

Glutamine analogs promote cytoophidium assembly in human and *Drosophila* cells

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Abstract

CTP synthase is compartmentalized within a subcellular structure, termed the cytoophidium, in diverse organisms including bacteria, yeast, fruit flies and the rat. Here we show that CTP synthase is also compartmentalized into cytoophidia in human cells. Surprisingly, the occurrence of cytoophidia in human cells increases upon treatment with a glutamine analog 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glutamine-dependent enzymes including CTP synthase. Experiments in flies confirmed that DON globally promotes cytoophidium assembly. Clonal analysis via CTP synthase RNAi in somatic cells indicates that CTP synthase expression level is critical for the formation of cytoophidia. Moreover, DON facilitates cytoophidium assembly even when CTP synthase level is low. A second glutamine analog Azaserine also promotes cytoophidium formation. Our data demonstrate that glutamine analogs serve as useful tools in the study of cytoophidia.

KEY WORDS: Azaserine, CTP synthase, Cytoophidium, 6-diazo-5-oxo-L-norleucine, *Drosophila*, HeLa cell, intracellular compartmentation

INTRODUCTION

Cytoophidia are filamentary subcellular structures that contain CTP synthase (Liu, 2010). Cytoophidia and equivalent structures have been independently observed in bacteria, budding yeast, fruit fly and rat, suggesting that the cytoophidium is an evolutionarily conserved subcellular structure (Ingerson-Mahar et al., 2010; Liu, 2010; Noree et al., 2010) (for a review see (Liu, 2011)). However, it is unknown whether CTP synthase-containing cytoophidia exist in human cells.

CTP synthase is a fundamental metabolic enzyme responsible for de novo synthesis of CTP, an essential nucleotide and precursor for the synthesis of RNA, DNA and sialoglycoproteins (Lieberman, 1956; Long and Pardee, 1967). CTP synthase catalyzes a set of three reactions: a kinase reaction, a glutaminase reaction and a ligase reaction (Levitzki and Koshland, 1971; von der Saal et al., 1985; Lewis and Villafranca, 1989; Endrizzi et al., 2004, 2005). The rate-limiting reaction is the glutaminase reaction in which glutamine is hydrolyzed to generate ammonia (Chakraborty and Hurlbert, 1961; Long and Pardee, 1967). The L-glutamine analogue 6-diazo-5-oxo-L-norleucine (DON) inhibits CTP synthase activity by irreversibly binding to its glutamine amidotransferase domain (Levitzki and Koshland, 1971; Levitzki et al., 1971). Cytoophidia (i.e. CTP synthase filaments) in *Caulobacter crescentus*, the curved bacterium, have been found to be quickly disassembled when treated with DON (Ingerson-Mahar et al., 2010).

Here we report the identification of cytoophidia in human cells using multiple antibodies against CTP synthase. We found that cytoophidia are

present in all phases of cell cycle and the position of cytoophidia is polarized. DON treatment does not abolish cytoophidium formation in human cells, in contrary to a previous observation in bacteria (Ingerson-Mahar et al., 2010). Instead, DON treatment increases the occurrence of cytoophidia in human cells and a variety of *Drosophila* tissues. Clonal analysis of multiple CTP synthase RNAi constructs in follicle cells suggest that the expression levels of CTP synthase protein are critical for the integrity of cytoophidia and DON treatment can induce cytoophidium formation even at low levels of CTP synthase. Similar to DON, another L-glutamine analog Azaserine also promotes cytoophidium assembly. Thus glutamine analogs provide useful tools in the study of cytoophidium assembly and dynamics.

MATERIALS AND METHODS

Cell culture

Human HeLa P4 cells were cultured in DMEM medium(with high glucose) plus 10 % FBS and incubated at 37°C, 5% CO₂. For imaging, all the cells were grown on coverslips in the 24-well cell culture clusters, with an initiated concentration of 5 X 10⁴ cells / well.

Fly stocks and genetics

All stocks were raised at 25°C on standard cornmeal. y w flies were used as a control in all our experiments if not indicated. Two GFP-CTP synthase protein trap stocks were CA06746 and CA07332 (gifts from Mike Buszczak and Allan Spradling) (Buszczak et al., 2007). Two inducible GAL4 driver stocks were: hsFLP, UAS-GFPnls; UAS-Dicer2, tub <GAL80> GAL4/SM5, Cy-TM6, Tb and hsFLP, UAS-GFPnls; UAS-Sp6, tub <GAL80> GAL4/SM5, Cy-TM6, Tb (gifts from Gary Struhl, Rebecca Frederick, and Allan Spradling). Three CTP synthase RNAi transgenic stocks were: UAS-CTP synthase^{HM04062} and UAS-CTP synthase^{JF02214} (TRiP, Harvard) (Ni et al., 2011); and UAS-CTP synthase^{GD12759} (Vienna *Drosophila* RNAi Center) (Dietzl et al., 2007).

Inducible RNAi

Female virgin flies with inducible drivers were collected and crossed to male CTP synthase RNAi transgenic flies. Female F1 flies were heat-shocked at 37°C for 45 min after eclosion. Flies were then fed on wet yeast

with or without DON for one or two days. Ovaries were dissected from non-Curly and non-Tubby female flies and fixed for immunohistochemistry.

Drugs

Cells in about 80% confluences were added with DON (D2141, Sigma) or Azaserine (Fluka, 11430) to make different final concentrations and cultured for 24 hours. Fruit flies were supplied with wet-yeast pastes made from a mixture of dried yeast and different concentrations of DON or Azaserine.

Immunohistochemistry

Human culture cells or dissected fly tissues were fixed in 4% paraformaldehyde in 1X PBS for 10 min at room temperature. The fixative was then removed and samples were rinsed by 1XPBS and incubated in wash solution (1XPBS + 0.5% horse or donkey serum + 0.3% Triton X-100). The samples were incubated in primary antibodies for at least 12 hours at room temperature. Samples were then briefly rinsed by wash solution and incubated in the DNA dye Hoechst 33342 and secondary antibodies for at least 12 hours at room temperature. Samples were then mounted onto slides for observations under confocal microscopy. Primary antibodies used in this studies included: Rabbit or goat anti- CTP synthase repectively (K-21 (sc-101925, 1:1000), y88 (sc-134457, 1:1000), C-13 (sc-131474, 1:1000), Santa Cruz BioTech Ltd., Santa Cruz, CA, USA); Rabbit anti-Miranda (1:100, gift from Chris Doe); mouse anti-Hu-li tai shao (HTS, Developmental Studies Hybridoma Bank, 1:50). Secondary antibodies used in this study were anti-mouse, rabbit, or goat antibodies that were labelled

with Alexa Flour® 488 or Cy5 (1:1000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

Microscopy

Images were acquired under 10X, 40X or 63X objectives on a laser-scanning confocal microscope (Zeiss LSM 510 META, Oberkochen, Germany).

RESULTS

CTP synthase forms cytoophidia in human cells

To examine whether cytoophidia exist in human cells, we carried out immunostainings on cultured human HeLa P4 cells using three different antibodies against human CTP synthase. In all cases similar filamentous structures were observed (Fig. 1). Because each antibody was against a different region of human CTP synthase, we believe the filamentary structures revealed by multiple CTP synthase antibodies represent cytoophidia in human cells.

DON promotes the formation of the cytoophidium in human cells

To verify if the formation of cytoophidia in human cells is sensitive to DON, we cultured HeLa P4 cells in media that contained DON at different concentrations. To our surprise, we found that occurrences of cytoophidia increased dramatically upon DON treatment (Fig. 2). When HeLa P4 cells were cultured in standard medium without DON, many cells showed a dot pattern when staining with CTP synthase antibody with only about 6% cells showing elongated filamentary cytoophidia. However, 25% of cells showed obvious cytoophidia when treated with 2 $\mu\text{g/ml}$ DON for two days while more than 40% of cells had detectable cytoophidia when supplied with 4 $\mu\text{g/ml}$ DON (Fig. 2E). When treated with DON, it appeared that cells with cytoophidia were not evenly distributed. Very often we observed clusters of cells with cytoophidia, segregating apart from clusters of cells without cytoophidia (Fig. 3A). In elongated HeLa cells, the cytoophidia localised preferentially near the anterior-posterior axis (Fig. 3, B and C). The polarised organisation of cytoophidia could simply reflect that the bulk

volume of cytoplasm in these cells distributed near the anterior-posterior axis, but it might be due to an unknown underlying mechanism.

The shapes of cytoophidia in HeLa P4 cells were dynamic, varying from straight filaments to ring formation (Fig. 4). Both ends of a filament-shaped cytoophidium can fuse together to form a closed ring (Fig. 2, C and D). Although the ring-shaped cytoophidia were rarely seen in the control group, about 10% of cytoophidia occurred in were in ring-shape when HeLa P4 cells were treated with 2 or 4 $\mu\text{g/ml}$ DON (Fig. 2E). Cytoophidia can form at different phases of the cell cycle and were localized in the mid-plate or spindle area at metaphase (Fig. 5).

DON induces apoptosis and promotes cytoophidium assembly during *Drosophila* oogenesis

We next examined the effect of DON treatment on *Drosophila* oogenesis and development. Adult female flies fed on wet yeast for a few days grew very large ovaries (Fig. 6, A and B). By contrast, flies fed on DON laced yeast (1000 $\mu\text{g/ml}$) developed tiny ovaries with many egg chambers undergoing apoptosis (Fig. 6, A and D). Treatment of DON at lower concentrations (e.g. 250 $\mu\text{g/ml}$) led to medium-size ovaries with fewer apoptotic egg chambers (Fig. 6, A and C). Consistent with our previous observation, more cytoophidia were formed in apoptotic egg chambers than in non-apoptotic egg chambers (Liu, 2010). We have previously reported that macro-cytoophidia occur predominantly in early and mid-stage germline cells. After stage 11 nurse cell cytoplasmic nutrients are transferred to the oocyte, a process known as dumping, very few macro-cytoophidia are detectable, although there are still numerous micro-

cytoophidia remain in the oocyte (Fig. 6, E and F). However, in DON treated flies, long and thick macro-cytoophidia were very pronounced in mature egg chambers (Fig. 6, G-J). The macro-cytoophidia were often longer than 100 μm , a length which is seldom seen under normal conditions. Higher concentrations of DON induced denser macro-cytoophidia and micro-cytoophidia, suggesting that the effect of DON is concentration-dependent (Fig. 6, E-J). Macro-cytoophidia were induced in mature eggs even when flies were fed with 100 $\mu\text{g/ml}$ DON for 6 hours. The induced macro-cytoophidia in mature oocytes share many properties with those in earlier egg chambers, such as structural discontinuities and multiple bundles in one structure (Fig. 6J).

CTP synthase is critical for the integrity of cytoophidium

To determine if the expression level of CTP synthase is important for cytoophidium formation, we analysed three RNAi stocks expressing CTP synthase hairpin constructs under the control of an upstream activation signal (UAS) promoter. We chose follicle cells as a system because almost every follicle cell contains one cytoophidium of similar length within the same egg chamber (Liu, 2010). Using an inducible GAL4 driver, mosaic expression of the RNAi hairpin was monitored by expression of GFP in the nuclei, i.e. GFP⁺ cells also express CTP synthase RNAi hairpins. Using antibodies against CTP synthase, we found that GFP⁺ cells were lacking CTP synthase-containing cytoophidia, confirming that CTP synthase was knocked down. Similar results were obtained from expression of all three CTP synthase RNAi constructs (Fig. 7, A, C and E). The disappearance of CTP synthase-containing cytoophidia could be due to the removal of CTP synthase from the cytoophidium, while leaving other components of the

cytoophidium intact. Alternatively, the whole cytoophidium structure may be disassembled when CTP synthase protein concentration is low. We cannot distinguish between these two possibilities because CTP synthase is the only component of the cytoophidium that yet known. Preliminary observations suggest that several antibodies with unknown antigens cross-react with the cytoophidium. This raises the possibility that additional molecular components of the cytoophidium exist (data not shown). We found that none of these cross-reacting antibodies could either detect cytoophidia when CTP synthase was knocked down, which argues that it is the decreased level of CTP synthase that disrupts the structure of cytoophidia.

DON induces cytoophidium formation from low levels of CTP synthase

When flies were fed on DON overnight, we started to observe short cytoophidia forming in CTP synthase knock down cells (Fig. 7, B, D and F). The lengths of the de novo-formed cytoophidia in CTP synthase RNAi cells (GFP+) were about one third of those in neighbouring cells that expressed normal levels of CTP synthase (GFP-). These results indicate that DON promotes cytoophidium assembly even when CTP synthase level is low.

DON globally promotes cytoophidium assembly in *Drosophila*

DON treatment showed a profound effect on larval development. Low doses of DON delayed development, while higher dosages arrested larval growth at the first instar (Fig. 8). This was presumably due to the fact that DON, as a glutamine analog, affects several glutamine-dependent metabolic enzymes including CTP synthase.

To determine whether DON-induced cytoophidium formation is limited to oogenesis, we examined other tissues in *Drosophila*. We found that DON treatment could promote cytoophidium assembly in a wide-range of tissues, including larval testes, ovaries, salivary glands, guts, trachea, adult testes and brains. In larval central nervous system, we found that CTP synthase was highly abundant in post-embryonic neuroblasts and epithelial stem cells (Fig. 9, A, C, E and G). Although some small filaments could be seen scattered in some cells, most cells show a rather diffuse distribution of CTP synthase. However, a low dosage of DON was able to promote prominent cytoophidium assembly in neuroblasts located in brain lobes, thoracic and abdominal ganglia of the third instar larvae (Fig. 9, B, D, F and H). A densely stained zone with clearly detectable cytoophidia appeared in epithelial neuroblasts upon DON treatment (Fig. 9, B and D). There was predominantly only one large cytoophidium per neuroblast and the lengths of cytoophidia observed correlates with cell sizes. In large-size neuroblasts in the brain lobes, cytoophidia were about 8 μm long, similar to the diameter of the cell, while small-sized epithelial neuroblasts contained much shorter cytoophidia. It is worth noting that many micro-cytoophidia occur in each cell, which appear faint under low-resolution microscopy.

Azaserine has a similar effect on cytoophidium formation as DON

To test whether the effect of DON on cytoophidium formation is related to glutamine hydrolysis, we treated flies and human cells with a second glutamine analog Azaserine. In both systems, we observed Azaserine to have comparable effects as DON. For example, cytoophidia with weak intensity were detected in a patch of cyst cells in adult testes (Fig. 10, A

and C). After Azaserine treatment, big and bright cytoophidia became very clear in germline stem cells, gonialblasts, and spermatogonia in the *Drosophila* testis (Fig. 10, B and D). Without any treatment, cytoophidia were only detectable with low intensity in very few cells in the male accessory glands (Fig. 10, E and G). After Azaserine treatment, very abundant and bright cytoophidium could be easily observed in accessory gland cells where most cytoophidia associated with the hexagonal membrane (Fig. 10, F and H).

DISCUSSION

Human cytoophidia

Three recent studies independently reported the identification of CTP synthase-containing structures (i.e. cytoophidia or CTP synthase filaments) in a variety of organisms, suggesting the cytoophidium is evolutionarily conserved (Ingerson-Mahar et al., 2010; Liu, 2010; Noree et al., 2010) (review see (Liu, 2011)). Thus it is not surprising that CTP synthase is also compartmentalized into cytoophidia in human cells.

Similar filamentous structures have been reported in several studies. A study by Willingham et al. (1987) revealed a unique filamentous structure in the cytoplasm of cultured cells from mouse, rat, bovine, and human origin but not avian origin using a monoclonal antibody (Willingham et al., 1987). This structure seems to be packed by small filaments as revealed under electron microscopy. The abundances of this structure are variable among different types of cells. Unfortunately, the antigen of the so-called “nematin” antibody used in that study was unknown.

Nuclear inclusions that contain Poly(A)-binding protein nuclear 1 (PABPN1) were found in rat oxytocin-producing neurons (Villagra et al., 2008). These inclusions with closed packed filaments mainly localise in the interchromatin region. The aggregation of PABPN1, ubiquitin, proteasomes and poly(A) RNAs is also the pathological hallmark of oculopharyngeal

muscular dystrophy (Tome and Fardeau, 1980; Calado et al., 2000; Abu-Baker and Rouleau, 2007).

Using human autoantibodies, Chan and co-workers identified a type of cytoplasmic structures with rod- and ring-shape in human HEp-2 cells and some other cell types (Carcamo et al., 2009). They found that the expression of these structures is independent of the cell cycle. They recently verified that the “rods and rings” contain CTP synthase, suggesting that rods and rings are equivalent structures of cytoophidia (reviewed in (Liu, 2011)).

DON treatments in prokaryotes and eukaryotes

It was puzzling that DON treatment disrupted cytoophidia in *Caulobacter crescentus* while it promoted the formation of cytoophidia in human and *Drosophila* cells (Ingerson-Mahar et al., 2010). This can be due to the difference between prokaryotes and eukaryotes. However, DON also delocalized CTP synthase filamentous structure when expressing *Caulobacter crescentus* mCherry-CTP synthase in the eukaryote, *Schizosaccharomyces pombe* (Ingerson-Mahar et al., 2010).

Another explanation for the difference lies in the exposure time of DON. In the *Caulobacter crescentus* study, the exposure of DON treatment could be very short (the exact time has not been specified in that study (Ingerson-Mahar et al., 2010)), while we treated human cells and fruit flies for at least 10 hours. The induction of cytoophidium formation by DON might be a compensation mechanism responding to its inhibition of CTP synthase activity. Although we have not determined how long it takes for the

induction of cytoophidia in human and *Drosophila* cells, it would be particularly interesting to see whether long time exposure to DON can induce cytoophidium formation even in bacteria.

Cytoophidia and cancer

In 1978, Weber and co-workers found that CTP synthase activity in hepatomas was elevated (Williams et al., 1978). Subsequent studies demonstrated that unregulated CTP levels and increased CTP synthase activity are features of many forms of cancer such as leukemia, hepatomas, and colon cancer (Williams et al., 1978; Kizaki et al., 1980; Weber et al., 1980; Ellims et al., 1983; van den Berg et al., 1993; Whelan et al., 1994; van den Berg et al., 1995; Verschuur et al., 1998; Verschuur et al., 2000a; Verschuur et al., 2000c; Verschuur et al., 2000d; Verschuur et al., 2000b; Verschuur et al., 2001). In addition, CTP synthase has also been an attractive target for drug development against viral (De Clercq, 2001) and parasitic disease (e.g. African sleeping sickness (Hofer et al., 2001; Fijolek et al., 2007), malaria (Hendriks et al., 1998), and infectious blindness (Wylie et al., 1996)). For this reason, DON has been tested as an anti-tumor, anti-virus and anti-parasite agent (Lyons et al., 1990; Hofer et al., 2001). Our RNAi study demonstrates that the occurrence of cytoophidia correlates to the expression level of CTP synthase. Further studies need to be done to determine whether the appearance and abundance of cytoophidia can be potential biomarkers for cancer.

Glutamine analogs for the study of cytoophidia

Glutamine analogs such as DON and Azaserine inhibit CTP synthase activity (Bartz et al., 1954; Hartman, 1963; Levitzki et al., 1971). These

drugs have been used extensively in the study of the enzymatic characters of CTP synthase. For example, Levitzki et al. (1971) used DON treatment to elucidate that DON reacts with only half of the total glutamine sites of CTP synthase and induces conformation changes of one subunit to another one (Levitzki et al., 1971). Robertson (1995) demonstrated that inactivation of CTP synthase by DON reduces the amount of tetramer in *Escherichia coli* (Robertson, 1995). Our study show that cytoophidia, as CTP synthase-containing structures, respond to DON treatment in a concentration-dependent manner. Thus DON treatment can be very useful for further studies concerning the composition, structure, dynamic, formation and function of cytoophidia.

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Figure legends

Fig. 1. Cytoophidia exist in human cells. HeLa P4 cells were stained using multiple antibodies against CTP synthase (green). DNA was stained with Hoechst 33342 (Magenta). A: Stained using a purified rabbit polyclonal antibody raised against human CTP synthase (K-21). B: Stained using a rabbit polyclonal antibody raised against a conserved region of CTP synthase (*Saccharomyces cerevisiae*) (y88). C: Stained using an affinity purified goat polyclonal antibody raised against a peptide mapping to the C-terminus of human CTPS (C-13). Scale bars, 10 μm .

Fig. 2. DON induces cytoophidia in human cells. A: Staining of CTP synthase reveals cytoophidia in some HeLa cells. Note that there are multiple cytoophidia in two cells in the centre but most cells show only dot-like structures. B: After treatment with 4 $\mu\text{g/ml}$ DON, a high proportion of cells show obvious cytoophidia. C: Cytoophidia adopting filamentary shapes. D: Cytoophidia in ring shapes. (E) DON treatment increase the occurrence of cytoophidia, especially ring shaped structures. Scale bars, 10 μm .

Fig. 3. Cytoophidia show a polarised distribution in human cells. A: HeLa P4 Cells with cytoophidia in filamentous or ring shapes are clustered to the right, while cells with dot pattern are clustered to the left. B, C: Cytoophidia are preferentially distributed near the anterior-posterior (AP) axis (white lines in B) in HeLa P4 cells. CTP synthase (green). DNA was stained with Hoechst 33342 (Magenta). Scale bars, 10 μm .

Fig. 4. Cytoophidia exhibit various shapes in human cells. HeLa P4 cells were stained by an antibody against CTP synthase (green). DNA was stained with Hoechst 33342 (Magenta). A-D: Cytoophidia with shapes of a heart, kisses and a ring. Cytoophidia show shapes of a flower (E), “80” (F), “P” (G) and “?” (H). I: ring-shape cytoophidia (same as Fig. 2D). J-N: Cytoophidia show shapes resembling numbers such as “8” (J), “9” (K), “10” (L), “-3” (M) and “1111” (N). O: A dot-shape cytoophidium localises near the mid-body between two cells. Scale bars, 10 μ m.

Fig. 5. Cytoophidia appear at different phases of the cell cycle. HeLa P4 cells were stained using an antibody against CTP synthase (green). DNA was stained with Hoechst 33342 (Magenta). A, B: Metaphase. C: Two cells, one at interphase and another at metaphase. D: Tetrapolar metaphase. E: Telophase. Scale bars, 10 μ m.

Fig. 6. DON induces apoptosis in *Drosophila* ovaries and promotes the formation of cytoophidia in mature eggs. A: Ovaries from flies fed with food containing 0 (left), 250 (centre), and 1000 (right) μ g/ml DON. DNA staining of ovaries from flies fed with food containing 0 (B), 250 (C), and 1000 (D) μ g/ml DON. While apoptosis is rarely seen in B, there are a few apoptotic egg chambers in C and many apoptotic egg chambers in D. CTP synthase of mature egg chambers from flies fed with food containing 0 (E), 250 (G), and 1000 (I) μ g/ml DON. Note both macro- and micro-cytoophidia increase dramatically upon DON treatments (compare E, G and I). F, H and J: Zoom-ins of E, G and I, respectively. Scale bars, 50 μ m.

Fig. 7. Cytoophidia in *Drosophila* follicle cells. Knock-down of CTP synthase is monitored by GFP signal in the nucleus when using inducible GAL4 driving UAS-CTP synthase RNAi (A, B). A: without DON treatment. B: with DON treatment. C, D: CTP synthase staining only. E, F: Zoom-ins of (C, D), respectively. Note that there are no obvious cytoophidia in GFP+ cells without DON treatment (A, C, E). Short cytoophidia appear in GFP+ cells with DON treatment (B, D, F). Scale bars, 10 μ m.

Fig. 8. DON treatment impedes *Drosophila* larval development. Larvae fed with food mixed with 0 (upper row) or 1000 (lower row) μ g/ml DON and recorded daily.

Fig. 9. DON induces cytoophidia in *Drosophila* neuroblasts. CTP synthase is enriched in neuroblasts with a diffused pattern without DON treatment (A, C, E, G). DON treatment induces obvious cytoophidia in neuroblasts (B, D, F, H). A-D: brain lobes. E-H: thoracic and abdominal ganglia. Scale bars, 50 μ m.

Fig. 10. Azaserine induces cytoophidia in *Drosophila* testes. Only weak staining of CTP synthase with cytoophidia in some cells in adult testis (A, C) and accessory glands (E, G). Upon Azaserine treatment, bright and big cytoophidia are apparent in an adult testis (B, D) and accessory gland (F, H). Scale bars, 50 μ m.

Figures 1-10

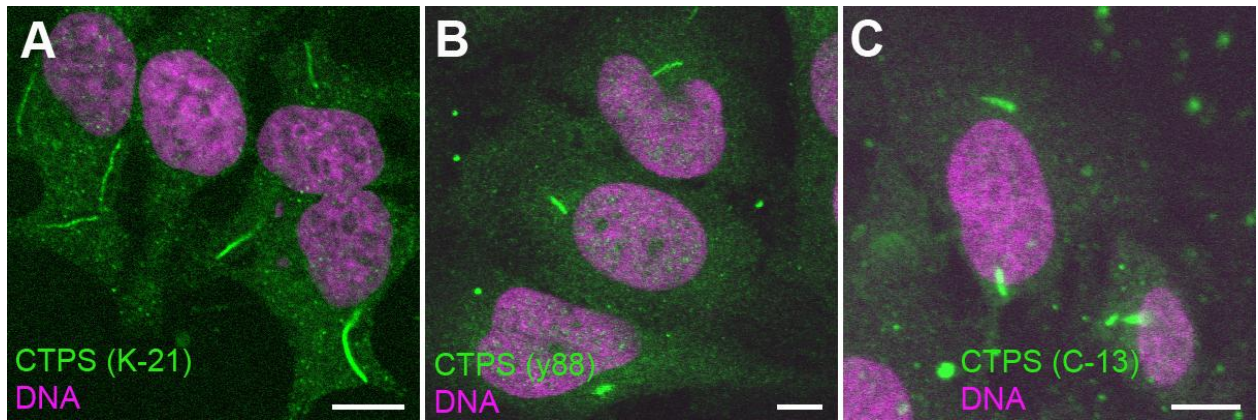


Figure 1

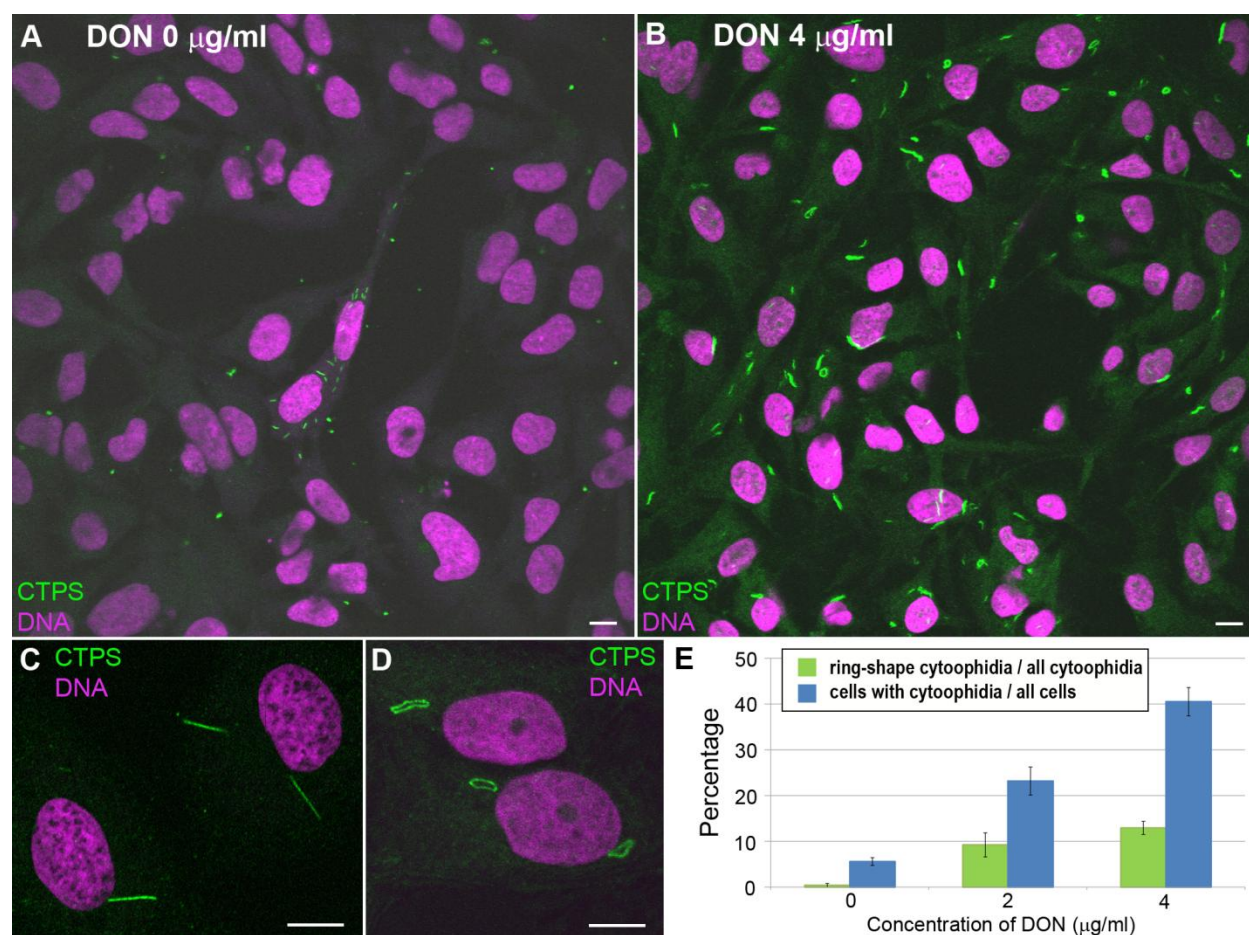


Figure 2

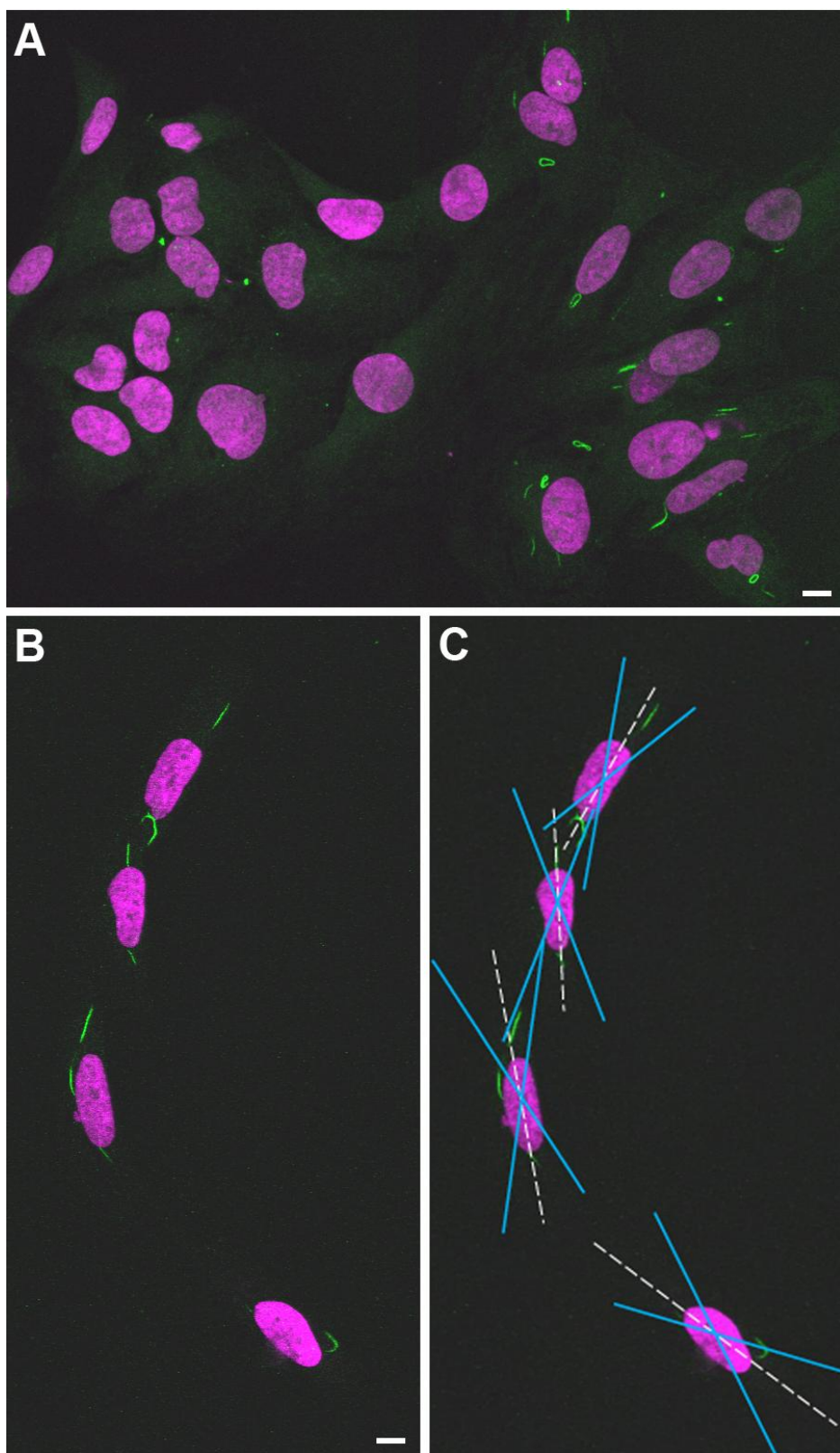


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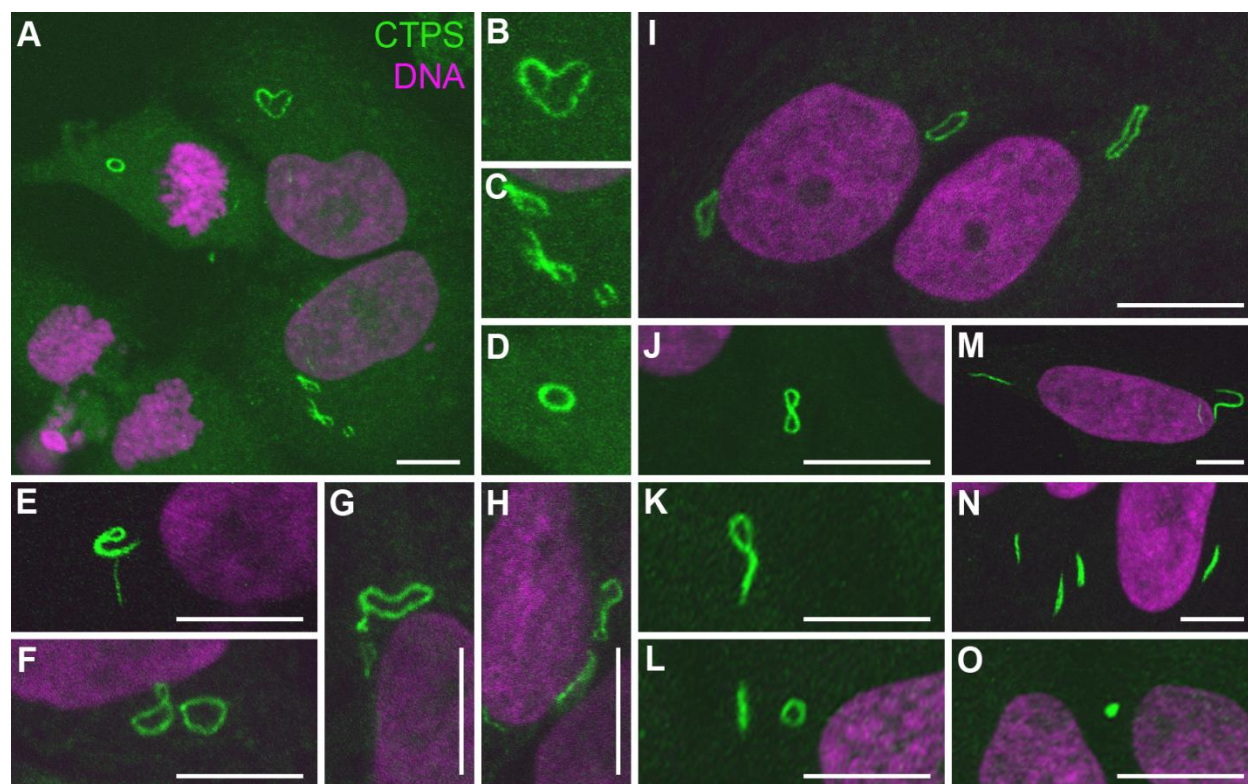


Figure 4

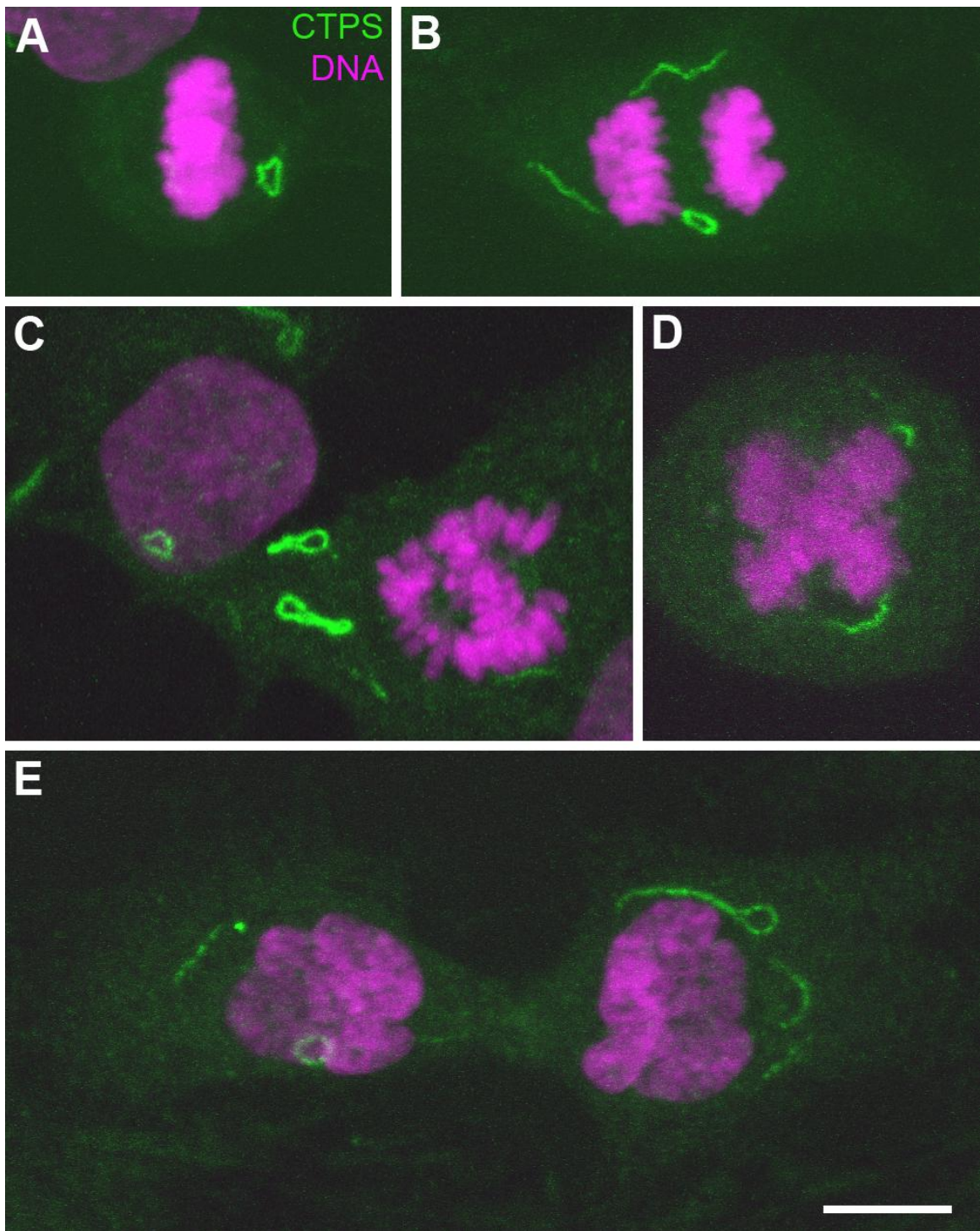


Figure 5

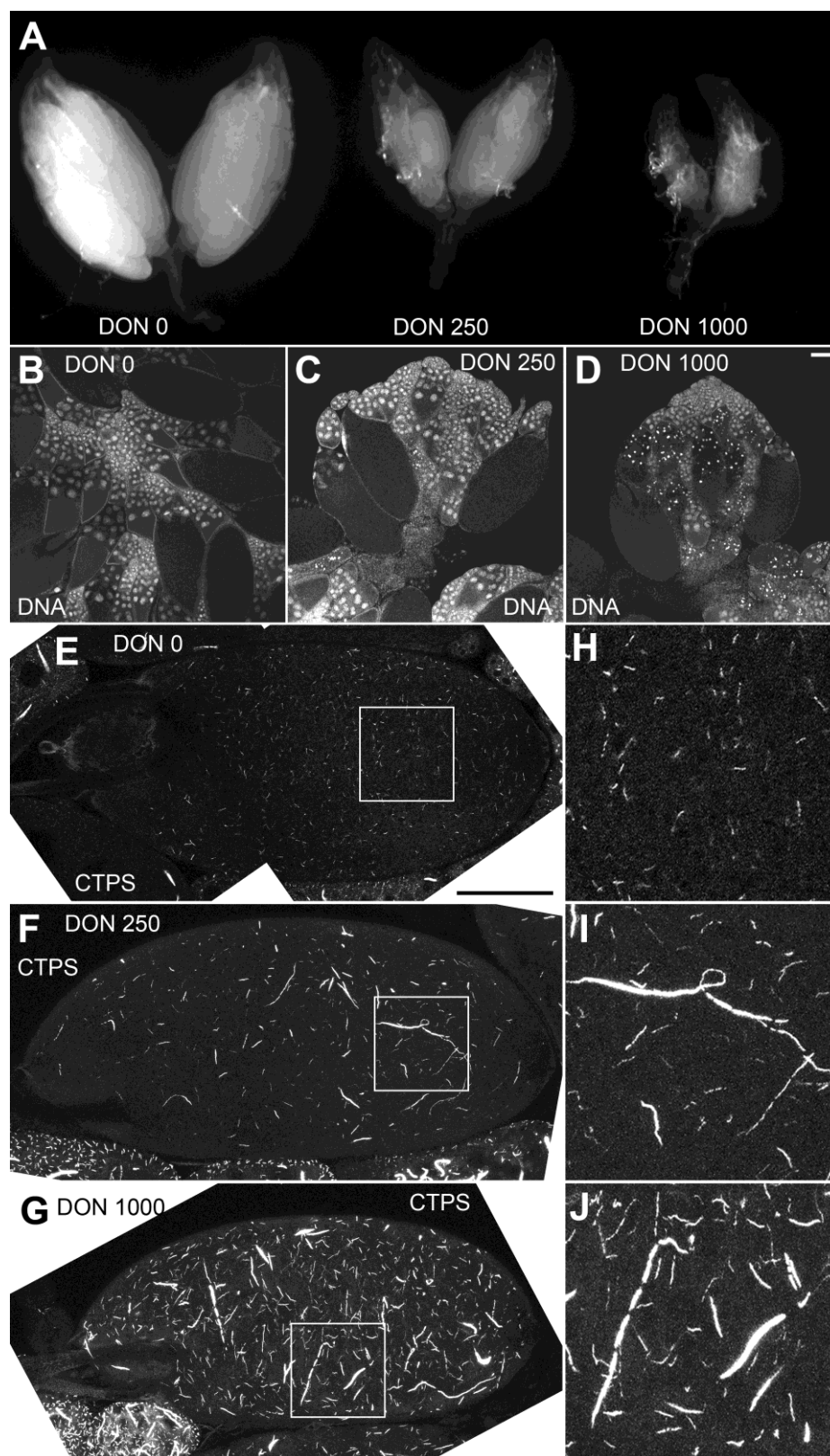


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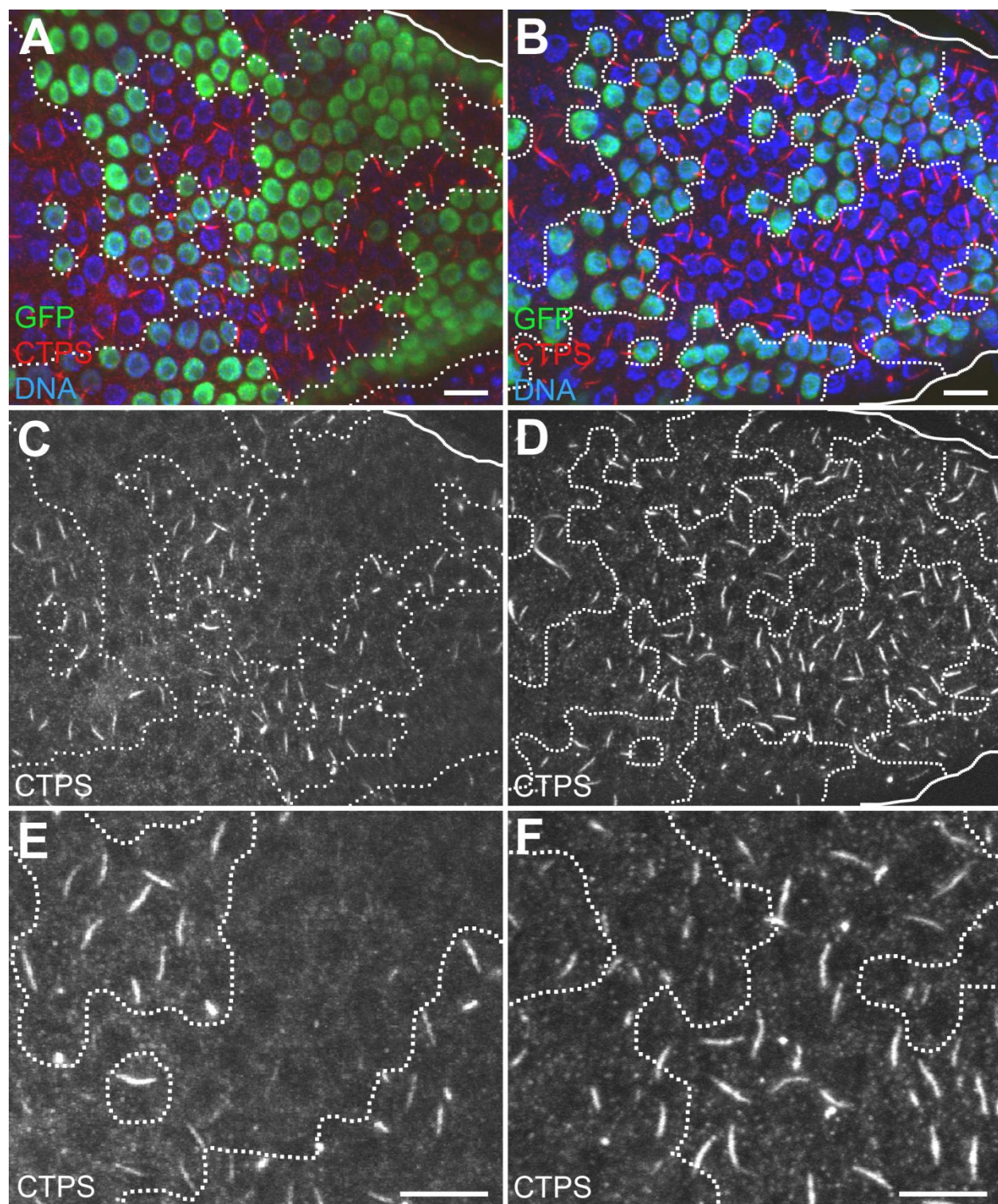


Figure 7

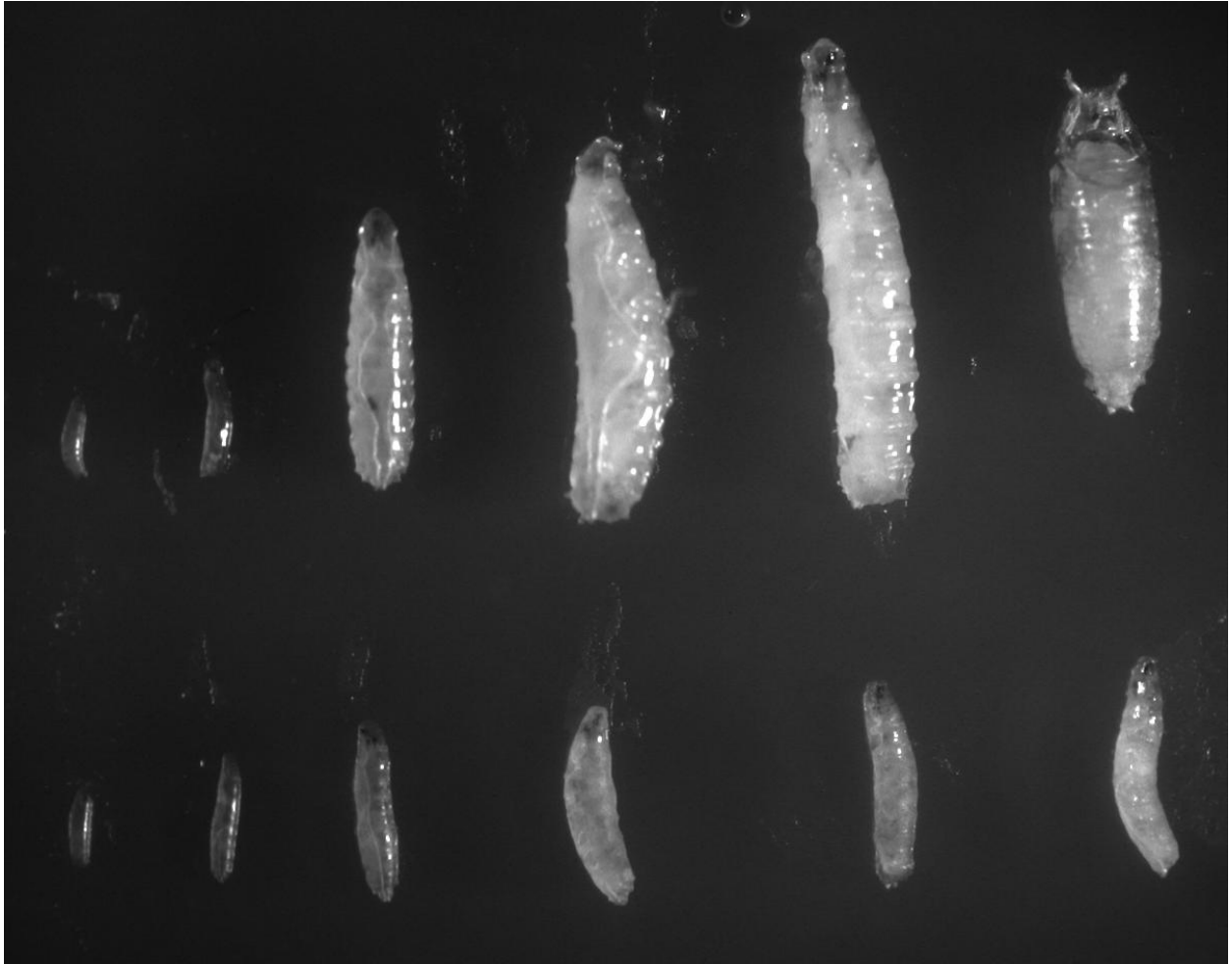


Figure 8

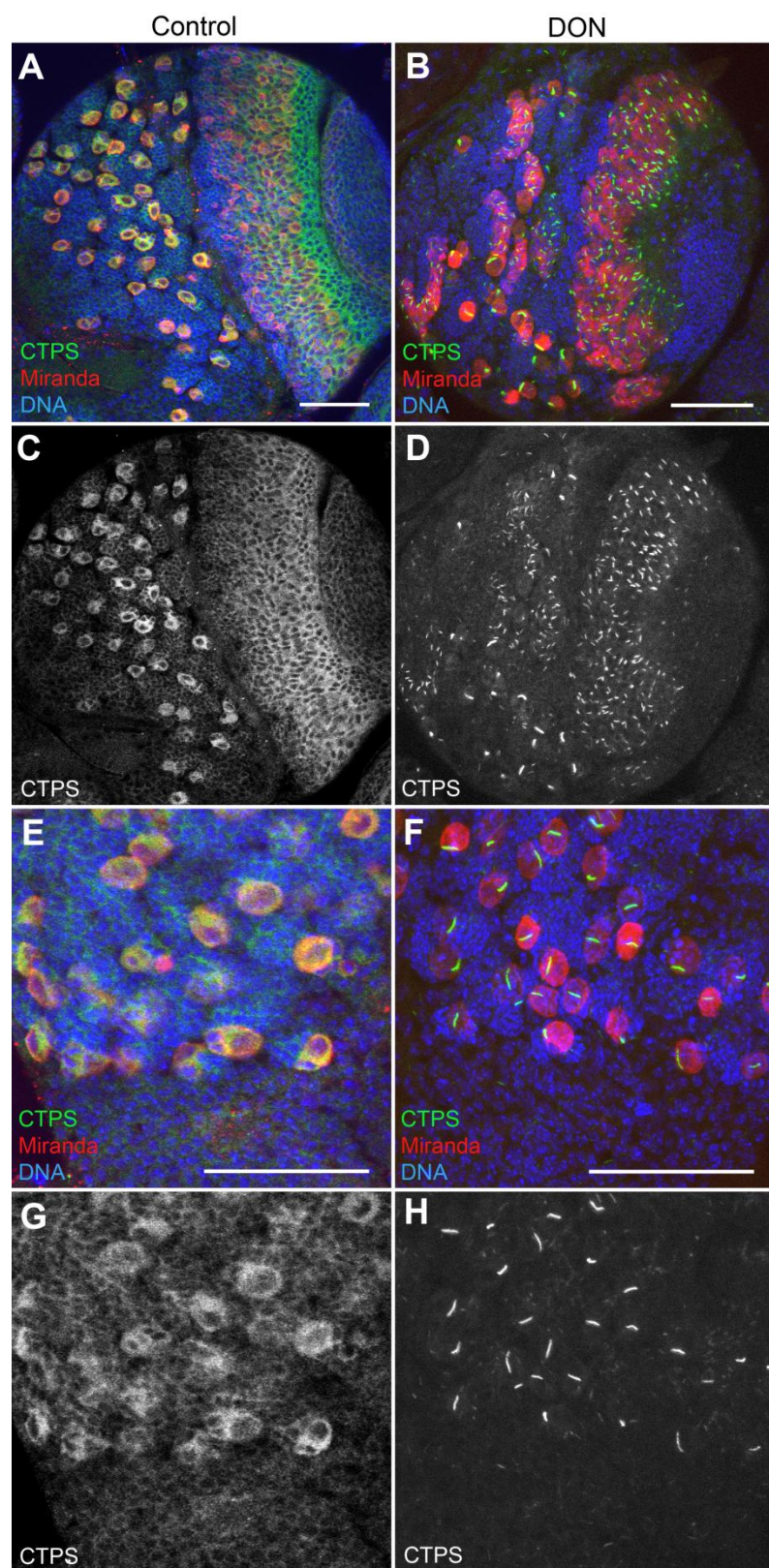


Figure 9

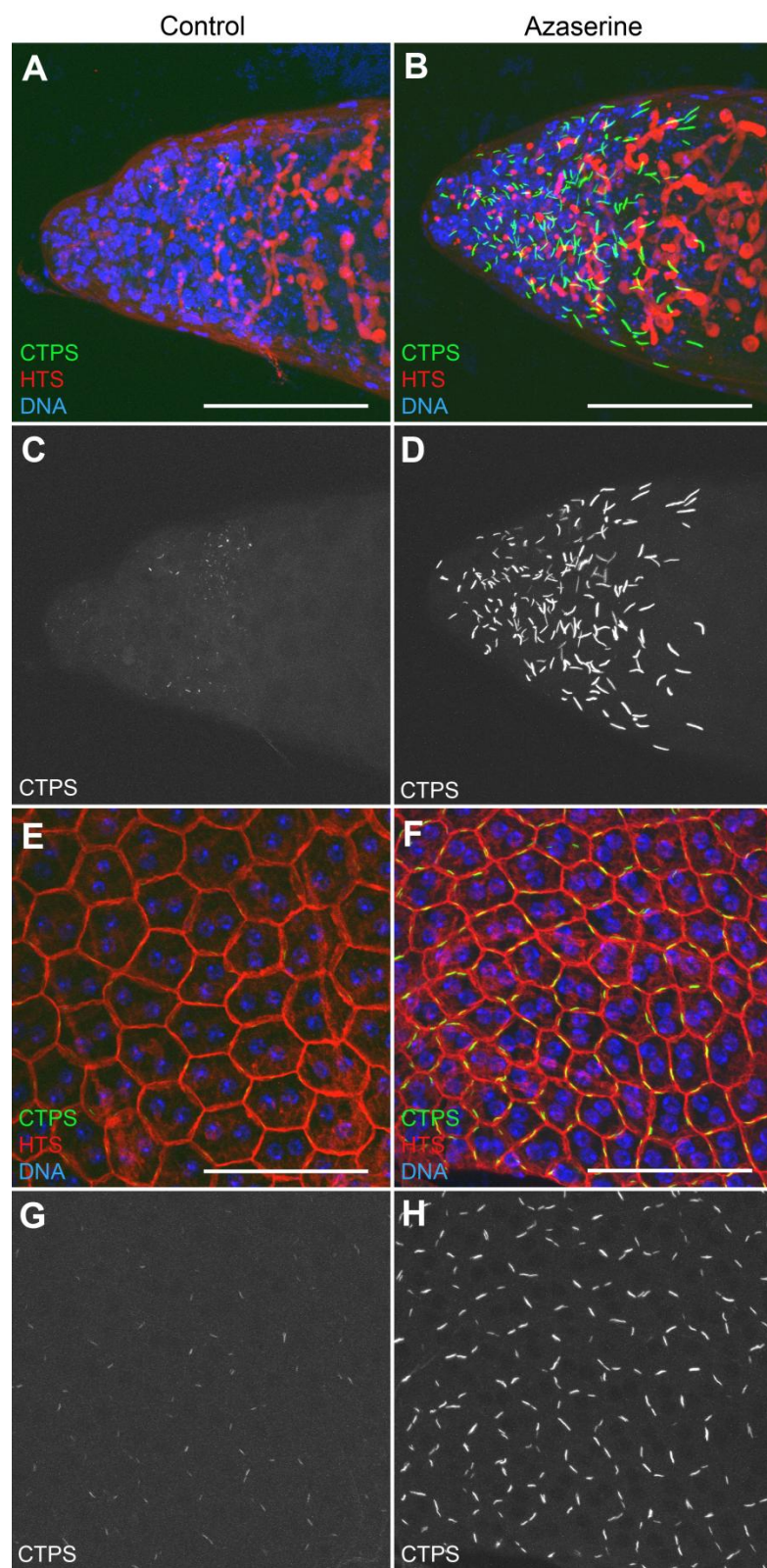


Figure 10