These studies can lead to an improved understanding of conserved and species-specific aspects of metal biology. Additionally, this knowledge might provide new opportunities to control insect populations and attenuate insect-borne disease.

This Frontiers Research Topic, *Metal Biology Takes Flight: The Study of Metal Homeostasis and Detoxification in Insects,* brings together a collection of articles contributed by researchers from

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seven different countries and with different expertise that address key aspects of metal regulation in insects. Studies of several species and from both laboratory and field settings are addressed in the collection, with authors approaching relevant questions from both experimental and theoretical perspectives. In primary research articles, (1) Tsujimoto et al. identify candidate metal transporters that might play a key role in protecting *Aedes aegypti*, the mosquito vector of Zika, chikungunya and other disease-causing viruses, from iron toxification following a bloodmeal, and (2) Walter-Nuno et al. identify key genes that protect the kissing bug *Rhodnius prolixus*, a blood-feeding insect that is a vector of Chagas disease, from blood iron toxicity. Also, (3) Prince and Rand identify genes that protect *Drosophila* from methyl-mercury toxicity. In review articles that focus on *Drosophila*, (4) Navarro and Schneuwly address metal imbalances with connections to Friedreich's Ataxia, Menkes and Wilson's diseases, whereas (5) Xiao and Zhou address metal imbalances with connections to Spondylocheirodysplasia-Ehlers-Danlos Syndrome disease. In a hypothesis paper, (6) Marelja et al. examine the iron-sulfur cluster and molybdenum cofactor regulation and the potential impact on iron and heme metabolism in *Drosophila*. Finally, several reviews compare metal homeostasis in multiple insect species, including (7) Merritt and Bewick, who highlight the genetic and epigenetic mechanisms used to deal with metal toxicities; (8) Whiten et al. detail the different mechanisms used to prevent iron toxicity in blood-sucking arthropods; and (9) Ben-Shahar describes the integration of responses to manganese toxicity.

In our view, insects are relevant and appropriate model systems in which to uncover new information about metal homeostasis and detoxification that will ultimately improve public health. The articles in this collection just scratch the surface of the possibilities of this field. In putting together this Research Topic, we have endeavored to strengthen ties between studies in model and non-model insects, and hope that the reported research, reviews, and hypotheses in this topic will inspire new studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Genetic Diversity in Insect Metal Tolerance

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Insects encounter a variety of metals in their environment, many of which are required at some concentration for normal organismal homeostasis, but essentially all of which are toxic at higher concentrations. Insects have evolved a variety of genetic, and likely epigenetic, mechanisms to deal with metal stress. A recurring theme in all these systems is complexity and diversity; even simple, single gene, cases are complex. Of the known gene families, the metallothioneins are perhaps the best understood and provide good examples of how diverse metal response is. Interestingly, there is considerable diversity across taxa in these metal-responsive systems, including duplications to form small gene families and complex expression of single loci. Strikingly, different species have evolved different mechanisms to cope with the same, or similar, stress suggesting both independent derivation of, and plasticity in, the pathways involved. It is likely that some metal-response systems evolved early in evolutionary time and have been conserved, while others have diverged, and still others evolved more recently and convergently. In addition to conventional genetics, insects likely respond to environmental metal through a variety of epigenetic systems, but direct tests are lacking. Ultimately, it is likely that classical genetic and epigenetic factors interact in regulating insect metal responses. In light of this diversity across species, future studies including a broad-based examination of gene expression in non-model species in complex environments will likely uncover additional genes and genetic and epigenetic mechanisms.

Keywords: insect metal response, metallothionein, antioxidant metabolic enzymes, epigenetic regulation, ABC transporter genes

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INTRODUCTION

Insects respond to environmental metals in multiple ways; here we highlight the genetics of this response and focus on the diversity of responses including different genes, gene families, and epigenetic responses.

Metal Biology

Metals present a biological challenge in that many are absolutely required for various cellular functions, but higher concentrations are generally toxic (Southon et al., 2013). Organisms must carefully balance metal uptake, exclusion, and excretion to ensure that metals are present in sufficient, but not toxic, concentrations. Biological metal concentrations are, however, low and environmental exposure issues are generally issues of contamination leading to excess metals and toxicity. The genetics of metal response and detoxification is strikingly dynamic with independent evolution of different mechanisms to achieve this balance across animals and even specifically within insects.

Metals have a suite of biochemical functions and metal responses are similarly diverse. Many metals, e.g., iron (Fe), copper (Cu), chromium (Cr), manganese (Mn), and zinc (Zn) have key biological roles including functions in energy production, carbohydrate and lipid metabolism, and gene regulation (Bondy, 2016). Excesses of these metals, however, have both direct and indirect toxic effects (Valko et al., 2005; Southon et al., 2013). High concentrations of metals bind to DNA causing direct damage or disrupting gene expression. Heavy metals can displace essential metals and, for example, disrupt enzyme function. Such disruption can lead to oxidative stress (OS) and a suite of changes in gene expression. Metal toxicity can also result from OS caused by redox cycling metals, e.g., Fe and Cu, directly promoting generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS; Valko et al., 2005, 2015). Similarly, even metals that are redox inert can result in OS toxicity by depleting antioxidants such as glutathione or by direct binding to proteins through sulfhydryl groups (Valko et al., 2015).

Gene Systems

Dynamism is a defining characteristic of the genetics of metal response with a diversity of genes and mechanisms present across taxa. Some systems appear to be evolutionarily ancient while others are more recent adaptations and can be variable even within populations. Laboratory, studies of the physiological responses to exposure to high-metal concentrations have identified a series of metal-responsive genes. These genes function to maintain metal balance by sequestering metals and promoting their expulsion from the organism and are up regulated in response to high metal concentrations. Changes in gene expression can be functions of binding of regulatory factors, changes in chromatin accessibility or transcript stability (Janssens et al., 2009). The changes in gene expression either result directly from metal binding or indirectly through changes in redox state (Valko et al., 2005, 2015). In addition, protein concentration and activity can be modified by control of translation or post-translational regulation or stability (Janssens et al., 2009).

Metal-responsive genes have also been identified through evolutionary studies of adaptation to high metal environments. Metal tolerance has evolved in a variety of insects with interesting differences that likely reflect differences in life history and ecology. Environmental metals are often highly persistent, placing long-term selective pressures on local populations (Stone et al., 2001; Migula et al., 2004). Lacking the ability to relocate, low dispersal organisms can be continuously exposed to metals for many generations placing a high selective pressure on adaptation through genetic mechanisms, with stress-resistant members of a population more reproductively successful in contaminated sites (Migula et al., 2004). Conversely, high dispersal organisms may not develop metal tolerance as dispersal can prevent local adaptation. Evolutionary studies explore standing genetic diversity in populations and complement physiological studies, generally identifying similar genes or pathways, but with the potential to uncover novel systems especially as more diverse taxa and environments are incorporated into studies. Such an evolutionary approach identified a broad, transcriptome-wide, metal response in springtails, *Orchesella cincta*, invertebrate hexapods, from contaminated mine sites (Roelofs et al., 2009). Individuals from control and contaminated sites were exposed to cadmium (Cd) in the laboratory and the reference population, but not the tolerant population, showed a strong transcriptome-wide stress response. Further, some stress-responsive genes were less inducible in tolerant springtails, suggesting that these genes were already overexpressed in this population. These differences in expression are likely evolutionary adaptation of the population from the contaminated site; changes in gene regulation that make a major contribution to the evolution of a stress-adapted phenotype.

Fundamentally, organisms have limited energy supplies and there are costs to living in stressful environments, even "tolerant" organisms must expend metabolic resources in accommodating stressors (e.g., the metabolic cost of sequestering or expelling excess metals; Stone et al., 2001; Morgan et al., 2007). Resistant individuals are better able to handle this stress, but resistance likely comes at a cost. For example, in the absence of metal contamination, metal tolerant mosquitoes have significantly lower viability, survivorship, and fecundity than controls; their adaptations to high metal are physiological burdens in the absence of metals (Mireji et al., 2010b). In addition, metal stress can limit an organism's ability to cope with other stressors and studies of multiple stressors have led to discoveries of novel mechanisms. For example, while carabid beetles living in a pollution gradient with high levels of Zn, Cd, Cu, and lead (Pb) were phenotypically indistinguishable from controls, they were less tolerant to desiccation or pesticide exposure (Stone et al., 2001). Multiple stressors had a profound effect; the stressed population was more susceptible to additional stressors, but it was unclear if this was a physiological effect or a function of reduction in genetic variability in the population as a result of long-term contamination. This cost of resistance may, in part, account for the dynamic nature of many genetic mechanisms of metal tolerance.

In addition to highlighting the compounding effects of multiple stressors, evolutionary studies also indicate that responses to different metal stressors may be distinct and complicated, with limited overlap. A laboratory study of. *magna* (Crustacea) exposed to Cu, Cd, or Zn, found a broad suite of genetic changes, but only four genes that were differentially expressed under all three metals; the different metal stressors resulted in distinct responses (Poynton et al., 2007). A similar study of *Fundulus heteroclitus* (Actinopterygii) exposed to a suite of contaminants showed broad changes in gene expression in different populations with only limited convergence: only two genes changed expression in all three comparisons and only one of these changed in the same direction (Fisher and Oleksiak, 2007). Limited parallel responses to metal stressors are observed between insects. Initial studies of the evolution of insecticide resistance, a stressor similar to metal contamination, in *Drosophila melanogaster* (Daborn et al., 2002; Le Goff et al., 2003), suggested that resistance was a single-gene phenomenon. However, later studies indicate that multiple genes are likely involved (Pedra et al., 2004; Schmidt et al., 2010; van Straalen et al., 2011). Additionally, populations do not necessarily adapt to a high metal environment. For example, 10 generations of

high exposure to Cd or Zn led to only a moderate increase in Cd tolerance, no Zn tolerance, and no evidence for cross-resistance in *Spodoptera exigua* moths (Kafel et al., 2014). Furthermore, moths from high Zn exposure were less tolerant to Zn, possibly reflecting long-term physiological stress and an inability to adapt to the stressor.

Substantial genetic diversity in environmental metal response, within and between, species is a recurring theme in these studies. Different species respond differently to the same stressors and different stressors elicit different responses within a single species and across species. Metal tolerance is rarely, if ever, a single gene affair, and a network view is most appropriate (van Straalen et al., 2011). This point notwithstanding, a few key genes and gene families have repeatedly been found to be involved in metal tolerance (**Table 1**).

CLASSICAL GENETIC RESPONSES

Metallothioneins (MTs)

Metallothioneins (MT), small, cysteine-rich, proteins that bind metals including Cu, Zn, and Cd through metal-thiolate bridges, are one of the more thoroughly studied metal-responsive gene families (Kägi, 1991). MT gene expression is up-regulated by metals in a variety of taxa and MT gene expression has been developed as a biomarker of metal contamination in insects and other animals (Mireji et al., 2010a; M'kandawire et al., 2017). MTs are ubiquitous in eukaryotes, generally present as multi-gene families; e.g., *D. melanogaster* have at least five (Atanesyan et al., 2011) and mosquitoes (*Anopheles gambiae*) at least two (Shaw et al., 2007). These gene families likely allow functional specialization. Specific loci are differentially regulated, at both transcription and translation (Janssens et al., 2009; Mireji et al., 2010a), with specific loci expressed in response to different stimuli in specific tissues and developmental stages (Atanesyan et al., 2011; Baurand et al., 2015; Qiang et al., 2017). Different MT proteins also have distinct biochemistry, at least in *Escherichia coli* expression systems (Achard-Joris et al., 2007). Interestingly, diversity within these gene families appears to have evolved independently in different taxonomic groups, through independent evolutionary radiations and losses, not an early diversification (Guirola et al., 2010). Even within insects and other invertebrates, the MT gene family is highly diverse likely the result of independent evolutionary events and convergence on a shared pattern of diversity (Shaw et al., 2007; Janssens et al., 2009).

Metallothioneins are well studied in *D. melanogaster* (e.g., Egli et al., 2006; Guirola et al., 2010; Atanesyan et al., 2011). These flies have five known *Mt* loci, *MtnA*, *MtnB*, *MtnC*, *MtnD*, *MtnE* (Atanesyan et al., 2011; Qiang et al., 2017). These loci are all induced by a shared transcription factor and shared promoter response element, but each has distinct expression patterns and likely distinct, but possibly overlapping, functions (Egli et al., 2006; Atanesyan et al., 2011; Qiang et al., 2017). Interestingly, given the diversity of this gene family across taxa, the five loci, and their expression products are conserved across 12 species of Drosophila (Guirola et al., 2010); the family can remain stable across considerable evolutionary time. The *D. melanogaster* MT loci are all primarily expressed in the intestine, but the different loci appear to respond to different metals, e.g., *MtnA* is preferentially induced by Cu and *MtnB* by Cd (Atanesyan et al., 2011; Qiang et al., 2017). The variable gene expression is reflected in different amounts of protein and the proteins have distinct biochemical characteristics, e.g., distinct binding affinities for Zn, Cd, and Cu (Egli et al., 2006; Qiang et al., 2017). *MtnE*, is the most recent *D. melanogaster* locus to be described (Atanesyan et al., 2011), has the broadest metal binding affinity, and may be a general-metal response element (Pérez-Rafael et al., 2012). *MtnB* gene expression, and *MtnB* mutant flies, were sensitive to Cu, Cd, and Zn. *MtnB*, *MtnC*, and *MtnD* expression also respond to elevated Fe levels (Qiang et al., 2017), but this response is indirect, a result of interruption of protein interactions with other metals, such as Zn, highlighting that metal toxicity can result from more complicated interactions than simple direct metal binding or displacement, adding an additional level of complexity to the genetics of metal response (Qiang et al., 2017). Further, expression of MT proteins has also been associated with induction, rather than repression, of OS, possibly a function of release under oxidizing physiological conditions of MT-bound metals that then interact with the superoxide dismutase (SOD) system (Achard-Joris et al., 2007). This counterintuitive association of a metal-tolerance gene family with induction of OS further highlights the complexity of these stress response elements.

Metallothioneins have also been studied in other insects and invertebrates. Expression of at least one of the two mosquito MT loci responds to Cd and Pb (Mireji et al., 2010a). Interestingly, in contrast to the multiple loci in fruit flies and mosquitoes, the springtail invertebrate hexapod, *Orchesella cincta*, has a single MT locus. Expression of this single locus is metal-responsive and expression differences appear to reflect local adaptation to metal contamination (Janssens et al., 2009; Roelofs et al., 2009; van Straalen et al., 2011). Further, regulatory variation in the *O. cincta* MT gene is sensitive to genetic background, i.e., an allele behaves differently in different backgrounds, indicating that expression is modified by *trans*-acting factors (van Straalen et al., 2011) and highlighting that even this "simple" system is complex, the function of multiple genes.

Antioxidant Metabolic Enzymes

Metal exposure is associated with OS and antioxidant enzymes have a substantial role in insect metal response. Metal exposure variously results in either enhancement or inhibition of antioxidant enzymes including mitochondrial and cytosolic SOD, catalase (CAT), and Glutathione-*S*-transferase (GST; Migula et al., 2004). Aluminum (Al) toxicity in *D. melanogaster* is a function of interactions with Fe, is mediated by ROS production, and results in increased expression of mitochondrial SOD, but not cytosolic SOD and is suppressed by overexpression of CAT, but not SOD (Wu et al., 2012) again highlighting the potential for indirect interactions and the complexity of genetic responses. Beetles exposed to a complex mix of pollutants including high levels of Pb, Zn, Cd, and Cu, had significant changes in the activities

TABLE 1 | Metal responsive genes in insects.

Gene family	Species	Known interacting metals
Metallothioneins	*Drosophila melanogaster*[1,2], *Anopholese gambiae*[3,4], *Orcista cincta*[5]	Cu, Cd, Zn
Cytosolic superoxide dismutase	*Pterostichus oblongopunctatus, Geotrupes stercorosus, Staphylinus caesareus, Phyllobius betulae*[6]	Pb, Zn, Cu, Cd
Mitochondrial superoxide dismutase	*Drosophila melanogaster*[7]	Al, Fe
Catalase	*Pterostichus oblongopunctatus, Geotrupes stercorosus, Staphylinus caesareus, Phyllobius betulae*[6], *Drosophila melanogaster*[7]	Pb, Zn, Cu, Cd
Glutathione-*S*-transferase	*Pterostichus oblongopunctatus, Geotrupes stercorosus, Staphylinus caesareus, Phyllobius betulae*[6]	Pb, Zn, Cu, Cd
Glutathione reductase	*Pterostichus oblongopunctatus, Geotrupes stercorosus, Staphylinus caesareus, Phyllobius betulae*[6]	Pb, Zn, Cu, Cd
Se-dependent glutathione peroxidase	*Pterostichus oblongopunctatus, Geotrupes stercorosus, Staphylinus caesareus, Phyllobius betulae*[6]	Pb, Zn, Cu, Cd
Se-independent glutathione peroxidase	*Pterostichus oblongopunctatus, Geotrupes stercorosus, Staphylinus caesareus, Phyllobius betulae*[6]	Pb, Zn, Cu, Cd
ABC transporter proteins	*Drosophila melanogaster*[8], *Lygus hesperus*[9]	Cd
α-tubulin	*Anopholese gambiae*[3], *Chironomus tentans*[10]	Cu, Pb, Cd
CYP6	*Anopholese gambiae*[11]	Cd, Pb

Sources: [1]*Egli et al., 2006;* [2]*Atanesyan et al., 2011;* [3]*Shaw et al., 2007;* [4]*Mireji et al., 2010a;* [5]*van Straalen et al., 2011;* [6]*Migula et al., 2004;* [7]*Wu et al., 2012;* [8]*Sooksa-Nguan et al., 2009;* [9]*Hull et al., 2014;* [10]*Mattingly et al., 2001;* [11]*Musasia et al., 2013.*

of antioxidant enzymes (Migula et al., 2004), but different species responded differently, possibly reflecting differences in life history leading to different effective exposures, or differences in genetic response in each species, or both. While exact responses differed across species, in general SOD activity was high, and CAT activity low, in highly contaminated areas and activity of glutathione enzymes were more variable. Strikingly, all enzyme activities were most variable in highly contaminated environments; stressful environments expose biological variability (Migula et al., 2004). This is exactly the kind of variability that would be required for evolutionary adaptation of these stressful environments (van Straalen et al., 2011).

Additional Gene Families

A wide variety of other genes and gene families have been directly or indirectly implicated in insect metal response (Calap-Quintana et al., 2017). The ATP-binding cassette (ABC) transporter proteins, a large family of membrane proteins characterized by an ATP-binding cassette, have a variety of biological roles, including metal detoxification. Metal-responsive ABC transporter genes are present in insects (Sooksa-Nguan et al., 2009; Hull et al., 2014) and, in *D. melanogaster*, increased gene expression is associated with tolerance to metal exposure (Sooksa-Nguan et al., 2009). Cadmium and Pb tolerant *A. gambiae* mosquitoes have significant reduction in expression of the CYP6 family of cytochrome p450 genes, a gene family associated with resistance to pyrethroid pesticides (Musasia et al., 2013), suggesting potential interactions between metal tolerance and pesticide sensitivity. Expression of the alpha-tubulin gene also responds to metal concentrations in both *A. gambiae* (Mireji et al., 2010a) and *Chironomus tentans* midges (Mattingly et al., 2001). This is not an exhaustive list, and as more efforts are put in to broad-based exploratory studies (see below) it will continue to grow.

EPIGENETIC SYSTEMS

Insect metal response likely extends beyond classical genetics to epigenetics – inherited phenotypic change that is not solely due to a change in DNA sequence. Epigenetic changes appear to contribute to metal tolerance in both animals (Salnikow and Zhitkovich, 2008) and plants (Hanikenne and Nouet, 2011), but have yet to be functionally tested in insects. DNA methylation to the 5th atom of cytosine (5-methylcytosine; 5 mC) is a relatively well-studied epigenetic modification in other organisms, and an attractive modification to test for an epigenetic component to metal stress in insects. In mouse cells, for example, 5 mC variation at the promoter of a MT gene causes differences in expression; 5 mC changes histone associations and represses gene expression (Majumber et al., 2006). Additionally, in the plant *Arabidopsis thaliana*, 5 mC variation is associated with variation for biotrophic bacterial pathogen resistance (Reinders et al., 2009). However, 5 mC is absent from *D. melanogaster*, and Diptera universally (Raddatz et al., 2013; Bewick et al., 2016), hindering functional testing and leading to speculation that epigenetic regulation by 5 mC has a smaller role in insects. Species belonging to other orders of insects do, however, possess varying levels and genomic locations of 5 mC (Kharchenko et al., 2011; Bewick et al., 2016; Glastad et al., 2016a,b, 2017). The functional importance of this variation specifically in response to metals, and stress generally, has yet to be tested. However, tractable species for epigenetic functional studies have been recently identified. An insect epigenetic response to metal stress will likely be complex, but systems and tools are now at hand to move insect functional epigenetics forward (Trible et al., 2017).

FUTURE DIRECTIONS

Complexity of genes, gene families and regulatory mechanisms is a recurring theme in insect metal response. Our current understanding strongly suggests that even more complex systems exist and calls for broad-based exploration across species taking advantage of recent technical advantages in transcriptomics, proteomics, and metabolomics (Morgan et al., 2007; Poynton et al., 2007; Shaw et al., 2007; Soetaert et al., 2007). The last two fields are particularly understudied although recent work indicates that moving beyond the transcriptome can uncover unexpected, but biologically important, interactions (e.g., Knee et al., 2013; MacMillan et al., 2016).

The evolution of different metal-tolerance mechanisms also suggests that we need to explore both with established model systems and novel systems. Recent advances in gene editing technologies should open up such novel systems for study (Janssens et al., 2009). In addition, existing work indicates that multiple stressors result in different responses than single stressors and genetic differences between individuals may only be apparent under a complex set of environmental conditions (Whitehead and Crawford, 2006; Alvarez et al., 2015). Studies of novel systems in the wild or other complex stress conditions is likely, then, to identify novel resistance mechanisms (Colbourne et al., 2011; Alvarez et al., 2015).

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The Impact of Environmental Mn Exposure on Insect Biology

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Manganese (Mn) is an essential trace element that acts as a metal co-factor in diverse biochemical and cellular functions. However, chronic environmental exposure to high levels of Mn is a well-established risk factor for the etiology of severe, atypical parkinsonian syndrome (manganism) via its accumulation in the basal ganglia, pallidum, and striatum brain regions, which is often associated with abnormal dopamine, GABA, and glutamate neural signaling. Recent studies have indicated that chronic Mn exposure at levels that are below the risk for manganism can still cause behavioral, cognitive, and motor dysfunctions via poorly understood mechanisms at the molecular and cellular levels. Furthermore, in spite of significant advances in understanding Mn-induced behavioral and neuronal pathologies, available data are primarily for human and rodents. In contrast, the possible impact of environmental Mn exposure on brain functions and behavior of other animal species, especially insects and other invertebrates, remains mostly unknown both in the laboratory and natural habitats. Yet, the effects of environmental exposure to metals such as Mn on insect development, physiology, and behavior could also have major indirect impacts on human health via the long-term disruptions of food webs, as well as direct impact on the economy because of the important role insects play in crop pollination. Indeed, laboratory and field studies indicate that chronic exposures to metals such as Mn, even at levels that are below what is currently considered toxic, affect the dopaminergic signaling pathway in the insect brain, and have a major impact on the behavior of insects, including foraging activity of important pollinators such as the honey bee. Together, these studies highlight the need for a better understanding of the neuronal, molecular, and genetic processes that underlie the toxicity of Mn and other metal pollutants in diverse animal species, including insects.

Keywords: manganese, manganism, dopamine, *Drosophila melanogaster*, *Apis mellifera*, insects, arthropoda

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INTRODUCTION

Manganese (Mn) is an essential trace element that acts as a metal co-factor in diverse biochemical and cellular functions (Mertz, 1981; Santamaria and Sulsky, 2010). However, chronic environmental or occupational exposures to high levels of Mn are often neurotoxic, and a well-established risk factor for severe, atypical parkinsonian syndrome (manganism) in humans (Lucchini et al., 2009; Racette, 2014; Dorman, 2017). The exact cellular and molecular mechanisms that mediate the specific neurotoxic effects of Mn exposure to neuronal pathways associated with motor and cognitive functions are still not well understood. However, studies in humans,

and in primate and rodent animal models suggest that chronic Mn exposure leads to its accumulation in the basal ganglia, pallidum, and striatum regions of the mammalian brain, with subsequent neurotoxic effects on the dopaminergic, GABAergic, and glutamatergic signaling pathways (Olanow, 2004; Fordahl et al., 2010; Peneder et al., 2011; Karki et al., 2013; Sidoryk-Wegrzynowicz and Aschner, 2013). Surprisingly, more recent studies have indicated that chronic exposures to Mn at levels that are below the known risk threshold for manganism, could still cause behavioral, cognitive, and motor dysfunctions in humans, non-human primates, and rodents (Schneider et al., 2006; Claus Henn et al., 2010; Al-Lozi et al., 2017). Yet, whether these effects are mediated by the same neuroanatomical regions and neural signaling pathways that induce manganism remains somewhat unresolved (Gwiazda et al., 2002; Khalid et al., 2011; Li et al., 2017).

Although much of the research focus on metal biology in general, and Mn neurotoxicity in particular, has been in mammalian models, emerging data indicate that invertebrate species such as the worm *C. elegans* and insects such as the fruit fly *Drosophila melanogaster*, the honey bee *Apis mellifera*, and others are also highly sensitive to Mn toxicity, with negative impacts on behavior and higher brain functions (Orgad et al., 1998; Ben-Shahar et al., 2004; Mogren and Trumble, 2010; Chen et al., 2015a,b; Horning et al., 2015; Søvik et al., 2015, 2017). Nonetheless, in spite of significant advances in understanding the direct impact of environmental Mn exposure on human health, its environmental impact on neural functions and behavior of other animals, especially invertebrates, remains mostly unexplored. Consequently, the long-term effects of environmental Mn on food webs via its impact on the physiology of plants and pollinators are likely to play an important role in the health of ecosystems (Gall et al., 2015).

MANGANESE AND INSECT BIOLOGY

Like vertebrates, all insects require Mn as a metal co-factor for the catalytic actions of diverse enzymes (Burnell, 1988; Schramm, 2012), including the universal mitochondrial Mn-superoxide dismutase (Holley et al., 2012). In addition to its more general role as an enzymatic metal co-factor, Mn also plays a direct role in various molecular and physiological processes specifically associated with insect development and behavior. One of the first studies to describe the effect of Mn on insect physiology was the description of increased melanism in moth larvae exposed to food laced with Mn (Harrison, 1928). Later studies of the chemical composition of the insect cuticle revealed that in some species, Mn and zinc (Zn) constitute up to 10% of the total dry weight of the cuticle (Eric Hillerton et al., 1984; Quicke et al., 1998; Morgan et al., 2003; Broomell et al., 2008). Studies of the process of cuticle sclerotization in these species revealed that Mn and Zn are required for the formation of mechanically hard cuticular regions in the ovipositor of females, the female organ used for egg laying, which helps them penetrate hard plant materials such as fruit skins, as well as for development of the abrasion-resistant cutting edge of chewing mandibles in insects that feed on hard-to-chew foods such as dry seeds (Eric Hillerton et al., 1984; Cribb et al., 2008; Andersen, 2010).

Although essential, chronic dietary exposure to Mn is often toxic and detrimental to the fitness of most arthropod species, including insects, via effects on embryonic development, feeding behaviors, reproduction, immunity, and general survivability (Olsén, 2011; Kula et al., 2014; Ternes et al., 2014; de Barros et al., 2017; Martinek et al., 2018). For example, the collembolan *Folsomia candida* is highly sensitive to Mn in its diet (Kuperman et al., 2004). However, other arthropods, such as the fly *Megaselia scalaris,* show few adverse effects of Mn exposure, even at levels as high as 2,600 mg Mn/kg (Sorensen et al., 2009). While the exact mechanisms that affect the sensitivity threshold of insects to Mn exposure are mostly unknown, studies suggest that some species can actively avoid the consumption of Mn-contaminated foods (Rokytova et al., 2004), while others evolved mechanisms for efficient excretion of dietary Mn and/or its sequestration in specific body parts (Kula et al., 2014; Martinek et al., 2017, 2018).

To date, the majority of formal environmental risk assessments of toxic exposures to metals such as Mn have been characterized in the contexts of inhaled particles under specific human occupational conditions and practices, or controlled laboratory inhalation exposure studies by using mammalian animal models (Tjalve and Henriksson, 1999; Dorman et al., 2002; Antonini et al., 2006; Elder et al., 2006; Erikson et al., 2007; Bailey et al., 2017; Bevan et al., 2017). However, emerging data indicate that metal exposure via drinking water and its accumulation in aquatic environments represents a considerable risk as well (Kavcar et al., 2009; Bouchard et al., 2011). Yet, the possible broader ecological neurotoxic impact of Mn exposure either via inhaled or oral pathways on animal physiology, behavior, and overall fitness, remains mostly unknown.

Evaluating true environmental risks for insects is further complicated by of the microscale features of their ecological niches, and their diverse feeding ecologies and complex life cycles. Nevertheless, although the direct sources of Mn accumulation in aquatic and terrestrial environments are often unknown, geographical proximity to anthropogenic activities associated with metal mining and processing, and the commercial use of Mn-containing fertilizers, is a well-established risk factor. Subsequently, because the salt forms of Mn, and similarly toxic metals, are often water soluble, they readily enter food chains via their accumulation in both marine and fresh water environments. Not surprisingly, several studies have found that aquatic insects exhibit rapid uptake and tissue accumulation of Mn and other divalent metal ions present in their environment (Poteat et al., 2012), which often have a direct negative effect on their fitness (Hernroth et al., 2004; Krång and Rosenqvist, 2006; Oweson et al., 2008). Although it is reasonable to assume that, due to its solubility, the toxic impact of Mn on insects and other invertebrates is primarily restricted to aquatic environments; data suggest that Mn exposure of insects with an aquatic larval stage could still carry fitness costs in the terrestrial adult phase. Furthermore, insects with a complex life cycle could bridge the negative impacts of metal exposure across the aquatic and terrestrial ecosystems and their associated food webs (Custer

et al., 2008; Dittman and Buchwalter, 2010; Kraus et al., 2014).

Natural and anthropogenic sources of environmental Mn could also have direct negative impacts on terrestrial insects. Because many insects seem to have no aversive behavioral response to the presence of Mn and other metals in their food (Mogren and Trumble, 2010), one possible oral path to exposure is presented by the accumulation of specific metals in plants and their subsequent consumption by phytophagous insects (Devkota and Schmidt, 2000; Rodrigues et al., 2008). These exposure risks are further amplified in insect pollinators, which seem to be highly sensitive to metals (Ben-Shahar et al., 2004; Moroń et al., 2012; Vanbergen and Initiative, 2013; Søvik et al., 2015), most likely via the consumption of nectar and pollen by adult insects (Behmer et al., 2005), as well as throughout development in bee species that provision their larvae with pollen and nectar (Somerville and Nicol, 2002; Moroń et al., 2014). Because metals can accumulate in the nectar of flowering plants, insect pollinators seem to be especially sensitive to environmental metals, including Mn (Haarmann, 1998; Meindl and Ashman, 2013; Søvik et al., 2015). This particular concern is alarming because of the increase in global pollinator duress due to the negative pressure of various pathogens (Cox-Foster et al., 2007; Naug, 2014), parasites (Martin et al., 2012), and possibly insecticides (Woodcock et al., 2016; McArt et al., 2017), which together lead to major costs in pollinator fitness, which could carry major economic and ecological consequences (Khoury et al., 2011). Metal toxicity is further amplified in social pollinators, such as the honey bee, which consume nectar in the form of concentrated honey, which leads to a significant accumulation of contaminants such as heavy metals in both honey and bee tissues via prolonged exposure throughout development (Leita et al., 1996; Hladun et al., 2016; Herrero-Latorre et al., 2017; Klein et al., 2017).

MANGANESE AND INSECT BEHAVIOR

The recognition that excessive exposure to metals such as Mn could also have an impact on insect neurophysiology and behavior is not new. For example, some of the early laboratory studies of insect muscle physiology revealed that the membrane of these cells is highly permeable to Mn^{2+} and other divalent metal cations, possibly via the action of Ca^{2+} channels (Fukuda and Kawa, 1977). Specifically, several studies demonstrated that increased levels of Mn^{2+} in the extracellular bath significantly reduced the excitability and contractility of visceral muscles in diverse insect species (Deitmer, 1977; Cook and Mark Holman, 1979). However, whether under natural conditions, environmental exposure to Mn negatively affects the fitness of individual insects via its impact on muscle physiology and associated flight-related behaviors remains mostly unexplored.

Exposure to Mn can also have direct effects on the behavior of insects. Although some insect species seem to be able to detect toxic levels of Mn, and therefore behaviorally avoid the consumption of tainted foods, most insects seem to be unable to sense the presence of metals, and some increase the consumption of foods and water that contain harmful levels of some metals (Ben-Shahar et al., 2004; Mogren and Trumble, 2010; Søvik et al., 2015, 2017). Although the direct impact of Mn on insect behavior has been studied in just a few species, data suggest that Mn exposure affects general locomotion, as well as innate behaviors associated with feeding drive and food choices. One of the first clues that Mn might be involved in innate food choices in insects came from forward genetic screens for food choice behaviors in the fruit fly *Drosophila melanogaster*. One of the genes identified is the solute carrier *Malvolio* (*Mvl*), which contributes to the decision of flies to consume high sugar foods (Rodrigues et al., 1995). Subsequently, it was shown that *Mvl* is a divalent metal transporter homologous to the mammalian NRAMP transporters, and that supplementing standard fly food with Mn is sufficient to rescue abnormal food choices in adult flies (Orgad et al., 1998; D'Souza et al., 1999; Southon et al., 2008). Similarly, studies in the honey bee revealed that the brain expression of *Mvl* increases with the age-dependent division of labor exhibited by workers in honey bee colonies, and is associated with age-dependent decrease in the appetitive response threshold to sugar. Furthermore, feeding young bees with Mn resulted in a dose-dependent lowering of their response threshold to sugar, and a precocious transition from in-hive behaviors to foraging (Ben-Shahar et al., 2004). A follow up study revealed that Mn-treated bees were also poor foragers with shorter foraging career than untreated controls, further indicating that Mn exposure could lead to neurodevelopmental and cognitive deficits in pollinators (Søvik et al., 2015). Consequently, studies by us and others have shown that exposure of honey bees and other pollinators to Mn and other toxic metals could affect their behavioral responsiveness to sucrose, foraging activity, and possibly increase their foraging on metal-contaminated nectars due to abnormally low appetitive response thresholds (Ben-Shahar et al., 2004; Hladun et al., 2012, 2013, 2016; Meindl and Ashman, 2013; Søvik et al., 2015). Although the specific molecular and cellular mechanisms that mediate the effects of environmental exposure to Mn on the behavior of insect pollinators remain mostly understudied, we describe some recent insights into the cellular and molecular bases for its effects on the nervous systems of insects.

CELLULAR AND MOLECULAR TARGETS OF MANGANESE IN THE INSECT NERVOUS SYSTEM

Although the specific molecular and cellular mechanisms by which Mn exposure leads to abnormal behaviors are not completely understood (Racette et al., 2012; Andruska and Racette, 2015), human pathology and laboratory studies in rodent models indicate that environmental or occupational exposure to high levels of Mn are often associated with the symptoms of an atypical parkinsonian syndrome (Chen et al., 2014; Andruska and Racette, 2015). As in the classic Parkinson's Disease (PD), these studies clearly demonstrate that exposure to high levels of Mn leads to the specific loss of

dopaminergic neurons and associated signaling pathways in the mammalian striatum (Chen et al., 2006, 2014, 2015a; Zhao et al., 2009; Andruska and Racette, 2015). However, why Mn is specifically neurotoxic to these neuronal populations is not yet understood. Previous studies indicated that Mn directly interacts with the neurotransmitter dopamine (Parenti et al., 1988; Prabhakaran et al., 2008), which may explain, at least in part, the specificity of Mn neurotoxicity. At the subcellular level, several studies have suggested that Mn could affect dopaminergic neurons by directly interacting with PD-related proteins such as α-*Synuclein* and PARK9 (Gitler et al., 2009). Other studies in mammalian models suggested that Mn exposure leads to dopaminergic cell death via the upregulation of mitochondrial-derived oxidative stress (Kitazawa et al., 2002; Erikson et al., 2004; Ávila et al., 2014). Recently, increased oxidative stress in response to Mn exposure has also been identified in the insect *Drosophila melanogaster* (Mohandas et al., 2017). However, why this mechanism would specifically affect dopaminergic neurons is not clear. Mn is also likely to affect the dopaminergic system indirectly via its impact on the production of reactive oxygen species (ROS) in non-neuronal microglia (Zhang et al., 2009). Other indirect mechanisms include the inhibition of glutamate uptake by astrocytes, which subsequently could lead to dopaminergic excitotoxicity (Karki et al., 2014; Streifel et al., 2014; Sidoryk-Wegrzynowicz and Aschner, 2015), or the possibility of Mn-dependent epigenetic modifications (Tarale et al., 2017). Overall, these findings suggest that chronic exposure to high levels of Mn disrupts dopaminergic signaling, possibly by modulating the homeostatic relationship between excitatory and inhibitory neurotransmission pathways (Sidoryk-Wegrzynowicz and Aschner, 2013).

Similarly to humans and rodents, studies in insect models such as the fruit fly and the honey bee have indicated that chronic exposure to Mn is toxic to flies in a dose-dependent manner (Mohandas et al., 2017). Furthermore, these studies demonstrated that Mn accumulates in brain tissues even at sub-lethal exposure levels (Ben-Shahar et al., 2004; Søvik et al., 2015, 2017). However, in contrast to the effects of exposure to high levels of Mn, chronic exposure to low levels are associated with a surprising increase in brain levels of dopamine in flies and bees, as well as a transcriptional upregulation of the rate-limiting enzymes in the biosynthesis pathways of dopamine (Søvik et al., 2015). A broader analysis of the brain neurogenomic response to chronic Mn exposure, at levels that are sufficient to induce precocious foraging in honey bees, revealed a unique transcriptional response that was different from that induced by other known pharmacological inducers of foraging behavior such as cGMP (Whitfield et al., 2006).

Furthermore, *in vivo* knockdown of the Mn^{2+}transporter *Malvolio* specifically in dopaminergic neurons leads to dramatic changes in the neural architecture of the dopaminergic circuit, which can be rescued by feeding flies Mn^{2+} (Søvik et al., 2017). Because the effects of Mn on dopaminergic signaling seem to be conserved across insects and mammals, future genetic studies in *Drosophila* and other insect models will likely reveal important mechanistic insights into the impact of Mn exposure on neurobiological functions across animals, including humans. Together, the findings that Mn-induced changes in insect brain gene expression patterns represent a unique transcriptional network indicate that the effects of Mn on neuronal and behavioral phenotypes is not due to general neurotoxicity (Sinha et al., 2006).

CONCLUSION

Emerging data indicate that although high and low levels of chronic Mn exposure can lead to negative neurological and behavioral outcomes associated with dopaminergic functions, the cellular and molecular mechanisms that mediate their respective phenotypic outcomes are very different. Nonetheless, whether environmental exposure to Mn represents a broad and acute environmental risk for insects and related ecological networks remains mostly anecdotal. Yet, it is very unlikely that insects in affected habitats would not be negatively impacted by the presence of metals. Therefore, further studies of the impact of metals such as Mn on the behavior, physiology, and neural functions of insects would serve several important functions. First, although the specific molecular and cellular processes that are affected by the disruption of Mn homeostasis in the insect brain remain poorly understood, the apparent specific effects of both Mn depletion and saturation on dopaminergic signaling and its associated behavioral phenotypes suggest that this specific, highly conserved neuromodulatory network is key for understanding the role Mn is playing in regulating brain functions and behavior in health and disease in insects, with direct mechanistic implications for other animals, including humans. Therefore, insect models should be used to improve our general mechanistic understanding of the impact of Mn exposure on neural functions at the cellular and molecular levels. Second, understanding better the effects of Mn on insect behavior and physiology could be used for effective biomonitoring of Mn in affected habitats. Finally, studies of the biological impact of Mn exposure on insect behavior and physiology will help us understand better the broader ecological and environmental costs associated with anthropogenic environmental accumulation of metals, and their indirect impact on human society and health by negatively affecting insect pollinators and other important nodes of key food webs.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Notch Target Gene E(spl)mδ Is a Mediator of Methylmercury-Induced Myotoxicity in *Drosophila*

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Methylmercury (MeHg) is a ubiquitous environmental contaminant and neurotoxicant that has long been known to cause a variety of motor deficits. These motor deficits have primarily been attributed to MeHg targeting of developing neurons and induction of oxidative stress and calcium dysregulation. Few studies have looked at how MeHg may be affecting fundamental signaling mechanisms in development, particularly in developing muscle. Studies in *Drosophila* recently revealed that MeHg perturbs embryonic muscle formation and upregulates Notch target genes, reflected predominantly by expression of the downstream transcriptional repressor Enhancer of Split mdelta [E(spl)mδ]. An E(spl)mδ reporter gene shows expression primarily in the myogenic domain, and both MeHg exposure and genetic upregulation of E(spl)mδ can disrupt embryonic muscle development. Here, we tested the hypothesis that developing muscle is targeted by MeHg via upregulation of E(spl)mδ using genetic modulation of E(spl)mδ expression in combination with MeHg exposure in developing flies. Developmental MeHg exposure causes a decreased rate of eclosion that parallels gross disruption of indirect flight muscle (IFM) development. An increase in E(spl) expression across the pupal stages, with preferential E(spl)mδ upregulation occurring at early (p5) stages, is also observed. E(spl)mδ overexpression in myogenic lineages under the Mef2 promoter was seen to phenocopy eclosion and IFM effects of developmental MeHg exposure; whereas reduced expression of E(spl)mδ shows rescue of eclosion and IFM morphology effects of MeHg exposure. No effects were seen on eclosion with E(spl)mδ overexpression in neural and gut tissues. Our data indicate that muscle development is a target for MeHg and that E(spl)mδ is a muscle-specific mediator of this myotoxicity. This research advances our knowledge of the target pathways that mediate susceptibility to MeHg toxicity, as well as a potential muscle development-specific role for E(spl)mδ.

Keywords: *Drosophila*, notch, methylmercury, MeHg, muscle, enhancer of split, mdelta, myotoxicity

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INTRODUCTION

Methylmercury (MeHg) is one of the most toxic forms of mercury, which has been studied extensively for its properties as a developmental neurotoxicant (Clarkson et al., 2007). Among the wide range of neurological deficits that MeHg causes, several involve motor deficits which resemble cerebral palsy, including ataxia, muscle weakness, rigidity, abnormal muscle tone and reflexes,

and involuntary movements (McKeown-Eyssen et al., 1983; Harada, 1995; Roegge and Schantz, 2006). These motor deficits have primarily been attributed to MeHg targeting of neurons (Sager et al., 1982, 1984; Eto et al., 2010; Patel and Reynolds, 2013). However, few studies have examined whether MeHg targeting of the skeletal muscle system could also contribute to these motor deficits, particularly in a developmental context.

Studies in adult rats and zebrafish, however, have shown that MeHg can decrease muscle fiber size and dysregulate muscle mitochondrial shape and inhibit mitochondrial enzymes (Usuki et al., 1998; de Oliveira Ribeiro et al., 2008; Cambier et al., 2009). More recent studies in *Drosophila* have associated MeHg susceptibility to genes in core muscle developmental pathways and have suggested that developing muscle may also be a target of MeHg toxicity, through the modulation of muscle-specific signaling pathways, including the Notch pathway (Engel et al., 2012; Engel and Rand, 2014a; Montgomery et al., 2014). Notch signaling is a conserved developmental program, which is involved in cell fate decisions (Beatus and Lendahl, 1998; Artavanis-Tsakonas et al., 1999; Udolph, 2012),cellular differentiation (Shawber et al., 1996; Kuroda et al., 1999), as well as cellular fusion (Gildor et al., 2012; Bao, 2014). When activated in muscle lineages, Notch signaling inhibits differentiation, and maintains progenitors and satellite cells by promoting quiescence and self-renewal (Vasyutina et al., 2007; Mourikis et al., 2012; Wen et al., 2012).

In *Drosophila*, the adult muscle progenitors (AMPs) respond to Notch signaling in a timing- dependent manner. Notch signaling is active in quiescent AMPs in embryonic stages, but promotes proliferation in during larval stages (Aradhya et al., 2015). During indirect flight muscle (IFM) development, the Notch receptor is expressed on membranes of both the developing muscle and myoblasts, but is only active in myoblasts and not in the fibers (Bernard et al., 2006). Notch signaling regulates the expression of Twist (Anant et al., 1998), a transcription factor expressed in myoblasts, and promotes their proliferation (Gunage et al., 2014). Notch signaling is thought to maintain the undifferentiated state and myoblast pool until myoblasts receive cues to differentiate, at which point Notch signaling is downregulated (Anant et al., 1998). Both an early knockdown of Notch and a sustained activation of Notch were shown to affect IFM development (Anant et al., 1998). Notch signaling also regulates molecules involved in myoblast fusion and adhesion in *Drosophila* (Gildor et al., 2012; Bao, 2014), and therefore is important in the process of myocyte fusion to form syncytial myotubes and myofibers. Recently, researchers have also discovered a satellite-like cell, located on the surface of *Drosophila* IFMs, which requires Notch signaling for proliferation upon muscle injury (Chaturvedi et al., 2017).

Notch signaling has been shown to be upregulated with MeHg exposure in *Drosophila* C6 and C3 cell cultures, in *Drosophila* embryos, and in neuronal cell cultures (Bland and Rand, 2006; Rand et al., 2008; Tamm et al., 2008; Engel et al., 2012; Engel and Rand, 2014a). Curiously, in C6 cells, RNAi knockdown of either the Notch receptor or its co-activator Suppressor of Hairless had no effect on Enhancer of Split [E(spl)] upregulation by MeHg (Rand et al., 2008). This suggests that MeHg may induce transcription of E(spl)s in a receptor-independent mechanism of modulating Notch signals in the *Drosophila* model. *Drosophila* embryos exposed to MeHg also exhibited preferential upregulation of the Notch-target gene *Enhancer of split mdelta* [*E(spl)mδ*], in comparison to other genes known to respond to the activated Notch receptor (Engel et al., 2012),which also implies a Notch-independent mechanism. E(spl)mδ transcripts and an E(spl)mδ reporter gene exhibit expression in the myogenic domain in fly embryos, and E(spl)mδ has been suggested to play an essential role in mesodermal development (Wech et al., 1999; Engel and Rand, 2014a). Genetic upregulation of E(spl)mδ in the mesoderm of *Drosophila* embryos was also shown to cause a similar disruption of muscle formation as exposure to MeHg (Engel and Rand, 2014a). We therefore hypothesized that developing muscle is a direct target of MeHg and that E(spl)mδ is a muscle-specific mediator of MeHg toxicity.

In this study, we utilized the *Drosophila* model to examine the potential targeting of myogenesis by MeHg through genetic protection of muscle against MeHg exposure. As we have previously shown that Multidrug Resistance Protein (MRP) expression can modulate MeHg tolerance and susceptibility (Prince et al., 2014), we upregulated MRP in myogenic lineages and assessed MeHg tolerance through eclosion assays and IFM development. As E(spl)mδ upregulation has been implicated in MeHg exposure and is thought to be expressed in embryonic myoblasts and myotubes (Engel et al., 2012; Engel and Rand, 2014a), we furthermore assessed the role that E(spl)mδ plays in developing tissues as well as in mediating MeHg-induced myotoxicity. E(spl)mδ expression was modulated in a tissue-specific manner and in conjunction with MeHg exposure, and then the consequences on muscle morphology and eclosion behavior were assessed.

METHODS

Drosophila Stocks

The following *Drosophila* strains were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, Indiana): Mef2-RFP (a recombinant of Mef2-GAL4 and UAS-RFP) (#26882); Mef2-GAL4 (#27390) (mesodermal driver); y^1w^{6723} (YW) (#6599), Canton S. (CS) (#1); ELAV(1)-GAL4 (#458); $MRP^{EY11919}$ [a Berkeley Drosophila Genome Project line that contains a 5′ promoter and upstream activation sequence (UAS)] (#20712) (Bellen et al., 2004); K33 (#6323); UAS-mδ RNAi/TM3, Sb (#26203), rebalanced with TM3,Ser,GFP; Attp2 control (#36303). UAS-mδ h8 was a gift from Sarah Bray (University of Cambridge, England) and is also available at Bloomington (#26677). NP1-GAL4 (gut epithelial driver) and Actin-GAL4/cyo,GFP (ubiquitous driver) were kindly provided by Benoit Biteau (University of Rochester, USA). The P3 E(spl)mδ deficiency line (Wurmbach and Preiss, 2014) was kindly provided by Annette Preiss (University of Hohenheim, Germany). P3 contains a deletion extending proximally from the K33 p-element and covers the entire E(spl)mδ gene; this deletion is homozygous lethal and therefore contains the TM6B balancer. UAS-E(spl)mδ ORF

(#F000084), UAS-E(spl)mγ ORF (#F000131), and UAS-E(spl)m3 ORF (#F000090) lines (Bischof et al., 2013) were obtained from FlyORF.ch (University of Zurich, Switzerland). The UAS-SNS RNAi line (#109422) was obtained from Vienna Drosophila Resource Center (Vienna, Austria). The E(spl)mδ-GFP line was generated as described in Engel and Rand (2014a,b). Briefly, E(spl)mδ-GFP contains a 1.9 Kb region of the E(spl)mδ promoter upstream of GFP, which was cloned into the pGreen H-Pelican *Drosophila* transformation vector (Engel and Rand, 2014a). pGreen H-Pelican contains insulator sequences to avoid positional effects of chromosomal insertion. Flies were kept on a 12/12-h light/dark cycle in a 25°C humidified chamber on a standard fly food made of cornmeal, molasses, yeast, and agar.

Eclosion Assays

Tolerance of various *Drosophila* lines to MeHg was assayed by a previously described eclosion behavior assay (Mahapatra et al., 2010; Rand et al., 2014). A mating population of approximately 300 flies were prepared in small population cages equipped with an exchangeable grape-agar plate with a spot of yeast paste. Populations were composed of indicated crosses of virgin GAL4 females (approximately 200) with corresponding UAS males (approximately 100). Grape plates were exchanged after 8–14 h to collect successive embryo layings. Embryos were allowed to develop to first instar (L1) stage at 25°C. L1 larval offspring were then seeded at a density of 50 larvae per vial on vials of food (Jazz Mix, Fisher Scientific, #AS153) containing 0–20 μM MeHg (methylmercury chloride, Sigma-Aldrich # 215465), and allowed to develop for 13 days. Replicates of three vials for each MeHg concentration were achieved by collecting L1 larvae from separate embryo collection plates from the same mating population. After 13 days, flies that successfully eclosed (hatched from their pupal cases) were then counted.

Tissue-specific effects of overexpression of E(spl)mδ, E(spl)mγ, and E(spl)m3 on eclosion behavior were assayed in a similar manner as above using standard fly food without MeHg.

Pupal Staging and Harvesting

Pupae were selected according to appearance of specific physical markers, as outlined in Bainbridge and Bownes (1981), since MeHg slows down *Drosophila* development, making time after pupal formation (APF) difficult to use (unpublished observations). Stages p5, p6, and p10 were chosen as they coincide with distinct phases of IFM development. Stage p5 occurs within 12.5–25 h of pupation (Bainbridge and Bownes, 1981), a time point at which myoblasts are migrating and fusing (Fernandes et al., 1991). Stage p6 occurs between 25 and 43 h of pupation (Bainbridge and Bownes, 1981), where myoblasts complete fusion and muscles elongate (Fernandes et al., 1991). Stage p10 occurs between 69 and 73 h of pupation (Bainbridge and Bownes, 1981), and coincides with muscle maturation. Pupae were determined to be at the p5 stage if they had undergone head eversion, and contained white Malpighian tubules within the interface of the thorax and abdomen. P6 pupae were selected upon the appearance of green Malpighian tubules within the abdomen, with a dark green "yellow body" at the anterior end of the Malpighian tubules, near the interface of the thorax and abdomen. P10 pupae selected upon the eyes turning a dark red color; orbital and ocellar bristles were present, but not thoracic bristles. Pupae were collected for either imaging or RT-qPCR, as described below.

Imaging

Pupae were dissected out of their pupal case, at the indicated stages of development, and placed dorsally upward on double-stick tape on a glass slide. All pupae were imaged on a Nikon AZ100 Multizoom microscope (MVI, Avon MA). A total of 10–15 pupae were examined per treatment group. Muscle phenotypes were examined upon treatment with either 10 or 15 μM MeHg, concentrations, which are typically known to effect eclosion rates. No obvious phenotypes are seen with a 5 μM MeHg exposure. A range of phenotypes was found with MeHg treatment, making quantifications of phenotypes difficult. Therefore, quantification of MeHg effects was left to the eclosion assay, and representative images are shown here. A broader representation of the range of the phenotypes seen with MeHg exposure can be viewed in the Figures S1, S2.

RT-qPCR

To examine MeHg effects on E(spl) gene expression, first instar larvae from a mating population of approximately 300 flies of the Canton S. strain were seeded at a density of 100 larvae/vial on fly food containing either 0 or 10 μM MeHg. For each of the 3 stages of development examined (p5, 6, and p10), pooled samples of 10 whole pupae were collected from 3 independent vials of either the 0 or 10 μM MeHg treatment groups. Replicates of three vials for each MeHg concentration were achieved by collecting L1 larvae from separate embryo collection plates from the same mating population. Pupae were homogenized in Trizol (Invitrogen) using a Kontes Pellet Pestle cordless motor (Fisher #NC0493674), and RNA from the indicated pupal stages was extracted using Trizol (Invitrogen). RT-qPCR was performed using the Biorad iScriptTM One-Step RT-PCR Kit with SYBR® Green kit (Biorad, # 170-8893), according to the manufacturer's protocol. Forty nanograms of RNA was used for each sample, and samples were run using a Biorad CFX Connect Real-time System. Fold change was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Samples were normalized to the ribosomal protein RP49 (aka, L32) reference gene to calculate ΔCt. RP49 was chosen as a reference gene as it is commonly used in qPCR gene expression analyses of *Drosophila* and other insect species (Daborn et al., 2002; Rand et al., 2008; Teng et al., 2012) and was also shown in this study to exhibit a variation within 0.5 Ct between all stages and treatments examined (Figure S3). The 10 μM treatment group was then normalized to the 0 μM control group to calculate ΔΔCt. Primer sequences were as follows, represented 5′/3′:

RP49: AGTATCTGATGCCCAACATCG/TTCCGACCAGGTTACAAGAAC
MRP: CTCAGTGGGCTAACGATCAAA/CAAATCCGAAGGCACCATAAAC
E(spl)mδ: CCGTTCAGGGTCAGAGATTTAT/CCTTGAGTTCGTCCAGATACAG (for determining mδ

knockdown efficiency with Actin > mδ RNAi) and AGGATCTCATCGTGGACACC/CAGACTTCTTCGCCATG ATG (for all other RT-qPCRs)
E(spl)mγ: GTCAATGAGGTCTCCCGTTC/GGTCAACAGG GAATGGCTGG
E(spl)m7: CGTTGCTCAGACTGGCGATG/ATCAGTGTGGT TCCAAAAGC
E(spl)mβ: GCTGGACTTGAAACCGCACC/AGAAGTGAGC AGCAGCCATC
E(spl)m3: AGCCCACCCACCTCAACCAG/CGTCTGCAGC TCAATTAGTC
Notch: GAATCTGCCCAGTCCGTAC/CCATTCATCCCGA GTCCT

Statistical Analysis

For RT-qPCR comparing 10 μM treatment to control treatment (0 μM), a two-tailed student-test was conducted. Fold change was calculated by comparing expression to that of the control treatment. A two-tailed student-test was also used for confirming upregulation or downregulation of Multidrug Resistance Protein (MRP) or E(spl)mδ; relative expression levels were expressed as a fold change, calculated by comparing expression to that of either the parent strain or the GAL4 crossed with the UAS parent strain, as indicated. All values are represented as an average of three replicates plus and minus standard error. $p \leq 0.05$ were considered to be significant.

For RT-qPCR comparing expression levels of two independent UAS-mδ constructs to that of the Mef2-GAL4 parent line, a one-way ANOVA with Tukey's multiple comparisons test was conducted. All values are represented as an average of three replicates plus and minus standard error. $p \leq 0.05$ were considered to be significant.

For eclosion assays, a two-tailed *z*-test was conducted, as the percent of flies successfully eclosed is a non-continuous value reaching 0 and 100% at the minima and maxima, respectively. Each MeHg concentration was treated categorically by comparing respective genetically manipulated strains or crosses to their relevant control strain or cross, as indicated. All values are represented as an average of three replicates plus and minus standard error. *p*-values of less than 0.05 or equal to were considered to be significant.

RESULTS

Effects of Genetic Protection of Muscle on MeHg Tolerance and Indirect Flight Muscle Development

We have previously shown that MeHg disrupts muscle development in *Drosophila,* both in embryos (Engel and Rand, 2014a) and in the indirect flight muscles (IFMs) during pupation (Montgomery et al., 2014). As neural and muscle development are highly dependent on each other (Fernandes and Keshishian, 1998; Landgraf et al., 1999), this finding could not distinguish whether or not MeHg-induced effects on muscle development may be a consequence of MeHg targeting of neurons. To test whether muscle is being directly affected, independent of effects that MeHg may have on neurons, we attempted to genetically protect muscle lineage cells by targeted upregulation of the Multidrug Resistance Protein (MRP) using the GAL4-UAS system (**Figure 1A**). MRP is a xenobiotic transporter that is known to excrete MeHg-glutathione complexes out of cells (Cernichiari et al., 2007). Tolerance to MeHg was assessed upon overexpression of MRP in muscle (Mef2-GAL4), neural (ELAV-GAL4), and gut (NP1-GAL4) tissues. We first evaluated eclosion behavior, which we have previously shown to be a sensitive read out of MeHg toxicity (Mahapatra et al., 2010; Rand et al., 2014). Eclosion is the very first behavior exhibited by the adult fly and requires a stereotypic peristaltic muscular contraction program known as extrication behavior (Reid et al., 1987). Eclosion rates are seen to decrease in a dose-dependent manner with developmental exposure to MeHg (**Figure 1**). Upon upregulation of MRP in gut tissue (NP1 > MRP), eclosion rates are slightly reduced at the 10 μM MeHg exposure in comparison to its control (NP1 > YW) (**Figure 1B**). Eclosion rates remain unchanged at all concentrations of MeHg when MRP is upregulated in neurons [ELAV(1) > MRP], in comparison to its control [ELAV(1) > YW] (**Figure 1C**). MRP upregulation in muscle (Mef2 > MRP), however, significantly increases the rate of eclosion on all concentrations of MeHg examined, in comparison to its control (Mef2 > YW) (**Figure 1D**).

To examine whether MRP upregulation could also rescue IFM perturbation by MeHg, we utilized the Mef2-RFP driver line to constitutively reveal muscle morphology via RFP expression while also driving MRP expression. Mef2-RFP > YW (control, **Figures 2A–C**) and Mef2-RFP > MRP (**Figures 2D–F**) 1st instar larvae were treated with MeHg concentrations known to inhibit eclosion, 10 and 15 μM MeHg, and allowed to develop to p10 pupae. Stage p10 was chosen as a representative culmination of muscle development. Normal flight muscle development is seen in the 0 μM treatment of both Mef2-RFP > YW (**Figure 2A**) and Mef2-RFP > MRP (**Figure 2D**) pupae, with the dorsal longitudinal muscles (DLM, closed arrows) and dorsal ventral muscles (DVM, asterisks) assuming a fully formed fiber morphology. With increasing concentrations of MeHg the IFMs of Mef2-RFP > YW are disrupted with muscles presenting in an aggregated mass (**Figures 2B,C**, open arrow). The flight muscles of Mef2-RFP > MRP are seen to be normal with exposure to 10 μM MeHg (**Figure 2E**), and only slightly disrupted at 15μM MeHg, but muscle fibers are clearly discernable (**Figure 2F**) (additional images in Figures S1A–C).

Effects of Developmental MeHg Exposure on E(spl) Expression in Pupae

We have previously shown that *E(spl)mδ* is upregulated in embryos exposed to MeHg (Engel et al., 2012). We therefore examined if similar effects on E(spl) expression with MeHg exposure occurs across timepoints that represent distinct stages of IFM development in the pupae. The expression of *E(spl)mδ* RNA across normal pupal development is shown to be dynamic, with the highest expression at the p5 stage (12.5–25 h after pupal

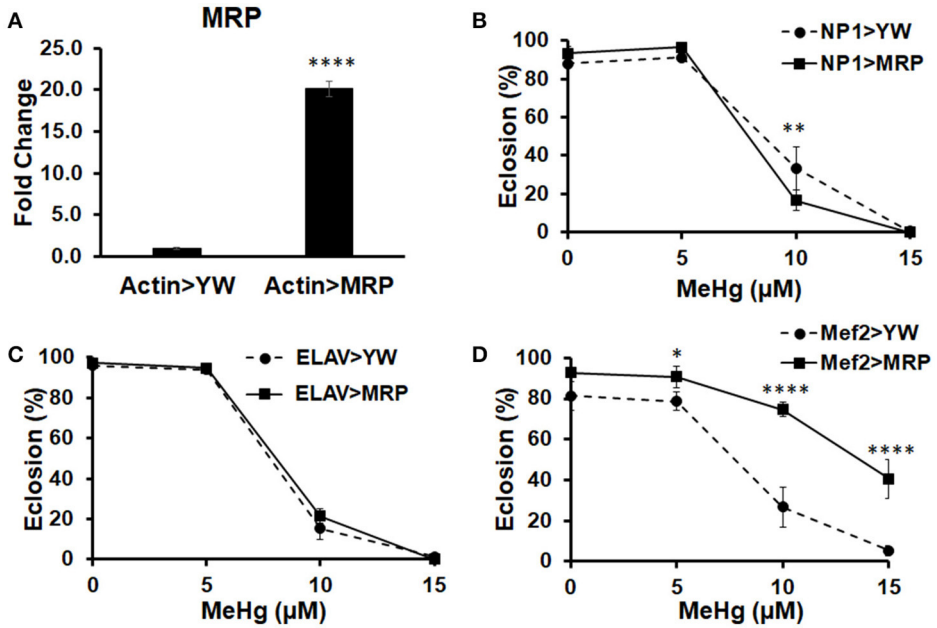

FIGURE 1 | Multidrug Resistance Protein (MRP) upregulation in myogenic lineages conveys MeHg tolerance during development. **(A)** Expression of MRP, a xenobiotic and MeHg transporter, was assessed by crossing UAS-MRP ($MRP^{EY11919}$) with Actin-GAL4 and conducting RT-qPCR on RNA extracted from pupal offspring (****$p < 0.0001$, *t*-test). Tolerance to MeHg during development was determined using an eclosion assay, with offspring of control (YW) or UAS-MRP flies crossed to various driver lines: **(B)** NP1-G4 (gut driver), **(C)** ELAV-G4 (neural driver), and **(D)** Mef2-G4 (muscle driver). Asterisks mark statistical significance in comparison to control at each treatment (*$p \leq 0.05$, **$p \leq 0.01$, ****$p < 0.0001$, *z*-test).

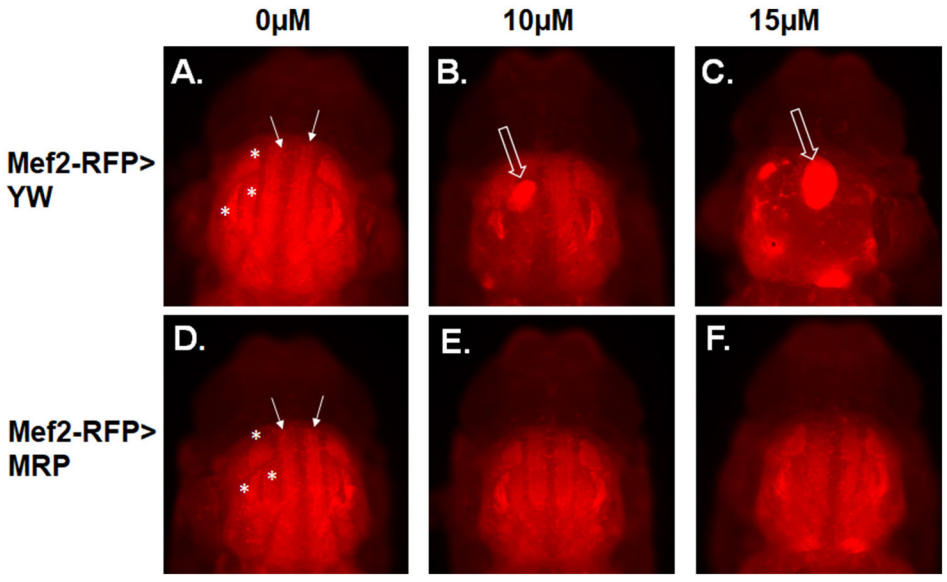

FIGURE 2 | MeHg effects on indirect flight muscle (IFM) development are rescued with MRP upregulation. Epifluorescence images of IFMs of pupae at stage p10. **(A–C)** Mef2-RFP > YW (control) and **(D–F)** Mef2-RFP > MRP. Pupae were imaged after treatment with the indicated concentration of MeHg from the 1st instar larval stage. Asterisks mark the dorsal ventral muscles (DVM) and closed arrows mark the dorsal longitudinal muscles (DLM). Open arrows indicate failure of muscle fiber development (see Figure S1 for additional images).

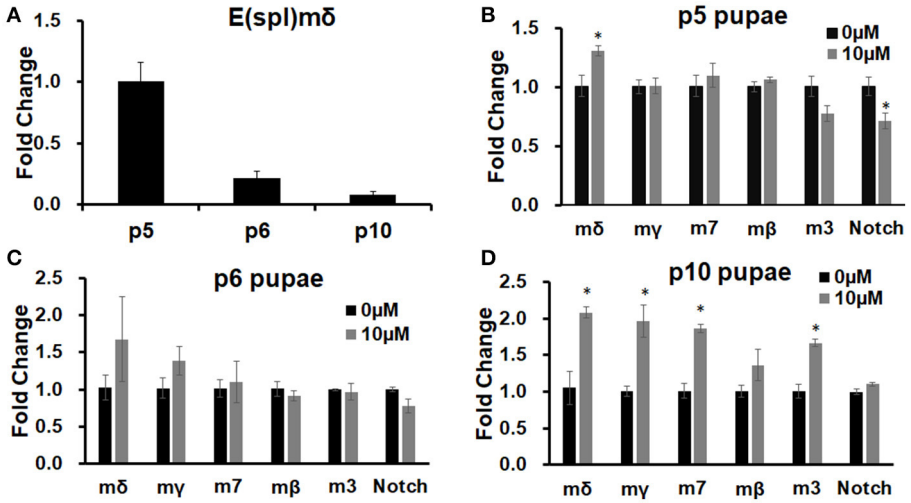

FIGURE 3 | E(spl) expression in pupae developmentally exposed to MeHg. RT-qPCR on RNA extracted from pupae was performed to assess expression levels of E(spl)mδ and other E(spl) transcription factors. **(A)** E(spl)mδ expression over pupal development in Canton S. **(B–D)** Canton S. 1st instar larvae were exposed to either 0 or 10 μM MeHg and allowed to develop to **(B)** p5, **(C)** p6, and **(D)** p10 stages of pupal development. Asterisks mark statistical significance from respective 0 μM treatment (*$p \leq 0.05$, *t*-test).

formation, APF; **Figure 3A**), a time when adult muscle precursor myoblasts (AMPs) are migrating and fusing with larval template muscles (Fernandes et al., 1991). *E(spl)mδ* expression shows a large decrease across the p6 (25–43 h APF) and into p10 (69–73 h APF) stages thereafter (**Figure 3A**). This is consistent with what has been reported for *E(spl)mδ* expression in the modENCODE temporal expression data set (Gelbart and Emmert, 2013). In comparison to control pupae, *E(spl)mδ* is significantly elevated with 10 μM MeHg exposure at the p5 stage ($p = 0.036$; **Figure 3B**). This upregulation occurs despite a slight decrease in *Notch* expression ($p = 0.046$; **Figure 3B**). Expression of other E(spl) genes is unmodified by MeHg exposure at this stage (**Figure 3B**). *E(spl)mδ* exhibits a trending increase, that does not reach significance, at stage p6 (**Figure 3C**), a time point where the majority of myoblasts are presumed to have fused with the IFM larval templates and muscle fibers begin extending to their tendon sites (Fernandes et al., 1991). The variation in the MeHg effect on *E(spl)mδ* levels seen at this stage likely stems from sampling within a developmental period where *E(spl)mδ* shows the greatest drop in endogenous expression (**Figure 3A**). Expression of *E(spl)mδ,* as well as *E(spl)mγ, E(spl)m7, and E(spl)m3* is significantly elevated, with respect to controls, with MeHg exposure at stage p10 (**Figure 3D**), a stage at which IFMs approach a mature fiber morphology. *Notch* and *E(spl)mβ* expression is not seen to be altered with MeHg exposure at p10.

E(spl)mδ Expression in Developing IFMs during Pupation

Because E(spl)mδ expression appears to localize to developing embryonic muscles (Engel and Rand, 2014a), we also wanted to see if E(spl)mδ is similarly expressed in developing adult flight muscles. IFM development was examined in pupae of various stages carrying a Mef2Gal4 > UASRFP and an E(spl)mδ enhancer-GFP reporter gene. RFP and GFP expression patterns were seen to largely overlap in the developing flight muscles in the thorax across the p5-p10 stages. At stage p5, Mef2-RFP and E(spl)mδ-GFP are seen to superimpose in the region where migrating myoblasts are presumably fusing with larval template (**Figures 4A–C**, brackets). E(spl)mδ-GFP is also seen to be expressed independently of Mef2 in the developing eye, a pattern that persists into p6 (**Figures 4B,E** green arrow). By stage p6, DLM fibers are clearly visible and expressing both RFP and GFP at a time where a majority of myoblasts have presumably fused with the larval templates and the growing fibers are extending out to their tendon sites of attachment (**Figures 4D–F**). By p10, the DLM (arrows) and DVM (asterisks) muscles are fully formed and visible with the perdurance of the RFP and GFP expression (**Figures 4G–I**). These data indicate the E(spl)mδ enhancer is activated in developing IFM muscle, suggesting that E(spl)mδ protein is likely expressed in this myogenic lineage.

Effects of E(spl) Overexpression in Muscle, Neurons, and Gut

To test whether E(spl)mδ upregulation by MeHg could directly affect muscle development and influence eclosion, we induced E(spl)mδ expression in the myogenic domain with the UAS-E(spl)mδ ORF responder (**Figure 5A**, Mef2-G4). For comparison, we also upregulated E(spl)mγ and E(spl)m3, by crossing Mef2-G4 with the corresponding UAS-ORF responders. The neuronal driver (ELAV-G4) and gut driver (NP1-G4) were also used for comparison of tissue-specific effects (**Figures 5B,C**). Upregulation of E(spl)mδ ORF in developing muscle almost completely inhibits eclosion (3% eclosion, **Figure 5A**). This effect is not specific to E(spl)mδ,

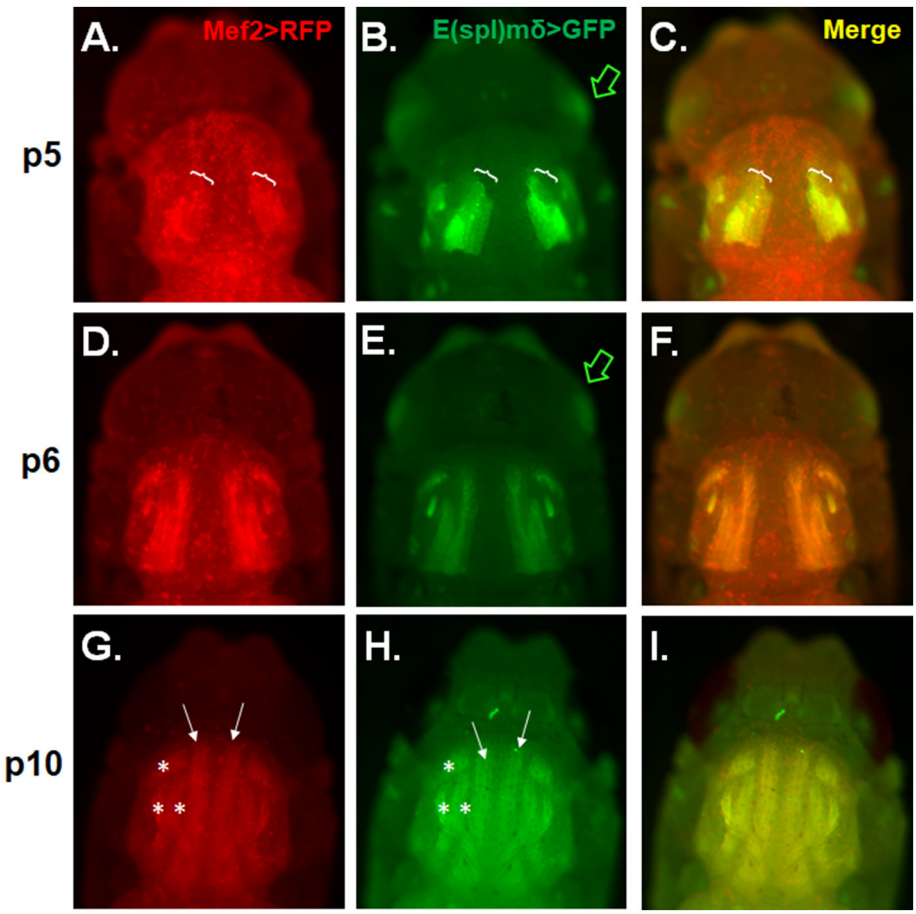

FIGURE 4 | E(spl)mδ is expressed in the developing IFM. Live epifluorescent imaging of developing IFMs in pupae carrying both Mef2-RFP and E(spl)mδ-GFP at the indicated stages **(A–C)** p5, **(D–F)** p6, and **(G–I)** p10. **(C,F,I)** Merged images of RFP and GFP represent overlapping regions of expression of Mef2 in developing IFMs and E(spl)mδ. Pupae were dissected from their pupal cases and imaged directly. Asterisks mark the dorsal ventral muscles (DVM) and closed arrows mark the dorsal longitudinal muscles (DLM). Brackets mark the regions of DLM development that encompass myoblast fusing to the larval templates. Green arrow points to E(spl)mδ expression in the developing eye.

as both E(spl)mγ ORF, and E(spl)m3 ORF greatly reduce eclosion rates (**Figure 5B**). However, both E(spl)mγ, and E(spl)m3 upregulation in muscle show slightly less of an effect on reducing eclosion rate (6 and 13%, respectively), compared to upregulation of E(spl)mδ (**Figure 5A**). In comparison, overexpression of these E(spl)s in neurons (**Figure 5B**) and gut tissue (**Figure 5C**) has no effect on eclosion rate.

Flight Muscle Morphology and Eclosion Behavior Upon Upregulation of E(spl)mδ in Myogenic Lineages

Since MeHg causes only a moderate increase in *E(spl)mδ* expression levels, we next sought to determine the sensitivity effects of E(spl)mδ upregulation upon development and eclosion. Using a second responder line, UAS-mδ h8, which shows a 13-fold expression increase compared to a 19-fold increase seen with UAS-mδ ORF (**Figure 6A**), we assessed dose dependent effects of E(spl)mδ expression on eclosion rates. The increasing levels of E(spl)mδ expression seen with Mef2 > mδ h8 and Mef2 > mδ ORF, respectively, is seen to correspond with a decreasing eclosion rate (**Figure 6B**).

We next examined if the dose-dependent E(spl)mδ effect could be discerned in IFM morphology and what stage of muscle development might be sensitive to E(spl)mδ upregulation. For comparison, we also examined IFM development upon RNAi knockdown of Sticks and Stones (SNS), a core mediator of myoblast adhesion and fusion (Bour et al., 2000). Expression in the Mef2-RFP control reveals a normal pattern of flight muscle development across the p5-p10 stages (**Figures 6C–E**). In contrast, extending fibers are not visible in Mef2-RFP > E(spl)mδ ORF p6 pupae (**Figure 6G**), prossibly reflecting a failure in the preceding events of myoblast fusion to larval templates and/or larval template integrity. This pattern of muscle development failure with E(spl)mδ ORF expression persists into stage p10

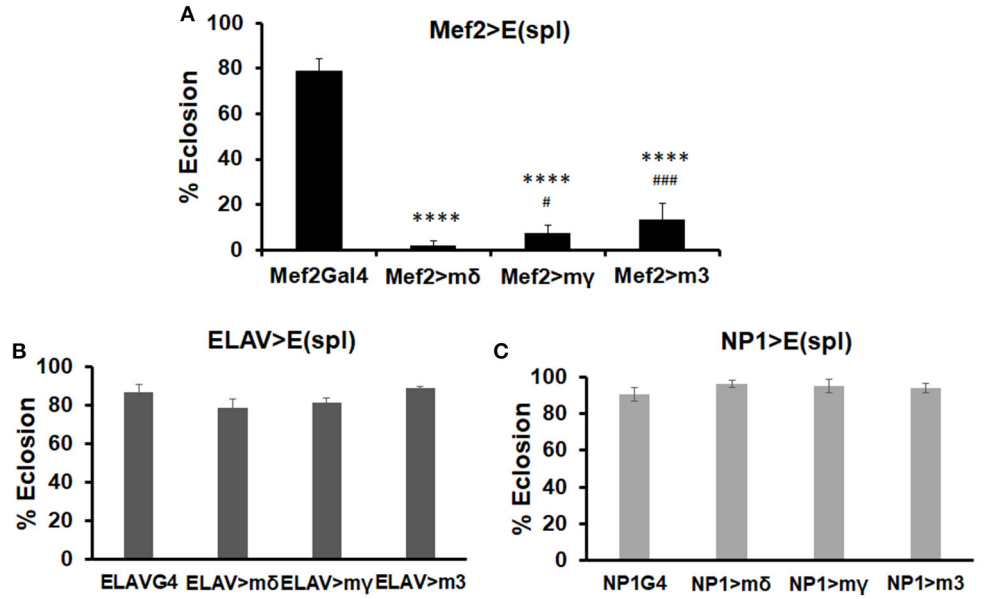

FIGURE 5 | Effects on eclosion rate upon upregulation of various E(spl)s in neurons, gut, and muscle. Developmental effects of genetic upregulation of E(spl)mδ ORF, E(spl)mγ ORF, and E(spl)m3 ORF were assessed by eclosion assay in the absence of MeHg exposure. UAS-E(spl) ORF responders were crossed with various drivers: **(A)** Mef2-G4 (muscle driver), **(B)** ELAV-G4 (neural driver), and **(C)** NP1-G4 (gut driver). The number of flies successfully eclosed were scored (****$p < 0.0001$, in comparison to Mef2-Gal4; #$p < 0.05$, ###$p < 0.001$, in comparison to Mef2 > mδ, *z*-test).

(**Figure 6H**). Despite the lower E(spl)mδ expression level in Mef2-RFP > mδ h8 pupae (**Figure 6A**), the pattern and severity of the muscle development phenotype could not be discerned from that seen with Mef2-RFP > E(spl)mδ ORF (**Figures 6I–K** vs. **Figures 6F–H**).

With SNS RNAi, pupae at p6 stage show a similar pattern as seen with f Mef2-RFP > E(spl)mδ pupae (**Figure 6M** compared to **Figures 6G,J**). Muscle fibers are seen to form by p10 in Mef2RFP > SNS RNAi pupae (**Figure 6N**, green arrows); however, DLM fibers appear much thinner than normal, and some DVMs are not apparent (**Figure 6N**, asterisks).

Effects of E(spl)mδ Knockdown on MeHg Tolerance and IFM Development

To examine if E(spl)mδ is a muscle-specific mediator of MeHg toxicity, we tested the effects of reducing *E(spl)mδ* expression in conjunction with MeHg exposure on eclosion and IFM development. Tolerance to MeHg upon reduction of *E(spl)mδ* expression was examined using an E(spl)mδ deficiency line (P3) as well as an RNAi against *E(spl)mδ*. The P3 E(spl)mδ deficiency line, which was necessarily maintained as a heterozygote, exhibits an *E(spl)mδ* expression level at 35% of the K33 control strain (**Figure 7A**). RNAi knockdown with the ubiquitous actin driver (Actin-G4) results in *E(spl)mδ* expression at 17% of control levels (**Figure 7C**). The P3 E(spl)mδ deficiency shows a greater tolerance to MeHg relative to its control strain (K33), as seen by an increase in eclosion rate at the 10 μM MeHg treatment level (**Figure 7B**). In comparison, RNAi knockdown of E(spl)mδ in the muscle domain, exhibits an even greater tolerance to MeHg, with increased eclosion rates at 10 and 15 μM MeHg exposures (**Figure 7D**). The greater tolerance to MeHg of both the P3 strain and the Mef2 > mδ RNAi cross is despite small but significant decreases in eclosion rates at the 0 μM MeHg treatment (**Figures 7B,D**).

Effects of E(spl)mδ knockdown on muscle morphology were also examined in p10 pupae exposed to 0, 10, and15 μM MeHg (**Figure 8**). At 10 μM MeHg, the Mef2-RFP > Attp2 control exhibits evidence of some IFM defects (**Figure 8B**, open arrow); whereas at 15 μM, the muscle fibers of Mef2-RFP > Attp2 are non-existent. Aggregates of RFP expressing bodies in the thorax suggest that myoblast fusion may have occurred but that DLM and DVM fiber elongation has failed (**Figure 8C**, asterisk). In comparison, muscle fiber development, is largely rescued from the effects of 10 and15 μM MeHg treatment with expression of E(spl)mδ RNAi in muscle (Mef2-RFP > mδ RNAi) (**Figures 8E,F**, asterisk and open arrow) (Additional images in Figures S2A–C).

DISCUSSION

We have shown here that protecting developing muscle from the effects of MeHg, through the upregulation of MRP in the muscle domain, can rescue the overall development of the fly. This can be seen both in an increase in the flies' ability to eclose and in a rescue of the effects of MeHg on adult flight muscle morphology. Using the same strategy to protect neuronal or gut tissue did not show an increased tolerance to MeHg, as measured by eclosion rates. These data suggest that muscle development can be targeted by MeHg through a mechanism independent of the effects of MeHg on neural development. Although the dose

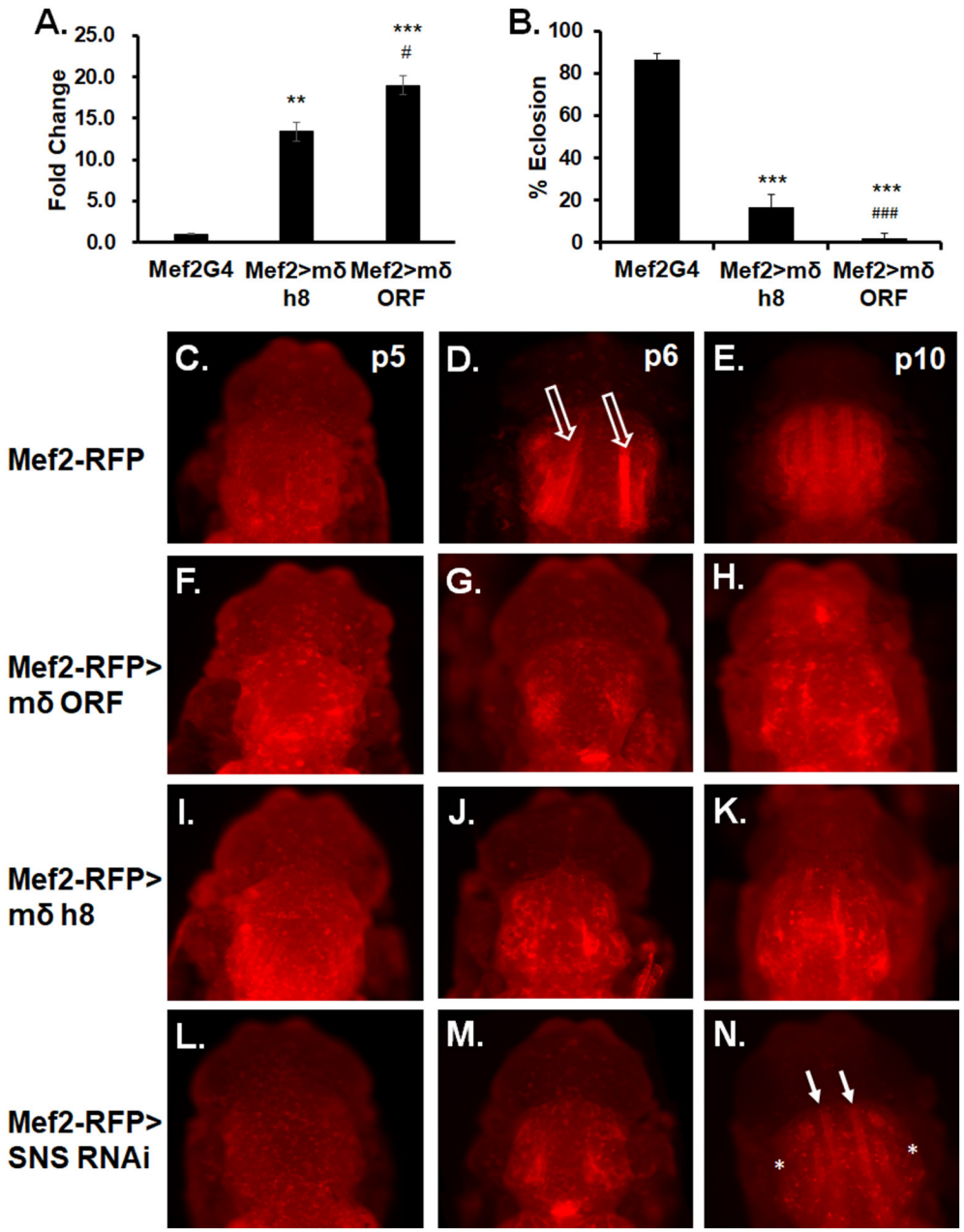

FIGURE 6 | E(spl)mδ overexpression in myogenic lineage perturbs IFM development. **(A)** Expression levels of *E(spl)mδ* using two independent constructs, UAS-mδ h8 and UAS-mδ ORF, was assessed by RT-qPCR with RNA from p5 pupae (**$p \leq 0.01$, ***$p < 0.001$ in comparison to Mef2-G4; #$p \leq 0.05$, in comparison to Mef2 > mδ h8, one-way ANOVA). **(B)** Eclosion of Mef2 > mδ h8 and Mef2 > mδ ORF (***$p < 0.001$ in comparison to Mef2-G4; ###$p < 0.001$ in comparison to Mef2 > mδ h8, *z*-test). **(C–N)** Developing IFMs in pupae at indicated stages were imaged by epifluorescence. Overexpression of E(spl)mδ was compared to knockdown of the myoblast fusion protein Sticks and Stones (SNS). **(C–E)** Mef2-RFP (control), **(F–H)** Mef2-RFP > mδ ORF, **(I–K)** Mef2-RFP > mδ h8, and **(L–N)** Mef2-RFP > SNS RNAi pupae were dissected from their pupal cases and imaged directly. Open arrows point to IFMs undergoing extension. White arrows point to partially formed DLM fibers. Asterisk mark absence of DVM formation.

levels used in this study, namely 5–20 μM MeHg in the fly food are high in comparison to typical human exposures [e.g., <0.1–1 ppm (<0.5–5 uM) MeHg in dietary fish], MeHg exposure levels used here are gauged with respect to observable effects elicited in this model system in order to gain insights into mechanism.

One mechanism by which MeHg may be disrupting muscle development is through modulation of the tissue-specific effectors in the Notch signaling pathway. The tissue specificity of transcriptional factors downstream of Notch remains an active area of research. Several studies have suggested that the function

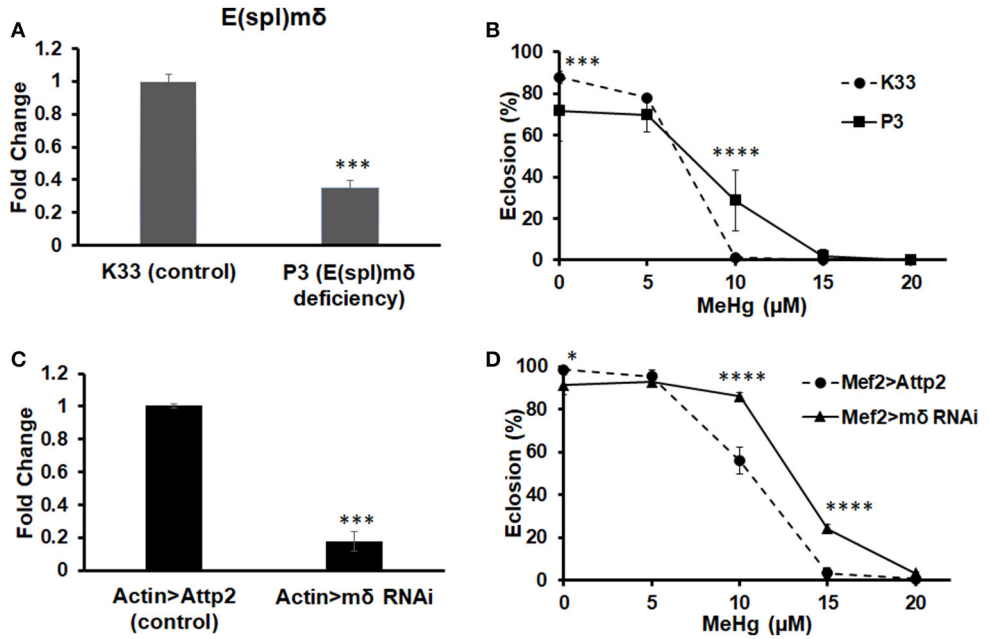

FIGURE 7 | Knockdown of E(spl)mδ conveys MeHg tolerance during development. Expression of *E(spl)mδ* was assessed by RT-qPCR with RNA extracted from p5 pupae of **(A)** E(spl)mδ deficiency (P3) and its background control strain (K33) and **(C)** Mef2 > E(spl)mδ RNAi and Mef2 > Attp2 (control) (***$p < 0.001$, *t*-test). Tolerance to MeHg was determined through an eclosion assay of **(B)** E(spl)mδ deficiency (P3) and its background control strain (K33) and **(D)** Mef2 > E(spl)mδ RNAi and Mef2 > Attp2 (control) strain (*$p \leq 0.05$, ***$p < 0.001$, ****$p < 0.0001$, *z*-test).

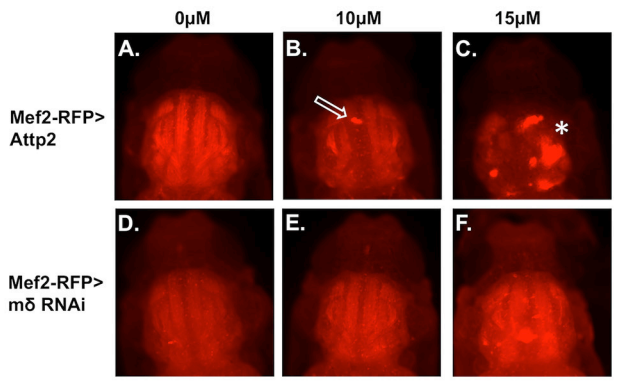

FIGURE 8 | Rescue of MeHg effects on IFM development with downregulation of E(spl)mδ in myogenic lineages. Developing IFMs in pupae, at the p10 stage after treatment with the indicated concentrations of MeHg were imaged by epifluorescence of **(A–C)** Mef2-RFP > Attp2 (control) and **(D–F)** Mef2-RFP > mδ RNAi. Pupae were dissected from their pupal cases and imaged directly. Asterisks and arrows mark failure of IFM development (see Figure S2 for additional images).

of the mammalian Hes/Hey and *Drosophila* E(spl) family of proteins are redundant (Fischer et al., 2004; Macdonald et al., 2005; Buas et al., 2010; Wurmbach and Preiss, 2014). However, the expression patterns of these proteins are distinct and may act in a timing and tissue-dependent manner, depending on tissue-specific regulators (de Celis et al., 1996; Wech et al., 1999). In *Drosophila*, Twist is thought to be a co-regulator of Notch in myogenesis, conferring a specific response to Notch signaling (Bernard et al., 2010). In vertebrates, overexpression of the Notch transcriptional repressors Hes6 and Hey1 have been shown to inhibit myogenic differentiation, while HeyL overexpression has little effect on differentiation (Cossins et al., 2002; Buas et al., 2009, 2010). Additionally, prior studies in *Drosophila* embryos, using transcript hybridization and a GFP reporter for a 1.9 kb E(spl)mδ enhancer, have suggested that E(spl)mδ is expressed in the myogenic domain (Wech et al., 1999; Engel and Rand, 2014a). Here, we demonstrate, using the same GFP reporter, that E(spl)mδ expression is likely localized to the myogenic lineage giving rise to the adult IFM. Furthermore, overexpression of E(spl)mδ in developing muscle, in contrast to a similar overexpression in neurons or gut tissue, can disrupt development and reduce eclosion rates. While it cannot be concluded from these results that E(spl)mδ plays an endogenous role in IFM development, our data suggest that in comparison to other tissues, E(spl)mδ may be a target of MeHg toxicity in developing muscle.

In response to MeHg, *E(spl)mδ* appears to be upregulated in *Drosophila* pupae across various stages of IFM development. It remains uncertain as to the mechanism of *E(spl)mδ* upregulation. Although *E(spl)mδ* is known to respond to activation of the Notch receptor (Wurmbach et al., 1999), MeHg may be upregulating *E(spl)mδ* in a Notch-independent manner. Previous data has shown that various E(spl) repressors are upregulated in *Drosophila* C6 cells in response to MeHg through a Notch-independent mechanism (Rand et al., 2008). A similar Notch-independent mechanism of E(spl) activation *in vivo*

remains to be demonstrated. However, in both *Drosophila* embryos and in *Drosophila* pupae, shown here, MeHg somewhat preferentially acts upon *E(spl)mδ* relative to other E(spl) genes (Engel et al., 2012). This preferential upregulation of a single *E(spl)* gene may also be indicative of an novel Notch-independent mechanism of MeHg activity. However, this mechanism will require further study both *in vitro* and *in vivo*.

Here, we examined gross morphogenic phenotypes in adult flight muscle (IFMs) to ascertain which global events and stages of muscle development might be affected by MeHg toxicity and point to possible underlying mechanisms. For example, strong upregulation of Notch signaling, via activated Notch expression, has previously been shown to cause persistent Twist expression in the AMPs of developing IFMs, leading to a complete loss of the IFMs (Anant et al., 1998). It is possible that a more restricted activation of the Notch target E(spl)mδ via MeHg may suppress AMP differentiation more moderately, giving rise to the phenotypes seen here. Notch signaling also modulates myoblast adhesion and fusion events in the progression of IFM formation, and has been shown to influence expression of the canonical myoblast fusion proteins SNS and Kirre (Gildor et al., 2012). Here, we find that altering SNS expression yields disrupted patterns of IFM development that partially mimic effects seen with MeHg. Remarkably, *Sns* and *Kirre* have been associated with MeHg tolerance and susceptibility through a genome wide association study in *Drosophila* (Montgomery et al., 2014). The possibility that E(spl)mδ may influence SNS or Kirre expression therefore also remains an attractive hypothesis to explore.

Despite several similarities, MeHg treatment and E(spl)mδ upregulation exhibit some phenotypic differences. Unlike in genetic E(spl)mδ upregulation, it appears myoblast fusion events do occur with MeHg treatment, as suggested by the large aggregates of Mef2 expressing bodies in the thorax. These differences may reflect the level of E(spl)mδ expression that is achieved with each approach, since eclosion rates were seen to be sensitive to E(spl)mδ dose. Alternatively, these differences may also be caused by MeHg targeting of additional factors involved in muscle development. Genes involved in neuromuscular junction formation and attachment to tendon cells have also been implicated in MeHg susceptibility (Montgomery et al., 2014). More research will be needed to elucidate these mechanisms at the level of cell-cell interactions.

Nonetheless, our data support the conclusion that E(spl)mδ is a mediator of MeHg toxicity in *Drosophila* muscle development. Furthermore, E(spl)mδ activity demonstrates tissue-specificity in that developing muscle appears to not only express E(spl)mδ, but is especially sensitive to genetic modulation of this transcription factor. Overall, these data elucidate an important mechanism by which modulation of the Notch target gene E(spl)mδ by the environmental toxicant MeHg can have tissue-specific implications. Establishing muscle development as a direct target of MeHg toxicity will bring greater understanding of the etiology of motor deficits typically seen with elevated environmental exposure to MeHg.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MR and LP. Performed the experiments: LP. Analyzed and interpreted the data: MR and LP. Drafted the paper: LP. Revised the paper for intellectual content: MR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2017.00233/full#supplementary-material

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REVIEW
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Copper and Zinc Homeostasis: Lessons from *Drosophila melanogaster*

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Maintenance of metal homeostasis is crucial for many different enzymatic activities and in turn for cell function and survival. In addition, cells display detoxification and protective mechanisms against toxic accumulation of metals. Perturbation of any of these processes normally leads to cellular dysfunction and finally to cell death. In the last years, loss of metal regulation has been described as a common pathological feature in many human neurodegenerative diseases. However, in most cases, it is still a matter of debate whether such dyshomeostasis is a primary or a secondary downstream defect. In this review, we will summarize and critically evaluate the contribution of *Drosophila* to model human diseases that involve altered metabolism of metals or in which metal dyshomeostasis influence their pathobiology. As a prerequisite to use *Drosophila* as a model, we will recapitulate and describe the main features of core genes involved in copper and zinc metabolism that are conserved between mammals and flies. *Drosophila* presents some unique strengths to be at the forefront of neurobiological studies. The number of genetic tools, the possibility to easily test genetic interactions *in vivo* and the feasibility to perform unbiased genetic and pharmacological screens are some of the most prominent advantages of the fruitfly. In this work, we will pay special attention to the most important results reported in fly models to unveil the role of copper and zinc in cellular degeneration and their influence in the development and progression of human neurodegenerative pathologies such as Parkinson's disease, Alzheimer's disease, Huntington's disease, Friedreich's Ataxia or Menkes, and Wilson's diseases. Finally, we show how these studies performed in the fly have allowed to give further insight into the influence of copper and zinc in the molecular and cellular causes and consequences underlying these diseases as well as the discovery of new therapeutic strategies, which had not yet been described in other model systems.

Keywords: *Drosophila melanogaster*, metal homeostasis, dMTF-1, methallothioneins, copper, zinc, *in vivo* modeling, human diseases

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INTRODUCTION

Drosophila melanogaster as a Model Organism

The discovery of the first mutant allele of the gene *white* by TH Morgan (Morgan, 1910) meant the beginning of the Age of *Drosophila* as a pivotal model for the study of genetics, developmental biology and, more recently, of neurobiology and human diseases. *Drosophila melanogaster* is a small low-cost organism with a fast life cycle and a relatively short life span (Helfand and Rogina, 2003). Several ground breaking discoveries with strong impact on vertebrate neuroscience

have endorsed *Drosophila* during the last century to become the model system of choice for many neuroscientists. Even before the genomic era, the fruit fly was pioneer in unveiling core elements of the nervous system development such as, among many others, Notch, Hedgehog, and Decapentaplegic (Jürgens et al., 1984; Wieschaus et al., 1984). The introduction of the P-element-mediated germline transformation (Rubin and Spradling, 1982) was another milestone in the history of *Drosophila* because it opened a new world of possibilities for genetic manipulations that culminated in 1991 and 1993 with the development of the flippase (FLP) and flippase recognition target (FRT) recombination (Golic, 1991) and the UAS/GAL4 (Brand and Perrimon, 1993) systems. Then, the publication of the first annotated version of *Drosophila* genomic sequence (Myers et al., 2000) confirmed that many human genes involved in all kinds of human diseases had an ortholog counterpart in the fly (Reiter et al., 2001). Moreover, genetic manipulation of those genes has successfully led to molecular, biochemical, tissue and behavioral defects that mirror the human conditions (Botella et al., 2009; Bouleau and Tricoire, 2015; Casci and Pandey, 2015; Xu et al., 2015). A remarkable example is the discovery of the molecular basis of behavior by characterization of genes controlling circadian rhythm and the biological clock (Konopka and Benzer, 1971). The impact of the characterization in the fly of the first clock gene, *period* (Bargiello and Young, 1984; Reddy et al., 1984) has been recently awarded with the Nobel Prize in Physiology or Medicine to Jeffrey C. Hall, Michael Rosbash and Michael W. Young. In addition, *Drosophila* has been instrumental in the characterization of the pathway controlling mitochondrial quality and integrity driven by the Parkinson's disease associated genes Pink and Parkin (Greene et al., 2003; Clark et al., 2006) or providing compelling information even if there was no identified paralogous gene as it is the case for α-synuclein (Feany and Bender, 2000).

The last decades of fly research have boosted the generation of tools in the form of mutants, variants of the UAS-GAL4 system such as the GeneSwitch and the GAL80 inhibitor (Duffy, 2002), the generation of RNAi constructs to target genes in a tissue/cell-specific manner (Dietzl et al., 2007; Ni et al., 2009), the alternative Q system (Riabinina et al., 2015), and, of course, the possibility to create mutants at will by means of the clustered regularly interspaced short palindromic repeat/ endonuclease CRISPR-associated 9 (CRISPR/Cas9) system (Bassett and Liu, 2014). The implementation of these methodologies will allow the fly to continue being the leading model in the analysis of genes involved in human diseases.

Drosophila melanogaster as a Model to Study Metal Regulation and Homeostasis

The number of manuscripts published using the fly to study the biology of metals is modest. However, the comprehensive work performed and being carried out in the labs of Walter Schaffner, Fanis Missirlis, James Camakaris, Bing Zhou, and Richard Burke has set up the foundations of the field, has provided invaluable translational evidences and has inspired many other fly researchers. *Drosophila* is extremely useful to analyze the regulation and toxicity of iron. Iron is essential to sustain life and it is the most important transition metal due to its cellular roles as electron donor in the oxidative phosphorylation and during photosynthesis. These roles have been detailed during the last decades as well as the impact of iron into several neurodegenerative diseases such as Friedreich's ataxia (FRDA), Parkinson's disease (PD) and neurodegeneration with brain iron accumulation disorders (Poujois et al., 2016). Importantly, the network of genes involved in iron biology and regulation is conserved between vertebrates and *Drosophila* with the exception of the Transferrin Receptor (reviewed in Mandilaras et al., 2013; Calap-Quintana et al., 2017). Fly studies have positively shown that targeting iron metabolism is sufficient to improve diseases conditions in models of FRDA (Navarro et al., 2015; Chen et al., 2016; Soriano et al., 2016), PD (Bonilla-Ramirez et al., 2011; Esposito et al., 2013; Zhu et al., 2016), or Alzheimer's disease (AD) (Rival et al., 2009; Liu et al., 2011; Ott et al., 2015). Besides iron, other functional studies have analyzed the regulation and homeostasis of other heavy metals (Maroni et al., 1986; Al-Momani and Massadeh, 2005; He et al., 2009; Ortiz et al., 2009; Meyer et al., 2014; Rovenko et al., 2014; Ternes et al., 2014; Chandra et al., 2015; Guan et al., 2015; Niehoff et al., 2015). Of special interest is the recent work of Anholt's lab (Zhou et al., 2017) analyzing the genetic networks involved in the resistance to Cadmium and Lead toxicity. This work highlights that, metal homeostasis is much more complex than anticipated and that, 60% of the identified genes have human orthologs. In line with this complexity, fly models have proven that dysfunction of one single metal might be accompanied with dyshomeostasis of other ones. For example, increased aluminum in the fly food also alters iron toxicity (Wu et al., 2012).

In this review, we will focus on the biology of Zinc (Zn) and Copper (Cu). We will summarize and discuss the main findings described in *Drosophila* regarding the cellular and organismal regulation of Zn and Cu with special insight into the nervous system, when possible. Moreover, we will also present a detailed review of the experiments performed with the fly to show the intimate relationship of both metals with human neurodegenerative diseases.

GENERAL FACTS ABOUT Zn AND Cu

Although the cellular roles of Cu and Zn are as important as those from iron, several aspects of their metabolism in the fruit fly are not as well characterized. Cu is an important

Abbreviations: Aβ, A-beta; AD, Alzheimer's Disease; APP, amyloid precursor protein; BCS, bathocuproine disulfonate; BMP, Bone Morphogenetic Protein; Cu, Copper; COX, Cytochrome c Oxidase; CRISPR/CAS9, clustered regularly interspaced short palindromic repeat/ endonuclease CRISPR-associated 9; ER, Endoplasmic Reticulum; FLP, Flippase; FRDA, Friedreich's Ataxia; FRT, Flippase Recognition Target; HD, Huntington's Disease; hh, Hedgehog; Htt, Huntingtin; KD, Knockdown; KSD, Kidney Stone Disease; LROs, Lysosomal Related Organelles; MRE, metal responsive element; Mtns, Methallothioneins; PD, Parkinson's Disease; Phm, peptidylglycine-hydroxylating monooxygenase;PrP, Prion Protein; qMtn, Quadruple mutant of MtnsA-D; ROS, Reactive Oxygen Species; SNPs, Single Nucleotide Polymorphisms; TGFβ, Transforming Growth factor beta; TGN, Trans-Golgi Network; TPEN, N,N,N'N'-tetrakis-(2-pyridylmethyl)ethylenediamine; Tyr, Tyrosinase; Zn, Zinc.

trace element necessary for living organisms because of its redox potential. Free Cu is able to inactivate cellular proteins by directly binding to cysteine residues (Letelier et al., 2009) and can promote oxidative stress and Reactive Oxygen Species (ROS), which will affect other cellular functions (Rotilio et al., 2000). Furthermore, Cu is an essential component of several enzymes (cuproenzymes) involved in diverse cellular processes. The existing literature has highlighted some of them: (i) Mitochondrial Cytochrome C oxidase (COX), a crucial gene for mitochondrial function; (ii) Peptidylglycine α-amidating monooxygenase (PAM) and Dopamine beta (β)-hydroxylase (DBH), involved in the biosynthesis of neurotransmitters; (iii) Lysyl oxidase (LOX), participating in the development of connectivity tissue; (iv) Tyrosinase (TYR), the rate-limiting enzyme in the biosynthesis of melanin, and (v) Superoxide Dismutase I (SOD1), one of the key cellular ROS scavengers (Zlatic et al., 2015; M Fetherolf et al., 2017). Altogether, Cu homeostasis is critical in skin pigmentation, integrity of blood vessels, myelination, maintenance of Purkinje cells, protection against oxidative stress and overall brain function via neurotransmitters (Harris, 2001; Opazo et al., 2014; Zlatic et al., 2015). Interestingly, Cu has also been described as a novel intracellular modulator of signal transduction pathways (Grubman and White, 2014). Accordingly, all this indicates the need of a strict regulation of Cu homeostasis.

Zn is also an essential trace metal with a huge panoply of biological roles and in turn with a strong impact on life. Opposite to Cu and iron, Zn is a redox-neutral element, which might exert antioxidant properties. It is a pivotal element for hundreds of enzymes in three different ways: as a key player of the catalysis (catalytic), as an enhancer of the reaction (coactivator) or by stabilizing the protein (structural) (Vallee and Falchuk, 1993). Zn plays key roles in metabolism of CO_2 and alcohol, immunological capacity, endocrine response, development of organs and tissues, DNA and protein synthesis and cell division. Zn also participates in the autophagy process (Liuzzi et al., 2014) and therefore, in proteostasis, one of the most important hallmarks of aging. Moreover, Zn has been also described as a signaling molecule itself with important functions in the Bone Morphogenetic Protein (BMP) or the Transforming Growth factor beta (TGF-β) pathways (Osredkar, 2011; Choi and Bird, 2014). Finally, its role as a cofactor for hundreds of transcription factors that contain Zn finger domains is of paramount importance. Remarkably, both, its scarcity and accumulation, lead to cellular damage that might culminate in cell death. Therefore, Zn levels need to be tightly regulated to keep a precise balance and ensure bioavailability of Zn in each cell type.

MTF-1 AND METALLOTHIONEINS: THE FIRST LINE OF DEFENSE

Cu and Zn homeostasis relies on its correct acquisition, coordination between import and export, distribution and usage. Understanding the mechanisms underpinning metal sensing and regulation as well as deciphering the regulatory machinery is crucial to fully elucidate the biology of these metals. The transcription factor MTF-1 and the metal-binding proteins named Methallothioneins (Mtns) are common genes involved in the metabolism of Cu and Zn (Choi and Bird, 2014; Krezel and Maret, 2017).

MTF-1

In mammals, the metal-responsive transcription factor-1 (MTF-1) is the major mediator of protection against accumulation of Zn, Cu and other heavy metals, by inducing the expression of genes that harbor several copies of the metal responsive element (MRE) in their promoters (Stuart et al., 1985). MTF-1 contains six zinc-finger domains and thus, requires Zn for its transcriptional activity. High Zn completely occupies all zinc fingers and then promotes the nuclear translocation of MTF-1. This property suggests, that MTF-1 might be working as a sensing molecule for Zn. Remarkably, MTF-1 also controls the response against oxidative stress, hypoxia, heat shock and even some nutritional alterations. Activation of MTF-1 by these other stressors is not completely elucidated, but seems to be indirect either by a release of Zn from Mtns or by phosphorylation of certain residues (Günther et al., 2012a). Once MTF-1 is in the nucleus, it interacts with other cofactors or additional Zn-dependent transcription factors to establish the specific expression profile according to the stressor (Krezel and Maret, 2017). In mammals, MTF-1 promotes the expression of several Cu and Zn-related genes. Interestingly, it also increases the expression of proteins involved in iron metabolism (ferroportin and hepcidin) or in sensing the cellular redox status (glutamate cysteine-ligase, thioredoxin reductases, and selenoproteins) (Günther et al., 2012a). In mouse, MTF-1 has an essential function since constitutive MTF-1 knockout mice die at day 14 of gestation (Günes et al., 1998), whereas excision of MTF-1 after birth only increased mice susceptibility toward heavy metal stress (Wang et al., 2004). The knockout lethality has been related to a liver dysfunction due to abnormal hepatocyte differentiation rather than to metal toxicity, since knockout of Mtns yielded viable mice (Masters et al., 1994).

In *Drosophila* (**Table 1**; **Figures 1**, **2**) such function falls on the shoulders of CG3743 (*dMTF-1*), that was first characterized by Walter Schaffner's lab (Zhang et al., 2001). Surprisingly, although MTF-1 is conserved throughout evolution, the degree of conservation between human and fly genes is only significant in the region containing the six zinc finger domains (81%), whereas the rest of the proteins share only a small 23% of homology (Zhang et al., 2001). Despite these differences, fly dMTF-1 is able to replace the human counterpart in mammalian cell cultures and the human gene restores the tolerance to metals in *dMTF-1* fly null mutants (Balamurugan et al., 2004). The seminal work from Walter Schaffner's group already showed in cell culture, that dMTF-1 was necessary and sufficient to activate the transcription of Mtns via MRE sequences (Zhang et al., 2001). Further works have shown that besides Mtns, iron (ferritin), Cu (*DmATP7* and *Ctr1B*), and Zn (ZnT35C) related genes are also transcriptionally activated by dMTF-1 (Southon et al., 2004; Selvaraj et al., 2005; Yepiskoposyan et al., 2006), suggesting that dMTF-1 participates in the acquisition and storage of metals. Similarly

TABLE 1 | *Drosophila* proteins involved in Cu homeostasis (names according to Flybase, http://flybase.org).

Fly gene	Cellular role	Human ortholog	Related disease fly model
Metal Transcription factor-1 dMTF-1 (CG3743)*	Control of metal-dependent transcription of Mtns and some Cu, Zn and iron genes	*Metal Transcription factor-1 (MTF-1)*	AD (Hua et al., 2011b) MD (Bahadorani et al., 2010a) FRDA (Soriano et al., 2016)
Methallotioneins MtnA (CG9470) MtnB (CG4312) MtnC (CG5097) MtnD (CG33192) MtnE (CG42872)*	Cellular metal (mainly Cu) detoxification	*MT-I MT-II MT-III MT-IV*	FRDA (Soriano et al., 2016) AD (Hua et al., 2011b)
Copper Transporter Ctr1A (CG3977) Ctr1B (CG7459) Ctr1C (CG15551)	Cellular Cu uptake	*Solute carrier family 31(SCL31A1) hCTR1*	AD (Lang et al., 2013) HD (Xiao et al., 2013) MD (Bahadorani et al., 2010a) PD (Saini et al., 2010)
ATPase 7 DmATP7 (CG1886)	Cellular Cu export Cu delivery	*ATPase copper transporting α and β (ATP7A and ATP7B)*	AD (Lang et al., 2013) HD (Xiao et al., 2013) MD (Bahadorani et al., 2010a; Southon et al., 2010; Mercer et al., 2017) WD (Mercer et al., 2017)
Antioxidant 1 copper chaperone Atox1(CG32446)	Chaperone. Cu delivery to DmATP7	*Antioxidant 1 copper chaperone (ATOX1)*	AD (Sanokawa-Akakura et al., 2010) FRDA (Soriano et al., 2016)
Copper Chaperone for superoxide dismutase Ccs (CG17753)	Chaperone. Cu delivery to SOD1	*Copper Chaperone for superoxide dismutase (CCS)*	Not tested
Synthesis of cytochrome c oxidase Scox (CG8885)	Chaperone. Cu delivery to cytochrome c oxidase	*Cytochrome c oxidase copper chaperone (SCO1)*	Not tested

*Gene symbols for human genes follow the regulation of Human Genome Organization Gene Nomenclature Committee (http://www.genenames.org). *dMTF-1 and Mtns have been added in this table since their main role in Drosophila melanogaster is the detoxification of Cu accumulation. AD, Alzheimer's disease; FRDA, Friedreich's ataxia; HD, Huntington's disease; MD, Menkes disease; PD, Parkinson's disease; WD, Wilson's disease.*

to the human protein, dMTF-1 has additional partners (Günther et al., 2012a) and phosphorylation sites (Gunther et al., 2012b) to allow the discrimination of target genes. Although MTF-1 and dMTF-1 cross-complement, some important differences have been observed. For example, dMTF-1 seems to be nuclear even under non-stress conditions (Gunther et al., 2012b) and it has a C-terminal domain that inhibits its hyperactivation (Gunther et al., 2012b). Moreover, dMTF-1 is essential to regulate Cu homeostasis via a cysteine cluster that works as a copper sensor (Chen et al., 2008).

Following the classical reverse genetic approach, mutant flies and flies overexpressing *dMTF-1* have been generated to analyze its functions in *Drosophila*. Opposite to mouse, mutant *dMTF-1* flies did not show any developmental defect, but presented a shorter longevity (Bahadorani et al., 2010b). Remarkably, they display an increased sensitivity toward accumulation of metals due to the lack of induction of the protective Mtns and the consequent accumulation of free metals in toxic concentrations (Egli et al., 2003, 2006b; Bahadorani et al., 2010b). In this line of events, *dMTF-1* overexpression in central and peripheral nervous systems extended longevity under normal conditions, although no effect was observed upon expression in the gut (Bahadorani et al., 2010b). These results indicate that the control of metal homeostasis has a crucial tissue-specific component.

Cu and Zn show an unusual relationship with *dMTF-1*. On the one hand, *dMTF-1* mutants are more sensitive to both Cu scarcity and supplementation, whereas overexpression increases resistance in both situations (Selvaraj et al., 2005; Bahadorani et al., 2010b). This dual role in detoxification and acquisition is likely related to the simultaneous control of the expression of the Cu importer *Ctr1B* and the Cu exporter *DmATP7* by dMTF-1 (Selvaraj et al., 2005; Yepiskoposyan et al., 2006; Burke et al., 2008; Bahadorani et al., 2010b). Activation of both might affect metal distribution and balance in a tissue-specific manner. On the other hand, dMTF-1 adult mutant flies are not sensitive to toxic concentrations of Zn. However, overexpression of dMTF-1 ubiquitously, in the gut or in the neurons makes the flies hypersensitive to Zn. Again, the explanation may rely in the induction of, at least, the zinc exporter ZnT35C by dMTF-1 (Yepiskoposyan et al., 2006). This induction will trigger aberrant ion distribution with depletion in some tissues and accumulation in others. In agreement, the overall amount of Zn was reduced in flies overexpressing dMTF-1 (Bahadorani et al., 2010b). These results suggested that nervous system and gut were particularly sensitive to perturbation of Zn homeostasis (Bahadorani et al., 2010b).

Metallothioneins

Mtns are ubiquitous, small cystein-rich proteins present from yeast to human with the ability to bind heavy metal ions and with thiol reactivity. Mtns are able to modulate their redox state in order to regulate metal bioavailability and buffer deleterious accumulation. Mammalian Mtns also respond to additional factors such as hormones, cytokines or oxidative stress. In humans up to 12 functional Mtns have been characterized, being MT-I to IV the most important family members (Palmiter, 1998; Krezel and Maret, 2017), whereas only five (A–E) have been found in the fruit fly (Egli et al., 2006a; Atanesyan et al., 2011). They carry out an essential metal detoxification because mutations and polymorphisms in Mtns are associated to human diseases ranging from diabetes, cancer, autoimmune diseases,

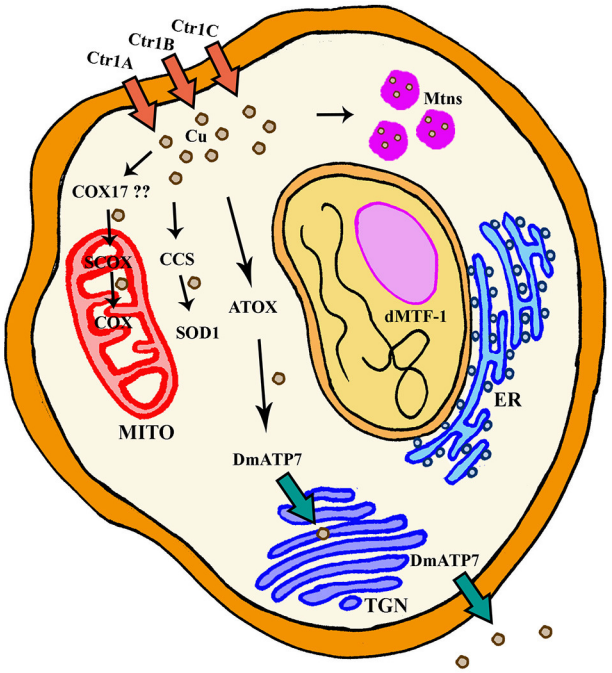

FIGURE 1 | General scheme of cellular Cu homeostasis in *Drosophila melanogaster*. ER, Endoplasmic Reticulum; Mito, Mitochondria; TGN, TransGolgi network.

neurodegenerative disorders and autism (Laukens et al., 2009; Raudenska et al., 2014). In mice, mutations in two Mtns do not induce lethality, but confer sensitivity toward Zn (Kelly et al., 1996).

Already in 1986, experiments in *Drosophila* larvae suggested the existence of genes with metal binding properties, whose expression was inducible by increasing concentration of metals (Maroni et al., 1986). Later on, the spectrum of stressors able to activate the expression of Mtns in flies was extended to thermal and oxidative stress (Bonneton et al., 1996). MtnA and B (firstly named as Mtn and Mto, respectively) were the first ones identified (Durliat et al., 1995) followed by MtnC and D (Egli et al., 2003) and more recently a fifth member (MtnE) was added (Atanesyan et al., 2011). In flies, all five Mtns (**Table 1**, **Figures 1**, **2**) depend on dMTF-1 (Egli et al., 2003; Southon et al., 2004; Atanesyan et al., 2011) and are broadly conserved among the *Sophophora* and *Drosophila* subgenus (Guirola et al., 2011). MtnA shows the highest expression in all developmental stages (Atanesyan et al., 2011; Graveley et al., 2011). In larvae, all Mtns are expressed in larval gut, fat body, Malpighian tubules or salivary glands (Durliat et al., 1995; Egli et al., 2006b; Atanesyan et al., 2011). Increasing metal content in the fly food enhanced the levels of Mtn in those tissues and expanded the pattern to new regions. Expression in adult tissues is very poorly described with the exception of the work from Durliat and collaborators (Durliat et al., 1995). In this case, it was shown that MtnA and B were expressed in the adult cardia, gut or Malpighian and no expression was detected in other tissues. However, and in light of the increasing importance of metal detoxification in human diseases, a deeper analysis of Mtn expression in adults using new technical approaches and the fly tool box is of high interest. In this sense, we have detected robust expression of most of the Mtns in adult heads by Real Time-PCR (our unpublished observations).

Metal specificity is a critical issue regarding the biology of Mtns. Single, double, triple and quadruple mutants of MtnA-D (qMtn) have been generated to address this question (Egli et al., 2006a,b). Similarly to MTF-1, all Mtn mutants are viable and fertile. The analysis of developmental hypersensitivity to toxic metal concentrations in the different mutant combinations has provided valuable information. Those experiments revealed that MtnA and B are, respectively, essential to detoxify Cu and Cd. MtnC and D did not seem to play a major role in protection (in agreement with their developmental expression) and Zn only had a minor impact even in the qMtn background. In contrast to *dMTF-1* mutants, qMtn flies were not sensitive to Cu depletion, suggesting that Mtns are not essential for Cu import or to transfer Cu. Regarding adults, qMtn flies show a strong hypersensitivity toward Cu, but no information about other metals has been reported. MtnE responds to a broader range of metal including Cu, Zn, cadmium, silver and mercury (Pérez-Rafael et al., 2012). In accordance to this less specific induction, MtnE displays a broader expression pattern. This result might indicate that it could be important in a context, in which the other Mtns fail. This hypothesis is supported by the induction of MtnE expression in qMtn mutant (Atanesyan et al., 2011). Interestingly, a constant exposure to metals attenuates the initial boost of Mtn expression and this effect has not been detected in other dMTF-1 target genes (Egli et al., 2006b). The binding abilities of all five Mtns are different and nicely correlate with their responsiveness to toxic metal concentrations. All show low affinity to Zn and higher affinity to bind Cu and cadmium (Egli et al., 2006a; Pérez-Rafael et al., 2012). Anyways, quantification of expression levels of MtnB and C either by Real-Time PCR or by induction of a genetic reporter has been successfully used to monitor Zn levels in flies (Georgiev et al., 2010; Saini and Schaffner, 2010; Qin et al., 2013; Yin et al., 2017). We can conclude that fly Mtns act primarily as Cu-thioneins in comparison to mammalian counterparts (Zn-thioneins) although several aspects of Mtn's cellular functions still need to be fully elucidated.

REGULATION OF Cu METABOLISM

Several proteins have already been described to participate in the Cu metabolism. In short, Cu is mainly imported via the copper transporter CTR1. Cu is then distributed by the copper chaperones, small proteins that deliver the metal to cuproenzymes such as SOD1 and cytochrome c oxidase, or to other members of the Cu regulatory network named ATP7A and ATP7B. These two P-type ATPases are also Cu donors to other Cu-containing enzymes and participate in Cu extracellular delivery (Madsen and Gitlin, 2007). *Drosophila* has been used to study Cu biology since the early fifties (Poulson et al., 1952). *Drosophila* S2 cells express all classical genes involved in regulation (import, delivery and export) of Cu and their

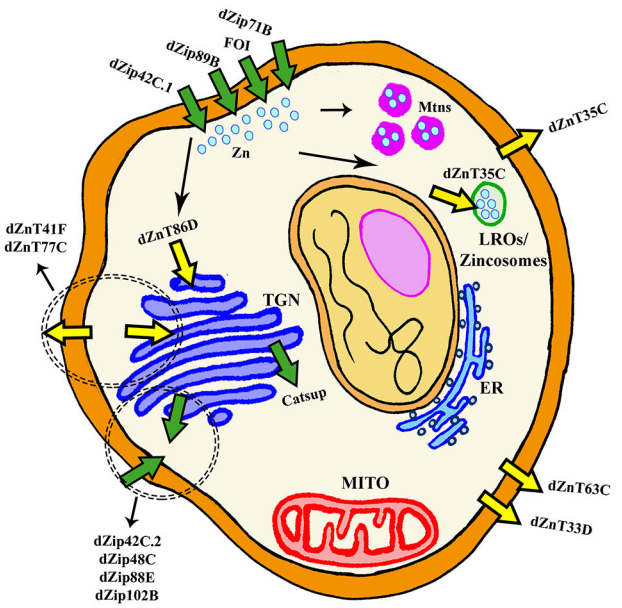

FIGURE 2 | General scheme of cellular Zn homeostasis in *Drosophila melanogaster*. ER, Endoplasmic Reticulum; Mito, Mitochondria; LROs, Lysosomal Related Organelles; TGN, TransGolgi network.

silencing altered Cu homeostasis as predicted, suggesting a conserved role. For example, knockdown (KD) of *Ctr1B* reduced Cu uptake and content, whereas downregulation of *DmATP7* induced Cu accumulation due to a deficient efflux. As a result, the systemic Cu homeostasis is very similar between humans and *Drosophila melanogaster* (Southon et al., 2013a). **Figure 1** and **Table 1** summarize the key fly proteins involved in Cu metabolism. One of the main handicaps of the studies about Cu biology is the lack of proper and direct ways to measure Cu and to monitor differences either in the amount or in the distribution of the metal. Richard Burke's lab has very elegantly and nicely showed that synchrotron x-ray fluorescence microscopy is able to detect Cu alterations in a small organism such as *Drosophila* even in some tissues including the fly brain (Lye et al., 2011).

Cu Uptake

Copper transporter 1 (CTR1) is the main responsible of cellular import of Cu. CTR1 belongs to the SLC31 family of transporters and forms a trimeric channel to allow Cu uptake (Nevitt et al., 2012). The fly genome contains three different orthologs (**Figure 1**): *Ctr1A*, *Ctr1B*, *Ctr1C* (Zhou et al., 2003). *Ctr1A* is highly expressed throughout development, whereas *Ctr1B* seems to be only relevant in late embryo and larvae with very strong expression in enterocytes (in order to uptake Cu from food). *Ctr1C* is very lowly expressed in all developmental stages and the expression seems to be mainly focused in male gonads (Zhou et al., 2003; Hua et al., 2010). All three proteins seem to be mainly localized in the plasma membrane (Selvaraj et al., 2005; Turski and Thiele, 2007; Binks et al., 2010; Steiger et al., 2010). In agreement, they are able to stimulate Cu import into *Drosophila* S2 cells (Zhou et al., 2003) and also *in vivo* (Balamurugan et al., 2007; Hua et al., 2010). Ubiquitous or panneuronal overexpression of any of the three genes is developmentally lethal, whereas eye-specific overexpression triggered a rough eye phenotype accompanied by neuronal degeneration (Balamurugan et al., 2007; Hua et al., 2010; Lang et al., 2013). This retinal degeneration is clearly related to Cu availability, since reducing Cu content in retinal neurons by increasing efflux (Balamurugan et al., 2007), enhancing Cu chelation by Mtns (Egli et al., 2006a), or with bathocuproine disulfonate (BCS) (Balamurugan et al., 2007; Hua et al., 2010) can rescue the phenotype.

Ctr1A seems to be the most important of all fly copper importers, since it displays the broadest expression pattern and its complete loss-of-function triggers developmental arrest (Turski and Thiele, 2007; Hua et al., 2010). In agreement with a reduced Cu import, *Ctr1A* deficient larvae and adults show lower activity of Cu-requiring enzymes such as Cytochrome c Oxidases (*Coxs*), tyrosinase (*Tyr*) and peptidylglycine-hydroxylating monooxygenase (*Phm*). This resulted in lower pigmentation of mouth hooks and body, and reduced amidation of neurotransmitters (Turski and Thiele, 2007; Binks et al., 2010). Importantly, Cu-supplementation in the food (Turski and Thiele, 2007; Hua et al., 2010) or genetic manipulation of intracellular copper levels (Binks et al., 2010) improved some of the defects. Furthermore, the human counterpart hCtr1 also improved loss of *Ctr1A* conditions, suggesting a strong functional equivalence between both proteins (Hua et al., 2010). Due to the lethality associated to the complete depletion of *Ctr1A*, tissue-specific experiments have also been performed. KD in the dorsal vessel generated a severe cardiomyopathy in the flies (Kim et al., 2010). This result demonstrates that cardiac defects present in several models of systemic Cu deficiency are a result of a tissue-specific effect. It is likely that, in a tissue highly dependent on mitochondria, strong reduction of *Cox* activity is underlying the cardiac defect. *Ctr1A* depletion in the nervous system showed that it is required for neuronal development (Binks et al., 2010). Interestingly, KD of *Ctr1A* in the prothoracic gland induced phenotypes reminiscent to a reduced Ras signaling. Accordingly, downregulation of *Ctr1A* was able to rescue the overactivation of Ras (Turski et al., 2012). *Ctr1B* is the only Cu importer identified as a direct target of dMTF-1 (Southon et al., 2004; Selvaraj et al., 2005). *Ctr1B* is upregulated by Cu deprivation (Zhou et al., 2003) in order to maintain the correct systemic Cu amount and, unexpectedly, also by toxic accumulation of mercury and cadmium (Balamurugan et al., 2009). Mutants are viable but show hypopigmentation due to defects in the activity of the Cu-containing enzyme *Tyr*. Since those mutants do not accumulate Cu, they fail to induce the expression of Mtns (Zhou et al., 2003; Balamurugan et al., 2007). This defect is really Cu specific since mutants successfully promoted Mtns expression upon feeding with cadmium (Balamurugan et al., 2007). However, under conditions of Cu scarcity, mutants are unable to complete development (Zhou et al., 2003). In agreement with the specific expression pattern in the fly gut, a panneuronal KD of *Ctr1B* does not affect cellular integrity (Binks et al., 2010). *Ctr1C* is the least studied and its expression seems to be restricted to the

maturing spermatocytes and the mature sperm. *Ctr1C* mutants are viable but their fertility is severely compromised in a Cu-dependent manner (Steiger et al., 2010). *Malvolio* (*Mvl*), the *Drosophila* homolog of *Divalent metal transporter-1* (*DMT1*) is mostly involved in the transport of dietary iron in the gut (Folwell et al., 2006) but also seems to influence Cu content in *Drosophila* S2 cells (increased when overexpressed and reduced when depleted) and in the fly gut (Southon et al., 2008). Mutant individuals are viable but sensitive to increased levels of Cu in the food. Since its function might be redundant with members of the Ctr1 family, it is logical that the loss-of-function does not cause a systemic Cu deficiency.

Cu Efflux

In mammals, the responsibility to secrete Cu from cells relies on two P-Type ATPases, ATP7A, and ATP7B. They both belong to a group of genes that hydrolyze ATP to pump substrates across membranes (Zlatic et al., 2015). The fly genome only encodes one ortholog of mammalian ATP7A and B, DmATP7 (**Figure 1**) which displays a high homology (>45%) to both ATPases (Southon et al., 2004). However, comparative genomic analysis of 12 *Drosophila* species shows that DmATP7 contains all the motifs existing in ATP7A required for localization and retention in the basolateral membrane, whereas ATP7B targeting motifs are not present (Southon et al., 2010). ATP7A has been described as a multitasking protein. It seems to be initially located in the transgolgi network (TGN), where it participates in the transfer of Cu into Cu-containing proteins and it is relocated to the plasma membrane upon increasing concentrations of Cu (Kaler, 2011), where it is involved in cellular efflux of Cu. Several functional reporter constructs (*DmATP7-lacZ*, *EYFP-DmATP7*, *UAS-DmATP7FLAG*, *gDmATP7:GFP*) have been used to study the endogenous expression pattern of DmATP7. DmATP7 is highly expressed at the basolateral membrane of the Cu cells of the midgut and in the central, peripheral and enteric nervous system in all developmental stages. It is also expressed in the larval mouthparts, developing tracheae, the gut and in the malpighian tubules but not in the optic lobes, salivary glands and fat body. In the adult nervous system the expression in peptidergic neurons is of special importance due to the role of Cu in the biosynthesis of amidated neurotransmitters (Norgate et al., 2006; Burke et al., 2008; Sellami et al., 2012; Mercer et al., 2017). In all cases, fusion proteins show clear localization at the plasma membrane. Importantly, DmATP7 presents a Cu-inducible and dMTF-1 dependent expression (Burke et al., 2008; Mercer et al., 2017). It is known that the Cu-induced trafficking of mammalian ATP7A and ATP7B from the TGN toward the plasma membrane is critical for their role in Cu homeostasis (Kaler, 2011). Unexpectedly, this trafficking was not observed in cultured embryonic S2 cells, larval neuronal Bm3-c2 cells (Southon et al., 2010) or *in vivo* in larval midgut cells (Burke et al., 2008; Mercer et al., 2017) with any of the DmATP7 constructs. However, DmATP7 is able to translocate from the TGN to the plasma membrane when introduced in mammalian cells (Southon et al., 2010). Therefore, fly models still need to clarify whether such translocation is not required/necessary in flies or whether just current methodological limitations avoid its detection. In agreement with the expected role in Cu transport and export, coexpression of DmATP7 counteracted the phenotypes induced by the overexpression of the Cu importer (*Ctr1B*) in the *Drosophila* retina (Balamurugan et al., 2007). Panneural overexpression of DmATP7 induced an altered morphology of neuromuscular junctions (Comstra et al., 2017) followed by a partial developmental lethality and a high percentage of unexpanded wings in the surviving adults due to a deficient activity of the corresponding neurotransmitters (Hwang et al., 2014). Similarly, ubiquitous overexpression induced body hypopigmentation due to the impairment of the Cu-requiring enzyme *Tyr* (Norgate et al., 2006). Since all these effects are rescued by Cu-supplemented food, we can conclude that overexpression also results in Cu deficiency due to increased efflux (Norgate et al., 2006; Hwang et al., 2014).

Cu Chaperones

In addition to transporters of Cu into and out of the cells, there is a third group of proteins with critical roles in Cu homeostasis. They are the so called "Cu chaperones" since their main role is to deliver Cu into aproproteins of cuproenzymes (Palumaa, 2013). *Drosophila* orthologs (**Figure 1**) of the mammalian Cu chaperones *ATOX1*, *CCS, COX17* and *SCO1*, and *SCO2* are found to be expressed in *Drosophila* S2 cells (Southon et al., 2004). *Atox1* is the fly homolog of the mammalian *ATOX1*, which delivers Cu to ATP7A and ATP7B. In agreement, loss of *Atox1* function in the flies phenocopies most of the defects of DmATP7 mutant flies (Hua et al., 2011a). *CCS* is the chaperone that delivers Cu to SOD1 (Cu/Zn superoxide dismutase). *Ccs* mutant flies display low levels of SOD1 activity, but also reduced levels of SOD1 protein. This important evidence suggests that *Ccs* plays a role in the stability of SOD1 (Kirby et al., 2008). SCO proteins are the Cu chaperones in charge of delivering Cu to the mitochondrial cytochrome c oxidase (COX). The *Drosophila* genome only encodes a single ortholog named *Scox* that is expressed during the whole development. Correspondingly, null mutants show a severely reduced COX activity that triggers developmental arrest at L2 stage. Hypomorphic alleles affect locomotion and female fertility, in agreement with its high expression in the egg chambers (Porcelli et al., 2010). It is likely that mitochondrial dysfunction is underpinning the loss-of-*scox* defects. However, additional studies will be needed to find out the complete mitochondrial mechanism. Unfortunately, although homologs of mammalian COX11 and COX17 have been found *in silico*, CG9065 and CG31648, and at least one of them is expressed in S2 cells (Southon et al., 2004), there is no report addressing their role in the Cu homeostasis or in the mitochondrial function.

Cuproenzymes and Other Cu-Related Genes

The classical enzymes described to require Cu in their activity, which have fly orthologs are: *Lysyl oxidase*, involved in connective tissue (Molnar et al., 2005); *Peptidylglycine-α-hydroxylating monooxygenase*, involved in amidation of peptides (Hwang et al., 2014); Multi-copper oxidases MCO1 and MCO3, involved in iron homeostasis (Mandilaras et al., 2013); Tyramine β-Hydroxylase, involved in neurotransmitter synthesis (Monastirioti et al., 1996);

tyrosine hydroxylase, involved in synthesis of melanine and dopamine (Wright, op. 1987) and Zn/Cu superoxide dismutase (SOD1), involved in protection against oxidative stress (Phillips et al., 1989). SOD1 is by far the most studied in the fly, due to its strong relation with oxidative stress, aging and amyotrophic lateral sclerosis (ALS) (Renton et al., 2014; Casci and Pandey, 2015).

Moreover, *in vivo* screenings and microarray experiments have helped to identify new candidate genes involved in the regulation of Cu. Syntaxin 5 was identified in a screening of genes that modulate mortality on excess Cu (Norgate et al., 2007). It is a soluble NSF attachment protein receptor (SNARE) gene involved in intracellular vesicle trafficking with high impact on Cu uptake (Norgate et al., 2010). Other similar examples are CG14036, CG11825, CG14545 (Norgate et al., 2007). VhaPPA1-2 and bib are a couple of genes that were first discovered as required for cuticle pigmentation and lately characterized as novel mediators of Cu biology that facilitate the membrane localization of Cu transporters (Mummery-Widmer et al., 2009; Wang et al., 2014). ADP-ribosylation factor 1, Adf1, contributes to maintain the golgi structure (Southon et al., 2011). Glutamatecysteine ligase catalytic subunit gene (Gclc) is the rate-limiting enzyme in glutathione (GSH) formation. GSH is a key cellular antioxidant molecule that also plays a role in Cu regulation (Mercer et al., 2016).

REGULATION OF Zn METABOLISM IN *DROSOPHILA*

Although Zn is an essential metal involved in a large panoply of key cellular processes, the *in vivo* physiological roles of Zn transporters are still not well characterized. In humans and in *Drosophila*, Zn homeostasis is mainly mediated by two large families of zinc transporters, the SLC39A or Zip (which import zinc into the cytoplasm) and the SLC30A or ZnT (which remove zinc from of the cytoplasm). Importantly, they are conserved throughout evolution from humans to flies (Lye et al., 2012). *In silico* and *in vivo* studies have identified up to 17 members of both families in the fly (Lye et al., 2013), whereas 24 proteins belong to those groups in mammals (Lichten and Cousins, 2009). In general, all Zn transporters in the fly are named by their cytological location (Yepiskoposyan et al., 2006) with the exception of *catecholamines up* (*catsup*) and *fear of intimacy* (*foi*) that retain their original names.

In the fly, most of the work to characterize the expression and function of dZip and dZnT genes has been performed in the Malpighian tubules and in the midgut because Zn mainly accumulates in the tubules and the gut is the primary site for the absorption of nutrients in *Drosophila* (Schofield et al., 1997). Therefore, global and systemic changes have been investigated in detail, but little is still known about local redistribution and availability of Zn within a given tissue or organ and even within the cell. Approaches based on genetic interactions have played an instrumental role in the identification of new genes involved in Zn homeostasis and in the analysis of the cellular and systemic roles of each transporter. Richard Burke's lab developed a brilliant strategy for this aim. They initially found that simultaneous overexpression of the main importer (*dZip42C.1*) and silencing of the main exporter (*dZnT63C*) triggered Zn entrapment in the cytosol, that lead to a Zn toxic phenotype (named Ztox) characterized by either retinal degeneration and loss of eye pigmentation when carried out in the developing eye (*gmr*-GAL4) or by scutellum loss, thorax cleft, hypopigmentation and bristles misaligned when performed in the developing thorax (*pnr*-GAL4) (Lye et al., 2012). Similarly, overexpression of *dZnT86D* or *dZip71B* also induce changes in the eye morphology and pigmentation (Dechen et al., 2015). In parallel, Bing Zhou's lab conducted similar studies within the gut (Qin et al., 2013; Yin et al., 2017). Importantly, all these phenotypes were modulated (rescued or worsened) by chemical or genetic manipulation of Zn levels (Lye et al., 2012, 2013; Qin et al., 2013; Dechen et al., 2015; Yin et al., 2017). The genetic interactions showed which combinations of Zn transporters modified the eye morphology, the thorax pigmentation or the response toward Zn overload/depletion. All this information along with the subcellular localization described by reporter lines has allowed dividing the Zn transporters into four subgroups.

Table 2 and **Figure 2** recapitulate all fly genes involved in Zn transport except dZnT49B. It shows sequence similarity to the *hZIP9* gene but no other information about the gene is available and it cannot be properly classified. It is important to note, that the results from the interactions between the Zn toxic genotypes and the collection of RNAi constructs provides additional evidences about the expression pattern of the Zn transporters and complements the information available in the Flyatlas (flyatlas.org). In this sense, the results already reported (Lye et al., 2012, 2013; Dechen et al., 2015) suggest that almost all transporters are expressed in the developing eye, in the thorax or in the PNS.

Importers of Extracellular Zn

The proteins, which have been classified as pure Zn importers into the cytosol from the extracellular matrix are dZip42C.1 (dZip1), dZip89B, dZip71B and FOI (**Figure 2**). All genes of this category are localized in the plasma membrane (van Doren, 2003; Dechen et al., 2015). dZip42C.1 has been described as the main protein responsible for dietary Zn absorption in the midgut enterocytes (Lang et al., 2012; Qin et al., 2013). In parallel to this function, dZip42C.1 also participates in Zn import into the adult nervous system (Lang et al., 2012). Unexpectedly, reduction of *dZip42C.1* in the gut did not trigger any deleterious effect, even under Zn depletion conditions. This strongly suggests the presence of other dZip proteins performing Zn uptake in the gut (Qin et al., 2013). Bioinformatic analysis showed that dZip89B and dZip42C.2 (that will be introduced in the next section) were the best candidates. In agreement, dZip89B increases cytosolic Zn levels when overexpressed (Lye et al., 2013; Dechen et al., 2015). Furthermore, flies lacking dZip89B are normal, although they show reduced Zn levels in the gut along with upregulation of dZip42C.1 and dZip42C.2. In addition, dZip89B has a broader expression pattern and works as a low-affinity Zn transporter (Richards et al., 2015). All this might explain the lack of sensitivity of dZip89B mutants to Zn deprivation. dZip71B performs the

TABLE 2 | *Drosophila* proteins involved in Cu homeostasis (names according to Flybase, http://flybase.org).

Fly gene	Cellular role	Human ortholog	Related disease fly model
dZip42C.1 (CG9428) *dZip89B (CG6898)* *dZip71B (CG10006)* *foi (CG6817)*	Cellular Zn uptake	*hZIP1, hZIP2, hZIP3* *hZIP1, hZIP2, hZIP3* *hZIP5* *hZIP6, hZIP10*	AD (Lang et al., 2012; Huang et al., 2014) FRDA (Soriano et al., 2016) KSD (Chi et al., 2015; Yin et al., 2017) PD (Saini and Schaffner, 2010)
dZip42C.2 (CG9430) *dZip88E (CG4334)* *Catsup (CG10449)* *dZip48C (CG13189)* *dZip102B (CG2177)*	Cytosolic Zn uptake from extracellular matrix and from organelles	*hZIP1, hZIP2, hZIP3* *hZIP1, hZIP2, hZIP3* *hZIP7* *hZIP11* *hZIP9*	FRDA (Soriano et al., 2016) PD (Chaudhuri et al., 2007)
dZnT63C (CG17723) *dZnT33D (CG31860)*	Cellular Zn export	*hZnT1, hZnT10* *hZnT2, hZnT3, hZnT4, hZnT8*	AD (Huang et al., 2014) FRDA (Soriano et al., 2016) KSD (Chi et al., 2015; Yin et al., 2017) PD (Saini and Schaffner, 2010)
dZnT35C (CG3994) *dZnT41F (CG11163)* *dZnT77C (CG5130)* *dZnT86D (CG6672)*	Cytosolic Zn export outside of the cell or into cellular organelles	*hZnT2, hZnT3, hZnT4, hZnT8* *hZnT2, hZnT3, hZnT4, hZnT8* *hZnT1, hZnT10* *hZnT5, hZnT6, hZnT7*	FRDA (Soriano et al., 2016) KSD (Chi et al., 2015; Yin et al., 2017) PD (Saini and Schaffner, 2010)

Gene symbols for human genes follow the regulation of Human Genome Organization Gene Nomenclature Committee (http://www.genenames.org). AD, Alzheimer's disease; FRDA, Friedreich's ataxia; KSD, Kidney Stones disease; PD, Parkinson's disease.

opposite role to dZip42C.1 and its co-workers (dZip42C.2 and dZip89B). It is mainly expressed in the Malpighian tubules and imports Zn from the body for detoxification and disposal. Therefore, it is instrumental in the excretion of Zn from the organism and accordingly, silencing of *dZip71B* triggers Zn accumulation in the body and these flies are more sensitive to Zn overload (Yin et al., 2017). When overexpressed, *dZip71B* acts as a very efficient and potent Zn transporter able to create a toxic Zn accumulation on its own (Richards and Burke, 2015). Interestingly, both reports highlight cell-specific regulation of Zn metabolism.

FOI is not expressed in the gut, but is one of the cellular Zn importers in several other tissues (probably cooperating with dZip42C.1). The gene *fear of intimacy* (*foi*) showed up in genetic screens aiming to identify mutations affecting the development of fly gonads and trachea (van Doren, 2003; Mathews et al., 2005), embryonic nervous system (Pielage et al., 2004), and fly muscles (Carrasco-Rando et al., 2016). FOI is able to transport Zn in yeast and mammalian cells (Mathews et al., 2005). The Zn-related activity of *foi* is required for the stability of the cell-adhesion protein E-cadherin, that is essential for the proper formation of the gonads (Mathews et al., 2005, 2006). In the myoblast development, the expression of target genes of zinc-dependent transcription factors, such as Minc and Kruppel, was severely altered in *foi* mutants. In agreement, the phenotypes could be rescued by overexpression of other dZip genes with the same cellular localization such as *dZip42C.1* and *dZip71B* (Carrasco-Rando et al., 2016). All these results support the role of *foi* as a Zn transporter and the impact of its loss-of-function in the activity of zinc-finger transcription factors.

Importers of Intracellular-Stored Zn

This category includes proteins that, besides having some capacity to uptake Zn from the outside of the cell, they have been shown to remove Zn also from intracellular compartments. This group encompasses the following proteins dZip42C.2 (dZip2), dZip88E, CATSUP, dZip99C, dZip48C, and dZip102B (**Figure 2**). dZip42C.2 is another Zn importer from the lumen into the enterocytes (Qin et al., 2013). Flies with reduced dZip42C.2 levels were normal but they display severe phenotypes (more than those with reduced dZip42C.1) including developmental arrest under low zinc conditions. This suggests, that dZip42C.2 might play more crucial roles than its counterpart dZip42C.1. Interestingly, expression levels of both seem to be regulated by Zn levels in the enterocytes (Qin et al., 2013). dZip88E was initially thought to be the fourth member of this group formed by dZip42C.1, dZip42C.2, and dZip89B to absorb Zn from diet (Lye et al., 2012). However, its recent characterization (Richards et al., 2017) and the results from genetic interactions (Dechen et al., 2015), suggest a completely different role. It is expressed in a small subset of cells in the gut and larval CNS displaying both, plasma membrane and intracellular localization (Dechen et al., 2015; Richards et al., 2017). Interestingly, even with this restrictive expression, mutant flies are hypersensitive toward Zn. It seems that dZip88E expression in these cells acts as a surveillance system to detect Zn toxicity and elicit a systemic response to counteract it.

Catsup is another gene that was isolated long before it was described as a dZip gene (Stathakis et al., 1999). The name of the gene comes from the high levels of catecholamines of the mutant flies due to the hyperactivity of the tyrosine hydroxylase (TH) enzyme. This result showed that *Catsup* is a negative regulator of dopamine synthesis. As a result, mutants display high synaptic activity and elevated mobility (Stathakis et al., 1999; Wang et al., 2011). *Catsup* has a very broad expression pattern (almost ubiquitous) and it is localized in the endoplasmic reticulum (ER) and Golgi apparatus (Groth et al., 2013). Accordingly, *Catsup* mutant flies accumulate misfolded proteins such as Notch and APPL in the Golgi apparatus and in the ER. Probably loss of *catsup* affects the normal processing and trafficking of proteins that, in turn, increases ER stress and caspase 3 activity (Groth et al., 2013). The genetic interactions suggest that *Catsup* participates in the transport of extracellular Zn and Zn efflux from Golgi apparatus (Dechen et al., 2015). Up to now, the relation of Zn with the synthesis of dopamine

and the accumulation of misfolded protein in *Catsup* mutants has not yet been established. *dZip99C* is a special case. It is classified in this group due to the results of genetic interactions (Dechen et al., 2015). However, recent research has found that it is an iron supplier and not a Zn transporter (Xiao et al., 2014). However, we cannot exclude that dZip99C is able to change Zn distribution indirectly. This would also explain the modification of Zn toxic phenotypes described in the literature (Lye et al., 2012, 2013; Dechen et al., 2015). Unfortunately, there is no functional data regarding dZip48C and dZip102B besides the information present in the FlyAtlas.

Cellular Zinc Exporters

The proteins that act as exporters of Zn outside of the cell are dZnT63C (dZnT1) and dZnT33D (**Figure 2**). *dZnT63C* was first discovered in the transcriptomic analysis of the fly response to metal toxicity (Yepiskoposyan et al., 2006). It shares homology with human ZnT1 (25.6% identity and 43.1% similarity) and it is the major responsible protein for Zn import from the gut into the body (Wang et al., 2009) although it is also expressed in other tissues such as developing eye (Lye et al., 2012), testis or salivary glands (Wang et al., 2009). As expected from the proposed function, flies with reduced *dZnT63C* accumulated Zn in the gut and have a Zn deficiency in the rest of the fly. In agreement, these flies are also sensitive to Zn deprivation. The gut tries to compensate the loss of *dZnT63C* by downregulating *dZip42C.1* and *dZip42C.2* to reduce Zn uptake. dZnT63C is also found in the Malpighian tubules where it acts in Zn reabsorption into the body (Wang et al., 2009; Yin et al., 2017). The genetic interactions performed in the eye and in the thorax of the fly to modify Zn dyshomeostasis phenotypes (Lye et al., 2013), have classified dZnT33D as another cellular Zn exporter, although some intracellular localization seems to be present as well. However, no additional information about its cellular roles is available.

Zinc Exporters into Cellular Compartments

This last group comprises ZnT transporters that mediate Zinc efflux from the cytoplasm into cellular compartments but that also might retain the ability of cellular export. The members of this group are dZnT35C, dZnT41F, dZnT77C, and dZnT86D (**Figure 2**). *dZnT35C* was first described as a gene upregulated by cadmium and Zn in flies (Yepiskoposyan et al., 2006). More importantly, it was also identified in the transcriptomic profile of *dMTF-1* mutants (Yepiskoposyan et al., 2006) as one of the targets of this transcription factor. Moreover, dZnT35C is sufficient to modulate total Zn content in the fly body. Null mutations and ubiquitous downregulation of *dZnT35C* leads to viable individuals that are more sensitive to Zn overload (Yepiskoposyan et al., 2006; Yin et al., 2017). This phenotype is in agreement with the suggested exclusive expression of *dZnT35C* in the Malpighian tubules and its role in Zn detoxification and secretion from the body (Yepiskoposyan et al., 2006; Chi et al., 2015; Yin et al., 2017). Loss of *dZnT35C* impairs Zn detoxification and reduces, in turn, Zn tolerance. The ability of KD of *dZnT35C* to improve the eye degeneration induced by *dZip71B* (Dechen et al., 2015) also suggests neuronal expression in photoreceptor cells. Although dZnT35C has been localized in the plasma membrane, a recent and very interesting result confers *dZnT35C* a new location and a new role. *dZnT35C* is pivotal for the formation of a specialized type of lyososomal related organelles (LROs) in the Malpighian tubules that store Zn (Tejeda-Guzmán et al., 2017). These LROs might be the fly equivalent of mammalian zincosomes (Zalewski et al., 1993). The possibility to transport metals via vesicle-like structures has already been reported for mammalian ATP7A (Lutsenko et al., 2007).

dZnT86D is a Zn transporter located in the Golgi apparatus. Its overexpression is able to produce Zn toxic phenotypes in the eye without any further genetic modification (Dechen et al., 2015). Therefore, is a very interesting tool to analyse the effects provoked by Zn accumulation in the Golgi as well as changes in the intracellular Zn distribution. The ubiquitous downregulation is lethal and also produces thorax and rough eye phenotypes when performed in a tissue-specific manner (Lye et al., 2012, 2013). All this suggests that Zn plays fundamental roles in the Golgi. The last two dZnT genes, *dZnT77C* and *dZnT41F*, were classified in this category according to the results of the genetic interactions (Lye et al., 2012, 2013; Dechen et al., 2015). However, very little is known regarding their roles in the organismal and cellular Zn metabolism. On the one hand, dZnT77C is also localized in the plasma membrane of midgut cells and collaborates with dZnT63C in supplying Zn to the body from the fly intestine (Qin et al., 2013). As expected, ubiquitous silencing also enhances Zn toxicity and worsens phenotypes derived from *dZnT63C* KD. On the other hand, *dZnT41F* is also expressed in the Malpighian tubules (Chi et al., 2015; Yin et al., 2017). The similarities between silencing of *dZnT41F* and *dZnT63C* in the Malpighian tubules suggested that *dZnT41F* also contributes to body Zn reabsorption (Yin et al., 2017).

Other Genes

Additional genes that do not belong to the ZnT/Zip families have also been identified. The single *Drosophila TRPM* gene (*dTRPM*) is a curious example. Georgiev and collaborators found that *dTRPM* is permeable to Zn. *dTRPM* deficiency triggered a strong decrease in cell size that clearly resembles the effects of cellular Zn deficiency in control flies. Further experiments suggested that *dTRPM* regulates Zn homeostasis in a specific cellular compartment that is pivotal to growth control (Georgiev et al., 2010). Other very interesting cases are the *Drosophila* aquaporin homolog *big brain* (*bib*) and *vhaPPA1-2*, a subunit of the vacuolar-type ATPase. KD of *vhaPPA1-2* or *bib* reduced sensitivity to high dietary zinc levels. Their study showed loss of these two genes altered the correct subcellular localization of zinc transporters. The defect in this new level of control of Zn homeostasis was probably due to a failure in the endosomal recycling of proteins back to the membrane that hence reduced the uptake of Zn (Wang et al., 2014). Very recently, *kuzbanian* (*kuz*) a gene that was already described as a target of *dMTF-1* (Yepiskoposyan et al., 2006) has been found to confer tolerance to Zn when overexpressed (Le Manh et al., 2017).

The unexpected finding of abnormal Zn levels in a heterozygous *fumble* mutant (Gutiérrez et al., 2010) lead to

the identification of an unknown recessive factor on the X chromosome displaying a strong influence on the organismal Zn content (Afshar et al., 2013). This mutation has been named *poco-zinc* in the unpublished observations of Fanis Missirlis'group (Tejeda-Guzmán et al., 2017). Although no definitive evidence was presented, all results point toward the *white* gene as the responsible for the *poco-zinc* phenotype. Following this initial discovery, authors realized that other mutants of genes involved in the transport of eye pigments, such as *scarlet* (*st*) or *carmine* (*cm*), also display reduced Zn levels. It seems that the collective action of most of these proteins is required for the biosynthesis of Zn storage granules in the Malpighian tubules of *Drosophila* (Tejeda-Guzmán et al., 2017) and thus to maintain systemic Zn homeostasis. Remarkably, the process of characterization of the *poco-zinc* locus has also highlighted the crucial importance of controlling the genetic background of flies in order to avoid misinterpretation of results when analyzing Zn metabolism and comparing different genotypes. Finally, in a recent publication from Norbert Perrimon's lab, a *Drosophila* cell-based RNAi-screen has allowed the identification of novel genes conferring sensitivity or protection toward Zn such as CG11897 (*red dog mine, rdog*) and some disease-related genes such as *IA-2 protein tyrosine phosphatase,* an ortholog of human PTPRN or *CG32000*, a putative ortholog of human ATP13A2 (Mohr et al., 2017).

THE ROLE OF Cu AND Zn IN *DROSOPHILA* MODELS OF HUMAN NEURODEGENERATIVE DISEASES

Cu also exerts some specific roles in the nervous system. Cu is necessary in flies and mammals for the biosynthesis of neurotransmitters (Sellami et al., 2012; Opazo et al., 2014) and thus, for brain activity. In addition, Cu modulates and even blocks the activity of receptors involved in neurotransmission such as NMDA, AMPA or GABA. It has been suggested that these synaptic roles are mediated by interplays between Cu and proteins like the amyloid precursor protein (APP), the prion protein (PrP), α-synuclein (α-syn) or neurotrophic factors. Interestingly, PrP proteins seem to be target genes of MTF-1 (Grzywacz et al., 2015). Moreover, Cu levels are altered in brains of patients suffering from AD, Huntington's Disease (HD) and PD. In AD and PD, Cu levels are overall reduced but enriched in the amyloid plaques, whereas Cu accumulates in HD brains (Fox et al., 2007; Davies et al., 2016; McAllum and Finkelstein, 2016). Cu binds directly bind to α-syn and promotes the formation of toxic oligomers (Davies et al., 2016). Similarly, Cu has also been found to facilitate amyloid beta (Aβ) deposition, formation of tau fibrills (McAllum and Finkelstein, 2016) as well as aggregation of Huntingtin (Htt) containing aberrant polyQ expansions (Fox et al., 2007). All these evidences and many more (Opazo et al., 2014; D'Ambrosi and Rossi, 2015) reveal new connections between Cu and neurodegenerative diseases. The critical role of Cu in several neurodevelopmental, neuromuscular and neurodegenerative disorders has been recently corroborated with the characterization of the cellular interactome of human ATP7A (Comstra et al., 2017). Similarly, Zn is also concentrated in glutamatergic synaptic terminals where it interacts with neurotransmitter receptors to modulate synaptic activity (Sensi et al., 2009, 2011). Although evidences about Zn levels in AD brains are contradictory, Zn metabolism is clearly disturbed in AD and several studies have demonstrated that Zn is crucial in the olimerization of Aβ. Furthermore, it has been suggested that combination of Cu and Zn dyshomeostasis increased toxicity of Aβ (Sensi et al., 2011; McAllum and Finkelstein, 2016). Mutations in ATP13A2 (*PARK9*) have been associated to PD. *ATP13A2* encodes a $P5_B$-type ATPase and although its specific substrate is still not clear, some evidences point toward Zn as a very good candidate (Tsunemi and Krainc, 2014).

Cu-Related Diseases

Menkes disease (MD) is the most important and best characterized neurological disorder related to Cu homeostasis. MD is a pediatric fatal metabolic syndrome accompanied with neurodevelopmental and neurodegenerative defects with a prevalence of 1:50,000 individuals (de Bie et al., 2007). MD is a recessive disorder produced by a loss of function of ATP7A. In the last 50 years, more than 370 mutations affecting ATP7A have been described. Depending on the severity of the symptoms, they have been ascribed, to different types of X-linked disorders named classical MD, occipital horn syndrome and spinal muscular atrophy (Zlatic et al., 2015). However, the mechanism of neurodegeneration is still poorly understood. Impairment of ATP7A functions induces accumulation of Cu into intestinal enterocytes but a deficiency of Cu in plasma, kidney, neurons and astrocytes due to the lack of transport of Cu into these tissues (Kaler, 2011). Mutations in ATP7A triggers several neurological phenotypes such as abnormal neuroblast migration, atrophy of gray and white matter as well as loss of neurons in the brain cortex and aberrant dendritic arborizations (Zlatic et al., 2015). The so called "oligoenzymatic hypothesis" (lack of Cu in cuproenzymes) does not explain the constellation of neurological manifestations and therefore, other proteins must be involved, too. In this sense, the existing mouse models very nicely recapitulate the human physiological and cellular defects (Lenartowicz et al., 2015) but they have failed to unveil new elements of the pathogenic mechanism. Moreover, it is still a matter of debate whether neurodegeneration in Menkes is a consequence of loss of ATP7A in neurons or of nutritional Cu. The potential of the fly will be very helpful to address particular roles of Cu in neurological disorders as well as to unveil novel neuronal specific proteins involved in Cu homeostasis and in the pathogenesis of MD.

As stated above, *DmATP7* is the fly ortholog of *ATP7A* (Southon et al., 2004). Remarkably, null alleles of *DmATP7* resemble human MD phenotypes (Norgate et al., 2006) and *DmATP7* is able to restore normal copper levels in MD patient's fibroblasts (Southon et al., 2010). All this shows a clear functional homology between human and fly proteins. Analysis of mutants shows that *DmATP7* is essential in early development as mutants never reach third instar larvae. Mutant larvae also display hypopigmentation (due to lack of *Tyr* activity) and lethargic behavior (because of compromised synthesis of neurotransmitters). Therefore, *DmATP7* plays a dual role

exporting Cu from the cells and delivering it to cuproenzymes (Norgate et al., 2006). Silencing of *DmATP7* in the fly digestive tract is sufficient to induce Cu retention in the gut (Binks et al., 2010) and to reduce Cu content in the nervous system. This systemic effect triggers, in turn, neurodevelopmental Menkes-like phenotypes such as enhanced pupal lethality and smaller brain size (Bahadorani et al., 2010a). However, the small proportion of adults that successfully completed development show normal lifespan, but with hypersensitivity to oxidative insult (Bahadorani et al., 2010a) likely because lack of a functional antioxidant enzyme Cu/ZnSOD. Reduction of *DmATP7* in different neuropeptidergic neurons had a strong impact on amidation of several neuropeptides but effects on fly behavior were more limited than anticipated (Sellami et al., 2012). *DmATP7* has recently been shown to interact with the oligomeric Golgi complex (COG) to control the development of synapsis via a novel mechanism (Comstra et al., 2017). All these results indicate that *DmATP7* plays cell and non-autonomous roles in Cu homeostasis. Moreover, survival of flies lacking *DmATP7* is improved by increasing Cu content in the food and by upregulating *dMTF-1*. This will consequently promote expression of *Ctr1B* to potentiate supply of Cu and of Mtns to reduce toxic effects of Cu accumulation in the gut (Bahadorani et al., 2010a). It is important to highlight that *Drosophila* only contains one ATP7 ortholog and therefore the fruit fly might also be an interesting organism to model Wilson's disease (WD), a human disorder caused by mutations in the ATP7B gene which produces increased intracellular Cu levels, accumulation of ROS, mitochondrial dysfunction and cell death (Bandmann et al., 2015). The fly models of MD and WD have gone one step beyond since they are being used as diagnostic tools to establish the pathogenicity of variants found in patients. The ability of given variants to improve the larval lethality of the fly null mutant helps to discriminate between pathological and control-like variants. In addition, subcellular localization of mutant forms have provided insight into the mechanism of copper dyshomeostasis in MD and WD patients (Mercer et al., 2017).

Several other neurodegenerative disorders have been shown to have an intimate relation with Cu (Ayton et al., 2013; McAllum and Finkelstein, 2016), although it is not clear if Cu is a friend or a foe. Usually WD patients manifest Parkinson-like symptoms (Bandmann et al., 2015). Therefore, it would be interesting to test the influence of Cu in PD models and whether manipulation of cellular Cu content might have a beneficial effect (**Table 1**). From the whole plethora of fly models (Botella et al., 2009), this has only been addressed in flies deficient for the protein Parkin (Saini et al., 2010). In this work, Cu-specific chelation by means of BCS was sufficient to increase longevity of *parkin* null mutants. Additional evidences suggested that reduction of ROS was mediating the neuroprotective effect of BCS in *parkin* deficient flies (Saini et al., 2010). It is likely that the combination of redox-activity of free Cu and the mitochondrial dysfunction in *parkin* mutant flies boosted the generation of ROS in these flies. The Cu-*parkin* relation was further corroborated by genetic interactions. Indeed, loss of *parkin* exacerbated the retinal degeneration triggered by overexpression of the Cu importer *Ctr1B* (Hua et al., 2010). Cu levels have been demonstrated to be lower in PD patients (Davies et al., 2016) although the rescue with BCS from Saini and collaborators goes in an opposite direction. Therefore, it would be of high relevance for the scientific community to study the impact of Cu biology in other PD fly models.

Modulation of Cu metabolism has also successfully improved the deleterious defects of toxic Aβ42 peptides in *Drosophila* models of AD (Lang et al., 2013; Singh et al., 2013; **Table 1**). Aβ proteins are the major component of plaques in AD patients. The Aβ42 isoform is more prone to aggregation due to the 2 extra aminoacids at the C-terminus (White et al., 1999). Reduction of cellular Cu content by silencing the expression of *Ctr1B* and *Ctr1C* (Lang et al., 2013), by overexpressing *DmATP7* (Lang et al., 2013) and *dMTF-1* or *MtnA* (Hua et al., 2011b) improved neurodegeneration, locomotion, longevity and oxidative stress markers. Similar effects were obtained by chemical chelation of Cu (Hua et al., 2011b; Singh et al., 2013). On the contrary, increasing cellular Cu or impairing intracellular Cu delivery worsened the phenotypes (Sanokawa-Akakura et al., 2010; Hua et al., 2011b). The work from Lang and collaborators raised a couple of interesting and paradoxical questions. First, AD phenotypes are rescued by panneuronal silencing of *Ctr1B* and *C*, genes supposed to be only expressed in fly gut and male gonads (Zhou et al., 2003; Hua et al., 2010). This indicates that better reporters to study expression patterns of these genes are needed. Second, the improvements were accompanied by increased amounts of higher-molecular weight forms of Aβ42. This result clearly questions the toxicity of the aggregates. However, similar genetic interventions in a fly model of HD expressing the mutant form of Htt (expanded polyQ tract) successfully reduced the levels of PolyQ aggregation by decreasing Cu content in flies (Xiao et al., 2013). Therefore, Cu metabolism might exert different and even opposite roles in distinct disease models. As a consequence, locomotion, survival and brain degeneration were also improved in this HD fly model (Xiao et al., 2013). These experiments revealed that Cu facilitates protein aggregation in HD. In agreement, a mutant form of Htt unable to bind Cu displayed a reduced toxicity (Xiao et al., 2013).

Other reports in the fly have identified genes with influence on neurodegenerative or neuropsychiatric diseases that resulted in new actors of Cu metabolism. Presenilins participate in the processing of Aβ proteins as they are the catalytic unit of the γ-secretase complex and their mutations underlie the majority of early-onset AD cases. Interestingly, secretase-independent roles have been reported for presenilins (Stiller et al., 2014). Silencing of *PSN*, the presenilin ortholog present in flies, in the fly gut reduced copper levels in flies and increased tolerance to excess dietary copper but also susceptibility to oxidative insult. PSN is suggested to contribute to the localization of Cu importers Ctr1A and Ctr1B in the plasma membrane (Southon et al., 2013b) but the exact action mechanism still needs to be completely elucidated. Misexpression of high molecular weight immunophilin FKBP52, which is a known interactor of the Cu chaperone *Atox1*, also modulates toxicity of Aβ42 (Sanokawa-Akakura et al., 2010). Finally, disruption of the dysbindin/BLOC-1 complex in the fly by means of *Drosophila* BLOC-1 mutants

is sufficient to alter Cu metabolism suggesting that external factors might modulate schizophrenia via mechanisms that are conserved from humans to flies (Gokhale et al., 2015).

Zn-Related Diseases

Zn also needs to be tightly regulated because excess or deprivation of Zn has severe health effects. Many reviews summarize the toxic consequences of Zn dyshomeostasis in humans and murine models (Fukada and Kambe, 2011; Hagmeyer et al., 2014; Kambe et al., 2014, 2015; Terrin et al., 2015). For example, Zn deficiency in the fetus affects organ growth that can result into preterm birth or even early death (Terrin et al., 2015). In mouse models, several behavioral tests have linked Zn deficiency with deficits in learning and memory or increased anxiety or depression-like disorders (Hagmeyer et al., 2014). Zn homeostasis is a pretty new field in the fly and although several new tools are being continuously implemented to further characterize the *Drosophila* Zn transporters, there are not many fly models of Zn-dependent diseases. In humans, mutations or even Single Nucleotide Polymorphisms (SNPs) in ZnT or Zip genes have been already associated to particular diseases (Fukada and Kambe, 2011; Kambe et al., 2015). For example, mutations in ZnT10 are responsible for hepatic cirrhosis that is accompanied with Parkinsonism or mutations in Zip4 triggers Acrodermatitis enteropathica. On the other hand, SNPs in ZnT8 are linked with increased susceptibility toward type I and II diabetes mellitus. From all human disorders directly related to Zn homeostasis, the Spondylocheirodysplasia-Ehlers-Danlos Syndrome-Like is the only one modeled in *Drosophila* (Xiao et al., 2014). The Ehlers-Danlos Syndrome is produced by loss of function of the human Zip13 transporter. However, the *Drosophila* ortholog *dZip99C* (CG7816) is involved in iron efflux and not in Zn transport. Loss of *dZip99C* affects iron storage by ferritin, increases cytosolic iron and, in agreement with the human counterpart, reduces collagen production. Interestingly, the fact that hZip13 but not hZip7 (a closely related gene that has been shown to import Zn) can complement *dZip99C* (Xiao et al., 2014), might suggest that the human disorder is also triggered by iron deregulation and thus, Zn defects are a secondary event.

Manipulation of Zn regulatory network has been a very successful approach to ameliorate neuronal dysfunction in *Drosophila* models of AD as well as to decipher the functional roles of Zn in this disease (**Table 2**). In an AD model based on overexpression of a mutant version of human Tau, results from a genetic screening showed that increasing or reducing the expression of a *dZip42C.1* and *dZnT63C* successfully modified Tau-related phenotypes in the fly (Huang et al., 2014). Reduction of Zn content by *dZnT63C* overexpression, silencing of *dZip42C.1* or chemical chelation improved longevity, brain vacuolization and retinal degeneration. Remarkably, authors proved in the fly that Tau phosphorylation was mediated by Zn and that hyperphosphorylation was a cardinal mechanism underlying toxicity. Furthermore, as Zn can directly bind Tau protein, authors mutated specific residues to suppress this interaction. This was sufficient to reduce Tau toxicity by suppressing Tau aggregation but without changing Tau phosphorylation. Thus, this work showed *in vivo* two Zn-dependent mechanisms underpinning Tau toxicity (Huang et al., 2014). In a second AD model, Zn chelation was sufficient to improve longevity, locomotion and photoreceptor degeneration in flies overexpressing Aβ42 protein. As expected, Zn supplementation exacerbated the phenotypes. This enhancement was suppressed in a special variant of Aβ42 in which Zn binding was abolished (Hua et al., 2011b). In agreement with the critical role of Zn metabolism in this model, other authors found that *dZip42C.1* level increases with age in the brain of Aβ42-expressing flies, whereas it goes down in controls. Such levels explain the accumulation of Zn in the brain of Aβ42 (Lang et al., 2012) and the toxic effects of Zn supplementation (Hua et al., 2011b). In line with these evidences, KD of *dZip42C.1* was sufficient to reduce neurodegeneration, prolong lifespan and improve memory loss as well as Aβ42 deposits in transgenic flies (Lang et al., 2012). All these results suggest that *dZip42C.1* is critically involved in the pathology of Aβ42. Paradoxically, overexpression of *dZip42C.1* also resulted in obvious memory recovery but the mechanism was not described in the manuscript.

Similar contradictory findings have been recently described in a *Drosophila* model of Friedreich's ataxia (FRDA). FRDA is the most important recessive ataxia in the Caucasian population. Impairment of transcription of the gene frataxin is the molecular cause underlying the disease (Campuzano et al., 1996). Most of the current evidences support a strong relation between frataxin and iron in several models (Kakhlon et al., 2008; Huang et al., 2009; Schmucker et al., 2011), including the fly (Soriano et al., 2013; Navarro et al., 2015; Chen et al., 2016). Moreover, genetic and chemical manipulation of iron biology was found to have a positive impact in FRDA phenotypes (Kakhlon et al., 2008; Whitnall et al., 2008; Navarro et al., 2015). Remarkably, flies displaying reduced levels of frataxin also seemed to have altered levels of other metals such as Zn and Cu. Although the contribution of other metals was already suggested by studies in human samples (Koeppen et al., 2012, 2013), this fly work was the first one showing that therapies based on such metals might be beneficial (Soriano et al., 2016). Indeed, Zn and Cu chelators improved frataxin deficiency without altering iron content. In this line, silencing either *dZip42C.1*, *dZip42C.2* and *dZip88E* or *dZnT35C*, *dZnT41F* and *dZnT63C* also improved FRDA conditions via reduction of the iron content (**Table 2**). All these results raise several interesting questions: Why are Cu and Zn accumulating in FRDA flies? Why does KD of genes with opposite function or acting in different cellular compartments trigger the same effect? Did they also modify the accumulation of Zn in FRDA flies? Is it possible that any of these transporters has also a mitochondrial function?

Studies of a *Drosophila* PD model took advantage of the antioxidant properties of Zn and explored the possibility to counteract the increased oxidative stress in the fly *parkin* mutant (Greene et al., 2005) by Zn supplementation. Interestingly, *parkin* mutant flies displayed altered levels of several Zn transporters such as *dZnT35C*, *dZnT63C,* or *foi* and had reduced Zn content in the head. Importantly, dietary Zn successfully increased eclosion frequency, survival rate and head zinc content of *parkin* mutants (Saini and Schaffner, 2010). Results showed that Zn boosted

expression of Mtns and that the ROS scavenging properties of Mtns mediated the rescue mechanism. In relation to PD, *Catsup* mutants, surprisingly, have increased tolerance against paraquat induced neurotoxicity (Chaudhuri et al., 2007). This result is interesting since *Catsup* mutants display high tyrosine hydroxylase (TH) activity and therefore increased dopamine pools (Stathakis et al., 1999), whereas reduction of *TH* expression and dopamine levels rescued PD phenotypes in two other fly models of the disease (Bayersdorfer et al., 2010). Although the mechanism driven by loss of *Catsup* was not addressed in the manuscript, we can speculate that the activation of stress pathways in the mutants (Groth et al., 2013) might contribute to counteract the paraquat effect.

In the literature, the relation between Zn and fly models of human diseases is mostly based on studies about neurodegenerative disorders (**Table 2**). Importantly, the fly has also been useful to mimic other human diseases and expand their knowledge beyond the state of the art. A recent report showed that Zn is also a crucial mediator in the formation of urinary stones. In human, deficiency of xanthine dehydrogenase is responsible of such kidney stones (Arikyants et al., 2007). In the fly, silencing of the ortholog gene (*Xdh* also known as *ry*, CG7642) also induces the presence of small stones in the intraluminal content of Malpighian tubules. Spectrometric analysis of such stones revealed high similarity to the human ones including the presence of abundant Zn. In line with this novel perspective, reduction of the expression of three ZnT transporters (*dZnT35C*, *dZnT41F*, *dZnT63C*) and of *dZip71B* that are highly expressed in the Malpighian tubules mitigated the formation of stones as well as other phenotypes associated to loss of *Xdh* (Chi et al., 2015; Yin et al., 2017). Evidences suggest that downregulation of these genes avoid the accumulation of Zn in the lumen of the tubules. In agreement, Zn chelation by N,N,N'N'-tetrakis- (2-pyridylmethyl)ethylenediamine (TPEN) also lead to similar results (Chi et al., 2015). A link between cancer and Zn has been established throughout the processing of the morphogen hedgehog (*hh*). Zn normally inhibits fly hh autoprocessing and thus upon Zn deficiency there is an overactivation of the pathway that might contribute to pathogenesis of several cancer types (Xie et al., 2015).

THE POTENTIAL OF *DROSOPHILA* FOR FUTURE STUDIES

However, there are some aspects of Cu and Zn biology that still remain obscure. For example, it is of paramount importance to understand the need of so many ZnTs and Zips to regulate Zn metabolism. The number of genes performing similar functions confers an unexpected level of complexity. Furthermore, establishing the precise spatial and temporal expression pattern of each gene regulating Cu and Zn homeostasis will facilitate the interpretation of many aspects of the metal biology. Frank Schnorrer's and Hugo Bellen's labs have created two large-scale transgenic collections that allow protein visualization. The first one is based on a fosmid library of clones in which target genes are C-terminally tagged within their genomic context (Sarov et al., 2016). The second one has modified the *Minos* mediated integration cassette (MiMIC) to introduce a dominant marker and a gene-trap cassette flanked by two inverted ΦC31 *attP* sites (Venken et al., 2011). The MiMIC collection also allows the replacement of the gene-trap cassette via recombinase mediated cassette exchange (RMCE) even without the need of microinjection (Nagarkar-Jaiswal et al., 2015a,b). A similar approach combining CRISPR and RMCE has been reported (Zhang et al., 2014). The labs of Christopher Potter, Matthias Landgraf and Benjamin White have very nicely mixed both, MiMIC, and CRISPR, strategies and have generated another collection of so called Trojan-GAL4 lines (Diao et al., 2015). This strategy takes advantage of MiMIC transposons located in introns between coding exons and exchanges their content with a T2A-GAL4 cassette. Because of it, the native gene product will be cleaved and translation of GAL4 will occur as an independent protein. A perfect complement to these reporters in the possibility to perform cell-type-specific transcriptional profiling without cell isolation (Southall et al., 2013; Marshall et al., 2016). This extremely powerful tool developed in Andrea Brand's lab might help to clarify the cells/tissues expressing the different Zn and Cu transporters.

Up to now, tissue-specific analysis of Cu and Zn relies on RNAi lines. However, they are not completely effective and the residual gene activity might be sufficient to hide some important aspects of gene function. To bypass this, Simon Bullock's lab has developed a fast approach to generate CRISPR mutants by means of the UAS/GAL4 system (Port and Bullock, 2016). Finally, the possibility to perform unbiased genetic screens, which confers *Drosophila* an important advantage over other higher eukaryotic model systems, will allow to identify new genetic factors involved in the regulatory networks of Cu and Zn and modifiers of phenotypes in the fly models of human diseases.

AUTHOR CONTRIBUTIONS

JAN and SS designed, wrote and approved the final manuscript.

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Iron Sulfur and Molybdenum Cofactor Enzymes Regulate the *Drosophila* Life Cycle by Controlling Cell Metabolism

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Iron sulfur (Fe-S) clusters and the molybdenum cofactor (Moco) are present at enzyme sites, where the active metal facilitates electron transfer. Such enzyme systems are soluble in the mitochondrial matrix, cytosol and nucleus, or embedded in the inner mitochondrial membrane, but virtually absent from the cell secretory pathway. They are of ancient evolutionary origin supporting respiration, DNA replication, transcription, translation, the biosynthesis of steroids, heme, catabolism of purines, hydroxylation of xenobiotics, and cellular sulfur metabolism. Here, Fe-S cluster and Moco biosynthesis in *Drosophila melanogaster* is reviewed and the multiple biochemical and physiological functions of known Fe-S and Moco enzymes are described. We show that RNA interference of *Mocs3* disrupts Moco biosynthesis and the circadian clock. Fe-S-dependent mitochondrial respiration is discussed in the context of germ line and somatic development, stem cell differentiation and aging. The subcellular compartmentalization of the Fe-S and Moco assembly machinery components and their connections to iron sensing mechanisms and intermediary metabolism are emphasized. A biochemically active Fe-S core complex of heterologously expressed fly Nfs1, Isd11, IscU, and human frataxin is presented. Based on the recent demonstration that copper displaces the Fe-S cluster of yeast and human ferredoxin, an explanation for why high dietary copper leads to cytoplasmic iron deficiency in flies is proposed. Another proposal that exosomes contribute to the transport of xanthine dehydrogenase from peripheral tissues to the eye pigment cells is put forward, where the Vps16a subunit of the HOPS complex may have a specialized role in concentrating this enzyme within pigment granules. Finally, we formulate a hypothesis that (i) mitochondrial superoxide mobilizes iron from the Fe-S clusters in aconitase and succinate dehydrogenase; (ii) increased iron transiently displaces manganese on superoxide dismutase, which may function as a mitochondrial iron sensor since it is inactivated by iron; (iii) with the Krebs

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cycle thus disrupted, citrate is exported to the cytosol for fatty acid synthesis, while succinyl-CoA and the iron are used for heme biosynthesis; (iv) as iron is used for heme biosynthesis its concentration in the matrix drops allowing for manganese to reactivate superoxide dismutase and Fe-S cluster biosynthesis to reestablish the Krebs cycle.

Keywords: aldehyde oxidase, DNA polymerase, electron transport chain, ecdysone, iron regulatory protein, quiescent mitochondria, magnetoreceptor, mitoflashes

INTRODUCTION

In the first known biochemical reactions on earth, molybdenum and iron-sulfur (Fe-S) clusters enabled electron transfers turning inorganic molecules into hydrogenated carbon molecules (Mortenson, 1964; Eck and Dayhoff, 1966; Hall et al., 1971; Ochiai, 1978; Wächtershäuser, 1988; Russell and Martin, 2004; Zhang and Gladyshev, 2008; Nitschke and Russell, 2009; Schoepp-Cothenet et al., 2012; Stüeken et al., 2015). Similar biochemistry remains active in living organisms carried out by a variety of metallo-enzymes. In this *hypothesis and theory* article, we present examples of Fe-S and molybdenum cofactor (Moco) enzymes from the dipteran fly *Drosophila melanogaster*, a genetically amenable and thoroughly characterized experimental model system (Bellen et al., 2010; St Johnston, 2013; Mohr et al., 2014). By looking at their multiple physiological functions, we propose that Fe-S enzymes are central in the development, life cycle transitions and aging of flies. Given the conservation of these phenomena in the evolution of the animal kingdom, we anticipate that many of our descriptions will be transferable to other organisms.

In the first part of the article, we present how Fe-S clusters are formed, a process that has been studied extensively by biochemists in prokaryotes (Roche et al., 2013; Blanc et al., 2015), yeast (Martinez-Pastor et al., 2017), plants (Balk and Schaedler, 2014), and humans (Paul and Lill, 2015; Rouault and Maio, 2017), but also by the biomedical community intent to find a therapy for patients with Friedreich's ataxia, caused by reduced expression of the frataxin (FXN) gene (Campuzano et al., 1996). Other Fe-S proteins are also implicated in human disease (Rouault, 2012; Beilschmidt and Puccio, 2014; Isaya, 2014). In *D. melanogaster*, the pioneering work of Maria Moltó and co-workers has almost exclusively focused on the *Drosophila* frataxin homolog describing what goes wrong when Fe-S biosynthesis is disrupted in flies (reviewed in Mandilaras et al., 2013; Tang and Zhou, 2013b; Zhu et al., 2014; Calap-Quintana et al., 2017). Furthermore, we describe the biosynthesis of Moco (Rajagopalan, 1997; Mendel and Leimkühler, 2015; Leimkühler, 2017), whose basic structure has two sulfur atoms of the tricyclic pyranopterin molecule molybdopterin (MPT) coordinating the Mo atom (Rajagopalan et al., 1982). Work on Moco enzymes in *Drosophila* started in the fifties and Victoria Finnerty studied the Moco biosynthetic pathway during the last quarter of the twentieth century (Kamdar et al., 1997). Her research program used the molybdoenzyme xanthine dehydrogenase (Xdh) encoded by the *rosy* gene, whose activity is required for the formation of eye pigments for reasons that are still not fully resolved (Phillips and Forrest, 1980; Wiley and Forrest, 1981; Ferre et al., 1986; Keith et al., 1987; Hilliker et al., 1992), and therefore mutants affecting Moco biosynthesis have evident eye color phenotypes (Kamdar et al., 1997). We complement this review with new discoveries in the role of proteins involved in Fe-S cluster and Moco biosynthesis by showing the original data, which are not published elsewhere (Marelja, 2013), and with a new hypothesis to explain previous observations that dietary copper decreases iron storage in *Drosophila* (Poulson and Bowen, 1952; Bettedi et al., 2011).

In the second part of the article, we review studies of the *Drosophila* molybdoenzymes Xdh, aldehyde oxidase (Aox), sulfite oxidase (Suox). Special attention is paid to the problem of how Xdh, a cytosolic enzyme that requires two Fe-S clusters, Moco and flavin for activity (Hughes et al., 1992; Doyle et al., 1996), finds its way into pigment granules of the eye, the only enzyme with such cofactors known to reside in the endomembrane system (Reaume et al., 1989, 1991). Our hypothesis is that exosomes are involved in the process. The *fly* literature on mitochondrial Fe-S enzymes required for respiration and the biosynthesis of ecdysone, heme, and lipoate is summarized. The role of cytosolic and nuclear Fe-S enzymes in DNA replication, transcription and translation is also reviewed, followed by a brief discussion of the regulation of the Fe-S cluster of iron regulatory protein-1A (IRP-1A) and cellular iron sensing in *Drosophila*. We then move to the question of how mitochondria sense iron, where we present a new hypothesis suggesting that the mitochondrial superoxide dismutase (Sod2) is a possible mitochondrial iron sensor. Our model of mitochondrial iron sensing also explains the previously observed superoxide bursts in mitochondria (Wang et al., 2008) and the connection between mitochondrial Fe-S cluster biosynthesis and lipogenesis (Tong and Rouault, 2007). Last, we revisit the question of whether Fe-S and/or Moco enzymes are involved in the circadian clock (Mandilaras and Missirlis, 2012).

In the third part of the article, we communicate how profoundly cell physiology depends on Fe-S enzymes. We review the shifts in cell metabolism from glycolysis to aerobic respiration during development (Tennessen et al., 2011, 2014) and during stem cell differentiation (Homem et al., 2014; Sieber et al., 2016), emphasize the requirement of Fe-S clusters for growth through the larval stage and into metamorphosis (Anderson et al., 2005; Uhrigshardt et al., 2013; Llorens et al., 2015; Palandri et al., 2015) and the decline of mitochondrial respiration during aging (Vann and Webster, 1977; Yan et al., 1997; Ferguson et al., 2005). We then discuss the interesting finding that in the female germ line, stem cell differentiation requires the mitochondrial ATP synthase, but not the respiratory chain enzymes (Teixeira et al., 2015). We critically evaluate the possibility that the presence of

quiescent mitochondria in the female germ line may serve as a protective hereditary mechanism against the accumulation of mutations in their genome (Allen, 1996). Finally, we describe specific functions of Fe-S or Moco enzymes in the major organs of the fly, concluding that they are a biochemically active component of the complex organization that characterizes living animals.

Fe-S CLUSTER AND MOCO BIOSYNTHESIS

Fe-S cluster assembly initiates in the mitochondrial matrix. We summarize the different steps of the assembly process (**Figure 1**); for detailed reviews of the pathway the reader is referred elsewhere (Roche et al., 2013; Balk and Schaedler, 2014; Blanc et al., 2015; Paul and Lill, 2015; Martinez-Pastor et al., 2017; Rouault and Maio, 2017). The mitoferrin transporter ensures mitochondrial iron uptake, whereas the L-cysteine desulfurase Nfs1 provides the inorganic sulfide as persulfide. Electrons are required for the cleavage of the persulfide group and assembly of the cluster, which are supplied from ferredoxins Fdx1 and Fdx2. The first assembly protein is IscU, which can accommodate [2Fe-2S] or [4Fe-4S] clusters. Fe-S clusters are transferred from IscU to other scaffold proteins like IscA1, IscA2, Iba57, Nfu, BolA1, or BolA2 that show specificity of cluster delivery to the target enzymes. For Fe-S cluster transfer between the assembly proteins, or between assembly proteins and target enzymes, specialized

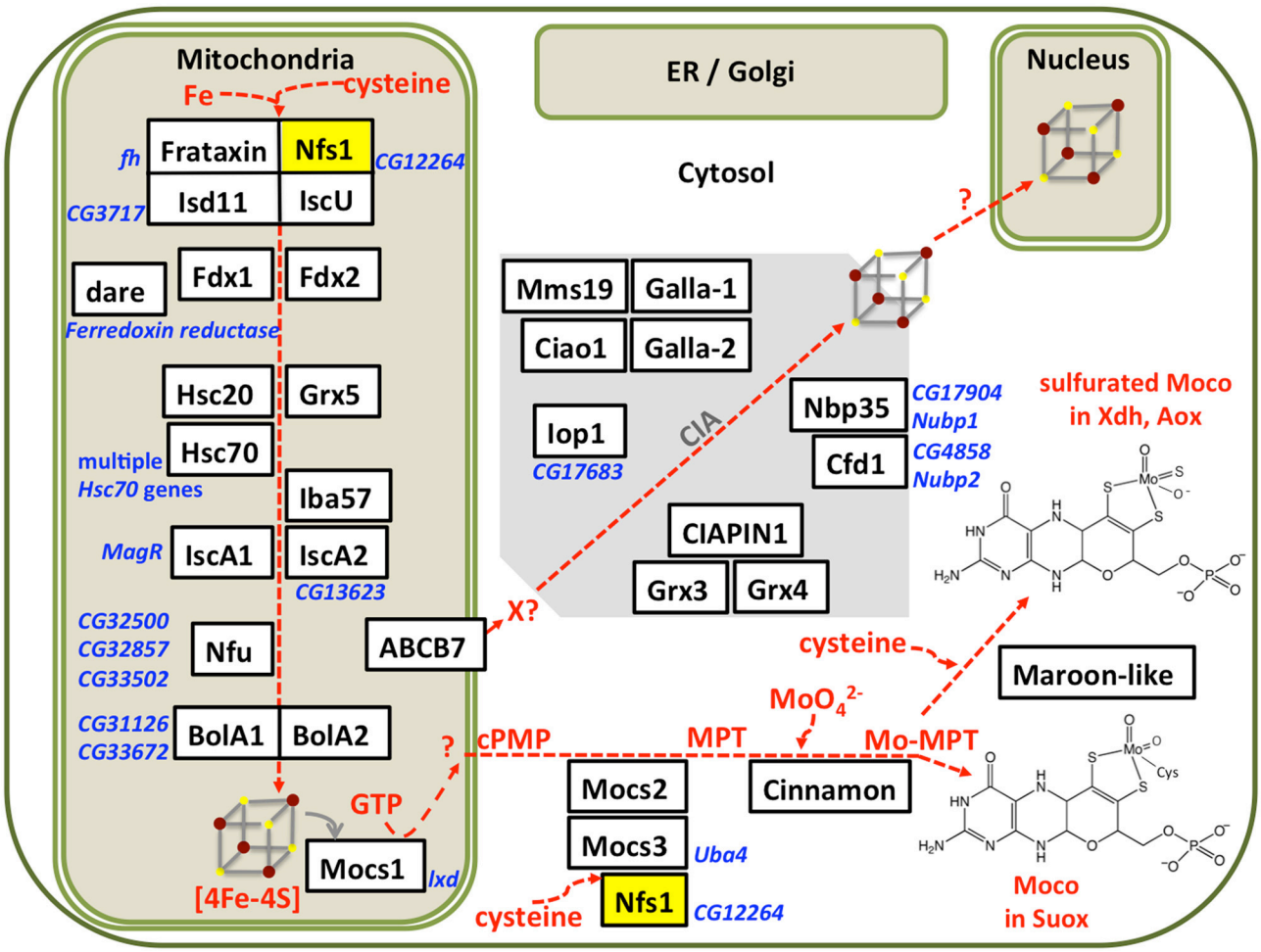


FIGURE 1 | Subcellular compartmentalization of the Fe-S cluster and Moco assembly processes. Iron and sulfur from cysteine are assembled on IscU in a process that requires electrons provided by ferredoxins (Fdx1, Fdx2, which are themselves Fe-S proteins). Chaperones and Grx5 are required for transfer of the Fe-S cluster to further scaffold proteins. The *Drosophila* homologs were identified by Blast searches, proposed names for the gene products (in white boxes) were based on the corresponding nomenclature in humans, current Flybase annotations are shown in blue font. Our own results (Marelja, 2013) confirm that the *Drosophila* genes *CG12264* and *CG3717* are the homologs of the human genes NFS1 and ISD11, respectively. Multiple Fe-S enzymes are present in mitochondria, cytosol, and nucleus, but so far none have been reported in the secretory pathway. All known components of the CIA pathway are conserved in *Drosophila*, but the first steps of CIA remain to be elucidated. Moco biosynthesis initiates in the mitochondria with the Fe-S protein Mocs1 and continues in the cytosol with Mocs2, Mocs3, and Cinnamon producing Moco used in Suox. Maroon-like sulfurates Moco for Xdh and Aox. Mitochondrial and cytosolic forms of the cysteine desulfurase Nfs1 (yellow box) provide sulfide to Fe-S cluster and Moco biosynthesis, respectively.

chaperones (Hsc20 and its cognate partner) and glutaredoxin-5 (Grx5) are required. A cytosolic Fe-S cluster assembly (CIA) pathway has been described, however it is unclear how the first clusters are formed on the CIA complex. The cytosolic electron donors cytokine-induced apoptosis inhibitor-1 (CIAPIN1) and glutaredoxins Grx3 and Grx4 have been identified (**Figure 1**). Only three genes involved in Fe-S biosynthesis have been individually studied in flies: (i) *Drosophila frataxin* whose exact biochemical function is still unclear (Cañizares et al., 2000; Anderson et al., 2005, 2008; Llorens et al., 2007; Kondapalli et al., 2008; Runko et al., 2008; Navarro et al., 2010, 2011, 2015; Shidara and Hollenbeck, 2010; Soriano et al., 2013, 2016; Tricoire et al., 2014; Calap-Quintana et al., 2015; Palandri et al., 2015; Chen et al., 2016b; Edenharter et al., 2017), (ii) *IscU* that encodes a protein assembly platform for Fe-S cluster biosynthesis (Dzul et al., 2017), and iii) *Hsc20* encoding one of two chaperones that mobilize the Fe-S cluster from IscU to downstream Fe-S proteins in the mitochondria (Uhrigshardt et al., 2013).

The *Drosophila* Nfs1/Isd11/IscU/Frataxin Complex

Two *Drosophila* Fe-S cluster assembly proteins have been purified to date, frataxin (Kondapalli et al., 2008) and IscU (Dzul et al., 2017). We characterized the core mitochondrial protein complex involved in Fe-S cluster assembly in flies (Marelja, 2013). Based on previous work with the human L-cysteine desulfurase NFS1 (Marelja et al., 2008, 2013), the *Drosophila* Nfs1 homolog (CG12264) was purified. The human and *Drosophila* enzymes share an amino acid sequence identity of 78% when the mitochondrial targeting sequences are removed from the calculation. All amino acids implicated in enzymatic function of the human protein are conserved in *Drosophila* Nfs1 (**Figure 2**).

The first 53 amino acids of the full length *Drosophila* Nfs1 were removed from an *Escherichia coli*-driven protein expression construct (for detailed Materials and Methods see Marelja, 2013). Nfs1$^{\Delta 1-53}$ was co-expressed with *Drosophila* Isd11 (CG3717 shows 44% amino acid sequence identity to human ISD11; Adam et al., 2006; Wiedemann et al., 2006) or with *Drosophila* IscU (Dzul et al., 2017), the scaffold protein where newly formed Fe-S clusters are initially assembled (Zheng et al., 1998; Gerber et al., 2003; Fox et al., 2015; Parent et al., 2015). The respective Nfs1$^{\Delta 1-53}$/Isd11 and Nfs1$^{\Delta 1-53}$/IscU complexes were affinity purified and recovered in a stable, soluble form. Higher molecular weight complexes were obtained when all three *Drosophila* proteins were co-expressed and also when human FXN (a gift from Kuanyu Li; Xia et al., 2012; Friemel et al., 2017) was added to the Nfs1$^{\Delta 1-53}$/Isd11/IscU complex (**Figure 3A**). Our own efforts to purify *Drosophila* frataxin as in Kondapalli et al. (2008) were unsuccessful (Marelja, 2013).

The characteristic yellow color observed for other L-cysteine desulfurases containing pyridoxal 5′-phosphate (PLP) as a prosthetic group was confirmed in absorption spectra of *Drosophila* Nfs1 at 420 nm (**Figure 3B**, solid black lines on both panels). Moreover, addition of the enzyme's substrate L-cysteine induced a decrease of absorbance at 420 nm and an increase of absorbance at 320 nm (**Figure 3B**, solid and dashed red lines), as reported for *Azotobacter vineladii* L-cysteine desulfurase NifS, showing that the α-amino group of cysteine binds to the PLP at the enzyme's active site (Zheng et al., 1993). For Nfs1$^{\Delta 1-53}$/Isd11, L-cysteine binding reached equilibrium at 1 min (**Figure 3B**, top panel). In contrast, binding of L-cysteine to the Nfs1$^{\Delta 1-53}$/Isd11/IscU complex was slower (**Figure 3B**, bottom panel), suggesting that the presence of IscU reduced substrate accessibility to Nfs1 *in vitro*. The *Drosophila* Nfs1$^{\Delta 1-53}$/Isd11 complex was also investigated for its ability to restore the growth deficiency of the *E. coli* Δ*iscS* strain CL100 (**Figure 3C**). As expected, CL100 transformed with the plasmid containing the endogenous *iscS* gene restored the growth defect. *Drosophila* Nfs1$^{\Delta 1-53}$/Isd11 partially complemented IscS function in the *E. coli* CL100 strain, whereas expression of IscU along with Nfs1$^{\Delta 1-53}$/Isd11 slowed down bacterial growth (**Figure 3C**). Notably, this *in vivo* result matches the *in vitro* observation that L-cysteine binding to Nfs1$^{\Delta 1-53}$ is slower in the presence of Isd11 and IscU (**Figure 3B**). These findings are consistent with the previous demonstration that the L-cysteine desulfurase activity of the human NFS1/ISD11 complex is reduced in the presence of ISCU (Tsai and Barondeau, 2010; Bridwell-Rabb et al., 2014).

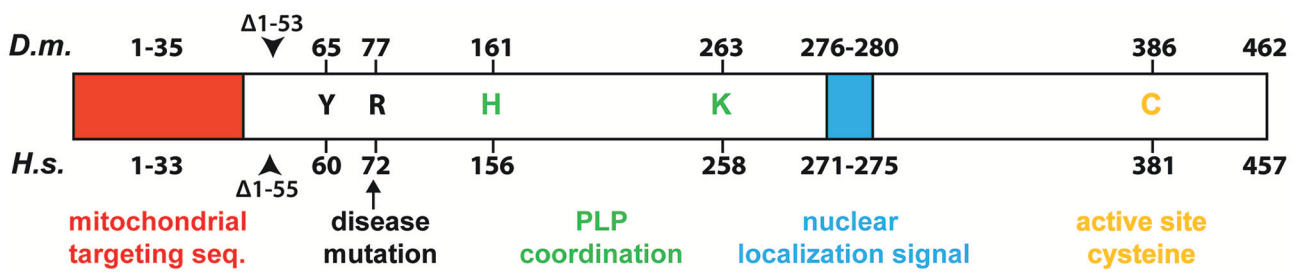


FIGURE 2 | Schematic comparison of *Drosophila* Nfs1 and human NFS1. Highly conserved residues that play a role for desulfurase catalysis and disease are indicated: the active site cysteine (C) that forms the persulfide intermediate (Zheng et al., 1994; Lauhon et al., 2004); the tyrosine (Y) was shown to be crucial for activity in *Saccharomyces cerevisiae* Nfs1p (Mühlenhoff et al., 2004); the point mutation p.Arg72Gln (arrow) in human NFS1 was identified to cause infantile mitochondrial complex II/III deficiency (Farhan et al., 2014); whereas histidine (H) and lyine (K) are involved in pyridoxal 5′-phosphate (PLP) coordination (Cupp-Vickery et al., 2003). The proposed N-terminal mitochondrial targeting sequences is shown by a *red box*, while the nuclear localization signal is shown as a *blue box*. The beginning of the truncated Nfs1/NFS1 versions used for biochemical purification are indicated by an arrow head (Marelja, 2013). Numbers indicate amino acid position in the *Homo sapiens* (*H.s.*) and *D. melanogaster* (*D.m.*) proteins.

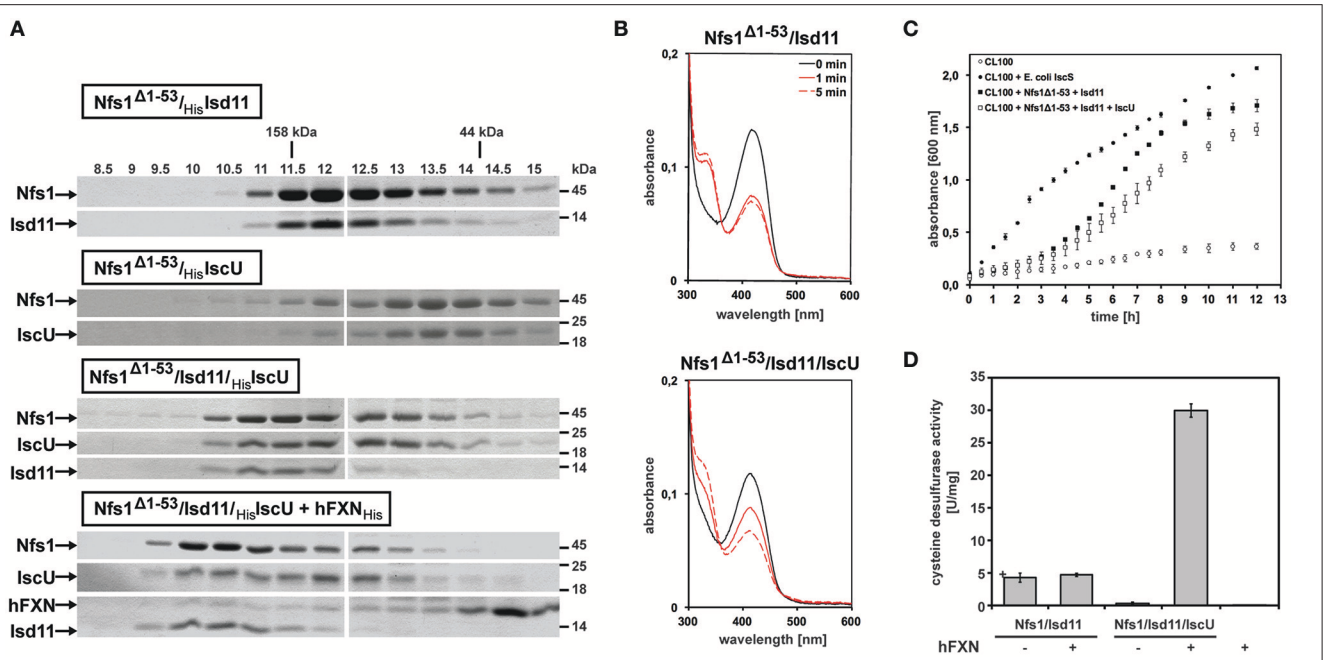


FIGURE 3 | *In vitro* characterization of *Drosophila* Nfs1, Isd11, IscU in complex with human frataxin. **(A)** Co-expression of Nfs1$^{\Delta 1-53}$ with Isd11 and/or IscU in *Escherichia coli*. After purification of the complexes with affinity chromatography, 30 μM of Nfs1$^{\Delta 1-53}$ was applied onto the analytical Superdex200 size exclusion column and compared by SDS-PAGE. The fractions show that the Nfs1$^{\Delta 1-53}$/Isd11 complex eluted earlier than the IscU/Nfs1$^{\Delta 1-53}$ complex, indicating its smaller size. As expected, the complex between all three proteins was larger. Human frataxin added to the purified Nfs1$^{\Delta 1-53}$/Isd11/IscU complex also bound as demonstrated by further increase in size. Gamma globulin (158 kDa) and ovalbumin (44 kDa) were standards (kDa ladder on the top; fractions in ml units). **(B)** UV-Vis absorption spectra of 10 μM Nfs1$^{\Delta 1-53}$ in complex with His_6-Isd11 (top panel) and Isd11/His_6-IscU (bottom panel) in the absence (*solid black line*) or 1 min and 5 min after addition of 4 mM L-cysteine (*red lines*). All spectra were recorded at 23°C in 500 μl 100 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA. **(C)** Growth curves of 100 ml cultures of the *Escherichia coli* CL100 strain (Δ*iscS*) transformed either with vectors pET15b and pACYCDuet-1 or with plasmids containing coding sequences for *Drosophila* Nfs1$^{\Delta 1-53}$, Isd11, IscU, or *Escherichia coli* IscS were recorded after addition of 100 μM IPTG for 12h at 30°C. **(D)** Desulfurase activity of Nfs1$^{\Delta 1-53}$/Isd11 and Nfs1$^{\Delta 1-53}$/Isd11/IscU complexes in absence or presence of human FXN. L-cysteine desulfurase activity was measured by determination of total sulfide produced. One unit is defined as the amount of enzyme producing 1 μmol of sulfide/min. Under anaerobic conditions, Nfs1$^{\Delta 1-53}$/Isd11 and Nfs1$^{\Delta 1-53}$/Isd11/IscU complexes were mixed in a 1:3 [Nfs1]:[FXN] ratio and incubated for 10 min at 23°C. The last lane (hFXN +) is a negative control to show that the human FXN preparation does not carry contaminant desulfurase activity. Error bars indicate the standard deviation of three measurements.

To further test this notion, we tested whether IscU altered the desulfurase activity of purified Nfs1 *in vitro* (**Figure 3D**). Sulfide production activity was determined with methylene blue in the presence of 1,4 dithiothreitol (Fogo and Popowsky, 1949; Urbina et al., 2001). Only weak activity was detected from the purified Nfs1$^{\Delta 1-53}$/Isd11 complex, possibly because the complex was unable to perform reaction turnovers. Addition of IscU, forming the IscU/Nfs1$^{\Delta 1-53}$/Isd11 complex, abolished sulfide production, consistent with observations presented above. Addition of human frataxin to the ternary IscU/Nfs1$^{\Delta 1-53}$/Isd11 complex led to a six-fold increase in sulfide production activity, showing that frataxin stimulated the desulfurase activity of Nfs1/Isd11 in an IscU-dependent manner (**Figure 3D**). Steady-state kinetic parameters were determined by varying L-cysteine concentration for Nfs1$^{\Delta 1-53}$ activity (after Nfs1$^{\Delta 1-53}$/Isd11/IscU/frataxin complex formation) showing a catalytic efficiency of 149 M^{-1} s^{-1}, k_{cat} 2.6 min^{-1}, and a K_M of 0.290 mM. Taken together, these data indicate that Isd11, IscU, and frataxin are required for the activation of Nfs1 in *Drosophila*, providing experimental evidence that the initial complex of the mitochondrial Fe-S cluster biosynthetic machinery is conserved in *Drosophila* similar to what is known in other eukaryotes, including species with non-respiring mitochondria (Tovar et al., 2003; Richards and van der Giezen, 2006).

Fe-S Cluster Biosynthesis Occurs in Distinct Cellular Compartments

Like the other proteins of the core complex described above, *Drosophila* Isd11 is localized in mitochondria (**Figure 4A**). Two mitochondrial ferredoxins (Palandri et al., 2015) and the *Drosophila* homolog of ferredoxin reductase (also known as adrenodoxin reductase encoded by the *dare* gene; Freeman et al., 1999) are required as electron donors for Fe-S cluster formation (Cai et al., 2017). Mitochondrial monothiol glutaredoxin-5 (CG14407) has not been investigated in *Drosophila*, but is likely required for Fe-S cluster biosynthesis (Rodríguez-Manzaneque et al., 2002; Wingert et al., 2005; Ye et al., 2010; Johansson et al., 2011), participating in the Fe-S cluster transfer to BolA-like proteins (Aldea et al., 1988; Frey et al., 2016; Melber et al., 2016; Uzarska et al., 2016; Nasta et al., 2017). Fe-S clusters are also transferred to Nfu-like (Tong et al., 2003; Melber et al., 2016; Wachnowsky et al., 2016) or Isa-like proteins (Jensen and

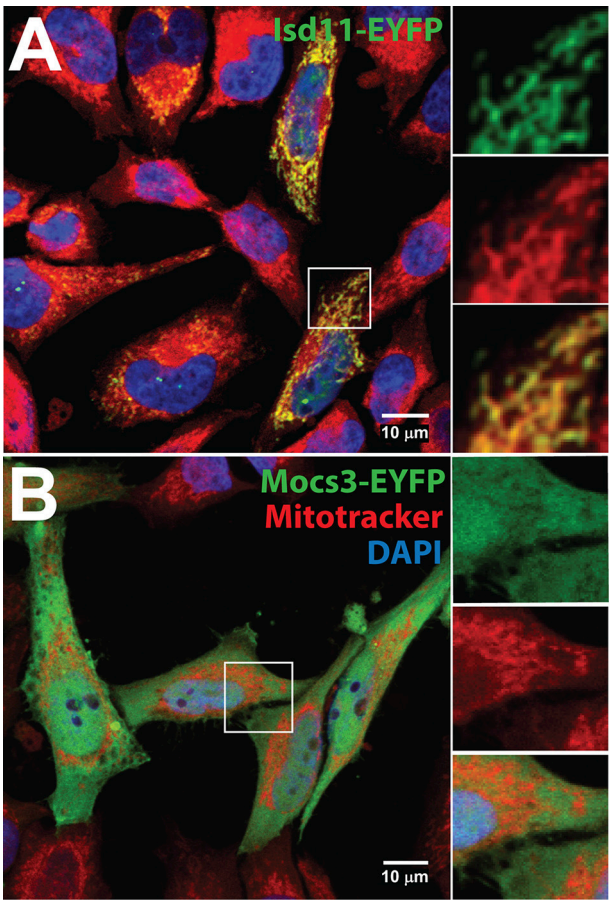


FIGURE 4 | Subcellular localization of Isd11 and Mocs3. Confocal microscopy of **(A)** Hela cells transfected with a C-terminal fusion of EYFP to Isd11, stained with Mitotracker Red to detect mitochondria and with DAPI to visualize nuclei. Insets show two channels separately to appreciate co-localization of the fluorescent signals from Isd11-EYFP and Mitotracker (merged, bottom inset); **(B)** Hela cells transfected with Mocs3-EYFP, which localizes in the cytosol and nuclei.

Culotta, 2000; Kaut et al., 2000; Muhlenhoff et al., 2011; Sheftel et al., 2012) with the chaperone activity of mitochondrial Hsc20 (Uhrigshardt et al., 2010; Sieber et al., 2016) and its cognate Hsc70 [not clear which of several candidate *Hsc70* genes present in the fly genome (Adams et al., 2000) functions in Fe-S cluster biosynthesis]. These scaffold proteins deliver the Fe-S clusters to target mitochondrial Fe-S enzymes.

Fe-S clusters are also assembled on proteins in the cytosol (**Figure 1**). Early work in this area suggested that core mitochondrial Fe-S cluster assembly proteins were also being directed to the cytosol (Land and Rouault, 1998; Tong and Rouault, 2000, 2006) and protein targeting sequences do not typically result in a unique destination for most proteins (Hegde and Bernstein, 2006). Furthermore, another set of cytosolic Fe-S cluster assembly (CIA) proteins has been described (reviewed in Roy et al., 2003; Hausmann et al., 2005; Paul and Lill, 2015). Briefly, an Fe-S cluster is assembled on CFD1 and NBP35 with electron donors provided by CIAPIN1. The clusters are transferred through IOP1 to a scaffold with CIA1, CIA2, and target proteins. These CIA proteins are conserved in *Drosophila* (**Figure 1**), but little work exists in the context of Fe-S cluster assembly. The fly Ciao1 homolog was shown to be required for viability (Radford et al., 2005) prior to assigning its biochemical function as part of the Fe-S scaffold complex in CIA (Balk et al., 2005a). Ciao1 received its name from the word "bridge" in the Chinese language (Johnstone et al., 1998), whereas the yeast homolog was conveniently designated Cia1 when its interaction with the hydrogenase-like Nar1 (IOP1 in the human nomenclature; Huang et al., 2007) was discovered (Balk et al., 2005b). The fly CIA2 scaffold (Zhao et al., 2015; Vo et al., 2017) homologs Galla-1 and Galla-2 (Yeom et al., 2015) associate with target nuclear Fe-S proteins Mms19 (Gari et al., 2012; Papatriantafyllou, 2012; Stehling et al., 2012; Nag et al., 2018) and Xeroderma pigmentosum D (Xpd) (Rudolf et al., 2006). The *Drosophila* CIAPIN1 homolog is required for oogenesis (Marzuk et al., 2013). CIAPIN1 carries an Fe-S cluster (Zhang et al., 2008) and receives electrons from a cytosolic reductase (Netz et al., 2010). Neither the reductase nor cytosolic monothiol glutaredoxins (Li et al., 2009; Mühlenhoff et al., 2010; Banci et al., 2015) have been characterized in *Drosophila*. Furthermore, it is important to stress that no experimental data exist to implicate the *Drosophila* proteins Cfd1, Ciao1, CIAPIN1, Galla-1, Galla-2, Iop1 Mms19, Nbp35 in the assembly of cytosolic Fe-S clusters; there inclusion here and elsewhere (Mandilaras and Missirlis, 2012) as members of the CIA was exclusively based on gene homology searches. Nevertheless, many projects are being undertaken in other systems to better describe the process of Fe-S cluster assembly in cytosol and nucleus and we hope to see contributions from studies in *Drosophila* in the near future.

Finally, it is noteworthy that no Fe-S enzymes have been reported in the secretory pathway, implying that the presence of thiol-reducing activity in the cytosol, nucleus and mitochondrial matrix is essential for their stability. In insects, such activity rests on the thioredoxin reductase system (Kanzok et al., 2001; Missirlis et al., 2001, 2002).

Excess Copper Inhibits Fe-S Cluster Biosynthesis

Copper and iron homeostasis are intimately linked (Fox, 2003). Physiologically, the two metals are required for aerobic respiration, albeit as separate cofactors (Villee, 1948). Poulson and Bowen made an early observation that when *Drosophila* larvae were fed a diet rich in copper their iron stores were being depleted (Poulson and Bowen, 1952). A key way in which the two metals depend on each other was uncovered when ceruloplasmin and other multicopper oxidases (MCOs) were shown to act as ferroxidases (Osaki et al., 1966) and their activity was linked to iron trafficking across membranes (reviewed in Kosman, 2010). *Drosophila* MCOs are linked to iron homeostasis in ways that are still not understood (Bettedi et al., 2011; Lang et al., 2012; Peng et al., 2015), but *MCO3* mutants fed on copper also show a reduction in ferritin iron content (Bettedi et al., 2011). It was therefore important to identify another hypothesis to explain copper-mediated cellular iron deficiency. The discovery

that excess copper displaces the Fe-S cluster of mitochondrial ferredoxin (Vallières et al., 2017), leading to disrupted CIA (Alhebshi et al., 2012), suggests that copper toxicity may directly inhibit Fe-S cluster biosynthesis also in *Drosophila*. Reducing Fe-S cluster biosynthesis in *Drosophila* with either RNA interference (RNAi) of *frataxin* or loss-of-function *Hsc20* mutants lead to mitochondrial iron accumulation and reduced ferritin expression (Anderson et al., 2005; Uhrigshardt et al., 2013; Navarro et al., 2015), offering a testable potential explanation of why excess dietary copper affects ferritin iron accumulation. In this respect, we also note that copper chelation ameliorated a fly model of Friedreich's ataxia (Soriano et al., 2016) and that the dithiol *Drosophila* glutaredoxin-1 was implicated in copper homeostasis (Mercer and Burke, 2016).

A Mitochondrial Iron Sulfur Enzyme Initiates Moco Biosynthesis

Turning to Moco biosynthesis, an ancient, ubiquitous and highly conserved pathway underpinning molybdenum biochemistry (Rajagopalan, 1997; Mendel and Leimkühler, 2015; Leimkühler, 2017), it can be divided into three major steps: (i) GTP is converted to cPMP, (ii) cPMP is converted to MPT by generation of the dithiolene group, and (iii) molybdate is then ligated to MPT forming Moco (**Figure 1**). Three fly genes involved in Moco biosynthesis have attracted individual attention: (i) *Mocs1* (formerly known as *low xanthine dehydrogenase*, *lxd*) encoding a mitochondrial Fe-S enzyme that converts 5′-guanosine triphosphate (GTP) to cyclic pyranopterin monophosphate (cPMP) (Keller and Glassman, 1964; Courtright, 1975; Duke et al., 1975; Bogaart and Bernini, 1981; Schott et al., 1986; Ho et al., 1992; Tahoe et al., 2002), (ii) *cinnamon* (*cin*) encoding a gephyrin homolog that inserts molybdate into MPT (Baker, 1973; Browder and Williamson, 1976; Kamdar et al., 1994; Wittle et al., 1999), and (iii) *maroon-like (mal)* that encodes a Moco sulfurase (Mitchell and Glassman, 1959; Hubby and Forrest, 1960; Finnerty et al., 1970; Marsh and Wieschaus, 1977; Kamleh et al., 2009).

Moco biosynthesis starts within mitochondria with a complex rearrangement reaction in which the C8 atom of the GTP purine is inserted between the 2′ and 3′-ribose carbon atoms (Wuebbens and Rajagopalan, 1993; Hover and Yokoyama, 2015). The human *MOCS1* gene is orthologous to the *lxd* locus (now renamed *Mocs1*) of *D. melanogaster* (Gray and Nicholls, 2000). Mutations in *lxd* affect molybdoenzyme activity in flies (Keller and Glassman, 1964). Alternative splicing at the *Mocs1* locus results in short (Mocs1A) and full length (Mocs1A-Mocs1B) proteins (**Figure 5A**). Mocs1A belongs to the superfamily of SAM-dependent radical enzymes (Hänzelmann and Schindelin, 2004), requiring a [4Fe-4S] cluster for the formation of a substrate radical by reductive cleavage of SAM. Studies on the human and bacterial homologs showed that Mocs1B participates in pyrophosphate cleavage after the formation of the 3′, 8cH$_2$GTP

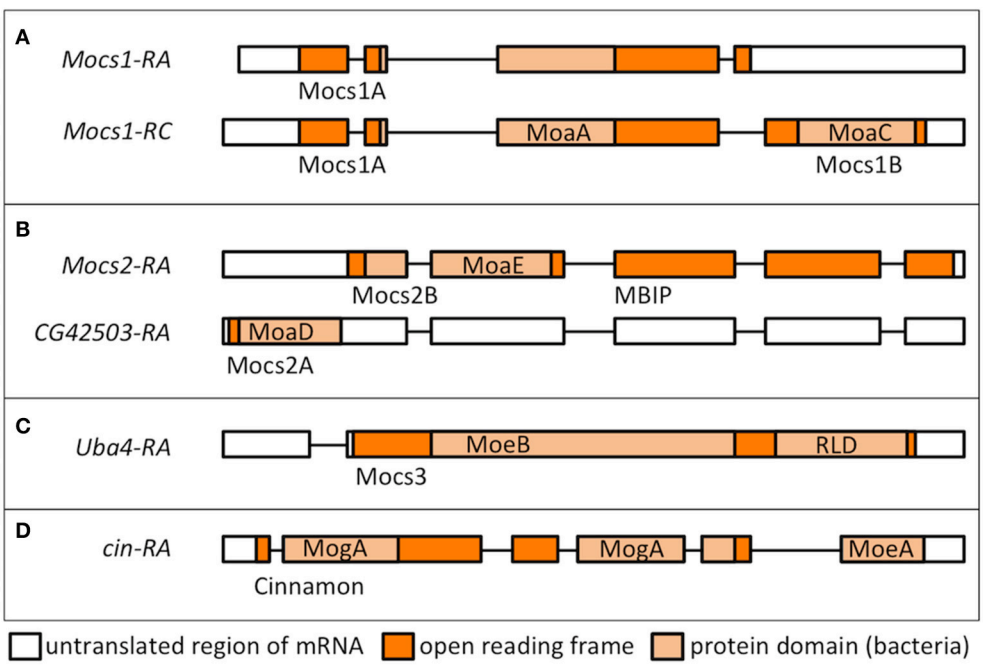


FIGURE 5 | Transcriptional units and proteins encoded by *Drosophila* genes involved in Moco biosynthesis. Transcript names are shown as per Flybase. Protein domains in light orange boxes are according to the nomenclature in bacteria (Leimkühler, 2017). Names of the *Drosophila* proteins as used in text are shown below each transcript. **(A)** Alternative splicing of *Mocs1* results in short (Mocs1A) and long (Mocs1A-Mocs1B) subunits, which complex together to form the active enzyme. **(B)** A bicistronic transcript results in Mocs2B (which also carries the unrelated to Moco biosynthesis MBIP domain, see text) and Mocs2A. **(C)** Mocs3 carries the MoeB-like domain and a rhodanese-like domain (RLD), shown to interact with Nfs1 (Marelja et al., 2013). **(D)** Cinnamon carries the MogA/Gephyrin G domain at the N-terminus prior to the C-terminal MoeA/Gephyrin E domain (in reverse order to the mammalian Gephyrins).

intermediate (Hänzelmann and Schindelin, 2006). Evidence was sought for Mocs1 having a role in lifespan determination of *Drosophila*, as polymorphisms in the gene were detected between short- and long-lived inbred lines, but the results were inconclusive as the polymorphisms could not be associated with clear effects on enzyme activity (Tahoe et al., 2002). Mutants in *Mocs1* showed differential sensitivity to dietary molybdate compared to wild type strains (Duke et al., 1975).

Molybdenum Cofactor Biosynthesis in the Cytosol

In the second step, two sulfur atoms are transferred to cPMP to form MPT. This reaction is catalyzed by the heterotetrameric MPT synthase, which is composed of two small Mocs2A and two large Mocs2B subunits encoded from a single locus (Stallmeyer et al., 1999; Leimkuhler et al., 2003; **Figure 5B**). The physiological sulfur donor for MPT synthesis is Mocs3 (Matthies et al., 2004, 2005), which resides in the cytosol (**Figure 4B**). *Drosophila* Mocs2 and Mocs3 are both required for Aox activity (**Figure 6**). Based on what is known in human cells (Marelja et al., 2008), Mocs3 is expected to receive sulfur from Nfs1, the same protein that acts as a sulfur donor for Fe-S cluster biosynthesis in mitochondria, to which it binds through a rhodanese-like domain (RLD; **Figure 5C**). Using Förster resonance energy transfer and a split-EGFP system, NFS1 was shown to interact in the cytosol of human cells with MOCS3 (Marelja et al., 2013). This result was corroborated by showing that human NFS1/ISD11 requires MOCS3 to complement Moco biosynthesis in the *E. coli* deletion strain used in **Figure 3C** (Bühning et al., 2017).

Exceptionally, the *Drosophila* MPT synthase is linked to c-Jun N-terminal kinase (JNK) signaling, since Mocs2B forms a fusion protein with the mitogen activated protein kinase upstream binding inhibitory protein (MBIP; **Figure 5B**), which is one of 13 subunits of the Ada Two A containing histone acetyltransferase complex (ATAC) transcriptional co-activator (Suganuma et al., 2010). In human cells, Mocs2B/MBIP were shown to regulate ferritin translation through inhibition of PKR, a double-stranded RNA-dependent protein kinase (Suganuma et al., 2012, 2016). Whether this form of regulating iron storage is conserved in *Drosophila*, whether it serves to regulate the availability of Fe-S clusters for Mocs1, and whether *Drosophila* Nfs1 has a cytosolic function are questions for future research.

In the last step, molybdate is ligated to the dithiolene group of MPT producing Moco (**Figure 1**). The first results for the enzyme inserting molybdate to the pterin structure came from the Xdh deficiency of the *cin* mutation in *Drosophila* (Baker, 1973). Cin is partially homologous to the two *E. coli* proteins MogA and MoeA, which are also found as domains G and E of the rat protein Gephyrin, albeit in reverse orientation to the *Drosophila* protein (Kamdar et al., 1994; Stallmeyer et al., 1995; Feng et al., 1998; **Figure 5D**). The MogA-like G domain binds MPT and catalyzes the MPT-adenylation from Mg-ATP; MPT-AMP is then transferred to the MoeA-like E domain for hydrolysis and molybdenum insertion (Kuper et al., 2000, 2004; Schwarz et al., 2000; Llamas et al., 2004, 2006). Furthermore, the central domain in Gephyrin binds and anchors inhibitory

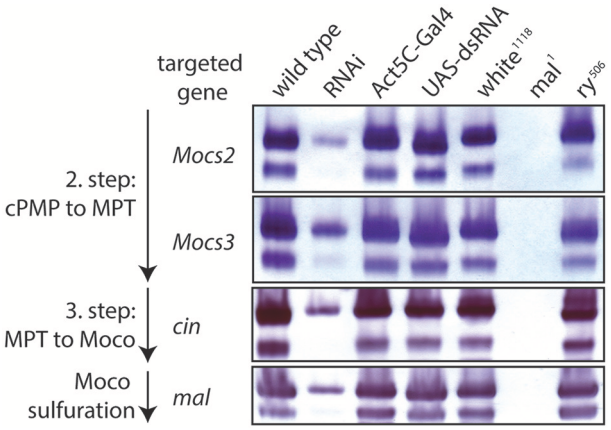


FIGURE 6 | RNAi of Moco biosynthesis genes disrupts Aox activity. In-gel Aox activity is shown as described before (Marelja et al., 2014). Extracts from wild type flies are shown in column 1 of the gels, followed by extracts from RNAi of, respectively, *Mocs2*, *Mocs3*, *cinnamon,* and *maroon-like* in column 2 (note reduced Aox activity, consistent with a requirement of these genes for Moco biosynthesis). Columns 3 and 4 are extracts from the parental flies of the Gal4/UAS system that show normal Aox activity (controls), as do extracts from *white* and *rosy* mutants in columns 5 and 7 (positive controls), whereas extracts from the *mal* mutant show no detectable Aox activity (negative control, column 6).

ligand-gated anion channels in the postsynaptic membrane of neurons (Feng et al., 1998; Stallmeyer et al., 1999). However, it is unknown whether this additional function is conserved for *Drosophila* Cin.

After molybdenum insertion into MPT, Moco is either inserted into Suox or further modified by exchanging an oxo ligand by a sulfido group (Hille, 1996, 2002; Hille et al., 2014). The sulfur incorporation was also first discovered in the fly *mal* mutant, which lacked Xdh and Aox activities (Hadorn and Mitchell, 1951; Glassman and Mitchell, 1959; Hubby and Forrest, 1960; Forrest et al., 1961; Courtright, 1967) but retained or induced Suox activity (**Figure 7**) and an apparently normal concentration of total Moco (Bogaart and Bernini, 1981; Warner and Finnerty, 1981; Wahl et al., 1982). *In vitro* reconstitution of Xdh and Aox activities with sulfide/dithionite treatment led to the suggestion that *mal* affected the sulfur modification after the Mo insertion (Wahl and Rajagopalan, 1982; Wahl et al., 1982). A sequence comparison with the L-cysteine desulfurase gene *nifS* from *Azotobacter vinelandii* suggested that the *mal* gene encodes the enzyme that catalyzes sulfuration of Moco used by Xdh and Aox (Amrani et al., 2000; Ichida et al., 2001). Oxo-containing Moco does not function in Xdh and Aox, but is the cofactor for Suox (**Figure 1**).

Fe-S AND MOCO ENZYMES

The fly offers an ideal system for interdisciplinary research to bridge findings from genetic manipulations and resulting phenotypes with detailed biochemical studies to build functional understanding for animal cell physiology, keeping in mind the diversity of cell types and subcellular microenvironments.

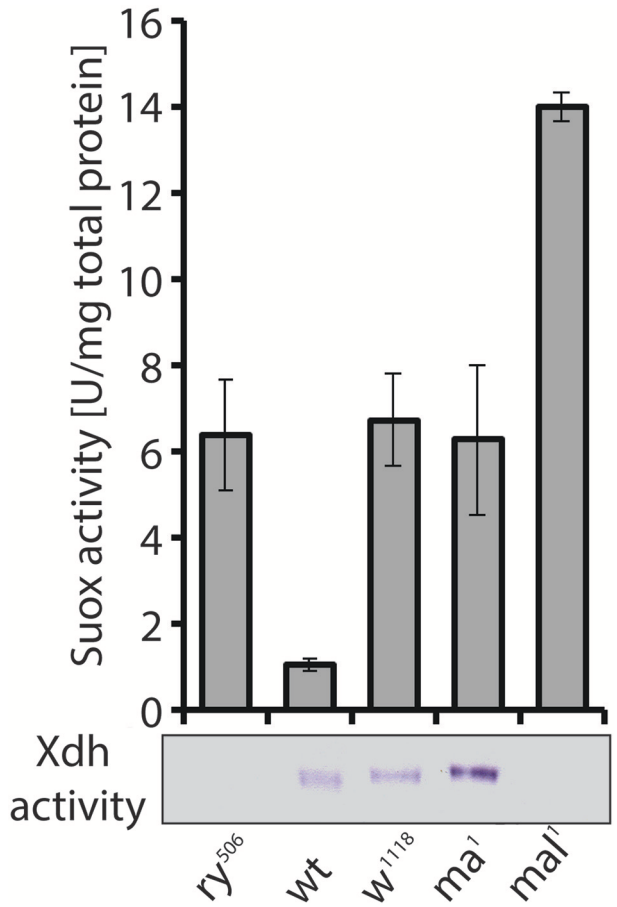


FIGURE 7 | Despite similar eye pigment phenotypes with *rosy* and *mal*, the *maroon* mutant shows increased Xdh activity. Suox activity measured in extracts from mutants (*ry, rosy; w, white; ma, maroon; mal, maroon-like*) and a wild type (*wt*) strain is shown in bars. In-gel Xdh activity of the same extracts is also presented. Loss of Xdh activity in *rosy* and *mal* explains the phenotypic changes in eye pigmentation, but it was surprising to find increased Xdh activity in *maroon* mutants, given their eye phenotype.

In this section, we review the enzymes that use Moco and/or Fe-S clusters. A representative list of these enzymes in *D. melanogaster* is provided (**Table 1**). We emphasize the physiological functions and biochemical pathways, as opposed to the detailed biochemistry of the cofactors at the active sites, which only in few cases has been the primary interest of *Drosophila* researchers.

The Molybdoenzymes: Xdh, Aox, Suox

In *D. melanogaster*, the *rosy* gene encodes Xdh (Chovnick et al., 1976; Keith et al., 1987). *Rosy* mutants accumulate xanthine and hypoxanthine and are devoid of urate (Hadorn and Schwinck, 1956; Morita, 1958; Glassman and Mitchell, 1959) and show a dull reddish-brown eye color because of the lowered concentrations of the red eye pigment drosopterin (bright red), and the increased chromogenic oxidation of the eye pigment dihydroxanthommatin (yellow brown) to xanthommatin (dull dark brown), either due to enzymatic activity (Phillips and Forrest, 1980; Wiley and Forrest, 1981; Ferre et al., 1986) or due to the decreased urate concentration (Hilliker et al., 1992). The use of *rosy* mutant strains to probe the structure and function of Xdh provides an early, elegant example in the field of biological inorganic chemistry (Hughes, 1992; Hughes et al., 1992; Doyle et al., 1996). Allelic series of mutants affecting residues coordinating the Fe-S clusters, the Moco, the flavin cofactor or the binding sites for NAD^+/NADH were used to decipher the complex mechanism of action for this prototypical molybdoenzyme. More recently, metabolic profiling of *rosy* mutants revealed additional changes in the tryptophan, arginine, pyrimidine, and glycerophospholipid metabolic pathways (Kamleh et al., 2008, 2009).

Two genes, *low pyridoxal oxidase* (*lpo*) and *aldehyde oxidase* (*Aldox*), were initially recognized to encode Aoxs in *D. melanogaster* (Courtright, 1967; Collins and Glassman, 1969; Dickinson, 1970; Browder and Williamson, 1976; Dickinson and Gaughan, 1981; Cypher et al., 1982; Nelson and Szauter, 1992). Genome analysis revealed, however, a cluster of four Aox genes (Garattini et al., 2008). We showed previously that (i) the *lpo* gene encodes Aox1, (ii) Aox2 activity is only present during metamorphosis, (iii) the activities associated with the classic *Aldox* locus correspond to two splice forms of the *Aox3* gene, and (iv) no major activity has been found associated with Aox4, the newest addition to a highly evolving protein family in Drosophilidae (Marelja et al., 2014). Aoxs show specific substrate specificities, but their *in vivo* substrates and physiological functions remain unclear (Dickinson and Gaughan, 1981; Cypher et al., 1982; Marelja et al., 2014).

Suox contains a cytochrome b5 (heme-containing) domain followed by 12–15 amino acids connecting it to the Moco domain. Suox catalyzes an oxygen atom transfer reaction to sulfite leading to its oxidation (and detoxification) to sulfate (Feng et al., 2007; Hille et al., 2011), an activity previously detected in *Drosophila* (Bogaart and Bernini, 1981; Braaten and Bentley, 1993). Based on sequence homology, we find that *CG7280* encodes for the *Drosophila* Suox.

A novel molybdoenzyme, mitochondrial amidoxime reducing component (mArc), has been described and has an identifiable *Drosophila* homolog, CG1665. The enzyme from humans has been proposed to reduce a broad range of N-hydroxylated compounds receiving electrons from cytochrome b5 (Gruenewald et al., 2008) and to reduce nitrite to nitric oxide (Sparacino-Watkins et al., 2014). A number of controversies over the function, subcellular localization and whether the newly discovered enzyme binds to sulfurated or oxo Moco, have been reviewed (Llamas et al., 2017).

Another relatively unexplored area in *Drosophila* molybdenum biology relates to the trafficking and homeostatic mechanisms for handling dietary molybdate. In wild type flies, Xdh and Aox activities are relatively stable, not responding to concentrations between 1 and 10 mM molybdate (Duke et al., 1975), although more recent studies documented a number of, so far unexplained, sex-specific physiological responses in carbohydrate and thiol metabolism at the same concentrations (Rovenko et al., 2014; Perkhulyn et al., 2017). Interestingly, 50 mM molybdate was tolerated by wild type flies, but was

TABLE 1 | List of known molybdoenzymes, iron sulfur enzymes and scaffold proteins.

Protein	Metal Cofactors	Cellular Compartment	Function	Key References
MOLYBDO-ENZYMES				
Aox 1-4 (aldehyde oxidases)	Moco 2 × [2Fe-2S]	Cytosol	Hydroxylation of xenobiotics	Dickinson and Gaughan, 1981; Marelja et al., 2014
mArc (mitochondrial amidoxime reducing component; CG1665)	Moco	Unknown	Unknown	Llamas et al., 2017
Rosy (xanthine dehydrogenase)	Moco 2 × [2Fe-2S]	Cytosol Pigment granule	Purine degradation Eye pigment formation	Keith et al., 1987; Reaume et al., 1991; Hilliker et al., 1992; Hughes et al., 1992; Kamleh et al., 2008
Suox (sulfite oxidase; CG7280)	Moco Heme	Mitochondrial intermembranes	H_2S detoxification	Bogaart and Bernini, 1981; Braaten and Bentley, 1993
MITOCHONDRIAL IRON SULFUR ENZYMES AND SCAFFOLD PROTEINS				
Acon (mitochondrial aconitase)	[4Fe-4S]	Mitoch. matrix	Krebs cycle	Cheng et al., 2013; Esposito et al., 2013
BolA 1-2	[4Fe-4S]	Mitoch. matrix	2nd Fe-S assembly scaffold	Uzarska et al., 2016
Fdx 1-2 (ferredoxins)	[2Fe-2S]	Mitoch. matrix	Electron transfer	Palandri et al., 2015
Fech (ferrochelatase)	[2Fe-2S]	Inner membrane	Heme biosynthesis	Sellers et al., 1998
IscA 1-2 (magnetoreceptor?)	[4Fe-4S]	Mitoch. matrix	2nd Fe-S assembly scaffold	Mandilaras and Missirlis, 2012; Qin et al., 2016
IscU	[2Fe-2S] or [4Fe-4S]	Mitoch. matrix	1st Fe-S assembly scaffold	Dzul et al., 2017
Las (lipoic acid synthase)	2 × [4Fe-4S]	Inner membrane	Lipoate cofactor biosynthesis	Harmer et al., 2014
Mocs1 (molybdenum cofactor synthesis 1)	[4Fe-4S]	Mitoch. matrix	Moco biosynthesis	Duke et al., 1975; Gray and Nicholls, 2000
mtDNA-helicase	[2Fe-2S]	Mitoch. matrix	Mitoch. DNA replication	Stiban et al., 2014
ND-24 (NADH dehydrogenase) **ND-51** **ND-75** **ND-23** **ND-20**	[2Fe-2S] [4Fe-4S] [2Fe-2S] & 2 x [4Fe-4S] 2 x [4Fe-4S] [4Fe-4S]	Facing matrix Inner membrane	Oxidative phosphorylation Respiratory complex I	Zhang K. et al., 2013; Fiedorczuk et al., 2016; Garcia et al., 2017
NFU 1-3	[4Fe-4S]	Mitoch. matrix	2nd Fe-S assembly scaffold	Tong et al., 2003
Nvd (cholesterol 7 desaturase)	[2Fe-2S]	Mitoch. matrix	Steroid biosynthesis	Yoshiyama et al., 2006
RFeSP (Rieske iron-sulfur protein)	[2Fe-2S]	Inner membrane	Respiratory complex III	Gontijo et al., 2011
SdhB (succinate dehydrogenase)	[4Fe-4S]	Inner membrane	Respiratory complex II Krebs cycle	Au and Scheffler, 1994; Kohler et al., 1995; Gray et al., 1996; Walker et al., 2006; Na et al., 2014; Van Vranken et al., 2014
CYTOSOLIC IRON SULFUR ENZYMES				
Cisd2 (CG1458) mitoNEET	[2Fe-2S]	Outer membrane	Fe-S cluster repair	Jones et al., 2014
Elp3 (lysine acetyltransferase) Elongator complex protein 3	[4Fe-4S]	Cytosol Nucleus	Acetylates synapse active zone RNA transcript elongation	Miśkiewicz et al., 2011
IRP 1A-1B (cytosolic aconitases/ Iron regulatory proteins)	[4Fe-4S]	Cytosol	Cellular iron homeostasis Intermediary metabolism	Muckenthaler et al., 1998; Lind et al., 2006; Surdej et al., 2008
Pixie (RNAase L inhibitor, ABCE1)	2 × [2Fe-2S]	Cytosol	Ribosome biogenesis Translation initiation	Andersen and Leevers, 2007; Kashima et al., 2014
Prat 1-2	[4Fe-4S]	Cytosol	Purine nucleotide biosynthesis	Clark, 1994; Merzetti et al., 2013
Su(r) (CG2194) dihydropyrimidine dehydrogenase	[4Fe-4S]	Cytosol	Pyrimidine degradation	Van Gelder et al., 1995; Rawls, 2006
NUCLEAR IRON SULFUR ENZYMES				
Chl1 (CG11403) DNA helicase	[4Fe-4S]	Nucleus	Heterochromatin organization	–
DNApol-α180 (DNA polymerase) **DNApol-α60** (DNA primase) **DNApol-δ** (DNA polymerase) **DNApol-ε255** (DNA polymerase) **DNA2** (CG2990) DNA helicase	[4Fe-4S] [4Fe-4S] [4Fe-4S] [4Fe-4S] [4Fe-4S]	Nucleus	Nuclear DNA replication	Kaguni et al., 1983; Peck et al., 1992; Sahashi et al., 2014
Nthl1 (CG9272) DNA glycosylase	[4Fe-4S]	Nucleus	Base excision repair	–
Xpd (xeroderma pigmentosum D)	[4Fe-4S]	Nucleus	Subunit of basal transcription and DNA repair factor TFIIH	Reynaud et al., 1999; Chen et al., 2003; Aguilar-Fuentes et al., 2006; Li et al., 2010

lethal to *Mocs1* mutant flies, implicating the Moco biosynthetic pathway as part of the detoxification mechanisms available to the fly (Duke et al., 1975).

The Curious Case of the *Maroon* Mutant

A second unusual aspect of Xdh, besides its implication in multiple metabolic pathways (see above Kamleh et al., 2008, 2009), relates to the enzyme's trafficking to the eye imaginal disk, where it accumulates in pigment granules (Reaume et al., 1989, 1991). Due to the similar eye color of the classic *maroon* mutant with that of the *rosy* and *mal* mutants (Bridges, 1918), we assayed *maroon* fly extracts for Xdh and Suox activity, expecting to find decreased Xdh activity consistent with the eye phenotype. To our surprise, increased activities of both enzymes compared to wild type flies were observed in *maroon* extracts (**Figure 7**). As Suox utilizes unmodified Moco, the increase in its activity was not entirely unexpected, because this form of Moco might accumulate as in *rosy* and *mal*. Accounting for increased Xdh activity was harder, however, given the similar eye color between *maroon*, *rosy,* and *mal*. The demonstration that the *maroon* gene encodes for Vacuolar protein sorting 16A (Vps16A; Grant et al., 2016), a protein implicated in granule formation (Pulipparacharuvil et al., 2005; Lorincz et al., 2016), suggests a possible defect in the tissue localization of Xdh in *maroon* mutants, as the enzyme obviously remains functional in whole fly extracts and is even induced (**Figure 7**). It will be informative to test in *maroon* mutants whether the Xdh activity is localized in peripheral tissues, such as the Malpighian tubules, and not in the eyes. If this prediction is correct, then *Vps16A* may represent the first known mutant that blocks the delivery of a vesicular structure to the eye. Another unresolved piece of this puzzle relates to the way in which Xdh, a cytosolic enzyme, is found in pigment granules in the eye (Reaume et al., 1989, 1991). The discovery of exosomes gives a possible answer to this conundrum (Hemler, 2003; Gross et al., 2012; Gradilla et al., 2014; Takeuchi et al., 2015; Beer and Wehman, 2017; Shibata et al., 2017; Tassetto et al., 2017). Clearly more experiments are required to explain how Xdh acts in the formation of eye color in flies, but complex non-cell autonomous processes relating to enzyme maturation, regulation, and transport are involved.

Mitochondrial Fe-S Proteins at the Heart of Mitochondrial Bioenergetics

Mitochondria form an important organelle of eukaryotic cells, typically containing their proper genome (Lane and Martin, 2010; Schatz, 2013; Allen, 2015) and performing various functions (Pagliarini and Rutter, 2013; Chandel, 2015; Munro and Treberg, 2017), of which the tricarboxylic acid (TCA) cycle and oxidative phosphorylation are famous (Vakifahmetoglu-Norberg et al., 2017). The idea that iron plays a part in the oxidation reactions of the living cell was firmly established by Warburg (1925). Beinert and Sands interpreted electron paramagnetic resonance spectra of succinate dehydrogenase (Sdh) as "non heme iron" (Beinert and Sands, 1960; Beinert, 2002). Later, Sdh and aconitase (both TCA cycle enzymes) were shown to carry [4Fe-4S] clusters (Ruzicka and Beinert, 1978; Cammack, 1982). The *Drosophila* enzymes are no exception to the rule (Duke, 1968; Fox et al., 1972; Au and Scheffler, 1994; Vincent et al., 2012; Esposito et al., 2013). Failure to build these Fe-S clusters will inevitably block the TCA cycle, and thereby development and growth (Au and Scheffler, 1994; Yan et al., 1997; Walker et al., 2006; Uhrigshardt et al., 2013; Na et al., 2014). Sdh is also known as Complex II of the respiratory chain that generates the inner mitochondrial membrane potential and proton gradient used by the F-ATPase for the production of ATP during oxidative phosphorylation (Alziari et al., 1985; Sardiello et al., 2005; Liu et al., 2011; Barry and Thummel, 2016). Complex I of the respiratory chain, otherwise known as NADH dehydrogenase, carries eight precisely spaced Fe-S clusters of different reduction-oxidation potentials assembled on five subunits of the complex (**Table 1**; Zhang K. et al., 2013; Fiedorczuk et al., 2016; Garcia et al., 2017). It is thus plain that without Fe-S clusters ATP cannot be produced in the mitochondrial process of aerobic respiration (Anderson et al., 2005; Llorens et al., 2007; Navarro et al., 2011; Edenharter et al., 2017).

Fe-S Enzymes Are Required for Heme, Ecdysone, and Lipoate Biosynthesis

Ferrochelatase is an enzyme that resides in the inner mitochondrial membrane accepting iron from the mitochondrial matrix and protoporphyrin IX from the intermembrane space to generate heme (Wu et al., 2001). Heme is another abundant iron-dependent protein cofactor (Ponka et al., 2017). Many ferrochelatases, including the *Drosophila* enzyme, carry a [2Fe-2S] cluster (Sellers et al., 1998). Therefore, both major forms of iron cofactors used in biology rest on the mitochondrial Fe-S cluster assembly machinery.

Furthermore, *Drosophila* ferredoxins carry a [2Fe-2S] cluster required for electron transfer during Fe-S cluster assembly, but also for the production of ecdysone in the larval prothoracic gland and other steroidogenic tissues (Uhrigshardt et al., 2013; Palandri et al., 2015). In this way, iron availability is linked to a key developmental signal that terminates growth and initiates metamorphosis (Yamanaka et al., 2013; Sandoval et al., 2014). Interestingly, glutathione production in the prothoracic gland is also required for steroidogenesis (Enya et al., 2017). The possibility that glutathione supports Fe-S cluster biosynthesis in this tissue should be considered (Song et al., 2006; Auchère et al., 2008; Qi et al., 2013; Ozer et al., 2015).

Lipoic acid or lipoate is a cofactor required in intermediary metabolism enzymes α-oxoglutarate dehydrogenase, pyruvate dehydrogenase, branched-chain oxoacid dehydrogenase, 2-oxoadipate dehydrogenase, and in the glycine cleavage system (Habarou et al., 2017). Biosynthesis of the lipoate cofactor is not well understood beyond prokaryotes (for comprehensive review see Cronan, 2016). Nevertheless, it is clear that lipoic acid synthase is required for the maturation of enzymes dependent on lipoate and uses two [4Fe-4S] clusters for its catalytic activity (Harmer et al., 2014). No study describing the *Drosophila* lipoic acid synthase has been published despite human disease related to lipoic acid deficiency (Mayr et al., 2014; Cronan, 2016; Habarou et al., 2017).

The Central Dogma of Molecular Biology Depends on Fe-S Enzymes

The central dogma of molecular biology, originally proposed by Crick (1958), radically changed the way biology is understood and taught (Cobb, 2017). DNA replication is the primary mode of information transfer during successive generations, whereas DNA transcription is the primary mechanism for specifying which proteins can be translated on ribosomes assembled in the cytoplasm. In addition to the nucleotide and amino acid building blocks, all three steps require energy and are interdependent, as nucleic acids and proteins are both essential for each process. Likewise, Fe-S clusters are also required at each step as cofactors of the DNA polymerase (see references in **Table 1** and Kaguni et al., 1983; Peck et al., 1992; Sahashi et al., 2014; Stiban et al., 2014), of the essential subunit of the basal transcription factor TFIIH Xpd (Reynaud et al., 1999; Chen et al., 2003; Aguilar-Fuentes et al., 2006; Li et al., 2010) and of Pixie, which is required for ribosome biosynthesis and the initiation of translation (Andersen and Leevers, 2007; Kashima et al., 2014). Thus, DNA replication, transcription, and translation rest on the CIA providing Fe-S cluster to DNA polymerase, Xpd, and Pixie, respectively.

Are Fe-S and/or Moco Enzymes Implicated in the Circadian Clock?

Plants alternate between photosynthesis and respiration during day-night cycles, whereas animal behavior shifts between an active stage that includes foraging, feeding and other motile behaviors, and sleep (Haydon et al., 2013; Mellor, 2016; Dubowy and Sehgal, 2017). Most animals anticipate the periodicity of sunlight and darkness through dedicated neuronal circuits whose rhythmic activity is sometimes referred to as the circadian clock (the genetic basis of which, was first discovered by Konopka and Benzer, 1971). In *Drosophila* the organization of the circadian circuitry has received considerable attention (Nitabach and Taghert, 2008; Hermann et al., 2013; Simoni et al., 2014). Similar to other animals, the circadian clock is interlinked with physiological functions in flies (Barber et al., 2016; Katewa et al., 2016; Rey et al., 2016; Kijak and Pyza, 2017; Klemz et al., 2017). Given the major role of Fe-S enzymes in intermediary metabolism and aerobic respiration, the question of whether Fe-S clusters are continuously present in key enzymes during the day-night cycle or whether some recycling of iron takes place in a rhythmic function has been posed (Mandilaras, 2012).

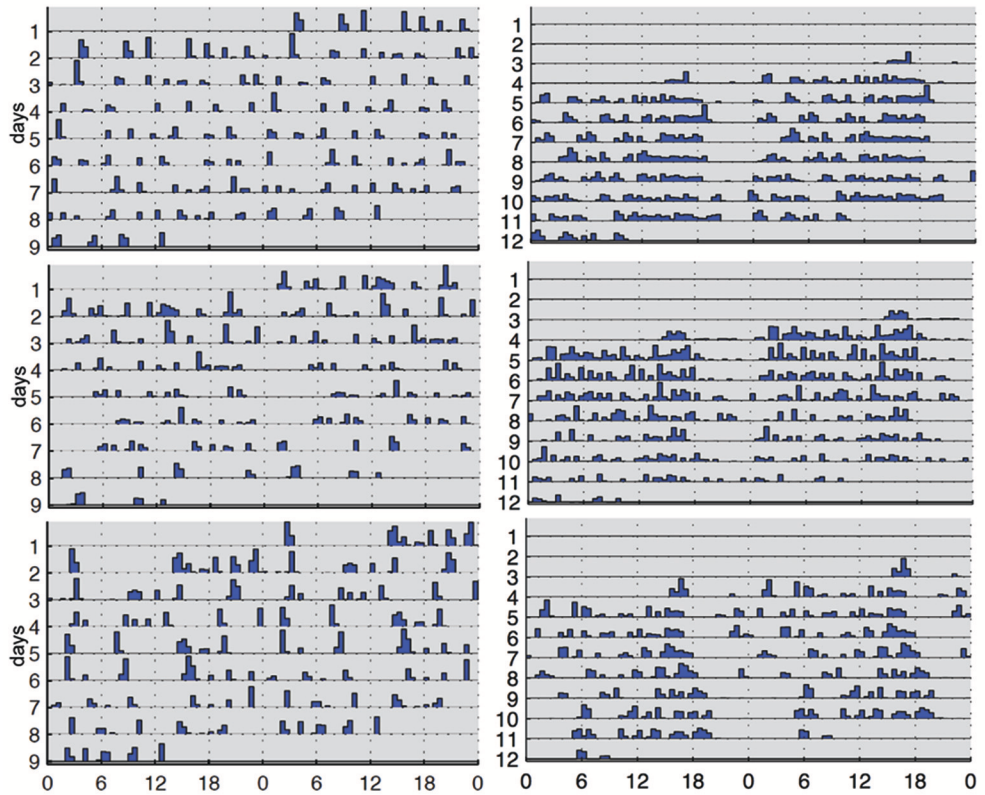
RNAi of *Nfs1* (the cysteine desulfurase required for the biosynthesis of Fe-S clusters and Moco; **Figure 1**) in the circadian clock neurons resulted in loss of rhythmic activity of flies monitored under constant darkness (Mandilaras and Missirlis, 2012). Ubiquitous RNAi of *Nfs1* caused lethality and eye-specific RNAi caused photoreceptor cell loss (Marelja, 2013). Two driver lines with overlapping, but not identical, expression patterns in the clock neurons, tim^{27}*-Gal4* and cry^{17b}*-Gal4* were recombined to the RNAi potentiator *UAS-Dicer2* (Dietzl et al., 2007) and used, showing that *Nfs1* RNAi driven by tim^{27}*-Gal4* resulted in a weaker arrhythmia than when driven by cry^{17b}*-Gal4* (Mandilaras and Missirlis, 2012). *IscU* RNAi driven by cry^{17b}*-Gal4* also resulted in arrhythmic flies, *IscU* RNAi driven by tim^{27}*-Gal4*, however, resulted in lethality (a more severe phenotype, but one that cannot be tested for rhythmicity). In contrast, *frataxin* RNAi with both drivers did not show an arrhythmic phenotype (Mandilaras and Missirlis, 2012). To probe these genetic results further, RNAi of *Isd11* and *Mocs3* using the same drivers and assay was undertaken. *Isd11* RNAi in circadian clock neurons showed no discernible phenotype, in contrast to *Mocs3* RNAi, which resulted in 56% arrhythmic flies when driven with tim^{27}*-Gal4* and 64% arrhythmic flies when driven with cry^{17b}*-Gal4* (**Figure 8**).

The other Fe-S cluster biosynthesis genes that were implicated in the *Drosophila* circadian clock were *Iba57* (*CG8043*) and *Cfd1* (*CG4858*; also referred to as *Nubp2*), both encoding components of the CIA machinery, and *IscA1*, which is an Fe-S scaffold protein predicted to be localized to mitochondria (Mandilaras and Missirlis, 2012). *IscA1* has recently found an exciting application in a new, non-invasive, technology to control experimentally the generation of neuronal action potentials, termed magnetogenetics (Long et al., 2015). It was also suggested that *IscA1* is a component of fly magnetoreception (Cyranoski, 2015; Qin et al., 2016), but see (Meister, 2016; Pang et al., 2017). The proposed cryptochrome-IscA1 protein complex is predicted to reside in the cytosol or nucleus, given that cryptochrome is a protein localized in the cytosol and nucleus (Stanewsky et al., 1998; Yoshii et al., 2008). Whether, in addition to their predominant presence in mitochondria, IscA1, IscU, and Nfs1 have cytosolic localization in flies, as recently shown for human HSC20 (Kim et al., 2018), requires experimental verification. It is an interesting possibility to keep in mind when considering the result that RNAi against *Mocs3*, whose product is the proposed cytosolic partner of Nfs1 (Marelja et al., 2008, 2013), resulted in arrhythmic flies (**Figure 8**). Of note, two Fe-S enzyme encoding genes, dihydropyrimidine dehydrogenase and phosphoribosylamidotransferase, show circadian expression and are localized in the cytosol (Van Gelder et al., 1995; Rey et al., in review).

The new results presented here and the findings in Mandilaras and Missirlis (2012) are only based on RNAi and require independent confirmation. That iron may play a role in the circadian clock and sleep disorders was also proposed based on a *Drosophila* model of the human Restless Legs Syndrome (Freeman et al., 2012, 2013). This line of research was unfortunately disrupted at Queen Mary University of London (Allen and Missirlis, 2012; Ashworth, 2012; Horton, 2012), but deserves further attention, given the interplay between iron metabolism and the circadian clock in humans (Earley et al., 2014; Furudate et al., 2014; Dye et al., 2016), pigs (Zhang et al., 2017), rodents (Yin et al., 2007; Bianco et al., 2009; Simcox et al., 2015; Janich et al., 2016; Okazaki et al., 2016), plants (Chen et al., 2013; Hong et al., 2013; Salomé et al., 2013), and even diatoms (Botebol et al., 2015).

Cellular Iron Sensing and Regulation Is Coupled to Fe-S Cluster Biosynthesis

D. melanogaster larvae or flies grown on diets with different iron content show cell-type specific responses (Poulson and Bowen, 1952; Georgieva et al., 1999; Mehta et al., 2009; Mandilaras et al., 2013). In mammals, IRPs regulate cytosolic



UAS-Dicer2; tim^{27}-Gal4				gene	*UAS-Dicer2; cry^{17b}-Gal4*			
N	**% rhythmic**	**period (h)**	**R. S.**	**RNAi**	**N**	**% rhythmic**	**period (h)**	**R. S.**
22	100	23.8 ± 0.4	2.8 ± 0.5	*Isd11*	11	100	23.8 ± 0.4	3.8 ± 0.5
45	44	23.3 ± 0.3	1.9 ± 0.2	*Mocs3*	19	36	22.8 ± 0.7	2.2 ± 0.2

FIGURE 8 | RNAi of *Mocs3* disrupts circadian activity in constant darkness. Flies were first entrained on 3 12:12 h light-dark cycles then representative actograms are presented during constant darkness. Statistical analysis is depicted for two independent drivers. N represents number of individuals tested, Rhythmic Statistic (R. S.) values are provided for flies scored as rhythmic: higher values demonstrate more robust rhythmicity (for detailed methodology see Mandilaras and Missirlis, 2012).

iron concentrations (Zhang et al., 2014; Muckenthaler et al., 2017; Papanikolaou and Pantopoulos, 2017; Rouault and Maio, 2017). *D. melanogaster* has two IRP-like proteins, showing a partial evolutionary conservation in its iron sensing mechanism (Muckenthaler et al., 1998; Lind et al., 2006; Freeman et al., 2013). Another conserved aspect in cellular iron homeostasis is that genetic manipulations that affect mitochondrial Fe-S cluster biosynthesis lead to cytosolic iron depletion (Anderson et al., 2005; Uhrigshardt et al., 2013; Navarro et al., 2015). The mechanism of this response is not fully conserved between yeast (Wofford and Lindahl, 2015) and vertebrates (Wingert et al., 2005; Ye et al., 2010), as the former lack IRPs, and it is not known how *Drosophila* cytosolic iron is regulated through the mitochondrial Fe-S cluster assembly machinery. Furthermore, transcriptional regulation of ferritin upon iron sensing appears to dominate the *Drosophila* iron response (Georgieva et al., 1999; Missirlis et al., 2007; Rosas-Arellano et al., 2016), suggesting that an undiscovered transcription factor responds to varying cytosolic iron concentration in insects. Of note, *Drosophila* ferritin is loaded with iron in the endoplasmic reticulum (Xiao et al., 2014; Xiao and Zhou, 2018) and is an essential gene (González-Morales et al., 2015), as is also the case for IRP-1A (Puri et al., 2008). More work is needed to understand cellular iron homeostasis in *Drosophila*.

How Do Mitochondria Regulate Their Iron Needs?

Mitochondria use a specialized ferritin (Missirlis et al., 2006) and at least one specialized transporter for iron import into the mitochondrial matrix, mitoferrin (Metzendorf and Lind, 2010; Navarro et al., 2015; Edenharter et al., 2017). The question how they sense and regulate iron concentration in the matrix

is not resolved. One unexplained observation in this respect is that, at least in yeast, the GTP to GDP ratio affects iron concentration within this compartment (Gordon et al., 2006). In what follows, we explore the idea that manganese Sod2 (Kirby et al., 2002; Duttaroy et al., 2003) may serve as a mitochondrial iron sensor (**Figure 9**). Our view of normally respiring mitochondria is the familiar setting with an active Sod2 protecting Fe-S clusters and maintaining the TCA cycle (**Figure 9A**; Missirlis et al., 2003a). An increase in superoxide (Wong H. S. et al., 2017) deactivates the Fe-S clusters in aconitase and Sdh (Gardner and Fridovich, 1991; Gardner et al., 1995) leading to an increase in ferrous iron in the mitochondrial matrix (Srinivasan et al., 2000; Jensen et al., 2004; Esposito et al., 2013). The literature on these reactions has been reviewed with a discussion of the accompanying consequences for cell metabolism (see superoxide/aconitase rheostat model; Armstrong et al., 2004). Increased mitochondrial iron can replace manganese on Sod2 and inactivate the enzyme (Yang et al., 2006; Naranuntarat et al., 2009). Sod2 inactivation would result in a positive feedback loop, as more superoxide would accumulate, fully inactivating aconitase and Sdh bringing the TCA cycle to a halt.

Heping Cheng and co-workers described a superoxide burst in mitochondria, termed mitoflash, lasting for several seconds (Wang et al., 2008; Shen et al., 2014). Some skepticism on whether the phenomenon is real exists, mostly arguing that perhaps the change in the fluorescence of the reporter used relates to pH changes (Schwarzländer et al., 2012, 2014), but the coupling of TCA cycle with oxidative phosphorylation could mean that both claims (a burst in superoxide and alkalinization of the mitochondrial matrix) occur at similar timeframes or in parallel (Wei-LaPierre et al., 2013).

During the inactivation of Sod2, citrate cannot be isomerized to isocitrate and is therefore exported to the cytosol for fatty acid synthesis (see below). Likewise succinate cannot be metabolized, but its precursor, succinyl-CoA is a substrate of aminolevulinate synthase (Alas) in the first step of heme biosynthesis (Ruiz de Mena et al., 1999; Ponka et al., 2017). Thus the superoxide burst couples the redirection of iron and intermediary metabolites to ferrochelatase for heme production (**Figure 9B**). This way, iron concentration in the mitochondrial matrix drops, manganese binds to and reactivates Sod2, and the mitochondria return to their conventional state. In the above discussion we have not considered the role of hydrogen peroxide, produced by Sod2, which should be treated as a distinct metabolite (Missirlis et al., 2003b; Munro and Treberg, 2017).

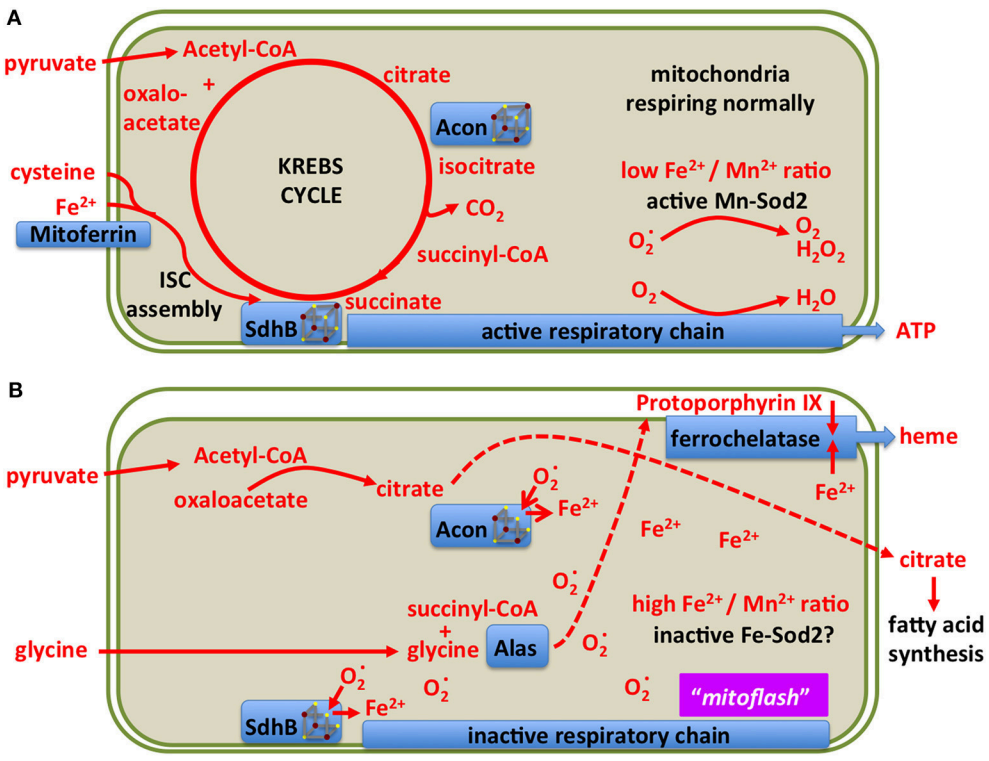


FIGURE 9 | A model showing two different states of mitochondrial metabolism. **(A)** Conventional state based on the TCA (Krebs) cycle feeding reducing equivalents into the respiratory chain for the production of ATP. Only metabolites and enzymes referred to in the text are shown for simplicity. **(B)** A superoxide burst is predicted to reduce the [4Fe-4S] clusters of aconitase and Sdh, releasing ferrous iron into the mitochondrial matrix and blocking the TCA cycle. The resulting increase in iron concentration may inactivate manganese Sod2 resulting in a sustained pulse of superoxide, previously referred to as a "mitoflash." In this state, mitochondria export citrate to the cytosol for fatty acid production, Alas uses up succinyl-CoA for protoporphyrin biosynthesis, ferrochelatase uses protoporphyrin and iron to produce heme. The consequent drop in matrix soluble iron reactivates manganese Sod2, allowing for Fe-S cluster biosynthesis to reactivate the TCA cycle enzymes.

Although the metabolites that are affected in the *Sod2* mutants remain to be described, a recent paper reported a four-fold induction of aminolevulinic acid in *Sod1* mutants, and curiously, a 20-fold induction of citrulline (Doran et al., 2017). Citrulline production depends on (the heme-containing) nitric oxide synthase (Nos), which uses as substrate arginine (Kuzin et al., 1996; Jaszczak et al., 2015). Superoxide-dependent inactivation of another manganese-containing enzyme, arginase, which degrades arginine (Samson, 2000), could lead to increased substrate availability for Nos. Indeed, it has been shown that arginase inactivation induces Nos activity (Caldwell et al., 2015). The above suggest another testable example of a similar hypothesis, where the interaction of superoxide with Fe-S clusters influences cell metabolism by releasing iron, which could transiently displace manganese from the active site of its target enzyme(s).

Fe-S Clusters and Lipid Metabolism

Citrate is a key precursor for fatty acid synthesis (Watson and Lowenstein, 1970; Halperin et al., 1975) and therefore it is not surprising that inactivation of aconitase leads to increased lipogenesis (reviewed in Tong and Rouault, 2007). The same metabolic connection has also been demonstrated in *Drosophila*, by driving *frataxin* RNAi in glial cells (Navarro et al., 2010) or in photoreceptor mutant clones (Chen et al., 2016b). Another outcome of increased mitochondrial iron due to loss of *frataxin* is the activation, in a way that is not yet understood, of sphingolipids. Sphingolipids, in turn, activate signal transduction pathways, like, in the example of photoreceptors, of Phosphoinositide-dependent kinase 1 and Myocyte enhancer factor 2 (Chen et al., 2016a,b). Moreover, murine mutants in Sod2 show increased lipid accumulation in their livers (Li et al., 1995; Chouchani et al., 2017), which we attribute to the inactivation of aconitase (**Figure 9**). It remains to be shown whether during normal physiology our hypothesis of two alternating mitochondrial states, one driving aerobic respiration, the other driving lipogenesis and heme biosynthesis, is valid or not.

PHYSIOLOGICAL RELEVANCE OF Fe-S AND MOCO ENZYMES IN DIFFERENT TISSUES

In this section we first look at the role of Fe-S enzymes during the life cycle of *D. melanogaster*, then focus on the main tissues of the adult fly where functions of Fe-S and Moco enzymes are known.

Development, Growth, and Aging: Stem Cells and Cellular Differentiation

The life cycle of *Drosophila* is separated in distinct stages. Egg-laying follows the insemination of oocytes in the female, embryogenesis gives rise to the larva, which feeds and grows until entry into metamorphosis, at the end of which adult flies emerge (Demerec, 1950). We discussed above that Fe-S enzymes drive aerobic respiration, which changes dramatically with the progress of embryogenesis (Lints et al., 1967; Tennessen et al., 2011, 2014), during larval growth (Heinrich et al., 2011; Merkey et al., 2011; Sen et al., 2013; Da-Ré et al., 2014) and adult aging (Lints and Lints, 1968; Ferguson et al., 2005; Dubessay et al., 2007; Klichko et al., 2014; Wolff et al., 2016). The decline in mitochondrial functions observed in late life, along with experiments showing that genetic manipulations leading to improved mitochondrial functions extended lifespan, suggested that mitochondrial metabolism governs the adult life span (Villee, 1948; Miquel, 1998; Fukagawa, 1999; Ross, 2000; Muller et al., 2007), but the finding that mitochondria isolated from old flies incubated with cytosol from young individuals restore respiration, whereas mitochondria from young individuals incubated with cytosol of old individuals fail to produce ATP questions this view (Vann and Webster, 1977; see also Sanz, 2016). On the other hand, there can be no doubt that defects in Fe-S cluster biosynthesis inhibit growth and dramatically shorten lifespan (Missirlis, 2003). It is also good to remember that the two sexes need to be considered separately when studying mitochondrial metabolism (Camus et al., 2012, 2015; Pomatto et al., 2017).

Increased glycolysis uncoupled from aerobic respiration is a characteristic of proliferative cells, for example during the early stages of *Drosophila* embryogenesis (Tennessen et al., 2011, 2014). This major metabolic switch is mediated through cell signaling (Thörig et al., 1981a,b; Markopoulou and Artavanis-Tsakonas, 1989; Homem et al., 2014; Barry and Thummel, 2016; Sieber et al., 2016; Slaninova et al., 2016; Mattila and Hietakangas, 2017). We already discussed that exit from the growth stage requires the concerted activity of Fe-S and heme enzymes in the prothoracic gland for ecdysone synthesis (Llorens et al., 2015; Palandri et al., 2015). Further, ecdysone is one of the signals inducing oxidative phosphorylation through the mitochondrial respiratory chain, sensed by neuroblasts and leading to their terminal differentiation (Homem et al., 2014) (see also Sen et al., 2013). Iron itself can directly influence stem cell differentiation, exemplified by hemocyte production in the lymph gland (Yoon et al., 2017). Thus, primary cell metabolism can define the fate of stem cells in a developing organism.

The Germ Line: Is a Lineage of Quiescent Mitochondria Set Aside for Reproduction?

Stem cell differentiation in the ovary was found to depend on the mitochondrial ATP synthase (Teixeira et al., 2015). Surprisingly, in the female germarium, oxidative phosphorylation played no role in the early differentiation steps of the female germ line (Teixeira et al., 2015). Why are differentiating stem cells in the ovary and the larval brain different? One idea, first proposed by Allen (1996), is that the female germ line is defined as a carrier of quiescent (non-respiring) mitochondria (Cox and Spradling, 2003; de Paula et al., 2013a,b; Sieber et al., 2016). A key experiment to test this hypothesis is whether quiescent mitochondria can be detected throughout the life cycle in the female germ line (Allen and de Paula, 2013). We showed that quiescent mitochondria are observed in the female germ line within gonads of third instar larvae (**Figure 10**). However,

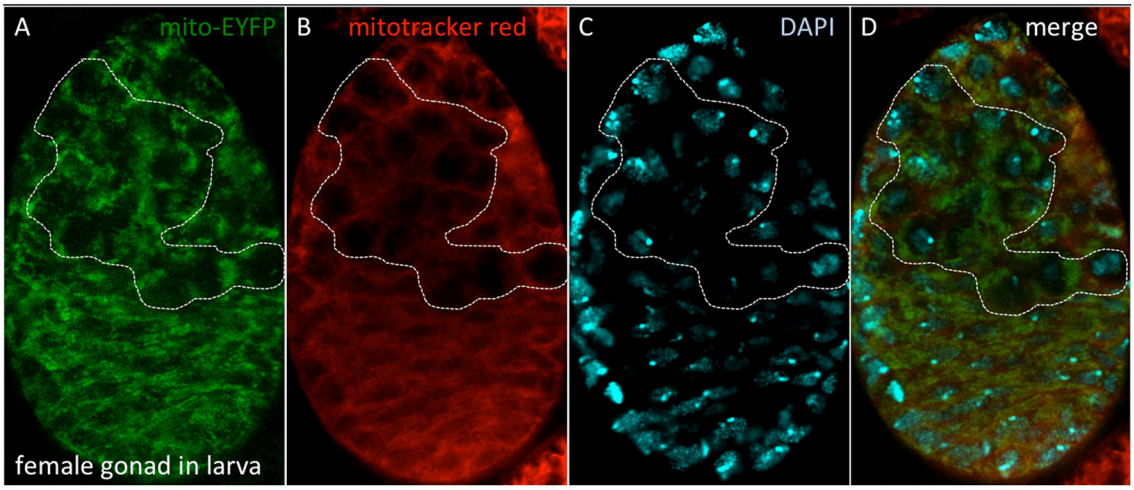


FIGURE 10 | Quiescent mitochondria in female gonads of *D. melanogaster* larvae. **(A)** Bloomington stock #7194 carries a P-element insertion including the *spaghetti squash* promoter driving expression of EYFP tagged at the N-terminal end with a mitochondrial targeting sequence. **(B)** Dissected gonads were incubated with Mitotracker Red, which accumulates in mitochondria with active membrane potential. **(C)** Nuclei were observed with DAPI. **(D)** Merged image. The dotted line depicts the female germ line, where quiescent mitochondria are observed. Methods as described in Cox and Spradling (2003).

similar experiments have proven harder to perform in embryos due to impermeability of the Mitotracker Red and reduced *spaghetti squash*-EYFP fluorescence (LaJeunesse et al., 2004), although the laboratory of Richa Rikhy recently succeeded to image mitochondria in living embryos using new constructs (Chowdhary et al., 2017). Of note, these quiescent mitochondria would constitute a third mitochondrial state, not described in **Figure 9**, since they lack the oxidative phosphorylation complexes and hence a source of superoxide to protect the mitochondrial DNA. This line of research was also unfortunately disrupted at Queen Mary University of London (Allen and Missirlis, 2012; Ashworth, 2012; Horton, 2012); for further insights see (Burrows, 2012; Mahul-Mellier et al., 2015; Lawrence, 2016; Edwards and Roy, 2017; Tsimilli-Michael and Haldimann, 2017).

Alan Spradling and co-workers have also described quiescent mitochondria in the oocyte (Cox and Spradling, 2003), but they have shown in addition an active mitochondrial membrane potential during stages 4–8 of oogenesis (Sieber et al., 2016). Insulin signaling acting through glycogen synthase kinase 3 is required to set mitochondria into a quiescent phase from stage 10 of oogenesis (Sieber et al., 2016). Is the finding of an active membrane potential during oogenesis sufficient to refute the theory of quiescent mitochondria? The first issue to resolve is whether mitochondria in the germarium respire or are quiescent. Our interpretation of the limited published data (Sieber et al., 2016) is that mitochondria in female germ cells are quiescent and this would offer an explanation for the unexpected finding that the early steps of the female germ line differentiation is independent of oxidative phosphorylation (Teixeira et al., 2015). However, another well-known phenomenon in the transmission of mitochondria through successive generations is bottleneck selection of functional mitochondria, which occurs during *Drosophila* oogenesis (Cox and Spradling, 2003, 2006; Hill et al., 2014; Ma et al., 2014; Chen Z. et al., 2015). It is therefore possible that the discovery by Ruth Lehmann and co-workers of ATP synthase induction in the first steps of such a differentiation program proceeds in order for mitochondria to test their ability to sustain a membrane potential during stages 4–8 of oogenesis as a selection of functional mitochondria that can be safely transmitted to the oocyte.

If Allen's hypothesis is proven correct, he may have resolved Darwin's still-standing question of why it is that two separate sexes evolved (Venton, 2013). The two sexes were required to keep a quiescent form of mitochondria in the resting gamete, while dispensing the mitochondria used by the motile gamete (DeLuca and O'Farrell, 2012; Politi et al., 2014). The mitochondrial DNA polymerase (an Fe-S enzyme, Stiban et al., 2014) participates in the elimination of paternal mitochondrial genomes in *D. melanogaster* by a mechanism that is not yet understood (Yu et al., 2017).

Eye Pigment Formation

The study of heredity defines the birth of the field of *Drosophila* research, famously through observations of the color of their eyes (Morgan, 1910, 1911). The study of how eye pigments are formed, inspired by the drive to understand the genetic control of development (Beadle and Ephrussi, 1936; Lewis, 1978), gave rise to biochemical genetics. Progress in biochemistry (i.e., Hadorn and Mitchell, 1951) made clear early on that the Moco is required for pigment biosynthesis (Glassman and Mitchell, 1959; Hubby and Forrest, 1960). Progress in electron microscopy revealed impressive changes in cell biology occurring in eye color mutants (Nolte, 1961; Shoup, 1966; Sullivan and Sullivan, 1975) and, as discussed above, how Xdh reaches the pigment cells of the eye remains unsolved (Reaume et al., 1989, 1991).

Malpighian Tubules and Urate Excretion

Malpighian tubules are the major excretory organ in flies (Beyenbach et al., 2010). The primary role of Xdh is in purine catabolism, mostly taking place in the Malpighian tubules (Dickinson and Gaughan, 1981; Reaume et al., 1989). Zinc ions are implicated in mineral excretion through this organ (Chi et al., 2015; Yin et al., 2017), while the same genes that govern pigment granule formation in the eye are also required for the formation of zinc storage granules in the Malpighian tubules (Tejeda-Guzman et al., 2018). Finally, the Malpighian tubules also show strong Aox activity (Dickinson and Gaughan, 1981), although the physiological function of this activity remains to be shown (Marelja et al., 2014).

Muscles for Flight and Heartbeat

D. melanogaster is able to fly several kilometers in the open desert (Dickinson, 2014). To do so, it uses specialized muscles (Iwamoto, 2011), which receive oxygen directly through the trachea (Lehmann and Schützner, 2010), respiring over 90% of the oxygen to sustain flight (Suarez, 2000). Mitochondria are key to this action (Levenbook and Williams, 1956) and ambient oxygen concentrations alter flight performance (Skandalis et al., 2011; Bosco et al., 2015; Shiehzadegan et al., 2017). If Fe-S respiratory enzymes are affected either by aging (Ferguson et al., 2005) or by mutation (Walker et al., 2006; Godenschwege et al., 2009; Martin et al., 2009; Vrailas-Mortimer et al., 2011; Oka et al., 2015), muscle pathology ensues. On the other hand, flies are highly resistant to hypoxia and indeed lacking manganese Sod2 results in short-lived adults (the majority dying within the first couple of days from pupal eclosion; Godenschwege et al., 2009), but this mortality is rescued by moving the flies in a hypoxic environment (Wicks et al., 2009). A survey into the genetic factors regulating natural variation in mitochondrial function in the *Drosophila* muscle revealed nuclear genomic control of naturally occurring variation in mitochondrial respiration (Correa et al., 2012; Jumbo-Lucioni et al., 2012), a process also regulated by calcineurin (Pfluger et al., 2015) and the mitochondrial contact site and cristae junction organizing system (Guarani et al., 2015). Finally, the requirement of Fe-S clusters and the respiratory chain has also been demonstrated in the *Drosophila* heart muscle (Tricoire et al., 2014; Martínez-Morentin et al., 2015).

The Insect Intestine Functions beyond Nutrient Absorption

The primary function of the intestine lies in digestion of food and absorption of nutrients (Shanbhag and Tripathi, 2009; Lemaitre and Miguel-Aliaga, 2013). As this epithelium is continuously renewed, *Drosophila* researchers have paid more attention to the regulation of stem cells (Ohlstein and Spradling, 2006, 2007; Biteau et al., 2008; Lin et al., 2008; Takashima et al., 2008; Jiang et al., 2009; Scopelliti et al., 2014; Reiff et al., 2015; Brand et al., 2016; Hudry et al., 2016; Resnik-Docampo et al., 2017). Furthermore, many microbes reside in the intestine (Leulier and Royet, 2009; Shin et al., 2011; Buchon et al., 2013) and their activity can influence fly behavior and physiology (Sharon et al., 2010; Hang et al., 2014; Fischer et al., 2017; Leitão-Gonçalves et al., 2017; Mistry et al., 2017; Wong A. C. et al., 2017). One of the unique functions of the fly intestine is its role in copper (Filshie et al., 1971; Dubreuil, 2004; Burke et al., 2008) and iron (Tang and Zhou, 2013a; Rosas-Arellano et al., 2016) homeostasis. The specific roles of Fe-S and Moco enzymes in this tissue remain to be shown (Uhrigshardt et al., 2013).

Secretory Glands, Fat Bodies, and Nephrocytes

The role of Fe-S proteins in secretory tissues, like the salivary glands and the fat bodies, and in hemolymph filtering tissues, like the Garland and pericardial nephrocytes is also not resolved. One common property of these tissues is that their nuclei undergo polyploidy (Nordman et al., 2011), hence nuclear Fe-S enzymes involved in DNA replication are expected to have an enhanced role.

The Nervous System

The ways in which iron and other metals relate to neurodegeneration have been reviewed (Zhu et al., 2014; Calap-Quintana et al., 2017), therefore a discussion on this topic will not be included here, except for the following points. Despite general agreement that *frataxin* is required for a functional nervous system, disagreement has been expressed on the cause, with different authors favoring oxidative stress (Llorens et al., 2007; Anderson et al., 2008; Kondapalli et al., 2008), iron toxicity (Soriano et al., 2013; Navarro et al., 2015), altered mitochondrial metabolism (Navarro et al., 2010; Tricoire et al., 2014; Calap-Quintana et al., 2015; Soriano et al., 2016), sphingolipid signaling (Chen et al., 2016b), and failure to maintain neuronal membrane potential (Shidara and Hollenbeck, 2010). We do not see any contradiction in the various positive claims made in the above-cited literature, whereas the negative claim that is often repeated—refuting a role for oxidative stress in explaining the phenotypes—normally arises because of failure to rescue the phenotypes with some transgenes as opposed to others. Similar failure could have various explanations: for example overexpression of Sod2 (Mockett et al., 1999) does not guarantee that the enzyme will be active in mitochondria with iron overload, at least our discussion of mitochondrial metabolism above (**Figure 9**) predicts otherwise. Furthermore, not all reactive oxygen species will act in the same way and their source and subcellular localization is also important to the effects they cause (Missirlis et al., 2003b). Last, cells handle iron in different ways: neurons and glia for example differ dramatically in their ability to store iron (Kosmidis et al., 2011, 2014) for reasons that are not understood. When ferritin mutants progress through early embryogenesis (they do so thanks to maternal contribution of iron-loaded ferritin to the oocyte) severe defects in the development of the nervous system ensue (González-Morales et al., 2015). Which step in brain development is most sensitive to the lack of ferritin has not been resolved. The blood-brain barrier regulates iron entry into the brain (Mehta et al., 2009), but we still do not know how iron traffics in the peripheral and central nervous systems or how Fe-S and/or Moco enzymes affect the circadian clock. Answers to questions of the basic cell biology of metal homeostasis are prerequisite for proposing

better therapeutics when neuronal functions are compromised in disease (Zhu et al., 2014; Calap-Quintana et al., 2017; Ruland et al., 2017; Poetini et al., 2018).

CONCLUSION

We cannot think of any biological function for which the fly will not require the biochemical participation of Fe-S clusters. We have attempted to describe our progress in understanding the role of Fe-S enzymes during the past 93 years since Otto Warburg firmly connected iron to respiration (Warburg, 1925) and also the Fe-S and Moco enzymes Xdh and Aox. We have used the fly as an example, but of course knowledge has been acquired from studying all forms of life as these enzymes are universal in character and may have formed at the origin of life (Hall et al., 1971; Russell and Martin, 2004; Nitschke and Russell, 2009; Schoepp-Cothenet et al., 2012; Varma et al., in review). We suggest that bioinorganic contributions to biology and bioenergetics be taken into account not only as having "house-keeping" roles, but also as an active component of the complex organization that characterizes all living systems (Frausto da Silva and Williams, 2001). Renewed attention on the inorganic chemistry underpinning *Drosophila* biology, together with the new analytical tools and methodologies available, should help integrate cellular metal homeostasis with metabolism (Dow, 2017; Navarro and Schneuwly, 2017). The humble fly has still much to contribute to our understanding of the workings of biology.

DEDICATION

Dedicated to Stefan Grimm (1963–2014) who discovered, while working at Imperial College London, a moonlighting function for IkBα binding to the outer mitochondrial membrane: protecting cells from suicide.

AUTHOR CONTRIBUTIONS

ZM: Performed the experiments on the mitochondrial Fe-S assembly core complex (**Figure 3**), determined the subcellular localization of fly Isd11 and Mocs3 (**Figure 4**), and performed the biochemical assays for aldehyde oxidase and sulfite oxidase (**Figures 6**, **7**) during his Ph.D. thesis with SL; ZM, SL, and FM: Wrote the sections on Fe-S cluster assembly, Moco biosynthesis and the function of molybdoenzymes; FM: Found the quiescent mitochondria in the female gonad of larvae (**Figure 10**) and is responsible for the hypothesis that Sod2 acts as a mitochondrial iron sensor and other theories expressed in the latter part of the article; All authors have read, reviewed, and endorsed the full content of this publication.

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REVIEW
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ZIP13: A Study of *Drosophila* Offers an Alternative Explanation for the Corresponding Human Disease

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The fruit fly *Drosophila melanogaster* has become an important model organism to investigate metal homeostasis and human diseases. Previously we identified dZIP13 (CG7816), a member of the ZIP transporter family (SLC39A) and presumably a zinc importer, is in fact physiologically primarily responsible to move iron from the cytosol into the secretory compartments in the fly. This review will discuss the implication of this finding for the etiology of Spondylocheirodysplasia-Ehlers-Danlos Syndrome (SCD–EDS), a human disease defective in ZIP13. We propose an entirely different model in that lack of iron in the secretory compartment may underlie SCD-EDS. Altogether three different working models are discussed, supported by relevant findings made in different studies, with uncertainties, and questions remained to be solved. We speculate that the distinct ZIP13 sequence features, different from those of all other ZIP family members, may confer it special transport properties.

Keywords: Drosophila, iron, zinc, ZIP13, SCD-EDS

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INTRODUCTION: SPONDYLOCHEIRODYSPLASIA-EHLERS-DANLOS SYNDROME AND ZIP13

Ehlers-Danlos syndrome (EDS) is a group of clinically and genetically heterogeneous disorders with defects in connective tissue characterized by skin hyperelasticity, tissue fragility, poor wound healing, and joint hypermobility (Yeowell and Pinnell, 1993; Beighton et al., 1998; Bowen et al., 2017; Brady et al., 2017). In the Villefranche Nosology, EDS is divided into six subtypes: the classical, hypermobile, vascular, kyphoscoliotic, arthrochalasis, and dermatosparaxis subtypes (Bowen et al., 2017; Brady et al., 2017). The pathogenesis of these diseases was not defined until recently. Defects of fibrillary collagens or collagen-modifying enzymes had been identified in all subtypes except the hypermobile subtype (Bowen et al., 2017; Zhang et al., 2017).

SCD–EDS is a very rare autosomal recessive disease caused by mutations in the hZIP13 gene (Fukada et al., 2008; Giunta et al., 2008; Calap-Quintana et al., 2017). An increased ratio of total urinary pyridinolines, lysylpyridinoline/hydroxylysylpyridinoline (LP/HP) was found in patients (Calap-Quintana et al., 2017), but the total amount of pyridinolines (LP plus HP) was not affected (Calap-Quintana et al., 2017). This indicates that the formation of LP was increased while HP was decreased. Same situation was observed in another disease, Ehlers-Danlos type VI (EDS VI), which shares several clinical signs with SCD–EDS (Steinmann et al., 1995). The hydroxylation of lysyl and prolyl residues

in collagens was measured *in vivo* and *in vitro*. Lysyl under-hydroxylation and prolyl under-hydroxylation are not confined to specific residues and occur along the whole molecule (Giunta et al., 2008). In normal people, these hydroxylation processes are catalyzed by lysyl hydroxylase (LH) and prolyl 4-hydroxylase (PH4) (**Figure 1**; Myllyharju, 2008). Notably, EDS VI is caused by mutations in the PLOD1 gene, which encodes lysyl hydroxylase (LH1) (Yeowell and Walker, 2000; Yeowell et al., 2000). These indicate SCD–EDS is closely related to collagen under-hydroxylation.

After excluding PLOD1, PLOD2, and PLOD3, ZIP13 mutations were finally identified as the culprit in SCD–EDS patients (Giunta et al., 2008). Bin et al. demonstrated that both the identified human mutation ZIP13^{G64D} and ZIP13$^{\Delta FLA}$ result in rapid degradation of the mutant protein in cultured primary cultured fibroblasts, and an imbalance of intracellular zinc homeostasis (Bin et al., 2014). Importantly, proteasome inhibitor MG132 treatment increased the total ZIP13 levels and restored the impaired intracellular zinc homeostasis of the SCD–EDS patient cells (Bin et al., 2014).

ZIP13 belongs to the ZIP (Slc39A) transporter family (To simplify, all Slc39A13 of various organisms are noted as "ZIP13" in this review). Most members of ZIP family transport zinc, but a few have also been found to transport other metals including iron. Most ZIP proteins have eight transmembrane domains (TMs) with a His-rich domain between TM3 and TM4, and hydrophilic residues in TM5 which may determine metal specificity (Taylor et al., 2007; Fukada and Kambe, 2011). The C- and N-terminal of ZIP proteins both face the extra cytoplasmic space (Lichten and Cousins, 2009; Jeong and Eide, 2013; Kambe et al., 2014). Based on amino acid sequence similarity ZIP family can be divided into four subfamilies: ZIPI, ZIPII, GufA, and LZT (Taylor and Nicholson, 2003; Fukada and Kambe, 2011).

Biochemical characterization of human ZIP13 protein by Bin et al. indicated that ZIP13 is located mainly in the Golgi apparatus, with both its N and C termini end in the luminal side (Bin et al., 2011). Zip13 is classified to be a member of the ZIP LZT subfamily, which is characterized by eight putative TMs and a unique hydrophilic region. ZIP13 additionally possesses some domains that are not found in other LZT family members (Taylor and Nicholson, 2003; Bin et al., 2011; Fukada and Kambe, 2011).

One thing puzzling is that ZIP13 lacks characteristic His residues at the N-terminal and in the region between TM3 and TM4 (Bin et al., 2011). It is believed that these regions in other ZIPs can bind zinc ions (Taylor et al., 2007). Potocki et al. evaluated metal ion binding properties of the cysteine-rich N-terminal domain fragment of the ZIP13 zinc transporter (MPGCPCPGCG–NH_2) and the results indicate that the binding ability of this fragment changes in the series Bi^{3+} >> Cd^{2+} > Zn^{2+} > Ni^{2+} (Potocki et al., 2011). This region of ZIP13, however, is not evolutionarily conserved in sequence. For example, in the *chicken* ZIP13, there is not this N-domain with several nearby "C"s or alternatively "H"s.

Structural information of ZIP13 is not available. Nevertheless, crystal structure of the extracellular domain (ECD) of

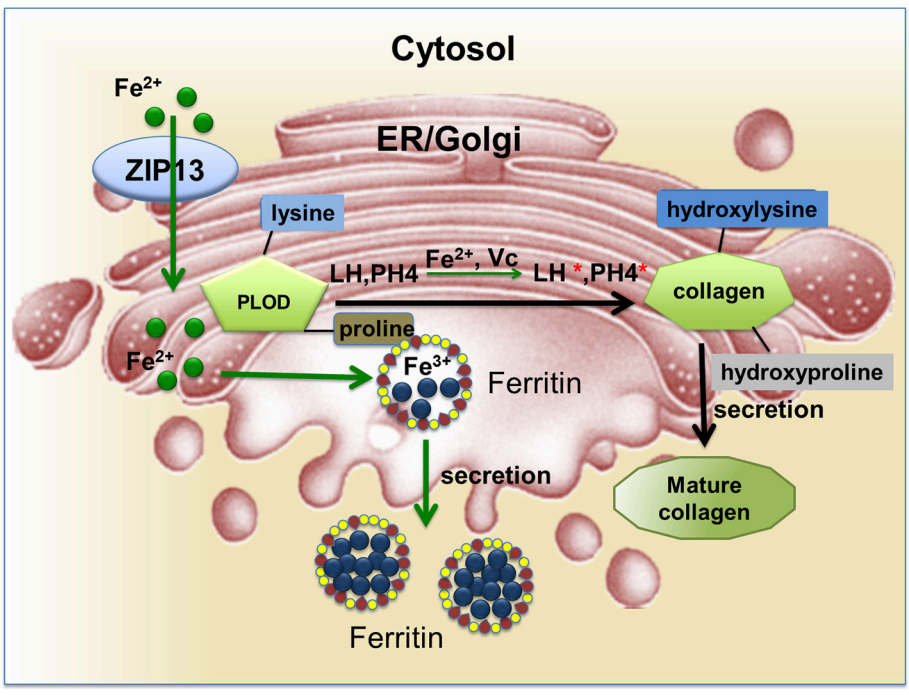

FIGURE 1 | LH and PH4 are critical enzymes for collagen hydroxylation during collagen maturation. The reactions require iron and ascorbate as cofactors. In this figure, ZIP13 is proposed as the iron exporter localized on the ER/Golgi and responsible for iron transporting from the cytosol into the secretory pathway. These iron ions will be utilized by proteins such as ferritin (in the fly and likely some other insects with secretion type of ferritin) and LH.

mammalian ZIP4 has been solved by Zhang et al., demonstrating that ZIP4-ECD is a crucial regulatory domain for optimal zinc transport (Zhang et al., 2016). Very recently the same group solved the crystal structures of a prokaryotic ZIP from *Bordetella bronchiseptica* (BbZIP), indicating that two metal ions were trapped halfway through the membrane, unexpectedly forming a binuclear metal center. This work provides the first structural framework for understanding the metal transport mechanism of the ZIPs (Zhang et al., 2017). But at this stage, it is not still clear how zinc is moved across the membrane, and the relationship between the two bound metal ions (Zhang et al., 2017). For ZIP13, the critical question is: how mutations in ZIP13, a likely zinc transporter, could lead to collagen under-hydroxylation? One further finding worthy of noting is that despite collagen under-hydroxylation in SCD–EDS patients, *in vitro* enzymatic assays revealed that LH and PH4 activities were normal in patients' fibroblasts (Giunta et al., 2008). It appears that certain things might differ under *in vivo* and *in vitro* conditions so that hydroxylation could happen *in vitro* but not so *in vivo*.

INITIAL EVIDENCES OF dZIP13 INVOLVING IN IRON METABOLISM

ZIP family members are key zinc importers in mammalian organisms. Studying zinc homeostasis in *Drosophila* is a relatively recent event, but large strides have already been made (Richards and Burke, 2016; Xiao and Zhou, 2016), thanks to several distinctive advantages of this model organism *Drosophila* genome encodes at least eight putative ZIP proteins (Lye et al., 2012; Qin et al., 2013). Bioinformatics indicates that the protein encoded by *Drosophila* CG7816 (ZIP99c) shares the highest overall homology with human ZIP13 (we named it dZIP13 accordingly based on sequence and functions; Xiao et al., 2014). Similar to hZIP13, dZIP13 also has several typical features of LZT family members (Jeong and Eide, 2013), including the predicted eight TMs, particularly amphipathic TM4 and TM5, the luminal location of both N and C termini and the highly conserved potential metalloprotease motif (HEXXH, where X is any amino acid) within TM5 (Jeong and Eide, 2013; Xiao et al., 2014). Notably, the single His residue of hZIP13 in a generally histidine-rich region (2–14 His) between TM3 and TM4, is also found in dZIP13 (Xiao et al., 2014). Genetic evidence suggested that human ZIP13 may function similarly to dZIP13 in *Drosophila* (Xiao et al., 2014), corroborating this protein is the hZIP13 ortholog in *Drosophila*.

Unexpectedly, we discovered in dZIP13 knockdown (dZIP13–RNAi) flies a lower total amount of iron, but relatively normal amount of zinc, in their bodies (Xiao et al., 2014). This indicates the occurrence of an iron dyshomeostasis when dZIP13 is repressed. However, this iron dyshomeostasis could either be interpreted as a primary event or a secondary event. If this is the result of a primary event, dZIP13 might directly affect iron homeosatsis; if not or in the secondary scenario, dZIP13 might directly affect intracellular zinc distribution in such a way (without affecting the total zinc level) that results in iron dyshomeostasis. How to distinguish these two possibilities?

Defects of dZIP13–RNAi flies are greatly rescued by treating with iron but not zinc supplementation (Xiao et al., 2014). This favors "the iron defect as the primary event" explanation. However, this is not a compelling piece of evidence since it could be argued that extracellular zinc supplement would be hard to rescue intracellular zinc redistribution defect conceivably happening in the dZIP13–RNAi flies. In other words, it could be contrived that dZIP13 affects intracellular distribution which cannot be rescued by external zinc changes, but the iron consequence as a result of zinc defect in this case could be rescued more readily by iron.

Ferric staining of the gut and native gel showed iron failed to efficiently incorporate into ferritin when dZIP13 was knocked down (Xiao et al., 2014). This demonstrates that dZIP13 is involved in iron loading to ferritin. This constitutes a good but still not definite evidence to support dZIP13 as an iron transporter. It could be argued that zinc dyshomeostasis in the ER/Golgi may disrupt iron incorporation into the ferritin proteins. dZIP13 as an iron transporter is such an unexpected finding and so much against intuition, because we have to propose it not only as an iron transporter but an iron exporter (instead of zinc importer as most other ZIP proteins). In this way, we need to be extremely cautious to reach this conclusion and have to challenge us repeatedly for alternative possibilities. Before we go further we need to introduce briefly iron homeostasis in the fly.

IRON HOMEOSTASIS IN *DROSOPHILA* IS DISTINCT FROM THAT IN MAMMALS

The maintenance of iron homeostasis by cells involves iron uptake, utilization, storage, and excretion. Many proteins participate in these processes in mammalian organisms, including transferrin (Tsf), transferrin receptor (TfR1), duodenal cytochrome b (Dcytb), divalent metal-ion transporter 1 (DMT1), ferritin, mitoferrin (Mfrn), mitochondrial ferritin (MtFt), ferropotin (FPN), ceruloplasmin (Cp), hephaestin, hepcidin, and so on (Hentze et al., 2010; Tang and Zhou, 2013a). Many but not all mammalian iron genes have homologs in the fly (Mandilaras et al., 2013; Tang and Zhou, 2013a). For the purpose of this review, we will only briefly summarize what we know about fly iron homeostasis. Please refer to Tang and Zhou (2013a) and Mandilaras et al. (2013) for a more complete review of iron metabolism in the fly.

For iron uptake in the fly, a proposed *Drosophila* DMT1/NRAMP2 ortholog, Malvolio (Mvl) (Rodrigues et al., 1995; Folwell et al., 2006), resides in the anterior and posterior parts of the middle intestine, as well as in the malpighian tubules, brain, testis and fat body (Folwell et al., 2006), may be responsible for iron uptake from extracellular environment into cells (Southon et al., 2008; Bettedi et al., 2011; Tang and Zhou, 2013b).Genome studies also suggest that there are candidate sequences for Dcytb in *Drosophila melanogaster* (Verelst and Asard, 2003). There are also transferrin homologs in the fly genome (Yoshiga et al., 1999; Adams et al., 2000; Tiklová et al.,

2010). Interestingly there is no obvious transferrin receptor in the genome (Dunkov and Georgieva, 2006).

For mammalian iron efflux, accompanied with iron loading to transferrin, intracellular Fe^{2+} is oxidized to Fe^{3+} under the action of the ferroxidases hephaestin (HEPH, intestinal, and CNS multicopper ferroxidase) or ceruloplasmin (CP, systemic multicopper ferroxidase; De Domenico et al., 2007). No obvious ferroportin homologs have been found in the *Drosophila* genome (Winzerling and Pham, 2006; Tang and Zhou, 2013a). However, four MCO candidate genes exist in the fly (Lang et al., 2012). Whether any one of these MCOs acts as ferroxidase is not clearly established yet.

Fer1HCH and *Fer2LCH* (Dunkov and Georgieva, 1999) encode the fly counterparts of mammalian ferritins. In contrast to mammalian cells, ferritin in *Drosophila* is not cytosolic, instead, it is secreted. Ferritin in the hemolymph is full of loaded iron. In *Drosophila* cells, intracellular iron would be incorporated into ferritin for storage and transporting via the ER/Golgi secretion path to the hemolymph and other cells (Tang and Zhou, 2013b). In this way, fly ferritin is responsible for dietary iron absorption and transport (Tang and Zhou, 2013b). Ferritin is retained in the ER or Golgi complex before secretion (Missirlis et al., 2007). ER/Golgi in the fly therefore turns out to be an iron-rich area and iron efflux via the secretory compartments is a key iron efflux pathway in this organism and likely many other insects (Xiao et al., 2014).

In comparison to the fly, in mammals, only a minor amount of ferritin (generally iron-poor) is found in the serum. As stated above, mammalian iron is exported by ferroportin and very little iron is moved across the secretory compartment. Nevertheless, the ER/Golgi still needs iron as some iron-containing enzymes are also stationed in the secretory pathway in mammals (Tang and Zhou, 2013b). In this context, there presumably should be an iron transporter on ER and/or Golgi membranes in both the fly and mammals.

FURTHER EVIDENCES FOR dZIP13 AS AN IRON EXPORTER

As mentioned, since different from that of mammalian organisms, *Drosophila* effluxes iron through the ER/Golgi secretory pathway via ferritin, a large amount of iron has to be loaded in the secretory pathway in the gut to satisfy the body iron demand. No previous candidates have been uncovered to fulfill this iron efflux role. dZIP13, surprisingly, is involved in the iron loading to ferritin. Does this mean dZIP13 relocates iron from the cytosol to the secretory pathway? Although that would be the most straight forward explanation, strictly speaking it is still not necessarily so. It could be contested that dZIP13 affects zinc homeostasis in the secretory pathway, which then affect iron loading to the ferritin. For example, zinc surplus in the ER/Golgi could cause stress, which affect iron loading to ferritin. Alternatively, excess zinc could compete with iron for ferritin binding so little iron is incorporated.

If zinc imbalance in the secretory pathway could so dramatically affect iron homeosatsis, we expect some other similarly resident proteins such as dZIP7 (Catsup) or dZnT7 (CG6672/ZnT86D) should also be involved in this process. Changing the expression of both these two genes by RNAi or overexpression leads to dissimilar phenotypes from those of altering dZIP13 expression, i.e., no obvious iron phenotypes in dZIP7- or dZnT7-modulated flies (Qin et al., 2013; Xiao et al., 2014). Genetic interaction studies of dZIP13 with dZIP7 or dZnT7 also reveal no significant effect of dZIP7 and dZnT7 on dZIP13 iron functions (Xiao et al., 2014). This supports strongly that the iron effects seen in dZIP13–RNAi flies are not due to zinc dyshomeostasis in the ER/Golgi compartments.

Direct evidence supporting dZIP13 as an iron exporter came from radioactive Fe transport assays (Xiao et al., 2014). dZIP13 expressed in *Escherichia coli* facilitated a linear time course of iron efflux. Isolated ER/Golgi from dZIP13–RNAi *Drosophila* larvae also exhibited reduced iron uptake rates whereas that from dZIP13–OE flies was significantly increased (Xiao et al., 2014). Taken together, all these evidences accumulatively indicate that dZIP13 is an iron exporter, and the iron phenotypes we saw in dZIP13–RNAi were indeed due to defective iron efflux.

IRON EXPORTING FUNCTION OF ZIP13 IN MAMMALS?

So far there is no direct evidence indicating that ZIP13 is an iron exporter in mammalian organisms. This is partially due to the fact that the possibility has rarely or probably never been explored. There remains a possibility that mammalian ZIP13 may function differently from dZIP13, although they share the greatest homology in protein sequence. We found when hZIP13 was expressed in the fly, phenotypes were reminiscent of, but much milder than, those of dZIP13 (Xiao et al., 2014). Defects of dZIP13–RNAi could be partially rescued by hZIP13 expressed in the fly (Xiao et al., 2014). This suggests that hZIP13 can function somewhat similarly to dZIP13 at least in the fly.

If ZIP13 supposedly exports iron, why no obvious iron phenotypes were observed in human SCD–EDS patients or rodent ZIP13–knockout (ZIP13–KO) models, as those in dZIP13–RNAi flies? This could be because in the fly the secretory pathway is loaded with iron, and the secretory pathway is the primary, if not the sole, iron exporting venue. In contrast in mammals, cellular iron is exported by plasma-membrane-resident ferroportin instead of the ER/Golgi path via secreted ferritin. Along this line of thinking, dZIP13 deficiency in the fly will result almost iron export shutdown, ending with overall iron absorption defect and deficiency. In mammals, iron is not exported via this avenue, so no general iron phenotype might be observed.

But iron is still needed in the mammalian ER/Golgi because some enzymes such as collagen crosslinking enzymes LH and PH4 need it as a cofactor. While it could be envisioned that the iron exporting function for *Drosophila* ZIP13 is highly demanding, that for mammalian ZIP13 is not so since much less iron is needed to pump into the secretory compartments in mammalian cells.

Overall, evolutionarily speaking, ZIP13 may also work as an iron exporter in mammalian cells to provide iron to ER/Golgi organelle. But direct evidences are still needed to support this assertion.

ZIP13 AS ALSO A ZINC TRANSPORTER?

Our studies in the fly indicate that dZIP13 is an iron exporter (Xiao et al., 2014). But does that mean dZIP13 has no zinc function? Probably not true. In the fly, dZIP13–RNAi phenotypes can be rescued by iron but not zinc. Nevertheless, we also noticed slightly altered zinc levels (Xiao et al., 2014), though we are not sure whether this is a primary or secondary event due to iron dyshomeostasis. In other studies, Fukada et al. found that ZIP13–KO mice show defects mimicking those of SCD–EDS patients (Fukada et al., 2008). Further studies showed that ZIP13 mainly expresses and functions in mesenchyme-originating cells, and locates in the Golgi apparatus in the mouse (Fukada et al., 2008). Zinc level in the Golgi was slightly increased in ZIP13–KO primary dermal fibroblasts as compared to that in wild-type cells, while zinc concentration in the cell nucleus was decreased (Fukada et al., 2008), suggesting that ZIP13 functions as a zinc transporter transporting zinc from the Golgi to the cytosol (Fukada et al., 2008). Consistent with being a zinc transporter, it was also reported that dietary zinc-deficiency up-regulates ZIP13 mRNA level in mice (Guo et al., 2011).

Biochemical characterization of human ZIP13 protein by Bin et al. demonstrated in cell culture studies that zinc importing activity of ZIP13, when assayed by metallothioneins (Mtns) expression or zinc probe FluoZin-3 after ZIP13 overexpression, is detectable when 100 μM zinc is added to the media (Bin et al., 2011). However, under normal growth conditions, overexpression of ZIP13 did not cause detectable zinc level changes (Bin et al., 2011). This suggests that ZIP13 is at least able to participate in zinc metabolism when zinc levels are high, but at normal conditions its zinc transporting efficiency might be less evident.

A research of zinc homeostasis and immune status in broiler chickens showed that both in jejunum and caecal tonsil, ZIP13 expression is the only measured zinc transporter impacted by dietary zinc source (Troche et al., 2015), suggesting that ZIP13 responds to zinc metabolism in chickens.

Another cell culture study by Jeong and his colleagues using antibody against native ZIP13 to determine the subcellular localization of endogenous ZIP13 indicated that ZIP13 localized to punctate vesicles dispersed throughout the cytoplasm (Jeong et al., 2012). To ascertain zinc uptake properties of Zip13, ZIP13 was overexpressed and a minor part was found mislocalized to the cell surface, enabling cellular zinc uptake assay resulted in zinc accumulation (Jeong et al., 2012). ZIP13-dependent zinc uptake was similar to other mammalian ZIP transporters and was not competed by excess extracellular iron (Jeong et al., 2012). These results indicate that ZIP13 imports zinc specifically. It is worthy of noting, however, that this experiment did not exclude iron transporting ability of ZIP13 for two reasons. The first is that zinc and iron may not necessarily compete for the same spot of transportation. Consistently, structure studies have found that ZIP may contain two metal binding sites (Zhang et al., 2017). More importantly, the competition experiment with extracellular iron would only address the iron importing function, but not the possible iron exporting function of human ZIP13, as claimed for dZIP13.

Considering all the evidences, zinc transporting of ZIP13 is entirely possible, although its zinc moving efficiency might be compromised compared to other ZIP transporters. This is consistent with the sequence data in that ZIP13 lacks a His-rich region in all its extracellular domains. According to biochemistry studies, the extracellular domains of ZIPs are likely involved in increasing transporting efficiency but not selective specificity (Jeong et al., 2012).

THE THREE CURRENT MODELS TO EXPLAIN HOW ZIP13 MUTATIONS CAUSE SPONDYLOCHEIRODYSPLASIA-EHLERS-DANLOS SYNDROME

As described before, collagen synthesis of SCD–EDS patients is abnormal in that crosslinking catalyzed by LH and PH4 is defective. During collagen synthesis, ER is the place where different collagen types are synthesized and modified (Myllyharju and Kivirikko, 2004). Among the various modifications, hydroxylation of collagen lysyl and prolyl residues is a highly conserved enzymatic process involving Fe^{2+} as the cofactor (Tuderman et al., 1977; Puistola et al., 1980). Externally supplied iron is needed for the enzymatic assays of the enzymes *in vitro* (Tuderman et al., 1977; Murad et al., 1985), suggesting iron is probably not tightly bound to these enzymes. On the other hand, zinc has been reported as an inhibitor of prolyl hydroxylase (Myllylä et al., 1977; Puistola et al., 1980).

Based on results from different studies as summarized in this review, three entirely different models have been proposed to explain the etiology of SCD–EDS. Since most ZIPs are involved in zinc transportations and the closest homolog of ZIP13 is ZIP7, which has been shown to be a Golgi-resident zinc importer, it is naturally to suggest that ZIP13 is responsible for zinc importing from ER/Golgi to cytosol in mouse and human cells (**Figure 2**; Fukada et al., 2008; Giunta et al., 2008; Bin et al., 2011; Jeong et al., 2012). Along this line, one model proposes that ZIP13 loss of function results in zinc accumulation in ER/Golgi, which competes with iron for binding to LH and PH4, two critical enzymes using iron as a cofactor in the secretory pathway for collagen hydroxylation (**Figure 1**; Myllyharju, 2008), leading to disruptive biosynthesis of collagens and SCD–EDS (**Figure 2**; Fukada et al., 2008; Giunta et al., 2008). Further supporting this, Fukada et al. found that zinc is slightly increased in the Golgi and decreased in the nucleus of ZIP13 KO cells (Fukada et al., 2008). They proposed that this zinc defect, somehow led to observed impairment in bone morphogenic protein (BMP) and TGF-β signaling pathway, and resulted in the nuclear shift of SMAD proteins and dysregulation of BMP/TGF-β-mediated genes expression critically involved in bone, tooth, and craniofacial skeletogenesis (Fukada et al., 2008).

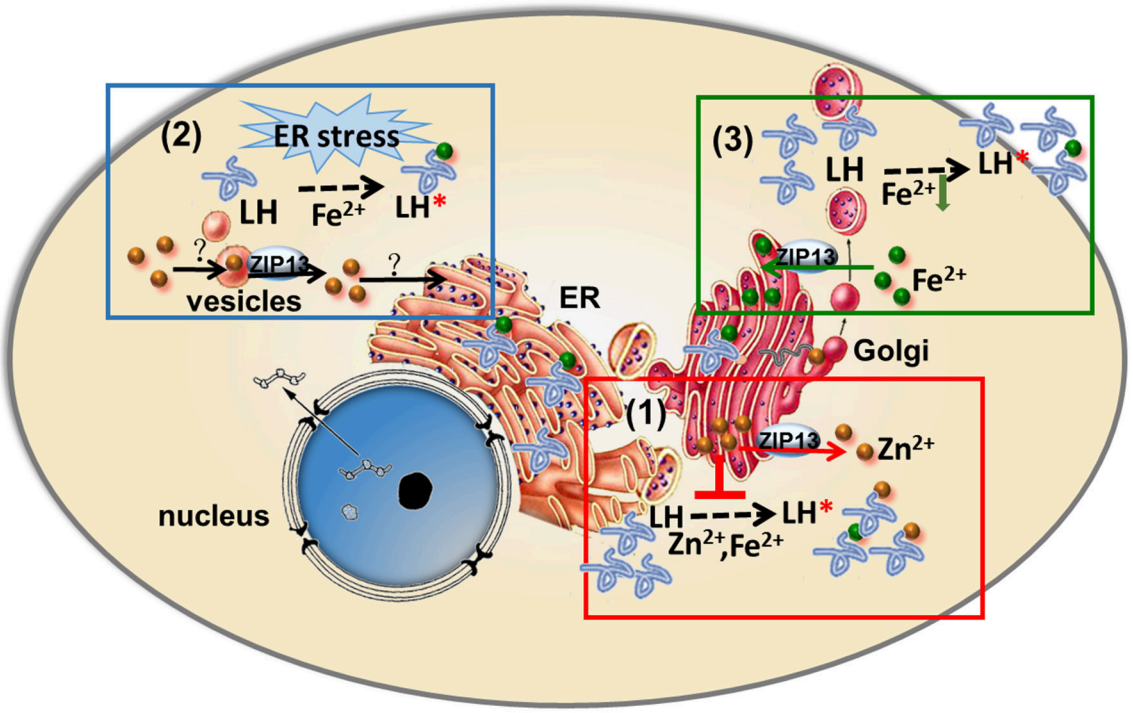

FIGURE 2 | Three models of ZIP13's action proposed three entirely different etiology for SCD-EDS. (1) SCD-EDS is suggested to arise from increased zinc accumulation in the ER/Golgi. ZIP13 imports zinc from ER/Golgi to cytosol, so the absence of ZIP13 results in zinc accumulation in ER/Golgi. The accumulated zinc competes with iron for binding to LH and PH4, leading to disrupted biosynthesis of collagens and SCD-EDS. (2) SCD-EDS is due to zinc deficiency in the ER/Golgi. ZIP13 is responsible for zinc releasing from vesicular stores for use in the secretory pathway, so ZIP13 loss of function leads to general ER dysfunction and the pathogenesis of SCD-EDS. (3) SCD-EDS is caused by iron deficiency in the ER/Golgi. ZIP13 exports iron from cytosol into the secretory pathway, so ZIP13 loss of function results in iron deficiency in the ER/Golgi compartments, leading to collagen crosslinking defect and SCD-EDS.

In contrast, Jeong et al. based on their cellular localization of ZIP13 to the vesicular compartment, claimed that ZIP13 might function to release labile zinc from vesicular stores for use in the ER and other compartments (Jeong et al., 2012), and proposed that ZIP13 loss of function could lead to general ER dysfunction and thus explained the biochemical findings of SCD–EDS (**Figure 2**; Jeong et al., 2012).

The above two models of ZIP13's action proposed two entirely different etiologies for SCD–EDS (**Figure 2**). In the first model, SCD–EDS is suggested to arise from increased zinc accumulation in the ER/Golgi [**Figure 2**, box(1); Fukada et al., 2008; Bin et al., 2011], while the second proposed that SCD–EDS is due to zinc deficiency in the ER/Golgi [**Figure 2**, box (2); Jeong et al., 2012].

The finding of dZIP13 as an iron exporter offers a new and more straightforward explanation for SCD–EDS [**Figure 2**, box (3)]. If hZIP13, or for that matter mammalian ZIP13 altogether, also works as an iron exporter, this would be the only iron transporter so far identified in higher organisms in the ER/Golgi, providing the necessary ions needed in these compartments. In the absence of ZIP13, the secretory compartments will lack iron, a cofactor for the enzymatic activities of LH and PH4 (**Figure 1**), resulting in collagen crosslinking defect.

The baffling finding that in cells from SCD–EDS patients LH and PH4 are normal could also be well-explained by ZIP13 being as an iron exporter. LH and PH4 activities are assayed *in vitro* with a buffer containing ferrous iron. Because SCD–EDS patients are not defective in the proteins of LH and PH4 *per se*, what is happening is that these enzymes do not "see" iron *in vivo*, but *in vitro* when iron is supplemented normal activities of these enzymes would certainly be observed.

If the new scenario is true or ZIP13 acts as an iron exporter, what unique features of ZIP13 might confer it such special transporting ability? The answer is largely unknown, however we can gain some insights from its sequence. The closest homolog of ZIP13 is ZIP7 (Jeong and Eide, 2013). But ZIP7 is an established zinc transporter moving zinc ion from Golgi to the cytosol (Huang et al., 2005). Sequence alignment of the LIV-1 subfamily members including human ZIP4, ZIP5, ZIP6, ZIP7, ZIP8, ZIP10, ZIP12, ZIP13, and ZIP14 shows that in addition to the five universally conserved residues found in all ZIP family members, most conserved residues within the LIV-1 subfamily are located in TM5 (Eide, 2004; Dempski, 2012). In addition, there are some conserved motifs among LIV-1 families, such as CPALLY motif and metalloprotease motif (**Figure 3**; Taylor et al., 2007). Notably, another conserved consensus sequence, DxxHNFxD in TM4 of LIV-1 subfamily, is changed to NxxDNFxH in ZIP13 (**Figure 3**; Xiao et al., 2014). The functional consequences of

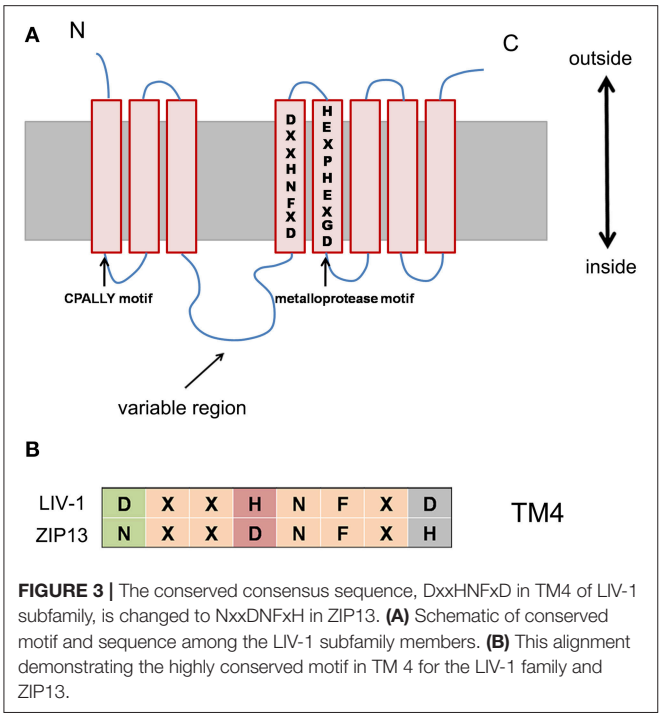

FIGURE 3 | The conserved consensus sequence, DxxHNFxD in TM4 of LIV-1 subfamily, is changed to NxxDNFxH in ZIP13. **(A)** Schematic of conserved motif and sequence among the LIV-1 subfamily members. **(B)** This alignment demonstrating the highly conserved motif in TM 4 for the LIV-1 family and ZIP13.

these sequence variations remain unknown and certainly worth exploring.

If SCD–EDS is indeed due to lack of iron in the ER/Golgi compartments, will iron supplement benefit these patients? We are not certain, but likely not very effective due to the following reasons. Firstly, alteration of iron levels within an organelle is normally less efficient than that in the cytosol. This is because it subjects to another level of control on top of that of the cytosol. In other words, it is a compartment within another compartment. Even when the cytosolic iron of a cell is altered, iron within an organelle of that cell may not be effectively changed. Mitochondria for example, have their own control of iron homeostasis on top of the other cellular iron control. Secondly, SCD–EDS mutations so far identified might be all null or close to null (Bin et al., 2014). In the absence of ZIP13 protein, it might be difficult to move iron into ER/Golgi even when the cytosolic iron level is higher. When we knocked-down dZIP13 expression in the fly, weaker collagen defects could also sometimes be observed. The collagen defects could be rescued by iron supplementation in the diet (Xiao et al., 2014). These flies, however, have partially functional dZIP13 due to incomplete suppression abilities of RNAi.

ZIP FAMILY MEMBERS WITH "UNCONVENTIONAL" TRANSPORTING PROPERTIES

dZIP13 turns out to be very unusual in that it not only transports iron, but the transport is in the opposite direction! Is there any precedent for this?

In fact, the ZIP is short for Zrt-, Irt-like protein (Lye et al., 2012; Jeong and Eide, 2013; Xiao and Zhou, 2016), including many zinc and iron transporters. There are quite a few examples of ZIPs acting as iron transporters. *Arabidopsis thaliana* Irt1 and Irt2 (iron regulated transporters) were discovered by complementation of yeast zinc importer *zrt1 zrt2* mutant (Zhao and Eide, 1996). Considering that Irt1 and Irt2 are iron transporters, while Zrt1 and Zrt2 are zinc transporters, it seems that subtle changes in the amino acids or physiological context could have an important effect on the cation selectivity of ZIPs (Dempski, 2012).

In mammals, ZIPs were initially believed to be zinc importers. Recently, certain members of ZIPs have been reported to be capable of transporting iron and other metal ions. ZIP8 and ZIP14 (Liuzzi et al., 2006; Gao et al., 2008; Zhao et al., 2010; Coffey and Knutson, 2017) are two notable examples. Both ZIP8 and ZIP14 belong to the LIV-1 subfamily. Overexpression of mouse ZIP14 (mZIP14) increases cellular accumulation of iron and zinc in HEK 293H cells while suppression of ZIP14 expression decreased the uptake of iron and zinc by mouse hepatocytes (Liuzzi et al., 2006). Girijashanker et al. indicated that Cd^{2+} (apparent Km of $1.10 \pm 0.02\,\mu M$) and Mn^{2+} (apparent Km of $18 \pm 2\,\mu M$) can also be transported by ZIP14 while Zn^{2+} uptake by this protein could be inhibited by Cd^{2+}, Mn^{2+}, and Cu^{2+} (Girijashanker et al., 2008).

ZIP8 is expressed on the plasma membrane of cells such as the testis (Dalton et al., 2005). Function analysis of ZIP8 in MFF cells and *Xenopus laevis* oocytes demonstrated that ZIP8 have high affinity for Zn^{2+} and Cd^{2+}, and mediates Cd^{2+} and Zn^{2+} uptake (He et al., 2006; Liu et al., 2008). Cadmium uptake is inhibited by Zn^{2+}, Cu^{2+}, Pb^{2+}, and Hg^{2+}, but not affected by Mn^{2+} or Fe^{2+} (Liu et al., 2008). The cell-surface expression of ZIP8 was enhanced by iron supplement in rat hepatoma cells (Wang et al., 2012), but the mechanism has not yet been elucidated.

Further study has demonstrated that, besides zinc and iron, many ZIP-family transporters can also transport cations such as copper, nickel, cadmium, and manganese (Dempski, 2012). For example, hZIP4, the gene whose mutations are involved in acrodermatitis enteropathica (AE) (Küry et al., 2002; Wang et al., 2002), was reported to transport copper and nickel in *X. laevis* oocytes (Antala and Dempski, 2012).

The above examples are all ZIPs transporting other metal ions in the inward direction. Is there any precedent that a ZIP member can export metal ions?

Bidirectional transport of zinc was first reported in *Saccharomyces cerevisiae* for the transporter Yke4p, encoded by yeast YIL023C (YKE4), which is the ortholog of mouse Ke4 (Kumánovics et al., 2006). Yke4p is a novel member of ZIP family localized to the ER membrane (Kumánovics et al., 2006). In high zinc medium, Yke4p eliminates the cytosolic zinc into the secretory pathway to avoid toxic zinc accumulation in the cytosol whereas under low cytosolic zinc conditions, Yke4p relocates zinc from the secretory pathway to the cytosol (Kumánovics et al., 2006). Current genetic and biochemical evidences indicate that Yke4p is capable of transporting zinc both into and out of the vesicular apparatus, depending on the zinc status in the cytosol (Kumánovics et al., 2006).

Outside of the ZIP family, a member of ZnT family is also capable of bidirectional zinc transport (Cragg et al., 2002; Valentine et al., 2007). Valentine et al. showed that human ZnT5 variant B [ZnT5B (hZTL1)], an isoform of ZnT5 expressed at the plasma membrane, operates in both the uptake and the efflux directions when expressed in *X. laevis* oocytes (Cragg et al., 2002; Valentine et al., 2007). They found that ZnT5B is expressed at the plasma membrane in Caco-2 cells and *X. laevis* oocytes, and can mediate zinc efflux against a concentration gradient but can also function in the opposite direction in the same system. Because dramatically elevated total intracellular zinc concentration was observed in Caco-2 cells and oocytes expressing ZnT5B at the plasma membrane, while zinc sequestration into specific intracellular compartments was not affected, ZnT5B mediating zinc uptake across the plasma membrane was suggested (Cragg et al., 2002; Valentine et al., 2007). These data demonstrate that ZnT5 can not only operate in an efflux mode, but also has the capability to mediate zinc uptake.

Besides zinc transporters, many ion exchangers move different ions in opposite directions. Na^+/Ca^{2+} exchanger (NCX), found in cardiac muscle cells and elsewhere in the body, transports three Na^+ ions into the cell in exchange for one Ca^{2+} ion transported out of the cell (Liao et al., 2012). The glucose transporter can exist in different conformations that expose the glucose binding site to either the extracellular fluid or the cytosol to ensure the counter transport of glucose in cells (Thorens and Mueckler, 2010). Glucose is transported from the extracellular fluid into the cytosol by transporters when cells need to uptake glucose for energy source. However, the direction of transport will be opposite if the glucose concentration is higher inside the cell than outside (Medina and Owen, 2002; Thorens and Mueckler, 2010).

CONCLUSIONS

Drosophila melanogaster has served as a good research platform to investigate the function of metal transporters and studies in this organism may advance our understanding of metal homeostasis and its relevance to human disease(Xiao et al., 2013). With the help of this platform, we identified that the primary role of dZIP13 is exporting iron from the cytosol into the secretory pathway. On top of the existing models, we suggest a more straightforward model to explain the pathogenesis of SCD–EDS; the activities of critical enzymes for collagen synthesis in these patients are affected due to iron deficiency in the secretary compartments. A critical issue remains to be solved is whether mammalian ZIP13 is also responsible to provide iron to the secretory compartments for collagen synthesis.

AUTHOR CONTRIBUTIONS

GX drafted the initial version of the manuscript; BZ drastically revised the manuscript. All authors made intellectual contributions, edited, and approved the manuscript for publication.

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REVIEW
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Ironing out the Details: Exploring the Role of Iron and Heme in Blood-Sucking Arthropods

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Heme and iron are essential molecules for many physiological processes and yet have the ability to cause oxidative damage such as lipid peroxidation, protein degradation, and ultimately cell death if not controlled. Blood-sucking arthropods have evolved diverse methods to protect themselves against iron/heme-related damage, as the act of bloodfeeding itself is high risk, high reward process. Protective mechanisms in medically important arthropods include the midgut peritrophic matrix in mosquitoes, heme aggregation into the crystalline structure hemozoin in kissing bugs and hemosomes in ticks. Once heme and iron pass these protective mechanisms they are presumed to enter the midgut epithelial cells via membrane-bound transporters, though relatively few iron or heme transporters have been identified in bloodsucking arthropods. Upon iron entry into midgut epithelial cells, ferritin serves as the universal storage protein and transport for dietary iron in many organisms including arthropods. In addition to its role as a nutrient, heme is also an important signaling molecule in the midgut epithelial cells for many physiological processes including vitellogenesis. This review article will summarize recent advancements in heme/iron uptake, detoxification and exportation in bloodfeeding arthropods. While initial strides have been made at ironing out the role of dietary iron and heme in arthropods, much still remains to be discovered as these molecules may serve as novel targets for the control of many arthropod pests.

Keywords: iron, heme, arthropod, mosquito, tick, bloodfeeding, peritrophic matrix, transporter

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INTRODUCTION: EVOLUTION OF ARTHROPOD HEMATOPHAGY

Estimates suggest there are more than 1 million insect and arachnid species inhabiting planet Earth, approximately 14,000 of which have adapted the ability to feed on vertebrate blood (Graça-Souza et al., 2006). Commonly known as hematophagy, this adaptation has arisen independently many times over the course of evolution (Graça-Souza et al., 2006). However, certain features are shared among the ancestors of bloodfeeding arthropods. Traditionally accepted ancestral pre-conditions to bloodfeeding include: (1) living in close proximity to vertebrates, (2) specialized feeding on skin remains, dung or fluids from animal carcasses, and (3) specialized mouthparts for piercing and cutting (Graça-Souza et al., 2006). It is quite interesting that hematophagous arthropods can consume anywhere from 2 to 100x their normal body weight during a single blood meal. Subsequently, this blood meal, comprised largely of proteins, is digested by enzymes secreted from midgut epithelial cells (Barillas-Mury and Wells, 1993; Jiang et al., 1997; Edwards et al., 2000; Brackney et al., 2010). The amino acids that result from blood meal protein digestion are

used for lipid, carbohydrate, and egg protein synthesis (Marquardt and Kondratieff, 2005). While the bloodfeeding process has obvious nutritional advantages for hematophagous arthropods, the digestive process releases the pro-oxidant molecules heme and iron in potentially toxic quantities. In response to this challenge, bloodfeeding arthropods have evolved a number of strategies to limit oxidative damage during blood digestion. This review seeks to compile and compare existing vertebrate and invertebrate literature as it pertains to iron and heme processing, with particular interest in applying knowledge gained in other systems to hematophagous arthropods.

PROTECTIVE ADAPTATIONS AGAINST OXIDATIVE STRESS AFTER A BLOOD MEAL: ANTIOXIDANTS

Over time, hematophagous arthropods have honed their antioxidant systems to avoid oxidative stress (Felton and Summers, 1995; Champion and Xu, 2017). Oxidative stress has traditionally been defined as the disturbance in balance between the production of reactive oxygen species (ROSs) and antioxidant defenses (Felton and Summers, 1995; Betteridge, 2000; Chaitanya et al., 2016), which may result in the oxidation of nucleic acids (Ryter and Tyrrell, 2000), lipids (Tappel, 1955; Vincent et al., 1988), and proteins (Aft and Mueller, 1984; Vincent et al., 1988). However, all cells and organisms naturally produce ROSs (Chaitanya et al., 2016). For example, prior to a blood meal, the mosquito *Aedes aegypti,* the most important vector of arboviruses worldwide, generate ROSs for the control of intestinal microbiota proliferation (Oliveira et al., 2011). Oliveira and Oliveira (2002) hypothesized that a decrease in mitochondrial ROSs may be necessary to avoid their interaction with the pro-oxidant products of blood meal digestion, heme and iron (Oliveira and Oliveira, 2002). This was confirmed by Gonçalves et al. (2009), who reported that bloodfeeding promoted the fusion of *Ae. aegypti* flight muscle mitochondria, which decreased the generation of ROSs (Gonçalves et al., 2009). Interestingly, mitochondrial decreases in H_2O_2 followed the digestion process, with mitochondrial function returning to pre-blood meal levels upon the completion of digestion (Gonçalves et al., 2009). Oliveira et al. (2011) found that this decrease in ROSs is triggered by heme and no other blood component (Oliveira et al., 2011). To further reduce ROSs generated post bloodfeeding and avoid a milieu prone to oxidative stress, *Ae. aegypti* and other hematophagous arthropods have deployed a number of defenses into the gut lumen and hemolymph (extracellular) as well as in the cytoplasm of midgut epithelial cells or other tissues in the body (intracellular).

Intracellular Antioxidants

Intracellularly, Cu, Zn, and Mn superoxide dismutases (SOD) catalyze the conversion of superoxide anions into hydrogen peroxide. Subsequently, catalase (CAT) and general peroxidases (POD) are responsible for detoxifying hydrogen peroxide (Felton and Summers, 1995). More specifically, catalase catalyzes the dismutation of hydrogen peroxide to oxygen and water. Paes et al. (2001) found that when compared to other tissues, SOD and CAT activity were highest in the adult female midgut of the kissing bug, *Rhodnius prolixus*. Likewise, subsequent inhibition of CAT resulted in increased H_2O_2 in midgut extracts (Paes et al., 2001). To further test the individual and collective roles of CAT and glutathione (GSH), each antioxidant was individually and collectively inhibited. For GSH and CAT, there was a 4- and 2-fold increase in H_2O_2 content in *R. prolixus* midgut extracts, respectively. Interestingly, when GSH and CAT synthesis were inhibited simultaneously, the authors saw a tremendous increase in midgut extract H_2O_2 content, confirming the importance of both antioxidants with regards to H_2O_2 control after a blood meal (Paes et al., 2001). In a more recent study, through gene expression comparisons, Oliveira et al. (2017) found that mRNA levels for the antioxidant enzyme CAT increased 6-fold at 24 and 36 h after a blood meal and decreased to values equivalent to sugar fed mosquitoes at the end of digestion (72 h). RNAi-mediated knockdown of CAT resulted in reduced oviposition and lifespan when adult female *Aedes aegypti* were challenged with the pro-oxidant H_2O_2 (Oliveira et al., 2017). This reduced fecundity as a result of RNAi mediated knockdown of CAT was also demonstrated in adult female *Anopheles gambiae, one* of the primary mosquito vectors of malaria parasites (DeJong et al., 2007). Interestingly, this study found that a single serine to tryptophan polymorphism resulted in decreased CAT activity and stability (DeJong et al., 2007). The antioxidant role of CAT in ovaries has also been documented in other Dipterans besides mosquitoes, as ovarian CAT was found to accumulate in developing oocytes of the bloodsucking sand fly, *Lutzomyia longipalpis,* 12–48 h after bloodfeeding (Diaz-Albiter et al., 2011). Beyond SOD and CAT, glutathione S-transferase (GST) catalyzes the conjugation between glutathione and other molecules (Freitas et al., 2007). Through competitive enzymatic-based assays and assays measuring changes in intrinsic fluorescence, *Ae. aegypti* recombinant GSTX2-2 was the first mosquito GST found to *in vitro* bind heme (Lumjuan et al., 2007). However, further studies are needed to determine antioxidant functionality for GSTX2-2 after a blood meal. In the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, GST activity was confirmed as antioxidant in the eggs and larvae of engorged females. More specifically, GST enzymatic activity significantly increased and O_2 consumption progressively increased during embryonic development and eventually peaked at hatch (Freitas et al., 2007). These authors reported a strong correlation between O_2 consumption and GST activity, as well as a positive correlation for the antioxidants CAT and GSH with GST in *R. microplus* eggs and larvae. These results suggest that in addition to the antioxidant parameters, which allow for avoidance of oxidative stress in the midgut after a blood meal, increased oxidative stress can be associated with embryogenesis and aging (Freitas et al., 2007).

Extracellular Antioxidants

While certain antioxidant molecules work in concert to intracellularly reduce oxidative stress, here we highlight recent advancements regarding extracellular antioxidants. Recently, Lima et al. (2012) suggested a new role for xanthurenic acid (XA) as a heme and iron chelator. XA is product of tryptophan degradation through the kynurenine pathway. Traditionally, in

insects, the kynurenine pathway is associated with eye pigment formation. However, reverse phase HPLC and mass spectrometry were used to identify xanthurenic acid (XA) as a component of *Ae. aegypti* midgut homogenates after a blood meal (ABM) (Lima et al., 2012). At 24 h ABM, XA reached maximum levels. This is also the time of peak of blood meal hemoglobin digestion, when large amounts of heme and iron are present in the midgut lumen. Both heme and iron-induced lipid peroxidation were inhibited by XA through the *in vitro* binding of XA to both heme and iron, suggesting an antioxidant role for XA in the *Ae. aegypti* midgut after a blood meal. Similarly, an antioxidant function has been demonstrated for a 15 kDa heme-binding protein (RHBP) in *R. prolixus* (Dansa-Petretski et al., 1995). Given that RHBP is a hemolymph-localized heme-binding protein, it will be further discussed in this context later in our review. However, using ^{32}P labeling of fat bodies (site of lipid production), Dansa-Petretski et al. (1995) provided evidence that RHBP serves as an antioxidant, allowing for proper functioning of lipophorin as a lipid shuttle from the fat bodies to other organs. Heme negatively affected the functionality of lipophorin, but functionality returned to normal levels when RHBP was present (Dansa-Petretski et al., 1995). More specifically, RHBP inhibited the heme-induced reduction of lipophorin ability to transfer phospholipids to the ovaries (Dansa-Petretski et al., 1995).

The above studies highlight various antioxidant molecules deployed by some bloodfeeding arthropods. Undoubtedly, many additional mechanisms remain to be uncovered, particularly in those bloodfeeding species that do not receive as much attention, such as lice, fleas and bed bugs. We note that the subject of redox homeostasis is even more complicated in context of the microbiota of bloodfeeding arthropods. This topic was the subject of a recent review (Champion and Xu, 2017), and thus will not be discussed further.

PROTECTIVE ADAPTATIONS AGAINST OXIDATIVE STRESS AFTER A BLOOD MEAL: SPECIALIZED MIDGUT DEFENSE MECHANISMS

Hematophagous arthropods have evolved specialized defense mechanisms to avoid heme toxicity during blood meal digestion such as the extracellular double plasma membrane structure, perimicrovillar membranes (PPM) (Lane and Harrison, 1979; Silva et al., 2004; Gutiérrez-Cabrera et al., 2015) which cover the midgut epithelia and facilitate generation of the heme crystal, hemozoin in the kissing bug (Oliveira et al., 1999, 2000, 2005; Pagola et al., 2000; Silva et al., 2004, 2007), hemosomes in the cattle tick (Braz et al., 1999; Lara et al., 2003), and Type I peritrophic matrix formation in larval and nymphal *Ixodes scapularis* ticks and adult Dipteran mosquitoes (except Tsetse adults which form Type II PM) (Peters, 1992; Narasimhan et al., 2014; Rose et al., 2014). Interestingly, all of the above-mentioned structures function in heme detoxification through various specialized forms of aggregation or crystalization (Graça-Souza et al., 2006). Below we have highlighted recent advancements in knowledge regarding each of these structures, but refer the reader to Graça-Souza et al. (2006) for a more detailed review of earlier literature (Graça-Souza et al., 2006).

In the kissing bug *R. prolixus*, transmission electron micrographs of the midgut showed large aggregates of crystalline heme during blood meal digestion (Oliveira et al., 1999, 2000). Interestingly, heme crystallization was also found in three other triatomine insects, *Triatoma infestans*, *Dipetalogaster maximus* and *Panstrongylus megistus* (Oliveira et al., 2007). Further investigation identified the perimicrovillar membranes, extracellular double plasma membranes which line the midgut epithelium, as necessary for heme crystallization into hemozoin for *R. prolixus*, *T. infestans*, *D. maximus*, and *P. megistus* (Oliveira et al., 2005, 2007; Silva et al., 2007). Specifically, lipid and protein constituents of the perimicrovillar membrane were necessary for proper hemozoin formation (Silva et al., 2007; Stiebler et al., 2010, 2014). Hemozoin formation assays and RNAi mediated knock-down of α-glucosidase provided evidence supporting the role of α-glucosidase in the nucleation step of hemozoin formation (Mury et al., 2009). α-glucosidase is a membrane-bound enzyme found in the midgut of *R. prolixus* with maximal activity 7–9 days post blood meal (PBM) (Silva et al., 2007). Interestingly, aggregated hemozoin also reached maximum levels at 7 days PBM (Silva et al., 2007). More recently, through kinetic analysis of hemozoin formation induced by the perimicrovillar membranes, Stiebler et al. (2010) identified three different stages of hemozoin formation in *R. prolixus*. Through Mössbauer spectroscopy, these authors provided evidence that at least 97% of all iron present in the midguts of *R. prolixus* 4 days post blood meal was in the form of hemozoin (Stiebler et al., 2010). Follow-up experiments provided evidence that lipids isolated from the perimicrovillar membranes are efficient catalysts of hemozoin formation *in vitro* (Stiebler et al., 2010, 2014). More specifically, *in vitro* experiments with two commercial phospholipids, unsaturated 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine (uPE) and to a lesser extent unsaturated 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (uPC) produced brick shaped and blunt ended β-hematin crystals similar to those produced by plasma-fed or blood-fed *R. prolixus* midgut lipids (Stiebler et al., 2014). This suggests that uPE and uPC (to lesser extent) play integral roles in the determination of crystal morphology for hemozoin in the midgut of *R. prolixus*. This is in agreement with previous findings, which reported 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine as the major phospholipid synthesized by *R. prolixus* midgut epithelium (Bittencourt-Cunha et al., 2013). A similar involvement of lipids in hemozoin formation has been well documented in the parasites *Plasmodium* and *Schistosoma mansoni* (blood fluke) (Oliveira et al., 2004; Corrêa Soares et al., 2007; Stiebler et al., 2010, 2014). Taken together, these results emphasize the importance of looking beyond protein components for the mechanistic basis of important biochemical reactions in bloodmeal detoxification.

R. microplus are unable to endogenously synthesize heme (Braz et al., 1999). For the first 7 days after a blood meal, hemoglobin-derived heme is directly absorbed for vitellogenesis (Lara et al., 2003). During this time, the largest amount of heme is concentrated at the basal lamina side of midgut digestive cells (closest to hemocoel) (Lara et al., 2003). This contrasts

with other hematophagous arthropods where digestion occurs in the midgut lumen. After the first 7 days PBM, the amount of heme absorption decreases and heme concentrates in midgut digestive cell organelles that specialize in heme sequestration, termed hemosomes (Lara et al., 2003). By the end of blood meal digestion, the mass of an individual hemosome was reported to be 90% heme (Lara et al., 2003). More recently, Lara et al. (2015) proposed a detailed model for heme movement in digestive cells of *R. microplus*. However, there remain unanswered questions regarding movement of heme from digestive vesicles to the intracellular hemosomes (Lara et al., 2015).

While digestive cell hemosomes (*R. microplus*) and heme crystal hemozoins in association with a perimicrovillar membrane (*R. prolixus*) have been documented as protective mechanisms against oxidative stress, *I. scapularis* ticks (vector of *Borrelia burgdorferi*) (Narasimhan et al., 2014) and most adult Dipterans (biting flies and mosquitoes) produce a peritrophic matrix in response to bloodfeeding (Dimopoulos et al., 1998; Shen and Jacobs-Lorena, 1998; Morlais and Severson, 2001; Shao et al., 2001, 2005; Hao and Aksoy, 2002; Devenport et al., 2005, 2006; Ramalho-Ortigão et al., 2007; Jochim et al., 2008; Dinglasan et al., 2009; Narasimhan et al., 2014; Rose et al., 2014).

The peritrophic matrix (PM) is a semipermeable extracellular layer, which lines the midgut of most invertebrates (Peters, 1992; Tellam et al., 1999). The peritrophic matrix is classified as Type I or Type II based on location of synthesis within the insect midgut (Peters, 1992). The Type I peritrophic matrix is completely synthesized by midgut epithelial cells (Tellam et al., 1999). In contrast, the Type II peritrophic matrix is constitutively secreted by the cardia, a specialized organ found between the foregut and midgut (Stohler, 1961; Moskalyk et al., 1996; Tellam et al., 1999). Type I PM formation is induced by midgut distension (Freyvogel and Jaquet, 1965; Richards and Richards, 1977) and it is comprised of proteins, proteoglycans, and chitin fibrils (Shao et al., 2001; Pascoa et al., 2002). Peritrophic matrix proteins are integral components of the PM, and are commonly referred to as peritrophins (Tellam et al., 1999). Peritrophins are characterized by the presence of a secretory signal peptide, multiple chitin-binding domains containing cysteine-proline dipeptides, and intervening mucin-like domains rich in proline, serine, and threonine (Tellam et al., 1999). The multiple chitin-binding domains of peritrophic matrix peritrophins function as cross-linkers for chitin fibrils, thereby providing structure and support for the peritrophic matrix (Schorderet et al., 1998; Shen and Jacobs-Lorena, 1998). In addition, PM peritrophins contain aromatic amino acid residues that facilitate binding with N-acetyl-glucosamine of the chitin fibrils (Toprak et al., 2010). Several Type I and Type II PM peritrophins have been identified and characterized from hematophagous insects (**Figure 1**).

Peritrophic matrix peritrophins can be further classified according to their chitin-binding domains (CBDs). Each CBD is comprised of cysteine residues that allow for disulfide bonds within the CBD, and the number of disulfide bonds within a CBD can range from three to five (Tellam et al., 1999; Toprak et al., 2010). The Peritrophin-A domain (PAD) is the most common type of CBD, and contains six interspersed cysteine residues (Toprak et al., 2010). Typically, an individual PAD contains 48–57 amino acid residues. However, the exact PAD sequence arrangement has been found to vary for different insect orders (Toprak et al., 2010), and as seen in *Anopheles gambiae* there are cases of modified peritrophin-A domains (mPADs). The mPAD of AgAper25a and AgAper29 are characterized as having two additional cysteine residues, however, these two cysteine residues are not considered significant (Toprak et al., 2010).

While less common, CBDs can also be classified as Peritrophin-B domain (PBD) or Peritrophin-C domain (PCD) according to the number of cysteine residues. An individual PBD contains eight interspersed cysteine amino acid residues, and is 81–88 residues long (Toprak et al., 2010). In contrast, an individual PCD contains 10 interspersed cysteine residues, and is typically 121–122 amino acid residues long (Toprak et al., 2010). As seen in the Tsetse (*Glosssina morsitans morsitans*), there are partial PCDs that contain 67–70 amino acids (Hao and Aksoy, 2002; Rose et al., 2014). In addition, as seen in *An. gambiae* AgAper25b, peritrophins can contain a Type III carbohydrate binding domains (CBDIII). While the CBDIII contains 10 interspersed cysteine residues (similar to PCD), this domain is typically 110–117 amino acid residues long.

In addition to binding chitin, one peritrophin has been reported to bind heme. *Ae. aegypti* intestinal mucin 1 (AeIMUCI) is a 275-amino acid glycoprotein that contains three chitin-binding domains and a mucin domain between CBD 1 and CBD 2 (Rayms-Keller et al., 2000; Devenport et al., 2006). Rayms-Keller et al. (2000) first identified *Ae*IMUC1 RNA in metal exposed *Ae. aegypti* mosquito larvae, metal fed adult females and blood-fed adult females (Rayms-Keller et al., 2000). Devenport et al. (2006), confirmed chitin and heme binding for AeIMUC1. Through deletion analysis using recombinant proteins, they also determined that chitin-binding and heme-binding functions are associated with the 3 CBDs of AeIMUC1 (Devenport et al., 2006). Most importantly, they confirmed AeIMUC1 as an integral PM peritrophin associated with the PM 12 to 24 h post bloodfeeding (Devenport et al., 2006). Taken together, results from previous AeIMUC1 studies suggest this protein is important for PM structural integrity (Devenport et al., 2006) and blood meal heme detoxification (Rayms-Keller et al., 2000; Devenport et al., 2006). However, studies suggest there are potentially more *Ae. aegypti* midgut peritrophic matrix peritrophins (Moskalyk et al., 1996; Pascoa et al., 2002).

With advancements in technology such as Multidimensional protein identification technology (MudPIT) (Schirmer et al., 2009), the total protein composition of tissues such as the PM can more readily be explored/discovered. For example, a similar approach to MudPIT was utilized by Dinglasan et al. (2009) to determine PM protein composition for *An. gambiae*. By utilizing an artificial protein-free meal enriched with latex beads for midgut distention and subsequent PM formation, Dinglasan et al. (2009) collected more than 750 PMs for sequential extraction, digestion and identification. In total, 209 PM and PM-associated proteins were identified via a mass spectrometry-based proteomic analysis. While the largest majority of identified proteins were associated with peptidase activity, nine PM proteins isolated in this proteomic analysis contained a secretory signal peptide and one or more chitin binding domains, and

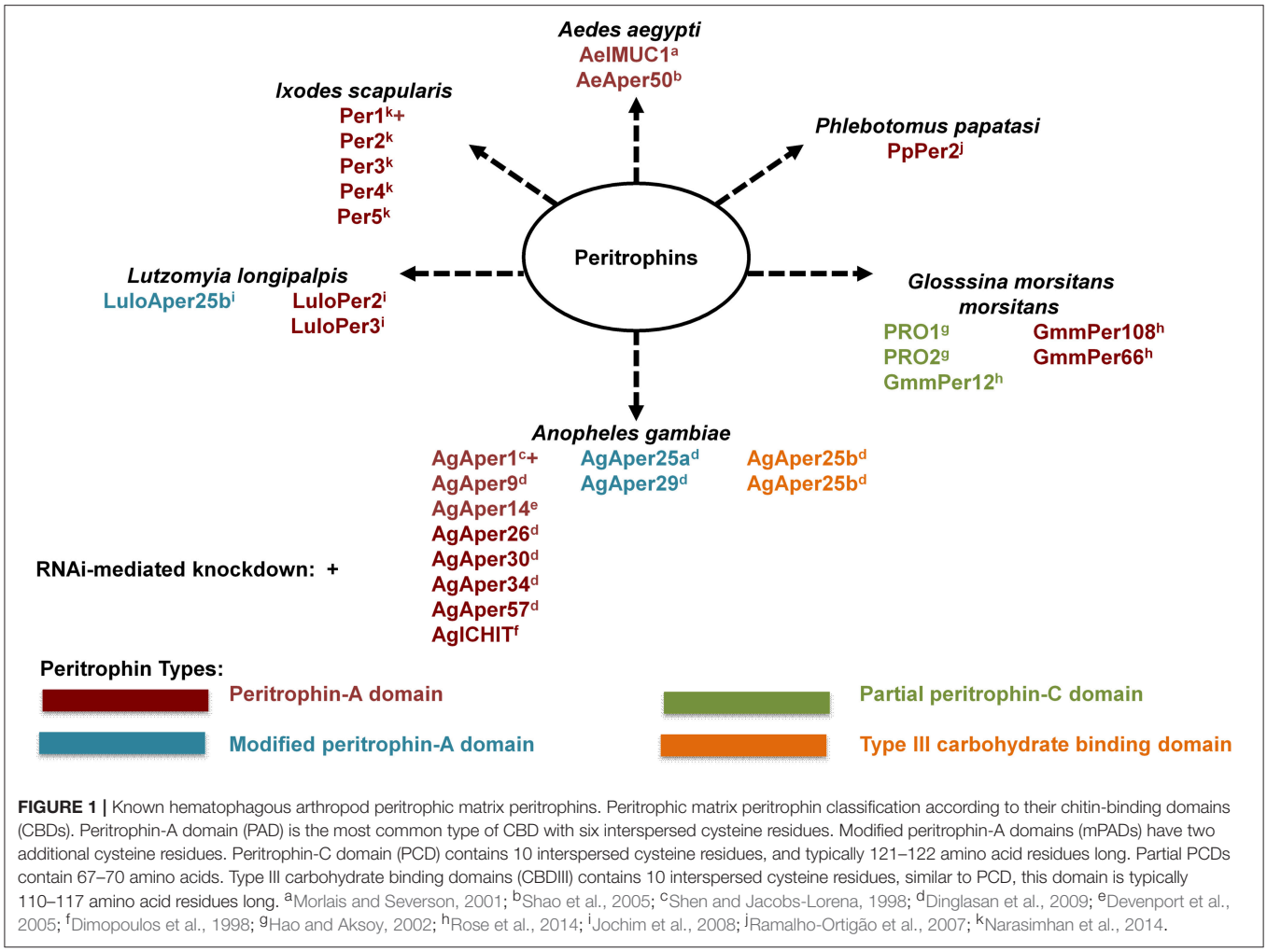

FIGURE 1 | Known hematophagous arthropod peritrophic matrix peritrophins. Peritrophic matrix peritrophin classification according to their chitin-binding domains (CBDs). Peritrophin-A domain (PAD) is the most common type of CBD with six interspersed cysteine residues. Modified peritrophin-A domains (mPADs) have two additional cysteine residues. Peritrophin-C domain (PCD) contains 10 interspersed cysteine residues, and typically 121–122 amino acid residues long. Partial PCDs contain 67–70 amino acids. Type III carbohydrate binding domains (CBDIII) contains 10 interspersed cysteine residues, similar to PCD, this domain is typically 110–117 amino acid residues long. [a]Morlais and Severson, 2001; [b]Shao et al., 2005; [c]Shen and Jacobs-Lorena, 1998; [d]Dinglasan et al., 2009; [e]Devenport et al., 2005; [f]Dimopoulos et al., 1998; [g]Hao and Aksoy, 2002; [h]Rose et al., 2014; [i]Jochim et al., 2008; [j]Ramalho-Ortigão et al., 2007; [k]Narasimhan et al., 2014.

therefore took the total list of identified peritrophins from 3 to 12 in *An. gambiae*. Dinglasan et al. (2009) also proposed a model which detailed *An. gambiae* PM formation and putative interactions among the various types of proteins identified in their study (Dinglasan et al., 2009).

Given the unprecedented level of exploration and discovery of *An. gambiae* PM protein composition, and the relation of the Dinglasan et al. (2009) results to *Ae. aegypti*, another important disease vector, this methodology should be further explored to determine PM protein composition for other hematophagous arthropods of medical importance. To date, only one study with the tsetse fly (*Glossina morsitans morsitans*) has utilized a similar methodology (Rose et al., 2014). Interestingly, this study utilized teneral (unfed) male tsetse flies to determine PM protein composition, as only two PM proteins had been identified previously, but were poorly characterized (Hao and Aksoy, 2002; Rose et al., 2014). Given that adult tsetse flies produce a Type II PM, it is constitutively expressed. Therefore, the requirement of blood meal or artificial meal induction, as seen in Type I PM formation, was found not necessary for PM protein composition analyses. To maximize the number of isolated proteins, the authors conducted in-gel and in-solution tryptic digests using 150 PMs. While their in-gel analysis yielded two highly visible bands at 26 and 21 kDa (identified as midgut trypsin), the most abundant and frequent protein hit was for a novel PM protein, GmmPer6 (Rose et al., 2014). As expected, the in-solution analysis yielded substantially more proteins (minimum of 195). Based on putative function, the largest majority of isolated proteins were associated with oxidation/reduction (17%). In total, five PM peritrophins were isolated in this study (two known and three novel). As in the in-gel analysis, GmmPer66 was one of the most abundant proteins isolate din the in-solution analysis with two other novel PM peritrophins isolated (GmmPer12 and GmmPer108) (Rose et al., 2014).

Despite the identification of these structural components, few studies have utilized reverse genetic approaches such as RNAi mediated knockdown to determine physiological importance of individual peritrophins. *I. scapularis* nymphs were administered dsRNA targeting *peritrophin-1* through their anal pore and subsequently allowed to feed until repletion on *B. burgdorferi*-infected C3H/HeN mice. While peritrophin-1 knockdown nymphs engorged at a comparable rate to control nymphs, knockdown of peritrophin-1 resulted in decreased thickness of the PM and compromised structural integrity (Narasimhan

et al., 2014). Interestingly, knockdown of peritrophin-1 was associated with decreased *B. burgdorferi* adherence/attachment to midgut epithelial cells. A similar effect was seen via RNAi-mediated knockdown of the signal transducer and activator of transcription (*stat)* gene which encodes STAT, the cytosolic component of the Janus kinase (JAK)/STAT pathway (Narasimhan et al., 2014), suggesting a modulatory role of STAT in peritritrophin-1 expression, and role of the intact PM in *B. burgdorferi* spirochetes adherence to midgut epithelium. RNAi-mediated knockdown of *An. gambiae* AgAper1 (most abundant CBD protein from Dinglasan et al., 2009) was used to determine its role in midgut epithelium response to microbiota in adult *Anopheles coluzzii* (Rodgers et al., 2017). Interestingly, knockdown of AgAper1 resulted in an increased immune response and translocation of bacteria from family *Enterobacteriaceae* into the body cavity. Therefore, these findings suggest that the PM of *An. coluzzii* serves as a barrier which blocks or limits dissemination of certain bacteria throughout the mosquito body (Rodgers et al., 2017).

Despite the highly successful strategies deployed by bloodfeeding arthropods to sequester heme during the process of blood digestion, heme and molecular iron are also critical nutrients that must be absorbed during digestion and transported throughout the body. In the following sections, we discuss recent progress on understanding heme and iron transport across cell membranes, as well as trafficking, packaging, signaling, and ultimate deposition into the ovaries for oogenesis, in order to systematically track the fate of blood meal heme and iron in medically important hematophagous arthropods.

HEME UPTAKE ACROSS CELL MEMBRANES

Despite its importance as a nutrient and signaling molecule (Hooda et al., 2014; Bottino-Rojas et al., 2015), surprisingly little is known about heme transport/uptake in arthropods. New developments in this area would seem to be promising targets, particularly since this subject has been explored in many other organisms including mammals, fish, yeast and worms, as moving heme from either its point of synthesis (mitochondria) or from the diet (extracellular space) to the cell cytoplasm involves crossing membranes.

The feline leukemia virus subgroup C receptor (FLVCR1) heme transporter has been characterized in both humans and mice (Quigley et al., 2004; Byon et al., 2013; Vinchi et al., 2014; Philip et al., 2015). Flvcr1a is a 12 transmembrane (TM) domain protein while Flcvr1b is a 6 TM domain protein which is thought to homo/heterodimerize to form a functional transporter. Recently, Mercurio et al. (2015) found that Flvcr1a and Flvcr1b were both required for development of committed erythroid progenitors with Flvr1a exporting heme through the plasma membrane into the extracellular space while Flvr1b exports heme into the mitochondria (Mercurio et al., 2015). In erythroid cells, heme regulation is particularly important because it ensures the balanced production of the globin chain components (Tahara et al., 2004a,b). Experiments using knockout mice showed that Flvcr1a and Flvcr1b both play a role in the expansion of committed erythroid progenitors as production of hemoglobin was reduced but only Flvcr1b is indispensable during terminal erythroid differentiation due to a block at the pro-erythroblast stage (Mercurio et al., 2015). RNAi in human lymphoblast K562 cells showed that the coordinated expression of both isoforms controls the cytosolic free heme pool; Flvcr1a deficiency resulted in cytosolic heme accumulation detrimentally effecting cell proliferation but promoted differentiation, while mitochondrial heme accumulation due to Flvcr1b loss was deleterious to both processes. While Flvcr1 has been characterized in vertebrates, a potential role in arthropod heme transport has not been explored.

In 2008, a new family of heme transporters was identified in the nematode *Caenorhabditis elegans*. Rajagopal et al. (2008) performed a genome-wide microarray analysis to identify genes transcriptionally regulated by heme and found F36H1.5 (hrg-4) and its 3 paralogues, R02E12.6 (hrg-1), F36H1.9 (hrg-5), and F36H1.10 (hrg-6) with only two, hrg-1, and hrg-4, found to be highly responsive to heme deficiency and the only genes that were not nematode-specific. White et al. (2013) identified a human/mouse hrg-1 ortholog localized on the macrophage phagolysosomal membranes in mice by immunofluorescence microscopy. Immunohistochemistry showed high levels of hrg-1 in the macrophages present in the tissues responsible for high levels of recycling of heme iron obtained from degraded red blood cells, the spleen, liver and bone marrow of mice and humans. Knockdown of the gene by siRNA in mice bone marrow-derived macrophages resulted in a reduction of the heme regulatory pool while overexpression increased cellular heme availability. Toh et al. (2015) identified and characterized an hrg-1 ortholog in the blood fluke *Schistosoma mansoni* (Smhrg-1), which had a low sequence identity and homology to the *C. elegans* genes hrg-1 and hrg-4 and was found localized via palladium mesoporphyrin IX fluorescence in the vitelline and ovary regions of the females of the species. However, heme transport by Smhrg-1 was only identified in these organs indicating that the heme transport mechanism after digestion to the vitelline and the ovary regions is not due to Smhrg-1 but some other unknown mechanism. A *Leishmania amazonensis* homolog of the *C. elegans* heme transporter hrg-4, LHR1, was identified by Huynh et al. (2012), and was found to localize to both the plasma membrane and the lysosomes as determined by measurement of GFP-LHR1 fusion proteins in cells via confocal laser fluorescence microscopy (Rajagopal et al., 2008; Huynh et al., 2012). Subsequent failure to generate a full knockout of LHR1 was interpreted to indicate the essential nature of this gene to organism survival. Partial knockout and overexpression resulted in reduced heme uptake and increased uptake respectively as measured by zinc mesoporphyrin (ZnMP). Taken together these three observations indicated that LHR1 accounts for the majority of the heme transport activity in *L. amazonensis*. Topology modeling identified four predicted transmembrane domains and cytoplasmic N- and C- termini in *C. elegans* hrg-1 and hrg-4 as well as LHR1 in *L. amazonensis* indicating similar protein structure between hrg family members in these distant eukaryotes (Rajagopal et al., 2008; Huynh et al., 2012). The fact that members of the hrg gene family are present in

humans, worms and single-cell protozoan parasites suggests that this gene family is very ancient, however orthologs of this gene family in bloodfeeding arthropods have not been described.

Recently, Lara et al. (2015) identified ATP binding cassette subtype B10 (ABCB10) as a heme transporter in the midgut cells of R. *microplus*. Incubation of midgut cells with Rhodamine 123 (a PgP protein transporter substrate), separately or combined with CsA (an ABC inhibitor) confirmed an ABC transporter was responsible for heme transport after a bloodmeal, while an anti-PgP-1 antibody identified the membrane of digestive vacuoles to be its location in the cells. RNAi of the RmABCB10 transporter in female bloodfed ticks and Zinc protoporphyrin IX (ZnPp) fluorescence showed reduced ZnPp in the hemosomes, but more in the digestive vacuoles, which confirmed RmABCB10 as the transporter characterized above. Lara et al. (2015) confirmed previous reports that identified ABC transporters as key components of detoxification of acaricides by showing that Tin protoporphyrin IX and amitraz transport to the hemosome are increased in the amitraz-resistant Ibirapuã strain when compared to wild type (Pohl et al., 2011, 2012, 2014; Lara et al., 2015; Koh-Tan et al., 2016). Mangia et al. (2016) expanded this to *Ixodes ricinus* by examining expression of ABCB1, ABCB6, ABCB8 and ABCB10 after ivermectin treatment in cultured cells (Mangia et al., 2016). The authors found that only ABCB8 showed changes in expression showing a low-dose stimulation but a high-dose return to control levels after exposure the increasing concentration of ivermectin. These results taken together indicate that ABC transporters transport acaricides in the detoxification pathway of multiple tick species with some family members also transporting heme out of the digestive vacuoles. Like FLVCR and HRG genes, orthologs of ABCB10 have not been described to date in other bloodfeeding arthropods.

Pereira et al. (2007) found evidence of heme transport into the midgut epithelium cells in *Ae. aegypti* and export of its degradation product biglutaminyl-biliverdin back into the lumen for excretion. The authors observed a change in color from red to green during bloodmeal digestion and upon analysis identified it as a bilin pigment. Heme degradation occurs via the cytosolic heme oxygenase reaction, thus heme must enter the midgut epithelium to be degraded and then the biliverdin biproduct must be secreted, although the mechanisms behind the transport of these 2 molecules are unknown. RNA sequencing following heme exposure in *Ae. aegypti* cultured cells identified several potential transport-related proteins that could be involved in this transport mechanism (Bottino-Rojas et al., 2015), however their activity has not been characterized to date. Although, strong evidence of the capacity of heme transport is available for bloodfeeding arthropods, only 1 transporter, RmABCB10 had been characterized to date.

HEME CATABOLISM

After heme is imported into the cell, a conserved heme degradation pathway is present in many organisms, during which heme is broken down into biliverdin IX α (BV α), iron and CO by heme oxygenase (HO) (for recent reviews, see Wegiel et al., 2014; Wilks and Heinzl, 2014). However, at least two bloodfeeding arthropods, *Aedes aegypti* and *Rhodnius prolixus*, deviate from this pathway as discussed below. In addition, many species of ticks have completely lost the ability to breakdown heme due to absence of key enzymes in the heme degradation pathway in their genomes (Braz et al., 1999; Paiva-Silva et al., 2006; Pereira et al., 2007; Perner et al., 2016). In *Ae. aegypti*, after BV IX, iron and CO are produced, two glutamine residues are added, as determined by electrospray ionization mass spectrometry (ESI-MS) (Pereira et al., 2007). A BV IX product with one glutamine residue was isolated with the final product in reverse phase HPLC indicating that the glutamines were sequentially added instead of simultaneously. This addition to BV IX yields a more soluble product, and was thought to reduce toxicity caused by the accumulation of the heme byproducts in the midgut by allowing for easier excretion (Pereira et al., 2007). The formation of a green pigment identified as biglutaminyl BV IX in the lumen indicates that this byproduct must exit the midgut epithelium cells to be eventually excreted, although the mechanisms behind the transport are unknown. This mechanism is so far unique to *Ae. aegypti*, the pathways utilized by other bloodfeeding dipterans are currently unknown.

In all cases previously reported, heme is broken down by HO prior to any modifications to the porphyrin ring, however the kissing bug contains a unique pathway in which two cysteinylglycine residues are added before oxidative cleavage of the porphyrin ring yielding the end product of dicysteinyl-BV IX ϒ (RpBV) as determined by ESI-MS (Paiva-Silva et al., 2006). While the biological advantage to these added residues is not clear, Paiva-Silva et al. (2006) hypothesized this change in structure is also enacted to increase solubility of the byproducts, easing excretion as confirmed by its elution with a more hydrophilic retention time during reverse-phase HPLC than BV IX α. Also, while RpBV has been identified as the end product, the method of residue addition and the order added are unknown. In mammals, all the degradation products produced by heme play key regulatory roles, for example CO has both anti-inflammatory and anti-apoptotic effects and BV may function as antioxidants, however these roles have not been explored in insects to date (Stocker et al., 1987; Doré and Snyder, 1999; Otterbein et al., 2000; Brouard et al., 2002; Sedlak and Snyder, 2004; Stocker, 2004; Al-Owais et al., 2015). Given at least two variations on heme degradation pathways in arthropods have been described so far, it is likely that there are more not yet discovered. **Figure 2** details the proposed mechanisms of the *Ae. aegypti* and *R. prolixus* heme degradation pathways.

In *Drosophila melanogaster*, heme oxygenase expression is necessary for the normal development of tissues. Whole body knockdown of HO in larval and pupal flies results in lethality, evidence of the necessity of heme-iron recycling in tissue development (Cui et al., 2008). Cui et al. (2008) observed the effects of tissue specific HO-knockdown utilizing the GMR-GAL4 driver system to target the eye imaginal disks which resulted in abnormal development of the adult eye tissue. Immunostaining the eye tissue of HO knockdown larvae revealed high concentrations of activated caspase-3, an apoptotic marker,

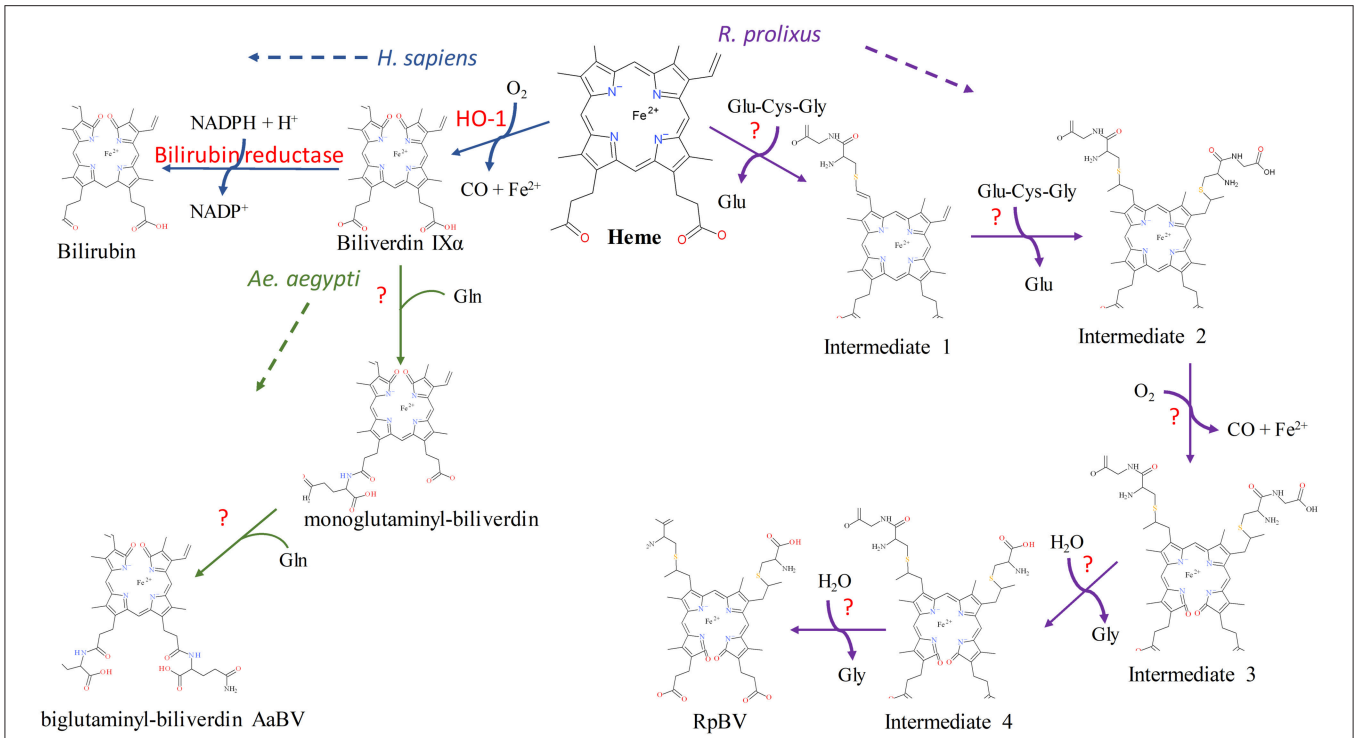

FIGURE 2 | Known heme degradation mechanisms. The arthropods *Ae. aegypti* and *R. prolixus* have alternate heme degradation mechanisms from the shared mechanism of humans, mice and many other organisms. All three mechanisms are detailed above. Enzymes utilized for each reaction are given in red, with unknown enzymes indicated (?). The order in which the modifications are sequentially added to the intermediates in both the *Ae. aegypti* and *R. prolixus* mechanisms is not known, however only one method is detailed here for simplicity. The structural drawings shown here were made using BIOVIA 2017 R2 (Dassault Systemes, San Diego).

as well as larger than normal iron deposits, both of which were thought to contribute to the rough eye phenotype observed. This observed phenotype was later linked to G1/S arrest of the cell cycle leading to cell death due to increased generation of reactive oxygen species (Ida et al., 2013). Ida et al. (2013) also observed a significant drop in proliferating cells and an increase of DNA damage detected in the eye imaginal disks of the larva after treatment with HO dsRNA. Ida et al. (2013) performed a genomic screen leading to the identification of eight genomic regions that suppressed the observed rough eye phenotype during HO knockdown. This indicates that specific genes on these isolated regions may interact with HO and help counteract its loss during a knockdown event, however further work is needed to isolate specific genes. Damulewicz et al. (2017b) found that *ho* is a clock-controlled gene as it oscillates in expression during the day peaking at the beginning of the light phase and in the middle of the night. One of the reasons behind this pattern of oscillation was due to HO's protection of the retina photoreceptors against ROS-induced degradation brought on during the transition of the night to day phase at the start of UV and white light exposure. DNA damage was reduced when HO was activated by hemin and increased when HO activity was inhibited by Sn PP (Damulewicz et al., 2017a,b). The decline in HO regulated two different canonical clock genes *period* and *clock*, increasing and decreasing respectively. This regulation is potentially mediated through CO as an increase in CO shows the same effect as the increase of HO expression (Damulewicz et al., 2017b). In conclusion, the results of the loss of HO in *Drosophila* tissues and whole body indicate that HO is very important for the successful development of these tissues. However, HO expression and its regulation of DNA damage response and canonical clock genes has not been examined in other insects. This would be particularly important to study in bloodfeeding arthropods as their ingestion of blood yields a much more iron rich diet than flies.

IRON TRANSPORT ACROSS CELL MEMBRANES

Iron-responsive genes including those implicated in membrane bound iron transport have been identified in multiple organisms, including arthropods (for recent reviews, see Galay et al., 2015 and Mandilaras et al., 2013). A recent study in *G. morsitans* for example, identified 150 iron-responsive genes, only two of which were previously identified, ferritin heavy chain and mRCK-alpha (Tang and Zhou, 2013b; Dashti et al., 2016). These genes were predicted by computational analysis of UTR regions searching for the characteristic stem loop structures present in iron regulatory element (IRE) regulated genes.

Of these genes, 29 had functions potentially related to iron trafficking, including cell envelope, transport and binding proteins that localized in the extracellular environment or the plasma membrane. Genes involved in iron trafficking are particularly important for bloodfeeding arthropods as the blood meal contains a huge influx of iron in the form of transferrin and hemoglobin, 1884.8 ng iron per female on average (Zhou et al., 2007).

Three genes in *D. melanogaster* have been identified as playing a role in membrane bound iron transport, *malvolio* (*mvl*), *zip13*, and *mco1*. Mvl is a homolog of the human natural resistance-associated macrophage proteins (*Nramp*). Both loss and gain-of-function *mvl* mutants in adult flies resulted in altered taste perception, particularly sugar and salt perception (Rodrigues et al., 1995). Folwell et al. (2006) found high expression of *mvl* in adult and larval midgut tissue as well as in the Malpighian tubules, while elucidating its role in iron acquisition at multiple developmental stages and its role in divalent cation reabsorption (Rodrigues et al., 1995; Folwell et al., 2006). Subsequently, an ortholog of *Nramp/mvl* was also characterized in the mosquito *Anopheles albimanus* (Martínez-Barnetche et al., 2007). This group determined that *anaNramp* localized to the head, midgut, Malpighian tubules and ovaries, the highest expression of which was in the Malpighian tubules (Martínez-Barnetche et al., 2007). These authors confirmed the role of *anaNramp* in Fe^{2+} transport by inducing full length cDNA expression in Xenopus embryos and measuring $^{59}Fe^{2+}$ isotope incorporation. An examination of the *anaNramp* 5′ and 3′ UTR sequences indicated that unlike *mvl*, no iron responsive elements (IRE) were present suggesting *anaNramp* may not be regulated by cytoplasmic Fe concentration. Few arthropods have been characterized that contain *h-Nramp* homologs, those that do contain the gene require further study as the regulation method behind their iron transport is unidentified.

Xiao et al. (2014) recently characterized the Drosophila ortholog of the human zinc iron permease 13 (*zip13*), an iron efflux pump that moves iron from the cytosol to the ER/Golgi (Xiao et al., 2014). RNAi knockdown of *dzip13* resulted in iron deficiency in the entire body of the fly with a reduction of about 50% of wild type iron levels except for iron in the cytosol of the gut cells which showed higher than wild type iron levels. On the other hand, *zip13* overexpression in the midgut resulted in increased iron content throughout the body. The authors then examined *ferritin* and *malvolio* as examples of genes involved in iron metabolism that could act as additional indicators of cytosolic iron levels. They found that when *zip13* expression was knocked down, ferritin expression increased and an Mvl expression decreased, with the opposite expression patterns observed when *dZip13* was overexpressed. The overexpression results matched ferritin and Mvl levels observed when larvae were fed an iron-supplemented diet indicating ferritin and Mvl expression levels seem to be a good indicator of cellular iron levels in Drosophila. With the exception of d*zip13* and *malvolio* no other iron transporters have been identified in *Drosophila*. While orthologs of *dzip13* and *malvolio* are present in many bloodfeeding arthropods, involvement in iron transport hasn't been confirmed in any to date, with the exception of *anaNramp* described above.

The multicopper oxidase enzyme family includes oxidases that target different types of substrates including iron, copper, ascorbic acid and bilirubin (Sakurai and Kataoka, 2007). dMCO1, while not an iron transporter, facilitates transport by acting as a ferroxidase at the membrane which oxidizes reactive aqueous ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) in the hemolymph allowing its binding to transferrin and similar iron binding proteins for transport in *D. melanogaster*. Lang et al. (2012) found dMCO1 localized to both the digestive system and Malpighian tubules, specifically the basal surfaces of each using RT-PCR on organ extracts to calculate expression of the transcript, followed by immunostaining tissues to visualize its location (Lang et al., 2012). dMCO1's role in iron homeostasis was confirmed by RNAi knockdown experiments yielding increased longevity when flies fed on high iron food and a decreased iron accumulation in the body. The MCO gene family is conserved in coleopterans, lepidopterans and dipterans (Lang et al., 2012; Liu et al., 2015; Peng et al., 2015; Ye et al., 2015). *An. gambiae* contains 5 putative multicopper oxidases, with AgMCO1 considered the functional ortholog to dMCO1, though experimental confirmation of this is lacking (Gorman et al., 2008). Very few putative MCO enzymes have been characterized to date in bloodfeeding arthropods and those that do contain these enzymes, require further work to identify which substrate they target and whether or not they are involved in iron metabolism.

IRON CHAPERONES

Metallochaperones facilitate metal ion storage in target proteins via specific protein-protein interactions at their docking surface tuned to recognize these partner proteins (Rosenzweig, 2002). Iron metallochaperones could be particularly important to iron homeostasis as they bind and safely transport ferrous iron around the cell, preventing oxidative damage from occurring (Philpott et al., 2017). Four cytosolic iron metallochaperones were discovered in humans that are thought to aid in ferritin iron loading: human poly(rC)-binding protein 1 (PCBP1) and its paralogues PCBP2, PCBP3, and PCBP4. When either PCBP1 or PCBP2 and ferritin are expressed in yeast cells, the amount of iron loaded into ferritin drastically increased when compared to ferritin expression by itself (Shi et al., 2008; Leidgens et al., 2013). This was confirmed by RNAi knockdown of PCBP1, PCBP2 or both proteins in human cultured cells and observation of the amount of ^{55}Fe incorporated into endogenous cytosolic ferritin, which led to similar reductions in iron uptake into ferritin compared to control cells indicating that both proteins are needed independently for efficient delivery of iron to ferritin (Shi et al., 2008; Leidgens et al., 2013). The other two family members, PCBP3 and PCBP4 also showed increased iron loading into ferritin (Leidgens et al., 2013). PCBP2 was also shown to interact with NRAMP2 via its cytoplasmic N-terminal region and with ferroportin, the main ferrous iron exporter, lending further credence to its function as an iron chaperone (Yanatori et al., 2014). Interestingly, HO1 was found

to complex with PCBP2 but not any of its paralogues (PCBP1, 3 or 4) or the NADPH-cytochrome P450 reductase (CDR) complex competitively, with PCBP2 affinity to HO1 seeing a significant reduction when in heme loaded cells or when PCBP2 is bound to ferrous iron (Yanatori et al., 2017). While these proteins have been characterized in humans, orthologs proteins have not been characterized in any arthropod species to date. If orthologs of the PCBP family of proteins exist in bloodfeeding arthropods, these could indicate a possible missing link in known mechanisms related to iron homeostasis as they may be involved in ferrous iron transport through the midgut epithelium cells, thus preventing the oxidative damage associated with free ferrous iron in the cell.

IRON PACKAGING AND FERRITIN SHUTTLING

Free iron can enter the cytosol through direct transport from outside the cell or be released internally following heme catabolism. Fe^{2+} is the reactive soluble form of iron while Fe^{3+} is both unreactive and insoluble. Ferritins chaperone and transport iron preventing large concentrations of cytosolic Fe^{2+} which can easily react with lipids, proteins and other cellular components causing oxidative damage. The Ferritin-like superfamily is present in many organisms including arthropods, with all individual members thought to be evolved from a rubrerythrin-like ancestor which played a role in the defense against reactive oxygen species (Andrews, 2010). Ferritin is typically composed of 24 subunits, which fold to create a large cavity that can store 1,500 + Fe^{3+} molecules as well as smaller amounts of other metals like zinc and magnesium (Gutiérrez et al., 2013). Insects have two ferritin subunits, very similar to vertebrate ferritin subunits, heavy-chain homolog (HCH) and light-chain homolog (LCH) (Pham and Winzerling, 2010). HCH, like dMCO1, also has ferroxidase activity and thus catalyzes the oxidization of Fe^{2+} to Fe^{3+} for storage in ferritin; LCH is involved in iron core formation. In most insects, ferritin contains a signal peptide directing it to the endoplasmic reticulum for translation where it stays until iron loaded ferritin is exported out of the cell via secretory vesicles (Nichol et al., 2002; Dunkov and Georgieva, 2006; Pham and Winzerling, 2010). As ferritin loading occurs in the ER and not the basal surface of the midgut epithelial membrane, dMCO1's ferroxidase activity is not utilized to facilitate loading of the iron into ferritin. Excellent reviews are available covering insect ferritins (Dunkov and Georgieva, 2006; Pham and Winzerling, 2010; Tang and Zhou, 2013b), so they are only mentioned briefly here.

Loss of ferritin following gene knockdown or knockout resulted in growth abnormalities ending in death in *D. melanogaster* (González-Morales et al., 2015). Lack of iron present in embryos either due to knockout of ferritin or lack of maternally derived iron also resulted in serious abnormalities often culminating in death during early development. When midgut specific knockdown was performed in *D. melanogaster*, iron accumulated in the iron cell region while systematic iron deficiency was observed throughout the rest of the body, confirming the importance of ferritin in serving as a transport mechanism (Tang and Zhou, 2013a). However, the exact mechanism of ferritin iron transport to non-intestinal tissues after export out of the midgut epithelium remains unknown, as while labeled ferritin or its substrate iron has been identified in multiple tissues across both *D. melanogaster* and *Ae. aegypti* their entry method into other cellular tissues remains uncharacterized (Zhou et al., 2007; Li, 2010; Tang and Zhou, 2013a).

In mosquitoes and other bloodfeeding arthropods ferritin is particularly important due to the large influx of iron they obtain during a blood meal. In *Ae. aegypti*, both ferritin subunits, HCH and LCH, increase in expression after an iron overload or a blood meal, indicating that ferritin may serve as a cytotoxic protector (Dunkov et al., 2002; Geiser et al., 2003). In mosquitoes, different cell types handle ferritin iron storage in different ways. Geiser et al. (2015) found that CCL-125 *Ae. aegypti* larval epithelial-like cells and 4a3b *An. gambiae* larval hemocyte-like cells experienced high levels of iron uptake using Calcein fluorescence assays upon exposure to high levels of iron (Geiser et al., 2009; González-Morales et al., 2015). However, the inductively coupled plasma mass spectrometry (ICP-MS) they performed showed low levels of cytoplasmic free iron present indicating that upon uptake, iron is immediately bound to proteins like ferritin and secreted (Geiser et al., 2009; González-Morales et al., 2015). When comparing the two cell types examined, Geiser et al. (2015) noted that after packaging of iron into ferritin occurred, the CCL-125 cells exported the ferritin out of the cell while 4a3b cells retained it. They speculated that since hemocyte cells are involved in the immune response, hoarding an essential nutrient like iron is likely done to prevent foreign cells access to it providing a more hostile environment for their growth. This immune response is observed in both *Ae. aegypti* and *Bombyx mori* (silkworm), when ferritin is upregulated during bacterial infection lending evidence that host-bacteria regulatory mechanisms involving ferritin production do exist in these organisms (Geiser et al., 2013; Otho et al., 2016). However, the signaling mechanism which determines when secretion occurs or which cell types secrete iron loaded ferritin or retain it has not been identified to date.

Ticks and insects have evolved different mechanisms to ensure free heme is dealt with during digestion, however they both utilize ferritin to package and transport the molecular iron absorbed from the blood meal (Donohue et al., 2009). Recently, Galay et al. (2013, 2014) characterized two distinct ferritins in the hard tick, *Haemaphysalic longicornis*. Hlfer1 and Hlfer2 both have unique functions: storage of iron in the midgut, and secretion from the midgut for transport to other organs with the subunit composition of each potentially controlling their different functions (Galay et al., 2013). Unlike *hlfer1*, the sequence of *hlfer2* lacks an IRE, indicating that it may not be regulated by cytoplasmic Fe concentration, and contains a signal peptide much like the secretory ferritin characterized by Hajdusek et al. (2009) in the hard tick *Ixodes ricinus* (Hajdusek et al., 2009; Galay et al., 2013). RNAi experiments showed the importance of both ferritins present to successful feeding and reproduction in both *H. longicornis* and *I. ricinus*. While intestinal cell types have been studied extensively, knowledge of

the regulation of ferritin subunits in non-intestinal cell types and whether multiple subunits work together in different situations is lacking.

Transferrin is an iron binding glycoprotein that is also conserved between vertebrates and arthropods. Unlike ferritin, transferrin can only carry two molecules of Fe^{3+} at a time. In mammals, transferrin is primarily utilized in iron transport in the blood to bring iron to the erythrocytes to be utilized in heme production (Zhang and Enns, 2009). *D. melanogaster* transferrin and *Ae. aegypti* transferrin 1 were both found to be expressed in larval, pupal and adult stages but not in embryos, with expression in *Ae. aegypti* found mainly in the fat body where it is secreted into the hemolymph with high levels of juvenile hormone acting as a negative regulator of its expression (Yoshiga et al., 1999; Harizanova et al., 2005). A second transferrin gene was described in *Ae. aegypti* with weaker iron binding due to key amino acid mutations in the binding pocket as compared to other members of the transferrin family (Zhou et al., 2009). Expression of both genes differ in the adult female mosquitoes, with *AaTf1* expression highest at 24 h post bloodmeal and *AaTf2* expression highest at 72 h post bloodmeal compared to sugar fed females (Zhou et al., 2009). This difference in expression suggests distinct roles in iron metabolism, however the precise role these genes play remains to be determined. Bacterial infection of *Ae. aegypti* also increases transferrin expression, particularly of *AaTf1*, suggesting that *AaTf1* may play a role in sequestering iron during pathogen infection (Zhou et al., 2009). Other studies have also shown transferrin upregulation in mosquito host response to pathogen infection, particularly *Wuchereria bancrofti*, in *Ae. aegypti* and *C. quinquefasciatus* females (Paily et al., 2007; Magalhaes et al., 2008). An early genome survey by Dunkov and Georgieva (2006) identified members of the transferrin family in several insect species; including four in *An. gambiae* and *Ae. aegypti*. However, little is known about the biological role or specialization of these transferrin genes. In summary, transferrin has been identified as an important regulator of iron homeostasis during blood digestion, larval and pupal development and bacterial infection in arthropods. However, most of the underlying data derives from inferred similarity with vertebrate transferrins. The exact signaling mechanism that activates transferrin expression upon pathogen infection has not been identified nor has the role of transferrin as an iron transporter been extensively studied in bloodfeeding insects.

HEME/IRON SIGNALING

In addition to its role as a nutrient, heme acts as a signaling molecule triggering many biological pathways. In *Ae. aegypti* cultured cells, heme was found to regulate the expression of several hundred transcripts, including those associated with redox stress, metabolism and transport related proteins, suggesting the existence of distinct signaling pathways regulated by heme (Bottino-Rojas et al., 2015). Analysis of these genes found that several immune genes were downregulated in response to heme exposure, indicating that the exposed cells may be more susceptible to immune challenge. This was confirmed both *in vitro* and *in vivo* by introduction of *E. cloacae* to heme incubated cultured cells and by orally challenging heme-fed females with *Serratia marcescens* resulting in diminished expression of immune genes both in cells and the midgut and a 2-fold increase in microbial growth. However, the mechanism by which heme is effects the immune pathways and the molecular mechanisms behind certain induced genes are not currently well understood.

While the role of heme signaling in regulating the immune response of bloodfeeding arthropods is just being appreciated, the role of heme in regulating reproduction in bloodfeeding mosquitoes is well established. In the arthropods *D. melanogaster* and *Ae. aegypti,* heme binding to the nuclear receptor E75 can result in the activation of the steroid hormone 20-hydroxyecdysone (20E), a key component in molting, metamorphosis and vitellogenesis (Segraves, 1994; Thummel, 1996; Kokoza et al., 2001; Martín et al., 2001). Cruz et al. (2012) found that heme was required for mediating 20E action via its stabilization of E75, implying its role as a signaling molecule to indicate the availability of a blood meal for vitellogenesis. E75's ligand binding pocket was characterized by Reinking et al. in *D. melanogaster*, where a single heme molecule was identified as a tightly bound prosthetic group (Reinking et al., 2005). The *Ae. aegypti* ortholog, like its *Drosophila* counterpart, has three isoforms, all three of which are vital to the regulation of multiple genes (Segraves and Woldin, 1993; Pierceall et al., 1999; Cruz et al., 2012). Aicart-Ramos et al. (2012) examined the heme binding of E75 hemoproteins in four different species of insect, *D. melanogaster, B. mori, O. fasciatus* and *B. germanica*. In the first two insects, heme is only tightly bound while in the latter two it is covalently attached (Reinking et al., 2005; Aicart-Ramos et al., 2012). In all four insects, heme is so tightly bound that E75 could not be purified without it. Reinking et al. (2005) also described E75 as a potential heme sensor, because upon treatment of cultured cells with an increased concentration of heme, the cells induced up to 8-fold increase of E75 expression indicating that the expression of E75 is directly proportional to the available heme level. Aicart-Ramos et al. (2012), however disagreed with this definition, stating that heme's tightly bound nature precludes it from acting as a heme sensor, since a heme sensor must be able to reversibly bind heme (for a detailed review of heme sensor proteins see Girvan and Munro, 2013). However, this appears to be a disagreement concerning the definition of a heme sensor, not experimental evidence disproving the evidence collected by Reinking et al. (2005), Aicart-Ramos et al. (2012), and Reinking et al. (2005).

The regulation of E75 by·NO and CO, common in many organisms, occurs when either gas binds the heme-E75 complex, which blocks the heme active site and prevents binding to E75's other regulatory targets (for detailed reviews on NO and CO signaling see Gullotta et al., 2012; Farrugia and Szurszewski, 2014; Jeffrey Man et al., 2014). Therefore, E75 is likely to act as a cellular oxidative state sensor since these gaseous molecules can only bind to ferrous iron not ferric, thus binding would only occur when the cellular state was reduced enough to allow the heme iron to be in its ferrous state (**Figure 3**). To determine E75's status as a

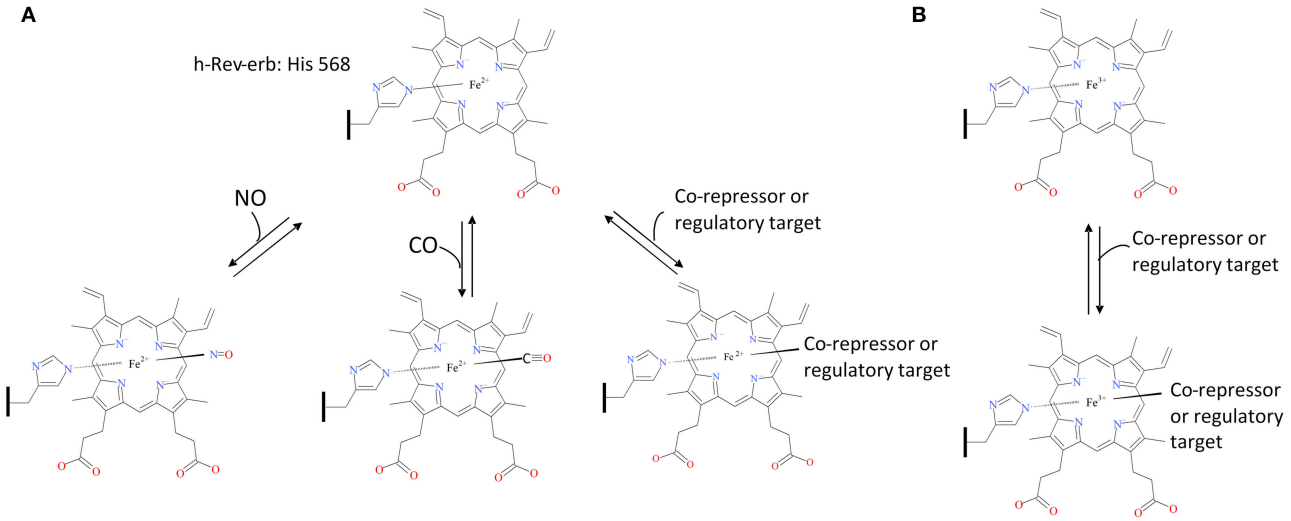

FIGURE 3 | E75-Heme as a cellular oxidative state sensor. The human ortholog of insect E75, h-Rev-erb, acts as a carbon monoxide and nitric oxide sensor when the cell exists in a reducing environment, inhibiting binding of Rev-erb to its co-repressors and regulatory targets **(A)**. Rev-erb's gas sensor ability is not present during a cellular oxidizing environment allowing uninhibited binding to its co-repressors and regulatory targets **(B)**. The structural drawings shown here were made using BIOVIA 2017 R2 (Dassault Systemes, San Diego).

redox sensor, further work must be completed to determine if the cellular environment can actually affect the binding capacity of the protein.

THE ROLE OF HEMOLYMPH HEME AND IRON-BINDING PROTEINS: HEME, IRON AND OOGENESIS IN ANAUTOGENOUS ARTHROPODS

In addition to the iron carrier proteins, transferrin and ferritin, many arthropods utilize heme/iron carrier proteins which chaperone maternal heme/iron to the developing eggs. This is especially important in ticks, as many species lack key enzymes in the heme synthesis pathway requiring heme to be acquired entirely exogenously (Braz et al., 1999; Perner et al., 2016). Perner et al. (2016) showed that hemoglobin was not necessary for egg production, but was essential for embryo survival in *Ixodes scapularis* (Perner et al., 2016). Female ticks were fed with whole blood or hemoglobin-free serum; eggs were laid by both sets, however only those laid by the bloodfed females hatched and developed normally. Hemoglobin was confirmed as the critical factor by performing rescue experiments with females fed on serum +10, 1, and 0.1% hemoglobin added prior to the rapid engorgement phase which showed that as little as one hundredth of the physiological concentration of hemoglobin was sufficient to rescue tick reproduction. Likewise, Perner et al. (2016) demonstrated that IrCP3 is the major heme-binding protein in *Ixodes ricinus* hemolymph (Perner et al., 2016). Expression profiling over *I. ricinus* developmental stages and tissues revealed that *ircp3* mRNA was consistently up-regulated by bloodfeeding and was predominantly expressed in the trachea-fat body complex, and to a lesser extent, in salivary glands and ovaries of adult females. The authors also confirmed IrCP3 as the most abundant protein in tick hemolymph via SDS PAGE and Western blot analysis. Interestingly, the hemolymph lipoglycoheme-carrier protein from the American dog tick, *Dermacentor variabilis,* was also found to bind heme suggesting that *D. variabilis* carrier protein may function to sequester heme derived from the digestion of the blood meal (Gudderra et al., 2001). Likewise, through spectrophotometric titrations, Maya-Monteiro et al. (2000) found an analogous heme lipoprotein (HeLp) to be a heme-binding protein abundant in the hemolymph of male and female *R. microplus* (To determine if HeLp is involved in extracellular transport, specifically to the ovaries, ^{55}Fe-heme-HeLp was injected into the hemocoel of female ticks. Decreased radioactivity was seen in the hemolymph of injected females by 210 min. Most interestingly, by 4 h after hemocoel injection, radioactivity was found associated with the ovaries of female *R. microplus*. However, *R. microplus* females maintained at 4°C after injections lacked clearance of ^{55}Fe-heme-HeLp from the hemolymph or incorporation into the ovaries. Therefore, this suggests active metabolism is involved (Maya-Monteiro et al., 2000). Vitellin (VN), the main yolk protein, has also been associated with heme-binding function in *R. microplus* ovaries (Logullo et al., 2002). Through heme-binding assays with purified VN, Logullo et al. (2002) demonstrated that a single VN bound 30 or more heme molecules. Given that both HeLp and vitellin have been ascribed heme-binding functionally and associated with egg development in *R. microplus*, this raises the questions as to whether these two proteins are necessary and sufficient to provide the developing *R. microplus* egg with heme reserves.

Walter-Nuno et al. (2013) found that the *R. prolixus* heme binding protein (RHBP) is an essential transporter of heme to the embryos. RNAi mediated knockdown of RHBP did not alter

fecundity, however the resultant eggs were not viable and were white in color. This is in contrast to the dark red embryos oviposited by controls. Taken together, the above studies shed light on the sheer importance of heme-binding and iron-binding proteins in terminal incorporation of heme and iron into the developing eggs of hematophagous arthropods, in particular ticks, which lack the *de novo* heme synthesis pathway. Whether corresponding heme carrier proteins are utilized by bloodfeeding arthropods that maintain the ability to synthesize heme remains unknown.

CONCLUSIONS

Although many arthropods have evolved the ability to feed on blood, relatively little insight into the internal processing of blood meal heme and iron is available. Much of the available information regarding iron processing in arthropods, more specifically insects, is gleaned from *D. melanogaster*. While certain life history traits make *D. melanogaster* a powerful model organism, they do not bloodfeed, highlighting the need to perform direct experimentation on the bloodfeeding arthropods.

Through this review, we provide a framework for future studies aimed at obtaining a better understanding as it relates to the fate of heme and iron processing in hematophagous arthropods (**Figure 4**). With recent advancements in scientific tools such as high-throughput protein and RNA analysis, as well as CRISPR/Cas9, we are optimistic that many questions related to the detoxification, acquisition, transport and ultimate fate of iron/heme in bloodfeeding arthropods can be effectively addressed. Below we have highlighted some key take away points and scientific knowledge gaps that await tackling.

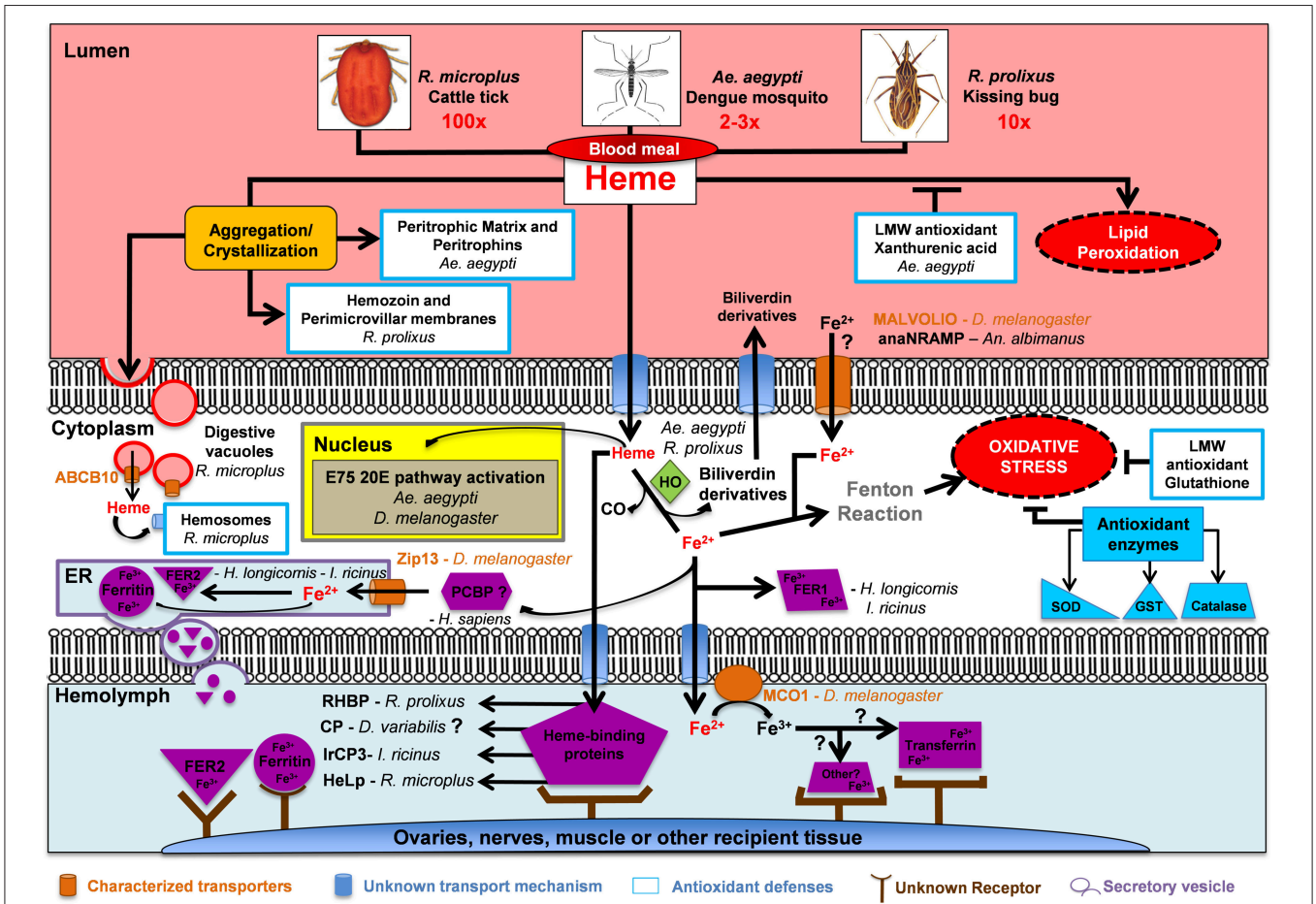

FIGURE 4 | Fate of blood meal heme and iron in selected hematophagous arthropods. Hematophagous arthropods ingest a large amount of blood during a single meal (2-3x, 10x, and 100x their normal body weight for *Ae. aegypti*, *R. prolixus*, and *R. microplus*, respectively). Hematophagous arthropods deploy a multifactor mechanism to avoid oxidative stress after a blood meal. This multifactor mechanism includes: (1) Aggregation/crystallization of heme for excretion; (2) Transport of heme and iron across plasma membranes; (3) Intracellular degradation of heme by Heme Oxygenase (HO); (4) Enzymatic (shown in dark blue) and non-enzymatic low molecular weight (LMW) antioxidant molecules; and (5) Heme and iron carrier proteins (shown in purple) which deposit these two molecules into ovaries, nerves, muscle or other recipient tissue. Abbreviations: *Anopheles albimanus* natural resistance-associated macrophage protein (anaNRAMP), ATP binding cassette subtype B10 (ABCB10), carrier protein (CP), endoplasmic reticulum (ER), ecdysone-induced protein 75 (E75), Ferritin (Fer), glutathione S-transferase (GST), heme lipoprotein (HeLp), *Ixodes ricinus* carrier protein 3 (IrCP3), multicopper oxidase 1 (MCO1), poly(rC)-binding protein (PCBP), *Rhodnius prolixus* heme binding protein (RHBP), superoxide dismutase (SOD), zinc iron permease 13 (Zip13), 20-hydroxyecdysone (20E).

- Given that catalase appears to be a key antioxidant in both midgut and ovarian oxidative stress avoidance for many hematophagous arthropods, further studies targeting this enzyme are needed to assess its potential for disrupting the midgut and ovarian oxidative balance on a larger scale when hematophagous arthropods take a blood meal.
- Heme aggregation/crystalization appears to be a common first line of defense in many hematophagous arthropods. Better understanding of this multifaceted protection playbook for each hematophagous arthropod can provide insight into key targets for breakdown of this system.
- In 2009, Dinglasan et al. set the stage for midgut PM protein composition exploration and discovery utilizing a highly sensitive MS-based approach. The success of this methodology was largely associated with the availability of an artificial protein-free meal. Such protein-free artificial meals for other hematophagous arthropods are needed to allow similar exploration of related and divergent PM structures.
- While peritrophic matrix peritrophins have been identified in many hematophagous arthropods, only two PM peritrophin has been knocked-down using RNAi (APER1 in *An. coluzzii* and peritrophin-1 in *I. scapularis)*. Further studies utilizing reverse genetic tools, such as RNAi and CRISPR/Cas9 are needed to determine the physiological function of PM peritrophins in medically important hematophagous arthropods.
- To date, only one quantitative analysis tracking the fate of blood meal iron in a hematophagous arthropod (*Ae. aegypti*) has been conducted (Zhou et al., 2007). A detailed road map for blood meal iron trafficking in other hematophagous arthropods is needed.
- Heme digestion and utilization regardless of its source, biosynthesis pathway or intake through diet, requires heme to pass across cell membranes. Despite, the characterization of membrane bound heme transport in vertebrates and nematodes, only one transporter has been characterized in a bloodfeeding arthropod to date indicating further work is needed to identify the heme import mechanisms utilized in these organisms.
- Heme catabolism by heme oxygenase 1 to biliverdin IX α, iron and CO is a conserved process in many organisms, however *Ae. aegypti* and *R. prolixus* deviate from this standard and produce alternate bilin pigments, thus there may be more deviations from the known standard yet to be discovered in other bloodfeeding arthropods.
- Heme oxygenase was shown to be essential to *D. melanogaster* tissue development as it regulates the DNA damage response and specific canonical clock gene expression. However, heme oxygenase's role in these two pathways has not been explored in bloodfeeding arthropods despite their diet consisting of a much more iron rich diet than flies.
- In *D. melanogaster*, three genes have been identified as membrane bound iron transporters or facilitators of this process, *mvl*, *zip13* and *mco1*. While orthologs of both *mvl* and *zip13* do exist in bloodfeeding arthropods, their involvement in iron transport has yet be examined in any to date, with the exception of *anaNramp*.
- The human poly(rC)-binding proteins (PCBP1-4) facilitate iron loading of ferritin. Homologues of these proteins in bloodfeeding arthropods could represent a missing link in iron homeostasis in these organisms.
- While ferritin or its substrate, dietary iron, has been localized in multiple tissues in both *D. melanogaster* and *Ae. aegypti* the mode of entry and recovery of iron from ferritin into cells and tissues remains uncharacterized.
- Transferrin, another conserved iron binding transport protein between vertebrates and arthropods, is found in many bloodfeeding arthropods. While transferrins have been shown to be upregulated after a bloodmeal or during pathogen infection, little is known about the biological role or specialization of these transferrin genes.
- Hemolymph heme-binding proteins (RHBP, HeLp, and IrCP3) allow for proper transport of heme and iron to developing eggs. However, further studies are needed to determine if orthologs for these proteins exist in other hematophagous arthropods and their role in proper shuttling of heme to ovaries. Loss of any heme and iron shuttling processes can decrease egg viability, and ultimately be utilized in novel mosquito population control techniques.

AUTHOR CONTRIBUTIONS

SW, HE, and ZA conceived the manuscript and edited the manuscript into its final form; SW and HE drafted the initial manuscript.

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Silencing of Iron and Heme-Related Genes Revealed a Paramount Role of Iron in the Physiology of the Hematophagous Vector *Rhodnius prolixus*

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Iron is an essential element for most organisms However, free iron and heme, its complex with protoporphyrin IX, can be extremely cytotoxic, due to the production of reactive oxygen species, eventually leading to oxidative stress. Thus, eukaryotic cells control iron availability by regulating its transport, storage and excretion as well as the biosynthesis and degradation of heme. In the genome of *Rhodnius prolixus*, the vector of Chagas disease, we identified 36 genes related to iron and heme metabolism We performed a comprehensive analysis of these genes, including identification of homologous genes described in other insect genomes. We observed that blood-meal modulates the expression of ferritin, Iron Responsive protein (IRP), Heme Oxygenase (HO) and the heme exporter Feline Leukemia Virus C Receptor (FLVCR), components of major pathways involved in the regulation of iron and heme metabolism, particularly in the posterior midgut (PM), where an intense release of free heme occurs during the course of digestion. Knockdown of these genes impacted the survival of nymphs and adults, as well as molting, oogenesis and embryogenesis at different rates and time-courses. The silencing of FLVCR caused the highest levels of mortality in nymphs and adults and reduced nymph molting. The oogenesis was mildly affected by the diminished expression of all of the genes whereas embryogenesis was dramatically impaired by the knockdown of ferritin expression. Furthermore, an intense production of ROS in the midgut of blood-fed insects occurs when the expression of ferritin, but not HO, was inhibited. In this manner, the degradation of dietary heme inside the enterocytes may represent an oxidative challenge that is counteracted by ferritins, conferring to this protein a major antioxidant role. Taken together these results demonstrate that the regulation of iron and heme metabolism is of paramount importance for *R. prolixus* physiology and imbalances in the levels of these key proteins after a blood- meal can be extremely deleterious to the insects in their various stages of development.

Keywords: iron, heme, hematophagy, insect, oxidative stress, genome, gene silencing, *Rhodnius prolixus*

INTRODUCTION

Due to its ability to change its oxidation state, iron as such or as heme, its complex with protoporphyrin IX, participates in redox reactions that are required by essential physiological processes such as cell signaling and energy metabolism (Ponka, 1999; Mense and Zhang, 2006; Muckenthaler et al., 2008). However, free iron or heme can be cytotoxic (Aft and Mueller, 1983, 1984; Gutteridge and Smith, 1988; Vincent, 1989; Schmitt et al., 1993; Ryter and Tyrrell, 2000). In the presence of oxygen, by means of the Fenton reaction, iron can produce reactive oxygen species, leading to cellular redox imbalance and an oxidative stress (Papanikolaou and Pantopoulos, 2005).

The control of iron and heme homeostasis is particularly critical for hematophagous insects, many of which are vectors of major human life-threatening or life-long diseases caused by the pathogens that are taken along with the blood meal. High amounts of iron and heme are released during the digestion of host blood. Thus, in the course of evolution, several mechanisms to avoid iron and heme overload and oxidative damage have appeared in these organisms, contributing to their adaptation to hematophagy (Graca-Souza et al., 2006). As a first line of defense against heme toxicity, some blood-sucking arthropods sterically isolate heme molecules, reducing their reactivity. In the hemipteran *Rhodnius prolixus*, most of free heme molecules derived from hemoglobin digestion are aggregated as hemozoin, which results in the reduction of free radical formation in the midgut lumen (Oliveira et al., 1999, 2002). Likewise, the cattle tick *Rhipicephalus microplus* accumulates the dietary heme in specialized membrane-bound organelles called hemosome, found in the midgut digestive cells (Lara et al., 2003).

Another mechanism to prevent iron and heme-induced damages is the expression of proteins that control iron availability by regulating its absorption, transport, storage and excretion as well as the biosynthesis and degradation of heme (Lane et al., 2015). Although some of these genes have already been described in insects, their role in the general physiology and in the adaptation of blood-sucking insects to the hematophagic habit are poorly understood (Mandilaras et al., 2013; Tang and Zhou, 2013b).

Here, we identified genes related to iron and heme metabolism in the genome of *R. prolixus*, one of the main vectors of Chagas disease (Mesquita et al., 2015) and evaluated the relevance of a selected group of these genes in the physiology of the insect. The expression levels of these genes in each of the midgut compartments were also measured and their role in the adaptation *of R. prolixus* to hematophagy was discussed.

MATERIALS AND METHODS

Gene Sequence Analyses

Gene sequence analyses were based on the genome assembly (Rproc1 version) and gene predictions (VectorBase 1.3 version) performed by Mesquita et al. (2015) and deposited in VectorBase database[1]. Uncompleted or fragmented genes were re-predicted using exonerate software version 2.2[2] (Slater and Birney, 2005) based on insect orthologous proteins sequences deposited in NCBI databases[3]. The re-prediction results were manually curated and edited when needed. Re-predicted sequences are available as Supplementary Material.

Analysis of Protein Domains

Transmembrane regions were predicted using TMHMM version 2.0[4] (Krogh et al., 2001). Signal peptide cleavage sites were predicted using SignalP software version 4.1[5]. Mitochondria targeting sequences were predicted using TargetP version 1.1[6] (Emanuelsson et al., 2007; Nielsen, 2017) and MITOPROT[7] (Claros and Vincens, 1996), while GPI-modification anchors were searched using PredGPI version 3.0[8] (Pierleoni et al., 2008).

Iron Responsive Elements (IREs) Prediction

Iron responsive elements stem-loop structures were screened in the upstream regions (1200 bp) of ferritin genes using the Search for IREs (SIRE) server version 2.0[9] (Campillos et al., 2010).

Multiple Sequence Alignments

Multiple alignments of protein sequences were performed using ClustalW with the default configuration (Thompson et al., 2002). Multiple sequences alignment figures were created using BioEdit software (Hall, 1999).

Phylogenetic Trees

Unrooted trees were calculated by the maximum-likelihood method with RAxML version 8 (Stamatakis, 2014) using PROTCAT+JTT models and bootstrap support with 500 replicates. FigTree software version 1.4 was used to draw the trees[10].

Insect Rearing

Insects were taken from a colony of *R. prolixus* maintained at 28°C and 80–90% relative humidity under a photoperiod of 12 h of light/12 h of dark. The animals used in this work were mated females fed on rabbit blood at 3-week intervals. *R prolixus* female injected with dsRNA were kept in individual vials maintained under the same conditions. In RNAi experiments, nymphs (first stage, N1) were artificially fed on heparinized blood supplemented with dsRNA (1 μg/μL) through a latex membrane stretched across the bottom of a water-jacketed glass feeder apparatus kept at 37°C.

[1] www.vectorbase.org

[2] https://www.ebi.ac.uk/about/vertebrate-genomics/software/exonerate

[3] http://www.ncbi.nlm.nih.gov

[4] http://www.cbs.dtu.dk/services/TMHMM/

[5] http://www.cbs.dtu.dk/services/SignalP/

[6] http://www.cbs.dtu.dk/services/TargetP/

[7] https://ihg.gsf.de/ihg/mitoprot.html

[8] http://mendel.imp.ac.at/gpi/gpi_server.html

[9] http://ccbg.imppc.org/sires/

[10] http://tree.bio.ed.ac.uk/software/figtree/

Ethics Statement

All animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro), which are based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals (ISBN0-309-05377-3). The protocols were approved by the Committee for Evaluation of Animal Use for Research (CAUAP) from the Federal University of Rio de Janeiro, under registry number 115/13. Technicians dedicated to the animal facility at the Institute of Medical Biochemistry (Federal University of Rio de Janeiro) carried out all aspects related to rabbit husbandry under strict guidelines to ensure careful and consistent handling of the animals.

Tissue Isolation and RNA Extraction

Anterior and posterior midguts (PMs) from starved and blood-fed females were dissected at different days after blood ingestion. Total RNA was extracted from individual tissues or pools of 3–5 midguts using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically at 260 nm on a Nanodrop 1000 spectrophotometer 3.7 (Thermo Fisher Scientific). Following treatment with RNase-free DNaseI (Fermentas International Inc., Burlington, Canada), 1 μg of RNA was used for cDNA synthesis with a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, United States) and random hexamers according to the manufacturer's instructions.

dsRNA Synthesis and Gene Silencing Assays

Fragments of 300–400 bp were amplified by PCR using cDNA from midgut epithelia from blood-fed females (48 h after feeding) produced as described above. The following conditions were used for amplification: one cycle for 5 min at 95°C, followed by 40 cycles of 30 s a 95°C, 30 s at 63°C, and 1 min at 72°C, with a final step of 10 min at 72°C. The oligonucleotides used for amplification of templates for dsRNA synthesis are listed at Supplementary Table 1. These primers contained a T7 polymerase binding sequence, required for dsRNA synthesis. Amplified cDNAs were used as a template for dsRNA synthesis using a MEGAScript RNAi kit (Ambion Inc., Austin, TX, United States) according to the manufacturer's instructions. The maltose-binding protein (MAL) gene from *Escherichia coli* (gene identifier 7129408) in a pBlueScript KS (Stratagene) was amplified by PCR using T7 minimal promoter primers under the following conditions: one cycle for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 45°C, and 45 s at 72°C, with a final step of 10 min at 72°C. The PCR product produced was used as a template for Mal dsRNA synthesis used as a control in the silencing assays. Following *in vitro* synthesis, all dsRNAs were purified according to the manufacturer's instructions. RNAi experiments were performed by injection of 1 μL of dsRNA (1 μg/μL) from the iron or heme–related genes HCH ferritin (RPRC009256), IRP (RPRC001246), HO (RPRC006832), and FLVCR (RPRC015407) into the thoraxes of starved adult females. Insects were fed with blood 48 h after injection and midgut epithelia were dissected at different times after feeding for RNA extraction as described above.

Quantitative RT-PCR Assays

Quantitative PCR was performed in a 7500 real-time PCR system (Applied Biosystems, CA, United States) using SYBR Green PCR Master Mix (Applied Biosystems, CA, United States) under the following conditions: one cycle for 10 min at 95°C, followed by forty cycles of 15 s at 95°C and 45 s at 60°C. PCR amplification was performed using the oligonucleotides specified in Supplementary Table 2. *R. prolixus* elongation factor 1 gene (RPRC007684) expression was used as an internal control for normalization (Majerowicz et al., 2011). *C*t values were calculated from *C*t (cycle threshold) values obtained from quantitative RT-PCR and were used to calculate relative expression and perform statistical analysis (Livak and Schmittgen, 2001). The relative expression values based on $2^{-\Delta\Delta C_T}$ were used in silenced genes analyses and $2^{-\Delta C_T}$ for gene expression to allow comparison between anterior and PMs. For evaluation of iron/heme-related gene expression in AM and PM, three independent experiments were performed. Each one analyzed 4–5 different individual samples (pools of 3–5 midguts each).

Analysis of Survival, Oviposition and Egg Viability

Two days prior to the blood meal, adult females were injected with 1 μL of dsRNA (1 μg/μL) into the thoracic cavity. dsRNA-injected and fed *R. prolixus* females (as described above) were kept in individual vials under controlled temperature and humidity conditions. The mortality and oviposition were followed for the 21 days. The eggs laid were collected daily and kept at 28°C and 80% humidity until hatching. Egg eclosion and nymphs molting (N1 to N2) were monitored daily for 30 days.

ROS Detection in the Midgut

Starved females were injected with 1 μg of Fer dsRNA, HO dsRNA or Mal dsRNA (control), 48 h before blood feeding. Two days after the meal, wings, legs and dorsal plaques were removed by dissection and the insect hemocoel was filled with 50 μM of the oxidant-sensitive fluorophore dihydroethidium (hydroethidine; DHE; Invitrogen) diluted in Leibovitz-15 media supplemented with 5% fetal bovine serum. The samples were incubated in the dark at 28°C for 30 min. After that, the incubation media was removed. The midguts were washed with 0.15 M NaCl and immediately transferred to a glass slide for fluorescence microscopy analysis. Quantitative evaluation of the oxidized-DHE fluorescence levels was performed by acquiring images under identical conditions using a 20× objective and 80 ms of exposure, to allow comparison of different samples. The images were acquired in a Zeiss Observer.Z1 with a Zeiss Axio Cam MrM Zeiss and the data were analyzed using AxioVision version 4.8 software. The filter set (excitation BP 546/12 nm; beam splitter FT 580 nm; emission LP 590 nm) was used for DHE labeling.

Statistical Analyses

Statistical analysis was performed using a one-way or two-way analysis of variance followed by Tukey's multiple comparisons *post hoc* test (GraphPad Prism software, San Diego, CA, United States). Details on the sample sizes, appropriate test used and results of the statistical analyzes are indicated in the respective legend figures. All experiments were carried out independently at least three times.

RESULTS

In the analysis of the genome of *R. prolixus* we identified 36 gene homologs to known to be iron and heme-related proteins previously found in other organisms. These genes were classified into three groups according to their functions: iron binding and storage, iron transmembrane traffic and heme metabolism.

Iron Binding and Storage Genes

Ferritins and Iron Responsive Binding Proteins

Ferritins are ubiquitous iron binding proteins involved in iron storage and transport. In vertebrates, they are also considered to provide antioxidant protection due to the high toxicity of free iron (Andrews and Schmidt, 2007; Lane et al., 2015). Ferritins are multimeric proteins composed of two types of subunits that, in insects, are named heavy and light chain homologs (HCH and LCH, respectively). HCH are characterized by conserved amino acid residues that compose the ferroxidase center responsible for the oxidation of Fe^{2+} whereas LCH is involved in iron nucleation (Andrews and Schmidt, 2007; Mandilaras et al., 2013; Tang and Zhou, 2013a). In contrast to vertebrate and plant ferritins that are cytosolic proteins, most insect ferritin genes display a typical signal peptide and are secreted proteins (Pham and Winzerling, 2010; Mandilaras et al., 2013; Tang and Zhou, 2013a).

Gene analysis of insects of different orders such as Diptera (*Drosophila melanogaster* and *Anopheles gambiae*), Hymenoptera (*Apis Mellifera*), Coleoptera (*Tribolium castaneum*) and Hemiptera (*Cimex lectularius*) reveals that all of them have one pair of genes encoding for secreted ferritin polypeptides (one gene for each subunit). Remarkably, the *R. prolixus* genome comprises three pairs of genes that encode for different secreted HCH and LCH subunits (RPRC007320, RPRC009256, RPRC012024 for HCH and RPRC000395, RPRC009258, RPRC012023 for LCH) (Supplementary Figures 1A,B). Secreted ferritin HCHs are very similar in aminoacid sequence (Supplementary Figure 1A) and are phylogenetic related (**Figure 1A**). The same is observed for LCH subunits (**Figure 1B** and Supplementary Figure 1B) These genes are localized in different regions of the genome, clustered in pairs (one HCH and one LCH) in a head-to-head position, as described for other insects (Dunkov and Georgieva, 2006) (**Figure 2**).

In addition to the secreted HCH ferritins, *R. prolixus* transcribes an HCH subunit that does not present a signal peptide, which is consistent with a cytosolic protein (RPRC013830) and a putative mitochondrial subunit RPRC009359 that has a mitochondria-addressing peptide. In both subunits, the amino acid residues involved in the ferroxidase activity are conserved as well (Supplementary Figure 1A). Phylogenetic analysis separated secreted and non-secreted ferritin HCHs into two groups (**Figure 1A**). Cytosolic and mitochondria subunits are grouped with non-secreted HCH insect ferritins. Futhermore, both cytosolic and mitochondrial genes have closelly-related orthologs in the *C. lectularius*, that also belongs to the order Hemiptera. No cytosolic or mitochondrial LCH genes were found in *R. prolixus* genome.

Besides regulation of transcription, ferritins and other iron/heme-related proteins such as the aminolevulinic acid synthase (ALAS), responsible for the first step of heme biosynthesis, are also regulated post-transcriptionally according to intracellular iron levels (Pantopoulos, 2004; Wilkinson and Pantopoulos, 2014). Under conditions of low iron availability, IRP (Iron Responsive Element Binding Protein) binds to a stem-loop structure found in the 5′ untranslated regions of the mRNAs, named iron-responsive element (IRE), sterically blocking mRNA translation of these proteins. When an iron atom binds to IRP due to increased iron levels, the protein reduces its affinity to the IRE (Pantopoulos, 2004; Wilkinson and Pantopoulos, 2014). We identified canonical IREs in the 5′UTR of all secreted HCH mRNAs (Supplementary Figure 2). Neither the LCH ferritins nor the cytosolic and mitochondrial HCH contain IREs in the 5′UTRs in their mRNAs.

In *R. prolixus*, two genes coding for IRP-like proteins were found (RPRC001246 and RPRC012271) (**Figure 3**). RPRC012271 codes for the mitochondrial IRP and thus may not be involved in the regulation of gene expression in the cytosol. Accordingly, RpIRPs are separated into two different branches of the phylogenetic tree that includes cytosolic and mitochondrial IRPs from other insects (Supplementary Figure 3). The presence of the putative cytosolic IRP RPRC001246 (herein named IRP1), with a high degree of similarity to other insect cytosolic IRPs and of the IRE-containing mRNAs indicates that the mechanism for translational control by iron availability has been conserved in this insect.

Transferrin Superfamily Proteins

The transferrin family is a diverse group that comprises soluble iron-binding proteins such as serum transferrin, ovotransferrin, the antibiotic protein lactotransferrin and membrane-bound proteins such as melanotransferrins (Hentze et al., 2010; Lambert, 2012). The canonical member of this family is the vertebrate serum iron transporter transferrin. As in vertebrates, insect transferrins are monomeric proteins that bind iron with extremely high affinity and have a described role in vitelogenesis and less characterized antibiotic activity (Yoshiga et al., 1997; Harizanova et al., 2005; Dunkov and Georgieva, 2006; Ursic-Bedoya and Lowenberger, 2007).

The *R. prolixus* genome has at least six genes that were identified as members of Transferrin superfamily (**Table 1**). The *R. prolixus* ortholog of the hemolymphatic iron-binding transferrin (Trf1) found in other insects is encoded by the RPRC10050 gene (**Figure 4**). Putative RpTrf1 presents a predicted signal peptide and the residues involved in iron binding, in the amino terminal region (N-lobe) but not in the carboxy terminal (C-lobe), suggesting that RpTrf1 is an

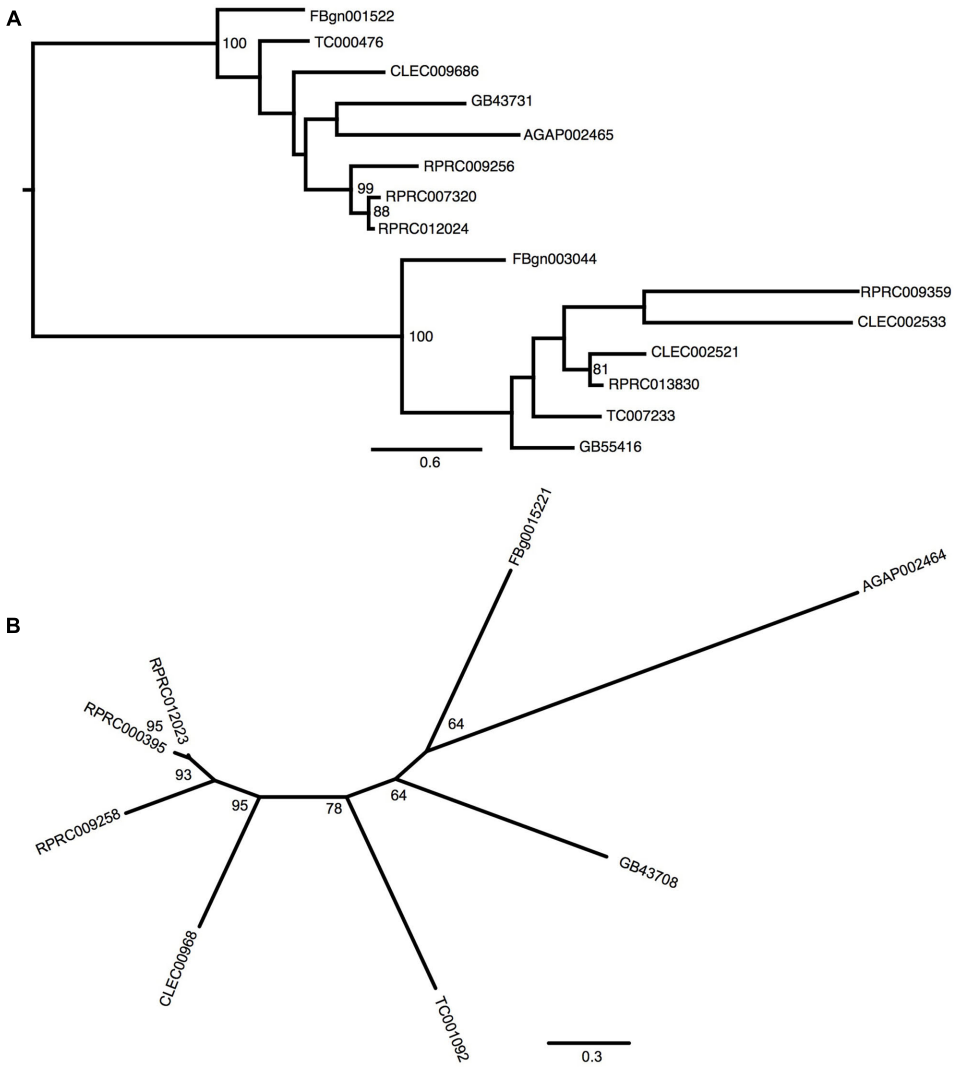

FIGURE 1 | Phylogenetic analysis of ferritins orthologs. Maximum-likelihood trees of ferritins HCH **(A)** and LCH **(B)** orthologs. Numbers on the branches are bootstrap support values from 500 replicates. Only numbers 50% or higher are shown. The sequence codes used were *Rhodnius prolixus* (RPRC), *Drosophila melanogaster* (FBg), *Tribolium castaneum* (TC), *Cimex lectularius* (CLEC), *Apis mellifera* (GB) and *Anopheles gambiae* (AGAP).

extracellular protein capable of binding a single iron atom, as previously observed for other insects (Supplementary Figure 4). Five other genes encode for proteins that were identified as members of the transferrin superfamily. The phylogenetic tree grouped them all in the same cluster, which diverges from the cluster that comprises the insect extracellular Trf1 proteins (**Figure 4**). Two of them (RPRC012861 and RPRC001152) have a large number of introns and a predicted GPI-anchor domain, typical of the membrane-associated melanotransferrins (MelTf) (**Table 1**) (Suryo Rahmanto et al., 2012). Finally, the three other genes (RPRC012860, RPRC004244 and RPRC007077) present low similarity with the putative melanotransferrin RPRC012861 (**Figure 4**). However, none of the proteins predicted by these three presents a GPI-anchor domain (**Table 1**). Furthermore, no transcripts encoded by these genes have been found in the transcriptomes from digestive and whole-body libraries suggesting that they could be pseudogenes (Ribeiro et al., 2014).

Iron Transmembrane Traffic

Although the role of transferrins and ferritins in iron transport and storage has been studied in insects, very little is known concerning the mechanisms by which iron is transported across cellular membranes.

Malvolio/DMT-1

In mammals, a unique protein named Divalent Metal Transporter-1 (DMT-1 or NRAMP-2) is responsible for cellular iron import from the intestinal lumen by the enterocytes (Gunshin et al., 1997). In this process, Fe^{3+} atoms provided by the diet are reduced by duodenal cytochrome b (Dcytb) to be transported to the cytosol by DMT-1 (McKie et al.,

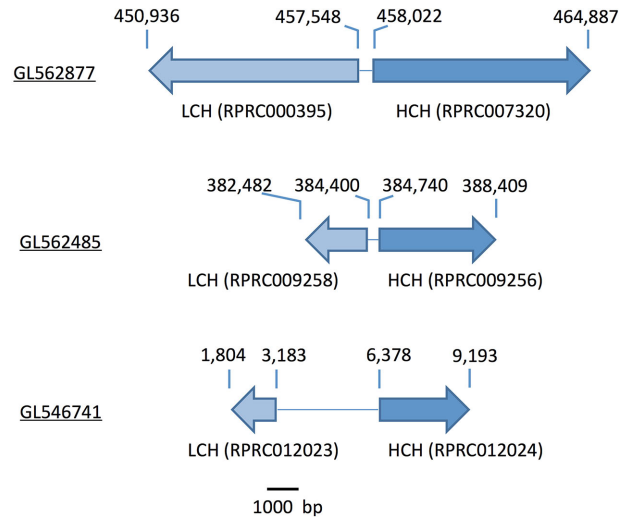

FIGURE 2 | Scheme of the genomic distribution of the secreted HCH and LCH ferritins subunits in the genome of *R. prolixus*. Scaffold numbers are underlined. The numbers above the arrows indicate the position within the scaffold. Ferritin subunits (gene codes) are indicated below the arrows.

2001). Studies on the physiological role of malvolio and its involvement in iron absorption have already been performed in *D. melanogaster* (Rodrigues et al., 1995; Folwell et al., 2006) and in the new world malaria mosquito *Anopheles albimanus* (Martinez-Barnetche et al., 2007). Unlike *Drosophila* which has a single DMT-1 homolog named malvolio (MLV), *R. prolixus* presents 2 paralogous genes (RPRC006515, named MLV1 and RPRC012012 named MLV2). In addition to *R.prolixus,* the Coleopteran *T. castaneum* and the Hemipteran *C. lectularius* also have paralogs of MLV. However, *T. castaneum* MLVs are not grouped with the close–related hemiptera MLVs suggesting that a gene duplication event occurred independently among these insects (**Figure 5**). Furthermore, putative RpMLV2 has two predicted transcripts (MLV2_RA and MLV2_RB). The proteins encoded by both MLV paralogous genes have conserved transmembrane domains as shown in Supplementary Figure 5.

Zinc/Iron-Regulated Transporter Proteins

Another class of proteins implicated in iron transport in animals is the metal transporter ZIP family. ZIP proteins are transmembrane proteins that can be localized in different compartments of the cell. In vertebrates, it has been already demonstrated that members of the ZIP family are capable of transporting both zinc and iron in different cell types (Liuzzi and Cousins, 2004; Calap-Quintana et al., 2017). So far, no description of ZIP transporters has been performed for *R. prolixus*. Seven encoding members of the ZIP family were found in the *R. prolixus* genome that are divided by homology into two major groups (**Figure 6**). RPRC003454 (ortholog of dmFOI and hZIP6/10), RPRC005566 and its paralog RPRC005564 (orthologs of dmZIP71B and hZIP5), the *D. melanogaster* catsup close-related RPRC009051 and RPRC002967 (ortholog of dmZIP99C and hZIP13) are clustered in the same group. On the other hand RPRC00118, RPRC013358 and its paralog RPRC013359 are in the second group that includes the fruit fly ZIP1, ZIP2 and ZIP3. RPRC013358 and RPRC013359, are localized side by side, in the same direction, as a cluster in the genome, suggesting that they are products of gene duplication (data not shown).

Mitoferrin

Mitoferrin (Mrfn) is a member of the mitochondrial solute carrier family. It is responsible for supplying the mitochondria matrix with iron required for heme synthesis as well as for the assembly of the iron-sulfur clusters that are components of a variety of proteins involved in energy metabolism pathways (Shaw et al., 2006; Lane et al., 2015). In vertebrates, two paralogous genes are found: mitoferrin 1 that is mainly expressed in erythropoietic cells and *mitoferrin 2* that is more ubiquitously expressed (Shaw et al., 2006). As observed in the other insect genomes that have been analyzed, the *R. prolixus* genome encodes a single putative mitoferrin (RPRC002819) with high a degree of similarity to other insect mitoferrins (**Figure 7**).

Heme Metabolism Genes

Heme Biosynthesis Pathway

Most of the living organisms synthesize heme with the exception of nematodes, such as *Caenorhabditis elegans* (Rao et al., 2005) and the ticks (Braz et al., 1999; Perner et al., 2016). Genes coding for all of the enzymes of the heme biosynthesis pathway were found in *R. prolixus* genome (**Table 2**), supporting previous report showing that this insect obtains heme not only from the diet but also by synthesis *de novo* (Braz et al., 2001).

Heme Degradation

Hematophagous insects have to deal with high amounts of heme released after host blood digestion in the lumen of their midguts. One of the molecular strategies involved in the cellular protection against heme-induced damage is the enzymatic degradation of heme catalyzed by HO. In most of the studied organisms, which include bacteria, plants and vertebrates, heme breakage results in formation of carbon monoxide, ferrous ion, and biliverdin IX alpha (Tenhunen et al., 1969; Wilks and Heinzl, 2014). Heme degradation pathways have been described in *R. prolixus* (Paiva-Silva et al., 2006) and in the mosquito *Aedes aegypti*, vector of Dengue, Zika and Chicungunya viruses (Pereira et al., 2007). the HO genes have not been characterized for any of these cases. A single gene encoding a HO was found in *R. prolixus* (RPRC006832) as in the other insect genome analyzed here. The predicted HO has all of the conserved residues necessary for heme interaction and degradation (**Figure 8**), as was determined in the structurally characterized recombinant *Drosophila* and human HOs (Schuller et al., 1999; Zhang et al., 2004).

Heme Transport_Feline Leukemia Virus Subgroup C Receptor (FLVCR)

In addition to the heme degradation catalyzed by HO in the cytosol of the *R. prolixus* gut epithelial cells, heme molecules released by hemoglobin proteolysis in the lumen of the insect gut reach the hemolymph (Dansa-Petretski et al., 1995), suggesting the existence of heme uptake and export from the gut lumen

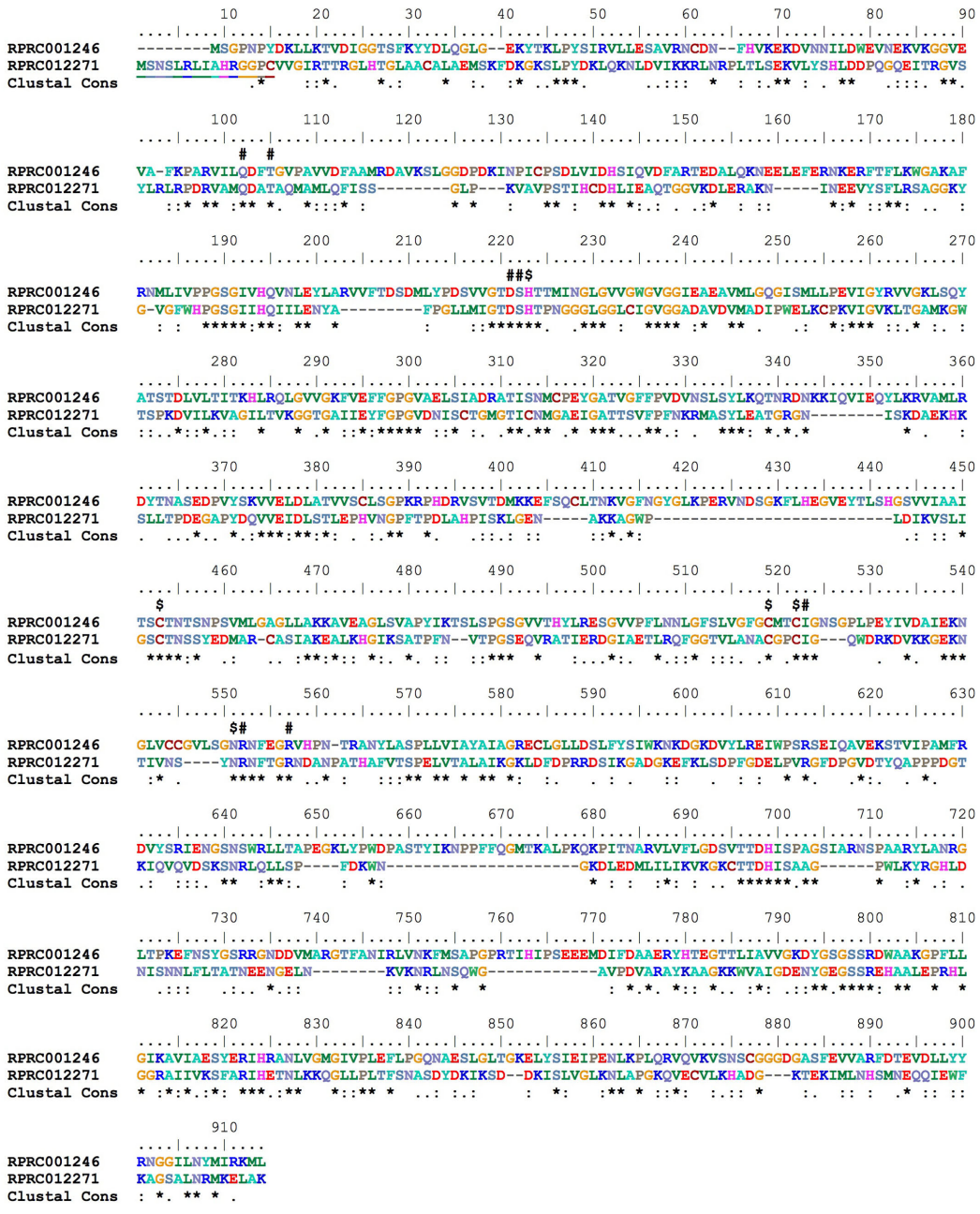

FIGURE 3 | Multiple amino acid sequence alignment of *R. prolixus* cytosolic (RPRC001246) and mitochondrial (RPRC012271) IRPs. Amino acid color code generated by ClustalW within BioEdit software was used. Consensus residues (Clustal cons) were also generated by ClustalW. "#" indicates alpha-methylisocitrate binding residues and "$" Fe-S cluster binding residues. Mitochondrial targeting peptide is underlined in mitochondrial IRP (RPRC012271).

through enterocytes to the hemolymph. However, the proteins involved in this process remain unknown.

In vertebrates, a transmembrane protein named Feline Leukemia Virus subgroup C Receptor (FLCRV-1) has been characterized as a cell-surface heme exporter (Quigley et al., 2000, 2004). FLVCR was conserved during evolution, from animals to plants and bacteria. An FLVCR ortholog was found in the *R. prolixus* genome (RPRC015407). In the alignment performed with other putative FLVCR located in different insect genomes, we identified the 12 transmembrane canonical domains that are typical of these proteins (**Figure 9**).

Expression Analysis of Key Genes Involved in Iron and Heme Metabolism in the Insect Midgut Compartments

As previously mentioned, midgut epithelium is the insect tissue that primarily addresses with the high amount of blood-derived heme while avoiding heme-induced tissue damage. During the

TABLE 1 | Transferrin superfamily genes identified in the *Rhodnius prolixus* genome.

Gene ID	Number of introns	Putative function	Signal peptide	GPI-anchor	Transcriptional evidence
RPRC010050	14	Hemolymphatic iron Transport (Trf1)	+	−	+
RPRC012861	10	Melanotransferin	nd	+	+
RPRC001152	12	Melanotransferin	−	+	+
RPRC012860	4	?	+	−	−
RPRC004244	1	?	−	−	−
RPRC007077	3	?	−	−	−

"+" present, "−" absent, n/d- non-determined (truncated protein); Transcriptional evidences are according to Ribeiro et al. (2014). Signal peptide and GPI-anchor were predicted as described in the section "Materials and Methods."

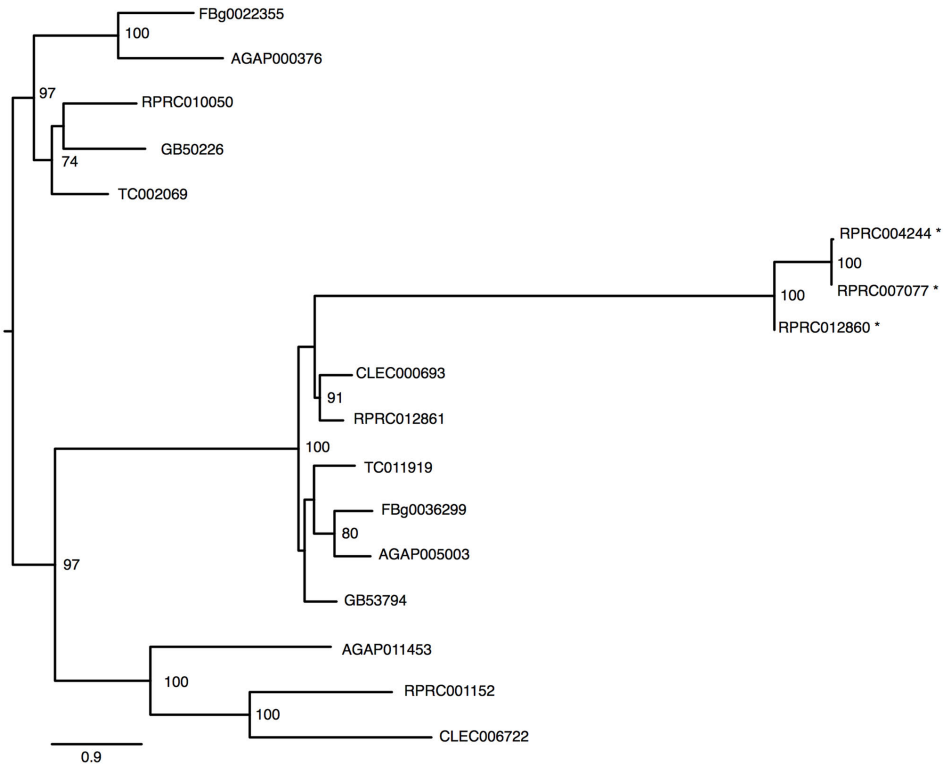

FIGURE 4 | Phylogenetic analysis of Transferrin superfamily genes orthologs. Maximum-likelihood tree of transferrins orthologs. Numbers on the branches are bootstrap support values from 500 replicates. Only numbers 50% or higher are shown. "*" indicate *R. prolixus* putative melanotransferrins pseudogenes. The sequence codes used were *R. prolixus* (RPRC), *D. melanogaster* (FBg), *T. castaneum* (TC), *C. lectularius* (CLEC), *A. mellifera* (GB) and *A. gambiae* (AGAP).

21 days of digestion, the PM is provided with blood coming from the anterior midgut (AM), the latter being the gut segment that stores the meal after ingestion. In the PM, massive digestion of proteins and complex lipids, such as tryacilglicerol continuously occurs since the 1st day of blood ingestion (Billingsley and Downe, 1986; Terra et al., 1988; Coelho et al., 1997). During the following days, the products of digestion are absorbed by the epithelial cells localized along the PM. Given that AM and PM have distinct functions in the insect physiology, it is worth speculating that midgut cells from these two regions could differentially express iron/heme-related genes during the course of digestion. Thus, we evaluated the expression of genes involved in different aspects of iron/heme metabolism: iron storage (ferritin), heme transport (FLVCR), degradation (HO), and regulation of gene expression (IRP) in the AM and PM, during one cycle of feeding and blood digestion.

In fact, we observed that the blood-meal modulated the expression of these genes, particularly in the PM where most of the blood digestion occurs and where intense release of free heme should occur (**Figure 10**). Expression of HO was induced in the PM during the very beginning of blood digestion, possibly preparing the midgut to degrade the first heme molecules that eventually reach the cytosol of the gut epithelial cells. After that, HO transcript levels were gradually reduced until day 10 when a second event of induction of HO expression was observed. Afterward the expression of this enzyme returned to the levels observed in the PM of fasting insects (day 0) (**Figure 10A**).

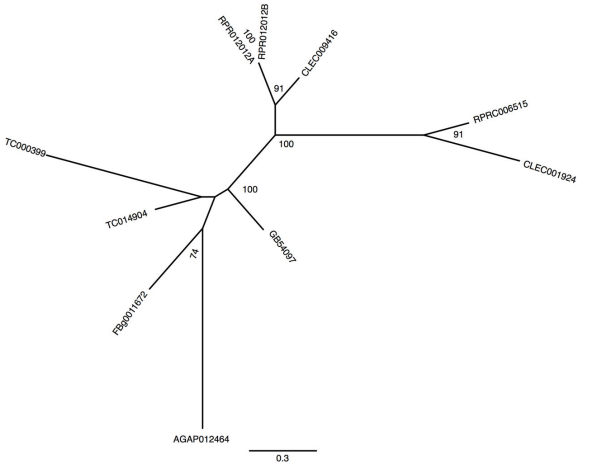

FIGURE 5 | Phylogenetic analysis of malvolio orthologs. Maximum-likelihood tree of Malvolio orthologs. Numbers on the branches are bootstrap support values from 500 replicates. Only numbers 50% or higher are shown. The sequence codes used were *R. prolixus* (RPRC), *D. melanogaster* (FBg), *T. castaneum* (TC), *C. lectularius* (CLEC), *A. mellifera* (GB) and *A. gambiae* (AGAP).

In agreement with this result. the expression of the secreted HCH ferritin subunit RPRC009256, the Fer subunit with the highest transcript levels in the midgut cDNA libraries (Ribeiro et al., 2014), followed HO expression, being highly upregulated in the PM (**Figure 10B**). Although less intense, a similar pattern was observed for the expression of IRP (**Figure 10C**), the protein involved in the post-transcriptional control of ferritin expression. These data suggest that both transcriptional and post-transcriptional regulation of the ferritin gene occurs in the PM, triggered by blood digestion. Interestingly, a significant modulation of IRP transcript levels was found in the AM, in the course of digestion. The heme transporter FLVCR presented a distinct gene expression profile (**Figure 10D**). The amount of FLVCR transcripts progressively increased in the PM until the 10th day, when their levels seemed to reach a plateau.

Altogether, these data reveal that intestinal cells show a complex regulation of the expression of iron/heme-related genes after a blood meal, probably to control the iron and heme availability in both the intra and extracellular celular compartment, while at the same time avoiding overload and the oxidative damage that could follow.

The Role of Genes Related to Iron and Heme Metabolism in Major Aspects of *R. prolixus* Physiology

To further determine the relevance of these genes in the physiology of the insect, we used dsRNA-mediated knockdown (KD) of the genes previously analyzed in **Figure 11**. Consistent silencing of all of the target genes was achieved, with efficiencies ranging from 60–80% and remaining for at least 7 days for both adults and nymphs (Supplementary Figure 6).

Silencing of iron-related genes (*Fers* and *IRP*) caused a significant impact on the survival of adult females (40 and 51% of survival, respectively), particularly during the 1st days of digestion when an intense heme degradation and iron release occurs (Coelho et al., 1997) (**Figures 11A,B**). In contrast, the KD of HO produced a more modest impact on survival as only 21% of the silenced insects died. In contrast, *FLVCR* KD caused intense mortality of adults (64% of mortality). However, lethality caused by silencing of the genes involved in heme metabolism was more pronounced in a later period of digestion, contrary to the early death observed after silencing of the iron-related genes (**Figures 11A–C**).

Regarding reproduction, gene silencing reduced the oviposition, at a varying rate, from 45% (dsHO) to 69% (dsFLVCR) (**Figure 11D**). Remarkably, despite the noticeable effect on oviposition, silencing of FLVCR caused only a minor impact on embryogenesis, as observed by the high rate of viable eggs produced by dsFLVCR-injected females (**Figure 11E**). On the other hand, the silencing of ferritin genes led to a reduction of 48% on oviposition but most of the laid eggs were not viable (95% of the total) (**Figures 11D,E**). The eggs laid by ferritin-silenced females had an altered morphology (**Figure 11F**), a pattern similar to eggs subjected to dehydration. This phenotype was not observed in the unviable eggs laid by females silenced for the other genes (data not shown).

To evaluate the impact of knockdown of the same genes during the insect development, nymphs (N1) were fed artificially with blood supplemented with each specific dsRNA and had their survival and molting monitored for 21 days. As observed in **Figure 11G**, the KD of all genes reduced nymphs survival. The FLVCR silencing produced the highest level of nymph mortality, as observed in adults (**Figures 11A,G**). Conversely, silencing of HO caused a more prominent effect on survival of nymphs when compared to adults (**Figures 11A,G**). Interestingly, in nymphs, HO KD led to high lethality in the early days after the blood-meal, when compared with adults (**Figures 11A–C,G–I**). Among the surviving insects, silencing of FLVCR impaired 80% of molting of N1 to N2 stage nymphs (**Figure 11J**). The KD of all of the other genes affected molting in slightly less dramatic manner, in the range of 55–65% inhibition (**Figure 11H**). Taken together, these data suggest that the control of intracellular balance of free heme is critical for the development of nymph stages.

Considering the early induction of HO and ferritin expression in the PM and the differences observed in the rate and the time course of mortality induced by the silencing of these genes in adults, we decided to evaluate the effect of silencing these genes on the production of ROS in the midgut epithelium after the blood repast. Knockdown of ferritin led to a dramatic increase of ROS production, observed by the increase in fluorescence of the ROS-sensitive probe DHE. On the other hand, the reduction of HO expression had no effect on the oxidative cellular status (**Figure 12**). These data showed that ferritin is a major antioxidant against iron induced damages in the midgut of fed adults. Moreover, in contrast to what is observed in mammalian models, insect HO does not contribute to the control of the redox balance in the presence of high levels of heme in fed adults.

Together these results show that imbalances in the levels of proteins involved in the control of homeostasis of heme or iron

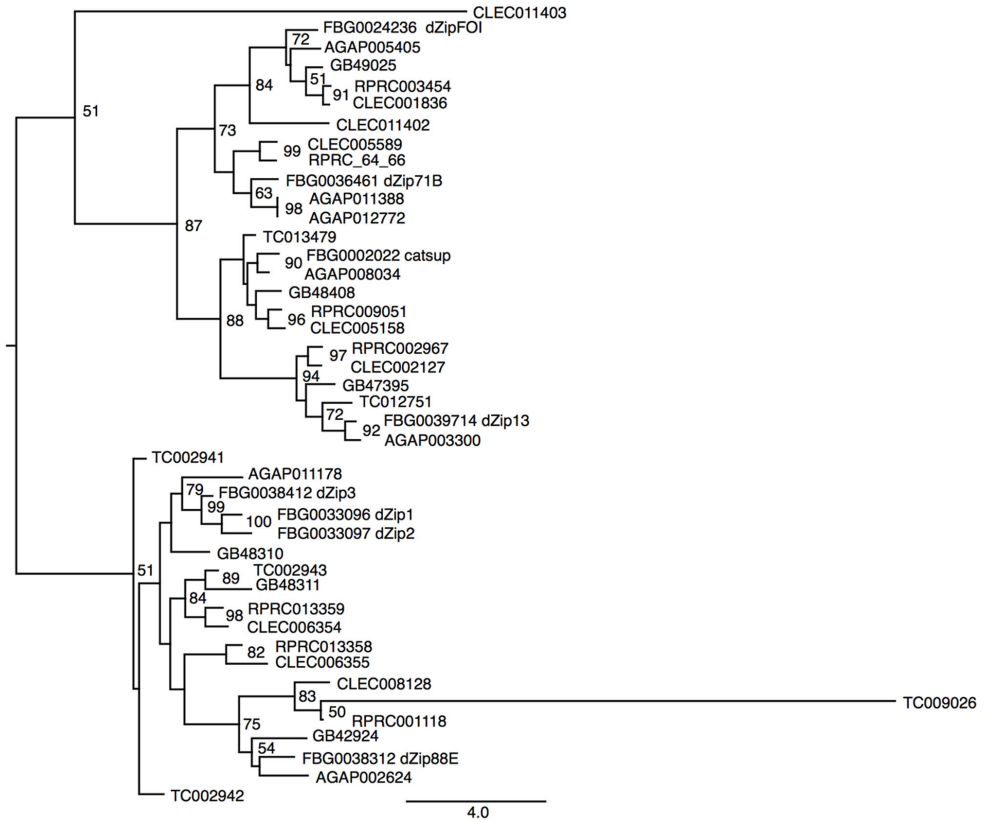

FIGURE 6 | Phylogenetic analysis of ZIP orthologs. Maximum-likelihood tree of ZIP orthologs. Numbers on the branches are bootstrap support values from 500 replicates. Only numbers 50% or higher are shown. The sequence codes used were *R. prolixus* (RPRC), *D. melanogaster* (FBg), *T. castaneum* (TC), *C. lectularius* (CLEC), *A. mellifera* (GB), and *A. gambiae* (AGAP). *D. melanogaster* gene names were included in the figure to allow the identification of ZIP subfamilies.

can cause extensive damage to insects in their various stages of development. These data also highlight that a fine tuning of the metabolism of these molecules is of pivotal importance to central aspects of the physiology of this blood sucking insect.

DISCUSSION

As a transition metal, iron participates in redox reactions that are essential to many physiological processes including cellular respiration, signaling and xenobiotic detoxification. Thus, iron/heme homeostasis is maintained by a vast array of proteins involved in absorption, intra and extra cellular transport, storage and efflux of these molecules (Andrews and Schmidt, 2007; Lane et al., 2015). The main iron and heme-related proteins involved in these processes were conserved in animals. Owing to the large-scale sequencing of genomes and transcriptomes, most of the proteins associated with iron and heme metabolism have been identified in many insects of different orders. However, the functional characterization and role of these proteins in general physiology have been performed mostly in the fruit fly *D. melanogaster*. In this work, 36 genes coding for proteins that control iron and heme intra and extracellular availability were revealed based on a survey of the *R. prolixus* genome. This effort was complemented by gene expression and RNAi-based experiments. A schematic representation of genes identified in this work is shown in **Figure 13**.

Iron Metabolism Genes

Among proteins related to iron metabolism, ferritins are the most extensive studied in insects, including blood-sucking species (Pham and Winzerling, 2010). In these organisms, ferritin is implicated in the storage and the transport of dietary iron to peripheral tissues (Zhou et al., 2007). Therefore, midgut cells express and secrete ferritins loaded with iron atoms into the extracellular compartment, i.e., the hemolymph. Interestingly, the *R. prolixus* genome encodes three distinct transcripts of each secreted ferritin subunits (HCH and LCH), while all other insects already studied encode only one transcript of each subunit (**Figure 1** and Supplementary Figure 1) (Dunkov and Georgieva, 2006; Mandilaras et al., 2013; Tang and Zhou, 2013b). However, this hemipteran has similar characteristic genome organization conserved in insects: in a cluster containing a pair of HCH and LCH genes, in a tail-to-tail position (**Figure 2**) (Dunkov and Georgieva, 2006). These data lead us to speculate that those genes were originated through gene expansion of an ancient single cluster during the evolution of the blood-feeding habit in the triatomine, either as a way to

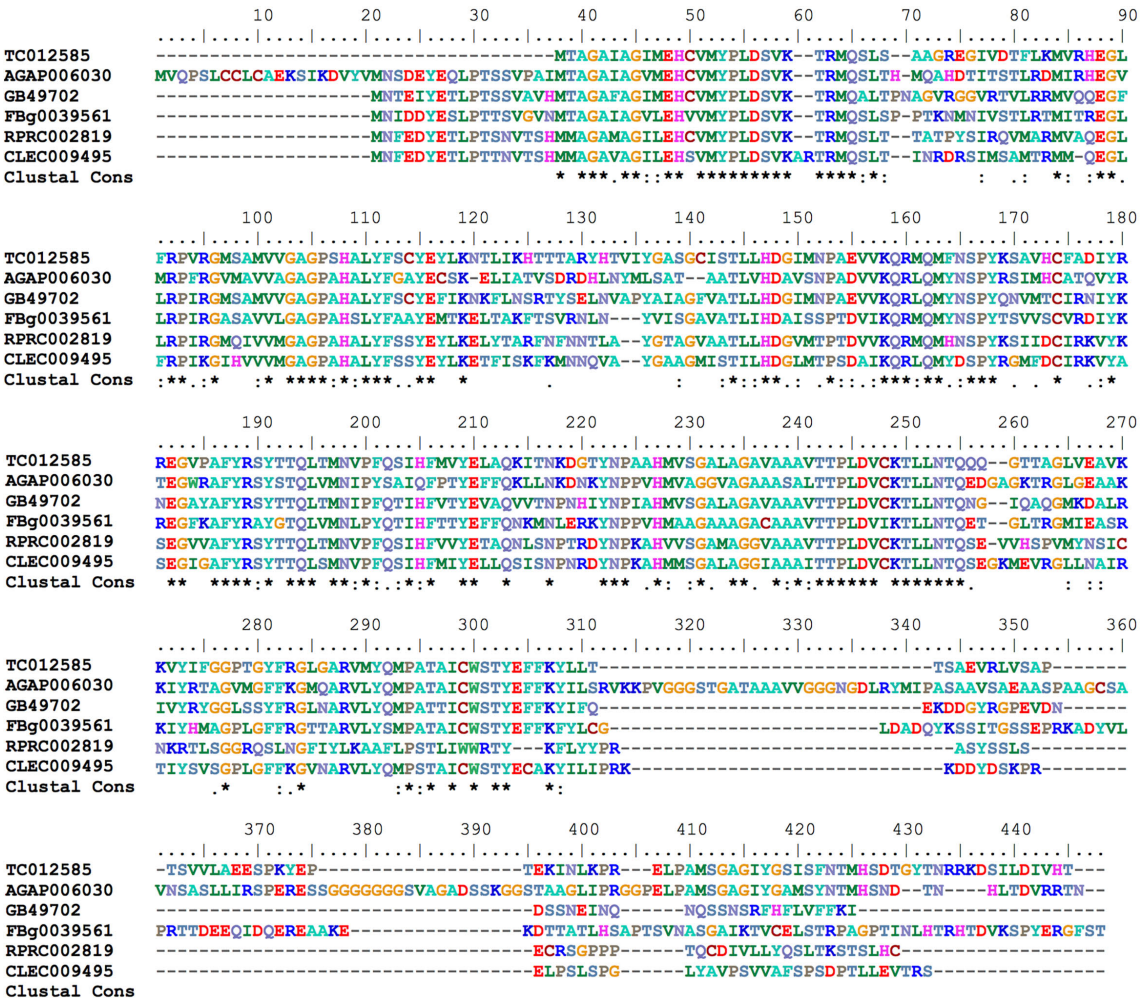

FIGURE 7 | Multiple amino acid sequence alignment of *R. prolixus* mitoferrin predicted amino acid sequences with insect orthologs. Amino acid color code generated by ClustalW within BioEdit software was used. Consensus residues ("Clustal cons" line) were generated by ClustalW.

increase gene expression or to allow for differential expression of each isoform, thus performing non-identical roles in the physiology of the insect. Furthermore, the organization of Fer subunits pairs in clusters, preserved during the evolution, allows the coordinated expression of both subunits, ensuring the HCH:LCH (1:1) stoichiometry observed in the ferritin multicomplexes.

In addition to the secreted HCH ferritins, this insect expresses a cytosolic HCH, as observed in vertebrates but not often in insects (**Figure 1** and Supplementary Figure 1). An exception is the *Philaenus spumarius*, a phloem-feeding Hemipteran that has a ferritin-like protein in the cytosol and nucleus of midgut cells (Collin et al., 1988). In our phylogenetic analysis, we found an ortholog of *R. prolixus* cytosolic *fer* gene in the hematophagous hemipteran *C. lectularius* (CLEC002521), suggesting that these genes may be present more frequently in insects than expected. As in vertebrates, insect cytosolic ferritins should act as intracellular chelators of free iron atoms, storing them in a non-oxidant condition.

The *Drosophila* genome contains a mitochondrial ferritin gene (Fer3HCH) (Missirlis et al., 2006). A mitochondrial HCH ferritin gene was identified in the *R. prolixus* genome. As for DmFer3HCH, the *Rhodnius* mitochondrial ferritin gene codes for a protein that has the mitochondrial targeting peptide and the conserved amino acid residues involved in ferroxidase activity and iron binding, indicating that this protein is functional. Phylogenetic analysis of ferritins genes revealed that the *R. prolixus* mitochondrial and the cytosolic ferritins are closely related, suggesting that they have a common ancestral. *Drosophila* mitochondrial ferritin has very low transcription levels in carcasses, being almost exclusive expressed in testes (Missirlis et al., 2006). Accordingly, analysis of *Rhodnius* transcriptomic data revealed that the triatomine mitochondrial ferritin transcripts were detected in whole-body but not in midgut libraries (Ribeiro et al., 2014).

The majority of the cytosolic labile pool iron is destined to mitochondria It is involved in the maintenance of intracellular energy and redox status by means of ATP and ROS production

TABLE 2 | *Rhodnius prolixus* genes encoding heme synthesis pathway enzymes.

Gene name	Abbreviation	Gene ID	Transcriptional evidences
5-aminolevulinate synthase	ALAS	RPRC011281	+
Delta-aminolevulinic acid dehydratase	ALAD	RPRC011504	+
Porphobilinogen deaminase	PBGD	RPRC003782	+
Uroporphyrinogen-III synthase	UROS	RPRC004613	+
Uroporphyrinogen decarboxylase	UROD	RPRC013534	+
Coproporphyrinogen III oxidase	CPOX	RPRC009673	+
Protoporphyrinogen oxidase	PPO	RPRC005054	+
Ferrochelatase	FECH	RPRC007112	+

Transcriptional evidences are according to Ribeiro et al. (2014).

during respiration, both pathways involving the participation of iron/heme-containing proteins. Thus, proper acquisition of cytosolic iron is required to regulate mitochondrial homeostasis. Iron is a component of several Fe-S protein clusters that are part of the electron transfer chain (ETC). Furthermore, iron is a substrate of ferrochelatase that catalyzes the last step of the heme biosynthesis pathway that occurs partially in the mitochondrial matrix (Papanikolaou and Pantopoulos, 2005). The transport of iron from the cytosol to the mitochondria is performed by mitoferrin. Mitoferrin was found in the *R. prolixus* genome but in contrast to mitochondrial ferritin, mitoferrins are expressed in the midgut of *R. prolixus* (Ribeiro et al., 2014). In *D. melanogaster*, the impairment of iron transport to mitochondria by mitoferrin knockdown reduces the mortality of flies submitted to an oxidative challenge (Edenharter et al., 2017). It has recently been demonstrated that *R. prolixus* reduces mitochondrial ROS production shortly after the blood meal as a protection against oxidative damage produced by iron and heme in the midgut (Gandara et al., 2016). This unique mechanism involves the TOR pathway but it is not fully understood. It would be interesting to investigate the involvement of mitoferrin in the modulation of is mitochondrial function under this condition.

One of the major post-transcriptional regulatory mechanisms involves the Iron Regulatory protein 1(IRP)/Iron Responsive Element (IRE) system. Under intracellular iron deprivation, IRP binds to IRE stem loops localized in the 5′UTR of the target genes blocking translation. The increase of the intracellular iron levels reverts the repressor effect exerted by the IRP. In vertebrates, genes directly related to iron/heme metabolism such as ferritins, ferroportin and ALAS are under IRP control (Muckenthaler et al., 2008).

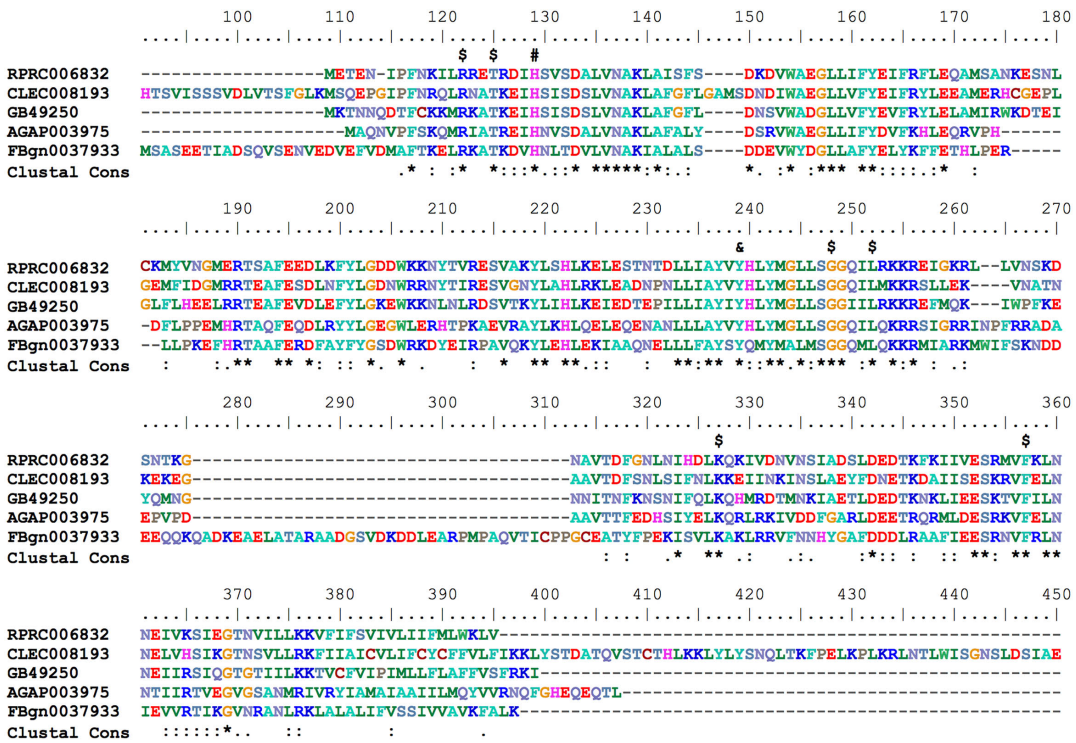

FIGURE 8 | Multiple amino acid sequence alignment of *R. prolixus* Heme Oxygenase. Amino acid color code generated by ClustalW within BioEdit software was used. Consensus residues ("Clustal cons" line) were generated by ClustalW. Conserved amino acids are indicated as follows: "$" heme binding pocket, "#" histidine that coordinates to heme iron and "&" tyrosine that binds to heme propionate. The CLEC008193 sequence was much longer than the other orthologs so its unaligned N-terminal (before His91) and C-terminal (after Glu409) sequences are not shown.

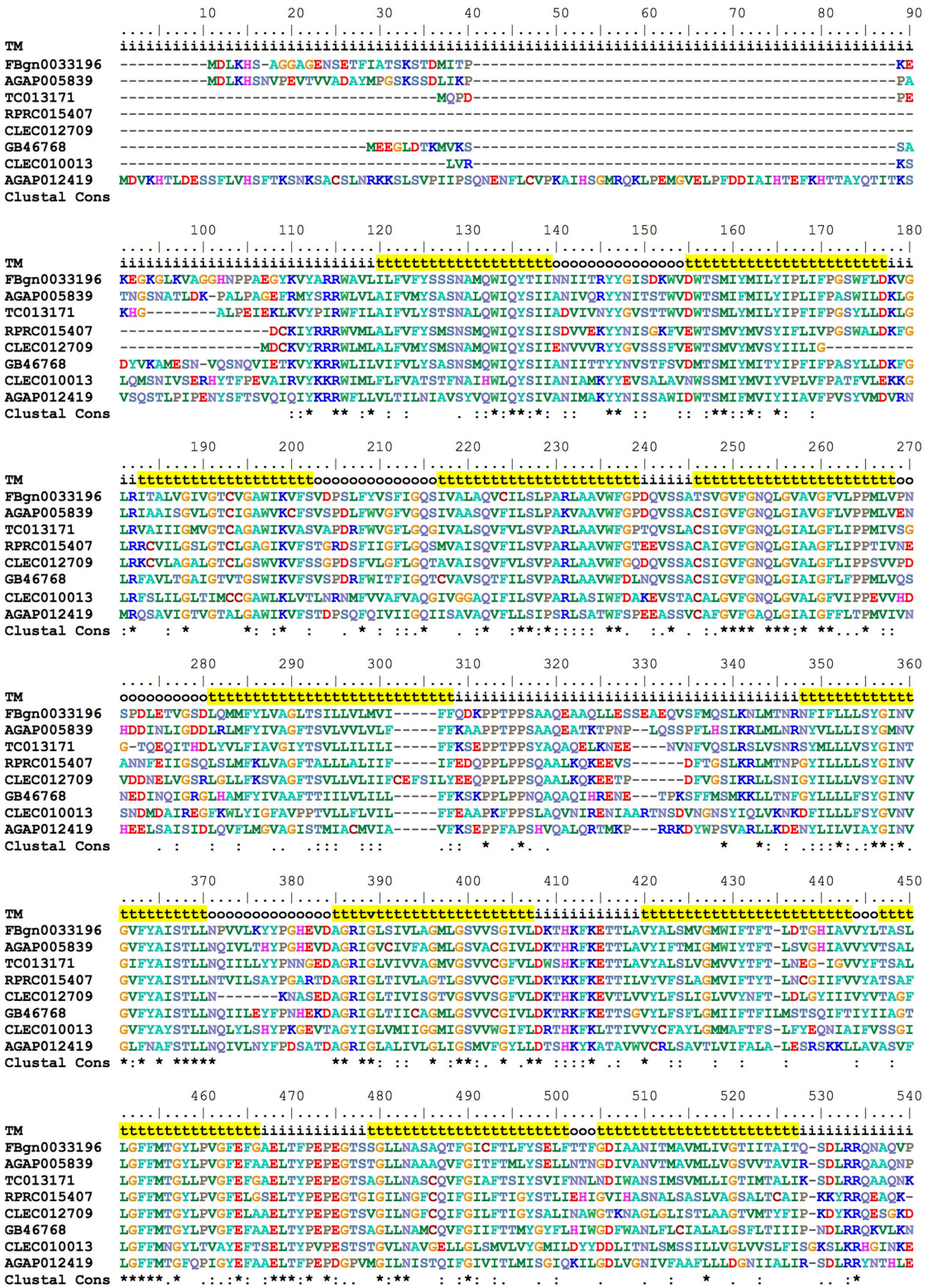

FIGURE 9 | Multiple amino acid sequence alignment of *R. prolixus* FLVCR. BioEdit "ClustalW" aminoacid color code was used. Consensus residues ("Clustal cons" line) were generated by ClustalW. "TM" lines indicate transmembrane regions by yellow background "t", inside (i) and outside (o) loops. The non-conserved C-terminal region (36 residues - position 541 to 576) was omitted to improve the conserved sequences comparison.

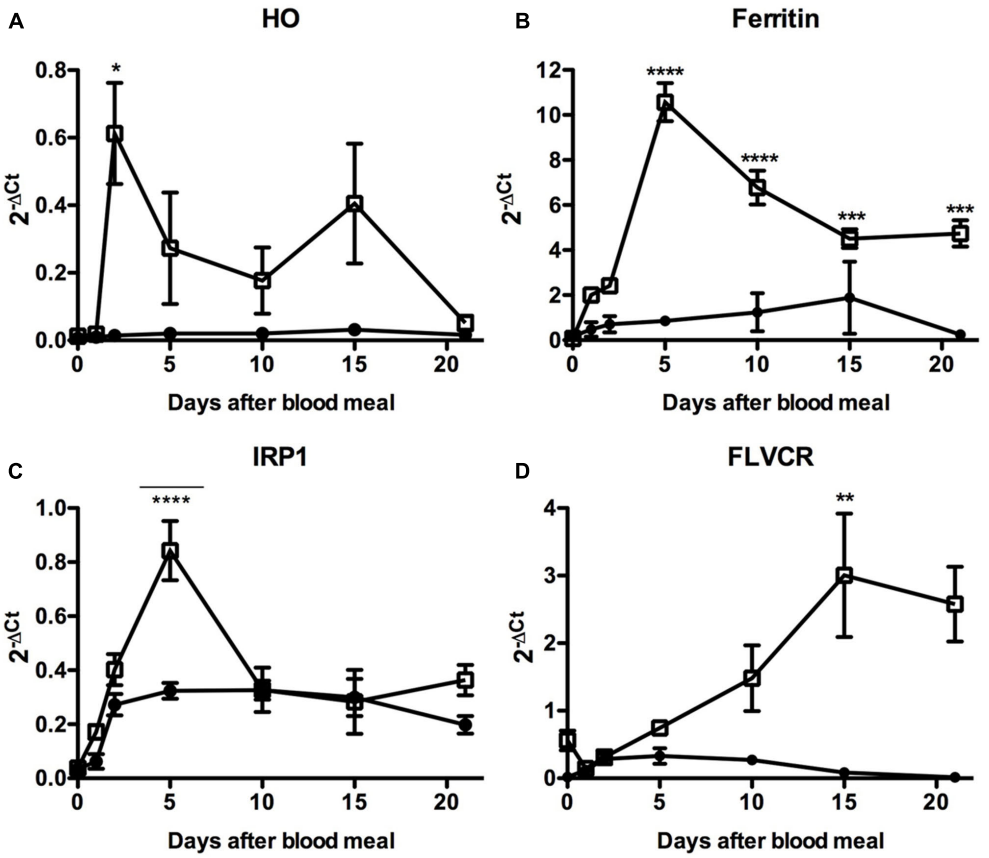

FIGURE 10 | Expression analysis of key genes involved in iron and heme metabolism in the insect midgut compartments. *R. prolixus* adult females were fed on blood and the anterior and PMs were dissected immediately before (day 0) or at the indicated times after the blood-meal for qPCR analysis. Heme oxigenase **(A)**, Ferritin **(B)**, Iron Responsive Protein 1 (IRP1) **(C)**, and Feline Leukemia Virus subgroup C Receptor (FLVCR) **(D)**. The elongation factor 1 gene (RPRC007684) was used as an endogenous control (n = 12–15). Data shown in all graphics are mean ± SE. One-way ANOVA followed by Tukey's multiple comparison test was used to evaluate differences in gene expression during the days after blood ingestion. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.05$, $^{****}p < 0.0001$. These data are resulted from three independent experiments.

Iron responsive protein orthologs of vertebrate IRP1 have been described in insects (Zhang et al., 2001; Lind et al., 2006) that comprise hematophagous species such as *A. aegypti* and *Anopheles gambiae* (Zhang et al., 2002). *R. prolixus* has two paralogs of IRP genes that are phylogenetically included in two clades. One of them codes for a clade of mitochondrial proteins that is close related with *D. melanogaster* aconitase and must not be directly involved in iron homeostasis. The cytosolic one resembles the IRPs of other insects. IRP/IRE regulatory system seems to be operative in the triatomine, as suggested by the presence of IREs in 5′UTRs of secreted ferritin HCH and by the exceptional similarity between the expression of IRP and ferritin HCH (RPRC009256) in the midgut throughout the digestion and phenotypes produced by KD of both genes. The LCH subunits as well as the cytosolic and mitochondrial HCHs lost their IREs in the 5′UTR region of their respective mRNAs, suggesting that they are not under IRP regulation.

Rhodnius genome analysis identified genes belonging to the transferrin superfamily. One of these genes is an ortholog of the canonical Tsf1 genes of other insects. This gene has a many introns and codes for a secreted protein able to bind a single iron atom as do most of the insect tranferrins. Other members of the transferrin superfamily are the melanotransferrins, cell surface GPI-anchored glycoproteins (Alemany et al., 1993). High expressions of these proteins are observed in human melanomas (Suryo Rahmanto et al., 2012). Although orthologs of these proteins have already been found in many animals, including insects, their function in iron metabolism is poorly understood. An exception is the description of the role of *D. melanogaster* melanotranferrin, named Tsf2 in the assembly of epithelial septate junctions (Tiklova et al., 2010). *R. prolixus* contains two typical melanotransferrin genes, which display a GPI anchor domain, and a membrane-addressing signal peptide. Such genes are expressed in the midgut of this insect (Ribeiro et al., 2014). A second group of genes located in the same branch of the transferrin family phylogenetic tree is comprised of three genetically related genes that contain a small number of introns in their gene structure and have no GPI anchor. In addition, there are no transcriptional expression evidences. for these genes, suggesting that they may be pseudogenes. The midgut epithelium

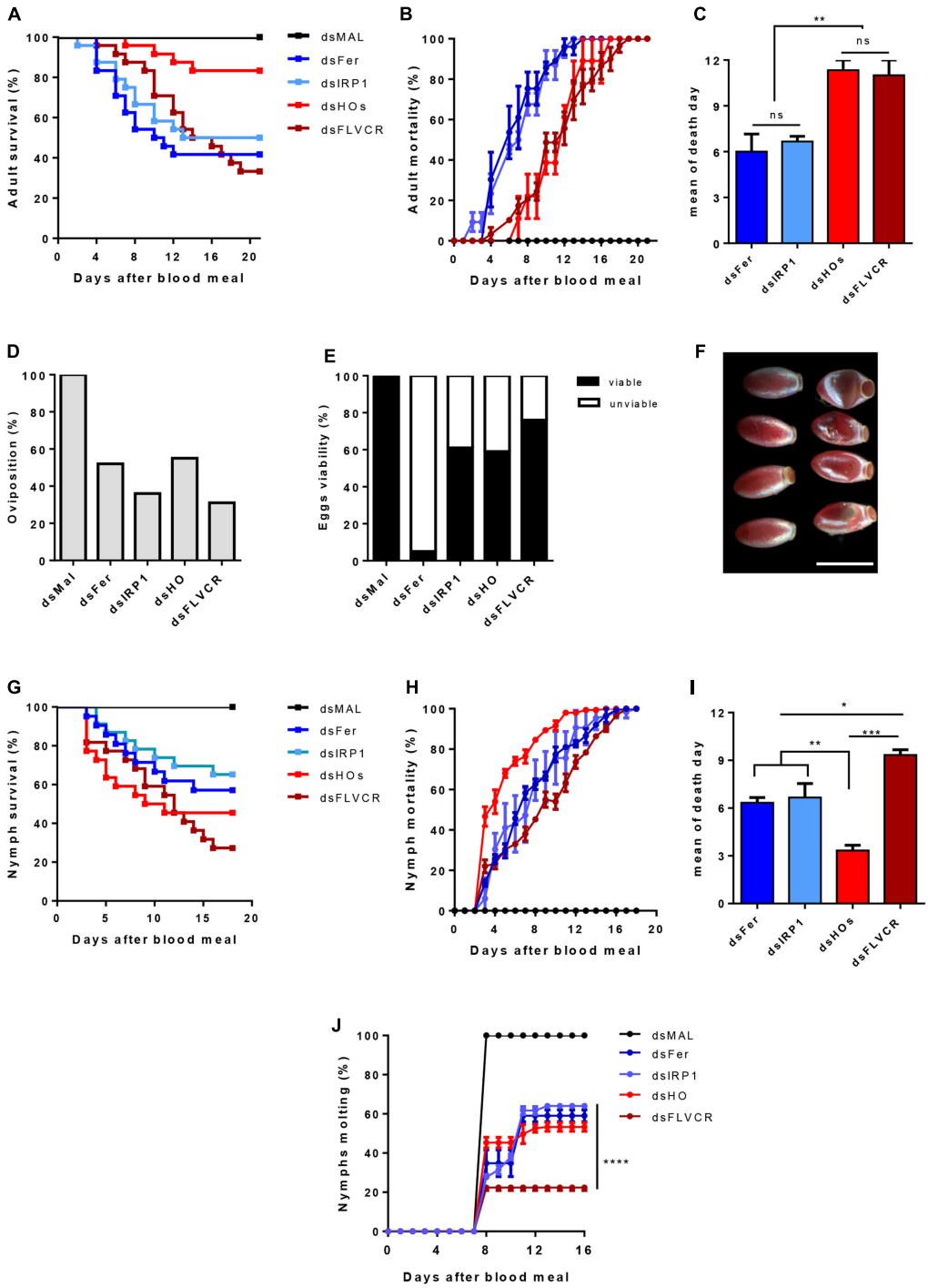

FIGURE 11 | The effect of iron/heme-related genes silencing in the insect physiology. Starved adult females were injected with 1 μg of dsRNA of Ferritin (Fer), IRP (IRP1), Heme Oxygenase (HO) and FLVCR. After 48 h, the animals were fed on blood. The adults survival curves **(A)** oviposition **(D)** and eggs viability **(E)** were monitored for 21 days (n = 24). Nymphs of the first instar (N1) were fed on blood supplemented with dsRNAs of the same genes. The nymphs survival curves **(G)** and the molting to the N2 instar **(J)** were monitored daily for 18 days (n = 30). The survival curves data were replotted as % of total mortality of adults **(B)** and nymphs **(H)** to show the time course of silenced-insects mortality. The mean day of death of adults and nymphs are shown in **(C,I)**, respectively. Data shown in graphics are mean ± SE **(F)** Representative images of the phenotypic morphology of eggs laid by dsMal and dsFer-injected females (scale bar = 1,5 mm). dsMal-injected or fed insects were used as a control. Log-rank (Mantel-Cox) test was used for survival curves comparison, $p < 0.0001$ in **(A)** and $p = 0{,}0001$ in **(G)**. One-way analysis of variance followed by Tukey's multiple comparison test was used to evaluate differences in the adults and nymphs mean death days, $^{*}p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$. Two-way analysis of variance followed by Tukey's multiple comparison test was used to compare molting curves of nymphs fed with iron and heme-related dsRNAs and control group (dsMAL) $^{****}p < 0.0001$. All the experiments described above were independently performed at least three times.

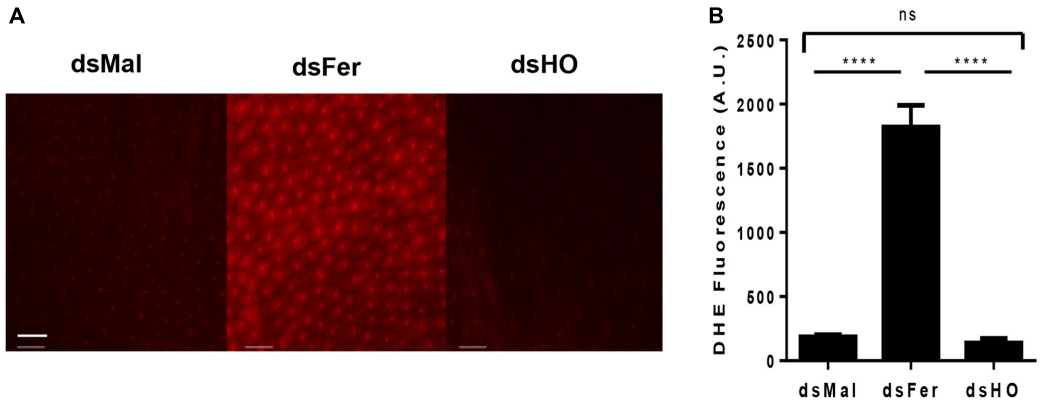

FIGURE 12 | Effect of gene silencing in the production of ROS in the midgut of adult insects. Starved adult females were injected with 1 μg of dsRNA of ferritin or heme oxygenase (HO). After 48 h, the animals were fed on blood. Dissected posterior midguts were incubated at 28°C for 30 min in a solution containing 100 μM DHE. dsMal-injected females were used as a control. The tissues were analyzed by Fluorescence Microscopy (Zeiss Axio Observer Microscope) using a 20× lens with 80 ms exposure time for all conditions. **(A)** The images are representative of a total of 12 intestines analyzed. **(B)** Quantification of fluorescence using Zeiss Axio Observer Quantification software. $P = 0.0016$. One-way ANOVA with Tukey post test. $^{****}p < 0.0001$ scale bar 2 μm.

is one of the most proliferative tissue in insects. Regeneration of the epithelial monolayer, by means of cell division and replacement, require the rearrangement of the protein complexes that maintain cell-cell contact and thus the tissue integrity. Hematophagous vectors, such as *R. prolixus*, suffer a constant biotic challenge by the interaction of their intestinal epithelium with the native microbiota or with the possible pathogens acquired during the blood-meal, thus demanding constant regeneration of damaged cells. In this way the study of the role of melanotransferrins in the maintenance of intestinal epithelial integrity and in the vectorial competence of these insects seems to be of great relevance.

Iron absorption across the apical membrane of mammalian enterocytes requires a divalent iron transporter (DMT1 or NRAMP2) and a ferric reductase (Dcytb). Ferric iron atoms from the diet must be reduced by the duodenal cytochrome b (Dcytb) to be transported by DMT-1. In other cell types, such as the erythrocytes, DMT1 is also involved in the transport of iron delivered by serum transferrin. In this case, iron is reduced by the endosomal-membrane reductase STEAP3 and transported to the cytosol by DMT1 as reviewed by Muckenthaler et al. (2017). Studies on the physiological role of insect DMT-1 orthologs of Malvolio have shown that MLV is involved in iron absorption and has a role in systemic iron homeostasis (Bettedi et al., 2011; Tang and Zhou, 2013b). Mlv is highly expressed in the anterior and posterior regions of the fly midgut and mutation of this gene caused depletion of iron stores in the intestine. As in vertebrates, *R. prolixus* has two MLV paralogs (MLV 1 and MLV2), as do *C. lectularius* and *T. castaneum*. However *T. castaneum* MLVs are not grouped to these closely related hemipteran MLVs suggesting that a gene duplication event occurred independently among these insects.

Another class of proteins involved in the transport of iron in animals is the ZIP-type family of metal carriers. They are transmembrane proteins capable of transporting zinc or iron and are located in different compartments of the cell. In invertebrates, the role of these proteins is still little explored. To date, the role of ZIPs in iron transport has been demonstrated only in *D. melanogaster*. In this insect, the ZIP13 protein acts as an exporter of iron for secretory pathways (Xiao et al., 2014). Seven ZIP orthologs allocated to two different phylogenetic clades were identified in *R. prolixus*. The specificity of these proteins to transport zinc or iron and their role in the homeostatic balance of these metals still needs to be investigated in this triatomine as well as in other insects.

Heme Metabolism Genes

Despite the high levels of heme provided by the digestion of host blood, *R. prolixus* synthesize heme in diverse tissues, including the midgut. The chemical inhibition of ALA dehydrogenase, a component of this pathway, abolishes oviposition, demonstrating the involvement of this pathway in the reproduction of this insect (Braz et al., 1999). As expected, genes encoding for all of the enzymes of the heme biosynthesis pathway were identified in the genome of *R. prolixus.*

Enzymatic degradation of heme, catalyzed by HO, is a central component in the control of heme homeostasis and is considered to be a major antioxidant mechanism against heme-induced damages, in several models. The breaking of the porphyrin ring produces the alpha isomer of biliverdin, CO and Fe^{2+}. A structural and functional characterization of HO was performed in *D. melanogaster* (Zhang et al., 2004). The HO knockdown increases the total heme content in the silenced flies and causes mortality in the larvae and pupae stages (Cui et al., 2008). *R. prolixus* as well as the mosquito *A. aegypti* degrade dietary heme in the midgut epithelial cells (Paiva-Silva et al., 2006; Pereira et al., 2007; Caiaffa et al., 2010). In contrast to what has been demonstrated in other organisms, the routes of heme degradation in these hematophagous insects studied so far are very unique. In the mosquito, alpha BV produced by HO is converted into biglutaminyl-BV, possibly to increase the solubility of this molecule (Pereira et al., 2007). In *R. prolixus,*

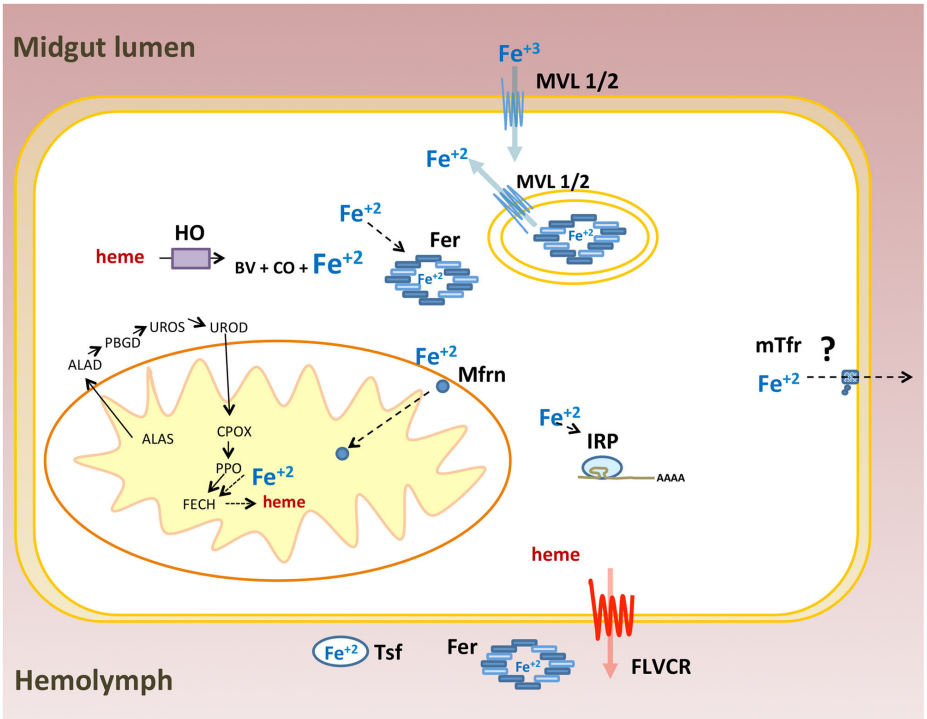

FIGURE 13 | Schematic representation of iron and heme related genes identified in the *Rhodnius prolixus* genome: Ferritin (Fer), Iron Regulatory Protein (IRP), Transferrin (Tsf), Melanotransferrin (mTsf) Malvolio (MLV1/2), Mitoferrin (Mfrn), Aminolevulinic Acid Synthase (ALAS), Delta-Aminolevulinic Acid Dehydratase (ALAD), Porphobilinogen deaminase (PBGD), Uroporphyrinogen III Synthase (UROS), Uroporphyrinogen Decarboxylase (UROD), Coproporphyrinogen III Oxidase (CPOX), Protoporphyrinogen oxidase (PPOX), Ferrochelatase (FECH), Heme oxygenase (HO) and Feline Leukemia Virus C Receptor (FLVCR). ZIP proteins were ommited.

the addition of two gluthathione molecules to the heme molecule is required prior to the porphyrin ring breakage, producing a bicysteinil-gamma biliverdin (Paiva-Silva et al., 2006). Based on the structural studies of HO from other organisms (Wilks and Heinzl, 2014), the specificity of the produced isomer is given by the orientation of the porphyrin ring of the heme within the active site of the enzyme. Thus, the production of a gamma isomer of BV suggests that the HO of this triatomine exhibits structural differences in the regions that coordinate the heme-enzyme interactions. Nevertheless, the *Rhodnius* enzyme conserves the residues involved in the heme interactions, suggesting that amino acid residues interactions not predicted in other HOs may allow an unusual active site conformation that accommodates the modified heme and produces the gamma isomer of BV.

Regarding heme transmembrane transport, a gene coding an ortholog of FLVCR, a member of the major facilitator superfamily of transporters, implicated in heme export in vertebrates (Khan and Quigley, 2013) was identified in the *R. prolixus* genome. FLVCR expression in the PM of fed females, increases during the digestion process. Yang et al. (2010) demonstrated that the efflux of heme performed by FLVCR is facilitated by the interaction with hemopexin, the major heme transport protein in the plasma of mammalians. Although speculative, it is tempting to propose that RHBP, the hemolymphatic heme transporting protein (Dansa-Petretski et al., 1995), may play the same role of mammalian hemopexin, thus allowing the distribution of heme molecules from the midgut to peripheral tissue, including the growing ovaries during oogenesis.

Finally, other genes related to iron/heme metabolism found in other organisms, including the insect *D. melanogaster* remained to be identified in *R. prolixus* genome. As examples are the two multicopper oxidases MCO-1 and MCO-3 with putative reductase activity, that are implicated in iron absorption by *D. melanogaster* midgut cells (Bettedi et al., 2011). The search for orthologs of these genes in the genome of *R. prolixus* by bioinformatics tools revealed a vast number of genes with conserved domains of the multicopper oxidase superfamily. Thus, the correct identification of the respective orthologs requires the use of additional biochemical and molecular approaches. The same occurred in the search for orthologs of ABCB10, a protein that stabilizes mitoferrin during iron influx process and ABCB7, involved in Fe-S complexes biogenesis (Lane et al., 2015). In contrast, orthologs of the component of mammalian mitochondrial apparatus frataxin; as well as the Heme Regulatory Genes (HRG) proteins, involved in heme traffick in parasites, worms and vertebrates (Korolnek and Hamza, 2014) were not found. Possible reasons for these results are low conservation in gene sequence, gene loss, genome assembly errors or low genome coverage.

Gene Expression and Silencing

Expression of ferritin, IRP, HO, and FLVCR genes, known to be capable of modulating the intracellular availability of iron and heme, is differently modulated in the insect gut throughout the digestion process. All of these genes had higher expression levels in the PM where the heme molecules are released after host hemoglobin proteolysis. It is well established in the literature that in many models (Mense and Zhang, 2006) intracellular changes in iron and heme levels can modulate the expression of several proteins related to heme metabolism, whether at transcriptional or post-transcriptional levels (Igarashi and Watanabe-Matsui, 2014). However, the signaling pathways involved in these processes are poorly understood in insects, being mostly focused on post-transcritional regulation performed by the IRP/IRE system.

An increase of IRP expression also occurs in the AM. It has already been shown that IRP can modulate genes not directly involved with iron/heme metabolism, such as citric acid cycle enzyme genes (Gray et al., 1996). We can speculate that IRP would be involved in the post-transcriptional control of genes required by the AM physiology, triggered by blood ingestion. HO has a peak of induction at the very beginning of blood digestion. In the late stage of digestion, a second event of induction is observed. The two peaks of HO expression may not have the same functional significance. The first peak is followed by an increase in ferritin and IRP transcripts levels, suggesting that in the first 10 days of blood digestion, intestinal HO activity may be an important source iron to be exported as ferritin to the peripheral tissues, particularly the growing ovary. The striking dependence of the embryo development on ferritin expression, revealed by the >95% inhibition of hatching upon ferritin silencing, provide additional support for this hypothesis. Previous studies revealed that maternally-provided heme is essential for the embryo development. Silencing of RHBP that transport heme to growing ovaries during oogenesis caused mitochondrial dysfunction and impaired embryogenesis during the early steps of embryo development (Walter-Nuno et al., 2013). However, heme molecules are not degraded during the embryogenic process, being recycled to be incorporated in newly synthesized of embryonic hemeproteins, Thus, heme is not a source of iron for the embryo. Altogether, these data indicate that the correct development of the embryos depends on an adequate iron delivery during oogenesis, performed by maternal ferritin, implicating this protein as the major transporter of iron for growing oocytes.

Of note, by day 15 after the blood-meal when there is the second peak of HO expression, most eggs (>80%) were already laid (Braz et al., 2001; Walter-Nuno et al., 2013), but there is still a significant amount of blood to be digested in the AM (Coelho et al., 1997). Therefore, the simultaneous breakdown of residual heme by HO in the intestinal epithelial cells and iron exportation to the ovary through ferritin would also prevent iron-induced damage to the gut.

Interestingly, silencing of either HO or FLVCR, both mechanisms to detoxify heme, induced death of adult females, but this occured only as a relatively late effect: >80% of the deaths occurring upon injection were after the 10th day post blood-meal. In contrast, a similar proportion (over 80%) of the lethality due to dsFerritin or dsIRP injection occurs before day 10 after the blood-meal. The late activation of FLVCR, together with the second, non-vitellogenic peak of HO, brings in the interesting possibility that these two proteins may work together, specially in the second half of digestion of the blood meal. In fact, the increase of FLVCR expression during the digestion and the severe phenotypical effects observed on survival of adults and nymphs and molting of FLVCR silenced insects. is in accordance with its role as an heme exporter, being a complementary mechanism to the route of degradation, aiming to control heme levels within enterocytes, avoiding heme overload and potential oxidative damages. In addition, the increase in heme transport from the midgut to the hemolymph can provide heme molecules to other tissues, such as the ovaries, where they are required for oocyte growth (Walter-Nuno et al., 2013).

The toxicity of iron is largely associated with its pro-oxidant properties. In the presence of oxygen, free iron promotes, through the Fenton and Haber–Weiss reactions, the generation of reactive species of oxygen, including the extremely reactive hydroxyl radical. The continuous production of ROS due to excess of iron, leads to chronic oxidative stress, causing tissue injuries and the appearance of the pathological symptoms observed with the progress of the diseases (Ryter and Tyrrell, 2000; Papanikolaou and Pantopoulos, 2005).

Not only free iron but also heme, its complex with protoporphyrin IX, is a potential pro-oxidant molecule. It promptly reacts to organic hydroperoxides producing alkoxyl or peroxyl lipid radicals, thus increasing lipid peroxidation (Ryter and Tyrrell, 2000). In addition, heme may interfere with permeability and selectivity of membranes upon insertion into the phospholipid bilayer (Schmitt et al., 1993).

This outcome points to a critical role of iron/heme-dependent ROS generation as a major potential source of oxidative damage, a conclusion that is strongly supported by the actual evaluation of oxidative species by use of the ROS-sensitive probe DHE, that revealed a dramatic increase in ROS upon ferritin silencing; this increase may be implicated as one cause of the high mortality observed in Fer and IRP silenced-insects. The apparent lack of effect of HO silencing might be due either to insuficient reduction of the enzyme or possibly to the preventive action of other antioxidant mechanisms that have been shown to operate at the same time. One is the heme aggregation (Oliveira et al., 1999, 2002) that quantitatively is the most important mechanism, sequestering most of the ingested heme (>95%) as the chemical inert crystalline hemozoin. The other would be the lowering of ROS production by endogenous pathways, particularly the mitochondria (Gandara et al., 2016). Additionally, the increase in heme concentration to levels capable of promoting ROS formation may be avoided by a compensatory efflux of heme by FLVCR.

Therefore, although there are a great number of vertebrates homologs have been found in *R. prolixus*, their biological roles and relevance have not been characterized in insects. Addressing in future research the function of these genes should result in better understanding of how insects control iron and heme homeostasis. Moreover, it may reveal new pathways and

mechanisms that have allowed *R. prolixus* and other blood sucking insects to adapt to hematophagy.

AUTHOR CONTRIBUTIONS

AW-N, MT, PO, and GP-S designed the project and experiments. AW-N, MT, and RM performed the experiments. AW-N, MT, RM, PO, and GP-S analyzed the data. AW-N, MT, RM, and GP-S wrote the paper. AW-N, MT, RM, PO, and GP-S revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2018.00019/full#supplementary-material

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Identification of Candidate Iron Transporters From the ZIP/ZnT Gene Families in the Mosquito *Aedes aegypti*

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Mosquito-transmitted viral pathogens, such as dengue and Zika, afflict tens of thousands of people every year. These viruses are transmitted during the blood-feeding process that is required for mosquito reproduction, the most important vector being *Aedes aegypti*. While vertebrate blood is rich in protein, its high iron content is potentially toxic to mosquitoes. Although iron transport and sequestration are essential in the reproduction of vector mosquitoes, we discovered that culicine mosquitoes lack homologs of the common iron transporter NRAMP. Using a novel cell-based screen, we identified two ZIP and one ZnT genes as candidate iron transporters in the mosquito *A. aegypti*, the vector of dengue, Zika, and chikungunya. We determined the organ-specific expression pattern of these genes at critical time points in early reproduction. The result indicates modulation of these genes upon blood feeding, especially a ZIP13 homolog that is highly up-regulated after blood feeding, suggesting its importance in iron mobilization during blood digestion and reproduction. Gene silencing resulted in differential iron accumulation in the midgut and ovaries. This study sets a foundation for further investigation of iron transport and control strategies of this viral vector.

Keywords: Aedes, iron transporter, ZIP, ZnT, ferritin

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INTRODUCTION

In recent years, diseases caused by mosquito-transmitted flaviviruses such as Zika and dengue fever have raised concern internationally (Bhatt et al., 2013; Gatherer and Kohl, 2015; Hennessey et al., 2016). Globally, the incidence of these diseases, especially dengue, show a steady increase over the past 50 years and additional effective measures to reduce the burden of infection are urgently needed (WHO, 2009; Bhatt et al., 2013). *Aedes aegypti* is the major vector of these viruses as well as other viruses and filarial parasites due to its adaptation to domestic settings (Powell and Tabachnick, 2013). Blood feeding is required for *A. aegypti* to initiate egg development and provide nutrients to its offspring; and it is during this act of bloodfeeding that the viruses are transmitted.

Vertebrate blood, while rich in protein, is potentially toxic in large quantities because of its high iron content that can catalyze the formation of oxygen radicals. Mosquitoes have evolved to use vertebrate blood for their reproduction by overcoming this dilemma. Conversely, mosquitoes could use high oxidative stress due to iron in blood meal to control pathogen infection. For example, activation of the Toll immune pathway in *A. aegypti* reduced dengue virus titer which is

correlated to reduction of oxidative response genes (Xi et al., 2008); and nitric oxide metabolites by oxyhemoglobin from blood limits development of malaria parasites in the mosquito midgut (Peterson et al., 2007). While digestion of blood proteins is extensively studied, utilization of blood-derived iron in mosquitoes is understudied. In fact, despite iron being an essential nutrient and co-factor for a number of biological processes, metabolism of iron is not fully understood, particularly in insects (Tang and Zhou, 2013). Iron is transported between and within cells in different forms—free ions or bound to protein carriers such as ferritin and transferrin (Pham and Winzerling, 2010; Garrick, 2011). In *Drosophila melanogaster* ionic iron is absorbed through Malvolio (Mvl), a homolog of mammalian NRAMP (natural resistance associated macrophage protein), which is a DMT (divalent metal ion transporter) (Orgad et al., 1998). NRAMP homologs function as iron transporters from bacteria to humans and are highly conserved (Nevo and Nelson, 2006). Despite the potential importance of NRAMP, the only other insect NRAMP to be functionally characterized was that from the malaria vector *Anopheles albimanus* (Martinez-Barnetche et al., 2007). Thus, more detailed molecular mechanisms for iron transport remain to be determined.

NRAMP has also been identified as a gene conferring resistance to intracellular pathogen infection in mammals (Vidal et al., 1993). Recently, Rose et al. (2011) reported that *Drosophila* Mvl serves as a receptor for Sindbis virus (SINV) entry *in vitro* and *in vivo*. The same study showed that suppressing Mvl expression by supplementation of iron reduced SINV infection in *A. aegypti* Aag2 cells as well as *Drosophila* DL1 cells. From this result they concluded that NRAMP is involved in SINV entry into the natural mosquito host. However, despite the availability of several culicine genomes, we were unable to identify an NRAMP ortholog in *A. aegypti* or other culicine mosquitoes. This raised the even larger question of how these bloodfeeding mosquitoes acquire iron from their diet. As a result, we screened members of the ZIP (Zinc-regulated transporter/Iron-regulated transporter-like) and ZnT (Zinc transporters) families for involvement in iron transport in *A. aegypti* under the hypothesis that culicine mosquitoes co-adapted these genes as a functional replacement for NRAMP. We identified three candidate iron transporter genes via an *in vitro* cell-based iron-specific reporter system. To further verify their iron transporting functions, expression analysis and RNAi phenotype assays suggest that these genes indeed related to iron transport in *A. aegypti*.

MATERIALS AND METHODS

Sindbis-EGFP Replication Assay

To generate recombinant Sindbis virus expressing EGFP, the EGFP ORF was cloned into the Sindbis virus expression plasmid pTE/3′2Jmcs as previously described (Myles et al., 2008). Following plasmid linearization, SP6-based RNA transcription and transfection into BHK-21 cells, virus was harvested as described (Myles et al., 2008). For challenge experiments, Aag2 cells were seeded into sealed 25 cm^2 flasks and incubated with 50 μM of the indicated source of iron for 72 h prior to virus challenge. Cells were infected by gently rocking for 30 min at an MOI of ∼0.01, at which time virus suspension was removed and growth media replaced; cells were imaged on a Zeiss Axiovert 200 at 24 and 48 h after infection.

Plasmid Assembly

To develop an iron inducible reporter plasmid, the putative *A. aegypti* ferritin light-chain (AAEL007383) promoter region was amplified using the primers listed in Supplementary Table 1. The resulting amplicon was digested with HindIII/NcoI and ligated into the HindIII/NcoI sites of pGL3-basic (Promega; Madison, WI), yielding the final reporter construct pLCH-FFluc. Construction of the normalization control plasmid pSLfa-PUb-Renilla luciferase was described previously (Haac et al., 2015).

dsRNA Preparation

To generate dsRNA targeting each candidate ZIP and Znt gene, PCR amplicons were generated using Phusion polymerase (New England Biolabs; Ipswich, MA), primers listed in Supplementary Table 1 and cDNA generated from *A. aegypti* Liverpool strain mosquitoes as template. Amplicons were used as a template for either the Replicator RNAi kit (Thermo-Fisher Scientific; Waltham, MA) with T7 polymerase and Phi6 replicase or the MEGASCRIPT T7 RNAi kit (Life Technologies; Carlsbad, CA), with two T7 promoters. After synthesis dsRNAs were nuclease treated to eliminate ssRNA as well as the DNA template, and then purified using the MEGAClear kit (Life Technologies; Carlsbad, CA).

Cells, Transfections, and Luciferase Assays

For all cell culture experiments, *A. aegypti* Aag2 cells were maintained in Leibovitz's L-15 media (Gibco), supplemented with 10% FBS (Atlanta Biologicals), 2% Tryptose phosphate broth (Gibco), and 1% Pen-strep (Corning) at 28°C.

For the RNAi screen of ZIP and ZnT genes for a role in iron transport, dsRNA transfections were performed using Lipofectamine 2000 (Life Technologies; Carlsbad, CA). Briefly, for each dsRNA, 4 μL Lipofectamine 2000 was diluted into 100 μL Opti-mem I Reduced serum media (Gibco); 1.6 μg dsRNA was diluted into a second 100 μL Opti-mem I. These mixtures were combined in the well of a 12-well plate and incubated for 5 min. Cells were counted, diluted to 5×10^6 cells/ml with 1 mL of cell suspension seeded into each well containing the transfection mix. After 3 days cells were aspirated into fresh media, counted and again diluted to 5×10^6 cells/ml. To introduce the reporter construct and normalization control, transfection mixes were prepared in a 96 well plate as follows: per well 0.5 μL Lipofectamine 2000 in 25 μL Opti-mem I, 100 ng pLCH-FFluc and 100 ng pSLfa/PUb-Renilla luciferase (Haac et al., 2015) in 25 μL Opti-mem I. The DNA/Lipofectamine mix was incubated for 5 min and then 100 μL of cells (5×10^5) were added to each well. Twenty-four hours after transfection lysates were prepared by aspirating the media, washing the cells once with PBS, then adding 45 μL per well of 1 × Passive Lysis Buffer (Promega; Madison, WI). Luciferase assays were performed using 20 μL lysate and the Dual Luciferase Assay Kit (Promega; Madison, WI) on a GloMax Multi-detection System (Promega;

Madison, WI). To normalize firefly luciferase values based on the number of living transfected cells, firefly luciferase values were divided by Renilla luciferase values for each sample. Normalized firefly luciferase values were compared using a one-way ANOVA and Bonferroni's multiple comparison test, as implemented in GraphPad v5.04.

For metal specificity, cells were transfected as above and simultaneously treated with the indicated concentration of FAC, $CuSO_4$, or $ZnSO_4$. For endocytosis experiments, Aag2 cells were seeded in a 96-well plate at 50,000 cells/well. After 24 h, cells were transfected as above except with 18 ng pGL3-AaLCH-FF + 6 ng pSLfa-PUb-RL. After another 24 h, cells were treated with 80 μM Dynasore, 75 μM EPIA, 100 μM Deferoxamine or DMSO alone with or without 100 μM FAC. Dynasore-treatment was stopped at 1.5 h to prevent cell death (media was replaced) and cells for all treatments were harvested after 8 h with luciferase assays and statistical analysis performed as above.

Bioinformatics and Phylogenetic Analyses

Amino acid sequences for mosquito (Vectorbase.org), Drosophila (Flybase.org) or human (Genbank) ZIP (SLC39), ZnT (SLC30), and NRAMP (SLC11) genes were downloaded from the respective databases and aligned using ClustalW (Larkin et al., 2007) in MEGA6 (Tamura et al., 2013). Refseq numbers for each sequence are provided in Supplementary Table 2. Multiple sequence alignments were used to infer phylogenetic trees using the Neighbor-joining method in MEGA6, with the pairwise deletion option and the bootstrap method for branch support (1,000 replicates). To search for Mvl/NRAMP orthologs in culicines, the *D. melanogaster* and *An. gambiae* NRAMP protein sequences were used as queries using either NCBI blastp (refseq) or Vectorbase blastp [*A. aegypti* peptides, AaegL3.5; *A. albopictus* peptides, AaloF1.2; C6/36 cells, NCBI-101; Cx. Quinquefasciatus peptides, CpipJ2.4] and tblastn [*A. aegypti* ESTs; AaegL3.5 geneset, AaegL3 contigs, AaegL3 scaffolds; C636 cells transcripts, NCBI-101; *A. albopictus* assembled transcriptome TSA:GAPW00000000.1; AaloF1.2 transcripts, AaloF1 contigs, AaloF1 scaffolds; CpipJ2 contigs, CpipJ2 scaffolds, CpipJ2.4 geneset). A single EST identified from *A. aegypti* larvae was used as a query in NCBI blastx. For all blast searches, Max *e*-value = 10, Word size = 3, Scoring Matrix = Blosum62 and the number of results per query was 50.

Mosquitoes

For all experiments, *A. aegypti* (Liverpool strain) was maintained in an insectary in humidified chambers at 28°C, 70% RH and 14H:10H (L:D) cycle. The mosquito colony was maintained on defibrinated sheep blood (Colorado Serum Company, Denver CO) using an artificial feeding system.

Organ-Specific Expression

To examine organ-specific transcript expression, midguts (Mg), Malpighian tubules (MT), ovaries (Ov) and carcasses (whole body without Mg, MT and Ov) were dissected from female mosquitoes in 1 × PBS and transferred immediately into 1.5-mL tubes containing TRIzol reagent (Thermo Fisher). Samples were

TABLE 1 | Blastp-based search of mosquito proteins using the Malvolio (*D. melanogaster* NRAMP ortholog) protein sequence as a query.

Hit	Description	Query	Aln length	*E*-value	Score	Identity
AGAP012464-PA	**Natural resistance-associated macrophage protein**	**Dmel_Mvl-PB**	**373**	**4E-163**	**1650**	**0.612**
An. gambiae						
AGAP010308-PA	Nucleolar complex protein 3	Dmel_Mvl-PB	56	1.8	91	0.25
AGAP013324-PA	Putative G-protein coupled receptor GPCR	Dmel_Mvl-PB	51	3	89	0.308
Cu. quinquefasciatus						
CPIJ005183-PA	Organic anion transporter, putative	Dmel_Mvl-PB	131	3.6	88	0.246
CPIJ010125-PA	Conserved hypothetical protein	Dmel_Mvl-PB	53	5.7	86	0.321
CPIJ001796-PA	Conserved hypothetical protein	Dmel_Mvl-PB	55	7.3	85	0.273
CPIJ014837-PA	Serine/threonine protein kinase lats	Dmel_Mvl-PB	34	6.5	85	0.382
A. aegypti						
AAEL013826-PA	Serine/threonine protein kinase lats	Dmel_Mvl-PB	40	2.4	90	0.4
AAEL004986-PA	Smg-7 (suppressor with morphological effect on genitalia protein 7)	Dmel_Mvl-PB	51	5	86	0.25
AAEL010052-PA		Dmel_Mvl-PB	20	2.8	86	0.4
AAEL012774-PA	Protease m1 zinc metalloprotease	Dmel_Mvl-PB	49	7.8	84	0.204
A. albopictus						
AALF008030-PA	Serine/threonine protein kinase lats	Dmel_Mvl-PB	40	0.51	98	0.4
AALF005921-PA		Dmel_Mvl-PB	42	0.89	92	0.468
AALF022221-PA		Dmel_Mvl-PB	42	0.89	92	0.468
AALF027921-PA	GPCR Leukokinin Family	Dmel_Mvl-PB	47	4.2	87	0.319
AALF021769-PA		Dmel_Mvl-PB	49	9.4	83	0.347
AALF012982-PA		Dmel_Mvl-PB	86	8.7	82	0.25
AALF026370-PA		Dmel_Mvl-PB	49	9	80	0.327

taken from sugar-fed (5 days after eclosion), 6 h post-bloodmeal (PBM) and 24 h PBM for three biological replicates.

To investigate effects of free iron on AaeZIP13 expression, female mosquitoes were fed with an artificial meal (AM) containing 150 mM NaCl, 20 mM $NaHCO_3$, 1 mM ATP and 1% low melt temperature agarose supplemented with 5 mM of FAC using an artificial feeding system. At 24 h after feeding, midguts were dissected for qRT-PCR analysis (pools of 20 individuals for each of three biological replicates).

RNA was isolated using the TRIzol method following the manufacturer's protocol (Thermo Fisher), and the total RNA was treated with RNase-free DNase (TURBO DNAfree kit: Thermo Fisher). Isolated RNA was quantified on a SpectraMax (Molecular Devices, Sunnyvale, CA). One microgram of RNA from each sample was used to synthesize cDNA using anchored oligo d(T) primers ($d(T)_{20}$-VN) with MultiScribe reverse transcriptase (Applied Biosciences) at 42°C for 2 h and reverse transcriptase was inactivated at 65°C for 20 min. cDNA was diluted to 1/50 in nuclease-free H_2O, which was used for qRT-PCR analysis.

Quantitative real-time PCR (qRT-PCR) was performed on CFX69 Touch Real-Time PCR Detection System (Bio Rad), using PerfeCTa qPCR FastMix II (QuantaBio). Primers listed in Supplementary Table 1 were designed using Primer3 server (version 4.0.0) (Untergasser et al., 2012) or PrimerSelect (DNASTAR), which amplify 107–247 bp fragments of cDNA and amplification efficiency (E) was empirically verified to be 0.9–1.0. Reactions were performed in 15 µL with 200 nM each primer and 3.75 µL of 1/50 diluted cDNA in triplicates. All reactions were performed with 30 s at 95°C, followed by 45 cycles of 5 s at 95°C, 15 s at 60°C and 10 s at 72°C, and melt curve analysis at 70–95°C. Expression was calculated relative to the reference housekeeping gene rpS7 by the dCt method (Liu and Saint, 2002). C_q values as well as calculated efficiencies for all primer sets/samples are presented in Supplementary Table 3.

Mosquito RNAi

To knockdown the expression of each candidate iron transporter in whole mosquitoes, female *A. aegypti* (2–3 days after eclosion) were injected with 1 µg of dsRNA using a Nanoject II microinjector (Drummond) with a needle drawn from glass capillary and were kept at 28°C rearing chamber for indicated time until each experiment was performed. Control group of mosquitoes was injected with 1 µg of dsRNA against exogenous EGFP sequence.

Semi-quantitative Intracellular Free Fe^{2+} Estimation by Calcein AM and Fecundity/Fertility Test

To determine the relative cytoplasmic iron content of dsRNA-injected mosquitoes, injected individuals were fed on defibrinated sheep blood (Colorado Serum Company) at 3 days post injection. From a part of each group (10–15 individuals), midguts and ovaries at 24 h PBM were dissected and treated with 2.5 µM of calcein AM (Thermo-Fisher) in 1 × PBS at room temperature on an orbital shaker. After 1 h incubation, calcein AM solution was removed and replaced with 1× PBS. Fluorescence images of the organs were captured at Ex: 450–490/Em: 500–550 with the same exposure time and gain setting within the same organ for each experiment using Leica M165 FC stereomicroscope equipped with DFC3000C digital camera. The images were analyzed by ImageJ (Schneider et al., 2012) for pixel intensity using manual polygon tool. Calculated values were compared using a Students two-tailed *t*-test as implemented in GraphPad v5.04.

To measure the effect of dsRNA treatment on fertility, the remaining individuals were kept until 72 h PBM and individually placed in a well of a 24-well plate with 2% agarose on the bottom as a wet surface for egg laying for 24 h. After laying eggs, each well was photographed by a digital camera (Olympus TG-4, microscope mode) to count eggs and water was added to the wells. After additional 5 days, each well of the plate, now with hatched larvae was photographed and the hatched larvae were counted. The number of eggs/larvae were compared with the control group using a Students two-tailed *t*-test as implemented in GraphPad v5.04.

Chemicals

Deferoxamine (mesylate) [DFO], 3-hydroxy-2-[(3,4-dihydroxyphenyl)methylene]hydrazide-2-naphthalenecarboxylic acid [Dynasore] and 5-(N-ethyl-N-isopropyl)-Amiloride [EIPA] were obtained from the Cayman Chemical Company. Iron (II) sulfate heptahydrate (F8633), ferric

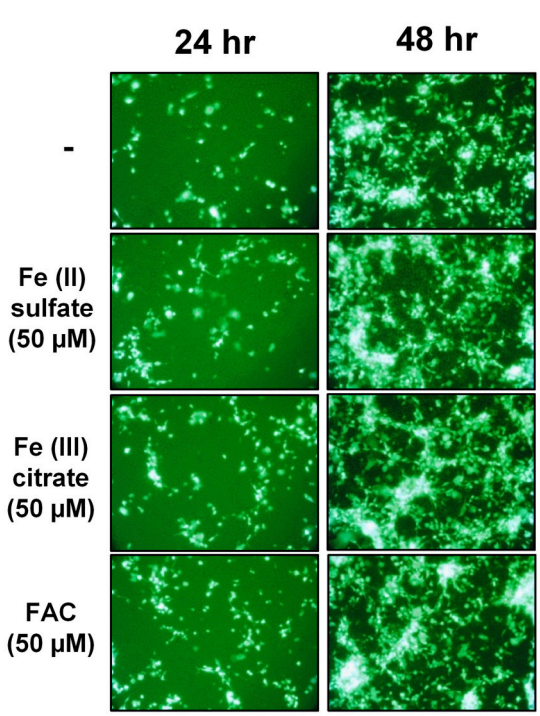

FIGURE 1 | Treatment with Fe^{2+} or Fe^{3+} does not interfere with SINV replication in *A. aegypti* cells. Aag2 cells were pre-treated with the indicated Fe compound and then challenged with a recombinant SINV expressing EGFP. Cells were photographed at 24 and 48 h post infection.

citrate (F3388), copper (II) sulfate pentahydrate (C8027), and zinc sulfate heptahydrate (Z0251) were obtained from Sigma-Aldrich. Ferric ammonium citrate (40600001) was obtained from Bioworld. Calcein acetoxymethyl (AM) was obtained from Life Technologies.

RESULTS

Rose et al. (2011) reported that the iron importer NRAMP (Mvl) serves as a receptor for SINV entry into *Drosophila*, vertebrate and even *A. aegypti* mosquito cells. In particular, Rose et al. (2011) suggested that treating *Drosophila* or *A. aegypti* cultured cells with 32 or 160 μM Fe^{3+} was sufficient to substantially inhibit the entry of SINV, due to a presumed down-regulation of NRAMP in response to Fe overload. We sought to confirm these findings, as a bioinformatic search of annotated genes, scaffolds, contigs, and available ESTs using either the *Drosophila* Mvl/NRAMP or *Anopheles gambiae* Mvl/NRAMP protein sequences failed to reveal an NRAMP ortholog in any of the sequenced culicine mosquito genomes (*A. aegypti, A. albopictus, Cx. quinquefasciatus)* (**Table 1** and Supplementary Table 4). [Note: a single EST described as coming from *A. aegypti* larvae was identified with an *e*-value of 6e-28 and 49% percent identity to *An. gambiae* NRAMP, but this EST was found to be 93% identical to NRAMP from Barley, suggesting it as a contaminant of the larval diet. No other *e*-value was less than 0.4]. To replicate the experimental protocol reported by Rose et al. (2011), we treated *A. aegypti* Aag2 cells with 50 μM of reduced (Fe II sulfate) or oxidized (Fe III citrate; ferric ammonium citrate FAC) iron and challenged the cells with a recombinant SINV expressing EGFP. We observed no difference in the number or brightness of SINV-EGFP infected cells at 24 or 48 h (**Figure 1**). We conclude that NRAMP is not likely a receptor for SINV in *A. aegypti*, or for any culicine mosquitoes that serve as a natural vector of this virus. As NRAMP is a highly conserved (when present, anyway) importer of dietary iron, and its absence in culicine mosquitoes suggests that alternative methods for the import of molecular iron must have evolved to compensate for the loss of this gene. Thus, we turned our attention to the identification of alternative iron transporters in *A. aegypti*.

Two large gene families, the ZIPs and ZnTs, are known to contain members that transport iron across membranes;

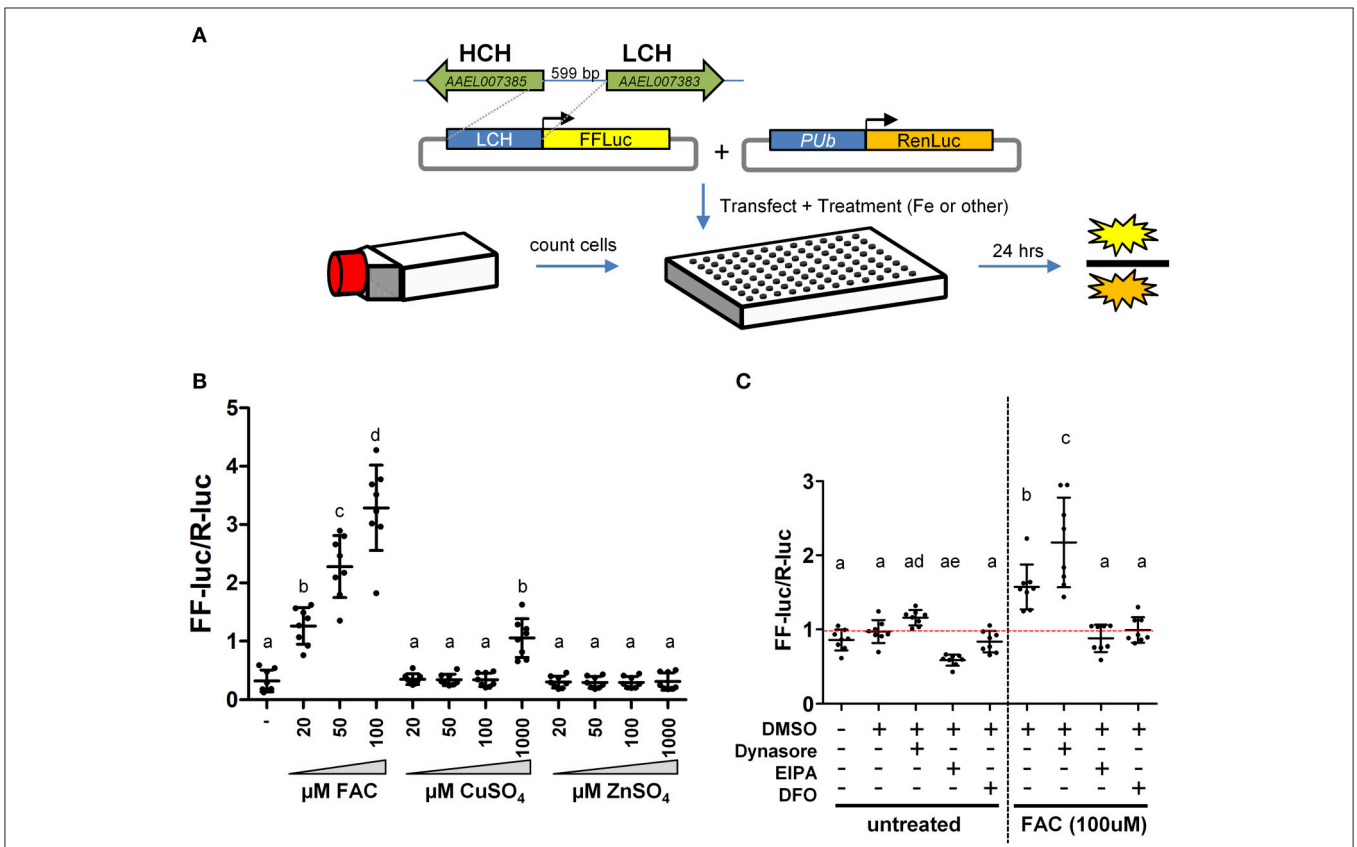

FIGURE 2 | A luciferase-based sensor induced by iron. **(A)** Schematic representation of the experimental workflow. *A. aegypti* cells were counted, mixed with transfection reagent/plasmid DNA and seeded into 96 cell plates with the indicated metal included in the cell culture medium. **(B)** Ratio of firefly luciferase (FF-luc) to *Renilla* luciferase (R-luc) in Aag2 cells after treatment with the indicated concentration of Fe, Cu or Zn. **(C)** Ratio of FF-luc to R-luc in Aag2 cells treated with Dynasore (80 μM, 1.5 h), EIPA (75 μM, 8 h), DFO (100 μM, 8 h). Luciferase values were determined after 8 h for all samples. For **B** + **C**, mean (horizontal line) and standard deviation (error bars) are indicated; lower case letter designations indicate statistically different groups following ANOVA ($P < 0.0001$) and Bonferroni's multiple comparison test ($P < 0.01$) (GraphPad v5.04). Red dotted line indicates the mean of the DMSO untreated sample.

A. aegypti encodes 10 members of the ZIP family and 8 ZnTs. In order to identify those that might be involved in the transport of iron, we constructed a luciferase-based sensor whereby the luciferase coding sequence was placed under the control of a promoter derived from the ferritin light chain gene (**Figure 2A**), a gene previously shown to be induced by iron treatment (Pham and Chavez, 2005). Following transfection of the plasmid into *A. aegypti* cells, we sought to establish the specificity of the reporter. Cells were incubated with increasing concentrations of Fe, Cu, or Zn and luciferase activity determined. We observed strong activation only in the presence of Fe (**Figure 2B**). Further experiments demonstrated that both reduced (Fe^{2+}) or oxidized (Fe^{3+}) iron served equally well to activate the reporter, and that at least 8 h was required to observe activation (Supplementary Figure 1). Next, we sought to determine whether endocytic pathways are involved in the uptake of free iron, as opposed to transport at the cell surface. *A. aegypti* cells were treated with the dynamin inhibitor Dynasore [inhibits both endo- and exo-cytosis; (Macia et al., 2006)] or the Na^+/H^+ exchanger inhibitor EIPA [inhibits macropinocytosis; (Fretz et al., 2006)]. As a control, cells were also treated with the iron chelator deferoxamine (DFO). In the presence of FAC, inhibiting dynamin filaments substantially increased reporter activity in the cells, while inhibiting Na^+/H^+ exchange decreased induction of ferritin. These experiments suggest that macro-pinocytosis may be used during iron import, and that dynamin-dependent processes such as exocytosis may be critical to reducing iron levels through export (**Figure 2C**).

Having confirmed that our luciferase reporter can detect changes in iron-dependent ferritin induction in mosquito cells, we designed double-stranded RNAs for each of the *A. aegypti* ZIPs and ZnTs and performed RNAi knockdown prior to transfection and measurement of the luciferase reporter. While a few genes produced small, but significant changes in a subset of experiments (Supplementary Figure 2), we repeatedly observed only three genes (AAEL014762, AAEL013490, AAEL000077) that when knocked down resulted in substantial changes in luciferase activity (**Figure 3**). Of these, two are members of the ZIP family (AAEL014762 and AAEL013490 are 1:1 orthologs of the fly and human ZIP13 and ZIP11 genes) and one a member of the ZnT family (AAEL000077, an ortholog of the fly ZnT86D and human ZnT7 genes) (**Figures 4A,B**).

We next investigated the organ-specific expression of each candidate iron transporter gene by quantitative real-time PCR (qRT-PCR) in the midgut (Mg), ovaries (Ov), and Malpighian tubules (MT) of the adult mosquito. Prior to bloodfeeding, AaeZnT7 is enriched in the Mg and Ov, AaeZIP11 is enriched in Mg and MT, and AaeZIP13 is enriched in Ov with the overall lowest expression (**Figure 5A**). Following a bloodmeal, the expression of all three transcripts increased. The most prominent up-regulation was observed for AaeZIP13 in the midgut, with an almost 45-fold increase in transcript levels compared to the resting state at 24 h PBM, while other genes merely increased up to 2-fold (**Figures 5B–D**). We further examined whether this up-regulation of AaeZIP13 upon bloodmeal is a specific response to free iron in the bloodmeal by feeding an artificial meal (AM) containing salt and FAC. AaeZIP13 expression was significantly

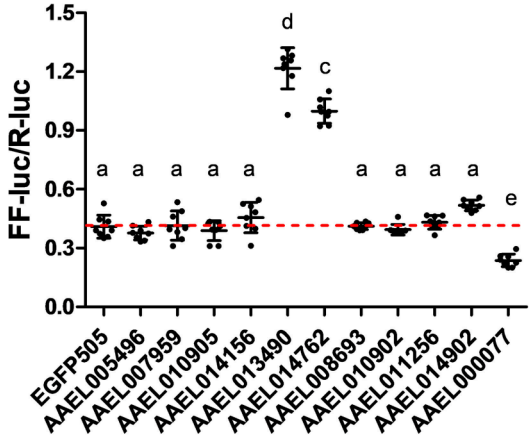

FIGURE 3 | Knockdown of ZIP or ZnT genes alters ferritin induction in *A. aegypti* cells. Ratio of firefly luciferase (FF-luc) to *Renilla* luciferase (R-luc) in Aag2 cells after treatment with the indicated dsRNA. Mean (horizontal line) and standard deviation (error bars) are indicated; lower case letter designations indicate statistically different groups following ANOVA ($P < 0.0001$) and Bonferroni's multiple comparison test ($P < 0.01$) (GraphPad v5.04). Red dotted line indicates the mean of the EGFP505 dsRNA treated sample.

up-regulated 24 h after the AM although the extent of up-regulation was much less than a real bloodmeal (**Figure 5E**). These results indicate that AaeZIP13 exhibits a strong response to the components in blood or blood feeding, which is likely related in iron transport during blood meal digestion.

We therefore sought to determine a physiological role of AaeZIP13 during blood-meal digestion and reproduction following RNAi-based gene silencing. Female mosquitoes were injected with dsRNA against AaeZIP13 or control (EGFP) and were fed on blood at 3 days post injection (dpi); midguts and ovaries were dissected for calcein AM treatment at 24 h PBM. Calcein AM freely diffuses across the plasma membrane and is converted to fluorescent calcein in living cells; the fluorescence is quenched when it is bound to iron (Fe^{2+}). Knockdown of AaeZIP13 resulted in a significant decrease in calcein fluorescence in the midgut, signifying an increase in cytoplasmic iron content. Conversely, we observed an increase in calcein fluorescence in the ovaries at 24 h PBM suggesting less iron accumulation at this time (**Figures 6A,B**). We also confirmed that dsRNA effectively reduced transcript levels in the Mg and to the lesser extent in the Ov at 24 h PBM (**Figure 6C**). As iron levels in the ovaries were altered upon AaeZip13 depletion, we further investigated the impact of AaeZIP13 gene silencing on fecundity and fertility. We detected no difference in fecundity (egg number per female) and fertility (hatch rate) (**Figures 7A,B**). Likewise, iron content in the ovaries following AaeZip13 dsRNA treatment at 72 h PBM was indistinguishable from the control (**Figure 7C**).

DISCUSSION

Contrary to the observation made by Rose et al. (2011), we found that ionic iron had no effect on SINV infection in *A. aegypti*

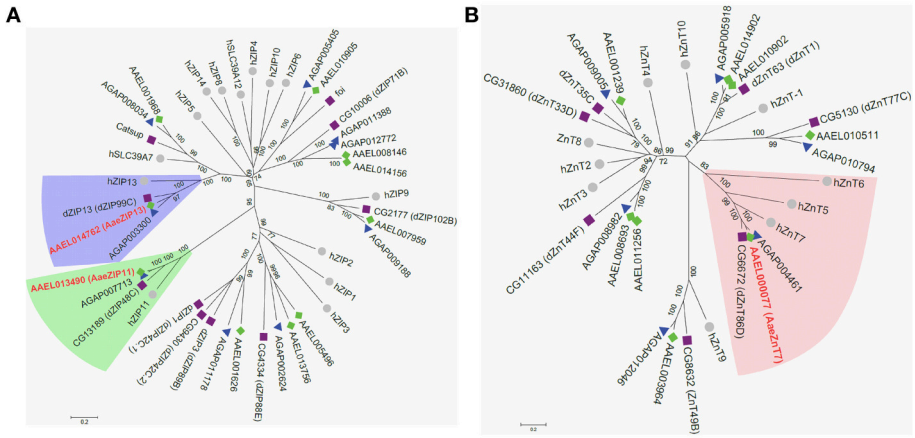

FIGURE 4 | Phylogenetic analysis of *A. aegypti* ZIP and ZnT genes. Neighbor-joining trees were produced in MEGA 6 following ClustalW alignment of ZIP **(A)** or ZnT **(B)** protein sequences from humans (•), *D. melanogaster* (■), and mosquitoes [*An. gambiae* (▲) and *A. aegypti* (♦)]. Clades containing Fe-sensitive transporters identified in our assay are highlighted (ZIP13, blue; ZIP11, green; ZnT7, peach). Putative *A. aegypti* iron transporters are in red-bold letters.

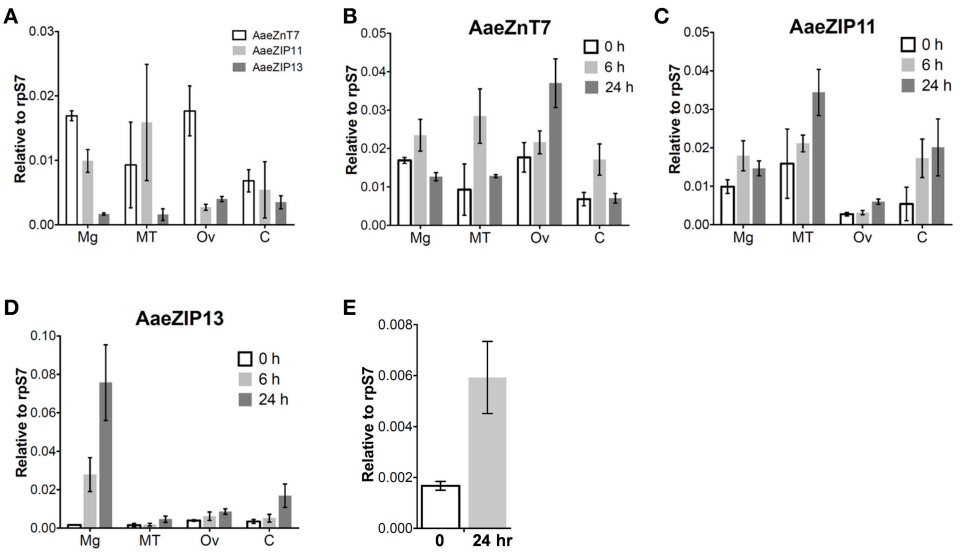

FIGURE 5 | Organ-specific transcript expression of candidate iron transporter genes by qRT-PCR. **(A)** Transcript expression in organs dissected from sugar-fed females (before bloodfeeding). Transcript expression of AaeZnT7 **(B)**, AaeZIP11 **(C)**, and AaeZIP13 **(D)** in organs dissected at the indicated times post-bloodmeal; graphs show mean ± standard deviation. **(E)** AaeZIP13 expression 24 h after Fe-only artificial meal. Values in 0 h in panel **(B–E)** are the same as values in panel **(A)**. Mg, midgut; MT, Malpighian tubules; Ov, ovaries; C, carcass (whole body without Mg, MT, and Ov). $N = 3$ pools, with 20 individuals per pool.

Aag2 cells. While it is possible that treatment with excess iron did not modulate NRAMP expression in mosquito cells, no homolog of NRAMP was found in the published genomes of three culicine mosquitoes. We conclude from these results that culicine mosquitoes, including *A. aegypti*, appear to lack NRAMP, the best studied importer of dietary iron to date. This raised the question of how *A. aegypti* acquire dietary iron from the gut lumen and led us to search for alternative iron transporters in this mosquito. Taking advantage of a ferritin gene known to be strongly induced by cytoplasmic iron levels, we constructed a luciferase-based iron-specific sensor-reporter construct. Though we did not test the response of this sensor to every divalent metal possible, Zn, and to a much lesser extent Cu, were some of the only metals likely to be present at physiologically relevant concentrations in our cell culture media. Inhibition of general export/import processes confirmed that the acquisition of iron is indeed an active process requiring endocytosis. This suggests that potential iron importers do not act to a large extent on the cell surface but instead in endocytic vesicles. Using our sensor-reporter, we screened genes in families with potential zinc and iron transporting function (ZIP and ZnT families), and identified three genes as candidate iron transporters; orthologs of

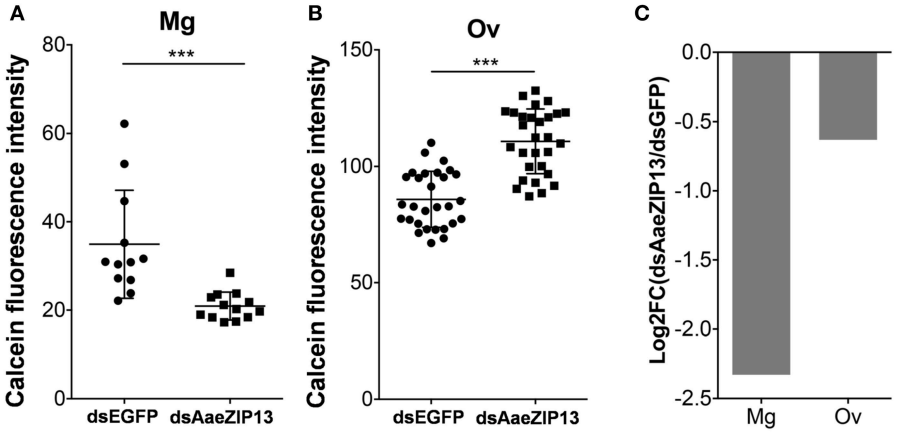

FIGURE 6 | Iron accumulation is disrupted following AaeZIP knockdown. Calcein fluorescence at 24 h post blood feeding in the midgut **(A)** and ovaries **(B)** from AaeZIP13 knockdown and control (dsEGFP) mosquitoes. For **(A)**, each dot represents a single posterior midgut; **(B)** each dot represents a lobe of ovary; lines indicate mean and standard deviation. Figures are representative of 5 independent experiments. *** indicates $p < 0.001$ by unpaired two-sided Student's *t*- test. **(C)** Knockdown efficiency of dsRNA injection at 4 day post dsRNA injection/24 h post blood feeding. Graph shows Log_2 transformed fold change (dsAaeZIP13/dsEGFP) values of qRT-PCR results (normalized to rpS7 expression). Mg, midgut; Ov, ovaries.

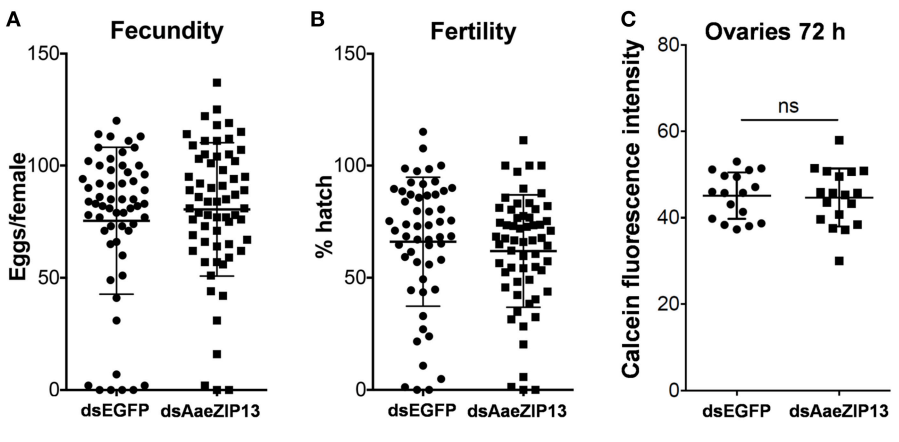

FIGURE 7 | Fecundity (egg number) and fertility (hatch rate) from AaeZIP13 knockdown and control (GFP) mosquitoes. **(A)** Fecundity (egg number per female); **(B)** Fertility (hatch rate per laid egg batch). Lines indicate mean ± standard deviation. Means are not significant by unpaired, two-sided *t*-test. **(C)** Calcein fluorescence at 72 h post blood feeding in the ovaries from AaeZIP13 knockdown and control (EGFP) mosquitoes.

human ZnT7 (AAEL000077), ZIP11 (AAEL013490), and ZIP13 (AAEL014762).

In *D. melanogaster* the ZnT7 ortholog (ZnT86D; CG6672) was described as a zinc transporter that localizes in ER/Golgi for zinc storage (Richards and Burke, 2016). When ZnT7 was overexpressed in the midgut or systemically suppressed, *Drosophila* exhibited lethality in the larval stage (Lye et al., 2012). Results of our qRT-PCR indicated that the transcript of AaeZnT7 is enriched in the midgut and ovaries, indicating the potential for involvement in the acquisition of nutrients from the blood meal. Upon bloodfeeding, AaeZnT7 transcripts increased in the Malpighian tubules and midgut at 6 h PBM, returning to resting level at 24 h PBM with a corresponding increase in transcripts at 24 h PBM in the ovaries. This pattern suggests several potential roles for AaeZnT7: importation of iron from the blood meal into the midgut epithethelial cells, and/or importation of iron from the hemolymph into the Malpighian tubules (for excretion of excess) and ovaries (for nutrition).

The *Drosophila* ZIP11 ortholog (ZIP48C; CG13189) is highly enriched in the Malpighian tubules (Chintapalli et al., 2010), but functional characterization data is lacking in this species. However, its mammalian counterpart has been characterized as a zinc transporter, which is most closely related to prokaryotic zinc transporters (Yu et al., 2013). AaeZIP11 transcripts were also found to be enriched in the Malpighian tubules, particularly following a bloodmeal, suggesting a potential role in excretion. As the key organ for insect excretion and detoxification, Malpighian tubules may play a critical role in excretion of excess iron accumulated in the hemolymph (Folwell et al., 2006; Beyenbach et al., 2010; Li et al., 2017). As a candidate exporter, AaeZIP11

may function in concert with AaeZnT7 (candidate importer) to transfer iron from the hemolymph to the MT lumen for excretion, and we are actively investigating this possibility.

While no previous role in iron transport has been reported for ZnT7 or ZIP11, the ortholog of ZIP13 in *D. melanogaster* (dZIP13/dZIP99C: CG7816) has recently been characterized as an iron transporter, critical to the export of iron from the cytoplasm of midgut epithelial cells into the ER lumen where loading into ferritin occurs (Xiao et al., 2014). In contrast to the minor blood-meal induced transcriptional changes we observed for AaeZIP11 and AaeZnT7, AaeZIP13 transcripts increased about 45 times that at the resting state after blood feeding, most prominently in the midgut. Iron-only AM feeding resulted in up-regulation of AaeZIP13 transcript in the midgut, which supports our hypothesis. The much less up-regulation of AaeZIP13 than with a real blood in this assay could be due to the lack of other complex signals induced by real blood meal (proteins, amino acids, heme, etc.) and associated hormonal controls. RNAi-mediated gene knockdown resulted in reduced calcein fluorescence, and thus increased cytoplasmic iron content in the midgut consistent with a role for AaeZIP13 in iron export from this tissue. This phenotype is similar to *Drosophila* dZIP13, whose knockdown resulted in reduction of iron in the body but accumulation of iron in the midgut (Xiao et al., 2014). This similarity suggests that the function of ZIP13 is conserved in Diptera. Retention of iron in the midgut delayed, but did not prevent, iron accumulation in the ovaries. This delayed iron accumulation in the ovaries had no detectable impact on fecundity and fertility of female mosquitoes. This could be due to residual ZIP13 activity in the midgut due to incomplete suppression of AaeZIP13 transcripts by RNAi. Alternatively, there may be other iron transporters serving in a redundant fashion to ensure that sufficient iron reaches the ovaries. The development of CRISPR/Cas9-based knockout strains may provide clarity in this regard, and these experiments are currently under investigation.

The apparent lack of a Mvl/NRAMP transporter in culicine mosquitoes suggests an alternative mode of dietary iron acquisition in these species. RNAi-based screening of the ZIP and ZnT families revealed several candidate iron transporters, but none that fit the profile anticipated of a midgut-associated dietary importer (though more evidence is clearly needed for AaeZnT7). One or more such transporters are presumed to be present, as it is known that human transferrin-bound iron is efficiently absorbed into the body of *A. aegypti* during bloodfeeding (Zhou et al., 2007). Moreover, gene(s) functionally replacing NRAMP may also play roles in mosquito behavior because highly conserved Mvl homologs are related to feeding behavior in *D. melanogaster* (Sovik et al., 2017), foraging behavior of honey bee (*Apis mellifera*) (Ben-Shahar et al., 2004), invading-reproducing behavior in its parasite verroa mite (*Verroa destructor*) (Cabrera et al., 2014) and potential correlation to parental care and social interaction of a carrion beetle, *Nicrophorus vespilloides* (Mehlferber et al., 2017). Since Mvl also transports copper, such gene(s) replacing NRAMP might as well have similar function (Southon et al., 2008; Navarro and Schneuwly, 2017). We suspect that another transporter family has been recruited to perform this function during culicine evolution, and are in the process of expanding our RNAi-based screen to identify additional candidates. The iron-specific sensor-reporter system we have developed is thus a valuable tool to detect iron-induced regulatory changes in mosquito cells and aid in the identification of genes and molecules involved in iron homeostasis and transport in this important disease vector mosquito.

AUTHOR CONTRIBUTIONS

HT, MA, KM, and ZA: design of the work; HT, MA, and ZA: performed experiments; HT and ZA: drafting manuscript; HT, MA, KM and ZA: approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00380/full#supplementary-material

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