Abstract:
Living organisms have evolved intricate systems to harvest trace elements from the environment, to control their intracellular levels and to ensure adequate delivery to the various organs and cellular compartments. Copper is one of these trace elements. It is at the same time essential for life, but also highly toxic, not least because it facilitates the generation of reactive oxygen species. In mammals, copper uptake in the intestine and copper delivery into other organs is mediated by the copper importer Ctr1. Drosophila has three Ctr1 homologs: Ctr1A, Ctr1B and Ctr1C. Earlier work has shown that Ctr1A is an essential gene that is ubiquitously expressed throughout development, while Ctr1B is responsible for efficient copper uptake in the intestine. Here, we characterize the function of Ctr1C and show that it functions as a copper importer in the male germline, specifically in maturing spermatocytes and mature sperm. We further demonstrate that loss of Ctr1C in a Ctr1B mutant background results in progressive loss of male fertility that can be rescued by copper supplementation to the food. These findings hint at a link between copper and male fertility, which might also explain the high Ctr1 expression in mature mammalian spermatozoa. In both mammals and Drosophila, the X chromosome is known to be inactivated in the male germline. In accordance with such a scenario, we provide evidence that in Drosophila, the autosomal Ctr1C gene originated as a retrogene copy of the X-linked Ctr1A, thus maintaining copper delivery during male spermatogenesis.

Introduction:
Copper (Cu) is an essential nutrient for all eukaryotic organisms. Several vital processes depend on the ability of this trace metal to undergo changes in redox state. Copper serves as a catalytic cofactor in redox enzymes in respiration (cytochrome c oxidase), protection from oxidative stress (superoxide dismutase), melanization (tyrosinase and laccase), neuropeptide and peptide hormone processing (peptidylglycine alpha-amidating mono-oxygenase) and others. While being essential for eukaryotic life, copper can also be highly toxic: it can disrupt the function of proteins by ectopically binding to them, and it catalyzes the formation of reactive oxygen species via the so-called Fenton reaction (1). Thus, eukaryotic organisms have evolved intricate systems to regulate copper homeostasis, including high affinity copper uptake systems, copper trafficking proteins (metallochaperones), metal scavenging proteins (metallothioneins), copper exporters (P-type ATPases) and transcriptional regulators (2-4).

Given copper's importance, organisms have evolved efficient copper uptake, as well as storage systems, that allow them to survive periods of copper scarcity. Copper can be taken up via lower affinity, less specific cation transporters, such as divalent metal transporter 1 (DMT1) (5,6). There is however a dedicated high-affinity transport system that is conserved from yeast to humans: the Ctr family of copper importers - small proteins that assemble into homotrimeric membrane complexes, forming a pore that allows an ATP-independent transport of copper (7). In yeast, three Ctr proteins are known. Two of these, yCtr1 and yCtr3, have been shown to localize to the plasma membrane and to import extracellular copper into the cytoplasm (8,9), while the third family member, yCtr2, transports copper stored in the vacuole back into the cytoplasm (10). In mammals, including humans and mice, two Ctr proteins are present, Ctrl and Ctrl2. Ctrl1 is an essential gene, localizing to the plasma membrane where it facilitates Cu import into the cytoplasm (11-14). Recently, the mouse Ctrl1 protein was also shown...
The results reveal that Ctr1C is an overexpression of Ctr1C to Ctr1A and Ctr1B, and compares the effects of ectopic plasma membrane when ectopically expressed Ctr1C. We show that Ctr1C localizes to the present a detailed analysis of the function of third instar larvae and adult males (18). Here, we demonstrate that Ctr1C can functionally replace Ctr1A in Ctr1A null mutants. Using a GFP knock-in mutant of Ctr1C and genomic Ctr1C-GFP transgenes, we demonstrate that the protein is localized in the plasma membrane.

Recently, Drosophila has been introduced as a model system for the study of copper homeostasis. Three Ctrl-like genes are present in Drosophila: Ctrl1A, Ctrl1B, and Ctrl1C (18). Ctrl1A is constitutively and ubiquitously expressed, its loss resulting in developmental arrest and a general failure of copper-dependent processes (Cu-enzyme activity, neuropeptide maturation, heart function) (19). Ctrl1B is responsible for copper uptake from the intestine. Ctrl1B mutants are viable, but they have strongly reduced organismal copper levels and they are sensitive to copper starvation. Ctrl1B is transcriptionally activated upon copper starvation by the metal responsive transcription factor MTF-1 (18, 20, 21).

In contrast to Ctrl1A and Ctrl1B, the third family member, Ctrl1C, has not been further studied after an initial characterization in which it was shown to be able to complement copper import-deficient yeast and to be expressed in Drosophila third instar larvae and adult males (18). Here, we present a detailed analysis of the function of Ctrl1C. We show that Ctrl1C localizes to the plasma membrane when ectopically expressed and compare the effects of ectopic overexpression of Ctrl1C to Ctrl1A and Ctrl1B. The results reveal that Ctrl1C is a bona fide copper importer that is indistinguishable in its overexpression effects from those of Ctrl1A and Ctrl1B. We also show that Ctrl1C can functionally replace Ctrl1A in Ctrl1A null mutants. Using a GFP knock-in mutant of Ctrl1C and genomic Ctrl1C-GFP transgenes, we demonstrate that the expression of Ctrl1C is restricted to maturing spermatocyte cysts and mature sperm and that the protein is localized in the plasma membrane. We show that in a Ctrl1B mutant background, i.e., in a situation of low intraorganismic copper, Ctrl1C is required for male fertility, revealing a hitherto unrecognized aspect of copper biology.

Finally, we explore the scenario that Ctrl1C has originated as a retrogene during sex chromosome formation in the evolutionary precursor of the drosophilid lineage, providing a copper delivery function in the male germline where the X chromosome-linked Ctrl1A cannot function due to X chromosome inactivation in the male germline.

**Experimental Procedures:**

**Fly culture**

One liter of standard fly food was composed of 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent nipagin (15% in ethanol). For experiments, food was supplemented with CuSO₄ or bathocuproinedisulfonate (BCS) disodium salt hydrate (Sigma-Aldrich No. 14,662-5) to the indicated concentrations. BCS is a specific copper chelator used to deplete copper in the food. Flies were raised at 25 °C and 65% humidity.

**Plasmids, mutants and transgenic fly strains**

To generate UAS-Ctrl1C transgenes, a DNA segment corresponding to the Ctrl1C-PA coding sequence (Flybase designation FBpp0085111, coordinates: 3R: complement (26789170..26789982)) was cloned into pUASTattB (22), pUAST and pUASP vectors. To generate genomic Ctrl1C transgenes, an 8 kb DNA segment corresponding to coordinates 3R: complement(26792528..26784388) was cloned into a pAttP vector (22). For the Ctrl1C-GFP genomic rescue transgene, an EGFP ORF was inserted in frame at the C-terminal end of the Ctrl1C ORF into the Ctrl1C genomic transgene. Transgenic fly lines were generated using standard P element transgenesis and PhiC31-mediated transgenesis (22, 23). The Ctrl1C6D mutant was generated by ends-in homologous recombination followed by allelic substitution (24, 25). The Ctrl1C ORF is disrupted by precise integration of a DNA segment containing the EGFP-ORF followed by an SV40polyA signal sequence, deleting amino acids 1-13 of the Ctrl1C ORF. Plasmid sequences are available upon request.

**Determination of survival rates**

The survival rates in the actin-GAL4 overexpression assay (Figures 1A and 1B) were calculated relative to the numbers of eclosing siblings (adult flies hatching from their pupal cases) that carried a balancer chromosome (CyO, y+) instead of the GAL4 driver, having otherwise identical genotypes. For the complementation assay (Figure 1E), a null mutant allele of Ctrl1A (Ctrl1A25) was used (19). The indicated transgenes were ubiquitously expressed, using a moderately strong tubulin-GAL4 driver in hemizygous Ctrl1A25/Y males and the number of rescued animals was scored. Survival rates were calculated relative to fully viable Ctrl1A25/FM7; UAS-transgene/+; MKRS/+ sisters (these females carry the X chromosome balancer FM7 and the third chromosome balancer MKRS and show wild type survival).
Fertility assays
Males were kept separate from females before mating (Figures 4A and 4B: for 12 days; Figure 4C: for 7 days). Single males were each crossed to two y w virgin females. After a mating period (Figures 4A and 4B: 5 days; Figure 4C: a first period of three days, a second period of two days), the parents were discarded. The offspring was left to develop and was counted after eclosion.

Flop-out clones
UAS-Ctr1C(wt) UAS-GFP (Figures 2A, 2B) and UAS-Ctr1C-FLAG UAS-GFP (Figures 2C, 2D) clones were generated with the hs-flp actin-FRT-CD2-FRT-GAL4 flop-out technique (26). With this technique, the expression of UAS transgenes can be induced with a heat shock in cell clones. Ctr1C overexpressing clones were marked by the coexpression of UAS-GFP. Since the cell clones only differ in the expression of actin-GAL4, UAS-Ctr1C and UAS-GFP from their non-expressing neighbors, the antibody signal in non-expressing neighbors can serve as a direct internal control for antibody signal and specificity. To induce the clones, a 5 min heat shock at 37 °C was administered after a 24 h egg lay period. Salivary glands and fat bodies were harvested from 3rd instar wandering larvae.

Tissue preparation and immunohistochemistry
Wandering 3rd instar larvae (to harvest larval male gonads, fat bodies and salivary glands) and adult males (to harvest adult testes and seminal vesicles) were anaesthetized and dissected in ice-cold PBS, pH 7.4. In cases were unfixed tissues were analyzed (Figure 3C, 5C), the tissues were directly mounted in 87% glycerol and microphotographed. Tissues that were subjected to DAPI staining (Figure 3B, 5A) were fixed in 4% paraformaldehyde for 10 min at room temperature (RT) and washed three times for 10 min with PBS. In the middle wash step, DAPI was included at a concentration of 5 μg/ml. Tissues were mounted in 87% glycerol and microphotographed. Tissues subjected to antibody and DAPI stainings (Figures 2, 5B) were fixed in 4% paraformaldehyde for 10 min at RT. The fixed tissues were washed twice for 10 min with washing solution (0.3% Triton X-100 in PBS, pH 7.4). Blocking of the tissues was performed in 10% fetal calf serum (in washing solution) for 1 hour at RT. After blocking, the tissue was washed once for 10 min and incubated with primary antibody (in washing solution) over night at 4 °C. Subsequently, the tissue was washed twice for 10 min at RT. The tissues were incubated with secondary antibody (in washing solution) for 2-4 hours at RT. The secondary antibody was washed three times for 10 min. In the middle washing step, DAPI was included at a concentration of 5 μg/ml. The dissected tissues were mounted in 87% glycerol. Antibodies and concentrations: Figures 2A and 2B: rabbit anti-Ctr1C peptide (raised against the epitope GGRDQYNPRRYYREA), 1:500 and goat anti-rabbit Alexa 594 (Invitrogen, A11012), 1:1000; Figures 2C and 2D: mouse anti-FLAG (Sigma, F1804), 1:400 and goat anti-mouse Alexa546 (Invitrogen, A11030), 1:1000; Figure 5B: rabbit anti-GFP (MBL598), 1:500 and goat anti-rabbit Alexa 488 (Molecular Probes A-11008), 1:400. The anti-Ctr1C antibody only produced a signal when used against overexpressed Ctr1C protein and it strongly cross-reacted with a nuclear protein.

Microscopy
Eye pictures were recorded with a Leica MZ16 stereomicroscope and a Leica DFC280 camera, pictures of adult testes (Figure 3C) and mature sperm (Figure 5C) were recorded with a Zeiss Axioplan 2 microscope and an Axiocam MRm camera. Pictures of larval male gonads (Figures 3B, 5A) and of seminal vesicles (Figure 5B) were recorded with a Zeiss LSM 710 confocal microscope.

Quantitative analysis of transgene expression
To quantitate the strength of the GFP signal in seminal vesicles, FITC filter epifluorescence pictures from seminal vesicles were taken using a Zeiss Axioplan 2 microscope and a Zeiss Axiocam MRm camera. The exposure time was kept equal for all recordings. The average signal intensity for 50 x 50 pixel sections from pictures of individual seminal vesicles was determined with the histogram function of Adobe Photoshop. To measure beta-galactosidase activity (liquid lacZ assay), three 3rd instar wandering female larvae per sample were lysed by successive freeze-thaw cycles in liquid nitrogen. 100 μl of Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4) was added to each sample and the sample was vortexed for 20 s. 700 μl of Z-buffer (containing 50 mM β-mercaptoethanol) was added to each sample. Subsequently, 160 μl of 4 mg/ml ONPG (orthonitrophenyl-β-galactoside) substrate were added to start the reaction. The reaction was carried out at 30 °C for 5 min. At the end of reaction, 400 μl of 1 M Na2CO3 were added and the samples were centrifuged for 10 min at 13000 rpm. The OD of the clear supernatant was measured at 420 nm.

Annotation of Ctr1 genes in insects
Ctr1-type genes in the different insect genomes were identified by standard BLAST searches.
(NCBI BLAST and Flybase BLAST) against the sequenced genomes and against protein databases. Sequences found in the BLAST searches were annotated using the Artemis software (Sanger Institute). Sequence alignments were generated with the Clustal W2 online software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) with standard parameters.

**Fly genotypes used in the figures**

See Supplementary Data.

**Results:**

**Ctr1C functions as a copper importer**

In order to characterize Ctr1C, we employed two overexpression assays that had been previously used to study Ctr1A and Ctr1B (20,21,27). In these assays, the copper transporters are expressed under the control of UAS enhancers either ubiquitously, with the GAL4 driver actin-GAL, or specifically in the eye with GMR-GAL4. Strong ubiquitous expression of Ctr1C transgenes was lethal when the flies were raised on normal food (NF). Such flies could be rescued by growing them on food supplemented with the copper chelator BCS, which was comparable to the effects on viability of Ctr1A and Ctr1B overexpression (Figure 1A). The chelator rescue experiment suggests that the lethality is due to the copper toxicity imposed by overexpression of Ctr1C. A transgenic fly line expressing Ctr1C at lower levels was viable on NF, but lethal if raised on food supplemented with copper (Figure 1B), further demonstrating that the observed effect is copper-dependent. Similarly, eye-specific expression of strong Ctr1C transgenes led to severely disrupted eye morphology (the rigid, quasi-crystalline pattern of the ommatidia was lost, ommatidia were fused, leading to an uneven eye surface; in addition, overall eye shape was changed) on NF, comparable to the effect of Ctr1A or Ctr1B expression. This rough eye phenotype could be fully rescued by BCS supplementation (Figure 1C). Consistent with the viability results, a weaker Ctr1C transgene did not alter eye morphology on NF, but caused a strong rough eye phenotype on food containing elevated levels of copper (Figure 1D). These data provide evidence that Ctr1C can function as a copper importer in a manner similar to Ctr1A and Ctr1B.

The notion that Ctr1C functions as a copper importer is further underscored by the observation that Ctr1C can functionally replace Ctr1A: as shown in Figure 1E, ubiquitous expression of Ctr1C transgenes with a moderately strong GAL4 driver (tubulin-GAL4) restored the viability of the Ctr1A null mutant $Ctr1A^{25}$ (19,27).

Finally, in agreement with the proposed copper import function of Ctr1C described above and with previous data on the subcellular localization of Ctr1A and Ctr1B proteins (19,21), we found that Ctr1C predominantly localizes to the plasma membranes when ectopically expressed in cell clones in larval fat bodies and salivary glands (Figure 2). Of note, a Ctr1C-GFP genomic construct with endogenous spermatocyte cyst expression (see below) also showed clear plasma membrane localization (Figure 5A).

Taken together, these data demonstrate that Ctr1C functions as a *bona fide* copper importer.

**Ctr1C is expressed in the male germline**

To examine the *in vivo* role of Ctr1C, we used ends-in homologous recombination followed by allelic substitution (24,25) to generate a Ctr1C mutant (Figure 3A). In the resulting allele, $Ctr1C^{6D}$, the Ctr1C open reading frame is disrupted by a precise integration of a GFP transgene, allowing expression of GFP in place of the original Ctr1C ORF. Residual expression of truncated Ctr1C is highly unlikely because the remainder of the Ctr1C ORF is separated from the GFP ORF by a DNA sequence containing an SV40 polyA signal sequence and several stop codons. Moreover, the two ORFs are not in frame. Successful targeting of the Ctr1C gene was confirmed by PCR assays and sequencing (data not shown). Flies carrying the $Ctr1C^{6D}$ allele were homozygous viable and did not show sensitivity to either elevated or reduced copper levels in the food (data not shown), indicating that Ctr1C is not an essential gene.

Further analysis of the $Ctr1C^{6D}$ flies revealed that Ctr1C expression is essentially restricted to the male germline. In the male gonads of wandering third instar larvae, GFP could be observed in germline spermatocyte cysts, the signal gradually becoming stronger from early to late spermatocyte cysts (Figure 3B). In adult testes, maturing elongating spermatocyte cysts are labeled by GFP (Figure 3C). The finding that Ctr1C is expressed in the male germline was further confirmed by the expression pattern of genomic Ctr1C-GFP fusion transgenes (see Figure 5 and below).

**Ctr1C is required for male fertility**

In light of the male germline-specific expression pattern of Ctr1C, we next examined if the loss of Ctr1C would influence male fertility. Since $Ctr1C^{6D}$ mutants did not show reduced fertility compared to wt controls, we combined the mutant allele with a Ctr1B null mutant ($Ctr1B^{4}$). Ctr1B mutants exhibit strongly reduced organismal copper levels due to impaired
intestinal copper uptake in the absence of Ctr1B (20). Whereas twelve days old Ctr1B\textsuperscript{3-4} mutant males were normally fertile, Ctr1B\textsuperscript{3-4} Ctr1C\textsuperscript{6D} double mutants displayed almost complete sterility, indicating that in the absence of Ctr1B, Ctr1C is required for male fertility (Figure 4A). Importantly, this loss of fertility is dependent on copper status: If Ctr1B\textsuperscript{3-4} Ctr1C\textsuperscript{6D} double mutant males were kept on food supplemented with copper after eclosion (emergence of the adult fly from its pupal case), i.e. as adult flies, for twelve days, fertility was largely restored (Figure 4B). The fertility of Ctr1B\textsuperscript{3-4} Ctr1C\textsuperscript{6D} double mutant males could also be restored by the presence of genomic Ctr1B-GFP, Ctr1C and Ctr1C-GFP transgenes (Figures 4B, C), demonstrating that the observed mutant phenotype is a specific consequence of the concomitant loss of Ctr1B and Ctr1C.

In the experiments described above, fertility was assayed at an age of twelve days. At this age, Ctr1B\textsuperscript{3-4} Ctr1C\textsuperscript{6D} double mutant males show almost complete sterility. Nevertheless, Ctr1B\textsuperscript{3-4} Ctr1C\textsuperscript{6D} mutants can give rise to considerable offspring numbers at an earlier age. In the experiment shown in Figure 4C and in Supplementary Figure 1, males were crossed at an adult age of seven days. Single males were left together with female virgins for three days, then placed in a fresh food tube for another two days. For both mating periods, the offspring was counted. In the first mating period of three days, only a part of the Ctr1B\textsuperscript{3-4} Ctr1C\textsuperscript{6D} double mutants were completely sterile. In the second period of two days immediately following the first, almost all double mutants were sterile, revealing a rapid loss of fertility. We examined the testes of these sterile males, but could not find any obvious morphological differences to fertile control males (data not shown).

**Ctr1C is present in maturing spermatocyte cysts and in mature sperm**

To gain further insights into the male germline function of Ctr1C, we studied the expression and subcellular localization of the genomic Ctr1C-GFP fusion transgene. The transgene consists of the Ctr1C genomic locus (approximately 3 kb upstream and downstream of the Ctr1C gene) with a GFP ORF C-terminally fused to the Ctr1C ORF. As shown in Figure 5A, genomic Ctr1C-GFP recapitulates the male larval gonad expression seen with the Ctr1C\textsuperscript{6D} GFP knock-in mutant in male larval gonads. Confocal sections showed that Ctr1C-GFP localizes to the plasma membranes of late spermatocyte cysts but is absent from early cysts and the brightly DAPI-labeled somatic cap of the gonads (Figure 5A, middle and right panel), confirming the male germline expression pattern seen with the Ctr1C\textsuperscript{6D} allele. In some larval gonads, elongating spermatocyte cysts are already present. Here, the Ctr1C-GFP signal could be detected around the developing sperm heads (Figure 5A, middle panel) and in the elongating sperm tails (Figure 5A, right panel). In the adult testes, elongating spermatocyte cysts are labeled (data not shown), giving a signal pattern similar to the one shown in Figure 3C. Interestingly, the seminal vesicles (sperm storage organs situated immediately behind the testes, filled with mature sperm) of Ctr1C-GFP expressing adult males showed a strong GFP signal, indicating that Ctr1C-GFP protein is present in mature sperm (Figure 5B). In Ctr1C\textsuperscript{6D} mutants, no signal of (soluble) GFP could be detected in the seminal vesicles (data not shown), what was to be expected, since spermatzoa slough off most of their cytosol during maturation. The confocal sections in Figure 5B show that the GFP expression is not restricted to the DAPI-labeled sperm heads, but comprises a much larger domain, suggesting Ctr1C-GFP presence along the sperm tails. This finding was corroborated by squeezing out mature sperm from seminal vesicles to separate individual sperm tails (Figure 5C). Taken together with the expression data from Ctr1C\textsuperscript{6D} mutants (Figure 3) and the finding that the genomic Ctr1C-GFP transgene is functional because it can rescue the sterility of Ctr1B\textsuperscript{3-4} Ctr1C\textsuperscript{6D} double mutants (Figure 4C), these data suggest that Ctr1C functions in maturing spermatocyte cysts and possibly in mature sperm.

**X chromosome inactivation in the male germline precludes the expression of X-linked Ctr1C-GFP**

Recently it has been shown that the X chromosome is inactivated in the Drosophila melanogaster male germline during spermatogenesis (28). In a process termed male meiotic sex chromosome inactivation (MSCI), genes residing on the X chromosome are silenced (29,30). This finding provides a molecular basis for the earlier findings that there is a bias for retrogene formation with a gene parent on the X chromosome and that most retrogenes derived from a parent on the X chromosome have evolved testis-specific expression (31). Intriguingly, Ctr1C is encoded as a single open reading frame on an autosome, while Ctr1A, which is ubiquitously expressed (18,19), is encoded on the X chromosome (Supplementary Figure 2, Supplementary Table 1 and Supplementary Information) and thus presumably is inactive during spermatogenesis. It is therefore possible that Ctr1C originated as a retrogene in the drosophilid lineage (see below for a more detailed discussion). One of the predictions of such a hypothesis is that Ctr1C...
would not be expressed during spermatogenesis if transplanted onto the X chromosome. To test this hypothesis, we inserted genomic Ctr1C-GFP transgenes into AttP landing sites on the X (landing site 2A), on the 2nd (landing site 51D) and on the 3rd chromosome (landing site 85Fb) and compared the GFP signal intensities of mature sperm in the seminal vesicles of adult males (Figure 6A). Indeed, the GFP signal of Ctr1C-GFP in the landing site on the X was only slightly above the background of autofluorescence and far below the signal intensity of the Ctr1C-GFP transgenes at the two autosomal sites. Importantly, the difference in signal intensity is not due to general silencing effects at the landing site on the X chromosome, since ubiquitous expression of UAS-lacZ transgenes in females (to exclude dosage compensation effects) resulted in comparably high expression levels (Figure 6B).

Discussion:

**Ctr1C is a bona fide Cu importer**

In this study, we present a detailed characterization of Ctr1C, the third member of the *Drosophila* Ctr1 copper importer family. Ctr1C was initially identified along with Ctr1A and Ctr1B (18) and, like these, was able to complement yeast mutants defective in copper import (18). However, ectopically expressed Ctr1C could not facilitate copper uptake in *Drosophila* S2 cells (18), raising the possibility that Ctr1C might not function as a copper transporter at the plasma membrane, but rather in an intracellular compartment similar to yeast Ctr2. It is not clear why Ctr1C failed to mediate copper uptake in cell culture, but our *in vivo* data strongly suggest that Ctr1C functions as a copper transporter in a similar manner to Ctr1A and Ctr1B. Strikingly, ubiquitous expression of Ctr1C could rescue *Ctr1A* null mutants, demonstrating that Ctr1C can functionally replace Ctr1A. When strongly overexpressed, either ubiquitously or eye-specifically, Ctr1C led to copper toxicity phenotypes that were indistinguishable from those caused by Ctr1A or Ctr1B. The observed toxicity effects were dose-dependent, allowing us to estimate relative copper import efficiencies of differently expressing genomic P element insertions and genomic AttP landing sites. Of note, a Ctr1C transgene inserted into the genomic landing site AttP 51D showed similarly strong toxicity effects as a Ctr1A transgene in the same landing site, arguing for a similar copper import capabilities of the two proteins. In addition to these findings, our data on the subcellular localization of Ctr1C (showing plasma membrane localization of a Ctr1C-GFP fusion protein with endogenous expression pattern and expression levels) support a role of Ctr1C in cellular copper uptake.

**Evolution of the Ctr1 homologs in the insects**

The occurrence of three Ctr1 homologs in the genetically streamlined model organism *Drosophila* is striking and has prompted us to examine the genome sequences of the other eleven sequenced drosophilid species as well as nine genomes from more distantly related insect species for the presence of Ctr1-type copper transporter genes (Supplementary Figure 2, Supplementary Table 1 and Supplementary Information). We could identify Ctr1A homologs in all sequenced genomes. Ctr1B was present in all drosophilids and also in some of the analyzed non-drosophilid insects. Ctr1B, the major intestinal Cu importer, appears to be less well conserved than the ubiquitous Ctr1A, but more so than the male germline-specific Ctr1C, which was only found among drosophilids. Ctr1B differs from both Ctr1A and Ctr1C by its shortened N-terminus. Interestingly, the Ctr1B homologs show partial conservation of the exon-intron boundaries that are shared between human Ctr1 and insect Ctr1A genes (see below), making it plausible that the insect Ctr1B gene has arisen from a duplication of the genomic DNA segment containing Ctr1A.

Regarding Ctr1C, we wanted to investigate the possibility that Ctr1C originated as a retrogene and has acquired male germline function. An "out-of-X movement" of male germline specific retrogens is thought to be a general consequence of sex chromosome evolution, either due to sex chromosome inactivation in the male germline (28,31) or due to evolutionary processes leading to a general demasculinisation of the X chromosome (32); this phenomenon is also documented in mammals which independently evolved their sex chromosome system (33). The hypothesis of a retrogene origin of Ctr1C would thus be falsified if Ctr1C homologs were present in species not sharing the *Drosophila* X chromosome, or if the cDNA-type single open reading frame configuration of *Drosophila melanogaster* Ctr1C was not shared by other Ctr1C homologs. As noted above, we could find Ctr1A homologs in all examined insect species, but Ctr1C homologs were only present in the *Drosophila* genus which shares an ancestral X chromosome (34-36). Ctr1A homologs have a tightly conserved exon-intron structure. Of note, the exon junctions in front of the first and the second transmembrane domains appear to be conserved even between human Ctr1 and the insect Ctr1As. In marked contrast, all Ctr1C genes possess a single long ORF. In some species, including *Drosophila melanogaster*, a part of the variable intracellular loop sequence
can be spliced out but the splice sites of these apparent “neo-introns” are not conserved between the Ctr1C genes. Regarding chromosomal locations, all Ctr1A homologs among drosophilids display synteny; in all cases where scaffolds are mapped to chromosomes, Ctr1A is present on Muller element A (the ancestral X chromosome). The Ctr1C homologs also show synteny and map to Muller element E (an autosome). These data, taken together with our experimental finding that expression of a Ctr1C-GFP transgene in the male germline was at most marginal upon inserting it into the X chromosome, support the hypothesis of a retrogene origin of Ctr1C as a consequence of X inactivation in the male germline.

Copper, a new player in male fertility?
Analysis of the endogenous Ctr1C expression pattern and loss of function analysis revealed that Ctr1C plays a specific role in spermatogenesis: it is expressed in the maturing spermatocyte cysts and present in mature sperm. Most importantly, in a Ctr1B mutant background when intraorganismal copper levels are strongly reduced, lack of Ctr1C leads to sterility. Fertility can be restored if the adult males are kept on food containing additional copper. These findings demonstrate that Ctr1C contributes to copper delivery in the male germline and that copper is required for the maintenance of normal fertility. The importance of the trace element zinc for male fertility has long been documented (reviewed in (37)) and zinc was recently shown to be essential for spermatogenesis (38). Our findings are compatible with a specific role of copper in male fertility. At present we can only speculate about the nature of such a role. Sperm motility depends on the function of the mitochondrial respiratory chain (39); Drosophila sperm contain a large so-called mitochondrial derivative in the head region (40) and might have a high copper demand for proper mitochondrial function. Intriguingly, high levels of Ctr1 are present in mature spermatozoa in the mouse (41) and Ctr2 levels are high in testis (D. J. Thiele, unpublished). It is therefore possible that the requirement for Ctr proteins in male fertility is conserved between insects and mammals.

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References:

**Abbreviations:**

BCS: bathocuproinedisulfonate disodium salt hydrate
MSCI: male meiotic sex chromosome inactivation
Figure legends:

Figure 1
Ctr1C shows copper import activity comparable to Ctr1A and Ctr1B and can functionally replace mutant Ctr1A.

Strong ubiquitous expression of Ctr1C transgenes causes Cu-dependent lethality (A, B). The indicated transgenes were expressed under the control of UAS enhancers by actin-GAL4. Flies were raised on food containing the indicated concentrations of Cu or BCS. A Ctr1C transgene that was integrated into the same AttP landing site as the Ctr1A control (AttP 51D) showed slightly stronger lethality on normal food (NF) and a less pronounced rescue of survival on BCS than Ctr1A, while a strongly expressed transgene on a P element (Ctr1C C34) showed a similarly high lethality as Ctr1A and Ctr1B, but a complete rescue on BCS (A). A weaker P element transgene (Ctr1C D19) was viable on normal food (NF), but completely lethal on Cu (B). The Ctr1A and Ctr1B controls were also shown in (27). Error bars indicate standard errors. Data points with zero value are indicated with an asterisk (*).

Eye-specific expression of Ctr1C under control of GMR-GAL4 disrupts eye morphology in a Cu-dependent fashion, comparable to Ctr1A and Ctr1C transgenes (C, D). BCS-supplementation (250 µM) in the food completely rescued the severe rough eye phenotype that arises when strong Ctr1C transgenes (Ctr1C in AttP51D and the P element transgene Ctr1C C34) were expressed in flies raised on NF (C). The weaker P element transgene Ctr1C D22 only caused a rough eye phenotype on 100 µM Cu (D). The Ctr1A and Ctr1B controls were also shown in (27).

Hemizygous lethal Ctr1A^{25} null mutant males can be rescued by ubiquitous expression of Ctr1C transgenes by the tub-GAL4 driver (E). Survival rates with standard errors are given relative to viable Ctr1A^{25}/y; UAS-transgene/+; MKRS/+ siblings.

Figure 2
Clonal expression of Ctr1C reveals predominant plasma membrane localization
Ctr1C wt transgenes (A, B) and C-terminally tagged Ctr1C-FLAG transgenes (C, D) were expressed in cell clones together with GFP, using the hs-flp actin-FRT-CD2-FRT-GAL4 flp-out technique (26). An antibody that recognizes Ctr1C clearly labels the plasma membrane, but also structures in the cytoplasm (confocal sections of fat body cells in A, of salivary gland cells in B; lens magnification 25x). The antibody also detects a nuclear signal (indicated with arrows) which is not specific to Ctr1C since it is equally strong in Ctr1C^{6D} mutants (data not shown).

Ctr1C-FLAG predominantly localizes to the plasma membrane in salivary gland cells (C, D; lens magnification 60x). C: Confocal section through the flat top membrane of a salivary gland cell (strong staining throughout the surface), D: Section below the top of the same cell.

Figure 3
Ctr1C is expressed in the male germline
Structure of the Ctr1C^{6D} GFP knock-in mutant (A). The Ctr1C ORF was disrupted by precise integration of a GFP transgene at the Ctr1C start (position 1). The remainder of the Ctr1C ORF is separated from the GFP ORF by an SV40 polyA signal sequence and is not in frame with the GFP. Grey boxes indicate 5' and 3' UTRs, blue and green boxes indicate ORFs. Large arrows indicate the transcription start site, arrowheads the position of splice sites in the Ctr1C ORF.

GFP expression in Ctr1C^{6D} mutant flies in the late germline cysts of the larval male gonads (a: anterior somatic cap, p: posterior somatic cap, asterisk (*): late germline cysts) (B) and in the elongating germline cysts of the adult testis (t: tip of the testis, asterisk (*): elongating germline cysts) (C). Pictures were taken with a lens magnification of 25x (larval gonad) and 10x (adult testis).

Figure 4
Ctr1B Ctr1C double mutant males exhibit copper- and time-dependent loss of fertility
A: While Ctr1B^{4-4} and Ctr1C^{6D} single mutants are normally fertile, Ctr1B^{4-4} Ctr1C^{6D} double mutants show almost complete sterility. The graph shows average offspring numbers and standard errors from crosses of single males of the indicated genotypes to two y w virgins. Before mating, the males had been kept separate from females until an age of 12 days.

B: The sterility of Ctr1B^{4-4} Ctr1C^{6D} double mutant males can be rescued either by the presence of a genomic Ctr1B-GFP, by a genomic Ctr1C wt transgene, or by keeping eclosed adult males on 500 µM Cu food. Average offspring numbers and standard errors from single males of the indicated genotypes crossed to two y w virgins are given. Before mating, the males had been raised on normal food (NF) and after eclosion kept on either NF or 500 µM Cu food for twelve days.
C: A genomic Ctr1C-GFP construct rescues the sterility phenotype of Ctr1B<sup>++</sup> Ctr1C<sup>6D</sup> double mutant males, who themselves exhibit a rapid loss in fertility in an experiment using two consecutive mating rounds. Single males of the indicated genotypes at least seven days old were mated to two y w virgins. The parents were together for three days for a first mating period, then used for a second mating period of two days. The distribution of offspring numbers for the different genotypes is shown in Supplementary Figure 1.

Figure 5
A genomic, C-terminally tagged Ctr1C-GFP transgene reveals Ctr1C localization in the plasma membranes of maturing male germline cysts and in mature sperm

A: Localization of genomic Ctr1C-GFP in the male larval gonad. Ctr1C-GFP is present in the plasma membranes of the late spermatocyte cysts, around developing sperm heads (arrow in the middle panel) and in the plasma membranes of elongating spermatocyte cysts (arrow in the right panel). Pictures show confocal sections of a male gonad of an L3 wandering larva, the left panel at 25x, the middle and right panel at 60x. The three panels show different Z sections of the same gonad. The punctate staining visible in some cell bodies must be unspecific, since it was also seen in the Ctr1C<sup>6D</sup> mutant male gonads (Figure 3B).

B: Ctr1C-GFP is present in mature sperm. Seminal vesicles of adult males carrying a genomic Ctr1C-GFP transgene show GFP-labeled mature sperm. Sperm head nuclei are labeled by DAPI. The small inlay pictures show the DAPI signal of the elongated sperm nuclei alone.

C: Presence of Ctr1C-GFP in sperm tails. Mature sperm were squeezed out of seminal vesicles and microphotographed. The left panels show Nomarski (DIC) images, the right panels show FITC channel signals.

Control panels in B and C show seminal vesicles and mature sperm from flies that are identical in genetic background, but do not carry the genomic Ctr1C-GFP construct.

Figure 6
A Ctr1C genomic transgene is marginally expressed if inserted in the X chromosome
Ctr1C-GFP genomic transgenes were inserted in AttP landing sites on the X, the 2nd and the 3rd chromosome. The strength of the GFP signal in mature sperm in seminal vesicles was measured. While Ctr1C-GFP transgenes in AttP landing sites on the 2nd and 3rd chromosome exhibited equally strong GFP signals in seminal vesicles, but the GFP signal for the transgene inserted into an AttP landing site on the X chromosome was barely elevated over background (A). This effect is not due to generally lower expression of transgenes in the AttP landing site on the X chromosome, since ubiquitous expression of UAS-lacZ transgenes by arm-GAL4 in females (to exclude dosage compensation effects) resulted in comparably high lacZ signals in a liquid lacZ assay (B). Red lines indicate background fluorescence levels of an "empty"-landing site control (A) and background lacZ signal levels from an average of the four controls (B).
**Figure 1**

**A**

![Graph showing survival percentages for different genotypes under NF and 250 μM BCS conditions.](image)

**B**

![Graph showing survival percentages for GFP and Ctr1C (D19) under NF and 100 μM Cu conditions.](image)

**C**

![Images of fly heads under NF and BCS conditions.](image)

**D**

![Images of fly heads under NF and Cu conditions.](image)

**E**

<table>
<thead>
<tr>
<th>Ctr1A genotype</th>
<th>Transgene</th>
<th>Gal4 driver</th>
<th>Survival (%) (relative to control)</th>
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<td>tub-GAL4</td>
<td>61 ± 12</td>
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<tr>
<td>Ctr1A&lt;sup&gt;25&lt;/sup&gt;</td>
<td>UAS-Ctr1&lt;sub&gt;A&lt;sup&gt;AttP 51D&lt;/sup&gt;&lt;/sub&gt;</td>
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<td>0</td>
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<td>40 ± 16</td>
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</tbody>
</table>
Figure 3

A

\[ Ctr1C \]

\[ \text{GFP} \]

\[ \text{SV40 polyA} \]

\[ Ctr1C^{6D} \text{ (GFP knock-in)} \]

B

DAPI

GFP

Ctr1C^{6D}

C

GFP

Ctr1C^{6D}
**Figure 4**

**A**

![Graph A](image_url)

**B**

![Graph B](image_url)

**C**

![Graph C](image_url)
Figure 5

A

$Ctr1C$-GFP$^{genomic}$

B

$Ctr1C$-GFP$^{genomic}$

C

$Ctr1C$-GFP$^{genomic}$

Control
Figure 6

A

FITC signal intensity (seminal vesicles)

Control  Ctr1C-GFP\textsuperscript{genomic}  Ctr1C-GFP\textsuperscript{genomic}  Ctr1C-GFP\textsuperscript{genomic}

AttP2A (X)  AttP51D (2nd)  AttP86Fb (3rd)

B

Rel. absorption

AttP51D  AttP86Fb  AttP2A  AttP51D  AttP86Fb  AttP2A

(2nd)  (3rd)  (X)  (2nd)  (3rd)  (X)

-  -  -  -  -  -  -  -  -  -  -  -  UAS-lacZ

+  +  +  +  +  +  +  -  -  -  -  -  arm-GAL4
The Drosophila copper transporter Ctr1C functions in male fertility
Dominik Steiger, Michael Fetchko, Alla Vardanyan, Lilit Atanesyan, Kurt Steiner, Michelle L. Turski, Dennis J. Thiele, Oleg Georgiev and Walter Schaffner

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